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ARTICLES

Assessment Of Different Samples For Molecular Diagnosis Of Extra-Pulmonary Tuberculosis

Gasmelseed N., Aljak M. A., Elmadani A. E., Elgaili M. E. and Saeed O. K.

Use Of The Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) Technique To Analyse The *Anthocyanidin Synthase* (ANS) Gene Locus In Zimbabwean Sorghum Landraces With Different Seed Proanthocyanidin Profiles

Ngonidzashe Mangoma and Zephaniah Dhlamini

Full Length Research Paper

Assessment of different samples for molecular diagnosis of extra-pulmonary tuberculosis

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Extrapulmonary tuberculosis is an important clinical problem particularly in developing countries. The aim of this study was to assess two different samples (blood and fluid) for the diagnosis of extra-pulmonary tuberculosis (abdominal tuberculosis and tuberculous lymphadenitis). The study subjects were recruited from WadMedani Teaching Hospital during 2009-2013. Seventy five ascetic fluid and blood samples were collected from each suspected patient with abdominal tuberculosis and twenty five lymphatic aspirates and blood samples were collected from each suspected tuberculous lymphadenitis patient. DNA was extracted using DNP™ kit (CinnaGenInc) and polymerase chain reaction (PCR) was done using *IS6110* gene for both samples. In abdominal tuberculosis, 20/75 (27%) were positive for tuberculosis when ascetic fluid was used and 9/75 (12%) in case of blood samples. The comparison between ascetic fluid and blood samples, showed that, there was a significant difference in both results, P-value < 0.05. In tuberculous lymphadenitis, 13/25 (52%) and 3/25 (12%) were positive to tuberculosis when lymph aspirate and blood were used respectively. This study concluded that the best sample for diagnosis of abdominal TB and lymphadenitis is ascetic fluid and LN aspirate. This study recommends that ascetic fluid and lymph aspirate samples are recommended to be used in molecular diagnostic test.

Key words: Extra-pulmonary tuberculosis, molecular diagnosis, acetic fluid, lymph aspirate samples.

INTRODUCTION

Tuberculosis (TB) is among the top ten causes of global mortality, it is estimated that approximately one-third of the world's population is infected with tuberculosis bacillus, and each year eight million people develop the tuberculosis disease which annually kills 1.8 million worldwide (Ahmed et al., 2011). In Sudan, an estimated

annual risk based on the data of the 1986 national prevalence survey of TB is 1.8% which gives an incidence of 90/100,000 smear positive cases, and puts Sudan among the high prevalence countries for TB in the eastern Mediterranean region (Crofton, 2000).

The World Health Organization estimated that Sudan

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ranked twenty-third in the list of countries with the greatest number of estimated incident of TB cases (78 030), with an estimated rate, half again as high as the estimate for the whole world (220 vs.140 per 100 000 population) (El Sony et al., 2007). According to the Sudan annual health report 2007, the incidence of TB rate was 58 per 100,000, where Khartoum State reported the highest number of TB patients admission of 4878 cases, then Gezira State, 3105 cases, North Kordofan with 1853 cases, at the end of the list, North States became the fewest prevalence state of TB with 820 cases (Annual Health Statistical Report, 2007). In Gezira State, the incidence of TB cases from 2003 to 2008 was between 2706 - 3259, (WHO TB Program in Gezira State). The prevalence of TB in Sudan is 209 cases per 100,000 of the population and 50,000 incident cases during 2009 (Sharaf Eldin et al., 2011). Most of the cases of TB are pulmonary TB which is about 75%, the other 25% cases are extra-pulmonary TB (EPTB), (CDC, 2003).

The incidence of extra-pulmonary forms of tuberculosis varies from country to another, such that on the average between 1964 and 1989, 5 to 10% of the approximately seven million new cases each year in the developing countries were extra-pulmonary. This distribution also can be affected by origin of the individuals within a country (Talavera et al., 2001).

The diagnosis of extra-pulmonary tuberculosis is still now challenging for diagnostic routine laboratories. The aim of this study is to assess two different specimens (blood and ascetic fluid for abdominal TB, blood or lymph aspirate for tuberculous lymphadenitis) using polymerase chain reaction (PCR) to diagnose abdominal TB and tuberculous lymphadenitis in patients attending Wadmedani Teaching Hospital during the period of 2009-2012.

MATERIALS AND METHODS

Study subjects

All clinically suspected patients with abdominal TB or tuberculous lymphadenitis patients attending Wadmedani Teaching Hospital during the period of study from 2009 - 2012 were recruited for this study.

Inclusion criteria of subjects

Patients with symptoms of guarding and free fluid (ascetic), abdominal pain and mass, weight loss, fever sweating, diarrhea and vomiting, and patients with past history of pulmonary TB disease or/and history of TB contact were included. Patients presented with lymphadenopathy in different body regions including cervical, inguinal and auxiliary lymph node were included in the study.

Exclusion criteria of subjects

Patients with HIV infection, Chron's disease, malignancy, cirrhosis,

ulcerative colitis, chronic diseases were excluded from this study.

Sample collection

Ascetic fluid or lymph nodes aspirate and 2 ml of venous EDTA blood from each patient were collected after a written informed consent. Ethical approval for this study was given from National Cancer Institute Research Ethical Committee (NCI-REC).

Molecular methods

DNA was extracted from lymph node aspirate and ascetic fluid blood using DNP™ kit (cinna GenInc, Cat. No.DN115C form Iran). The kit was designed to isolate double stranded DNA from human and animal sources. Lymph node aspirate and ascetic fluid samples were treated with 4% sodium hydroxide, then they were centrifuged, homogenized, supernatant discarded, and the rest used for DNA extraction as described by manufacturer.

Polymerase chain reaction (PCR)

Identification detection of *Mycobacterium tuberculosis* was done by using a specific pair of primers (the sequence of these primers, T4 and T5, are: 5'-CCT GCG AGC GTA GGC GTC GG 3' and 5' CTC GTC CAG CGC CGC TTC GG 3', respectively) designed to amplify an insertion sequence *IS6110* gene in the *M. tuberculosis* complex, the expected band size was about 123 bp. The total volume of was 25 µl for each reaction (positive and negative control was done for each PCR run). All the amplicons were checked run using agarose gel electrophoresis. The presence of 123 bp for *IS6110* gene fragments indicated a positive test for *M. tuberculosis*.

Statistical analysis

A statistical analysis was performed with SPSS statistical package version 16.0. Descriptive analysis was done and correlations between different variables were calculated using Pearson Chi-square test.

RESULTS

Hundred suspected extra-pulmonary TB cases were recruited from WadMedani Teaching Hospital during the period of September 2009 to September 2012. Seventy five out of hundred (75%) of the cases were suspected with abdominal TB while 25/100 (25%) were suspected to have lymphadinitis TB. The mean of ages of suspected abdominal subjects was 46.7 ± 18.2 years with range between (min 3 - max 80 years old). The mean age of suspected tuberculous lymphadenitis patients was 34.7 ± 20.7 years with range between (min 2 - max 80 years) as shown in Figure 1. The description of the study subjects showed that in suspected abdominal TB and TB lymphadenitis, males were 45 (60%), 10/25 (40%) respectively as shown in Table 1. The majority of the study subjects were from rural areas: 56/75 (74.7%) and 18/25 (72%) respectively as shown in Table 2. Regarding suspected abdominal TB patients, ascites was the common presenting symptom in all study subjects and

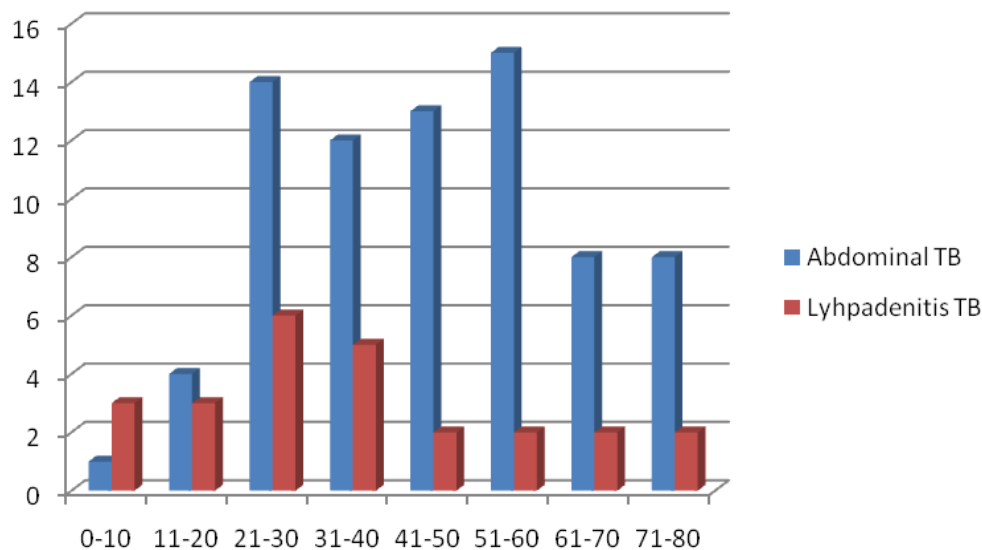


Figure 1. Description of study subjects by age groups.

Table 1. Description of study subjects by gender.

Gender	Abdominal TB (%)	Lymphadenitis TB (%)	total no. of subjects
Male	45 (60.0)	10(40.0)	55
Female	30 (40.0)	15(60.0)	45
Total	75	25	100

Table 2. Residence description of study subjects.

Sex description	Abdominal TB (%)	Lymphadenitis TB (%)	Total no. of subjects
Urban	19(25.3)	7 (28.0)	26
Rural	56(74.7)	18 (72.0)	74
Total	75	25	100

weight loss was 58/75 (77.3%), while lymphadenopathy was common presenting for TB lymphadenitis and night sweating, 10/25 (40%) were the common presenting symptoms in the suspected TB lymphadenitis study subjects.

Molecular detection of *M. tuberculosis* in abdominal TB study subjects

The *IS6110* gene is a multi-copy gene found only in *M. tuberculosis* complex. Most of the PCR studies have targeted *IS6110* gene sequence of *M. tuberculosis*

genome because of the presence of repetitive sequence of *IS6110* gene, this characteristic helps to increase the sensitivity of PCR over that obtained in the amplification of single DNA sequence. PCR was done for the ascetic fluid samples and blood for seventy five suspected abdominal TB, PCR resulted to 20/75 (26.7%) of ascetic fluid and 9/75 (12%) of blood samples were positive for *M. tuberculosis* as shown in Figure 2, PCR results indicate *M. tuberculosis* DNA with length 123 bp as in Appendix 1.

The comparison between ascetic fluid samples blood samples results, showed that there was a significant difference, between the two samples using person Chi-

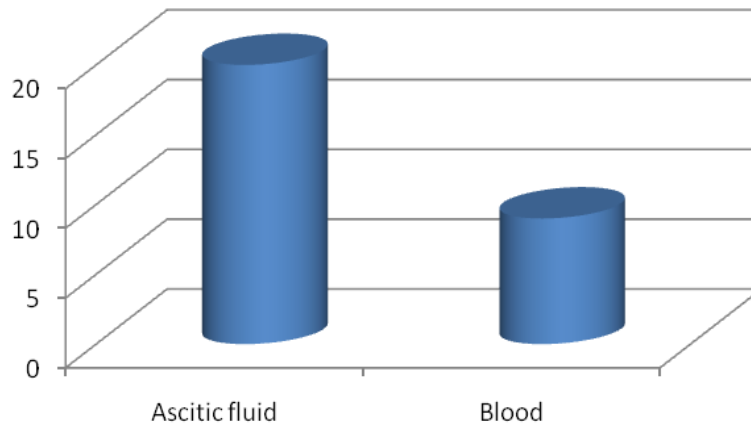


Figure 2. The positive PCR results in ascetic fluid and blood samples of study subjects. N=75.

Table 3. Comparison between ascetic fluid and blood.

		PCR results for blood		Total
		Positive	Negative	
PCR results for fluid	Positive	9	11	20
	Negative	0	55	55
Total		9	66	75

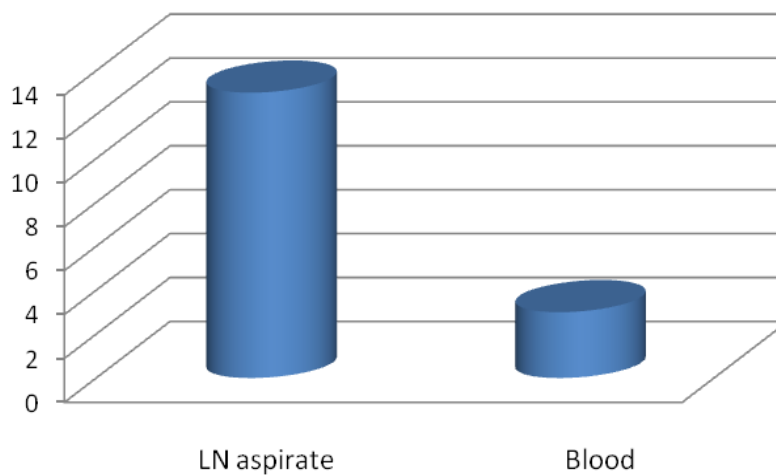


Figure 3. The positive PCR results in LN aspirate and blood samples by IS6110 in suspected LN TB patients. N=25.

Square test = 28.1, P- value = 0.000 as shown in Table 3.

Detection of *M. tuberculosis* in tuberculous lymphadenitis study subjects

PCR was done using IS6110 gene for 25 lymph node

aspirate samples, 13/25 (52%) of the study subjects were positive for *M. tuberculosis* indicating bands with 123 bp. For the blood samples 3/25 (12%) of the study subjects were positive for PCR as shown in Figure 3. In comparison between the lymph node aspirate samples and blood samples, there was a significant difference, Person Chi-square test = 28.1, P- value = 0.000 as

Table 4. Comparison between LN and blood samples results.

		PCR results for blood		Total
		Positive	Negative	
PCR results for LN aspiration	Positive	3	10	13
	Negative	0	12	12
Total		3	22	25

shown in Table 4.

DISCUSSION

Extra-pulmonary tuberculosis (EPTB) is considered as an important clinical problem, it can occur in isolation or along with a pulmonary focus as in disseminated tuberculosis. It has been observed that EPTB constituted about 15 to 20% of all cases of TB (Sharma and Mohan, 2004). Abdominal TB involve the gastrointestinal tract, peritoneum, lymph nodes or solid viscera, constitutes up to 12% of extra-pulmonary TB and 1-3% of the total TB cases. The disease can mimic many conditions, including inflammatory bowel disease, malignancy and other infectious diseases, thus diagnosis is therefore often delayed (Uzunkoy et al., 2004). In this study, in 100 suspected EPTB, 75 were abdominal TB, 20/75 (26.7%) of them were positive. Tuberculosis of the lymph node (tuberculous lymphadenitis) is the most common form of extrapulmonary tuberculosis. In developed countries, tuberculosis is implicated in as few as 1.6% of patients with lymphadenopathy (Sarwar et al., 2004). But in developing countries almost two third of the cases of lymphadenopathy are due to tuberculosis (Sarwar et al., 2004).

The mean age of the abdominal TB cases in this study was 47.8 ± 18.2 years, while the age range was found to be between 21-70 years which was similar to study done by Uzunkoy et al. (2004) in Turkey; they found that the mean age was 39 years (range 18-65). In Taiwan, Huan-Lin et al. (2009) found that patients with abdominal TB, had age range from 22 to 88 years, with a mean age of 50 ± 18 [9]. In the tuberculous lymphadenitis patients, the mean ages was 36.4 ± 16.6 years and the age range was 21-40 years, this was different from study done by in Sudan that showed found that a mean age of thirty patients was 26.9 ± 11.2 years (Sharaf-eldin et al., 2002).

Extra-pulmonary tuberculosis, which remained without bacteriologic or histological confirmation, and the diagnosis based on recognition of signs and symptoms is raised from involvement of particular organ or system. Molecular technique using nucleic acid amplification methods to detect *M. tuberculosis* in clinical specimens are increasingly in use as a tool for TB diagnosis. *IS6110* gene is used as a target by the majority of the

investigators performing PCR based diagnosis of TB (Kulkarni et al., 2006). The principal reason for using *IS6110* gene is because it is considered to be a good target for amplification as this insertion sequence is found in almost all members in high copy number in most strains of the *M. tuberculosis* complex, which was thought to confer higher sensitivity (Kulkarni et al., 2006; Sharma et al., 2013).

The aim of this study was to compare between different samples molecular diagnosis of extra pulmonary TB by using *IS6110* gene. In comparison between ascetic fluid and blood samples, the study showed that there was a significant difference ($P < 0.05$) between the two different samples, this means that ascetic fluid had a high positivity than blood and it may be due to the presence of the microorganism at the location of the lesion while low level of was found in the blood. This is consistent with a study done in Pakistan using ascetic fluid DNA sample and *IS6110* specific primer sequence for *M. tuberculosis*, 50/55 (90.9%) patients were positive (Phulpoto et al., 2010). Several studies had the same results with high positivity from ascetic fluid (Uzunkoy et al., 2004; Protopapas et al., 2003). Thus, ascetic fluid is a suitable sample for diagnosis of abdominal TB. Therefore, detecting *M. tuberculosis* from blood samples was less likely (Chhuttani et al., 1986).

Comparison between lymph node aspiration and blood samples for patient with lymphadenitis, showed that lymph node aspirate samples 13/25 (52%) were positive when compared with only 3/25 (12%) in blood samples, and there was a significant difference ($P < 0.05$), this is consistent with a study conducted in lymphadenopathy clinic, Central Police Hospital, Burri, Khartoum, Sudan, showing that circulating *M. tuberculosis* DNA was detected in the aspirate fluid of all the 28 patients (100%) with multiple lymph nodes (Sharaf-eldin, 2002). Another study detected *M. tuberculosis* from blood, in 191 suspected patients with extrapulmonary TB, they found a low rate of detection (Kolk et al., 1998). Same result was also found in a study done on sputum-smear positive patients with *M. tuberculosis* (Aguado et al., 1996).

Conclusion

For PCR diagnosis of abdominal TB and tuberculous

lymphadenitis, the best samples are ascetic fluid and lymph nodes aspirate, respectively.

Recommendation

Ascetic fluid and lymph aspirate are recommended to be used as sample for diagnosis of extra-pulmonary tuberculosis.

Conflict of Interests

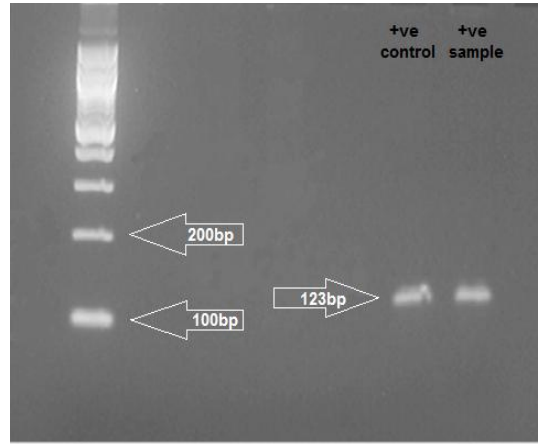
The author(s) have not declared any conflict of interests.

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Appendix 1. A band of 123 bp for positive sample using *IS6110*.

Full Length Research Paper

Use of the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique to analyse the *Anthocyanidin Synthase (ANS)* gene *Locus* in Zimbabwean sorghum landraces with different seed proanthocyanidin profiles

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Studies on the effects of mutations within flavonoid pathway genes on the resultant flavonoid profiles in sorghum are important in the identification and characterisation of varieties with nutritionally superior flavonoid profiles. In this study, we aimed at determining the effect of mutations at one important flavonoid pathway *locus*, the *anthocyanidin synthase (ANS)* gene, on grain flavonoid profile in sorghum. Sequence polymorphisms at this *locus* were determined in sorghum varieties with different seed proanthocyanidin profiles. The proanthocyanidin profiles of 61 local landraces were determined by the DMACA stain and butanol-HCl assay. The *Anthocyanidin synthase (ANS)* gene was then amplified using PCR from a subset of 11 landraces, and the amplicons subjected to sequence polymorphism analysis using the restriction fragment length polymorphism (RFLP) technique. Results show that 89% of the brown landraces, 4% of the red and none of the white landraces had detectable proanthocyanidins in their grain. Grain proanthocyanidins ranged from 0.1 to 1.8 AU at 550 nm per gram of sample. Using the PCR-RFLP technique, no sequence variations were detected at the *ANS locus*. Consequently, the different proanthocyanidin profiles observed could not be attributed, according to the methods used, to events at the *ANS gene locus*. These could be due to mutations at other *loci* or a combination of genetic and environmental factors.

Key words: Sorghum, flavonoid, flavonoid profile, mutation, restriction fragment length polymorphism (RFLP), condensed tannins, Zimbabwe.

INTRODUCTION

Sorghum bicolor (cultivated sorghum) is a cereal crop of the hot, semi-arid tropics, believed to have originated from Africa (Taylor, 2006; Waniska, 2000). The crop can do well in a wide range of climatic conditions, including

the arid and semi-arid tropics that characterise most of Africa. Despite this, sorghum has remained largely a subsistence crop in Africa, grown mainly by resource poor communal farmers for domestic consumption,

thus contributing to the generally low growth in sorghum production figures on the continent. Average yields have remained static, with an average less than 1.1 tonnes per hectare (Kumar et al., 2011; Taylor, 2006).

This apparent lack of growth in sorghum production in Africa is due to a number of reasons, including the use of traditional, and usually less efficient, methods of farming and lack of access to improved varieties with high yield potential. Studies have indicated that communal farmers would prefer varieties that show high pest and disease tolerance, drought tolerance, early maturity, palatability and storability (Nagaraj et al., 2013). Sorghum grain flavonoids play important roles in the uptake of the crop by communal farmers, due to their roles in conferring resistance to bird depredation and grain mold in developing sorghum grain, and in human and animal nutrition.

There are over 4000 known classes of structurally related flavonoids, and these include condensed tannins, anthocyanins, 3-deoxyanthocyanidins and flavanols (Patel, 2008). All sorghum varieties contain phenolic compounds in their grain, while only some contain condensed tannins (Dykes and Rooney, 2006). The types and quantities of flavonoids (flavonoid profile) that each variety accumulates in its grain determine how the variety would be utilised. Condensed tannins, and several other flavonoids, have important roles in human and animal nutrition and in the sorghum plant's physiology (Winkel-Shirley, 2001). However, some flavonoids, including condensed tannins, have both desirable nutritional as well as undesirable, anti-nutritional factors. Thus, a balance of flavonoids is required if sorghum is to fully reach its potential.

There are efforts to try and manipulate the flavonoid pathway in sorghum in order to identify and develop sorghum flavonoid pathway mutants that have desirable and nutritionally superior flavonoid profiles. Sorghum varieties for different applications would need to have flavonoid profiles specific to the application, e.g. flour milling and baking. Different applications would require the presence of different classes and quantities of flavonoids in sorghum grain. Such varieties can be obtained by screening a large number of mutants for those with desired flavonoid profiles.

It is important to understand how mutations within certain flavonoid pathway genes would affect the flavonoid profile in a given sorghum variety. The flavonoid pathway is a complicated pathway with over 10 known and unknown structural and regulatory genes (Winkel-Shirley, 2001). Studies on mutations at these different *loci* are yet to be carried out in sorghum, even though a

lot of such work has been carried out in other plants (von Wettstein, 2007; Abrahams et al., 2002; Debeaujon et al., 2001). Due to the complex nature of the flavonoid pathway, it is difficult to accurately predict how a mutation in a given flavonoid pathway gene would impact on the flavonoid pathway in sorghum. However, it is worthwhile to carry out studies that relate any genetic polymorphisms at different flavonoid pathway *loci* to different flavonoid profiles, with a view to understanding how mutations in a given flavonoid pathway gene would affect flavonoid accumulation.

In this study, we aimed at studying how sequence variations at the anthocyanidin synthase (*ANS*) gene *locus* relate to observed differences in condensed tannin profiles in different sorghum varieties. This gene, also called leucoanthocyanidin dioxygenase (*LDOX*) catalyses the conversion of leucoanthocyanidins into anthocyanidins and sits at the branch-point between the PA-specific branch and the anthocyanin-specific branch of the flavonoid pathway (Abrahams et al., 2002). Our interest is to find out if any sequence polymorphisms at this *locus* may explain observed differences in grain proanthocyanidin profiles. A number of flavonoid *ANS* gene mutants in Arabidopsis, namely *tt 11*, *tt17* and *tt 18* (*tds-4*) have been identified and characterised in Arabidopsis and alfalfa, both at the biochemical and molecular level (Misyura et al., 2012; Bowerman et al., 2012; Abrahams et al., 2002; Buer and Djordjevic, 2009). All these mutants were characterised by altered flavonoid profiles and deposition patterns in the grain.

MATERIALS AND METHODS

Sample selection

A total of 61 sorghum landraces were obtained from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) Research Station stationed at Bulawayo, Zimbabwe. Selection was based on availability and grain colour. Dwarf Wonder, a brown, inbred medium tannin line was the reference landrace in the study. Condensed tannins were assayed for in all 61 grain samples, while only 11 landraces, including Dwarf Wonder, were selected for *ANS* gene sequence studies.

DMACA screening assay for sorghum grain proanthocyanidins

The aldehyde dye, dimethylaminocinnamaldehyde (DMACA) reacts with proanthocyanidins (PAs) and their immediate precursors, flavan-3-ols and flavan-3,4-diols, producing a blue coloured complex. It does not react with anthocyanidins and anthocyanins, making it suitable for use in tracing condensed tannin biosynthetic activity in fruits, seeds and tissues (Bogs et al., 2005; Abrahams et

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Table 1. Expected *ANS* gene PCR amplicon restriction profiles.

Restriction enzyme	Frequency of cuts	Cut positions	Expected sizes of restriction products (bp)
<i>Hinfl</i>	1	1218	52, 1218
<i>Mbol</i>	4	167, 740, 929, 1231	39, 167, 189, 302, 573
<i>TaqI</i>	6	312, 576, 774, 1059, 1125	48, 54, 69, 77, 138, 344, 540
<i>AluI</i>	5	77, 125, 179, 719, 788, 926	66, 145, 198, 264, 285, 312

al., 2002). In this study, DMACA was used to screen 61 sorghum landraces on the basis of PA accumulation in the grain samples available. The DMACA assay was performed as described by Abrahams et al. (2002), with minor modifications. About 20 healthy, dry seeds from each landrace were soaked in DMACA solution (2% [w/v] DMACA in 3M HCl/ 50% methanol (v/v)) in a microcentrifuge tube for seven days, followed by washing thrice with 70% ethanol. Washed seeds were dried and then analysed for the development of a blue/blue-black colour, indicating the presence of PAs and/or their precursors.

Quantification of proanthocyanidins in sorghum grain samples

Sorghum grain PAs were quantified in a grain total flavonoid extract. To obtain the extract, exactly 1.0 g of each sorghum grain sample was ground to a fine meal using a mortar and pestle, and then total flavonoids in the meal were extracted in 10 ml of 1% (v/v) HCl in methanol, with shaking. After extraction, the mixtures were centrifuged at 5000 rpm for 30 min and the clear supernatants refrigerated at 4°C until they were analysed. Total condensed tannins in each extract were then quantified by the butanol-HCl assay as described by Hagerman (2000). All samples were analysed in triplicate, and the results expressed as absorbance units at 550 nm per grain of sample. This assay is based on the acid catalysed hydrolysis of proanthocyanidins in a mineral acid to produce pink/red coloured anthocyanidins that absorb maximally at 550 nm. An iron reagent is normally included in the reagent to enhance colour formation and stability.

Sorghum genomic DNA extraction

Sorghum genomic DNA was extracted from the frozen leaves of 3-week old etiolated sorghum seedlings. The frozen tissue was ground to a fine powder using a chilled mortar and pestle. Genomic DNA was then extracted from the crushed material using the ZR Plant/Seed DNA Miniprep kit (Zymo-Research, Pretoria, South Africa), following the manufacturer's instructions. A 10 µl aliquot of the extracted DNA was run on a 0.8% agarose gel to determine DNA quality and quantity.

PCR amplification of the sorghum *ANS* gene in selected landraces

The *ANS* gene primers used in this study were designed for use in *Arabidopsis* complementation studies (Liu et al., 2010). The primer sequences are *ANS* 1 (5' - CGCGGCGATAGTGAATTAGT) and *ANS* 2 (5' - ATTGATGGATGGACGAAAGC). PCR amplification was performed in 50 µl reactions with the Phire Hot Start II DNA Polymerase (Thermo Scientific Inc., Pretoria, South Africa) using the following program: initial denaturation at 98°C (30 s); 30 cycles consisting of denaturation at 98°C (5 s), annealing at 55°C (5 s), and extension at 72°C (30 s); and then a final extension at 72°C (60 s), in a GENEAMP® PCR System 9700 thermocycler machine (Life

Technologies). After cycling, a 10 µl aliquot of each reaction was electrophoresed on a 1% agarose gel for 1 h and PCR products were confirmed by viewing the gel under ultraviolet irradiation.

RFLP analysis of *ANS* gene amplicons

Restriction digestion analysis was performed directly on PCR amplicons as a preliminary screen for polymorphisms at the *ANS* gene locus in the selected landraces. The PCR amplicons were digested using the restriction enzymes *Hinfl*, *Mbol*, *TaqI* and *AluI* (Thermo Scientific Inc, Pretoria, South Africa), following the manufacturer's instructions. Enzyme selection was based on a virtual digest of the *ANS* gene sequence using several enzymes. Only those enzymes producing a sufficient number of well-spaced cuts were selected for use (Table 1).

RESULTS

Variation in seed PA content

The sorghum landraces used in this study showed significant variability in their seed proanthocyanidin profiles. Both qualitative and quantitative assays for proanthocyanidins were carried out, using the DMACA stain assay and the butanol-HCl assay, respectively.

Qualitative assay for PAs in sorghum grain samples

The DMACA assay was used to characterise available sorghum landraces on the basis of the presence or absence of PAs in their grain. It is a qualitative assay that detects PAs or their immediate precursors. With the DMACA assay, 89% of the brown lines, 4% of the red lines and none of the white lines tested positive for PAs and/or their precursors (Figure 1). A few samples gave inconclusive results (Figure 2). Being a qualitative assay, the DMACA assay was used in combination with the butanol-HCl, a quantitative assay for PAs in order to get more insight about each sorghum landrace.

Quantitative assay for PAs in sorghum grain samples

The butanol-HCl assay is based on the acid-catalysed depolymerisation of PAs into anthocyanidins. Due to the absence of a tannin standard, butanol-HCl assay results were expressed as absorbance units at 550 nm per gram of sample (AU@550 nm/g of sample). All 27 brown landraces had detectable grain PA levels, except for only

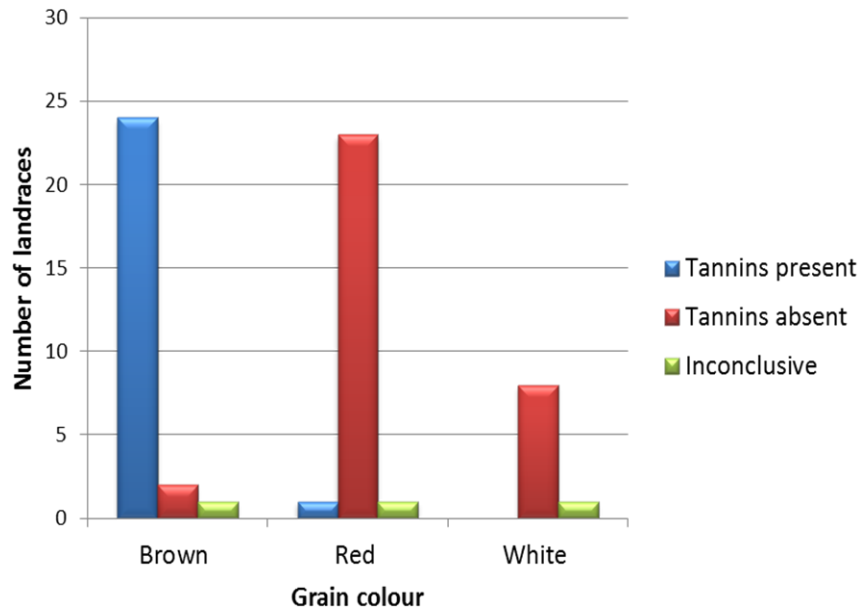


Figure 1. Number of tannin positive, negative and inconclusive sorghum landraces as determined by the DMACA assay.



Figure 2. Sorghum seed stained with DMACA dye. [+] - DMACA positive; [-] - DMACA negative; [+/-] - inconclusive result.

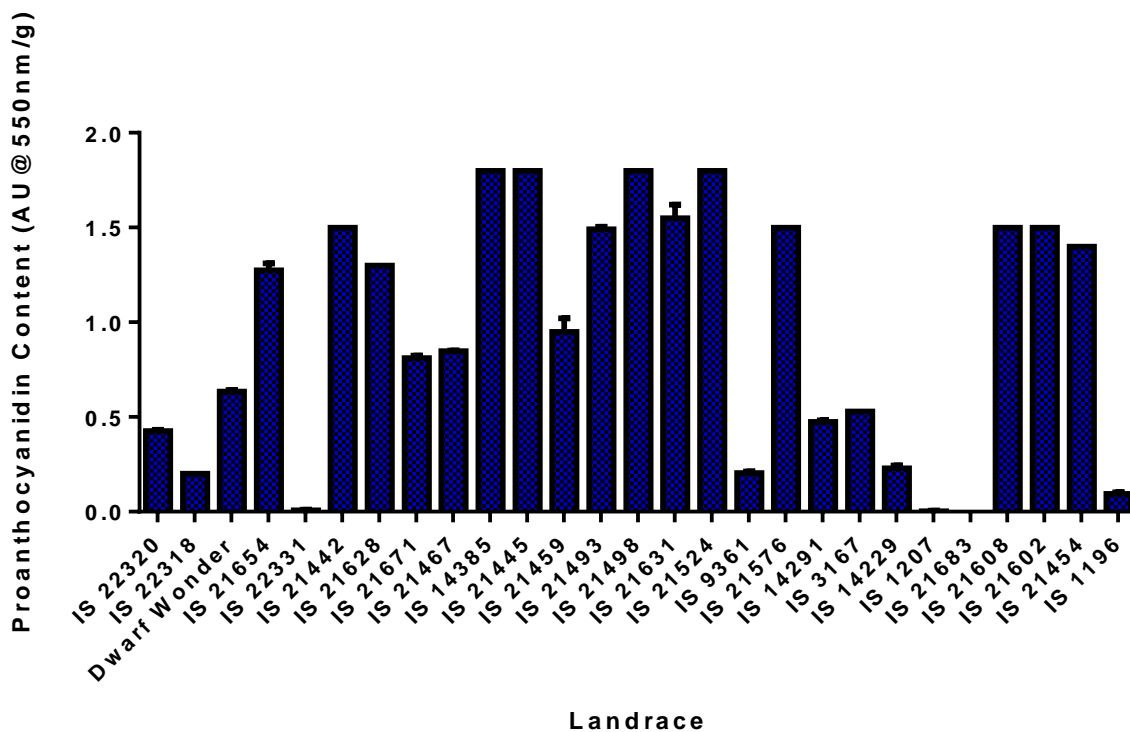


Figure 3. Levels of proanthocyanidins in brown sorghum grain samples.

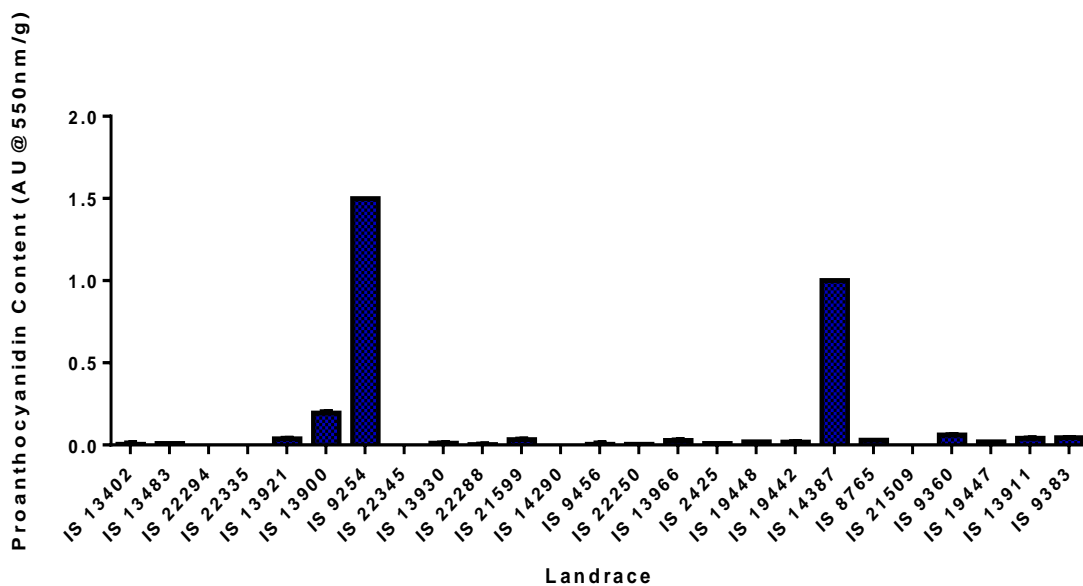


Figure 4. Levels of proanthocyanidins in red sorghum grain samples.

the three landraces IS 22331, IS 1207 and IS 21683 (Figure 3). The reference landrace, Dwarf Wonder, is a medium tannin line, thus in this study all landraces showing lower tannin levels were designated low tannin, while those accumulating higher quantities were desig-

nated high tannin lines. All but three red lines assayed for did not accumulate grain PAs. Of the three that did, IS 13900 accumulated very low levels while IS 14387 and IS 9254 accumulated high quantities (Figure 4). Meanwhile, all white lines tested PA negative by the butanol-

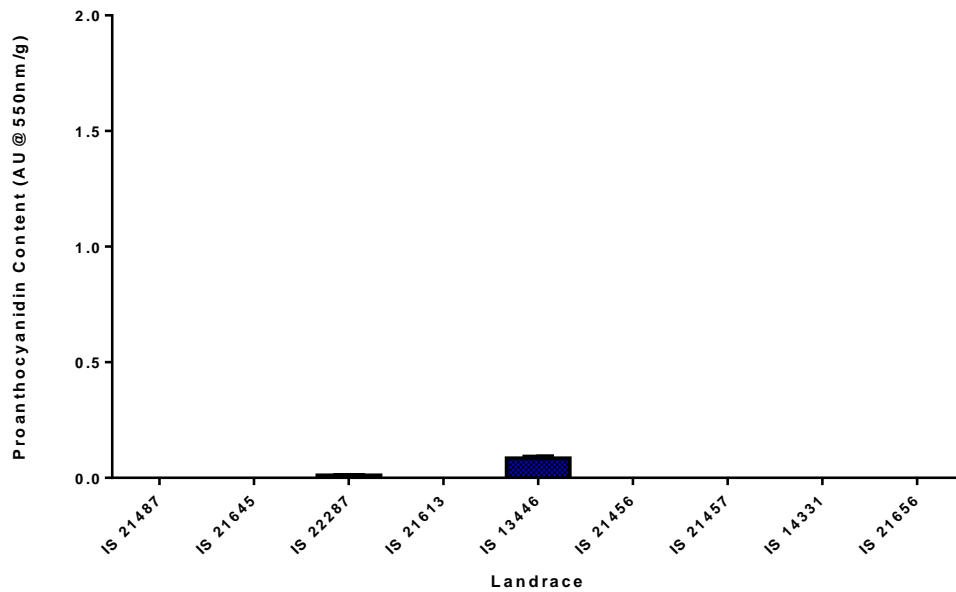


Figure 5. Levels of proanthocyanidins in white sorghum grain samples.

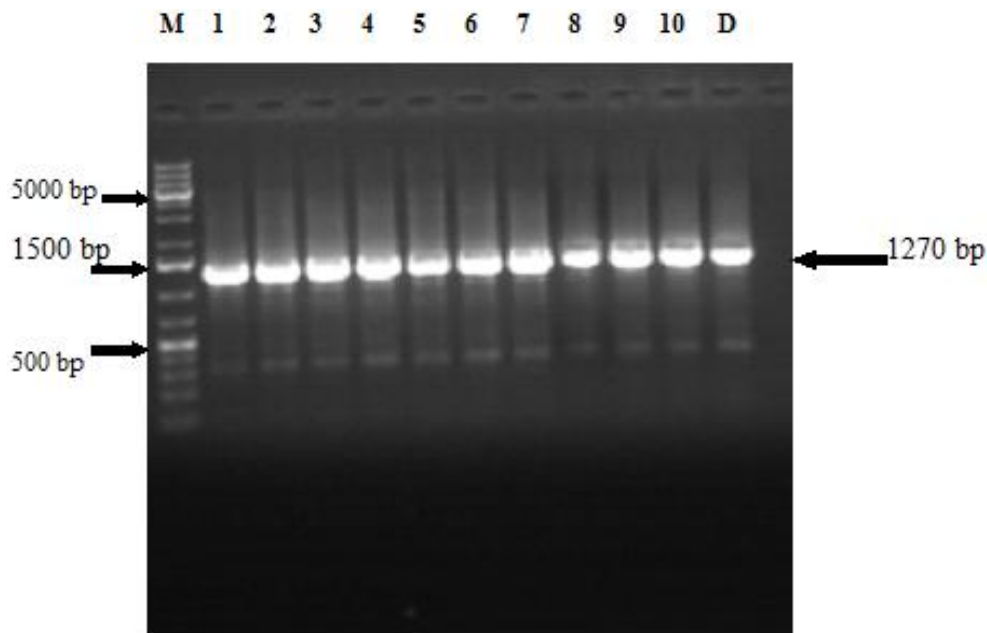


Figure 6. PCR amplification of the sorghum *ANS* gene. Lane marked M contains a 1 kb DNA ladder, lanes 1 - 10 contain the samples as follows: 1 - IS 22320; 2 - IS 13402; 3 - IS 21487; 4 - IS 22318; 5 - IS 13483; 6 - IS 22294; 7 - IS 1196; 8 - IS 22335; 9 - IS 21456; 10 - IS 21457; D - dwarf wonder.

HCl assay (Figure 5).

PCR amplification of the sorghum *ANS* gene

Good quality *ANS* gene PCR amplification products were

obtained in all samples (Figure 6). The *ANS* amplicon obtained was 1270 bp in size, as expected. This includes the whole of the gene’s coding sequence (CDS) as well as 33 and 36 bp of the 5’ and 3’ UTR, respectively. The *ANS* gene has been cloned in sorghum and the sequence filed under accession number XM_002451291

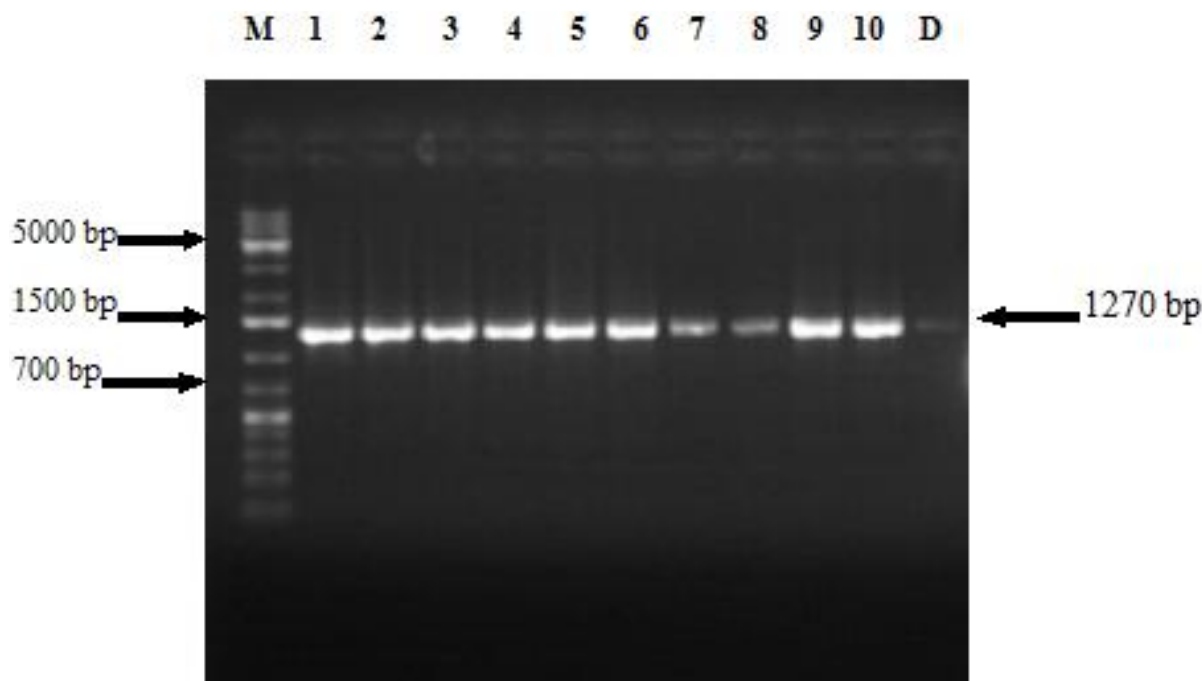


Figure 7. Sorghum *ANS* gene PCR product recovered from agarose gel. Lane marked M contains a 1 kb DNA ladder, and other lanes: 1 - IS 22320; 2 - IS 13402; 3 - IS 21487; 4 - IS 22318; 5- IS 13483; 6- IS 22294; 7- IS 1196; 8- IS 22335; 9- IS 21456; 10- IS 21457; D- dwarf wonder.

(www.ncbi.nlm.nih.gov). It is important to produce amplicons that include the whole of the CDS if any determinations involving how changes in a gene may affect its functionality are to be made. Also, high quality PCR amplicons were recovered and purified using the Zymoclean™ Gel DNA Recovery kit (Zymo-Research, Pretoria, South Africa). Only 2.5 µl of the recovered and purified PCR amplicons were electrophoresed on 1% agarose gel, producing good images (Figure 7).

***ANS* gene locus sequence polymorphism analysis**

The aim of this project was to investigate the relationship between differences in seed PA profiles in local sorghum landraces and sequence variations at a key flavonoid pathway locus, the *anthocyanidin synthase* (*ANS*) gene. This gene is vital for the biosynthesis of condensed tannins in sorghum. The method used in this study is the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. This combines the DNA amplifying prowess of the PCR to a specific gene and the specific cleavage of the DNA amplicons by the action of a group of nucleases called restriction endonucleases.

Restriction digestion analysis of DNA is used to detect polymorphisms that cause the disappearance of existing, or emergence of new, restriction enzyme sites. In this

study, the following restriction enzymes were used: *Hinf*I, *Mbo*I, *Taq*I and *Alu*I. The enzymes *Mbo*I, *Taq*I and *Alu*I are all 4-base cutters, while *Hinf*I is a 5-base cutter. Each *ANS* gene fragment was cut sixteen times by all the enzymes combined. High resolution restriction data was generated for the enzymes used. However, with all these restriction enzymes, no differences in restriction patterns were detected, implying that the restriction maps for the amplicons under study using the available restriction enzymes were the same (Figures 8, 9 and 10).

DISCUSSION

Selecting or breeding for flavonoids in sorghum is a fairly unexplored area. Unlike in other plants like *Medicago truncatula* (alfalfa), *Camellia sinensis* (the tea tree) and *Arabidopsis thaliana* where the flavonoid pathway is fully understood, a lot is still unknown in sorghum. This study is part of efforts aimed at the identification and characterisation of flavonoid pathway mutants in sorghum. In this study, 61 sorghum landraces which are part of the sorghum genebank, made up of Zimbabwean and other Southern African sorghum lines were used. The study showed that 44% of these landraces contained quantifiable condensed tannins in their grain. Another study which focused on the use of RAPD markers to determine the genetic relatedness of Zimbabwean sorghum varieties with different seed proanthocyanidin

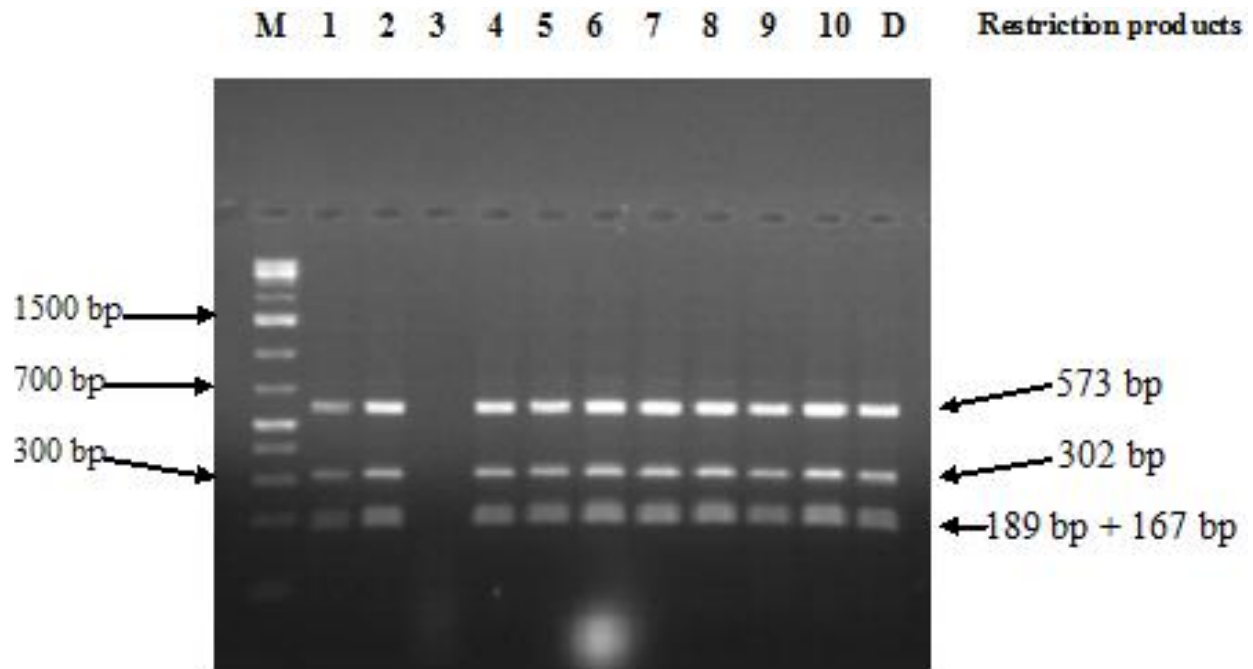


Figure 8. Sorghum ANS gene PCR product restricted by the enzyme *Mbo*I. Lane marked M contain a 1 kb DNA ladder, lanes 1 - 10 contain the samples as follows: 1 - IS 22320; 2 - IS 13402; 3 - IS 21487; 4 - IS 22318; 5 - IS 13483; 6 - IS 22294; 7 - IS 1196; 8 - IS 22335; 9 - IS 21456; 10 - IS 21457; D - dwarf wonder.

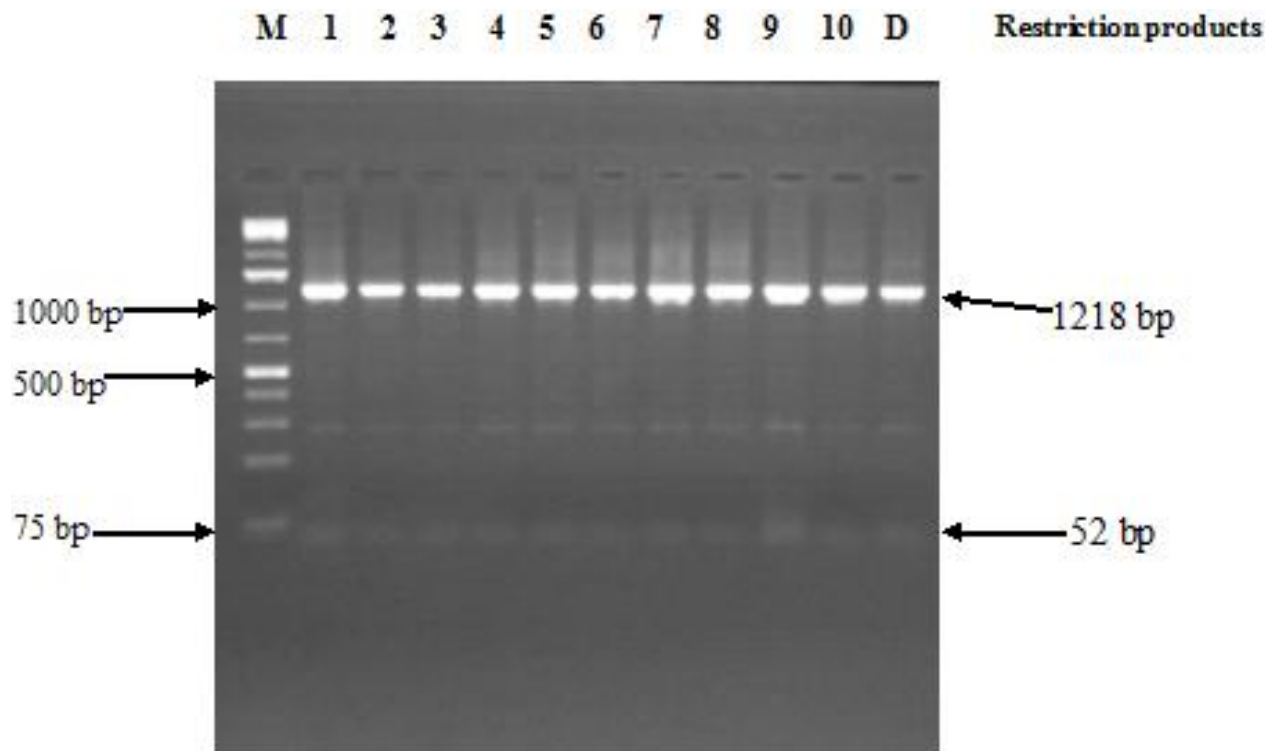


Figure 9. Sorghum ANS gene PCR product restricted by the enzyme *Hinf*I. Lane M contains a 1 kb DNA ladder, while: 1 - IS 22320; 2 - IS 13402; 3 - IS 21487; 4 - IS 22318; 5 - IS 13483; 6 - IS 22294; 7 - IS 1196; 8 - IS 22335; 9 - IS 21456; 10 - IS 21457; D - dwarf wonder.

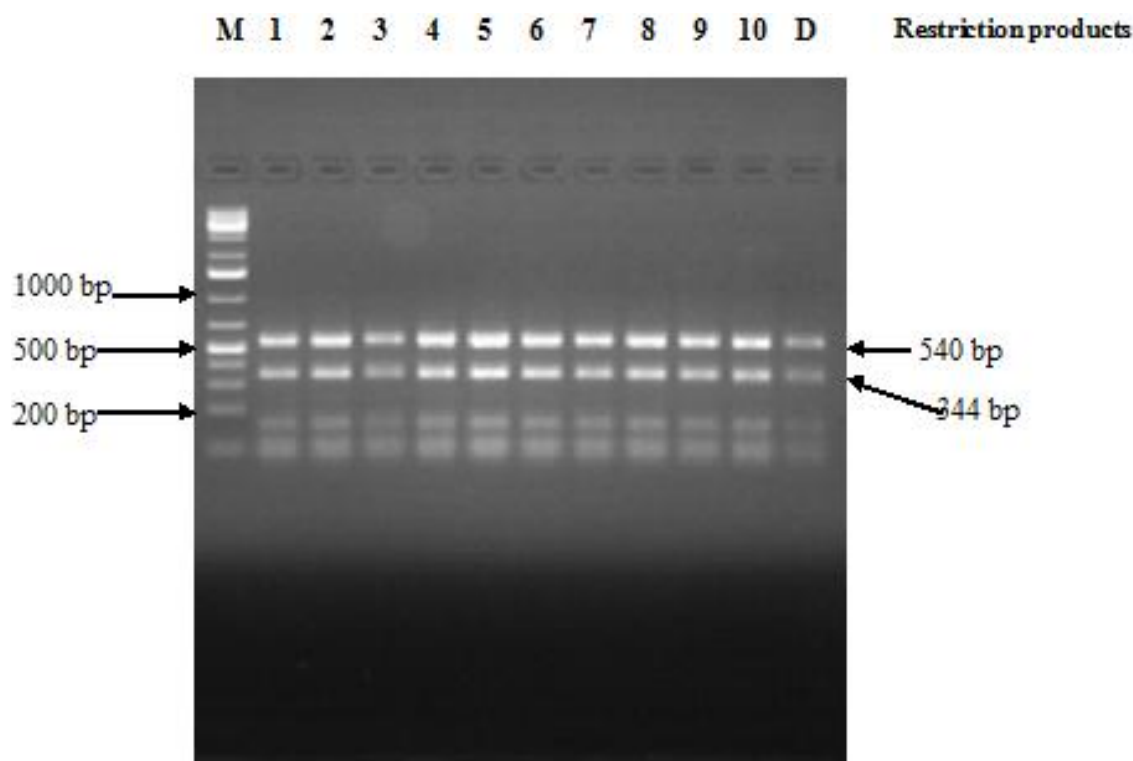


Figure 10. Sorghum *ANS* gene PCR product restricted by the enzyme *TaqI*. Lane marked M contain a 1 kb DNA ladder, lanes 1 - 10 contain the samples as follows: 1 - IS 22320; 2 - IS 13402; 3 - IS 21487; 4 - IS 22318; 5 - IS 13483; 6 - IS 22294; 7 - IS 1196; 8 - IS 22335; 9 - IS 21456; 10 - IS 21457; D - dwarf wonder.

levels concluded that 35% of the landraces used in the study had detectable proanthocyanidins in their grain (Dhlamini and Niang-Sithole, 2014). These results agree with the results obtained in this study, even though different batches of local landraces were used.

The DMACA assay was used not only as a preliminary screen for PAs in the selected sorghum landraces, but also to reveal those sorghum lines that have an altered PA distribution pattern. In other plants, altered seed DMACA staining patterns have been successfully used to identify flavonoid pathway mutants. In studies in *Arabidopsis*, several transparent testa (*tt*) and tannin deficient seed (*tds*) mutant lines were identified through the use of DMACA staining (Abrahams *et al.*, 2002). Further studies on these lines indicated that they were indeed flavonoid pathway mutants at several *loci* vital for PA biosynthesis and accumulation (Abrahams *et al.*, 2002). Mutations in some flavonoid pathway genes, e.g. *ANS* and *TT12*, have been shown to not only affect presence and quantities of certain flavonoids, but also final PA deposition patterns (Abrahams *et al.*, 2002; Debeaujon *et al.*, 2001).

Using the DMACA assay, five landraces showing unusual staining patterns were identified. These are IS 22331 (brown non-tannin), IS 13446 (chalky-white), IS

9254 (red high-tannin), IS 13900 (red low-tannin) and IS 9361 (brown high-tannin). All showed a patchy appearance after DMACA staining, typical of some flavonoid pathway mutants. The butanol-HCl assay was used to further characterise these lines, showing in the process that of those lines showing unusual DMACA results; IS 22331 (pale brown) and IS 13446 (chalky white) did not contain any seed PAs, while the 2 red lines IS 9254 and IS 13900 contained PAs. The black and white DMACA colouration given in IS 13446 is typical with many other chalky white grain samples. The chalky-white appearance of the grain of some sorghum varieties is due to either the presence of starch grains in the mesocarp or the presence of a pigmented testa layer containing insoluble tannins underneath a colourless pericarp (Rooney and Murty, 1982). A sectioning examination has shown IS 13446 to indeed possess a pigmented testa underneath a clear pericarp.

From this study, it can be concluded that most red (92%) and all white (100%) sorghum grain samples tested negative for PAs, while 89% of the brown lines tested positive for grain PAs (Figures 1, 3, 4 and 5). Many white sorghum varieties are mutated at *loci* higher than the flavonoid pathway at or before points when coloured flavonoids begin to appear in the grain, thus

explaining why they stain negative for tannins and do not accumulate any assayable tannins. In tannin sorghum, tannins accumulate either in the pigmented testa only (type II) or in both the pigmented testa and pericarp (type III), meaning the presence of a pigmented testa is vital for tannin accumulation (Earp et al., 2004).

The DMACA assay was used in combination with the butanol-HCl assay. This is because use of the DMACA assay alone in the biochemical characterisation of tannin-producing mutants in sorghum is insufficient. Firstly, DMACA not only reacts with condensed tannins, but also with their immediate precursors, that is, flavan-3-ols and flavan-3,4-diols (Abrahams et al., 2002). This means any mutant expressing any one of the precursors will stain positive, giving a false positive result for the presence of tannins when in actual fact the mutant will be accumulating only the precursors. Secondly, studies have shown that those mutants that accumulate minute quantities of the soluble PA precursors tend to leach those precursors into the DMACA staining solution before the reaction is complete, leading to a false negative result (Koupai-Abyazani et al., 1993). As a consequence, the butanol-HCl assay was used to confirm the results of the DMACA assay.

Even though the flavonoid pathway has several structural and regulatory genes, any mutation on any one of these many *loci* has the potential effect of altering the flavonoid profile of the mutant line carrying the mutation. It is important to identify such mutations and establish their exact effects on the flavonoid pathway and on flavonoid profiles. Such studies have been carried out in other plants like *Arabidopsis*, alfalfa and barley (Misyura et al., 2012; Abrahams et al., 2002; Bowerman et al., 2012; Buer and Djordjevic, 2009; von Wettstein 2007). However, similar studies are yet to be carried out in sorghum, hence this study is aimed at attempting to establish a link between sequence polymorphisms at the *ANS* gene *locus* and differences in proanthocyanidin profiles in sorghum.

Sequence polymorphisms at the *ANS locus* were determined using a combination of PCR and restriction digestion analysis (PCR-RFLP). Each *ANS* gene fragment was cut sixteen times by all the enzymes combined, translating to a DNA fragment of 65 bp in length. This covers the actual position of the cut and the other bases that make up the restriction site. Any mutation within this 65 bp region of the amplicon under study would have been detected by this method. However, with all these restriction enzymes no differences in restriction patterns were detected (Figures 8, 9 and 10).

This potentially means either of two things, firstly that there are no polymorphisms at this *locus* in the landraces under study, and as a result any observed differences in PA profiles may be due to mutations at other *loci* or due to environmental factors, or secondly that the RFLP

technique probably missed some potential SNPs due to its inherent shortcomings.

The RFLP technique has one major handicap when used in the detection of sequence variations among similar sequences. The method targets only those mutations that occur within restriction sites and ignores the rest of the gene. In this case, this method only focused on a 65-bp region in a PCR amplicon, 1270 bp in size. This translates to only 5% of the gene. Inasmuch as the method may be a very useful and cheap rapid screening tool for DNA mutations, it suffers from low coverage. The method can however be made more effective if more restriction endonucleases are used. This way the portion of the gene directly screened for any mutations significantly increased.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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