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Full Length Research Paper

Novel Alleles 8 and 9 Strains of *Mycoplasma mycoides* subsp. *mycoides* circulating in South East Nigeria and Comparison with Vaccine Reference Strain T1/44

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Control of contagious bovine pleuropneumonia (CBPP) is difficult in Nigeria due to lack of sufficient data. *Mycoplasma mycoides* subsp. *mycoides* is the causative organism of CBPP. This disease is endemic in Nigeria with serious economic impact. This study was undertaken to characterize *M. mycoides* subsp. *mycoides* (*Mmm*) in cattle in south-east Nigeria using single-locus sequence analysis based on polymorphism analysis of non-coding genes and also comparison of the obtained sequences with the vaccine reference strain (T1/44) that is presently used in Nigeria. Twenty one Polymerase chain reaction (PCR) confirmed *Mmm* isolates from Anambra and Enugu states of Nigeria were used for this study. The amplicons were amplified using one locus of the PG1 reference strain (LocPG1-0001) and sequenced using the corresponding primers. The sequences were edited using BioEdit[tm] software and a consensus sequence obtained. Thereafter, the consensus sequence was aligned using ClustalW (BioEdit[tm]). The consensus sequences obtained were compared with the PG1 reference strain and allele numbers were assigned based on any nucleotide changes. Three allelic numbers were obtained; Allele 1, 8 and 9. Allele number 8 and 9 are new findings as they were not previously reported in Nigeria. Points of polymorphism were observed between the vaccine strain and the field isolates. The 21 sequences were deposited in the Genbank with the following accession numbers; MW487814-MW487834.

Key words: Alleles, multi-locus sequence analysis, *Mycoplasma mycoides subsp. mycoides*, Nigeria, polymorphism.

INTRODUCTION

Mycoplasma mycoides subsp. *mycoides* (*Mmm*) is the causative organism of contagious bovine pleuropneumonia (CBPP) (Thiaucourt et al., 2006). It is a highly contagious and fatal disease of cattle, causing

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serious economic losses in Nigeria and some African countries (Egwu et al., 1996; Fadiga et al., 2013). CBPP is a trans-boundary disease (TAD) and is one of the bacterial diseases in the OIE list A diseases (OIE, 2000). The disease negatively affects cattle production in Nigeria and Africa (Egwu et al., 1996). There is an upsurge in the incidence of CBPP in Nigeria especially in the south east region (Anyika et al., 2021). So many pastoralists have adopted 'the live with the disease' perspective in Nigeria (Chima et al., 2001). This has resulted in the heavy usage of antibiotics for the treatment of the disease and reporting it (Chima et al., 2001). Several authors have documented the economic impact of CBPP in Nigeria (Aliyu et al., 2003; Fadiga et al., 2013; Tambi et al., 2006). Successful isolation and identification of the causative organism, is the first major step in designing an effective control program of the disease. *M.mycoides* subsp. *mycoides* is a member of the *M. mycoides* cluster, made up of six mycoplasma subspecies, closely related and at a point thought to be homogeneous (Cottew et al., 1987). However, with the advent of different molecular technologies, the identification and differentiation of the different *Mycoplasma* strains is possible (Miles et al., 2006; Lorenzon et al., 2003). Miles et al. (2006) developed an efficient polymerase chain reaction protocol that could differentiate the European strains of *M. mycoides mycoides* from the African strains, by identification of an 8.8 kb deletion in the genome of most *M. mycoides* Subsp *mycoides* strains of European origin. Lorenzon et al. (2003), identified 15 different allelic profiles of *Mmm* from 48 different strains using a technique that involves sequencing multiple loci, termed Multi Locus Sequence Analysis (MLSA). Similarly, Yaya et al., (2008) also identified three main groups and 31 different allelic profiles of *Mmm* using the same method. Multi-locus Sequence analysis is a technique that is based on sequencing multiple loci. It is a very robust approach (Yaya et al., 2008).

In Nigeria, Nwankpa (2008) identified three different allelic profiles of *Mmm* circulating in Northern Nigeria. However, the study was not extended to other parts of the country. This current study is aimed at characterizing *Mmm* isolates from the south-eastern part of Nigeria and comparing the sequences obtained with the vaccine strain (T1/44). This is very important in designing an effective control programme of CBPP in Nigeria. Several reports have indicated the in efficiency of the current vaccine (T1/44) in protecting cattle against CBPP in the field (Thiaucourt et al., 2006; Yaya et al., 1999).

MATERIALS AND METHODS

Study area

This study was conducted in three selected South Eastern states of Nigeria (Anambra, Enugu and Imo states). It is one of the six geopolitical zones in the country. The region consists of Anambra, Enugu, Imo, Ebonyi and Abia states (Figure 1). Anambra state lies between latitude 5° 32' and 6° 45' N and longitude 6° 43' and 7° 22' E; Enugu state lies between latitude 5° 27'and 6° 33' N and longitude 6° 28' and 7° 32' E and Imo state is located between latitude 4° 45' and 7° and 15' N and longitude 6° 50' and 7°25' E. South-east Nigeria has an estimated cattle population of 4.5 million from the total of 16.3 million estimated cattle population in Nigeria (Ikhatua, 2011).

Confirmation of *Mmm* isolates using polymerase chain reaction (PCR)

Twenty one *Mycoplasma* isolates from the two South-Eastern states of Nigeria (11 from Enugu state, 10 from Anambra state), positive on Pleuropneumonia like organism (PPLO) agar were confirmed using conventional PCR according to protocols by Miles et al. (2006). DNA was extracted from a 5 ml *Mmm* broth culture using QIAamp® DNA Mini kits according to manufacturer's instructions. Lyophilized T1/44 vaccine (NVRI, Vom Nigeria) was used as the positive control for this reaction. All the PCR reactions were carried out in a total volume of 25 µl, that consisted of dH_2O, 5x FIREPol® master mix (12 mM MgCl, 1 mM dNTP mix, FIREPol® DNA polymerase and 1 µl IS1296F: Primer (5' to 3'): CTA AAG AGC TTG GAG TTC AGT G and 1 µl R (all) (sequence 5' to 3'): CCA GCT CAACCA GCT CCA G (Miles et al., 2006).

DNA amplification was performed using GeneAmp® PCR system 2720 (Perkin Elmer, Courtaboeuf, France) with an initial denaturation step at 95°C for 5 min, followed by 32 cycles of denaturation at 95°C for 30 s, annealing at 62°C for 30 s and extension at 72°C for 1 min and 20 s. The final extension stage was maintained at 72°C for 5 min. The PCR product was then ran on 2% agarose gel impregnated with ethidium bromide (0.5 µg/ml) at 130 volts for 30 min. Finally, the DNA migration was viewed under ultra-violet light and photographs taken. The production of a band equivalent to 1.1 kbp and at the same distance with the positive control (T1/44) was considered confirmatory for *M. mycoides mycoides.*

Molecular characterization of M*mm* isolates

All the twenty one PCR confirmed isolates were characterized using Single-Locus Sequence Analysis according to protocol of Yaya et al. (2008). It was carried out using the locus: Loc- PG1-0001(non-coding region). This locus was selected due to its ability to effectively differentiate *M. mycoides mycoides* strains from West Africa (Yaya et al., 2008). In brief, PCR reaction was performed using GeneAmp® PCR system 2720 (Perkin Elmer, Courtaboeuf, France) in a final volume of 50 µl reaction mix. Samples were amplified with the following primers: Loc-PG1-0001-F: 5'AACAAAAGAGATCTTAAATCACACTTTA 3' and Loc-PG1-0001-R: 5' CCTCTTGTTTAACTTCTAGATCAGAAT 3 (Yaya et al., 2008). Thermal cycling involved an initial step of denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s and extension at72°C for 90 s. The final extension step was maintained at 72°C for 7 min. The PCR amplification products was then analyzed by electrophoresis through 1.5% agarose gel (QA-Agarose, MP Biomedicas, IllKirch, France) at 100V and viewed after staining with Ethidium bromide on a ultra-violet trans-illuminator. Subsequently, samples with the expected band size of 538 base pairs (Figure 2) were sent to Macrogen™ Europe B.V, The Netherlands, for sequencing.

Sequence analysis of amplicons

The sequences obtained from Macrogen™ Europe B.V with the

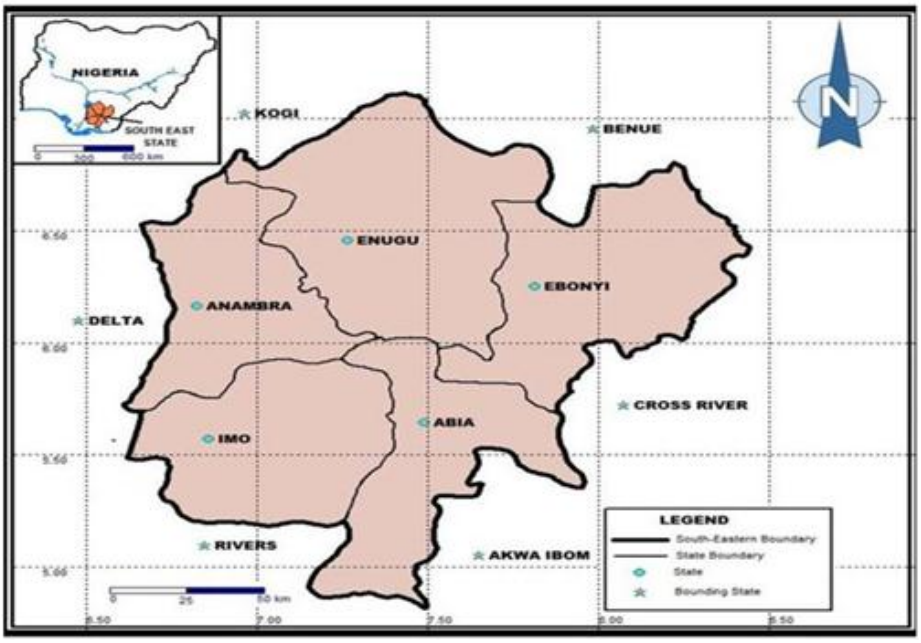

Figure 1. Map of Nigeria showing the South-eastern states of Nigeria.

forward and reverse primers of each sample was assembled using BioEdit™ software. Thereafter, the extremes of each sequence or those showing aberrant features were cut off by carefully examining the chromatographs, after which a consensus sequence was obtained. The consensus sequences obtained from the different isolates for the selected locus (Loc-PG1-0001) was aligned using ClustalW (BioEdit™). Subsequently, the sequences obtained were compared with the PGI reference genome in the Genbank and those obtained from the 51 strains of *Mmm* by Yaya et al. (2008). If the feature of a strain corresponds to one of the strains in the work of Yaya et al. (2008), its allele number was given to the strain. However, if there are differences, a new allele number was given. The strains were characterized using allele numbers. Furthermore, the consensus sequences obtained for each sample was also compared with the vaccine reference strain (T1/44). Points of polymorphism between the field isolates and vaccine strain were documented.

RESULTS

Multi-locus sequence analysis on Loc-PG1-0001

Twenty one of the PCR confirmed *M. mycoides* subsp *mycoides* isolates were of the expected band size of the selected locus, Loc-PG1-0001 as indicated by the production of a band size equivalent to 538 bp (Figure 2).

Alleles identified in the non-coding sequences Loc-PG1-0001

Three allele numbers were identified on the locus Loc-PG-0001 (allele 1, 8 and 9) (Figures 3 and 4). There was no point mutation at position 1523 on the PG1 reference genome. All the isolates had A (Figure 3) (Allele 1). However, there were point mutations on two positions: position 1525 and 1751 on the PG1 reference genome. Isolate A8 had A at position 1525 of the PG1 genome while the others had T (Figure 3) (Allele number 8). Similarly, isolate A3 had A at position 1751 while the other isolates had T (Figure 4) (Allele number 9). Allele 1 was found in both Anambra and Enugu state while Allele 8 and 9 were only found in Anambra state. Alleles 8 and 9 is a new finding as they were not observed on the 51 strains previously described by Yaya et al. (2008) neither in work done by Nwankpa (2008) in Northern Nigeria. Consequently, a new allele number (allele numbers 8 and 9) was given to them.

MLSA allelic sequence comparison of field Isolates with the vaccine reference strain (T1/44).

After alignment of the twenty one field isolates sequences with the vaccine reference strain sequence (T1/44), points of mutation were observed at two positions: positions 1525 and 1750 (Figure 5). Isolates A3 and A8 had A instead of T at position 1525 and 1750 respectively. All other isolates were identical with the vaccine (T1/44) at Loc-PG-0001 of the PG 1 reference strain (Figure 6).

Genbank Accession Numbers

These twenty one sequence data have been submitted to

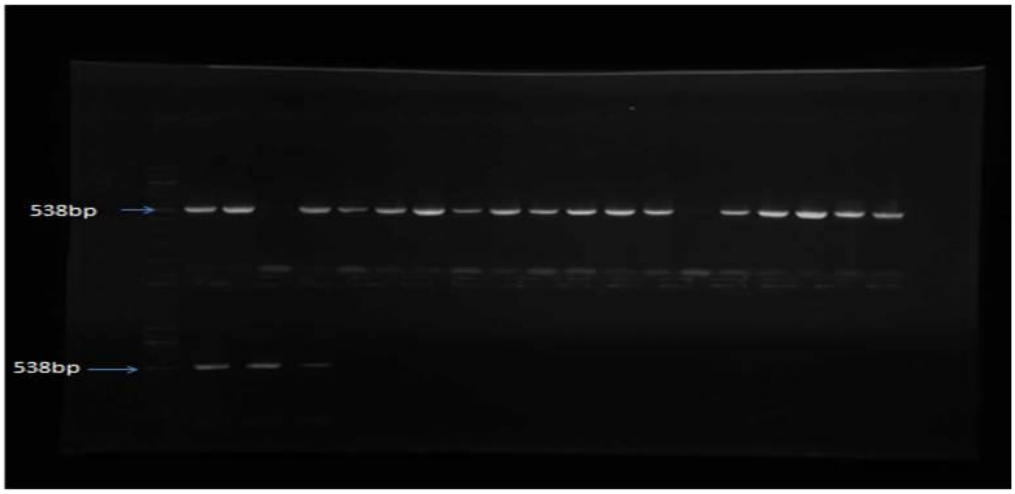

Figure 2. PCR confirmed isolates prior to sequencing with the expected band size of 538bp for the locus: LocPG-0001.

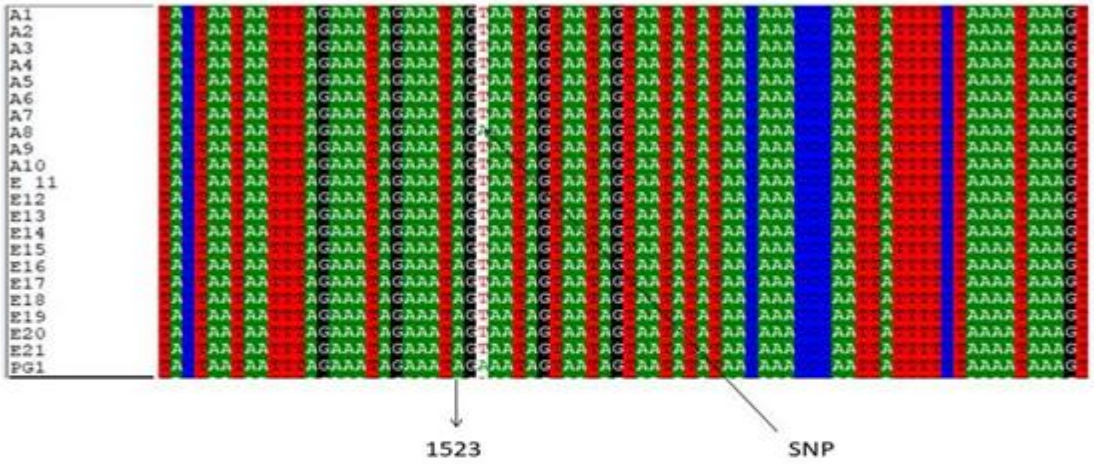

Figure 3. Alignment of sequences on locus Loc-PG1-0001; polymorphism at position 1525 of PG1 reference strain. Isolate A8 had A while the other isolates had T.
Key: SNP: single nucleotide polymorphism.

the DDBJ/ Genbank database under accession numbers; MW487814-MW487834. Addresses are as follows:

DNA Data Bank of Japan (DDBJ)
http://www.ddbj.nig.ac.jp
GenBank: http://www.ncbi.nlm.nih.gov

DISCUSSION

Contagious bovine pleuropneumonia is a trans-boundary animal disease, with economic effects estimated at billions of Naira in Nigeria (Fadiga et al., 2013). Since the introduction of the disease in Nigeria, its control has faced many challenges, especially due to lack of efficient data. Countries that have successfully eradicated CBPP relied heavily on data and other programmes such as stamping out policy with commiserate compensation of farmers (Egwu et al., 1996, Thiaucourt et al., 2006). Control of this disease has become a challenge in Nigeria. Studies have documented an upsurge in CBPP outbreaks in South Eastern region of Nigeria mainly due to transhumance from the northern part of the country (Anyika et al., 2021).

This work reports the first molecular analysis of *Mmm* isolated from south east, Nigeria and comparison with vaccine reference strain T1/44. The use of molecular biology tools has greatly improved the capability to

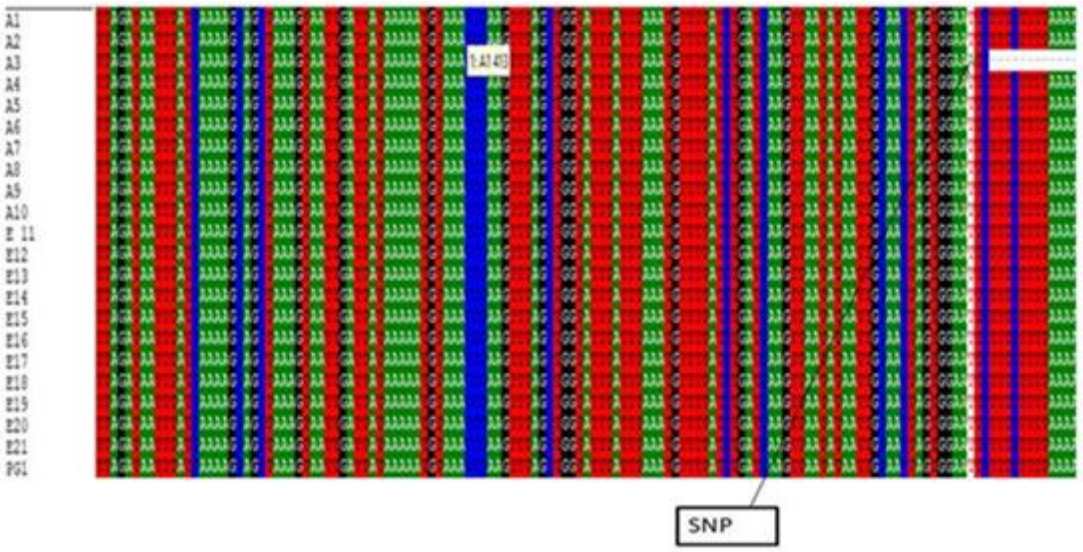

Figure 4. Sequences alignment on locus Loc-PG1-0001; polymorphism at position 1751 of PG1 reference strain. Isolate A3 had A while the other isolates had T.
Key:SNP: single nucleotide polymorphism.

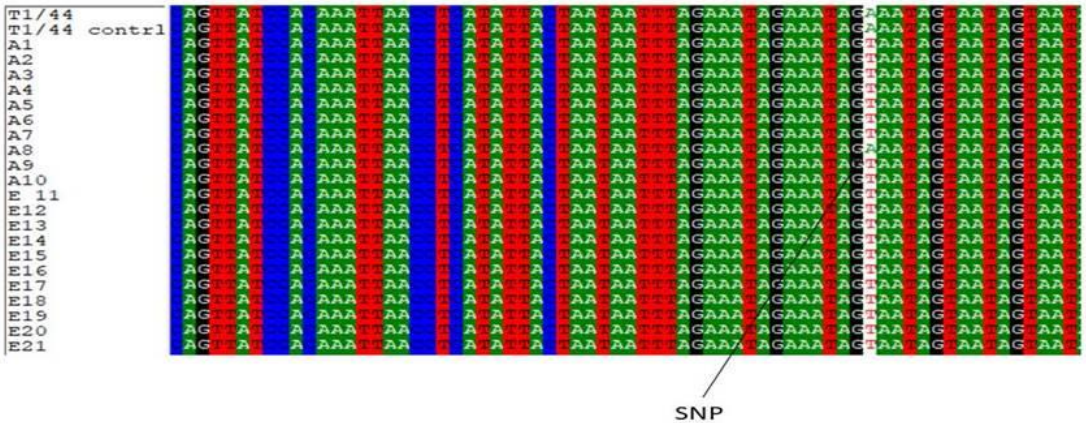

Figure 5. Sequence alignment on the vaccine reference strain (T1/44); polymorphism at position 1525 of T1/44 reference strain. Isolate A8 had A while the other isolates had T.
Key:SNP: single nucleotide polymorphism.

detect, identify and characterize strains (Bashiruddin et al., 1994; Thiaucourt et al., 2000a). Three allelic numbers were observed in this study (Allele 1, 8 and 9). Allele 1 was found in both Anambra state and Enugu state while Allele number 8 and 9 were identified only in Anambra State. Allele 1 was earlier reported in the study by Nwankpa (2008) in Northern Nigeria. According to his work, it was found circulating in cattle in both Kaduna and Taraba State of Nigeria. This could be possible, as most cattle slaughtered in south eastern region of Nigeria are bought and transported from the Northern parts of the country. There is a large consumption of beef in south east Nigeria. This could also be the reason why Allele 1 earlier identified by Nwankpa (2008) in Northern Nigeria was also found in the south eastern region. However, Allele 8 and 9 are new strains of *Mmm* that have not been earlier reported in both the works of Yaya et al. (2008) and Nwankpa (2008). This could be a new strain of *Mmm* circulating within the south eastern region of Nigeria. It is however, important to extend this study to other parts of Nigeria to determine if there are such similar *Mmm* profiles in other regions of the country. The new Allele numbers (1, 8 and 9) identified in this study, may be under profile A strains according to groupings by Yaya et al. (2008), which established thirty one allelic profile using eight loci. These profiles are divided into

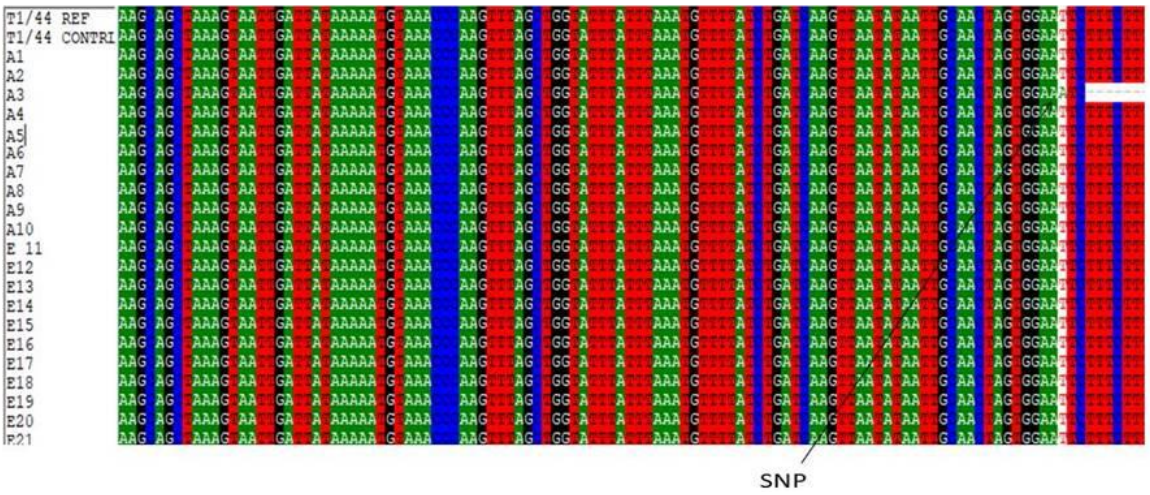

Figure 6. Sequence alignment of vaccine strain T1/44; polymorphism at position 1750 of T1/44 reference strain. Isolate A3 had A while the other isolates had T.
Key: SNP: Single nucleotide polymorphism.
Genbank Accession Numbers.

seven groups: A, B, C, D, E, F and G. Profile A had the largest number of strains with thirteen profiles and are from African origin especially from West Africa. Similarly, Mahamadou et al. (2021) also identified strains under profile A with a novel profile A15 in Niger. Finally, the determination of two new allele numbers not described in previous works of Nwankpa (2008) in Northern Nigeria, is a significant finding in this study.

There are several vaccine strains of CBPP; KH3J, T1SR and T1/44 (Aliyu et al., 2003; Egwu et al., 1996). Presently, T1/44 vaccine strain is used in Nigeria for the control of CBPP (Aliyu et al., 2003). In this study, points of mutation were observed between the field isolates and the vaccine reference strain (T1/44). Perhaps, these observed differences (polymorphism) may account for the low effectiveness of the vaccine (T1/44) to fully protect cattle from field infections as reported by several authors (Thiaucourt et al., 2000a; Thiaucourt et al., 2006; Yaya et al., 1999). For instance, isolate A3 from Anambra state had A at position 1560 of the T1/44 reference strain while other isolates had T. Perhaps, this change in Nucleotide sequence, could account for failure of the vaccine to confer maximum immunity against CBPP. However, more studies need to be carried out to ascertain these claims. Several studies have reported similar differences between the field isolates of CBPP in Nigeria with the Vaccine strain. Folashade (2014) reported difference between the vaccine strain and field strain using restriction fragment length polymorphism. Similarly, Nwankpa (2008) also reported differences between the sequences of the field isolates form some Northern states of Nigeria and the vaccine strain (T1/44), he observed points of polymorphism among some of the field isolates from Northern Nigeria and the vaccine strain (T1/44).

The efficacy of CBPP vaccine in protecting cattle against infection has been widely studied (Thiaucourt et al., 2000a; Wesonga and Thiaucourt, 2000: Yaya et al., 1999). Some of these studies have indicated the inability of the vaccine to effectively protect cattle against contagious bovine pleuropneumonia (March and Brodie, 2000). This underscores the need for newer approach in the control of CBPP in Africa. Perhaps, there is the need to develop a polyvalent CBPP vaccine, which will contain two or more different strains of *M. mycoides* Subsp. *mycoides* alongside the T1/44 currently used. We believe that a better CBPP vaccine would increase the chances of eradicating the disease from the continent.

Conclusion

This study identified two novel alleles: Allele 8 and 9 and observed sequence differences between the field isolates of *M. mycoides mycoides* and the vaccine reference strain (T1/44) in south east Nigeria.

RECOMMENDATION

This molecular method should be extended to other parts of the country, to effectively characterize other circulating strains of *Mmm*. This should improve the surveillance and control of this disease in Nigeria.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Influence of phosphorus-solubilizing microorganisms and phosphate amendments on pearl millet growth and nutrient use efficiency in different soils types

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Two greenhouse growth trials were performed to assess inoculation with phosphorus (P)-solubilizing microorganisms (PSM) in combination with alternative sources of phosphate in pearl millet (*Pennisetum glaucum*) cultivation: One using sandy soil and the other using clayey soil. The treatments comprised five P sources, with or without inoculation with PSM B119 (*Bacillus megaterium*) and B2084 (*Bacillus subtilis*) strains. Amendment of alternative sources (granulated, branned organomineral, and Bayovar rock) of P along with PSM inoculation produced more plant dry mass on sandy soil, which was not observed on clayey soil. Phosphorus use efficiency (PUE) did not differ between inoculated and non-inoculated treatments, and it was higher with the alternative P sources, compared to triple superphosphate (TSP) treatments. Available P content in the soil was higher with TSP, in sandy soil, and with PSM inoculation. Overall, acid and alkaline phosphatases and β-glucosidase activity was higher in clayey soil, compared to sandy soil, which contains little organic matter; it was also higher with alternative P sources, compared to TSP, and in inoculation treatments, showing the potential of using PSM inoculation and alternative P sources to achieve higher sustainability and productivity in agriculture.

Key words: *Pennisetum glaucum,* alternative phosphorus sources, fertilization, organominerals.

INTRODUCTION

Phosphorus (P) is closely related to several soil characteristics, particularly weathering degree, buffering potential, mineralogy, and organic matter (OM) content. P is an essential macronutrient to plants as nitrogen (N) and potassium (K). Soils with high-buffer-capacity soils are characterized by high clay content, iron and aluminum oxides and 1:1 clay mineral (Batjes, 2011; Du et al., 2020). This results in strong P adsorption and low P uptake (approximately 10 to 25%) by plants growing on tropical soils (Hanyabui et al., 2020). Therefore, P

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fertilization in dosages exceeding the demand of agricultural crops in tropical soils is necessary to overcome adsorption losses. These factors, combined with the important role of P in the development and yield of several agricultural crops, have attracted research interest to optimize soil P management to achieve optimal use by plants.

Currently, Brazil is a major consumer and importer of phosphate fertilizer (55% of consumed P), due to the characteristics of local soils and absence of high-quality phosphate rock mines (Lapido-Loureiro and Melamed, 2009). Fertilizer import reached the highest level in the country's history in the first half of 2020, accounting for 29.4 million tons. This contributed to the rising cost of grain production and added this issue to the most relevant aspects in the Brazilian agribusiness (Globalfert, 2021). Meanwhile, studies conducted by Embrapa indicated that almost half of the P applied as inorganic agricultural fertilizer in the past 50 years remains fixed in the soil where it is unavailable to plants, amounting to approximately 30 Tg and corresponding to a reserve of over US$ 40 billion (Withers et al., 2018).

The main phosphate fertilizers used in Brazil are soluble P sources such as superphosphates (single and triple) and monoammonium and diammonium phosphates. The national sources include deposits essentially of igneous origin, associated with carbonatites; beneficiation of these rocks is difficult, due to their complex mineralogy, low uniformity, and low apatite and high carbonate content, which entail challenges regarding industrial utilization. By contrast, rock phosphates from other countries, such as those from Tunisia (Gafsa), Israel (Arad), Peru (Bayovar), and Morocco (Benguerir) are of sedimentary origin, easy to mine, and highly reactive as they are composed of open poorly consolidated aggregates of microcrystals with a large specific surface area (Lapido-Loureiro and Ribeiro, 2009).To improve the use of less soluble P sources, alternatives for P use and management are required to achieve a more sustainable, productive, and economically viable form of agriculture by using alternative nutrient sources, organomineral fertilizers, and biological materials.

The P availability from rock phosphates can be improved during crop cultivation, for example, through soil microorganisms (Coutinho et al., 1991; Silva et al., 2017; Ribeiro et al., 2018; Mattos et al., 2020). Applying P solubilizing microorganisms (PSMs) together with different P sources may help optimize the use of P and other fertilizers; therefore, this approach constitutes a promising alternative with low environmental impact and with the potential to increase crop productivity (Spolaor et al., 2016; Oliveira- Paiva et al., 2020; Rosa et al., 2020; de Sousa et al., 2020). This is possible due to processes elicited by microorganisms, such as biosynthesis of phytohormones and metabolites (organic acids, phosphatase enzymes, etc.) (Duca et al., 2014; Tahir et al., 2017; Oliveira-Paiva et al., 2020), induction of tolerance to biotic and abiotic stresses (Yan et al., 2016; Takishita et al., 2018), production of siderophores (Ali et al., 2014), and solubilization of soil nutrients such as P and K (Gupta et al., 2015; Shen et al., 2016; Patel and Archana, 2017). Production of organic acids by microorganisms (Mendes et al., 2014; Abreu et al., 2017) and the release of protons in $NH4^+$ assimilation reactions or other proton-releasing metabolic reactions (Prabhu et al., 2019) stand out among P solubilization mechanisms. In addition to the mechanisms of mineralization of soil organic P by the production of phosphatases, especially of the phytase group, chelating agents are produced by plants and microorganisms (Sharma et al., 2013).

Promising results regarding interactions between microorganisms and phosphate rocks have already been described for different crops, including maize (Manzoor et al., 2016; Silva et al., 2017), wheat (Kaur and Reddy, 2015), millet (Ribeiro et al., 2018), sorghum (Ehteshami et al., 2018; Mattos et al., 2020), and forage legumes (Zineb et al., 2020). In maize, the presence of PSMs, combined with the benefits of organomineral sources, resulted in increased availability of P to plants. This positive effect can also be proven by the available P content in the soil, which is higher in treatments with organominerals and microorganisms and similar to treatments with triple superphosphate (TSP), with comparable results for millet (Almeida et al., 2016). Therefore, the objective of this study was to evaluate the effect of inoculation of PSM on millet plants grown on different soils fertilized with different P sources.

MATERIALS AND METHODS

Two experimental setups were established in the greenhouse of Embrapa Milho and Sorgo, in Sete Lagoas, MG (19°28' S, 44°15' W) from May/2018 until January/2019 using two soil types: one with a very clayey texture from the Experimental Farm of Embrapa Milho and Sorgo (typical Dystrophic Red Latosol) with the following chemical and physical characteristics before any treatment: pH H_2O = 5.2, aluminum (Al) = 0.56; calcium (Ca) = 1.1; magnesium (Mg) = 0.1 ($cmol_c\ dm^{-3}$); total cation exchange capacity (T) = 9.7 ($cmol_c\ dm^{-3}$); P-Mehlich 1 = 1.2; potassium (K) = 15.1 (mg dm^{-3}); base saturation (V) = 12.7%; organic matter (OM) = 3.64%, and clay content = 74%; the other soil type was sandy and originated from the Trijunção Farm (Dystrophic argisolic Red-Yellow Latosol), with the following characteristics before any treatment: pH H_2O = 6.2, Al = 0.04; Ca = 1.2; Mg = 0.3 ($cmol_c\ dm^{-3}$); T = 3.1 ($cmol_c\ dm^{-3}$); P-Mehlich 1 = 3.4; K = 11.3 (mg dm^{-3}); V = 49.3%; OM content = 0.91%, and clay content = 14.0%. Pots capable to support 5 kg filled with 4 kg of soil were used. Requirement for liming and fertilization (apart from P) was calculated to reach V = 70% and to meet the demand of the crop in a greenhouse test (Resende et al., 2020), respectively, with application of poor analysis (p.a.) reagents one month before the experiment. During this preliminary period and during the experiments, the pots were irrigated to maintain humidity at 80% field capacity.

The experimental design was entirely randomized in a 5 ×2

factorial design with five P treatments, that is, (1) control without P fertilization, (2) granulated organominerals (GO; mixture of 45% poultry litter, 5% additives to improve granulation, and 50% Bayovar phosphate), (3) branned organominerals (BO; mixture of 45% poultry litter, 5% additives to improve granulation, and 50% Bayovar phosphate), (4) Bayovar phosphate rock, and (5) triple superphosphate (TSP); with or without PSM inoculation, thus comprising 10 treatments. Each treatment was performed using four replicates. The P_2O_5 dosage was 458 mg dm^{-3} per pot, and the P_2O_5 contents in P sources utilized to calculate fertilization rates were 27% of total P_2O_5 for Bayovar, 16% of total P_2O_5 for organominerals, and 41% of citric acid soluble P_2O_5 for TSP.

The granulation process involved mixing the raw materials in a mass: mass ratio, homogenizing and placing them in a pelletizer disc with constant speed and inclination, and adding water manually. After granulation, the fertilizer was sieved to segregate granules with diameters of 2-4 mm in order to meet the granulometric requirements specified by Brazilian Department of Agriculture, Livestock and Food Supply (MAPA), in accordance with the Normative Instruction No. 23 of August 31, 2005. Then, the granules were dried to constant mass at 40°C using an oven with forced air circulation.

Phosphate fertilization was conducted one month after soil incubation by producing “cross-shaped” furrows in the soil. The inoculant contained the P-solubilizing bacteria B119 (*Bacillus megaterium*) and B2084 (*Bacillus subtilis*) were obtained from the Microorganism Collection of Embrapa Maize and Sorghum. One isolated colony of each strain grown on BDA plates (200 g L^{-1} potato, 20 g L^{-1} dextrose and 15 g L^{-1} agar) was transferred to TSB medium (Trypticase Soy Broth) and incubated overnight at 28° C. The concentration of bacteria was determined by measurement of absorbance at 560 nm (10^8 cells mL^{-1}) using a spectrophotometer UV/Vis UV1800 (Shimadzu, Japan). Liquid inoculant was applied on top of the fertilizers or on the soil (control treatment) at 10 ml pot^{-1}. The applied fertilizers and inoculant were covered by thin layer of soil, after which 20 pearl millet seeds (*Pennisetum glaucum*) ADR500 obtained from a breeding company was sown. After germination the seedlings were thinned to six plants per pot. The plants were grown for three successive crop cycles until the budding stage. Phosphate fertilization was performed only in the first crop to assess residual effects on the following crop cycles. Inoculation with PSM was executed on the first crop and was repeated before planting the third crop. N fertilizer was applied at three times weekly, 15 days after sow (50 mg dm^{-3} per pot) were provided per crop cycle.

Plants of each crop cycle were harvested at the booting stage by cutting the shoot part close to the ground. After the third crop cycle, the roots were collected in addition. The collected material was weighed, green mass was determined, and samples were placed in a forced circulation oven at 65°C. After reaching constant mass, dry mass and macronutrients in shoot part (and roots, in case of the third crop) were determined (Silva, 2009). The data were used to calculate extraction and use efficiency (UE) of N (NUE), P (PUE), and K (KUE), according to the ratio between plant biomass and total nutrient accumulation (Tomaz and Amaral, 2008). Soil samples were collected at each harvest to determine available P using a sodium bicarbonate extraction solution ($NaHCO_3$), 0.5 N at pH 8.5, according to Olsen et al. (1954).

Soil microbial activity was evaluated based on acid and alkaline phosphatases (Tabatabai, 1994) and β-glucosidase (Eivazi and Tabatabai, 1988) through colorimetric determination of released p-nitrophenol after incubation at 37°C with buffered solution of p-nitrophenyl phosphate and p-nitrophenyl-β-D-glycopyranoside, respectively.

Data were checked for normality and analyzed using One-way Analysis of Variance, and means of treatments were compared using Tukey's test at 5% probability, as implemented in Sisvar software (Ferreira, 2011). Data were analyzed comprehensively, considering the crops as subplots in order to assess differences between crops. Correlation analyses were performed according to Hair Jr. et al. (2006).

RESULTS AND DISCUSSION

Production of dry mass of shoot parts and roots

Dry mass production in shoot parts of millet plants was affected by P source and by PSM inoculation in all crop cycles on sandy soil (Figure 1), with no significant effect of the interaction (data not shown). Dry mass in clayey soil was affected by the P sources in all crops, but by the microorganisms only in the third crop and by the Bayovar source (Figure 1); no interaction effect was observed.

The first crop exhibited higher production of dry mass of shoot parts in the TSP treatment, with or without PSM inoculation, followed by GO and BO and by the control without addition of P and Bayovar (Figure 1). In the first crop on sandy soil, higher production of dry mass was observed in the TSP treatment with no PSM inoculation.

Bars bearing the same capital letter for P sources and the same lowercase letter for microorganism inoculation do not differ significantly at $p > 0.05$ (Tukey’s test), for each soil type. The second and third crops accumulated more shoot dry mass in treatments with GO, BO, and Bayovar rock, compared to the TSP and control treatments, on both soil types, as well as compared to the PSM treatments, especially on sandy soil (Figure 1). These results highlight the importance of residual effects of organomineral sources containing rock phosphate, and even Bayovar, compared to TSP because in addition to the improvement in dry mass production throughout the crops (Figure 1), organomineral sources and Bayovar also showed lower depletion of available P in soil compared to TSP (data not shown). Furthermore, the importance of PSM, especially in sandy soil and for alternative P sources (organominerals and Bayovar) is shown in Figure 1, and confirms the positive effects of phosphate rock-PSM association observed in previous studies (Mattos et al., 2020). Maize grown on soil fertilized with rock phosphate for three years showed biomass production and grain yield comparable to those of plants fertilized with TSP (Silva et al., 2017). Based on metataxonomic data, Silva et al. (2017) hypothesized that, in the long term, rock phosphate fertilization promoted higher relative abundance of microbial taxa related to P solubilization/acquisition in the soil and, consequently, increased P release and availability to plants.

In general, the performance regarding shoot dry mass production was very similar among organomineral sources, as well as between these and Bayovar, with the exception of crop 1, where the Bayovar treatment

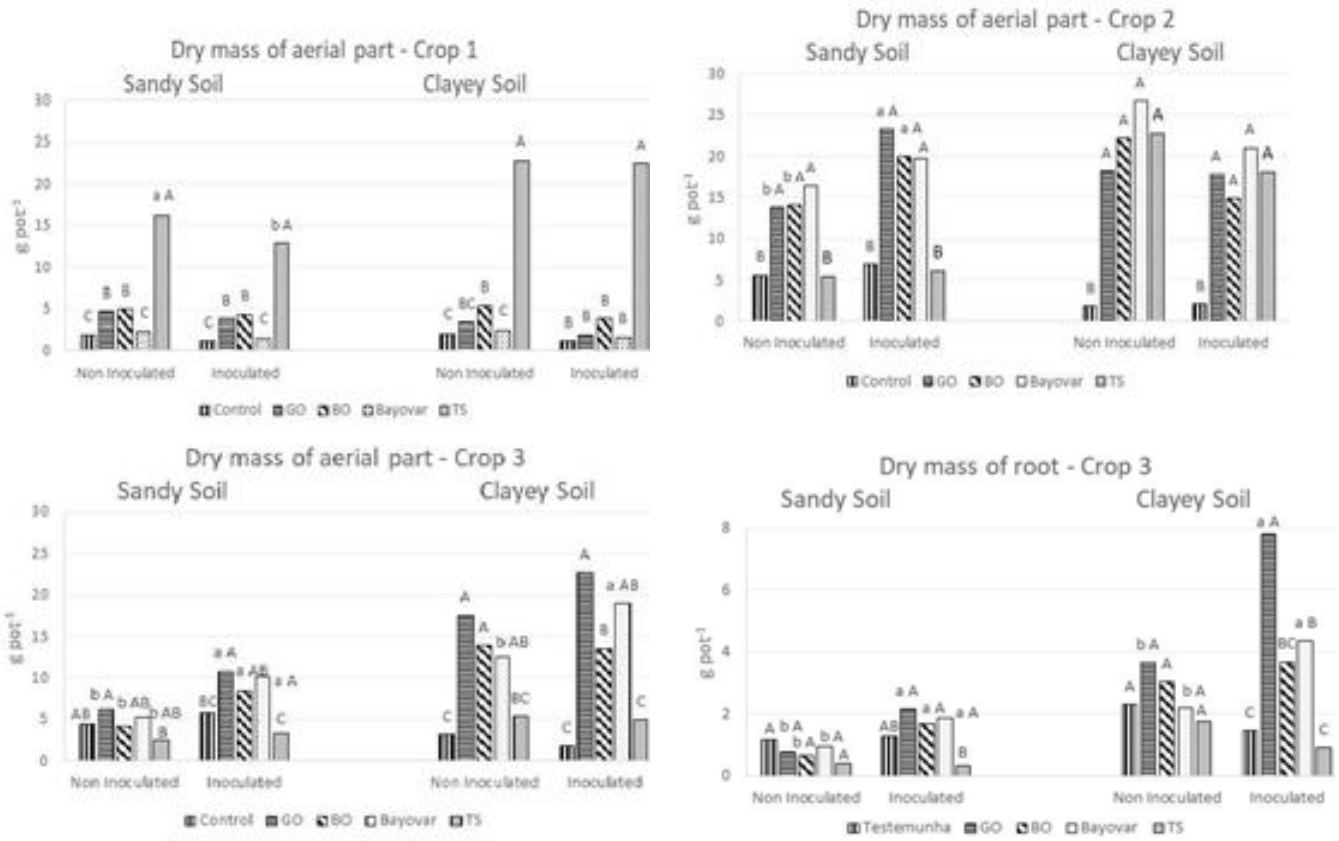

Figure 1. Dry mass of shoot of the three crop cycles and root dry mass of the third crop grown on sandy and clayey soils as a function of P sources and inoculation with phosphate (P) solubilizing microorganisms.

produced less than the organominerals in both soil types, and of crop 3 on clayey soil where the BO treatment produced less (Figure 1).

Higher production of dry matter of shoot parts only in the TS treatment on clayey soil in the first cycle was probably due to rapid P availability as this fertilizer is a soluble phosphate with high P concentration (45% of total P_2O_5). This characteristic leads to marked differences in the speed of P release to plants, as well as in the potential for fixation of this element in the soil (Korndörfer et al., 1999) and reduced availability to subsequent crops.

Similarly, the organomineral fertilizers and the combination of Bayovar rock with PSM inoculation resulted in higher production of dry masses of shoot parts, especially in the second and third crop cycles, compared to the TSP treatment. As soluble P in TSP is released faster to the soil, plants, may consume it largely during the first crop cycle and was thus less available thereafter. Furthermore, the fast initial release of P in TSP may also favor its adsorption to the soil and thereby reducing its availability to plants (Novais et al., 2007). Thus, because organomineral phosphates are less soluble, P release is slower, favoring plant uptake rather than soil adsorption (Novais et al., 2007; Almeida et al., 2012). These characteristics of organomineral fertilizers may have helped overcome the limitations of soil P dynamics, particularly regarding P availability in second and third crop cycles allowing increased P uptake by plants, thus resulting in higher dry mass production. Silva et al. (2021) observed that the combination of rock phosphate with P- solubilizing bacteria helped to increase the supply of P to millet, especially in management systems where chemical fertilizers such as TSP are not admissible. Indeed, the slow release of P from rock phosphate may be an advantage in tropical soils, as it minimizes the loss of soluble P by adsorption to soil particles.

The combination of natural phosphates with PSM may be a viable alternative to improve P availability and recycling. These microorganisms can solubilize and mineralize P from inorganic and organic sources and thereby increase its availability to plants (Richardson, 2001); additionally, they produce other metabolites which promote plant growth, which also explains the observed larger dry mass of shoot parts in the inoculation treatments, especially in the second and third crop cycles. Re-inoculation before the third crop showed the importance of repeated application to ensure the desired effects to achieve higher crop yields,

especially regarding sandy soil where biological activity is lower due to more limiting conditions for growth and survival of soil microbiota (Sessitsch et al., 2001).

Root dry mass production was significantly affected by P sources and by PSM inoculation, as well as by the interaction, with the organomineral and Bayovar treatments producing higher root mass, compared to the TSP treatments. Moreover, PSM inoculation led to higher production of root mass, especially in sandy soil (Figure 1). The bacteria used in the present study favorably affect plant root development (Ribeiro et al., 2018; de Sousa et al., 2020), and de Sousa et al. (2020) observed that, under controlled conditions, inoculation of maize seedlings with these *Bacillus* strains resulted in increased biomass and nutrient content of shoot parts and root surface area, compared to the non-inoculated control. Under field conditions, the same authors noticed that inoculation with these two *Bacillus* strains resulted in increased grain yield of maize plants in soils fertilized with TSP. For example, inoculation with *B. megaterium* B119 resulted in 26% increase in the production and accumulation of P in grains, compared to non-inoculated plants and to 23% increase in P accumulation in grains, compared to plants that received only phosphate fertilization (de Sousa et al., 2020). In millet plants, positive effects of inoculation with endophytic *Bacillus* strains on dry weight of shoot parts and roots, in addition to plant N, P, and K content, were reported, reaching increments of 55% in the shoot part biomass, and N, P, and K content increased by 30, 50, and 70%, respectively (Ribeiro et al., 2018).

The positive effects of inoculation with *Bacillus* strains may be related to the synergistic effects of multiple growth-promoting factors, such as production of phytohormones and solubilization of plant nutrient sources. The bacterial strains used in the current study are considered effective for the production of the phytohormone indoleacetic acid (IAA), with strains B119 and B2084 producing 61.67 and 30.16 µg mL^{-1} IAA, respectively (de Sousa et al., 2020). Solubilization of rock phosphate by B119 has been reported (Gomes et al., 2014), whereas B2084 is an endophytic bacterium which can solubilize tricalcium phosphate ($Ca_3(PO_4)_2$), in addition to producing high concentrations of organic acids *in vitro* (Abreu et al., 2017).

A larger root system increases the plant's efficiency to acquire nutrients and water from the soil. The production of IAA increases the root surface, allowing the penetration of larger volumes of soil, which leads to increased absorption of nutrients, especially those of low mobility in soil, such as P. In addition, the increase of root mass allows higher exudation of carbon compounds, which stimulates microbial activity in the rhizosphere.

Combined analysis of the three crop cycles

The overall means of the three crop cycles (Table 1) showed that the alternative sources GO, BO, and Bayovar inoculated with PSM stimulated the production of dry mass of the shoot part of plants on sandy soil. Such effect was not observed in plants on clayey soil (Table 1). This indicated a major effect of PSM inoculation in sandy soil with low clay and OM content, that is, where conditions for plant growth are more limiting. Increased root growth in the inoculation treatment in sandy soil (Figure 1) shows the potential of this approach which may help make use of a bigger soil volume and thereby increase water and nutrient absorption and plant productivity.

No effect of PSM inoculation on NUE, PUE, and KUE was observed, however, between P source treatments, a difference was observed regarding P and K (Table 1). Considering P and the two soil types, although there was no statistical difference between the treatments with and without inoculation, the PUE values of the inoculated treatments were higher, except for the TSP treatment. These results suggest a beneficial effect of PSM to increase UE by plants. The higher PUE of the control may be irrelevant, as dry mass production was not increased (Figure 1), that is, the plants absorbed P more efficiently but were less productive, which is not of agronomic interest.

Regarding P sources in both soil types, plants receiving GO, BO, and Bayovar were more efficient in absorbing P than those receiving TSP. This suggests the potential of alternative sources of phosphate nutrients. The low performance of the TSP source stands out because of the low PUE values, even though it resulted in good dry mass production, especially in clayey soil (Figure 1 and Table 1).

PUE was lower in sandy than in clayey soil (Table 1) which has lower buffering capacity and thus facilitates higher nutrient accumulation by plants (Novais et al., 2007).This suggests higher P export by plants in sandy soils. PSM inoculation may help increase P uptake, which is important regarding sandy soils in which biological activity is lower due to the low OM content (typically < 10 g kg^{-1}) and, in general, enzyme activity increases with increasing soil OM content, thus suggesting higher stability of enzymes adsorbed to humic materials and growth of microbial communities in the soil (Marinari and Antisari, 2010; Oliveira et al., 2015). This influence is evidenced by the results in Table1, as enzymatic activity in clay soil was higher than in sandy soil, regarding the three evaluated enzymes.

Significant correlation at 5% probability occurred in plants on clayey soil between DM and UEN (r = 0.91), DM and PUE (r = 0.81) as well as DM and UE of K (r = 0.80). OM is an important source of N for plants, and higher OM content in clayey soil (36.4 g kg^{-1}) may explain for this significant correlation.

The Olsen extractor (Olsen et al., 1954) was used to analyze soil P availability, as acid extractors such as

Table 1. Mean values of three crop cycles regarding shoot part dry mass, utilization efficiency of nitrogen (NUE), phosphorus (PUE), and potassium (KUE), soil available phosphorus (P) by Olsen extraction, and activity of acid phosphatase, alkaline phosphatase, and β-glucosidase as a function of P source and inoculation with phosphate solubilizing microorganisms.

Soil	Source	DM	NUE	PUE	KUE	P-OLSEN	ACI PHOS	ALK PHOS	β-GLUCOS
		g pot^{-1}	g shoot /g nutrient accumulated in the shoot			mg dm^{-3}	µg p-nitrophenol h^{-1}g^{-1} soil	µg p-nitrophenol h^{-1} g^{-1} soil	µg p-nitrophenol h^{-1} g^{-1} soil
Sandy	Test*	3.94^{f**}	30.45^{a}	995.94^{a}	138.43^{a}	1.58^{c}	126.99^{bc}	27.99^{e}	17.65^{c}
	Test +Inoc	4.61^{ef}	29.67^{a}	1178.62^{a}	125.65^{a}	2.21^{c}	132.20^{abc}	55.80^{cd}	21.81^{bc}
	GO	8.20^{bcd}	33.59^{a}	517.49^{bc}	166.96^{a}	9.08^{c}	158.56^{ab}	58.42^{cd}	25.89^{ab}
	GO +Inoc	12.64^{a}	42.91^{a}	614.26^{bc}	136.79^{a}	5.89^{c}	187.69^{a}	79.92^{ab}	32.00^{a}
	BO	7.76^{cd}	28.01^{a}	474.30^{c}	153.39^{a}	9.73^{c}	158.04^{ab}	56.65^{cd}	23.47^{bc}
	BO +Inoc	10.93^{ab}	39.62^{a}	613.22^{bc}	122.43^{a}	8.56^{c}	141.60^{abc}	98.02^{a}	27.99^{ab}
	Bayovar	7.94^{cd}	32.83^{a}	500.81^{bc}	141.64^{a}	6.18^{c}	106.78^{bc}	38.03^{de}	21.80^{bc}
	Bayovar +Inoc	10.47^{abc}	47.11^{a}	659.13^{b}	154.43^{a}	3.64^{c}	143.72^{abc}	72.93^{bc}	24.03^{bc}
	TSP	8.03^{bcd}	26.79^{a}	157.73^{d}	134.79^{a}	93.38^{b}	94.40^{c}	29.68^{e}	22.91^{bc}
	TSP +Inoc	7.40^{de}	33.08^{a}	155.19^{d}	137.32^{a}	144.80^{a}	112.75^{bc}	45.14^{de}	23.43^{bc}
Clayey	Test	2.33^{c}	25.12^{a}	1300.12^{a}	23.97^{d}	2.11^{b}	409.56^{c}	275.67^{b}	43.97^{e}
	Test +Inoc	1.67^{c}	41.24^{a}	1330.28^{a}	52.66^{d}	1.80^{b}	664.74^{b}	308.88^{b}	49.23^{de}
	GO	13.05^{ab}	32.35^{a}	766.55^{b}	142.02^{c}	5.41^{b}	715.11^{b}	321.22^{b}	69.54^{bcd}
	GO +Inoc	14.10^{ab}	44.15^{a}	787.75^{b}	134.28^{c}	5.56^{b}	1047.78^{a}	434.88^{a}	114.84^{a}
	BO	13.83^{ab}	27.49^{a}	660.75^{b}	160.21^{bc}	6.16^{b}	681.26^{b}	294.08^{b}	70.98^{bcd}
	BO +Inoc	10.71^{b}	31.11^{a}	684.65^{b}	115.24^{c}	6.87^{b}	1024.74^{a}	444.25^{a}	90.81^{ab}
	Bayovar	13.92^{ab}	28.67^{a}	677.56^{b}	147.33^{c}	6.58^{b}	631.31^{b}	259.86^{b}	62.55^{cde}
	Bayovar +Inoc	13.85^{ab}	35.71^{a}	771.39^{b}	143.53^{c}	5.72^{b}	946.21^{a}	423.32^{a}	76.62^{bc}
	TSP	16.95^{ab}	31.88^{a}	385.28^{c}	225.50^{a}	56.53^{a}	564.07^{bc}	243.10^{b}	68.23^{bcde}
	TSP +Inoc	15.10^{ab}	41.30^{a}	374.19^{c}	208.82^{ab}	47.55^{a}	655.63^{b}	330.07^{b}	78.01^{bc}

*Test: non-inoculated control; GO: granulated organomineral; BO: branned organomineral; Inoc.: inoculated; TSP: triple superphosphate.**Same lower-case letter in the row does not differ at 5% probability level (Tukey's test) in each soil type. Dry mass (DM), soil available phosphorus (P) by Olsen extraction (P- Olsen), activity of acid phosphatase (ACI PHOS), alkaline phosphatase (ALK PHOS), β-glucosidase (β-GLUCOS).

Mehlich 1 are not indicated for phosphate rock P sources. P availability in the two soils was higher in the TSP treatment and with PSM inoculation in sandy soil (Table 1), showing the potential of solubilizing microorganisms to increase soil P availability.

Higher values of available P were observed in sandy soil compared to clayey soil, corroborating the results of Novais et al. (2007) with higher critical levels of P in sandy soils (Table 1). As TSP is a soluble phosphate and, therefore, more rapidly available in the soil (Korndörfer et al., 1999), treatment with this fertilizer resulted in a higher concentration of P in the soil in the first crop and, consequently, it increased the P availability to plants; thus, P incorporation into cellular structures results in an increase in the dry mass of shoot parts of the first crop, that was higher than other sources of P fertilizers (Figure 1 and Table 1). In line with this, the correlation of PUE and available P were stronger in sandy soil (r = 0.86) than in clayey soil (r = 0.76).

The increased effect of inoculation with TSP corroborated the results of other studies showing that inoculant is more effective in the presence of fertilizers (Oliveira-Paiva et al., 2020; de Sousa et al., 2020). The activity of acid and alkaline phosphatases in soil can be a good indicator of organic P mineralization potential of and biological activity (Margalef et al., 2017). Acid phosphatase activity differed significantly between P sources and inoculation treatments. Combined analysis of the three crop cycles revealed that, in sandy soil and with PSM inoculation, acid phosphatase and β-glucosidase activity was highest in the GO treatment, and that of alkaline phosphatase was highest in the BO treatment (Table 1). In clayey soil, high activity of acid and alkaline phosphatases was found in the Bayovar, GO, and BO treatments with PSM inoculation, and β-glucosidase activity occurred only in the GO plus inoculation treatment. These results differed statistically from those of the other treatments; these three sources provided less available P, which may have increased enzyme production in inoculation microorganisms in response to stimuli from the plants. Regarding organominerals, the existence of a carbon source can stimulate microorganisms activity (Zucareli et al., 2018).

PSM inoculation increased the enzyme activity in most treatments, which was expected, as soil enzyme activity is directly linked to the presence and origin of microorganisms (Tabatabai, 1994, Mendes and Reis Junior, 2004). Phosphatases are enzymes released by plants and microorganisms, which contribute to the cleavage of organic P, thereby releasing it into the soil solution to be absorbed by plants (Abou-Baker et al., 2011), and alkaline phosphatase is produced mainly by microorganisms. Approximately 80% of the P in Brazilian soils is organic, thus the role of phosphatases is crucial because organic P is not readily available to plants. Pinho et al. (2016) examined the activity of phosphatases in millet cultivated on latosol under greenhouse conditions and supplied with TSP, Bayovar, and GO, and they observed that inoculation of organomineral fertilizers with PSM contributed to increased cycling activity of available P in the soil, which corroborates our results.

Mendes et al. (2018) emphasized the importance of β-glucosidase in the early detection of soil changes in response to different management practices and its crucial role in improving soil physical properties, in addition to carbon cycling in soil organic matter, which may also influence soil P and N dynamics (Ndossi et al., 2020). Similar results were observed by Silva (2018), who evaluated enzyme activity in PSM-inoculated Yellow Red Acrisol on which sugarcane was grown, and one year after planting, acid phosphatase activity increased in soil fertilized with organominerals, TSP, and Bayovar; alkaline phosphatase activity increased in soils fertilized with organominerals, and β-glucosidase showed higher activity with organominerals and Araxá phosphate. Thus, soil enzyme activity is a potential indicator of soil quality due to its high sensitivity to external factors, compared to physical and chemical attributes, as well as for ease of evaluation (Utobo and Tewari, 2015). Soil biological attributes react rapidly to any changes in the environment and can therefore serve as indicators of soil quality and sustainability of agroecosystems (Schloter et al., 2018).

Sandy soils have low levels of OM, typically below 10 g kg^{-1}, and, in general, enzyme activity increases with increasing soil OM content, suggesting higher stability of enzymes adsorbed to humic materials (Marinari and Antisari, 2010; Oliveira et al., 2015). This influence is evidenced by the results presented in Table 1, showing that the enzyme activity in clay soil is higher than in sandy soil, regarding the three evaluated enzymes. In sandy soil, significant correlations were observed at 5% of probability between the production of dry mass of shoot parts and acid and alkaline phosphatases and β- glucosidase.

Conclusion

PSM inoculation may be used for increasing crop productivity and PUE. Furthermore, the potential for using alternative P sources such as GO, BO, and rock phosphates for improving crop production is also highlighted. However, future studies are required to evaluate the use of alternative P sources and the use of bioinoculants under field conditions and in other crops.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Diversity and distribution of arbuscular mycorrhizal fungi associated with Bambara groundnut (*Vigna subterranea* (L.) Verdcourt) in Benin

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Bambara groundnut, despite its attributes in providing protein to human, is facing soil fertility and degradation problems, always leading to little performances. Arbuscular mycorrhizal fungi (AMF) constitute a microorganism group used by many researchers to improve productivity of crops in poor soils. This study aimed to evaluate the distribution and diversity of AMF associated to Bambara groundnut in different agro-ecological zones in Benin. A survey was conducted through 20 villages chosen based on Bambara groundnut yield, cropping area and its production across five agro-ecological zones (from AEZ 1 to AEZ 5). Soil and root samples were collected to assess spore density and diversity, root colonization levels and soil chemical properties. Results revealed significant difference (p <0.0001) among agro-ecological zones in terms of density of AMF, which varied from 2825 to 5713 spores per 100 g of soil, depending on the AEZ. The highest density was recorded in the cotton zone in the northern Benin. The diversity of AMF also varied, depending on the AEZ. In total, 14 morphotypes belonging to five genera (*Glomus, Gigaspora, Acaulospora, Scutellospora* and *Diversispora*) were identified in the different studied zones with *Glomus* genus the most frequently recorded in all AEZ. Correlation tests among the different parameters have, in general, revealed that, the zones with the low rates of the different parameters had those with the highest frequencies of mycorrhization. It also appears that spore density did not correlate with diversity index, mycorrhization frequencies and intensities, but soil chemical parameters significantly did.

Key words: Bambara groundnut, mycorrhiza, agro-ecological zone, density, Arbuscular mycorrhizal fungi (AMF).

INTRODUCTION

Nutritional proteins are one of the major metabolism needs, which are rarely satisfied in some developing

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countries, especially by Africans. Indeed, in Africa, animal proteins are less available for people due to economic constraints.

Therefore, focusing on plant proteins becomes the preferred way to satisfy protein needs in Africa (Mazahib et al., 2013). Among protein-providing crops, legumes take a large part in supplying safe proteins in food systems by increasing proteins levels for human adequate nutrition (Vertès et al., 2015). Moreover, legumes have ability to fix atmospheric nitrogen, and then transfer it into, and fertilize the soil. Like other legume species, Bambara groundnut (*Vigna subterranea* (L.) Verd.) contains high level of lysine and plays an important nutritional role when it is eaten combined with cereals in rural peoples' diet (Massawe et al., 2005; Bamshaiye et al., 2011). Moreover, Bambara groundnut takes part in increasing phosphorus bio-availability in the soil (Ndiang et al., 2012; Touré et al., 2013; Gbaguidi et al., 2015).

Despite its attributes, Bambara groundnut always remains a neglected and underutilized species often scoring low yields in Benin (Dansi et al., 2012). In fact, the low crop low yields are caused by low cation exchange capacity, low sum of bases, mineral deficiency (nitrogen and mainly assimilable phosphorus) and low organic matter contents of the soil, which are not favourable for increased growth of many crops in Benin soils (Igue et al., 2013). However, Arbuscular mycorrhizal fungi (AMF) constitute a microorganism group mainly having beneficial effects on plant growth (by mobilization of some nutrients) and tolerance to many biotic and abiotic stresses after initializing symbiosis with plant roots (Smith and Read, 2008; Saïdou et al., 2012; Haougui et al., 2013).

Many researchers have reported use of endomycorrhizal symbiosis to improve productivity of many plants (Aboubacar et al., 2013; Usharani et al., 2014; Haro et al., 2015; Do Rego et al., 2015). In Benin, Houngnandan et al. (2009) evaluated indigenous *Glomus* species diversity in the clear forest of *Isoberlina doka* (Craib et Stapf) at Wari-Maro in Benin centre. Tchabi et al. (2008) and Balogoun et al. (2015) also have studied the diversity of endomycorrhizal fungi associated respectively with cotton and cashew tree. These different researches resulted in identification of some species of Glomeromyceta associated with many crops in Benin. To the authors' knowledge, no research was carried out in West Africa, especially in Benin focusing on AMF species and strains associated to Bambara groundnut. Therefore, the aim of this work was to study AMF community present in Bambara groundnut rhizosphere in the different agro-ecological zones in Benin.

MATERIALS AND METHODS

Study areas

Earlier researches showed that Bambara groundnut was grown in Benin with more production areas localized in the northern part of the country (Gbaguidi et al., 2016). Statistical data on Bambara groundnut production also showed that it is cultivated in six agro ecological zones in Benin, namely the Far North Benin (AEZ 1), Cotton region of North Benin (AEZ 2), Food crop region of South Borgou (AEZ 3), West zone of Atacora (AEZ 4), Cotton region of the centre (AEZ 5) and Bar lands (AEZ 6) (Figure 1). However, the production of the crop is not so much important across all sixth AEZ and therefore the study covered only five AEZs.

Survey in the field

Based on Bambara groundnut sowing date in the different areas, the survey was carried out in September 2018 in order to be sure to find Bambara groundnut in the field and especially at flowering phase. Two regions were selected per agro-ecological area. These regions were taken on the base of Bambara groundnut yield, cultural surface affected to this species and its production in these regions the last five years. The online database of FAO (Country Stat) has been used for obtaining this information on Bambara groundnut in each region and it helps for sampling place choice. Each sampling site was georeferenced with GPS.

Laboratory work

Density and diversity assessment of AMF, assessment of mycorrhization level and soil chemical characteristics determination were done at the Laboratory of Soil Microbiology and Microbial Ecology (LMSEM) of the Faculty of Agronomic Sciences (FSA) in the University of Abomey-Calavi.

Soil and root sampling from the fields

Per sampling site, composite samples have been made. Four soil samples were collected (20 soil samples) at four different points using a custom-made corer at 20-cm depth. All of these samples were mixed together and the mixture was used to obtain a composite sample. At each soil sampling site, four plants were also been pulled up for root sampling.

Spore extraction, counting and morphological identification

AMF spores were extracted using the wet sieving and centrifugation method of Gerdmann and Nicholson (1963). One hundred grams of composite soil sample from Bambara groundnut rhizosphere was weighed, mixed in water, stirred thoroughly, let decant before being sieved through serial sieves with different mesh sizes (2 mm, 250 µm, 150 µm, 63 µm and 50 µm). This process was repeated four times. The sediments from the different sieves were collected in tubes and finally centrifuged after adding sucrose solutions (5mL of 20 and 60% w:v) at 4000 rounds/min for 4min at 4ºC (Oehl et al., 2003). After this process, suspended spores were collected and counted under a binocluar magnifying glass using gridded Petri dish (5cm of diameter) to make the counting easier. Based on morphological criteria (colour, presence or absence of suspensor bulb and hyphae, etc.), some AMF morphotypes were identified, counted and grouped into genera by using identification and description keys from the International Collection of Vesicular and Arbuscular Mycorrhizal fungi (INVAM, http//www.invam.caf.wdu.edu). Spore density was expressed as numbers of AMF spores per 100 g of dry soil. AMF spore density in each sample was obtained by summing abundances of all species recorded in the sample.

AMF diversity assessment

Biological diversity indexes; that is, Shannon diversity Index (H')

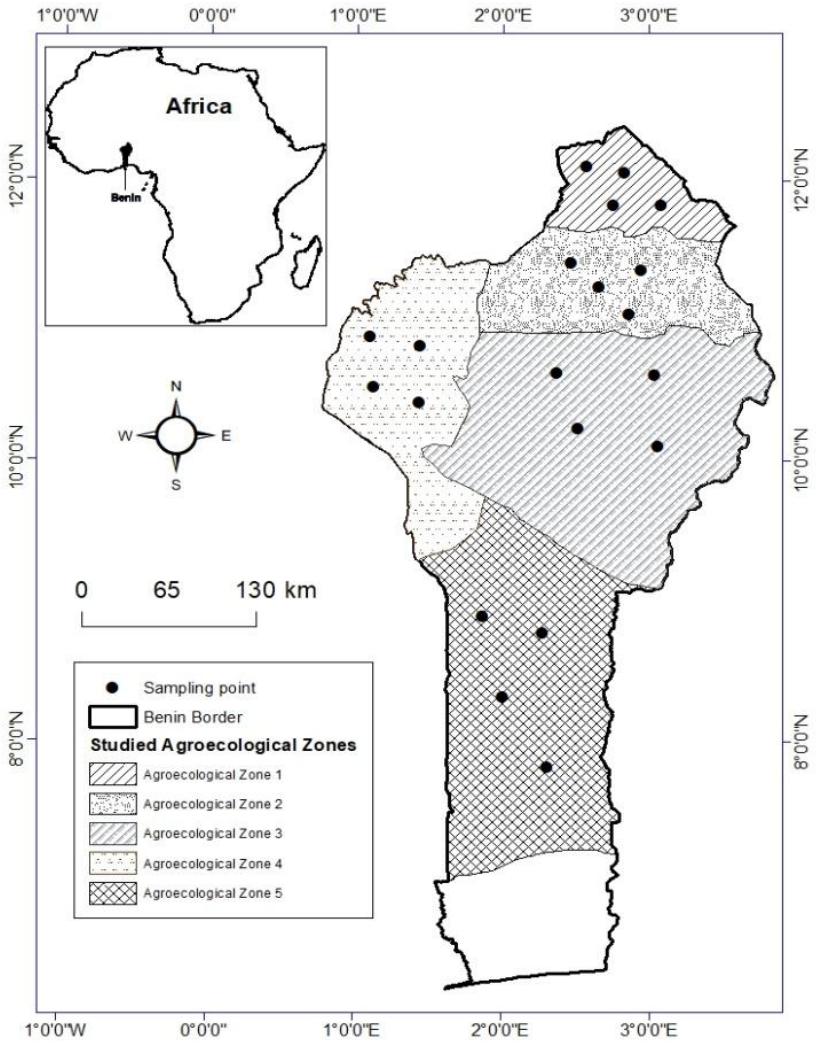

Figure 1. Benin geographic map with different sampling places.

(Shannon and Weaver, 1962), Simpson diversity Index (1-D) (Simpson, 1949) and Hill diversity index (1-Hill) were calculated for each agro ecological zone to evaluate AMF diversity.

Assessment of mycorrhization

Fresh roots of Bambara groundnut collected during the survey were used. After staining with trypan blue dye using the method described by Phillips and Hayman (1970), 20 fragments of fine Bambara groundnut roots were cut and placed on a glass slide with cover slip, and were observed with an optical microscope (XSP-BM-2CA, AliExpress) to observe different structures of AMF from different samples. Assessment of AMF colonization was performed using intersection method described by Trouvelot et al. (1986). The mycorrhization rates were assessed by two parameters of arbuscular mycorrhizal colonization namely: mycorrhization frequency and mycorrhization intensity.

Soil chemical analysis

From each Bambara groundnut field soil collected during the survey was subsampled (20), air dried and sieved for determination of chemical properties including the pH by potentiometric method in water (soil: water ratio 1:2.5) using a digital pH meter, organic carbon (C) by the Walkley–Black (1934) method, total N by Kjeldahl method and assimilable P by Bray I method.

Statistical analysis

One-way Analysis of Variance (ANOVA) was performed to assess the effects of agro-ecological zone (AEZ) on AMF spore density and diversity parameters, but also soil chemical parameters. When significant differences ($P < 0.05$) were found, post hoc comparisons of means among AEZ were made using a Student-Newman–Keuls' test. This ANOVA was performed with non-transformed data after ensuring conformity of these data with ANOVA assumptions. Furthermore, a Factorial Component Analysis (FCA) was performed in order to characterize different zones with identified spore genera. For choosing axes, the two principal's components must present more than 50% of the total information. Additionally, a Pearson correlation test was performed among all of the studied parameters in order to determine the relationships between them. All statistical

Table 1. Total AMF spore density and specific density by colour.

Studied factor	Modality	Density (number of spores/100g of soil)	Spores density by colour			
			Black	Brown	White	Yellow
AEZ	AEZ 1	5175.94 ± 46.68^{b}	3144±56.26^{a}	777.5±7.37^{b}	1142.94±13.82^{c}	111.5±0.71^{a}
	AEZ 2	5713.00 ± 34.48^{a}	3020.25±65^{a}	1180.06±9.52^{a}	1400.25±18.9^{b}	112.31±1.04^{a}
	AEZ 3	5169.81 ± 21.36^{b}	2855.19±4.61^{b}	333.94±9.96^{c}	1945.75±9.12^{a}	34.94±0.51^{d}
	AEZ 4	2825.63 ± 27.85^{d}	1861.56±35.34^{d}	316.25±3.37^{c}	543.81±4.17^{e}	104±2.61^{b}
	AEZ 5	3248.13 ± 53.16^{c}	2347.94±74.35^{c}	223.94±2.07^{d}	611.31±10.74^{d}	64.94±1.5^{c}

AEZ: Agro-ecological zone. Means with the same letters are not significantly different (*P*>0.05) based on Student Newman-Keuls test.

Table 2. Specific AMF spore density by size.

Studied factor	Modality	Spores density by size			
		250 µm	150 µm	63 µm	50 µm
AEZ	AEZ 1	154.38±1.71^{b}	463.44±9.87^{c}	2641.19±45.59^{c}	1913.94±41.83^{a}
	AEZ 2	269.81±4.95^{a}	805.25±17.28^{a}	3169±17.01^{b}	1468.81±26.43^{b}
	AEZ 3	161.94±3.84^{b}	536.38±9.85^{b}	3533.69±26.97^{a}	937.81±8.87^{c}
	AEZ 4	107.81±0.74^{c}	361.94±4.56^{d}	1575.56±38.97^{d}	780.31±12.59^{d}
	AEZ 5	104.13±1.14^{c}	534.13±12.23^{b}	1638.5±18.27^{d}	971.38±33.58^{c}

AEZ: Agro-ecological zone. Means with the same letters are not significantly different (*P*>0.05) on the basis of Student Newman-Keuls test. Different letters represent SNK groups ranking.

analyses were carried out using SAS software version 9.2.

RESULTS

Density of spores associated to Bambara groundnut in different agro-ecological zones

Analysis of variance results showed that all of the recorded mean spore densities were significantly different (p <0.001) among surveyed AEZs (Table 1). In different Bambara rhizosphere soil samples, important spore densities were recorded. The highest spore density (5713 ± 35 spores per 100 g of soil) was recorded in AEZ 2 while AEZ 4 has recorded the lowest spore density (2826 ± 28 spores per 100g of soil). Thus, the ANOVA results showed that the mean spore density recorded was significantly different (p <0.001) among different surveyed AEZs. Additionally, when identification criteria were considered, it appeared that black spores were more frequent compared to other coloured spores; and the AEZ 1 recorded the highest black spore density (3144 ± 56spores per 100g of soil) followed by AEZ 2 (3021 ± 62 spores per 100g of soil) (Table 1). Based on spore size, it generally appeared that spores with small size were most abundant. It was noticed that spore size of 63µm showed the highest spore density compared to other spore sizes (250µm, 150µm and 50µm); and the AEZ 3 had recorded the highest spore density of 63µm (3534 ± 27spores per 100g of soil) followed by the AEZ 2 (3169 ± 17 spores per 100g of soil (Table 2).

AMF community composition

In the Bambara rhizosphere studied, 14 mycorrhizal fungal morphotypes have been identified according to morphological criteria (colour, presence or absence of suspensor bulb and hyphae, etc.). After identification of these morphotypes based on the INVAM identification key, results indicated that the AMF belonged to five genera (*Glomus, Gigaspora, Acaulospora, Scutellospora and Diversispora*), four families (Glomeraceae, Acaulosporaceae, Diversisporaceae and Gigasporaceae) and two (02) orders (Diversisporales and Glomerales), all in Glomeromycetes Phylum. Relative abundance of these genera broadly among AEZs showed that *Glomus* species are the most abundant genera recorded in all surveyed AEZs, and were represented by 66.82% of all recorded genera. Other genera found in surveyed AEZs were *Gigaspora, Scutellospora, Acaulospora* and *Diversispora* genera scoring 18.46, 8.25, 4.89 and 1.58%, respectively (Figure 2). Furthermore, analysis of this fungal distribution in the different AEZs indicated that species belonging to *Glomus* and *Diversispora* genera are mostly recorded in AEZ 1 and AEZ 4, while species

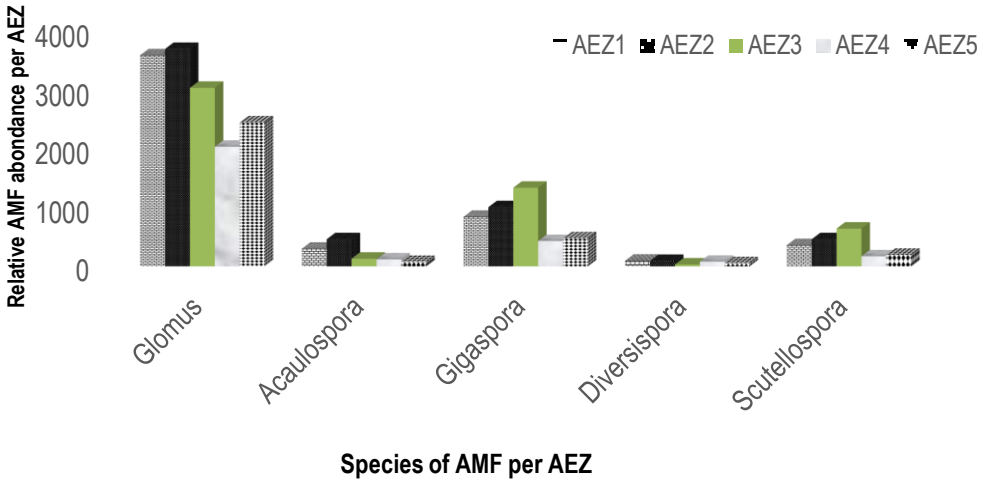

Figure 2. Relative AMF genera abundance in all surveyed zones in Benin.

of *Gigaspora* and *Scutellospora* genera were most common in AEZ 3 (Figure 3). All identified genera were found in AEZ 2 and AEZ 5.

AMF diversity

Statistical analysis showed that for all diversity indexes, there were significant differences ($p<0.0001$) among AEZs (Table 3). Shannon index (H') indicated that the AMF species were most diverse in AEZ 4 based on its highest index value (1.46) as compared to AEZ 3, which recorded the least species diversity (Table 3). When Simpson index (1-D) is considered, it appeared that AEZ 2 showed the most diverse species with the highest Simpson index (0.677) and AEZ 5 showed the least species diversity (0.533). This is true because H' index considered small populations, whereas the 1-D index considered larger populations. It was indicated earlier that AEZ 4 was a less abundant zone compared to AEZ 2, which was the most abundant zone in term of spores. The Hill index (1-Hill) also showed that all of surveyed zones are mainly diverse, like both previous diversity indexes (Table 3).

Assessment of natural mycorrhization level

As shown in Figure 4, there was a significant ($p<0.0001$) difference amongst the studied AEZs in terms of the mycorrhization frequency. The highest frequency (45.63%) was recorded in AEZ 1, compared to other AEZs. There was no significant difference amongst other AEZs and the lowest rate of mycorrhization frequency was recorded in AEZ 4 (17.25%). In terms of mycorrhization intensity, there were significant differences amongst AEZs; and the AEZ 3 recorded the significantly highest value (8.06%), while the lowest rate was from AEZ 4 (2.39%).

Chemical characteristics of soil from surveyed agro-ecological zones

The results showed significant differences ($p<0.001$) amongst the different AEZs based on studied soil parameters (Table 4).

In general, soils from different surveyed zones have relatively low pH (between 5 - 6.5 in all of the surveyed zones), poor in nitrogen (%N < 0.075% in all of surveyed zones) and phosphorus (P < 40 ppm in all of surveyed zones) according to Baize (2000) classification guideline. Furthermore, AEZ 2 had the recorded highest level of studied parameters; that is, Carbon (0.63%), Nitrogen (0.063%) and Phosphorus (29.50 ppm) but was third in pH level as compared to other zones (Table 4). Lowest levels of chemical parameters of soil were mostly recorded in AEZ 1.

Assessment of relationships between all of studied parameters

In AEZ 1, there was a significant positive correlation between Nitrogen and parameters like mycorrhization frequency ($r = 0.958$), Hill index ($r = 0.974$) and Simpson index ($r = 0.999$) (Table 5). This means that increasing of Nitrogen level in soil will induce enhancement of mycorrhization frequency and also AMF diversity. Additionally, it appeared there was a positive significant correlation between all of the diversity indexes (Table 5).

For AEZ 2, there was a significant negative correlation ($r = -0.970$) between Nitrogen (N) and mycorrhization frequency (F%) as shown in Table 5. So, increasing of N

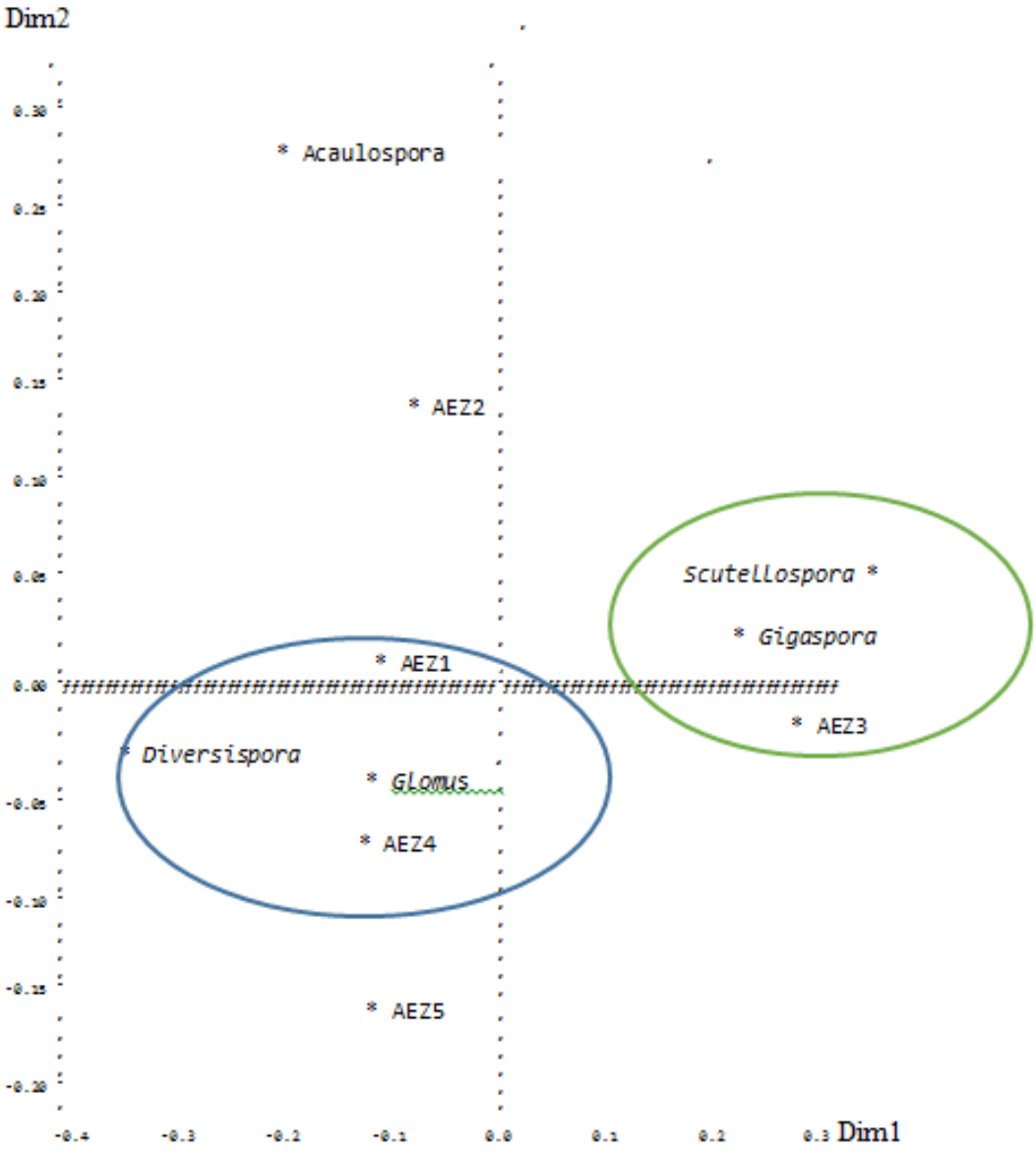

Figure 3. Typology of AEZ according to AMF species.

level by one unit led to 97% decrease of F%. Additionally, the Shannon and Hill indexes are positively correlated and both of them increased or decreased in the same way.

In AEZ 3, a significant positive correlation (p = 0.0285) between soil pH and spore density was recorded (Table 5). So increasing or decreasing soil pH could affect spore density in soil. Additionally, a significant negative correlation (r = -0.952) appeared between P and I%. So, a P increase leads to I% decrease in 95.2%. Positive correlation was found among diversity indexes.

For Zone AEZ 4, mycorrhization frequency is negatively correlated with Phosphorus (r = -0.99) and all of diversity indexes (Table 5). Increase of phosphorus level in soil leads to a decrease in root colonization frequency of mycorrhizae. Moreover, negative correlation between diversity index and mycorrhization frequency can suggest that fungal species which really mycorrhize Bambara groundnut roots were few present in this zone. We have earlier seen that this zone has acceptable diversity; but species which are most capable of forming mycorrhizal infections are rarely observed. If diversity declined, efficient fungi became abundant and could induce high mycorrhization frequency levels. Additionally, it appeared that Phosphorus positively affected the Shannon index and also all of diversity indexes are positively correlated (Table 5).

For AEZ 5, correlation results were in general not

Table 3. Different diversity index values in each of the zones.

Studied factor	Modality	H'	1-D	1-Hill
AEZ	AEZ 1	1.358 ± 0.02^{ab}	0.639 ± 0.007^{ab}	0.597 ± 0.013^{a}
	AEZ 2	1.402 ± 0.03^{ab}	0.677 ± 0.009^{a}	0.636 ± 0.016^{a}
	AEZ 3	1.149 ± 0.04^{c}	0.604 ± 0.013^{bc}	0.471 ± 0.033^{b}
	AEZ 4	1.469 ± 0.08^{a}	0.654 ± 0.033^{ab}	0.639 ± 0.043^{a}
	AEZ 5	1.240 ± 0.02^{cb}	0.553 ± 0.007^{c}	0.476 ± 0.176^{b}

AEZ: Agro-ecological zone. Means which have the same letter are not significantly different (*P*>0.05) based on the Student Newman-Keuls test.

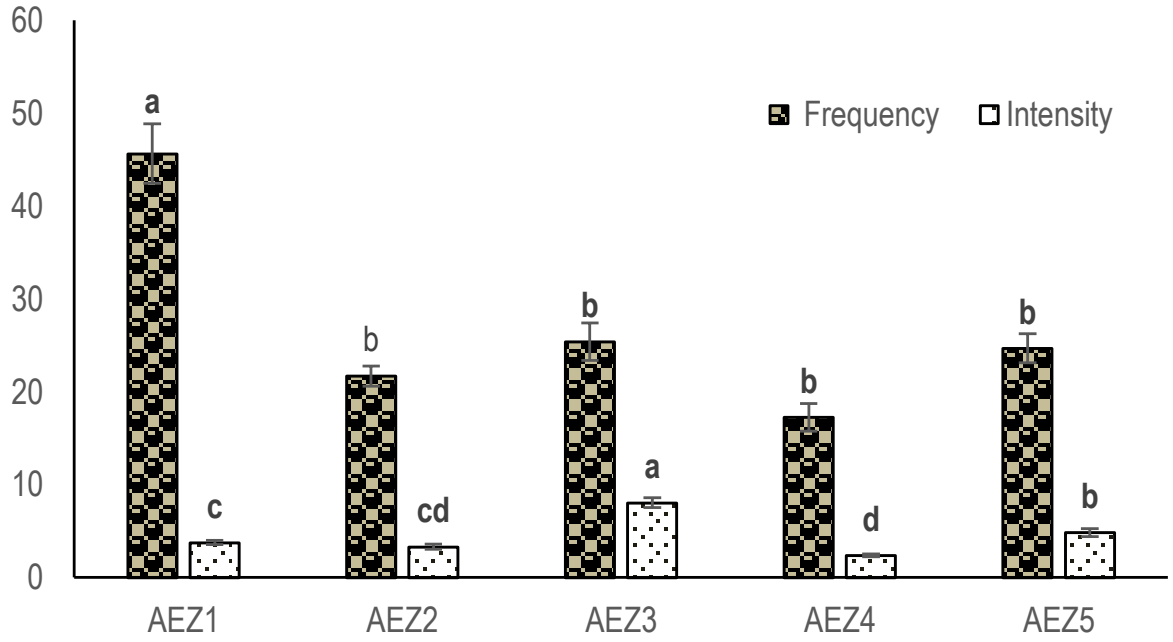

Figure 4. Mycorrhization percent frequency and intensity according to AEZ.

Table 4. Soils characteristics of surveyed zones.

Studied factor	Modality	pH	Phosphorus (ppm)	Nitrogen (%)	Carbon (%)
AEZ	AEZ 1	5.48±0.03^{d}	26.64±0.43^{b}	0.04±0.0009^{c}	0.35±0.006^{d}
	AEZ 2	5.82±0.03^{c}	29.50±0.39^{a}	0.063±0.0015^{a}	0.63±0.012^{a}
	AEZ 3	6.15±0.04^{b}	26.61±0.78^{b}	0.07±0.0014^{a}	0.54±0.008^{b}
	AEZ 4	6.25±0.09^{b}	21.98±0.38^{c}	0.068±0.0013^{b}	0.52±0.019^{b}
	AEZ 5	6.58±0.18^{a}	28.84±0.46^{a}	0.037±0.0016^{c}	0.45±0.015^{c}

AEZ: Agro-ecological zone. Means which have the same letters are not significantly different (*P*>0.05) on the basis of a Student Newman-Keuls test. Different letters represent the SNK groups ranking.

significant except mycorrhization frequency and Shannon index which have shown significant positive correlation (r = 0.986; p = 0.0135) (Table 5). It can be notice that only correlations statistically significant have been taken into account here.

DISCUSSION

Density of spores associated to Bambara groundnut

Most of the surveyed zones have a high level of spore

Table 5. Summary of different significant correlations between studied parameters in all surveyed zones.

Correlation of Pearson	AEZ 1	AEZ 2	AEZ 3	AEZ 4	AEZ 5
Nitrogen * Frequency	0.958*	-0.970*	ns	ns	ns
Nitrogen * Simpson	0.999***	ns	ns	ns	ns
Nitrogen * Hill	0.974*	ns	ns	ns	ns
Frequency * Phosphorus	ns	ns	ns	-0.990*	ns
Intensity * Phosphorus	ns	ns	-0.952*	ns	ns
Shannon * Phosphorus	ns	ns	ns	0.969*	ns
Density * pH	ns	ns	0.972*	ns	ns
Frequency * Simpson	0.960*	ns	ns	-0.956*	ns
Frequency * Shannon	ns	ns	ns	-0.987*	0.986*
Frequency * Hill	ns	ns	ns	-0.970*	ns
Shannon * Hill	0.991**	0.986*	0.995**	0.996*	ns
Simpson * Hill	0.967*	ns	0.996**	0.985*	ns
Shannon * Simpson	ns	Ns	0.983*	0.984*	ns

***: Very highly significant (p<1‰), **: Highly significant (p<1%) *: Significant (p<5%) ns: Not significant at 5%.

densities. These spore densities under Bambara groundnut range from 2826 to 5713 spores per 100g of soil. These recorded densities were less than those recorded by Bossou et al. (2019) under maize (6260 spores per 100 g dry weight soil) but higher than those by Johnson et al. (2013) under cowpea (202 ± 42 per 100 g dry weight soil), by Balogoun et al. (2015) in cashew orchards and by Houngnandan et al. (2009) in *Isoberlinia doka* habitats (237 to 258 spores per 100g of soil). These high spore levels could be due to different factors; and Brundrett (2009) indicated that the presence and natural distribution of glomales were controlled not only by floristic composition but also by environmental conditions. Additionally, legumes had an ability to promote development of fungal propagules by releasing some exudates in their rhizosphere, which favoured development and increase of microorganisms including mycorrhizal fungi (Scheublin et al., 2004). Indeed, a crop species can directly influence mycorrhizal fungal spore's abundance and composition (Eom et al., 2004; Lovelock et al., 2003). The results in the current study have shown that the largest spore amounts were recorded in AEZ 2, which is a cotton cropping area. In this part of Benin, the land-use system is mostly an agroforestry system in which many forest species such as *Vitellaria paradoxa*, *Parkia biglobosa* and *Tamarindus indica* (Gnangle et al., 2012) were kept in fields during land occupation. Indeed, species such as *Parkia biglobosa* and *Tamarindus indica* are also legumes harbouring in their soil and root habitat significant fungal flora that increase in population over many years; and these floras are beneficial for subsequent crops (Guissou et al., 1998). In addition, the cropping fields constitute a continual rotating environment where involved crops promote, with times, a high spore's abundance (Houngnandan et al., 2009). Furthermore, in the current study, zones where high spore densities were found are mostly characterized by sandy or sandy loamy soils.

This could also explain high densities recorded in these zones because sandy soils are said to favour high glomales populations (Dalpé, 1989). Ferruginous soils on sandstone and the presence of many rocks and concretions (in AEZ 4) as well as heavily degraded soils (in AEZ 5) have limited fertility and may be the cause of low spore densities in these soils compared to others zones. It is also important to note that the samples were taken during the flowering period. Knowing that the crops establishment is done during the raining period, the unimodal climate (a long dry season followed by a long rainy season), and the period from sowing to flowering associated with favourable environmental conditions (moisture and presence of the roots of the plant) could have allowed an activation and multiplication of spores that could explain the high densities recorded. Indeed, Bohrer et al. (2003) reported that spores number is higher in the soil exposed to relatively long water stress conditions (dry season) thanks to the production of spores.

Furthermore, small-sized spores are most abundant compared to those with large size in all surveyed zones. These results are supported by research findings of Bossou *et al.* (2019) and Johnson et al. (2013) who indicated that AMF spore densities were proportionally inverse with respect to their sizes. These study results are consistent with findings from Bossou et *al.* (2019) who showed a largest abundance of black spores followed by white spores in all surveyed zones. However, these results are in opposite with findings from Johnson

et al. (2013) who reported that black spores became first in terms of density but followed by brown spores. These different results could be explained due to differences in plant species, which were not the same in these different studies areas.

AMF diversity

The fourteen different morphotypes collected and identified based on the morphological characters belong to the genera of *Glomus, Gigaspora, Acaulospora, Scutellospora* and *Diversispora*. From these genera, *Glomus* and *Gigaspora* were more frequently recorded from all surveyed AEZs. The richness of these genera was higher than those obtained in Benin under different crop habitats including corn (Bossou et al., 2019), cashew (Balogoun et al., 2015) and *Isoberlinia doka* (Craib and Stapf) (Houngnandan et al., 2009). On the other hand, this genera richness was low as compared to that obtained by Tchabi et al. (2008) in the sub-Saharan savannahs (08 genera), by Johnson et al. (2013) under cowpea in all AEZs in Benin and by Diop et al. (1994) in Senegal (15 species). Results from the present study showed that the spore diversity of fungi varies relatively little from one AEZ to another, with *Glomus* the most abundant genus in all zones. Indeed, this predominance of *Glomus* was reported in AMF morphotypes in various tropical soils (Tchabi et al., 2008; Houngnandan et al., 2009) and in agricultural soils from temperate zones (Oehl et al., 2003; Mathimaran et al., 2005). Abundance of this genus in different agro-ecosystems might indicate that it is the most adaptable and available in different environments (Daniell et al., 2001; Brito et al., 2012). Diversity analysis has also shown that AEZ 1 and AEZ 4 were characterized by genera *Glomus* and *Diversispora* and AEZ 3 by *Gigaspora* and *Scutellospora.* This might indicate that some genera are typical to each zone. This is an important aspect to consider for the determination of the type of inoculum appropriate for each zone.

Mycorrhization frequency and intensity

Mycorrhization frequencies and intensities varied amongst zones. The area recording the highest mycorrhization frequency had neither a good infection level nor the highest levels of soil chemical parameters. At the same time, the area with the highest levels of spore density and different soil chemical parameters recorded low mycorrhization frequency and intensity. This could indicate that low levels of certain soil parameters (nitrogen, carbon, phosphorus and pH) favour the colonization capacity of Bambara groundnut roots by the fungal species present. Indeed, these results corroborate a previous report from Houngnandan et al. (2009) who showed that plots with low phosphorus levels were those with high frequencies of mycorrhization. Conversely, Liu et al. (2012) have shown that high levels of Nitrogen and Phosphorus have reduced Glomeromycetes populations in the soil. There is a very weak correlation between density, frequency and intensity of mycorrhization. It is said that living spores of AMF present in the soil may not function as propagules, but they may be quiescent (inactive because soil conditions are unsuitable) or have an innate period of dormancy-mechanisms, which may help them survive during periods of adverse soil conditions (Brundrett, 2009). In addition, soil parameters might diversely influence establishment of the symbiosis between the mycorrhizal fungi and Bambara groundnut roots.

Correlation between different studied parameters

There is much evidence supporting the hypothesis of a large and diversified influence of soil properties on AM fungi (Sano et al., 2002; Johnson et al., 2005; Mechri et al., 2008; Gryndler et al., 2009). It appeared that AM species may survive and function well within a range of soil and environmental conditions. In the current study, almost no significant correlation was found between soil chemical parameters and spore density, with the exception of soil pH in AEZ 3, where positive correlation with density occurred. Tchabi et al. (2008) reported similar results showing spore production increase with soil pH. In contrast, Houngnandan et al. (2009) found correlation amongst most parameters and indicated a negative correlation not only between Phosphorus, Nitrogen and Soil carbon with spore density but also between mycorrhizal frequency and spore density. In an earlier report, Subramanian et al. (2006) have already indicated that application of Phosphorus can influence spore production either positively or negatively.

In the current study, there was no relationship between mycorrhization frequency and intensity. Indeed, the zone with the highest frequencies did not necessarily have strong intensities of mycorrhization and vice versa. However, negative correlation was denoted between mycorrhization frequency or intensity with diversity index. These might stipulate that efficiency of mycorrhization depends on the diversity of species or genera present in the plant rhizosphere. In this study, negative correlations between Nitrogen and Phosphorus with the frequency and intensity of mycorrhization were found. This might show that increase occurrence of some soil parameters may lead to reduction of mycorrhization level. Furthermore, species diversity was positively correlated with some soil parameters (Nitrogen and Phosphorus). This could stipulate that certain species have preferences and tolerance levels in terms of physico-chemical soil parameters; and this could lead to their appearance or disappearance in different soils. These results were opposite to those of Liu et al. (2012) who did not indicate

any relationship between soil parameters and diversity. Also, Johnson et al. (2013) found that AMF diversity indexes are negatively correlated with both available and total Phosphorus. All these results may be natural, because AMF are living organisms and have different preference or tolerance levels to some environmental factors.

Conclusion

The present study has shown that there is an important spore density of AMF associated with Bambara groundnut that differs significantly from one AEZ to another. In addition, it appeared that soil parameters diversely influence both mycorrhization frequency and intensity, but also AMF diversity. Also, it should be noted that having high spore densities does not imply higher levels of symbiosis. Furthermore, it appeared that AEZ 1 and AEZ 4 were characterized by *Glomus* and *Diversispora* genera; but *Gigaspora* and *Scutellospora* characterized AEZ 3. So it would be appropriate that further studies be carried out in order to confirm these results. Also, achievement of molecular characterization will allow most accuracy in species identification. This will enable an evaluation of effectiveness and efficiency of different collected species in order to develop some ecological technologies in Bambara groundnut fertilization based on AMF.

CONFLICTS OF INTERESTS

The authors have not declared any conflicts of interests.

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Full Length Research Paper

Antibody responses after Oxford AstraZeneca (Covishield) vaccine among healthcare workers in Dhaka Medical College, Dhaka, Bangladesh

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Oxford AstraZeneca (Covishield) vaccine is the 1st vaccine administered in Bangladesh to prevent the spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The antibody response after 1st and 2nd doses of this vaccine was assessed in health care workers of Dhaka Medical College Hospital, Bangladesh. Blood sample was collected from healthcare workers (teachers, clinicians and medical staff) after 28 days of 1st vaccination and 14 days after 2nd vaccination. Quantitative post-vaccination antibody responses were measured using the chemiluminescent immunoassay, ADVIA Centaur (Siemens, Munich, Germany) SARS-CoV-2 IgG (COV2G) assay (output index was ≥1.00). Vaccine related antibody was found in 126 (41%) participants after 1st dose of AstraZeneca vaccine. After 2nd dose of vaccine, reactive level of antibody was found in 172 (93%) participants. Antibody responses were significantly higher in previously infected participants compared to participants who had no history of previous COVID-19 after 1st dose (51.92±50.85 vs 23.67±41.07, p=0.001) as well as 2nd dose of vaccine (64.12±97.76 vs 35.04±64.84, p=0.001). No difference in antibody response was observed among participants with or without comorbidities. Oxford AstraZeneca Covishield vaccine induces a strong immune response after two doses of vaccination.

Key words: SARS-CoV-2, vaccine, comorbidities.

INTRODUCTION

Severe acute respiratory syndrome corona virus type 2 (SARS-CoV-2), which causes the corona-virus-disease-19 (COVID-19) beginning in 2019 in Wuhan, China has rapidly spread throughout the whole world (WHO, 2020). World Health Organization (WHO) has declared COVID-19 as a global pandemic on March 11, 2020 (Del et al., 2020). COVID-19 pandemic has widespread impact on health, including substantial mortality among older adults and those with pre-existing health conditions and impacts on the global economy, caused by physical distancing

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measures, with the greatest consequences for the most vulnerable in society (Zhou et al., 2020). Considering the rapid spread and high mortality of COVID-19, an effective vaccine is urgently needed to control this pandemic. Vaccines can play an important role in increasing population immunity, preventing severe disease, and reducing the ongoing health crisis (Li et al., 2020).

Multiple vaccines have been developed that offer protection against COVID-19 by generating immune responses against the spike antigen of SARS-CoV-2. On the 8 of December 2020, the United Kingdom (UK) started its national vaccination programme with the Pfizer–BioNTech BNT162b2 vaccine (Medicine and Health Care Products, 2020a) followed by the approval of the Oxford-AstraZeneca ChAdOx1 nCoV-19 vaccine, first used outside a clinical trial on the 4 of January 2021 (Medicine and Health Care Products, 2020b). Bangladesh has started its COVID-19 vaccination program in 7th February 2021 with the plans to administer more than 30 million doses over the next few months. The AstraZeneca-Oxford University vaccines, manufactured under license by the Serum Institute of India, had been primarily given to front-line health workers including doctors, nurses and hospital staffs.

Fewer immunogenicity data or comparative data for the Oxford AstraZeneca (Covishield) vaccine are available outside of clinical trials. No study regarding determination of immunogenicity after 1st and 2nd doses of AstraZeneca (Covishield) vaccine has been done yet in Bangladesh. So this study was designed to determine the antibody response among teachers, clinicians and medical staff after 1st and 2nd dose of Oxford AstraZeneca (Covishield) vaccine in Dhaka Medical College.

MATERIALS AND METHODS

Study design and participants

The study was designed to get preliminary idea about vaccine related antibody production. The study was performed in the Department of Microbiology, Dhaka Medical College, Dhaka, Bangladesh. The duration of the study was from February, 2021 to June, 2021. A total of 308 healthcare workers including teachers, clinicians and medical staff of Dhaka Medical College and Hospital, who have taken at least one dose of AstraZeneca Covishield vaccine, participated in this study. Participants who were reverse transcription polymerase chain reaction (RT-PCR) positive for SARS COV-2 infection and recovered were recorded using a data collection sheet. Co-morbidity history of diabetes mellitus and hypertension and history of hospitalization due to Covid-19 were also recorded.

Participants were given Covishield (AstraZeneca vaccine manufactured by Serum Institute of India) in two doses at 8 weeks interval. Side effects after vaccination were noted for all participants.

Blood sample was collected from each participant after 1st and 2nd doses of vaccination. First blood sample was collected after 28 days of 1st vaccination with a window period 7 days and second sample was collected 14 days after 2nd vaccination with a window period of 14 days. Antibody level from the serum was determined using ADVIA Centaur (Siemens, Munich, Germany) SARS-CoV-2 IgG (COV2G) assay, a chemiluminescent immunoassay intended for semi-quantitative detection of IgG antibodies to SARS- COV-2.

According to manufacturer's instructions, serum samples were considered reactive when the output index was ≥1.00 and nonreactive when the output index was <1.00.

All the participants provided written informed consent and the protocol was approved by ethical review committee of Dhaka Medical College.

Statistical analysis

All statistical analysis was performed using IBM SPSS Statistics Version 18 (IBM Co. NY, USA). All variables are presented as means or medians with standard deviation. Categorical variables are shown as numbers with percentages. Student's t test was used to compare between COVID-19 positive participants and non-infected participants. Statistical significance was defined as $p < 0.05$.

RESULTS

In total, 308 participants provided blood specimens and a completed questionnaire for this follow up. Among the participants, 197 (64%) were male and 111 (36%) were female. The mean age was 45.08 years (±9.2 years). Most (61.03%) of the participants were in between 30 and 50 years (Table 1).

In this assessment, 144/308 (46.75%) participants reported no significant medical history and 86 (27.92%) and 51 (16.55%) participants gave the history of hypertension and diabetes mellitus, respectively (Table 1). None of the participants developed complications like blood clotting or other serious adverse effects after vaccination.

Among 308 participants, all the participants gave blood sample after 28 days of 1st dose of AstraZeneca vaccine. After 14 days of 2nd vaccination 185 participants gave blood sample. After 2nd dose of vaccine antibody titre increased in 110 (59.45%) and reduced in 75 (40.54%) participants. Overall antibody titre after 1st dose and 2nd doses were 33.67 ± 46.70, 95% CI: 28.80- 38.65 and 35.40±64.84, 95% CI: 27.50-45.19, respectively.

Out of 308 participants reactive level of antibody was found in 234 (76%) participants after 1st dose of vaccination. Among them, 108 (35.06%) participants were previously COVID-19 positive confirmed by RT-PCR. So vaccine related antibody was found in 126 (41%) participants after 1st dose of AstraZeneca vaccine. After 2nd dose of vaccine out of 185 participants, reactive level of antibody was found in 172 (93%) participants (Table 2).

Out of 108 previously COVID-19 positive participants, blood sample was collected from 62 participants after 2nd dose of AstraZeneca vaccine. These 62 participants, all developed reactive level of antibody after 2nd dose.

In this study, out of 308 participants, 108 (35.06%) COVID-19 positive participants had mild to moderate

Table 1. Demographic status and post vaccination symptoms of participants.

Variable	Total	Percentage
Participants (n)	308	
Male	197	64
Female	111	36
Age (years)		
<30	10	3.25
30-50	188	61.03
>50	110	35.71
Medical history		
Hypertension	86	27.92
Diabetes Mellitus	51	16.55
Bronchial Asthma	10	3.25
Others	9	2.92
Post vaccination symptoms		
Fever	119	38.63
Body ache	133	43.18
Back pain	20	6.49
Vertigo	8	2.59
Headache	7	2.27
Cough	2	0.65
Weakness	3	0.97
Local pain	12	3.89
Allergy	2	0.64
Nausea	3	0.97
Joint pain	2	0.65

symptoms and 31 required hospital admission. These previously infected participants mounted greater antibody response after 1st dose of vaccine compared to participants who had no history of previous COVID-19 infection (51.92±50.85 vs 23.67±41.07, p=0.001) and 2nd dose of vaccine (64.12±97.76 vs 35.04±64.84, p=0.001) compared with previously non-infected participants. No difference in antibody response was observed among participants with or without comorbidities.

DISCUSSION

The ongoing COVID-19 pandemic caused by SARS-CoV-2 has resulted in remarkable mortality and morbidity globally (Krammer et al., 2021). Rapid vaccine-induced population immunity is a key global strategy to control COVID-19 pandemic. Vaccination programs must maximize early impact, particularly with accelerated spread of new variants (Ramasamy et al., 2021).

This study analyzes the antibody response after two doses of Oxford AstraZeneca COVID-19 vaccines in a well-defined group of hospital employees. Until now, limited data has been available looking at antibody response to either a single or double dose of BioNTech/Pfizer or AstraZeneca vaccine in comparison to natural infection or immune-naïve people (Baden et al., 2021).

The data presented by Parry et al., 2021 showed no antibody-response in 13% of individuals after a single dose AstraZeneca in elderly people. In this study, 24% participants were non-reactive after single dose of AstraZeneca vaccine. The reason of lower level of antibody after first dose of vaccination might be due to the fact that, in addition to geographical and ethnic variations, the present vaccine which was used here are produced in serum institute of India and maintenance of cold chain during and after transportation might have some role.

In this study, vaccine related antibody was found in 41% participants 4 weeks after 1st dose of AstraZeneca (Covishield) vaccine. In phases 1 and 2 randomised control trial in humans, one dose of ChAdOx1 nCoV-19 (Covishield) vaccine elicited a significant increase in IgG

Table 2. Antibody response after 1st and 2nd dose of vaccination.

Antibody response	First dose (%)	Second dose (%)
Reactive	234 (76)	172 (93)
Non-reactive	74 (24)	13 (7)

antibodies (91%, peaked by day 28) against SARS-CoV-2 spike protein, as measured by ELISA in 127 participants (Folegatti et al., 2020). Again, a cross-sectional study was done in India among 552 healthcare workers, where 79.3% showed seropositivity after the first dose (Covishield) vaccine (Singh et al., 2021). In another cohort study, 82.1% participants had a positive post-vaccine anti-spike IgG (Wei et al., 2021). The reported higher levels of antibody after 1st dose than the present study might be due to already infected persons were not segregated in those studies. But in the present study, 35% health care workers were already infected before vaccination and were RT-PCR positive and they were not included in calculating antibody response after 1st dose of vaccination. If they were included, total antibody reactivity would be 76%.

In our study after 2nd dose of vaccine, reactive level of antibody was found in 93% participants. Post-vaccination antibody responses were studied in health care workers from Oxford University Hospitals (OUH), four teaching hospitals in Oxfordshire, UK where 97.1% developed a positive anti-spike IgG antibody test by >14 days post-first vaccination and all healthcare workers develop antibody after AstraZeneca second vaccination (Eyre et al., 2021).

There is a broad range of side effects reported after vaccination with BioNTech/Pfizer or AstraZeneca, ranging from local symptoms to systemic post-vaccination symptoms such as fever or headache. These occurred in up to 68.5% of participants after the second dose of BioNTech/Pfizer and up to 58.7% after first dose of AstraZeneca (Menni et al., 2021). In this study, 38.63 and 43.18% participants reported with fever and headache, respectively. There is no correlation between the reported severity of post-vaccination symptoms and immune response measured by antibody levels. Müller et al. (2021) could not find such a correlation either after the first or second dose of BioNTech/Pfizer vaccine.

Menni et al. (2021) reported that the occurrence of side effects is more common in women and in younger people. The majority of participants in this study reported at least one post vaccination symptom, but these reports are not comparable between individuals.

According to world Health Organization (19 April 2021), the AstraZeneca vaccine is safe and effective in protecting people from the extremely serious risks of COVID-19, including death, hospitalization and severe disease. Based on the statement of the WHO Global Advisory Committee on Vaccine Safety on AstraZeneca COVID-19 vaccine, the risk of blood clotting events (Thrombosis with thrombocytopenia syndrome, TTS) with Vaxzevria and Covishield vaccines appears to be very low (WHO, 2021). Data from the UK suggest the risk is approximately four cases per million adults (1 case per 250000) who receive the vaccine, while the rate is estimated to be approximately 1 per 100 000 in the European Union (EU). In our study, no participants reported such type of symptoms after vaccination.

This study demonstrates that previously infected individuals mounted higher immune response after two doses of Covishield vaccines compared with those with no previous infection. Earlier studies reported higher antibody response to a single dose of vaccine in previously infected individuals (Rinott et al., 2021). The immune response to the vaccine after the first dose is substantially pronounced in individuals with pre-existing immunity and it is similar to the immune response developed after the second dose in individuals not previously infected (Krammer et al., 2021). The reason behind it might be due to the fact that memory cells are developed in individuals after natural infection and the memory cells are stimulated by the first dose of vaccine and produce more antibodies. In a related UK-wide study and a study from Israel, high levels of protection from infection following natural infection were observed that were comparable to those seen after two doses of vaccination without prior infection (Pritchard et al., 2021; Goldberg et al., 2021).

In this study, after 2nd dose of vaccination antibody titer were reduced from titer observed after 1st dose in 75 (40.54%) participants. Exact cause of this phenomenon is unknown. The reason of decreased antibody titer after 2nd dose than 1st dose might be due to antivector antibody was formed after 1st dose which neutralized the vaccine vector after 2nd dose. Another reason might be during the study period, high infection rate was prevailing in Dhaka city, the participants might have been contracted with the virus within few days prior to collection of blood after 2nd vaccination and the viruses might have neutralized the antibodies produced after 1st dose. Substantial level of antibodies was produced among the participants who received two doses of AstraZeneca (Covishield) vaccine.

Conclusion

The Oxford AstraZeneca (Covishield) vaccine produced

significant levels of antibodies among doctors and other staffs without any severe adverse events.

Limitations

Participants were not followed up whether they got infected with SARS-cov2 virus who developed reactive level of antibodies.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Assessment of antiplasmodial and immunomodulatory activities of endophytic fungal metabolites from *Azadirachta indica* A. Juss

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This study reported the antiplasmodial and immunomodulatory activities of extracts of endophytic fungi isolated from *Azadirachta indica*. The extracts were evaluated for potential *in-vivo* and *in-vitro* antiplasmodial activity using murine malaria models and microtechnique methods respectively. Immunomodulatory potentials of the extracts were assessed using cyclophosphamide-immunomyelosuppression in mice and hypersensitivity and hemagglutination reactions in rats, using sheep red blood cells (SRBC) as the antigen. The extracts inhibited the growth of *Plasmodium berghei in-vivo* and *Plasmodium falciparum in-vitro*. At 100 and 200 mg/kg oral doses, extracts of AIL1, AIL3, AIS1 and AIS2 recorded parasite inhibition of 95.62-97.87, 73.47-85.71, 83.11-98.63 and 94.31-100% respectively. *In vitro* inhibition of schizont maturation was concentration-dependent; extract of AIS2 at 1 mg/ml gave the highest activity (86.67%). A dose-related increase in the mean total white blood cell (WBC) and a significant $p < 0.001$ increase in neutrophil counts compared to the positive control was shown by the extracts at 100 and 200 mg/kg with a significant $p < 0.05$ increase in the hypersensitivity reaction to the SRBC antigen and an increase in the antibody titer value, to SRBC in rats. Thus, extracts of the isolated fungi exhibited immunomodulatory activity in both the innate and adaptive immune components of the immune system which correlated positively with the antiplasmodial activity.

Key words: *Azadirachta indica*, endophytes, antiplasmodial, immunomodulatory activity.

INTRODUCTION

Infectious diseases are a significant burden on the public health and economic stability of societies all over the world. They have for centuries been among the leading causes of death and disability and presented growing challenges to health security and human progress (Nii-Trebi, 2017). The emergence of new diseases, re-emergence of old diseases, development of resistant strains, side effects of some currently available drugs including toxicity and other undesirable effects in allergic patients are a few major problems that require immediate attention to combat these diseases with effective drugs of high therapeutic index (Nii-Trebi, 2017). Malaria is a life-threatening disease caused by parasites that are transmitted to people through the bites of infected female Anopheles mosquitoes. It is preventable and curable (WHO, 2001).

In 2019, the global tally of malaria cases was 229 million, an annual estimate that has remained virtually unchanged over the last 4 years and the disease claimed some 409 000 lives in 2019 (WHO, 2020). The World Health Organization (WHO) is calling on countries and global health partners to step up the fight against malaria, a preventable and treatable disease that continues to claim hundreds of thousands of lives each year (WHO, 2020).

Plants are still considered as one of the important sources of biologically active compounds in natural products research (Marcellano et al., 2017). Many plant species have been utilized globally in traditional healing and have been studied extensively for their pharmacological properties. Medicinal plants are also reported to host some fungi that are involved in the co-production of active metabolites (Alvin et al., 2014). Endophytes are microbes that colonize the living internal tissues of plants without causing any immediate disease symptoms or overt negative effects (Bacon and White, 2000).

Endophyte biology is an emerging field. The backbone of the endophytes is plants. The microorganisms like bacteria and fungi live in the tissues, leaves and roots of plants for some reason like habitat, food and protection (Preethi et al., 2021). The importance of studying the endophytes is their secondary metabolite production. The secondary metabolites have been isolated, and many studies have been carried out (Okoye et al., 2015, Ujam et al., 2020, 2021). These are biologically active compounds which include alkaloids, flavonoids, steroids and phenols (Preethi et al., 2021). The production of selected bioactive secondary metabolites by medicinal plants and by the endophytes provided for countless drugs selected as important therapeutic options innumerable diseases. The endophytes still have wide potential to be explored what could expand even more the phenomenal contribution to health and well-being. Considering the multi-resistant pathogenic micro-organisms and the producing capacity of antimicrobial metabolites by endophytes, it is indispensable for the search of antibiotic substances with new mechanisms of action, less toxic effect, and/or medication enhancement through these inexhaustible bioactive metabolites source (Demain and Sanchez, 2009).

Focus has also been on the exploration of secondary metabolites of fungi to obtain new bioactive molecules with potential applications in the medical, pharmaceutical, industrial, agricultural, and environmental fields (Okoye et al., 2015; Ujam et al., 2020, 2021). The discovery of novel antimicrobial secondary metabolites and bioactive compounds from different types of endophytic microorganisms is an important alternative to overcome the increasing levels of drug resistance to various pathogenic microorganisms (Godstime et al., 2014).

Azadirachta indica has been reported to be a fast-growing evergreen popular tree found in India, Africa, and America (Panjak et al., 2011). Extracts of the fresh leaves of this plant is reported in folk medicine for their antimicrobial, antimalarial, anthelminthic, antiviral, antiulcer actions etcetera (Orwa et al., 2009; Panjak et al., 2011). Several reports in recent years show that the endophytic fungi from neem produce several bioactive compounds (Li et al., 2007; Wu et al., 2008). This study was carried out to determine the acute toxicity, antiplasmodial and immunomodulatory activities of endophytic fungi metabolites isolated from *A. indica.*

MATERIALS AND METHODS

Plant material

Fresh leaves and stem of *A. indica* were collected from Agulu, Awka, Anambra State, South-East Nigeria and authenticated by a taxonomist, Mrs. Anthonia U. Emezie of the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Nigeria. A voucher specimen (PCG474/A/046) was deposited in the herbarium of the Faculty.

Experimental animals

Albino rats and mice of both sexes were procured from the animal facility of the Faculty of Veterinary medicine, University of Nigeria, Nsukka, Nigeria. Animals were handled in compliance with the National Institute of Health Guidelines for the care and use of laboratory animals (Pub. No.85-23, revised 1985) as approved by the Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Nigeria.

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Malaria parasite

Chloroquine-sensitive *Plasmodium berghei*, used in the study was obtained from the University of Nigeria, Nsukka, Enugu State Nigeria. Parasite's viability was maintained through a weekly passage in mice, by aseptic inoculation of a known amount of parasite into healthy mice every week.

Drugs

Cyclophosphamide (500 mg) was used as a standard immunosuppressant. Cycloxan® (Biochem–Pharmaceutical Industries Ltd., Mumbai). NONI® (10 mg/kg) and Artemether/ lumefantrine (120/80 mg) were used as standard immunostimulatory and antimalarial drugs respectively. Drug dilutions were made using sterile water for injection according to manufacturers' instruction.

Antigen

The antigen used in the work was fresh Sheep Red Blood Cell (SRBC) obtained from Animal Farm in Agulu, Awka, Anambra State. The SRBCs were washed three times in a large volume of pyrogen-free sterile normal saline by repeated centrifugation at 2500 rev/s for 10 min on each occasion. The washed SRBC was adjusted to a concentration of approximately 1×10^9 cells/ml and used for both immunization and challenge.

Endophytic fungal extracts

Information regarding the fungal endophyte extract samples (AIL1, AIL3, AIS1, and AIS2), isolation, identification, and extraction were previously described by Ujam et al. (2020).

Acute toxicity (LD_{50}) study

Acute toxicity study of the endophytic fungal extract was assessed in mice following Lorke's method (1983).

***In vivo* antiplasmodial assay**

The *in vivo* antiplasmodial activity of the extract against blood schizonts of *P. berghei* was evaluated following Peter and Reyley's curative test method (Peter and Anatoli, 1998). Donor albino mice were infected with chloroquine-sensitive *P. berghei* and rising parasitemia of 30% determined using thin blood film, the blood sample was collected using an EDTA bottle. The collected blood sample was diluted using phosphate-buffered saline (concentration of 137 mMNaCl, 10 mM Phosphate, 2.7 mMKCl, pH 7.4) such that 0.2 ml contained 10,000 infected red blood cells. To avoid variability in parasitemia, all the animals used were infected from the same source. Fifty adult albino mice were used to assess the antiplasmodial effect of the four endophytic fungi isolate (AIL1, AIL3, AIS1, and AIS2) extracts. Animals were inoculated with 10,000 *P. berghei* infected red blood cells and allowed for three days to establish infection. On day 3 the mice were randomized into Ten groups of 5 mice each such that the mean parasitemia levels of the groups are almost similar. Groups 1 and 10 served as the negative and positive controls and were given distilled water (10 ml/kg) and Artemether-lumefantrine respectively (0.3/0.2 mg/kg), while groups 2 and 3; 4 and 5; 6 and 7; 8 and 9 were treated with two doses (200 and 100 mg/kg) of AIL1, AIL3, AIS1, and AIS2 extracts respectively. Treatment was carried out once daily from day 1 to day 4. On day 4, blood was collected from the tail vein of the mice, and blood films made using a clean glass slide (Devi et al., 2000). The dry blood films were fixed with methanol and subsequently stained with 10% Giemsa for 10 min. They were washed with clean tap water and allowed to air dry. To ensure optimal film quality each film was duplicated. The slides were microscopically examined using x100 magnification in oil immersion (Model Olympus microscope) and the level of parasitemia was assessed. Treatment was continued from day 4 to day 7 and the above procedure was repeated (Dikasso et al., 2006). The percentage curative activity of parasitemia was calculated using the following formula:

$$\%\ \text{Cure for Parasitamia on day 3} = \frac{\text{Basal parasitamia count-Parasitemia count on day 3}}{\text{Basal parasitamia count}} \times \frac{100}{1} \quad (1)$$

$$\%\ \text{Cure for Parasitamia on day 7} = \frac{\text{Basal parasitamia count-Parasitemia count on day 7}}{\text{Basal parasitamia count}} \times \frac{100}{1} \quad (2)$$

***In vitro* antiplasmodial assay**

The antiplasmodial assay was carried out based on the *in-vitro* microtechnique method by Rieckmann et al. (1978) with little modification. The blood samples were collected from the malaria-infected subject by a certified medical laboratory scientist on the consent of the patient. The blood samples were screened for *Plasmodium falciparum* infections. One to two drops of the blood samples obtained were used to prepare thick and thin smears on clean slides. Prepared slides were stained for 10 min with 10 % Giemsa solution prepared in phosphate buffer of pH 7.3 and examined microscopically for parasites (Molta et al., 1992). The patient who had mono-infection of *P. falciparum* was included in the *in vitro* drug susceptibility test (WHO, 2001).

The fresh blood samples were centrifuged at 2000 rpm for 10 min, the blood plasma was removed and the blood pellets were suspended and washed thrice in a sterile Roswell Park Memorial Institute (RPMI) medium before use for parasite cultivation (Flyg et al., 1997). Artemether - Lumefantrine was used as the standard drug.The ethyl acetate extracts were first dissolved in DMSO and two-fold dilutions were carried out to prepare the following concentrations of 1.0, 0.5, 0.25, 0.125 mg/ml. A 1 ml volume of the extracts at various concentrations was first distributed into the plates after which 1 ml of culture medium was added into the well plates. The plates were incubated in a 5% CO_2 incubator (Thermo Fisher Scientific, Inc. Corporate Office is located in Waltham, MA,

USA) at 37°C for 24-30 h. After incubation, the contents of the plates were harvested and the red cells were transferred to a clean microscopic slide to form a series of thick films. The films were stained for 10 min in 10% Giemsa solution of pH 7.3. Schizont growth inhibition per 200 asexual parasites was counted in 10 microscopic fields. The control parasite culture freed from extracts was considered 100% growth. The percentage inhibition per concentration was calculated using the formula:

$$\% \text{ Inhibitio of schizont growth} = \frac{(\% \text{ parasitaemia in control wells} - \% \text{ parasitaemia of test wells})}{(\% \text{ parasitaemia of the control})} \times 100 \quad (3)$$

(WHO, 2001; Ngemenya et al., 2006)

Immunomodulatory assay

Preliminary evaluation of the immunomodulatory potential of the endophytic fungi extracts was carried out using the cyclophosphamide-induced immunosuppression method as previously reported by Ujam et al. (2021).

Delayed type hypersensitivity response (DTHR)

Hypersensitivity reaction to SRBC was induced in rats following the method reported by Gabhe et al. (2006) with modifications. SRBCs collected in Elsevier's solution, were washed three times with pyrogen-free sterile normal saline and adjusted to a concentration of 1×10^8 cells/ml for sensitization and challenge. Animals were divided into five groups of five animals each, one group served as control while the remaining served as experimental groups for the treatment with AIL1, AIL3, AIS1, AIS2 extracts. Animals were sensitized by injecting 0.1 ml suspension of 10% freshly prepared SRBCs (1×10^8 cells /ml) on days one and six. The experimental groups received 200 mg/kg of test extracts for eight days whereas the control group was administered with equal volume distilled water. On day 8, 2 h after giving the extracts, animals were challenged by injecting 0.1 ml of SRBC intradermally in the left hind footpad. The thickness of the footpads was measured using a micrometer screw gauge before the challenge and at 24 h after the challenge. The difference between 0 and 24 h values of footpad thickness was taken as a measure of DTH reaction and the mean percent edema was determined using the formula:

$$\% \text{ Edema} = \frac{\text{Mean final reading-Mean initial reading}}{\text{Mean initial reading}} \times 100 \quad (4)$$

Haemagglutination inhibition activity

The crude extracts of AIL1, AIL3, AIS1, AIS2 at 200 mg/kg were administered to the animals (test groups) orally for eight days and the vehicle (normal saline) was administered to the control animals. Each group consists of five rats and was immunized intraperitoneally by injecting 0.1 ml suspension of freshly obtained Sheep Red Blood Cells (SRBCs) (1×10^8 cells/ ml) on days 0 and 6. Blood samples were collected by a retro-orbital puncture on day 8 after the immunization. Antibody levels were determined by the haemagglutination technique. 5 µl of 10% SRBC suspension was added to 5 µl of two-fold diluted serum samples in a glass test tube. After 18 h of incubation, the highest dilution giving haemagglutination was considered as the antibody titer. The antibody titer was expressed in a graded manner, the minimum dilution (1/2) being ranked as 1, and mean ranks of different groups were compared for statistical significance.

$$\% \text{ Inhibition} = 100 - (a/b \times 100/1) \quad (5)$$

Where a = mean rank of the control group and b = mean rank of the treated group.

Statistical analysis

Results of the study were presented as mean ± Standard error of the mean (SEM) of sample replicate, n=5. Raw data were analyzed by one-way analysis of variance (ANOVA) followed by post-hoc Turkey's test and independent students't-test. Bivariate correlation was used to assess the relationship between the antiplasmodial and immunomodulatory activity. The analysis was done using the statistical package for Social Sciences (SPSS) version 20 for windows. Statistical significance was established when $p<0.05$. The graphical illustration was carried out using Microsoft Excel, 2010.

RESULTS

The extracts of the endophytic fungi coded AIL1, AIL3, AIS1, and AIS2 isolated from the leaves and stems of *A. indica* were examined in this study. The acute toxicity result showed the extract of AIL1 did not produce any mortality or obvious signs of toxicity at the first stage doses (10-1000 mg/kg) but was toxic at the second dosage levels (2000-5000 mg/kg) causing mortality of the animals (mice). However, the extracts of the fungi AIL3, AIS1, AIS2 showed no mortality of the mice even at 5000 mg/kg dose (Table 1).

The results of the curative *in vivo* anti-plasmodial study showed that at 100 and 200 mg/kg dose level, the extracts of AIL1, AIL3, AIS1, and AIS2 recorded percentage parasite inhibition of 95.62-97.87; 73.47-85.71; 83.11-98.63 and 94.31-100% respectively (Table 2). The fungal extracts significantly ($p<0.001$) inhibited the growth of the plasmodium parasite after 3 days of treatment. On the 7th day, of the curative experiment, the parasites were further inhibited and total clearance of the parasites was displayed by AIS2 extract. The result of the *in-vitro* antiplasmodial assay against *P. falciparum* is shown in Figure 1. The parasite growth decreases as the concentration of the extracts increases, the negative

Table 1. Acute toxicity/oral median lethal dose (LD_{50}) study of the fungal extracts.

S/N	Extract codes	D_0 (mg/kg)	D_{100} (mg/kg)	The oral median lethal dose (LD_{50}) in mice (mg/kg)
1	AIL1	1000	2000	1414
2	AIL3	10	5000	>5000
3	AIS1	10	5000	>5000
4	AIS2	10	5000	>5000

Do = Highest dose that gave no mortality, D_{100} = Lowest dose that produced mortality.

Table 2. *In-vivo* antiplasmodial activity of crude extracts of endophytic fungal isolates against *Plasmodium berghei.*

Animal groups	Endophytic fungal extracts/Doses (mg/kg)		Mean parasitemia count			% cure	
			B	D_3	D_7	D_3	D_7
1	AIL1	200	44.67 ± 0.33	1.00 ± 0.58[a]	1.00 ± 0.58	97.87	97.76
2		100	45.67 ± 2.03	2.00 ± 0.58[a]	2.00 ± 0.58	95.62	95.62
3	AIL3	200	49.00 ± 0.00	7.00 ± 3.46[a]	3.50 ± 0.00[*]	85.71	92.86
4		100	47.67 ± 2.03	13.50 ± 4.33[a]	8.50 ± 0.29[*]	73.47	84.27
5	AIS1	200	49.00 ± 0.58	1.56 ± 1.00[a]	0.67 ± 0.88[*]	95.74	98.63
6		100	45.00 ± 1.16	7.60 ± 0.88[a]	2.00 ± 1.00[*]	83.11	95.56
7	AIS2	200	47.00 ± 1.73	1.50 ± 0.29[a]	0.00 ± 0.00	94.98	100.00
8		100	47.00 ± 0.58	2.67 ± 0.67[a]	0.00 ± 0.00	94.31	100.00
9	AL	9.8	49.00 ± 1.16	4.50 ± 0.87[a]	3.50 ± 0.87	90.81	92.86
10	DW	10 ml/kg	43.00 ± 2.00	51.00 ± 1.16	59.00 ±3.84	-	-

Values are expressed as mean ± sem, n = 5, * indicates significant difference, *** = a ($p < 0.001$), ** = ($p<0.01$), * = ($p<0.05$) b = basal, d3 = day 3 and d7 = day 7 after inoculation. Dw =distilled water (negative control), AL = artemether-lumefantrine/20mg-120mg (positive control), - = no activity.

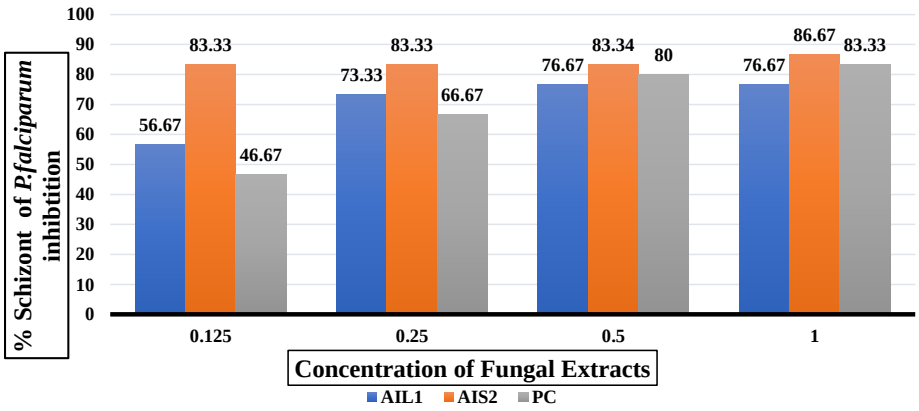

Figure 1. *In-vitro* antiplasmodial activity of the extracts of three endophytic fungal isolates against *P. falciparum.* Positive control= artemether-lumefantrine.

control (distilled water) had 100% parasite growth. Percentage inhibition of schizont maturation was a concentration-dependent extract of AIS2 at 1 mg/ml gave the highest activity (86.67%) which was comparable to

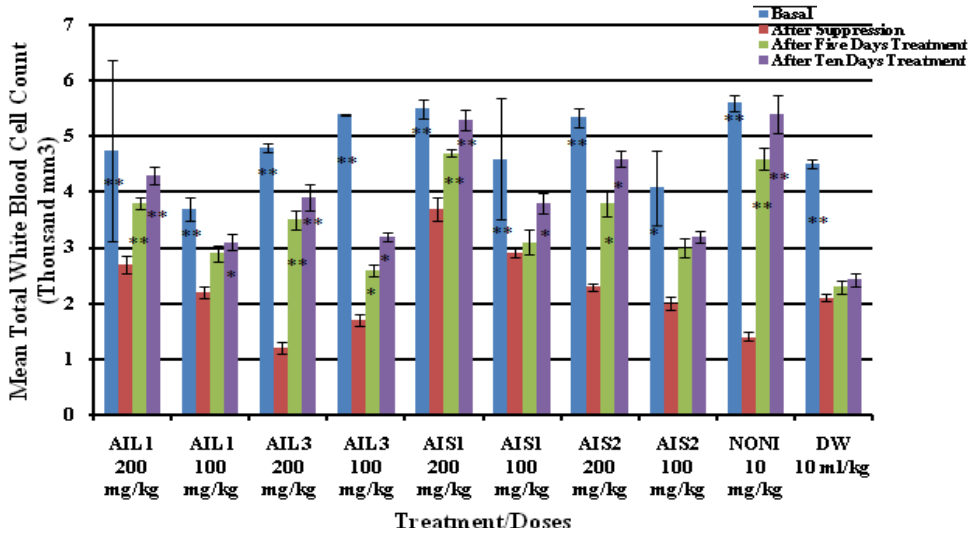

Figure 2. Mean total white blood cell counts (TWBC) for the curative experiment *p<0.05, *p<0.01 significantly different from WBC level at suppression.

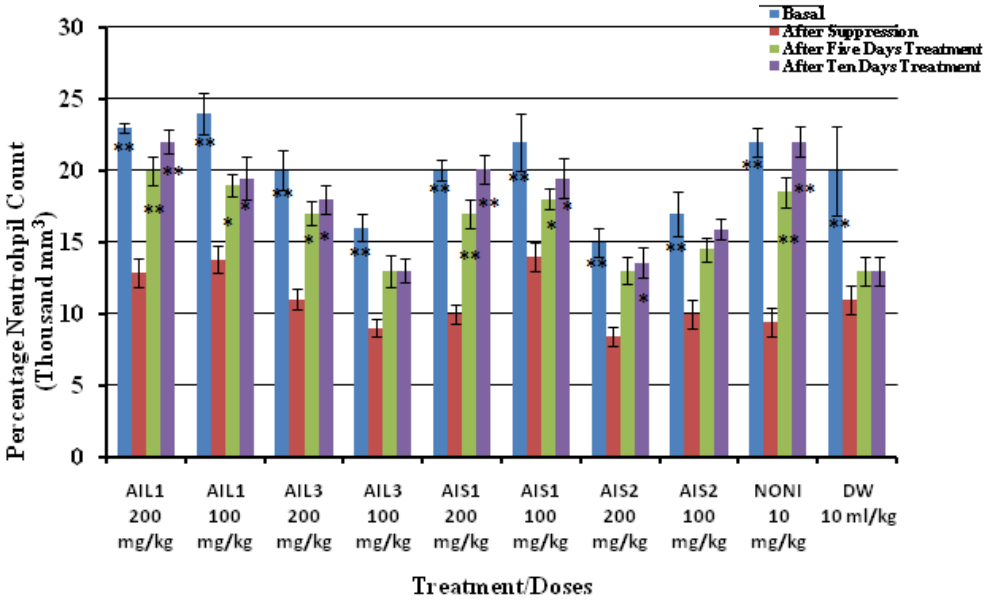

Figure 3. Mean differential white blood cell (Neutrophil) count for the curative experiment. *p<0.05, and **p<0.01 significantly different from the neutrophil level at suppression.

the activity of the positive control (artemether-lumefantrine) 83.33%.

The result of the curative immunomodulatory study of the AIL1, AIL3, AIS1, and AIS2 extracts is shown in Figures 2 and 3. The mean basal total white blood cell (TWBC) count of the mice ranged from 4.08- 5.60 × 10^3/ mm^3. Following induction of cyclophosphamide, the TWBC count significantly $p < 0.01$ decreased compared to the basal white blood cell count, ranging from 1.20 to 2.90 × 10^3 / mm^3 (Figure 2). After five days of treatment, extracts of AIL1, AIL3, AIS1, and AIS2 raised the TWBC count of the mice, mean TWBC count ranged from 2.30 to 4.70 × 10^3 /mm^3. Further increase of the total WBC counts was exhibited by the extracts after ten days of treatment, mean TWBC count was from 2.90 to 5.40 ×10^3/mm^3 (Figure 2).

The mean basal neutrophil count of all the groups in the experiment ranged from 14.90 to 24.00 × 10^3/mm^3 (Figure 3). After administration of cyclophosphamide the mean neutrophil count of the mice significantly decreased

Table 3. Mean total white blood cells counts (thousand mm^3) for the prophylactic experiment.

Treatment groups	Doses (mg/kg)	Basal blood sample	Blood sample after 10 days treatment	Blood sample after the suppression
AIL1	200	5.50 ± 0.15	10.60 ± 0.14^{aaa}	9.40 ± 0.14
AIL3	200	6.60 ± 0.15	8.60 ± 0.10^{aaa}	6.80 ± 0.10 ^{b}
AIS1	200	5.40 ± 0.10	7.80 ± 0.06^{aaa}	7.20 ± 0.04
AIS2	200	4.50 ± 0.16	9.79 ± 0.12^{aaa}	6.50 ± 0.06 ^{bb}
Distilled water	10 (ml/kg)	8.30 ± 0.13	10.30 ± 0.18^{aaa}	5.42 ± 0.16^{bbb}

Values were presented as mean ± Standard error of the mean of five (5) replicates (n=5). $^{a}P<0.05$ and $^{aa}P<0.01$ significantly different from basal WBC level. $^{b}P<0.05$ and $^{bb}P<0.01$ significantly different from WBC level on day 10. Distilled water = Negative control.

Table 4. Mean Percentage Neutrophils Counts for the Prophylactic Experiment.

Treatment groups	Doses (mg/kg)	Basal blood sample	Blood sample after 10 days administration of extracts	After suppression blood sample
AIL1	200	19.00 ± 0.71	25.00 ± 0.71^{aa}	24.00 ± 0.63
AIL3	200	17.00 ± 0.95	20.00 ± 1.22^{a}	17.00 ± 1.14
AIS1	200	22.00 ± 0.63	26.00 ± 1.22^{a}	24.00 ± 1.22
AIS2	200	16.00 ± 1.30	19.00 ± 1.05^{a}	15.00 ± 0.20 ^{b}
Distilled water	10 ml/kg	21.00 ± 1.22	22.60 ± 0.84	12.00 ± 1.14^{bbb}

Values were presented as mean ± Standard error of the mean (SEM) of five (5) replicates (n=5). $^{a}p<0.05$, and $^{aa}p<0.01$: significantly different from basal neutrophil level. $^{b}P<0.05$ and $^{bb}P<0.01$: significantly different from the basal neutrophil level on day 10.

compared to the basal blood sample, $p< 0.01$, ranging from 7.00 to 14.20 $\times10^3/mm^3$.Comparison of the neutrophil count of mice after 5 days with the counts after suppression showed that AIS1, AIL1, and AIL3 extracts and NONI (positive control) significantly ($p < 0.01$) increased the neutrophil count while AIS2 extracts showed an increase at ($p<0.05$). The Negative Control (DW) exhibited no significant increase ($p > 0.05$) (Table 3). After 10 days of treatment, AILI, AIL3, AIS1 extracts, and NONI further increased the neutrophil count of the mice ($p<0.005$). At 200 mg/kg the fungal endophyte extracts showed a higher increase compared to the 100 mg/kg dose level (Figures 2 and 3).

In the prophylactic immunomodulatory study, the mice were pre-treated with the extract for ten days and there was a significant increase $p< 0.05$ in the TWBC (Table 3). Extracts of AIL1, AIL3, AIS1, and ASI2 significantly raised the leucocytes of the mice after the ten days administration and inhibited the suppressive effect of cyclophosphamide on the TWBC (Table 3) and Neutrophil count (Table 4) of the mice. Percentage inhibition of cyclophosphamide effect by AIS2, AIL3, AIL1, AIS1 extracts were 32, 63, 75 and 87% respectively (Figure 4).

Oral administration of 200 mg/kg of AIL1, AIL3, AIS1, and AIS2 extracts caused a significant inhibition of Delayed-Type Hypersensitivity Reaction (DTHR) induced by SRBC in rats. Percentage inhibition of DTHR ranged from 66, 53, 76, 69 and 53% for AIL1, AIL3, AIS1 and AIS2 extracts respectively (Table 5). The Mean HI antibody titers in the sera of all the groups obtained after vaccination were expressed as HI titer (log^2) and presented in (Table 6). A positive correlation ($r = 0.893$) was established between the immunomodulatory and antiplasmodial activity and was also statistically significant at $p < 0.05$.

DISCUSSION

A. indica is found throughout the geographical area of southeast Nigeria is a medicinal plant used in folk medicine. In this present study, the antiplasmodial and immunomodulatory properties of four endophytic fungi (AIL1, AIL3, AIS1, and AIS2) isolated from *A. indica* were studied in swiss albino rat and mice.

All (100%) the endophytic fungi (AIL1, AIL3, AIS1, and AIS2) extracts at 10 to 1000 mg/kg (phase 1) produced no physical signs of toxicity in the mice 24 h after administration and became increasingly pronounced as the dose increased towards 5000 mg/kg body weight. However, AILI extract showed mortality at Phase II of the experiment. An acute toxicity test gives clues on the range of doses that could be toxic to the animal. It could also be used to estimate the therapeutic index (LD_{50}/ED_{50}) of drugs and xenobiotics (Rang et al., 2001).

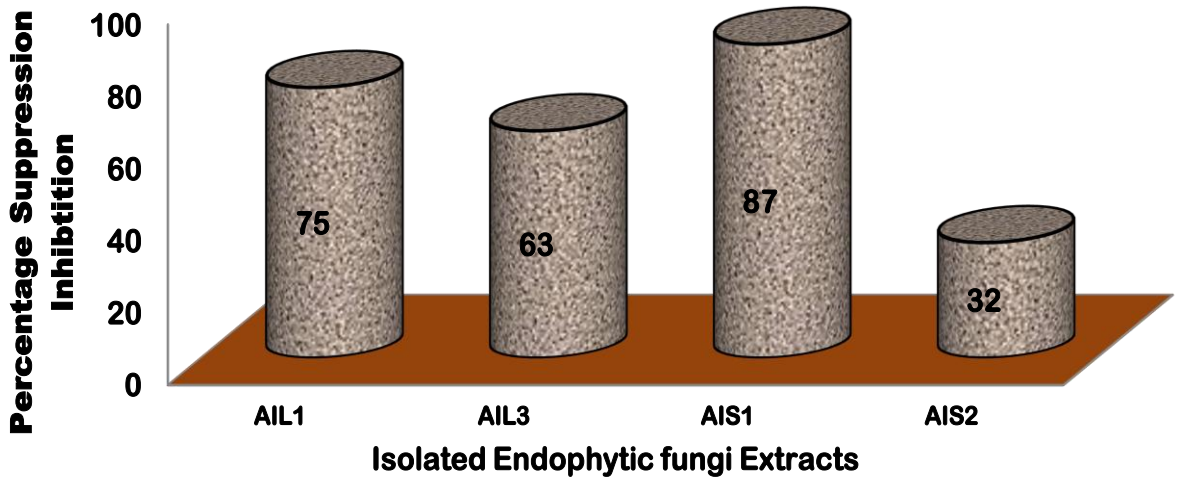

Figure 4. Percentage inhibition of the suppression by cyclophosphamide by the endophytic fungi extracts (prophylactic effect).

Table 5. Delayed type hypersensitivity response (DTHR) of the extracts in rats.

Treatment group	Dose (mg/kg)	DTHR edema (cm^3)	Inhibition (%)
AIL1	200	0.32 ± 0.03	65.59
AIL3	200	0.43 ± 0.10	53.76
AIS1	200	0.29 ± 0.04	68.81
AIS2	200	0.44 ± 0.12	52.68
Distilled water	10 ml/kg	0.93 ± 0.05	-

Values are expressed as mean ± Standard error of mean, n = 5 per group. DTHR: Delayed-Type Hypersensitivity Reaction, - = No inhibition.

Table 6. Antibody titre value of rats against different extracts.

Fungal extracts/ control	Doses (mg/kg)	Antibody titer value after seven days	Antibody titer	Haemagglutination inhibition (%)
AIL1	200	6 log2 ± 0.58	1.806	66.67
AIL3	200	4.5 log2 ± 0.29	1.355	55.56
AIS1	200	5.5 log2 ± 0.87	1.656	63.64
AIS2	200	5 log2 ± 0.58	1.505	60.00
Distilled water	10 ml/kg	2 log2 ± 0.00	0.602	-

Values are expressed as mean ± Standard error of the mean, n = 5 per group.

The mice were treated orally with endophytic fungi extracts and the route was chosen because of its sensitivity and rapid results. LD_{50} greater than 5000 mg/kg is thought to be safe (Erhirhie et al., 2018).

The present study demonstrates the antiplasmodial potentials of the fungal endophyte extracts against *P. berghei in vivo*. *P. berghei* has been used in studying the activity of potential antimalarials in mice (Kifle and Atnafie, 2020) and in rats (Pedroni et al., 2006). It produces diseases similar to those of human plasmodium infection (Peter and Anatoli, 1998; Shimada et al., 2019). Average percentage parasitemia decreased in the groups treated with the extracts comparable to the effect of the positive control (artemether-lumefantrine) while a daily increase of parasitemia was in the negative control group. *In vivo* models are usually employed in anti-malarial studies because they take into account the possible prodrug effect and probable involvement of the

immune system in the eradication of the pathogen (Waako et al., 2005; Mulisa et al., 2018).

The findings of this study agree with other studies carried out on the plant extracts of *A. indica* but the results from this fungal endophyte study showed a higher percentage of parasite inhibition. The *A. indica* stem bark and leaf extracts have been documented to have recorded about 56-87% and 51-80% parasitemia inhibition respectively (Akin-Osanaiye et al., 2013). Also, other researchers have reported earlier the antimalarial activity of different parts of *A. indica* tree (leaf, stem bark, and seed) against *P. berghei* and *P. falciparum* responsible for causing mammalian malaria (Deshpande et al., 2014).

In our *in vitro* experiment, AIL1 and AIS2 extracts inhibited the maturation of the schizont stage of the *P. falciparum* parasite, with percentage inhibition comparable to that of the positive control (Arthemether-lufamentrine) used in the study. Interestingly, AIS2 extract cleared the plasmodium parasites from the mice blood at treatment. The *A. indica* leaf extract has also been reported by Udeinya et al. (2008) to have both schizonticidal and gametocytocidal activities.

Basic research on natural substances with immunomodulating properties is performed by stimulating cells of the immune system including neutrophils, macrophages, T and B cells, NK cells (Yuandani et al., 2021). The present study demonstrates, for the first time, the immunostimulatory potential of the endophytic fungi of *A. indica*.

Cyclophosphamide administration induces acute and transient myelosuppression, primarily through damage to rapidly proliferating hematopoietic progenitors and their mature progeny leading to a decline in the number of peripheral blood cells (Sheeja and Kuttan, 2006). Cyclophosphamide induced immune-suppressive mice model was used because the dynamic and complex nature of the immune system in which a drug elicits its effect can be detected more reliably after immune challenge (Ahlmann and Hempel, 2016).

From our findings, injection of cyclophosphamide caused a significant drop of total white blood cells (TWBC) and the differential white blood cells (neutrophil) in mice. Nevertheless, treatment of the mice with the fungal endophyte extracts enhanced the proliferation of the TWBC, and neutrophil count was observed in groups of mice treated with the extracts compared to the group given the distilled water (negative control) group. The increase in the TWBCs count observed may have resulted from stimulation of leucocytosis by the extracts and enhanced production in the bone marrow (Okokon et al., 2004). The extracts worked in a dose-dependent manner. The present study demonstrated, for the first time, the immunostimulatory property of the extracts of endophytic fungi isolated from *A. indica.*

Pre-treatment of the mice with the fungal endophyte extracts increased its TWBC and neutrophil count and thus, protected them against cyclophosphamide-induced leucopenia. This indicates that the extracts possess the ability to prevent the occurrence of infection. Extracts of the endophytic fungi inhibited the delayed-type hypersensitivity reaction evoked by SRBCs in the rat. Hence, the extracts can modulate the cell-mediated adaptive immune response in rats as shown by the inhibition of DTHR. This indicated the stimulatory effect of the fungal endophyte on chemotaxis-dependent leucocyte migration. In the early hypersensitivity reaction, the antigen-antibody formed immune complexes, which are known to induce local inflammation with increased vascular permeability, edema, and infiltration of PMN leucocytes. Similar results have been obtained by other researchers (Dhasarathan et al., 2010; Eze et al., 2014).

Furthermore, the humoral immune response (HIR) to sheep red blood cells was measured by the haemagglutination (HI) test. The fungal endophyte extracts demonstrated an increase in the hemagglutination titer in mice. Antibody molecules that are secreted by plasma cells mediate the humoral immune response. This augmentation of the humoral response to SRBC indicated enhanced responsiveness of the macrophages and T and B lymphocyte subsets involved in antibody synthesis (Gabhe et al., 2006, Kaur et al., 2014). The immunoregulatory properties of the antibody have been recognized since the earliest passive immunization experiments, and the potential to modulate the immune response by deliberate immunization with antigen bound by antibody has been demonstrated in numerous instances over the decades (Brady et al., 2000; Alber et al., 2001; Antoniou and Watts, 2002; Rafiq and Clynes, 2002). The extracts of the endophyte fungi were found to have a significant immunostimulant activity on both the specific and non-specific immune mechanisms. From the positive significant correlation between antiplasmodial and immunostimulatory activities displayed by the fungal endophyte extract, it could be inferred that the activities shown by these fungal extracts were influenced by immunomodulatory property.

Conclusion

The extracts of the isolated fungal endophytes showed antiplasmodial and immunomodulatory activities. They modulated both cellular and humoral immunity. There was a positive correlation between antiplasmodial and immunostimulatory activities displayed by the fungal endophyte extract. Consequently, plant immunomodulators demonstrating therapeutic and immunomodulatory mechanisms of action possibly will be a perfect target for drug development.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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