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Biodegradation of Antinutritional Factors in Whole Leaves of *Enterolobium cyclocarpum* by *Aspergillus niger* Using Solid State Fermentation

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Abstract

In the tropics, native pastures and crop residues constitute the major feed resources for ruminants. There are available seasonally and of low quality. They contribute to the limitation of ruminant production which is predominantly managed by small holders. *Enterolobium cyclocarpum* (EC) is a hardy multipurpose tree (MPT) that has potential as supplement for ruminant feeding. However, anti nutritional factors (ANFs) contained in the leaves of EC could have negative effects on its nutritive value. Fungal biodegradation by solid substrate fermentation (SSF) of four ANFs namely: tannin, saponin, phytic acid and oxalate in leaves of EC was investigated. *Aspergillus niger* was used to inoculate whole leave samples of the EC. The levels of ANFs in the leaf substrates were estimated after 0, 7, 14, 21 and 28 days. Inoculation of the whole leaf samples of the EC with *A. niger* caused reductions in the ANFs. Percentage reduction in EC was 42.7%, 28.7%, 25.5%, and 26.5% for tannins, saponins, phytic and oxalate respectively. At the same time, it significantly ($p<0.05$) increased crude protein levels in the leaves of EC up to day 14 beyond which there was no further significant increase. The results of ether extract, acid detergent fibre and neutral detergent fibre (NDF) showed decrease as period of fermentation increased. It could be concluded from this study that the solid state fermentation of whole leaves of *E. cyclocarpum* by *A. niger* was sufficient to degrade antinutritional factors and caused significant improvement in the substrate nutrient composition. The reduction in antinutritional factors: tannins, saponins, phytic acid and oxalate, protein enrichment and simultaneous degradation of fibrous fractions (ADF and NDF) and ether extract showed the importance of fungal fermentation in improving the nutritive value of MPTs.

Keywords: *Enterolobium cyclocarpum*, fermentation, chemical composition, antinutritional factors

1. Introduction

Enterolobium cyclocarpum (Jacq.) Griseb is a tropical multipurpose tree species, whose foliage has great potential as a ruminant feed resource. The tree is fast growing with no widespread disease and pest problem (Ezenwa, 1999). Biomass yield is high, the foliage is rich in protein and other nutrients (Arigbede et al., 1997; Babayemi et al., 2004) and as a leguminous multipurpose plant, it has the potential of fixing atmospheric nitrogen in the soil (Idowu et al., 2013). The leaves are used to feed sheep (Navas Camacho et al., 1993) and serve as fodder (Carranza Montano et al., 2003). However, in the southwest of Nigeria, *E. cyclocarpum* foliage has not been accepted by sheep, goats and cattle possibly due to the presence of antinutritional factors (Babayemi, 2006; Idowu et al., 2013) which makes them low in palatability (Arigbede et al., 1997).

Tannins and saponins have marked effects on palatability, acceptance and digestibility and are among notable anti-nutritional factors (ANFs) of multipurpose trees and shrubs (MPTS). Tannins are usually associated to a decrease in palatability and consequently discourage grazing (Ngwa et al., 2003). They produce an astringent taste, which alters palatability of diets, thus reducing overall feed intake (Patra & Saxena, 2010). Tannins owe their astringent action to the fact that they precipitate protein and render them resistant to attack by proteolytic

enzymes, internally they form a pellicle of coagulated protein over the lining of the alimentary tract (Hari Babu & Savithramma, 2014). High tannin levels reduce preference level of plants by cattle, sheep and goats (Perevolotsky et al., 2003). Animal feed selection depends heavily on the palatability of the feed (Elga lamy et al., 2011). Saponins are naturally occurring triterpenes or steroid glycosides which exhibit surface active properties and are found in a wide variety of plants (Enujingha et al., 2014). They are characterized by bitter taste, foamy characteristics and some produce adverse effects (Sanusi et al., 2013). They affect fermentation and methanogenesis in ruminants (Hess et al., 2003) and have haemolytic effect on red blood cells (Prohp & Onoagbe, 2012). Their ability to form non- absorbable saponin-vitamin complexes has been suggested as a possible mechanism for producing saponin-induced growth depression (Enujiugha et al., 2014). Other anti-nutritional factors such as phytates and oxalates limit utilization of nutrients in MPTS. They form complexes with minerals making them unavailable to livestock. In spite of the negative effects, biological treatments offer an economic and effective option for improving the nutritive value of ANFs containing materials.

Solid state fermentation using fungal cultures such as *Aspergilli* can improve the nutritive value of feedstuffs (Mathivanan et al., 2006; Yamamoto et al., 2007). In solid state fermentation process, the moist solid substrate is used and without free-flowing water, and thus the adherence and penetration of microorganisms are clearly associated with the physical properties of the substrate such as the crystalline or amorphous nature, the accessible area, surface area ,porosity, particle size, etc (Junjun et al., 2014). *Aspergillus niger* was effective in lowering the level of antinutrients in *Jatropha curcas* kernel cake to levels that do not elicit toxic response in the West African Dwarf goats (Belewu & Sam, 2010). The fungus is a eukaryotic microorganism belonging to a group of filamentous fungi, which are naturally capable of secreting large amounts of protein and metabolites (Meyer et al., 2010). A preliminary study on ground leaves of *E. cyclocarpum* using *A. niger* showed marked decreases in tannin, saponin, phytic acid and oxalate (Ayuk et al., 2004). In this study, the effect of *A. niger* on anti-nutritional factors in larger amounts (1 kg) of whole *E. cyclocarpum* leaves was investigated.

2. Materials and Methods

2.1 Preparation of Leaves

The leaves of *E. cyclocarpum* were harvested from an established arboretum in Wasimi, South Western Nigeria. Three kilogram of whole leaf samples was autoclaved using Hearson Giant autoclave, immediately after harvest in polythene sacks at 121 ^{o}C for 15 minutes. One kilogram of autoclaved sample was weighed into sterile nylon bags, allowed to cool, ready for inoculation.

2.2 Preparation of Inoculum

A. niger originally isolated at the Department of Yam pathology, International Institute of Tropical Agriculture, was maintained on potato dextrose agar (PDA) slants. Serial dilutions were made to obtain spore or cells per ml (Ayuk et al., 2004). A loopful of *A. niger* was harvested into sterile water for serial dilution. Potato dextrose agar (PDA) media were then seeded with the *A. niger* suspension and incubated at 30 ^{o}C for 72 hours. The colony forming units were counted and a loopful gave 2×10^{7} cfu/g substrate.

Each autoclaved cold 1kg sample was inoculated with three -day -old one loopful *A. niger* grown in 100 ml potato dextrose broth. Inoculated samples were tied in polythene nylon bags and incubated at 30 ^{o}C. The samples were run in triplicates for each incubation period and five periods (0, 7, 14, 21 and 28) were used for the study. Samples were turned once daily to ensure mixing of samples with inoculums, and for aeration. At the end of each incubation period, samples were oven dried at 60 ^{o}C, thereafter, chemical composition, tannin, saponin, phytate and oxalate were determined.

2.3 Chemical Analysis

Harvested leaves of *E. cyclocarpum* were used for determination of chemical composition. The oven dried samples were ground to (A.O.A.C, 1990) pass through 1mm screen for chemical analysis. The Kjeldahl procedure was used to determine total nitrogen (N) and crude protein was calculated by multiplying N by 6.25(Nx6.25). Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined (Goering and Van Soest, 1970).

2.4 Determination of Anti-Nutritional Factors

Tannin was determined using methods of Price et al. (1978). Methods for determining saponin, phytic acid and oxalate were as described by Brunner (1984).

2.5 Statistical Analysis

Data were analyzed using the analyses of variance procedures of SAS (1988). Results were expressed as means of three replicates. Significant differences were compared using the Duncan Multiple Range Test at $P<0.05$.

3. Results

3.1 Effects of A. niger on Anti-Nutritional Factors of E. cyclocarpum

Variations of antinutritional factors (tannin, saponin, phytic acid and oxalate) are presented in Table 1. The *A. niger* caused significant reductions in all ANFs estimated.

Minimal levels of tannin, saponin, phytic acid and oxalate were obtained after 28 days fermentation. The effect of each anti-nutrient differed as reflected in changes over five incubation periods.

Table 1. Changes in tannin, saponin, phytic acid and oxalate (mg/g^{-1}DM) of *E. cyclocarpum* following solid substrate fermentation with *A. niger*

Time(days)	T	S	PA	O
0	2.18 ± 0.23^{a}	27.65 ± 1.08^{a}	8.95 ± 0.23^{a}	12.13 ± 0.65^{a}
7	1.89 ± 0.01^{b}	24.42 ± 0.76^{b}	8.05 ± 0.13^{b}	11.33 ± 0.55^{ab}
14	1.70 ± 0.09^{bc}	22.01 ± 0.08^{c}	7.10 ± 0.20^{c}	10.47 ± 0.75^{bc}
21	1.50 ± 0.02^{c}	21.81 ± 0.30^{c}	6.95 ± 0.06^{cd}	9.94 ± 0.35^{c}
28	1.25 ± 0.05^{d}	19.69 ± 0.54^{d}	6.67 ± 0.19^{d}	8.92 ± 0.10^{d}
Mean	1.70	23.12	7.54	10.56

Means with different letters in each column are significantly ($P<0.05$) different.

3.2 Percent Decrease of Antinutritional Factors in the E. cyclocarpum by A. niger

Decrease in tannin (13.30 %) was most obvious compared to decrease in oxalate (6.60%) after 7d fermentation. All ANFs reduced after 7-28d fermentation (Table 2). The differences between saponin and phytic acid for 14(20.40% and 20.67%) and 21(21.22% and 22.35%) were not marked.

Table 2. Percentage decrease in Tannin, Saponin, Phytic acid and Oxalate (mg g^{-1} DM) in *E. cyclocarpum* leaves by *A.niger*

Time d	[a]T	S	PA	O
0	0	0	0	0
7	13.30	11.68	10.06	6.60
14	22.02	20.40	20.67	13.69
21	31.19	21.12	22.35	18.05
28	42.66^{1st}	28.79^{2nd}	25.39^{4th}	26.46^{3rd}

Levels of T, S, PA and O at day zero (O) were 2.18 mg/g, 27.65 mg/g, 8.95 mg/g respectively on dry weight basis.

(a) T: tannins; S: saponin, PA: phytic acid and O: oxalate.

3.3 Chemical Composition

Variation in chemical composition of *E. cyclocarpum* is presented in Table 3. Fermented *E. cyclocarpum* leaves at day 7 and 14 contained more crude protein (22.02 and 23.24% respectively). These values represent only 9.22 % and 13.98% increases, respectively. However, crude protein contents after 21 and 28 days were less (19.74 and 18.95%, respectively) than unfermented (day 0), representing 1.25% and 5.20% decreases in protein respectively.

Table 3. Changes in chemical composition of *E. cyclocarpum* following solid state fermentation with *A. niger*

Time(days)	CP	EE	ADF	NDF
0	19.99 ± 0.083^{b}	0.88 ± 0.74^{a}	24.65 ± 1.65^{a}	70.20 ± 3.15^{a}
7	22.02 ± 2.84^{a}	0.86 ± 0.48^{a}	22.88 ± 0.64^{ab}	54.95 ± 1.74^{b}
14	23.24 ± 1.87^{a}	0.76 ± 0.13^{ab}	22.05 ± 0.97^{b}	53.13 ± 2.28^{b}
21	19.74 ± 0.96^{b}	0.66 ± 0.34^{bc}	21.78 ± 1.30^{b}	52.92 ± 1.24^{b}
28	18.25 ± 1.35^{b}	0.55 ± 0.82^{c}	21.11 ± 1.75^{b}	52.32 ± 0.67^{b}
Mean	20.79	0.74	22.49	56.70

4. Discussion

4.1 Changes in Antinutritional Factors in E. cyclocarpum Leaves

The results showed the ability of *A. niger* to secrete enzymes capable of degrading tannins, saponins, phytic acid and oxalates. Jacqueline and Visser (1996) observed that decrease in various toxins levels could be due to the production of various enzymes during the vegetative and reproductive phases of the fungi. The synergetic enzymatic effects of mixed culture of *Absidia spinosa* and *Mucor rouxii* have been shown to lower antinutritional factors to bearable level (Sanusi et al., 2013). The significant decrease in tannins, saponins, phytic acid and oxalates confirms reports of Ayuk et al. (2004) for ground leaves of *E. cyclocarpum.* The reduction in some antinutritional factors such as phytate, saponin, and tannin when treated with *Rhizopus oligosporus* has been reported (Belewu, 2008). Belewu and Sam (2010) showed that *A. niger* was effective in lowering the level of antinutrients in *Jatropha curcas* kernel cake to levels that do not elicit toxic response in the West African Dwarf goats. Chipkar and Demuyakor (2012) also reported decrease in the crude phorbol ester and phytic acid levels in all *Jatropha curcas* fermented seed/kernels.

Tannin is an antinutritional factor found in *E. cyclocarpum*. It is known to be bitter and form high polyphenol complex with protein thereby making it unavailable in the diet (Nupo et al., 2013). The protein complexes are by-passed in the rumen and are digested at the lower segment of the gastrointestinal tract (Sanusi et al., 2013). They have antimicrobial activities and are known to inhibit the activities of digestive enzymes and nutritional effects of tannins are mainly related to their interaction with protein (Nwhocha et al., 2014). The percent reduction in tannin after 28 days of fermentation for *E. cyclocarpum* was between 13.30-42.66% and was lower than that of ground samples of *E. cyclocarpum* (30.47-69.96%) in solid state fermentation using *A. niger*. The fact that whole leaf samples were used in the present study could be responsible for lower percent reductions of tannins. Intact cell wall components might have hindered degradation by hampering mycelial penetration. Two mixed strains of *A. niger* have been shown to degrade tannin and concentration decreased by 66.1% (Sun et al., 2009).

The presence of saponins could confer bitter and astringent characteristics on the material. Saponins are known astringent components of food materials and this could lead to reduced palatability (Enujiugha et al., 2014). They also have defaunating properties. In this study, presence of saponins did not prevent growth of *A. niger* rather saponins were degraded. However, percent reductions of saponin herein, were lower than those obtained earlier in solid state fermentation with *A. niger* using ground leaves (Ayuk et al., 2004).

In phytic acid, percent reductions (10.05-25.47%) were lower than in tannin and saponins after 28days of fermentation. The values were also lower than those obtained from previous study with *A. niger* using ground leaves. Phytic acid is a strong chelator and is the principal storage form of phosphorous and other minerals and trace minerals in many plant tissues (Santos, 2011). The unique phytate ion structure, with 12 replaceable protons and high density of negatively charged phosphate groups (responsible for its characteristic properties), allows it to form stable complexes with multivalent cations (Dost & Tokul, 2006). Phytase is an acid phosphohydrolase that catalyses the hydrolysis of phosphate from phytic acid to inorganic phosphate and myo-inositol phosphate derivatives (Roopesh et al., 2006). The genus Aspergillus (*A. niger* in particular) continues to be favored for production of phytase, other enzymes and organic acids (Santos, 2011). This is not only due to its GRAS (Generally recognized as safe) status, but also due to its great secretory potential and in depth knowledge regarding its growth cultivation (Shivana & Govindarajulu, 2009). Oxalate is found in nature in some plants in the form of soluble and insoluble salts and as oxalic acid, the simplest organic acid (Ilelaboye et al., 2013). It affects calcium and magnesium metabolism and reacts with protein to form complexes which have

an inhibitory effect on peptic digestion (Akande et al., 2010). Oxalate reductions after 7-28 days of fermentation in *E. cyclocarpum* (6.60-25.47%) were similar to reductions in phytic acid but lower than obtained previously using ground leaves (Ayuk et al., 2004).

During SSF process, the availability of surface area plays an important role in microbial attachment, mass transfer, subsequent microbial growth and metabolite production, and is most associated with the particle size of the substrate (Prakasham, 2006). Lower effects of *A. niger* on the antinutritional factors compared to previous studies could arise from reduced surface area and difficulty in penetrating intact leaves. In general, bigger particles, with less surface area, cause a trend towards poorer accessibility to the microorganism, but inter-particular porosity is greater, while smaller particles, with larger surface area, are preferred for better accessibility to the microorganism, but the porosity is less (Ghuan, 2013). Furthermore, smaller particles are favorable for anaerobic fermentation, due to forming a higher solid medium density and a less void fraction (Ghuan, 2013). But, too small particles may diminish the heat transfer and carbon dioxide exchange rates because of medium compaction (Molaverdi et al., 2013).

Fermenting foods can make poorly digested, reactive foods into health giving foods (Hassan et al., 2014). The antinutritional factors measured herein, were reduced to a level that could impact some qualities of ruminal undegradable protein thereby enhancing the utilization of its protein (Sanusi et al., 2013).

4.2 Chemical Composition

Solid state fermentation (SSF) represents a technological alternative for processing a great variety of legumes and/or cereals to improve their nutritional quality and to obtain edible products with palatable sensorial characteristics (Reyes-Moreno, 2004). *A. niger* significantly ($P < 0.05$) increased crude protein in leaves of *E. cyclocarpum* up to day 14 beyond which there was no further increase (Table 3). The results are lower than those obtained by Belewu and Sam (2010) and Sanusi et al. (2013) using Jatropha curcas kernel cake but higher than those obtained by Oboh (2006) with Cassava peels. The increment in the protein content could be due probably to the addition of microbial protein during the process of fermentation (Belewu & Sam, 2010; Meyer et al., 2010). During fermentation, microflora enzymes hydrolyze bonds among bound protein antinutrient and enzyme to release free amino acids for synthesis of new protein (El-Hag et al., 2002; Ahn et al., 2005; Hotz & Gibson, 2007). Compared with the conventional feedstuff, the advantages of bio-feed lie in not only the high content of protein, but also the various microorganism metabolites produced in the process of fermentation, especially the enzymes interrelated (Sun et al., 2009).

Optimum period for good yield of protein in this study lies between 7-14days. Balogolapan (1996) reported 12 days as the optimum for some cassava products. In another study, Iyayi and Losel (2000) reported between 12 to 15 days as the optimum for cassava products. However, in studies with rice straw and maize stover, all fungi significantly increased crude protein concentration after 30 days (Karunananda et al., 1992), although incubation period was fixed at 30 days for all fungi.

The ether extract values decreased significantly ($P < 0.05$) after 21days beyond which there was no further decrease. Reduction in ether extract contents indicates lipolytic activity is present in *A. niger*.

E. cyclocarpum treated with *A.niger* resulted in residues having significantly reduced ADF and NDF values after 28 days fermentation. The decrease in the value of neutral detergent fibre (hemicellulose, cellulose and lignin) and acid detergent fibre (lignin and cellulose) could be indicative of the degradation of the cell wall component of the substrates produced by extra cellular enzymes (Sanusi et al., 2013). The ADF and NDF values are in agreement with those obtained by Makkar et al. (1994) for Quercus incana leaves but lower than those of maize straw reported by Karunanda et al. (1992).

Despite low reductions in ANFs compared to previous study (Ayuk et al., 2004) using ground leaves, *A.niger* caused significant improvement in substrate nutrient composition. These findings are in conformity with the reports of Sun et al. (2009) that the fermented apple pomace using mixed strain of *A.niger* carried not only lower content of anti-nutritional factors, but also higher nutritional values.

In most cases, chemical composition for the substrate was not significantly altered beyond day 14. According to Iyayi and Losel (2000), the period between 0 and 15days represents the period when growth of the microorganisms is most vigorous. Beyond this period, the microorganisms very quickly use up the materials in the medium and growth is slowed down (Iyayi & Losel, 2000). It is also probable that the fermenting micro flora used the nutrients for metabolism or it leached into the fermentation media – a commonly observed phenomenon (Onoja, 2009).

Although, NDF and ADF were not substantially degraded beyond day 14 this could be an added advantage in the

supply of rumen digestible carbohydrates. It may be necessary to use *in sacco* dry matter degradability to assess effect of biological treatment.

5. Conclusion

This study has showed the effectiveness of solid state fermentation of whole leaves of *E. cyclocarpum* using *A.niger* in biodegrading the antinutritional factors therein namely; tannin, saponin , phytic acid and oxalate and improving the nutritive value of the leaves. The reduction of antinutritonal factors, protein enrichment and decrease in ADF and NDF values in the whole leaves of *E. cyclocarpum* highlights the importance of fungal biodegradation as a cheap option for improving nutritive value of foliage for ruminants.

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