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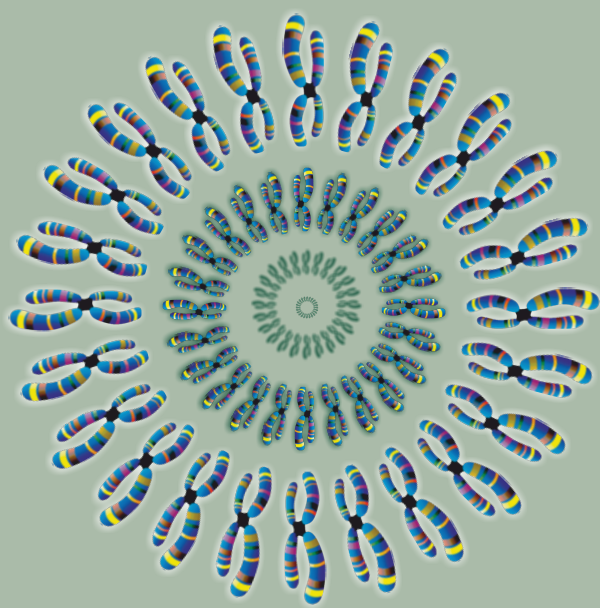

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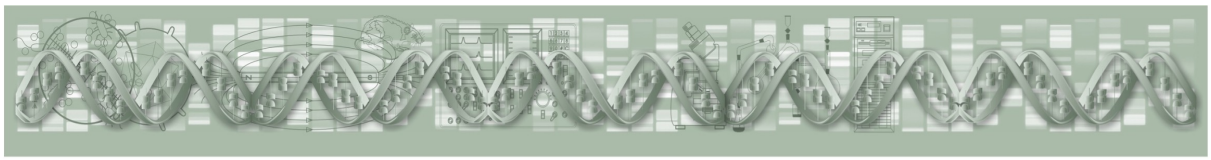

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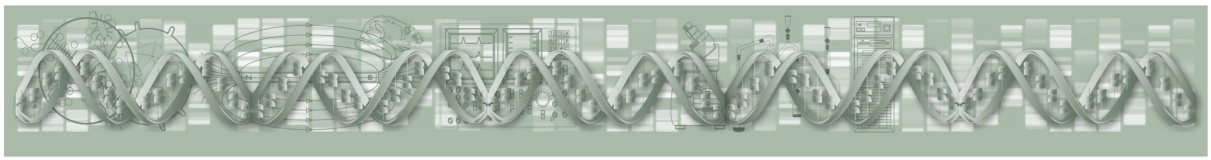

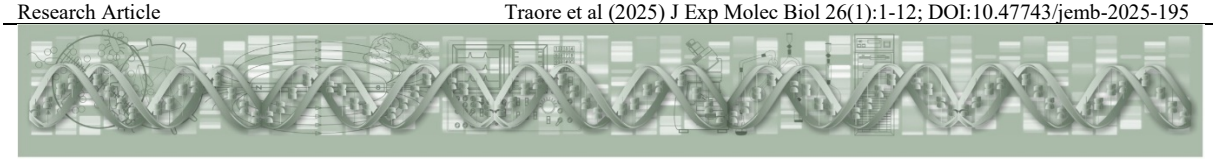

INVOLVEMENT OF HLA-DRB1*11 AND HLA-DRB1*12 ALLELES IN THE OCCURRENCE OF DENGUE FEVER IN BURKINA FASO

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Abstract

Dengue has become the world's most common arbovirosis. In some individuals, genetic factors can increase the risk of developing severe dengue fever. Human leukocyte antigen (HLA) genes are one of human disease's most extensively studied gene groups. The present study investigated HLA DRB1*11 and HLA DRB1*12 polymorphisms in dengue cases and their susceptibilities in developing dengue in a population in Ouagadougou, Burkina Faso. This was a case-control study involving 56 patients with clinically and biologically confirmed dengue fever and 65 others who had never been in contact with DENV, for a total of 121 individuals. A blood sample was taken from each study participant. After extraction of genomic DNA using the salting-out technique, characterization of carriage of the HLA-DRB1*11 and 1*12 alleles was carried out using multiplex polymerase chain reaction (PCR). The χ^2 test, odds ratio (OR), and confidence interval (CI) were calculated using SPSS software to estimate associations and assess the level of risk. Allele frequencies in the general population were 64.4% and 62.8% for HLA DRB1*11 and HLA DRB1*12, respectively. The HLA-DRB1*12 allele was present in 28.9% of cases and 33.9% of controls. The HLA-DRB1*11 allele was present in 32.2% of both cases and controls. In this study, no direct association was found between the presence of the HLA-DRB1*11 and HLA-DRB1*12 alleles and the surveillance of dengue infection. Furthermore, the absence of the HLA-DRB1*11 allele was associated with protection against the development of severe disease (OR = 0.03; 95% CI [0.11 - 0.80]; and p = 0.01). No risk of developing severe dengue fever was found in individuals carrying the HLA-DRB1*11 and HLA-DRB 1*12 alleles. However, further study of other HLA alleles involved in the development of severe dengue may provide more information.

Keywords: Dengue, HLA-DRB1*11, HLA-DRB1*12, risk factors, Burkina Faso

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Background

Dengue is a vector-borne viral infection that occurs mainly in urban areas of the intertropical zone. Once confined to Southeast Asia and the Americas, the disease has progressively spread disproportionately to all WHO regions worldwide. The main dengue vector is Aedes aegypti, followed by Aedes albopictus. The dengue virus (DENV) belongs to the Flavivirus genus of the Flaviviridae family. It is divided into four serotypes: DENV-1 to DENV-4 and causes dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (1). Dengue has become the world's most common arbovirosis (2). In 5 to 30% of confirmed cases, the condition can be severe and complex. The main indicators of disease severity include thrombocytopenia, plasma leakage, haemorrhage and hypovolemic shock, commonly known as DHF and DSS (3). Dengue fever is common in tropical and subtropical regions worldwide, with a predilection for urban and semi-urban areas. The global incidence of dengue has risen dramatically, and half the world's population is now at risk of contracting the disease. Between 100 and 400 million infections are recorded each year worldwide, but only around 20% of these are generally symptomatic (4). The dengue situation in the African region remains relatively undocumented. However, the disease affects several West African countries, including Burkina Faso, particularly its capital Ouagadougou (5).

In some individuals, genetic factors can increase the risk of developing severe dengue fever (2). Human leukocyte antigen (HLA) genes are one of the most studied groups of genes in human disease (3). In humans, HLA molecules are encoded by the major histocompatibility complex (MHC) and are located on chromosome 6. Class I and II molecules are involved in the presentation of peptide antigens to host T cells to activate the immune system. They harbour the most important genetic polymorphisms in the host and play an important role in the selection of target antigens and in determining the nature and intensity of the immune response(1). The MHC carries around 220 genes for proteins, more than half of which are directly involved in immunity.

The genes of the HLA system are organized into three regions: HLA class I, HLA class II and HLA class III. Class I comprises HLA-A, HLA-B and HLA-C, while class II comprises HLA-D and its subtypes HLA-DO, HLA-DP, HLA-DQ and HLA-DR (6). The HLA system has approximately 28,938 alleles, including 21,040 class I alleles and 7,898 class II alleles (7). Numerous studies have revealed that allelic polymorphism in the genes of the HLA system is associated with various diseases. HLA class I molecules are the most studied. As for HLA class II molecules, HLA-DRB and HLA-DQ are the most widely studied genes. Two alleles of the HLA DRB gene, namely: HLA-DRB1*11 and HLA-DRB1*12, are associated with protection against or susceptibility to dengue fever in different populations (8,9). These associations may also vary according to ethnic and geographical distribution (8). To this end, it is important to provide sufficient information on the likely implications of these alleles in the occurrence and/or protection of the host against the severe form of dengue in each endemic region. This will strengthen control strategies and serve as a lead in the production of a possible effective dengue vaccine.

However, there are no known studies in the literature implicating the HLA-DRB1*11 and HLA-DRB1*12 alleles in the development of dengue fever in Burkina Faso. The aim of this study was therefore to determine the association of the HLA-DRB1*11 and HLA-DRB1*12 alleles with the occurrence of dengue fever in the population of Burkina Faso.

Materials and Methods

This study aimed to determine the involvement of the HLA-DRB1*11 and HLA-DRB1*12 alleles in the occurrence of DENV infection and its progression towards severe forms of the disease in Burkina Faso.

This was an analytical case-control study. The study lasted 6 months, i.e., from September 2022 to February 2023. The study population consisted of 122 individuals subdivided into two groups: 57 cases and 65 controls. They included patients of different age groups, including children, and donors representing different professions and social statuses.

Blood samples were taken from three different laboratories, including those at Hôpital Saint Camille de Ouagadougou (HOSCO), Clinique Princesse Sarah (CPS) and CERBA. Patients' sociodemographic data, such as sex, age and place of residence, were recorded by the collection forms.

A case was defined as any patient presenting with at least two clinical signs suggestive of dengue with a positive RDT for DENV and confirmed by ELISA and presenting at least two signs suggestive of dengue fever, while controls were RDT- and ELISA-negative for DENV and presented no signs associated with dengue fever. Patients with an already well-known pathology were not included in this study.

Sampling mode

Sampling consisted of interviewing patients on a collection sheet. A blood sample was then taken from each study participant and distributed in two tubes: a dry tube and an EDTA tube. The collected samples were centrifuged at 4,000 G for 5 min, and then the plasma was used for the Rapid Diagnostic Test (RDT) for DENV. Sera from samples negative for Ag NS1 in the DENV RDT and control cases were tested by ELISA before being collected in Cryotubes and stored at -20°C.

Genomic DNA extractions

Genomic DNA was extracted using the rapid salting-out technique based on cell lysis, protein digestion and precipitation, impurity washing and DNA elution (10).

Amplification of DNA extracts by conventional PCR

For the detection of HLA-DRB1*11 and HLA-DRB1*12 by PCR, primers described by Ma et al. (11)were used with slight modifications. To ensure PCR reliability, a primer pair for amplification of the human growth factor (HGF) gene was also included as an internal control. This made it possible to confirm the presence of DNA in samples where none of the alleles studied were detected.

The technique involves multiplex PCR where the target alleles and the HGF internal control are amplified at the same time. This is performed with the GeneAmp PCR System 9700 (Applied Biosystems, USA) using a reaction volume of 25 µL. The reaction mixture consisted of 13 µL of molecular biology water, 4 µL of 5X master mix, 0.5 µL of each primer pair at a concentration of 0.2 µM, and finally the addition of 5 µL of DNA extract at 10 ng/µL per reaction.

The sequences of the primer pairs used are listed in Table 1.

Table 1. Primers and amplicon size

Genes	Primers	Amplicon size (bp)
DRB1*11	F : 5'GTTTCTTGGAGTACTCTACGTC3' R : 5'CTGGCTGTTCCAGTACTCCT3'	176
DRB1*12	F : 5'ACTCTACGGGTGAGTGTT3' R : 5'ACTGTGAAGCTCTCCACAG3'	244

HGF	F : 5'CAGTGCCTTCCCAACCATTCCCTTA3' R :5'ATCCACTCACGGATTTCTGTTGTGTTTC3'	432

PCR program

For amplification, the PCR program used consisted of an initial denaturation of the DNA for 10 minutes at 94°C, followed by 35 cycles, each consisting of a denaturation step at 94°C for 1 minute, a hybridization step at 56°C for 1 minute and finally an elongation step at 72°C for 1 minute. A final extension was performed at 72°C for 7 minutes.

Electrophoresis and revelation of PCR products

A 1X Tris Acetate EDTA (TAE) buffer was used to migrate PCR products by electrophoresis under non-denaturing conditions in a 2% agarose gel.

Validation of PCR product results

The PCR of a sample is validated if a band of amplification of the HGF gene is visible at the development stage. The PCR of a sample is invalid if the HGF gene amplification control band is not observed. The presence or absence of HLA DRB1*11 and HLA DRB1*12 alleles is linked to whether bands of the expected size are observed, namely, 176 bp for HLA DRB1*11 and 244 bp for HLA DRB1*12 (Figure 1).

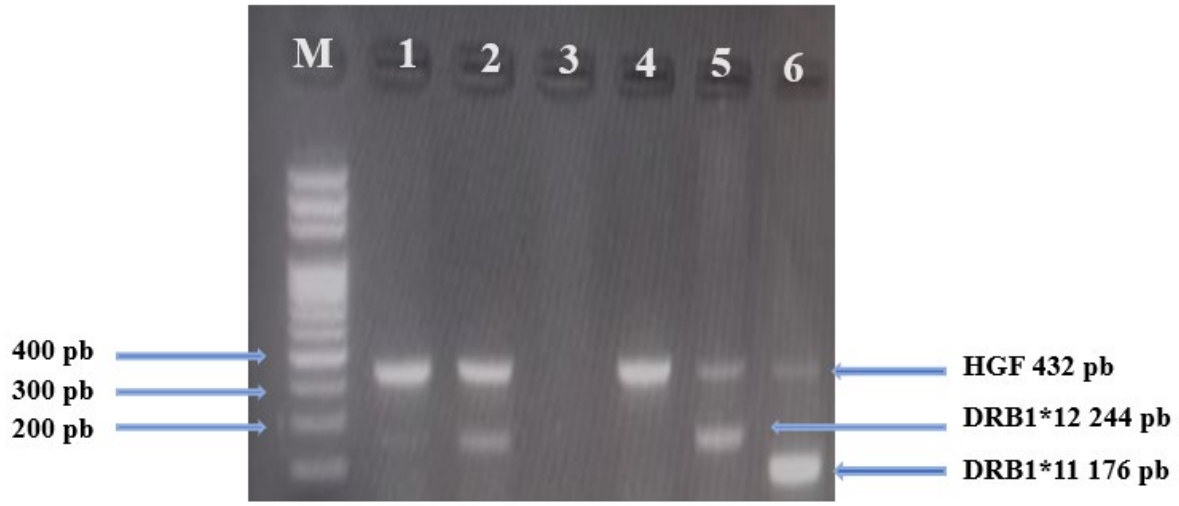

Figure 1. Electrophoresis gel of PCR products

M: *Molecular weight marker.* ***(1,2 and 5):*** *Presence of HLA-DRB1*12;* ***(3):*** *Invalid* ***(4):*** *Absence of HLA-DRB1*11 & HLA-DRB1*12* ***(6):*** *Presence of HLA-DRB1*11*

Data processing and analysis

Data were entered using Excel 2016 and then analyzed using R version 1.4.1717 and SPSS version 20. The chi-square test was used to compare frequencies. Odds ratios and 95% confidence intervals were calculated to assess risk. The results were considered statistically significant at $p < 0.05$.

Ethical considerations

This study was approved by the Ethics Committee for Health Research **CERS N° 25 48 89 37**. Written informed consent was obtained from patients and donors. We ensured the confidentiality of our database by storing it on a password-protected computer.

Results

Sociodemographic characteristics of the study population

This study involved 56 people with clinical signs of dengue confirmed by diagnostic tests in the medical biology laboratory and 65 people who had never been in contact with DENV, giving a total of 121 people. In our study population, 47.9% (58/121) were men, and 52.1% (63/121) were women, with a sex ratio of 0.67. Among DENV patients, half (28/56) or 23.1% were men (Table 2).

The most represented age group was between 16 and 30 years, i.e., 38.0%. This age group also had the highest incidence of dengue fever (18.2%). In our study population, the youngest was 2 years old, and the oldest was 75 (Table 2).

Table 2. Sociodemographic characteristics of the study population

Variables	Cases n (%)	Control n (%)	Total n (%)	P value
Gender				
Male	28 (23.1)	30 (24.8)	58 (47.9)	0.673
Female	28 (23.1)	35 (29.0)	63 (52.1)	
Age (years)				
0-15	6 (5.0)	15 (1.4)	21 (17.4)	0.115
16-30	22 (18.2)	24 (19.8)	46 (38.0)	
31-40	16 (13.2)	18 (14.9)	34 (28.1)	
41-60	8 (6.6)	8 (6.6)	16 (13.2)	
> 60	4 (3.3)	0 (0.0)	4 (3.3)	
Total	56 (46.3)	65 (53.7)	121 (100.0)	

n = numbers, % = frequencies

Virological and serological characteristics of patients

Data on serological and virological parameters are summarized in Table IV. The search for specific IgM and IgG antibodies tells us that in the study population, among the positive cases, 14.3% (8/56) had a primary infection with the DENV virus, (7/56) 12.5% had at least one previous infection and (26/56) 46.4% were in the secondary infection phase with another type of DENV or the recovery phase (Table 3).

Table 3. Prevalence of Ag NS1, IgG and IgM type Ac in the study population

	Cases	Control	Total
	n (%)	n (%)	n (%)
AgNS1	18 (32.14)	0 (0.0)	18 (100.0)
Ac-IgM-/IgG-	0 (0.0)	56 (100.0)	56 (100.0)
Ac-IgM-/IgG+	26 (46.4)	0 (0.0)	26 (100.0)
Ac-IgM+/IgG-	8 (14.3)	0 (0.0)	8 (100.0)
Ac-IgM+/IgG+	7 (12.5)	0 (0.0)	7 (100.0)

n: effectifs, **%**: sequences, **Ag**: antigen, **Ac**: antibodies, **NS1**: nonstructural protein 1
IgM -: Immunoglobulin M negative, **IgM +**: Immunoglobulin M positive, **IgG -**: Immunoglobulin G negative, **IgG +**: Immunoglobulin G positive.

Categorization of dengue cases in the study population

Table 4 shows the frequency of severe dengue cases among patients. Analysis of this table shows that 21.4% of patients developed the severe form of the disease. That is a total of 12/56. Men were the most affected, with a percentage of 12.5% (7/12), compared with 8.9% (5/12) for female patients.

Table 4. Categorization of dengue cases in the study population

Variables	Females n (%)	Males n (%)	Total n (%)	P value
DF	23 **(**41.1**)**	21 (37.5)	44 (78.6)	0.515
DS	5 (8.9)	7 (12.5)	12 (21.4)	
Total	28 (50.0)	28 (50.0)	56 (100.0)	

n: effectiveness, %: frequency, DS: severe dengue, DF: dengue fever

Frequency of HLA DRB1*11, HLA DRB1*12 alleles and their involvement in dengue infection.

In our population, the most frequent allele was DRB1*11 with a frequency of 64.4% and the DRB1*12 allele was 62.8%. No risk was found for separate carriage of the DRB1*11 and DRB1*12 alleles (OR = 0.65; 95% CI [0.30-1.39]; and p = 0.27) and the occurrence of dengue fever. However, for combinations of carriers of these two alleles, the absence of the HLA-DRB1*11 allele showed a protective effect against infection by the dengue virus (OR = 0.03; 95% CI [0.11 - 0.80]; and p = 0.01) (Table 5).

Table 5. Frequency of HLA DRB1*11 and HLA DRB1*12 alleles and their involvement in dengue infection

HLA Variables	Cases n %	controls n %	OR 95% CI	P value
DRB1* 11				
Present	39 (32.2)	39 (32.2)		Ref.
Absent	17 (14.0)	26 (21.5)	0.65 (0.30 - 1.39)	0.27

DRB1* 12				
Present	35 (28.9)	41 (33.9)		Ref.
Absent	21 (17.4)	24 (19.8)	1.02 (0.48 – 2.14)	0,948
DRB1*11 & 1*12				
DRB1*11+ & 1*12+	26 (21.4)	19 (15.7)	-	Ref.
DRB1*11+ & 1*12−	13 (10.7)	20 (16.5)	0.47 (0.19 – 1.18)	0.11
DRB1*11− & 1*12+	**9 (7.4)**	**22 (18.1)**	**0.03 (0.11 – 0.80)**	**0.01**
DRB 1*11− & 1*12−	8 (6.6)	4 (3.3)	1.46 (0.38-5.57)	0.58

n: effective, **%**: frequencies, OR 95% CI: odds ratio and 95% confidence interval

Frequency of HLA DRB1*11 and HLA DRB1*12 alleles and their involvement in recent dengue infection

From the analysis in Table 6, none of the DRB1*11 and 1*12 alleles were associated with DF in patients with primary DENV infection. This was observed both for separate carriage and for co-carriage of these two alleles.

Table 6. Frequency of HLA DRB1*11 and HLA DRB1*12 alleles and their involvement in recent dengue infection

HLA Variables	AgNs1+/IgM-/IgG- n %	Controls n %	OR 95% CI	P value
DRB1* 11				
Présent	11 (61,1)	39 (60,0)		Ref.
Absent	7 (38,9)	26 (40,0)	0.95 (0.33-2.78)	0.93
DRB1* 12				
Présent	9 (50,0)	41(63,0)		Ref.
Absent	9 (50,0)	24 (37,0)	1.71(0.53-4.90)	0.32
DRB1*11 & 1*12				
DRB1*11+ &1*12+	7 (38,9)	19 (29,2)		Ref.
DRB1*11+ & 1*12−	4 (22,2)	20 (30,7)	0.55 (0.14-2.16)	0.38
DRB1*11− & 1*12+	2 (11,1)	22 (33,9)	0.25 (0.05-1.33)	0.09
DRB 1*11− & 1*12−	5 (27,8)	4 (6,1)	3.39 (0.7016.38)	0.12

***n**: effects, %: frequencies, OR 95% CI: odds ratio and 95% confidence interval,* **NS1 +**: nonstructural protein 1 antigen test positive, **IgM -**: immunoglobulin M negative, **IgM +**: immunoglobulin M positive

Frequency of HLA DRB1*11 and HLA DRB1*12 alleles and their involvement in severe forms of dengue infection

With regard to the frequencies of these two alleles, we found no increase in the risk of developing severe dengue fever in carriers of the DRB1*11 and DRB1*12 alleles, when considering the separate or combined carriage of these two alleles (Table 7).

Table 7. Frequency of HLA DRB1*11 and HLA DRB1*12 alleles and their involvement in severe forms of dengue infection

HLA Variable	DF n %	DS n %	OR 95% CI	P value
DRB1* 11				
Present	30 (53.6)	9 (16.1)		Ref
Absent	14 (25.0)	3 (5.4)	1.40 (0.32 – 5.98)	0.650
DRB1* 12				

Present	26 (46.4)	9 (16.1)		Ref.
Absent	18 (32.1)	3 (5.4)	2.07 (0.49 – 8.75)	0.319
DRB1*11 & 1*12				
DRB1*11+ & 1*12+	19 (43.1)	7 (58.5)		Ref.
DRB1*11+ & 1*12−	11 (25.0)	2 (16.6)	2.02 (0.35-11.52)	0.42
DRB1*11− & 1*12+	7 (15.9)	2 (16.6)	1.29 (0.21-7.76)	0.78
DRB 1*11− & 1*12−	7 (15.9)	1 (8.3)	2.58 (0.27-24.90)	0.40

***n**: effectifs, %*: frequencies, DS: severe dengue. DF: Dengue Fever *OR 95% CI: Odds Ratio and 95% confidence interval*

Discussion

This study aimed to determine the involvement of HLA-DRB1*11 and HLA-DRB1*12 alleles in the occurrence of DENV infection and its progression to severe forms of the disease in Burkina Faso.

Dengue is a re-emerging viral disease almost worldwide, with seasonal frequencies in Burkina Faso.

According to our study, 14.3% (8/56) of patients were infected with DENV for the first time, while 85.7% (48/56) were infected for at least a second time or with a DENV serotype different from that of the previous infection. These results are similar to those of a study carried out in Burkina Faso in 2016, in which the secondary infection rate was 89.9%. (12). This high rate of secondary infection in our study population could be explained by the endemic nature of dengue fever in Burkina Faso.

According to gender, in our study, women were infected with dengue fever at the same rate as men, i.e., 23.1%. This result indicates that the disease affects both sexes almost equally. Our results are in line with those obtained in a study carried out in Burkina Faso, which estimated that women were slightly more infected than men, with no significant difference (5). The same is true of those obtained in a study carried out in Cuba on 120 individuals, including 54 women and 66 men. (13).

Our results show that 21.4% of the study population suffered from severe dengue fever. Of these. A total of 12.5% were men, and 8.9% were women. No significant difference was observed ($p = 0.515$). Severe dengue affects both men and women. However, in another study carried out in Vietnam, despite the low representation of men among dengue cases, female subjects had a higher risk of developing the severe form of the disease (14). This discrepancy between our results could be explained by our relatively small sample size and the almost equal representation of both sexes in our study population.

The HLA system is one of the most diverse genetic systems in humans. Our data indicated that the frequency of the HLA DRB1*11 allele was 64.4%, slightly higher than the frequency of the DRB1*12 allele (62.8%) in the general population. These results are similar to those obtained in a study carried out in Cuba, where the DRB1*11 allele was represented at 4.8% and the DRB1*12 allele at 0.5% (13). Similarly, another study of 318 descendants of black Africans in Brazil found that 13.05% had the DRB1*11 allele, while 1.72% had the DRB1*12 allele (15).

However, our results are not consistent with those obtained in the Tunisian population, where 49% of participants experiment with the DRB1*12 allele and only 14.36% experiment with DRB1*11 (16). The same is true for the results obtained in a population from Burkina Faso, where HLA DRB1*12 was the most represented, with a proportion of 56.63%, compared to 24.49% for HLA DRB1*11 (17).

This discrepancy between our results and those of other studies may be linked to the number of participants in each study. Most studies of both alleles report a higher frequency of the DRB1*11 allele (http://www.allelefrequencies.net/).

Various studies have investigated the possible association of HLA genes with the occurrence of dengue fever. Two alleles of the HLA DRB gene, namely: HLA-DRB1*11 and HLA-DRB1*12, are associated with protection against or susceptibility to dengue fever in different populations. In a study conducted in Mexico, the HLA-DRB1*11 allele was identified as a risk factor for the development of severe dengue fever. No association was found for the HLA-DRB1*12 allele (9). However, in another study conducted in Cuba, carriers of the HLA-DRB1*11 and HLA-DRB1*12 alleles did not present any risk of developing classic DF (18). Our results indicate that separate carriage of the DRB1*11 and DRB1*12 alleles is not associated with dengue risk.

These different studies therefore show different results depending on the population studied. Consequently, more in-depth studies in different populations are needed to establish meta-analyses that can be used for very detailed risk analyses.

Carrier combinations of these two alleles.

Deletion of the HLA-DRB1*11 allele was associated with a protective effect against the dengue virus (OR = 0.03; 95% CI [0.11 - 0.80]; and p = 0.01). Thus, a dengue sufferer with this mutated HLA DRB1 * 11 allele is less likely to develop a severe form of the disease.

To date, the pathophysiology of dengue fever is unclear. Evidence suggests that the disease is caused, at least in part, by an inappropriate immune response to the virus. The virus' dominant antigen (envelope protein E) is responsible for the entry of the virus into target cells and also induces protective immunity. This protein can also stimulate cross-reacting antibodies and CD4 and CD8 lymphocytes.

The antigenic determinants of the E protein can be processed and presented by MHC class II antigens. In this way, mutated HLA-DRB1*11 molecules can present these viral antigens to CD4 lymphocytes, generating an effective immune response and preventing dengue fever.

Conclusions

This study explored the frequency of HLA-DRB1*11 and DRB1*12 alleles and their involvement in the occurrence of dengue fever in Burkina Faso. The HLA-DRB1*11 allele was the most represented in the study population, with a frequency of 64.4%, compared with 62.6% for the DRB1*12 allele. No direct association between the carriage of these two alleles and the occurrence of dengue infection was proven. However, loss of the HLA-DRB1*11 allele was associated with protection against severe dengue fever.

In addition, further studies on other alleles of the HLA gene in large populations could provide additional information. If these results are corroborated, mutated HLA-DRB1*11 could indeed be an important genetic factor in resistance to DHF in Burkinabe populations.

Abbreviations list

ADN	**:**	Acide désoxyribonucléique
Ag NS1	:	Antigene Non-Structural Proteine
ARN	:	Acide ribonucléique
BET	:	Bromure d'éthidium
CERBA	:	Centre de Recherche Biomoléculaire Pietro Annigoni
CMH	:	Complexe majeur d'histocompatibilité
CPS	:	Clinique Princesse Sarah
DENV	:	Virus de la Dengue
DF	:	Dengue Fever ou fièvre dengue
DHF	:	Dengue Hémorragic Fever ou fièvre hémorragique dengue
DNTPs	:	Désoxynuléotides triphosphates (ATP, TTP, CTP, GTP)

DSS	:	Dengue Shock Syndrome
EDTA	:	Ethylene–Diamine–Tetra–Acetic acid
ELISA	:	Enzyme-Linked Immunosorbent Assay
GPI	:	Glycosyl-phosphatidyl-inositol
HGF	:	Human Growth Factor
HLA	:	Human Leucocyte Antigen
HOSCO	:	Hôpital Saint Camille de Ouagadougou
ICAM	:	Intracellular Adhesion Molecule
IFN	:	Interféron
KIR	:	Killer-immunoglobulin-like receptor
LAIR	:	Leucocyte-associated inhibitory receptor
MgCl2	:	Chlorure de magnesium
NK	:	Natural Killer
OMS	:	Organisation Mondiale de Santé
SPSS	:	Statistical Package for Social Sciences
TDR	:	Test de Diagnostic Rapide

Declarations

1. Ethics approval and consent to participate

This study was approved by the Ethics Committee for Health Research **CERS N° 25 48 89 37**. Written informed consent was obtained from patients and donors. We ensured the confidentiality of our database by storing it on a password-protected computer

2. Consent for publication

Not Applicable

3. Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

4. Competing interests

The authors declare that they have no competing interests.

5. Authors' contributions

Study concept and design: LT, OFK, BB, AMD, ATY, FWD and JS. Sampling and laboratory analysis: LT, OFK, ASAT, AKO, RK, MRY, FT, MS, NS, SOTB. Statistical analysis and data interpretation: LT, OFK, ASAT and AKO. Drafting of the manuscript: LT, OFK and ASAT. Critical revision of the manuscript for important intellectual content: RK, MRY, FT, MS, NS, SOTB BB, AMD, ATY, FWD and JS. Administrative, technical, and material support: LT, OFK, ASAT, AKO, FWD and JS. Study supervision: BB, AMD, ATY, FWD and JS. The corresponding author declares that the manuscript has been read and approved by all named authors and that the order of authorship in the manuscript has been approved by all of us.

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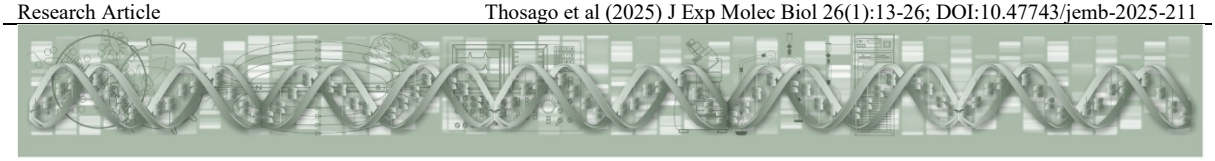

ISOLATION AND MOLECULAR CHARACTERIZATION OF RHIZOBIAL STRAINS ISOLATED FROM SOYBEAN NODULES IN LIMPOPO PROVINCE, SOUTH AFRICA

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Abstract

Limited nitrogen in the soil is a major constraint to sustainable crop production in most developing countries including South Africa. Soybean productivity in South Africa is limited by drought, poor soil fertility, and the ineffectiveness or unavailability of native strains. Most soil in South Africa contains low or ineffective rhizobium strains for biological nitrogen fixation in legume crops. The study aimed to isolate and characterize compatible rhizobial strains for soybeans in response to soil moisture conservation technologies and *Bradyrhizobium japonicum* inoculation in Limpopo province, South Africa. The study used a phylogenetic analysis of 21 bacteria' 16S rRNA gene sequences isolated from soybean root nodules in the Limpopo province. Experiments were conducted at Syferkuil farm and Lebopo sites in Limpopo province. DNA was extracted to perform PCR amplification of the 16S ribosomal RNA using primer fD1 and rD1. Sequencing was done at Inqaba Biotec, Pretoria, and edited using Bioedit and Mega X programs. A total of 21 bacterial isolates were isolated from soybean root nodules. The isolated strains from Syferkuil and Lebopo sites had both medium-growing and fast-growing strains; however, they were dominated by fast-growing strains. Phylogenetic results showed four categories of bacterial genera: *Agrobacterium, Bradyrhizobium, Bacillus*, *and Rhizobium*. Application of local rhizobium strains and efficient strains could enhance productivity and contribute to the low input cost of soybean production in Limpopo province.
Keywords: *Agrobacterium deltaense*, *Bradyrhizobium diazoefficiens*, closed ridges, *Paenibacillus pocheonensis*, plant growth-promoting

Introduction

Soybean (*Glycine max* (L.) Merrill) is regarded as an ancient legume crop that provides high-quality plant protein worldwide. The world population of 9.8 billion is expected to reach 12.6 billion in 2100 (United Nations 2020). Particularly in sub-Saharan Africa (SSA), where food consumption may rise by more than 300% by 2050, this increase in population may lead to acute food insecurity (Engelbrecht et al. 2020). The crop can provide both high-quality plant protein, and calories and may aid in feeding the world's growing population (Messina 2022). Soybean is used as food, fodder, and biofuels since it contains an average of 20% seed oil and 40% protein (Engelbrecht et al. 2020). Soybean converts atmospheric nitrogen (N_2) to biologically functional ammonia (NH_3), thereby reducing N fertilization application and also ensuring a sustainable production process economically and environmentally (Medeiros et al.

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2020). However, soybean production and N fixation are mainly affected by various variables such as high input costs, dearth of suitable microsymbionts in the soil, soil temperature, pH, and local rhizobia population (Mburu et al. 2022, Jaiswal and Dakora 2019). Furthermore, several rhizobia species naturally occur in the soil, and their populations decline due to persistent cultivation and continuous application of pesticides to manage diseases and pests, which affect the efficacy of nodulation and yield (Ogola et al. 2020).

Moreover, soybean root nodules can host many rhizobial and nonrhizobial endophytes (NREs) (Mayhood and Mirza 2021). *Rhizobium, Bradyrhizobium, Allorhizobium, Mesorhizobium, Sinorhizobium*, and *Azorhizobium* are among the rhizobial genera that coexist with host legume plant (Muchhadiya et al. 2024). Rhizobial genera such as *Bradyrhizobium, Rhizobium*, *Mesorhizobium*, and *Ensifer* (formerly *Sinorhizobium*) can effectively fix N with soybeans (Nakei et al. 2022). *Bradyrhizobium japonicum* was shown to be the most common endosymbiont in soybean root nodules and is highly preferred in the rhizosphere (Mayhood and Mirza 2021). The soil bacteria in the genus *Allorhizobium* can coexist symbiotically with legumes, converting atmospheric N into ammonia that the plant can use. In many legumes, but not in soybeans, the *Rhizobium* group of bacteria is common for fixing N. They only nodulate and fix N in soybeans when *Bradyrhizobium* and *Rhizobium* undergo horizontal gene transfer. The genus *Mesorhizobium* is a member of the class *Alphaproteobacteria* of the phylum *Pseudomonadota* and the family *Phyllobacteriacea*e in the order *Hyphomicrobiales* (Li et al. 2024). The bacterium *Sinorhizobium meliloti* lives freely in soil and interacts in symbiosis with leguminous plants belonging to the genera *Medicago, Melilotus,* and *Trigonella* to fix N (Kearsley et al. 2024). The genus *Azorhizobium* comprises Gram-negative soil bacteria. In symbiosis with plants belonging to the genus *Sesbania*, they fix N. While non-rhizobial endophytic bacteria (NEB) such as *Paeniacillus, Bacillus, Enterobacter, Flavobacterium, Planococcus,* and *Variovorax* are rarely found (Mayhood and Mirza 2021). Non-rhizobial endophytic bacteria were reported to enhance rhizobia's symbiotic efficacy improving nodulation and N fixation in legume plants (Subramanian et al. 2015). *Enterobacter* belongs to the Enterobacteriaceae family. *Bacillus* and *Paenibacillus* are two genera of Gram-positive aerobic endospore-forming bacteria (AEFB) that are almost universal and widely distributed in the majority of rhizospheric soils. The rhizosphere is home to species of these two genera that fix N in the atmosphere, solubilize phosphorus in the soil, absorb micronutrients, and produce phytohormones and antimicrobial metabolites (Govindasamy et al. 2010). *Planococcus* is a halophilic bacterium that produces a variety of secondary metabolites (Waghmode et al. 2020). The genus *Variovorax* contains bacteria that can enhance legume growth and health in several ways, such as: nodulation, seed yield, stress resistance, and metal uptake. Both fast-growing and slow-growing rhizobial genera nodulate soybean crops (Ayuba et al. 2021). High symbiotic efficacy among native rhizobial strains that are compatible with soybean cultivars indicates that these strains are competitive and beneficial and might be used as inoculants in soybeans (Klogo et al. 2015). One of the main reasons for low soybean yield is the absence of locally suitable and effective rhizobial inoculants (Ayuba et al. 2021). Native bacteria are compatible with local soybean varieties and adapted to local environmental conditions to improve crop productivity (Mortuza et al. 2020). Nonetheless, the presence and effectiveness of the indigenous rhizobia prevent introduced rhizobial strains from successfully nodulating a host legume (Akley et al. 2023, Yamakawa et al. 2003). Rhizobia distribution and diversity are heavily influenced by geography and understanding their phylogeny could illuminate their evolutionary origins (Abd El-Ghany et al. 2020). Prior inoculation, it is necessary to understand the ecology of native rhizobia in the soil (Mason et al. 2017). Characterizing soybean-associated bacteria from local environments can discover new rhizobial strains that may be used as biofertilizers (Mortuza et al. 2022). To provide guidelines for the manufacture of soybean inoculants, studies on the

prevalence of rhizobial strains in local soils, their genetic diversity, and the type of rhizobia preferred by soybeans in South Africa are required (Naamala et al. 2012).

Several studies were conducted using these molecular techniques such as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), rapid amplified polymorphic DNA (RAPD), DNA–DNA hybridisation, sequence analysis of the 16S rDNA gene and multilocus sequence analysis (MLSA). The MLSA method, which use housekeeping gene analysis was a quick and accurate way to identify strains within the *Bradyrhizobium* genus at the species level (Rodriquez et al. 2024). In order to identify strains of the *Bradyrhizobium* genus, Delamuta et al. (2012) used MLSA, examining the 16S rRNA gene together with five housekeeping genes (recA, atpD, glnII, gyrB, and rpoB). Conversely, because of its limited sequence variation, 16S rDNA gene sequencing is accurate at the genus level but inaccurate at the intra- and inter-species levels (Martens et al. 2008). Housekeeping genes are more specific, and they can distinguish between rhizobial strains that belong to closely related lineages (Martens et al. 2008).

Low soil N significantly restrict crop productivity in SSA countries, including South Africa. Most smallholder farmers cannot afford to buy synthetic fertilizer due to cost. Nitrogen contributes by legume-rhizobia symbiosis, which presents an opportunity for N-input in agriculture, which can assist smallholder farmers in improving crop productivity. Low agricultural productivity in arid and semi-arid areas has been attributed to ineffective soil moisture conservation techniques (Mak-Mensah et al. 2021). Closed ridges maximize rainfall by improving infiltration, moisture retention, and surface drainage, and reducing runoff and soil erosion (Verma et al. 2020). Low-cost agricultural technologies are gaining popularity worldwide as people seek sustainable food production strategies (Mburu et al. 2022). The study aimed to isolate and characterize efficient rhizobia from soybean variety in response to soil moisture conservation technologies and *Bradyrhizobium japonicum* inoculation in Limpopo province.

Materials and Methods

Study site

The field experiments were conducted at the University of Limpopo experimental farm (Syferkuil) (23°53′10″S, 29°44′15″E) and farmers ‘s field (Lebopo cooperatives) (24°01′52.0″S, 29°44′16.0″E) in Limpopo province, South Africa during 2019/2020 growing season. The rainfall received during the 2019/2020 growing season was 260 mm. The soil at Syferkuil was classified as the sandy loam of Hutton form, Glenrosa family, with a pH of 6.0 to 7.1 (Table 1). Lebopo soil was classified as by high lixisols (IX), with a clay-enriched lower horizon, low cation exchange capacity (CEC), and high saturation of bases (Mohlala 2021).

Treatments and experimental design

The field experiments were laid out in a split-plot design fitted into a randomised completely block design. The experiments consist of soybean variety (PAN 1664R), two levels of soil moisture conservation techniques [flat and closed ridges], and two levels of inoculation (with and without) using *Bradyrhizobium japonicum* strain WB74 $1X10^9$ colony-forming gram manufactured by SOYGRO (PTY) LTD. The Closed ridges were manually constructed using a hoe, spade and rake. The height and width of closed ridges were 60 and 30 cm, respectively. Treatments were PAN166R x Closed ridges, PAN1664R x Flat planting, PAN1664R x inoculated with *Bradyrhizobium japonicum*, Closed ridges x inoculated with *Bradyrhizobium japonicum*, PAN1664R x inoculated with *Bradyrhizobium japonicum.* The main plot was assigned to soil moisture conservation techniques while the subplot was assigned to the inoculation level. The plot size was $15m^2$ with an interplot distance of 1m. The planting depth was 2cm, with an inter-row spacing of 60 cm and intra-row spacing of 20 cm.

Physio-chemical properties

Soil samples were collected from Syferkuil and Lebopo at 0-30 cm depth. The pH meter from Mettler Toledo was used to analyse soil pH (KCl). The Mclean titration method was used to calculate exchangeable acidity. Phosphorus (P), and potassium (K), were determined using Bray 1 and mehlich-3 extraction (Matcham et al. 2023) magnesium (Mg) and calcium (Ca) were all determined using atomic absorption (Diwakar et al. 2023). Nitrogen (N), Zinc (Zn) and manganese (Mn) were determined using the Kjeldahl method (Aguirre 2023). Organic carbon was determined using Walkley-Black method (Mustapha et al. 2023).

Seed inoculation with *Bradyrhizobium japonicum* strain WB74

The most popular and least expensive approach involves applying *Bradyrhizobium japonicum* inoculant to the seeds right before sowing. As the manufacturer advised, the inoculant was applied to seeds just before planting. Live cultures of the *Rhizobium* group of bacteria (*Bradyrhizobium japonicum*) are present. For use in soybean growing, this product contains at least 2× 109 (at least 2 billion) live bacteria (*Bradyrhizobium japonicum*) per gram of peat substrate (Serafin-Andrzejewska et al. 2024). To achieve a concentration of $109CFUg^{-1}$ of rhizobia, *Bradyrhizobium japonicum* inoculation was carried out at a rate of 10g of inoculants per kg seed, and seeds were coated using an adhesive made of sucrose solution. After evenly stirring 1 kg of seed with 15 mL of 5% sucrose solution using a wooden spatula, 10g of the peat inoculant was added. The mixture was then swirled once more and allowed to air dry for 15 to 20 minutes before planting (Kabiru et al. 2024). Un-inoculated seeds were planted first, followed by inoculated seeds, to prevent contamination in the trials.

Collection of root nodules

Soybean nodules were sampled at 50% flowering stage followed by Kabiru et al. (2024). A spade was used to make a 20 cm-distance dig on each side of the plant. Four (4) plants were sampled per plot, and root nodules were gently washed with tap water using a 2-mm mesh sieve and separated from the plant. Non-active nodules were not included during the characterization of rhizobia. Healthy nodules were separated from the root and stored for morphological and molecular analysis.

Isolation of *Bradyrhizobium* genera

The nodules were surface sterilized by submerging them in 95% ethanol for 5 seconds and transferring them to a 3% sodium hypochlorite solution for 5 minutes. They were rehydrated for two hours, washed with sterile distilled water and dried. Sterile distilled water was used to rinse the nodule 5–6 times. A sterile petri dish was used to crush each surface-sterilized nodule before nodule suspension was streaked over a yeast-mannitol agar (YMA) plate. Rhizobia was recovered from nodules that had been surface-sterilized (Tak et al. 2020).

Morphological characterization of root nodules

Growth rates were observed on Yeast Extract Mannitol Agar (YEMA) plates. Depending on the strains, YEMA plates were used to grow the isolates for 2 to 10 days.

Genomic DNA preparation

Isolates were grown in YM broth until the late log phase to extract bacterial genomic DNA. According to the manufacturer's instructions, DNA was extracted from bacteria using the GenElute bacterial genomic DNA extraction kit (Sigma-Aldrich, USA).

DNA amplification and sequencing

Using 16S ribosomal enzyme, primer pairs fD1 and rD1, DNA was isolated for PCR amplification (Efstathiadou et al. 2021). The 16S rRNA universal primer for bacteria was used for amplification (Fukuda et al. 2016). A 25 µL reaction volume containing 6 µL of template DNA, 5 µL of Flexi buffer, 2.5 µL of MgCl, 0.5 µL of dNTPs, 0.5 µL of each of forward and reverse primers, 0.5 µL of Taq polymerase, and 9 l of nuclease-free water were used for amplification. Amplifications were carried out using 30 cycles of initial denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 1 min, and final extension at 72 °C

for 1 min in an Eppendorf Master cycler Gradient apparatus (Applied Biosystems, USA). Electrophoresis on 1% agarose gel in tris-borate buffer containing 0.5 mg/mL ethidium bromide, PCR-amplified DNAs were seen. Sequencing was done at Inqaba Biotec, Pretoria, and edited using BioEdit and Mega X programs (Kumar et al. 2018).

Phylogenetic analysis

Phylogenetic analysis grouped the isolates and compared them with the reference strains in the National Centre for Biotechnology Information (NCBI) and EzBiocloud database library with good bootstrap support (≥70%), confirming their close relationship. Numbers in the parenthesis represent NCBI accession numbers. Genera were identified using Nucleotide Basic Local Alignment Search Tool (BLAST) analysis of the 16S rRNA gene. The phylogenetic tree was constructed to scale with branch lengths indicating the number of nucleotide substitutions per site.

Results and discussions

Effect of soil chemicals characteristics on rhizobia

Bradyrhizobium japonicum was less prevalent than fast-growing *Rhizobium* genera in Syferkuil and Lebopo. *Rhizobium* genera, including *Rhizobium tropici,* were isolated from acidic environments at Lebopo, while *Bradyrhizobium japonicum* was isolated at Syferkuil under slightly acidic to neutral-alkaline conditions. *Rhizobium* genera were found to be capable of surviving in acidic soil (Zinga et al. 2017). Bradyrhizobia genera have been reported to be impacted by the pH of the soil (Temprano-Vera et al. 2018). The study by Puozaa et al. (2019) reported that the soil physiochemical characteristics significantly influence the diversity of microbes found in specific environments. This study's, pH level impacted some crucial nutrients such as N, P, Mg and K, which might have restricted microbial activity toward nodule development and count (Table 1). Lack of Mg and P impacted the biological nitrogen fixation (BNF) process, development of nodules, rhizobial bacteria, legume growth, and a free-living Rhizobia colony in the rhizosphere (Mburu et al. 2022).

Table 1. Chemical properties of soil analysis at Syferkuil and Lebopo sites

Locations	P	K	Ca	Mg	Zn	Mn	EA	pH (KCl)	OC	N
	mg/kg						cmol/L		%	
Syferkuil	0.13	0.36	2.81	0.62	0.001	0.016	2.625	6.94	<0.050	<0.325
Lebopo	0.006	0.53	0.83	0.31	0.006	0.022	0.12	5.48	<0.533	<0.2

Growth rates of isolated bacterial strains on soybean root nodules

Growth rates of the isolates revealed that 35% had moderate growth (6–10 days) and 65% had fast growth (2–3 and 3-5 days) (Table 2). The diversity in growth period could be associated with the genetic morphology of the isolated strains. *Priestia megaterium, Rhizobium miluonense, Rhizobium tropici, Neobacillius cucumis,* and *Paenibacillus pocheconensis* grew within 2-3 days, while *Agrobacterium deltaense, Agrobacterium pursense, Neorhizobium alkalisoli,* and *Neorhizobium huautlense* grew within 3-5 days, and *Agrobacterium* and *Bradyrhizobium* genera grew within 6-10 days (Table 2). These findings demonstrated the diversity of the fast growers and slow growers. However, the fast-growing rhizobia in this study was only fair to poor symbionts. Fast-growing rhizobia include rhizobium, *Neorhizobium* and *Agrobacterium* genera (Tounsi-Hammami et al. 2019), similar to this study's findings. Fast-growing strains have benefits such as high efficacy, commercial production potential, easier soil formation, and N_2 fixation efficiency.

Table 2. Molecular identification of soybean strains from nodules

Reference no	Locations	Treatments	Isolates growth (days)	Strains	Probability
EX3P3951-1	Syferkuil	Ridge × Inoc	6-10	*Agrobacterium deltaense*	98.18%
EX3P3252	Syferkuil	Ridge × Inoc	3-5	*Agrobacterium deltaense*	98.9%
EX3P3351-2	Syferkuil	Ridge × Inoc	3-5	*Agrobacterium pusense*	99.92%
EX3P752	Syferkuil	Ridge × Inoc	6-10	*Bradyrhizobium diazoefficiens*	99.3%
EX3P5051	Syferkuil	Ridge × without	3-5	*Neorhizobium alkalisoli*	99.77%
EX35851-2	Syferkuil	Flat × Inoc	3-5	*Neorhizobium huautlense*	98.8%
EX3P1652	Syferkuil	Flat × Inoc	6-10	*Bradyrhizobium diazoefficiens*	100.0%
EX3P5851-1	Syferkuil	Flat × Inoc	3-5	*Agrobacterium pusense*	99.3%
EX3P6351-2	Syferkuil	Flat × inoc	3-5	*Agrobacterium pusense*	99.92%
EX3P5852-1	Syferkuil	Flat × inoc	2-3	*Priestia megaterium*	99.86%
EX3P5752	Syferkuil	Flat × without	6-10	*Bradyrhizobium diazoefficiens*	99.8%
T43	Lebopo	Ridge × Inoc	2-3	*Rhizobium tropici*	99.47%
TP4312b	Lebopo	Ridge × Inoc	2-3	*Rhizobium tropici*	99.635
4252A	Lebopo	Ridge × Inoc	2-3	*Rhizobium tropici*	99.40%
TP2652	Lebopo	Flat × Inoc	6-10	*Bradyrhizobium diazoefficiens*	99.7%
TP5751-2	Lebopo	Flat × Inoc	6-10	*Bradyrhizobium diazoefficiens*	99.8%
TP5751	Lebopo	Flat × Inoc	6-10	*Bradyrhizobium diazoefficiens*	100.0%
T5951-1	Lebopo	Flat × Inoc	3-5	*Agrobacterium pusense*	98.54%
TP5951-3	Lebopo	Flat × Inoc	2-3	*Rhizobium miluenense*	99.55%
TP27b	Lebopo	Flat × Inoc	2-3	*Neobacillus cucumis*	94.00%
T3951	Lebopo	Flat × without	2-3	*Paenibacillus pocheonensis*	98.92%

Molecular identification and phylogenetic analysis of isolates using 16s rRNA gene sequence

Four significant clusters: *Agrobacterium, Rhizobium, Bradyrhizobium*, and *Bacillus* genera (Figure 1) were also discovered in soybean and *Hedysarum spinosissimum* root nodules (Sbabou et al. 2016, Zhao et al. 2018). In Cluster I, isolates such as Ex3P3351-2, EX3P5851-1, and EX3P6351 from Syferkuil and T5951-1 from Lebopo were significantly related to [*Agrobacterium fabrum* (AE007869), *Agrobacterium salinitolerans* (MRDH01000011), and *Agrobacterium pusense*] with 98.18-99.92% sequence identity. Syferkuil isolates EX3P3951 and EX3P3352 were connected to [*Agrobacterium deltaense* (MRDI01000025), *Rhizobium oryzihabitans*, (MT023790)], and [*Beijerinckia fluminensis (*MW559665.1)] (Figure 1). The results of the current study revealed the presence of *Agrobacterium genera* in soybean root nodules under closed ridge and inoculation rather than flat inoculation at Syferkuil. Reports indicated that Agrobacteria were isolated from numerous legumes (mungbean, cowpea, common bean, and soybean) root nodules and are widely distributed in soils across all environments. However, these *Agrobacterium* genera either fail to demonstrate their ability to nodulate upon re-inoculation or they nodulate but fail to effectively fix N. The role of *Agrobacterium* genera in symbiosis and BNF process on legumes is still not well understood (Delamuta et al. 2020). The mechanisms through which these isolates are integrated into nodules are currently unknown. Tounsi-Hammami et al. (2019) indicated that *Agrobacterium* genera are incapable of creating nodules on the host plant, while on the other hand, Rosariastuti et al. (2022) argued that the development of root nodules in legume crops might be influenced by symbiotic plasmids (Sym) resulting from *Agrobacterium* genera. The isolation of non-pathogenic, opportunistic agrobacterial genera from surface-disinfected nodules and their cohabitation with rhizobial strains inside root nodules in various legumes worldwide was reported (Mahdhi et al. 2016). The current study showed less conservation of 16S sequences among *Agrobacterium* genera. Overall, findings suggest that the 16S rRNA gene in the genus *Agrobacterium* genera is highly conserved.

Cluster II, *Rhizobium miluonense* (jgi.1052910), *Rhizobium rhizogenes* (BAYX01000035), *Rhizobium hainanense* (FMAC01000030), and *Rhizobium freirei* (AQHN01000056)] had a connection with *Rhizobium* genera with 64% bootstrap and 99.40-99.635% similarity. EX3P5051 and EX3P5851-2 from Syferkuil linked with *Rhizobium* genera. [*Neorhizobium huautlense* (AF025852) *Neorhizobium alkalisoli* (EU074168) and *Neorhizobium vignae* (GU128881)] with 90% bootstrap and 99.77% sequence identity. In soybean root nodules, *Neorhizobium huautlense, Neorhizobium alkalisoli,* and *Neorhizobium vigne* were associated with EX3P5851-2 isolate under flat and inoculation, EX3P5051 from the ridge and without inoculation at Syferkuil. *Neorhizobium* genera were found in soils and soybean root nodules (Mayhood 2020). *Beijerinckria fluminensis* was discovered from Syferkuil utilising rRNA sequence analysis in soybean root nodules. *B. fluminensis* isolates might be associated with previous crops planted, such as potatoes. Studies also isolated *B. fluminensis* in potato rhizosphere fields and mung beans (AL-Shwaiman et al. 2022; Sansanwal et al. 2023). Several studies have reported that rhizobium genera can nodulate soybean crops (Mayhood & Mirza 2021). T5951-3 belonging to *Rhizobium* genera (*Rhizobium miluonense, Rhizobium rhizogenes,* and *Rhizobium hainanense*) isolated from flat and inoculation at Lebopo was identified in cluster II. Three isolates from ridges and inoculation at Lebopo (TP4312b, T43, and 4252) were associated with *Rhizobium freirei*, whilst EX3P3951 and EX3P3252 were connected to *Rhizobium oryzihabitans* from Syferkuil. In the current study, locations, not the host species and influenced these Rhizobium strains. *Rhizobium* genera are site-specific in its spread (Guanzon et al. 2023). *Rhizobium freirei*, the symbiont of common beans, is renowned for its tolerance to low pH (Tullio et al. 2019), which is typical of Lebopo.

TP2652, TP5751-1, and TP5751 from Lebopo, EX3P1652, EX3P5752, and EX3P752 from Syferkuil were grouped with *Bradyrhizobium centrosematis* (KC247115), *Bradyhizobium nitroreducens* (AB542368), *Bradyrhizobium. diazoefficiens* (BA000040), and *Bradyrhizobium niftali* (MK673807) in cluster III with a sequence identity of 100% (Figure 1). Since suitable strains of inoculating soybean in South African soils are uncommon and do not naturally occur, results may suggest that symbiotic genes were transmitted from the application of inoculant to native soil bacteria with a variety of *Bradyrhizobium* genera genetic backgrounds (Naamala et al. 2016). *Bradyrhizobium* genera, on the other hand, has regularly been discovered among native rhizobia in Africa (Chibeba et al. 2017), which may be another explanation for why these genera were discovered. The 16S rRNA sequence showed that isolated strains were closely grouped according to their sites. Geographical locations significantly affect the distribution and diversity of rhizobia (Abd El-Ghany et al. 2020). The findings from this study also showed that soybean and the inoculated microsymbiont had the best mutualisms. Rhizobia and legumes have been reported to have a host-specific interaction (Abd El-Ghany et al. 2020). The nucleotide sequences of the 16S rRNA genes revealed that the strains of nodule bacteria in soybean shared 97.07%-98.98% similarity with *Bradyrhizobium japonicum* (Naamala et al. 2016, Shakirov et al. 2023). However, the results reported by Ayuba et al. (2021) showed that isolates with the closest phylogenetic relationships to *Bradyrhizobium diazofficiens* were not the most efficient N fixers, indicating that functional features might differ greatly among close phylogenetic relatives. *Bradyrhizobium* genera were isolated from flat inoculation with *Bradyrhizobium japonicum* rather than closed ridges and inoculation in Syferkuil and Lebopo. The results contradict Basediya et al. (2018), who claimed that there were more nodules under ridge and furrow planting than flat planting. The 16S rRNA gene showed considerable nucleotide identity of *Bradyrhizobium* genera, indicating that other genes were required to provide in-depth investigation at the species level.

In cluster IV, *Bacillus* genera were associated with TP27b and TP3951 from the Lebopo site and EX3P5852-1 from the Syferkuil site (Figure 1). Isolate TP3951 from the Lebopo site was connected with *Paenibacillus aceris* (KU879057) and *Paenibacillus silvestris* (MN381952)

with 61% bootstrap and *Paenibacillus aestuarii* (EU570250) with a bootstrap of 100%. Isolate TP27b, and EX3P5852-1 were grouped with *Priestia aryabhatti* (EF114313), *Bacillus zanthoxyli* (KX865140) and *Prestia megaterium* (EF114313) with a bootstrap 100% (Figure 1). These non-rhizobial bacteria's partial 16S rRNA gene sequencing revealed the existence of *Bacillus,* and they do not fix N or nodulate. *Bacillus* genera were also isolated from soybean root nodules or rhizosphere (Zhang et al. 2018). Bacillus is a rhizosphere bacterium with many nodule endophytes (Zhang et al. 2018). Korir et al. (2017) reported 16S rRNA partial gene sequencing for the molecular characterization of endophytic isolates, and the distributions were genetically varied on numerous species of *Bacillus* genera including *B. megaterium, B. subtilis, B. aryabhatta*i, and *P. polymyxa. Priestia megaterium* isolate (EX3P5852-1) was isolated from the flat and inoculated in Syferkuil. *Priestia megaterium* is crucial for increasing phosphate solubility and auxin biosynthesis. The isolated strains possess multiple plant growth-promoting traits (Sansanwal et al. 2023*).* This could be significant for enhancing soybean production.

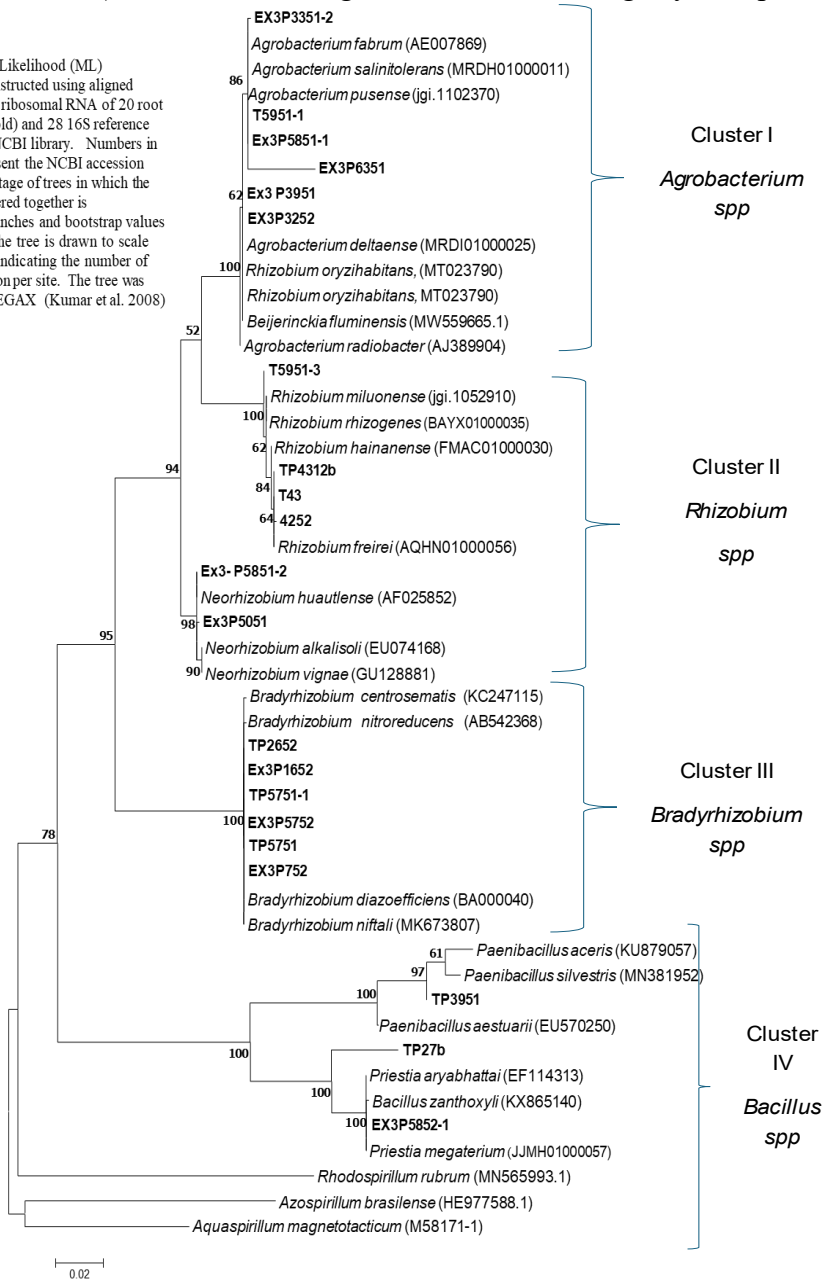

Figure 1. Maximum Likelihood (ML) phylogenetic tree constructed using aligned sequences of the 16S ribosomal RNA of 20 root nodule isolates (in bold) and 28 16S reference sequences from the NCBI library. Numbers in the parenthesis represent the NCBI accession numbers. The percentage of trees in which the associated taxa clustered together is shown next to the branches and bootstrap values ≥ 70% are shown. The tree is drawn to scale with branch lengths indicating the number of nucleotide substitution per site. The tree was constructed using MEGAX (Kumar et al. 2008)

Conclusions

Phylogenetic analysis revealed four (4) genera: *Agrobacterium, Bacillus, Bradyrhizobium,* and *Rhizobium.* Nucleotide BLAST analysis based on the 16S rRNA sequence resulted in the identification of the following species: *Agrobacterium deltaense, Agrobacterium pusense, Bradyrhizobium diazoefficiens, Neorhizobium huautlense, Neorhizobium alkalisoli, Paenibacillus pocheonensis, Priestia megaterium, Rhizobium miluonense* and *Rhizobium tropici* in soybean root nodules. *Agrobacterium deltaense, Agrobacterium pusense, Neorhizobium huautlense, Neorhizobium alkalisoli, Paenibacillus pocheonensis, Priestia megaterium, Rhizobium miluonense* and *Rhizobium tropici* are classified as fast-growing rhizobia. In contrast, *Bradyrhizobium diazoefficiens* is classified as a slow-growing rhizobia. *Beijerinckia fluminensis* was isolated from soybean root nodules in Syferkuil. Flat and inoculation with *Bradyrhizobium japonicum* improved the population density of *Bradyrhizobium diazoefficiens* in soybean. The isolated strains (TP2652, EX3P1952, TP5751-1, EX3P5752, EX3P5752, and TP5751) can be used to enhance soybean productivity and contribute toward low input cost in Limpopo province. Research is needed on pot or field experiments to confirm the efficacy of identified rhizobial strains on growth, nodulation, and yield of soybean variety.

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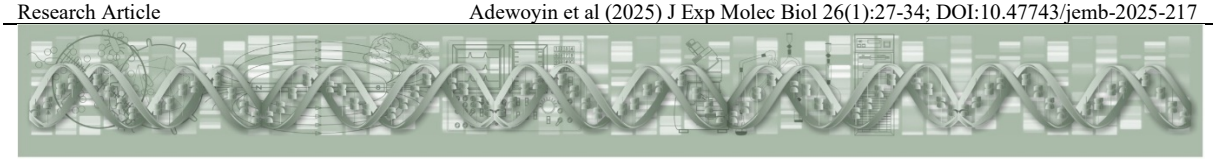

EVALUATION OF *CANDIDA* SPECIES ON FOOD AND NON-FOOD CONTACT SURFACES AMONG STUDENTS RESIDING IN SCHOOL HOSTELS IN A TERTIARY INSTITUTION, ILE IFE, OSUN STATE, NIGERIA

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Abstract

Candida species are significant opportunistic fungal pathogens that can cause various diseases, particularly among individuals with compromised immunity. While there is plenty of literature on Candida in clinical and public settings, incidence on surfaces within student residence halls is still not well understood. The distribution and frequency of Candida species were assessed on surfaces that come into contact with food and non-food items at the Obafemi Awolowo University residence halls in Ile-Ife, Nigeria. Seven out of nine residence halls of the university were studied using a cross-sectional investigation. Sterile swabs were used to collect surface samples from 50 rooms, which were subsequently processed on Hi Chrome Candida Differential Agar and Sabouraud Dextrose Agar. Data analysis was done using the Chi-square test with a significance level of $p < 0.05$. It was found that Candida species were fairly common, with an occurrence rate of 86%. The contamination rate on non-food contact surfaces was 64% compared to 76% on food contact surfaces. Its dominating species was Candida glabrata, which comprised 28%, followed by Candida albicans 6%, and Candida tropicalis was also 6%. More pronounced co-infections were noticed at the postgraduate hall: food-contact surfaces between the Candida albicans and Candida glabrata comprised 28.6%. The Moremi Hall recorded the greatest percentage of total contamination at 26%. The results stress the need for improved hygienic practices, routine check-ups, and public health programs to avoid opportunistic infections among students, as well as the need to turn attention to the possible danger of fungal contamination in communal living facilities.

Keywords: *Candida* species, fungal pathogens, contamination, food and non-food contact surfaces

Introduction

Candida species are a leading cause of fungal infections (Staniszewska 2020). These fungal inhabitants constitute a part of the human flora, establishing residence on skin, and mucous membranes within the oral cavity, gastrointestinal, and genitourinary tracts (Romo and Kumamoto 2020). While Candida thrives harmoniously within the body's cavities, its

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opportunistic inclinations can pave the way for infections affecting diverse anatomical regions causing infections called candidiasis, often manifesting as secondary infections in individuals with compromised immune systems (Arya and Rafiq 2023). It can affect the oral cavity, vagina, penis, or other parts of the body. In the context of the oral cavity, candidiasis takes the form of "thrush" evident by the appearance of characteristic white patches on the tongue, throat, and other oral surfaces (Arya and Rafiq 2023). This disrupts the aesthetics of the mouth and can induce discomfort, with symptoms extending to soreness and difficulties in swallowing (CDC 2022). While *Candida* prevalence in clinical and public settings has been extensively studied, its presence in communal living environments such as university residential halls remains underexplored. Communal spaces, characterized by shared facilities and diverse hygiene practices, create an ideal environment for microbial proliferation. Studies by Cristina et al. (2023), and Belizario et al. (2021), underline the significant fungal burden in non-clinical indoor settings. However, limited data exist on *Candida* prevalence in student hostels, where individuals with varying lifestyles and immune statuses cohabit.

While Candida infections often manifest superficially within urinary or oral mucosal cavities, these fungi possess the alarming ability to infiltrate the bloodstream, paving the path for deep-seated tissue infections (Pappas et al. 2018). The spectrum of invasive candidiasis encompasses varying degrees of severity, spanning from symptomatic candidemia, which stands as the most frequently diagnosed form of invasive candidiasis (Barantsevich and Barantsevich 2022), to the extremities of sepsis, where mortality rates soar beyond 70% (Pappas et al. 2018). With substantially high mortality rates attributed to Candida infections and their potential for antifungal resistance, the urgency to investigate their prevalence in student residential halls is important. This study aims to bridge this gap by evaluating the prevalence and distribution of *Candida* species on food and non-food contact surfaces in student residential halls at Obafemi Awolowo University, Ile-Ife, Nigeria.

Materials and Methods

STUDY A

Obafemi Awolowo University (OAU), situated in Ile-Ife, Osun State, Nigeria, provided the premises for the study. The study concentrated on seven of the university's nine residential halls, which encompass a variety of postgraduate, male, and female lodgings. Purposefully choosing these halls allowed for diversity in student demographics, such as gender and style of residence.

STUDY POPULATION

Out of the nine halls of residence, 7 were purposely selected for the research. Food-contact and non-food-contact surface samples were collected from fifty (50) rooms with a variety of students with distinct habits and lifestyles living within the residential halls of Obafemi Awolowo University, Ile-Ife were enrolled using a random sampling technique. Verbal consents were obtained from all the students and confidentiality was assured by using codes. The study was conducted between July and August 2023 by approaching students in their rooms.

SAMPLE COLLECTION

Before the commencement of the research work, permission and informed consent were taken from the student. Samples were collected using sterile swab sticks under strict aseptic conditions. The samples were obtained from surfaces in both food contact and non-food contact. The category of food contact surfaces comprises various items, namely plates, pots, frying pans, cups, bowls, knives, forks, spoons, spatulas, and handheld graters, which were collected from both washed and unwashed items. The non-food contact surfaces encompass gas cookers, hot plates, cupboards, the floor space surrounding the cooking area, plate racks, and tabletops.

ISOLATION AND IDENTIFICATION

The swab samples were then inoculated onto Sabouraud Dextrose Agar (SDA) and incubated aerobically for 24 hours at 37°C. The isolates were first identified by Gram staining before further culturing on Hi Chrome Candida Differential Agar (M1297A) for differentiation of Candida species based on colour. Gram staining aids in the identification and helps in distinguishing yeast-like organisms from other microorganisms. The identification procedure was based on the physical traits of the colonies and their colour responses on the selective agar.

DATA ANALYSIS

Chi-square analysis was used to survey the data and find the prevalence on the different surfaces of Candida species. The level at which the difference was declared to be statistically significant was if $p < 0.05$ to determine the association between variables. This study aimed at investigating whether the type of surface or residential hall and the prevalence of Candida species significantly correlated.

Results

A total of 50 rooms were sampled across seven residential halls at Obafemi Awolowo University, Ile-Ife. Out of the 50 rooms sampled, 43 (86%) were positive for *Candida* species. This high prevalence highlights the significant contamination of surfaces within the student residential halls as shown in Table 1.

Of all the halls of residence, Moremi Hall has the highest number of samples 14 (28%) recorded in this research work. The Postgraduate and Akintola Halls of Residence both accounted for 14% of the data, ETF Hall accounted for 12%, Angola Hall accounted for 10% and Alumni Hall accounted for the lowest samples, 2%. Food contact surfaces had a frequency of 76%, but non-food contact surfaces had a slightly lower prevalence of 64%, according to our analysis of the prevalence of Candida species on these surfaces. Candida was most common on tabletops (24%) and floors (16%), then on gas cookers (8%) and cabinets (4%), as shown in Table 1.

The amount of Candida found within these 50 rooms has been ordered into 2 groups, food contact surfaces and non-food contact surfaces as shown in Tables 2 and 3 respectively.

Table 1. The prevalence of Candida species in halls residence among Food contact and Non-food contact surfaces in Obafemi Awolowo University

HALL	OCCUPIED BY	NO. OF ROOMS EXAMINED	NO. OF ROOMS POSITIVE FOR CANDIDA SPECIES
AKT	Female	7 (14.0)	7 (14.0)
ALM	Female	1 (2.0)	1 (2.0)
ANG	Male	5 (10.0)	5 (10.0)
ETF	Male	6 (12.0)	6 (12.0)
FAJ	Male	10 (20.0)	6 (12.0)
MORE	Female	14 (28.0)	13 (26.0)
PG	Mixed	7 (14.0)	5 (10.0)
TOTAL		**50 (100.0)**	**43 (86.0)**

KEY: AKT = Akintola, ALM = Alumni, ANG = Angola, ETF = Education Trust Fund, FAJ = Fajuyi, MOR = Moremi, PG = Post Graduate Hall

From Table 2, the result shows the prevalence of Candida on Non-Food Contact Surfaces and non-food contact surfaces and their respective contributions of overall 64%. For instance, among the contact areas which are not in direct contact with food, tabletops carried a higher risk of 24% contamination, followed by floors at 16%, a gas stove at 8%, and cabinets at 4%. The high incidence may be caused by the frequent interaction of these surfaces with cooking supplies and personal belongings of pupils. The presence of Candida on particular non-food contact surfaces also varied statistically significantly as shown by the Chi-square analysis. The correlations are very strong for knives and spatulas, for example, $X2 = 50.000$, $p = 0.000$, and $X2 = 25.818$, $p = 0.000$, respectively. Such items are highly polluted and thus require more focused cleaning procedures.

Table 2. The Prevalence of Candida species in Halls Residence among Food Contacts in Obafemi Awolowo University

		NON–FOOD CONTACT POSITIVE FOR CANDIDA SPECIES				
HALL	**ROOMS POSITIVE FOR CANDIDA SPP (%)**	**GAS COOKERS (%)**	**HOT PLATES (%)**	**TABLE TOPS (%)**	**FLOORS (%)**	**CUPBOARDS (%)**
AKT	7 (14.0)	1 (2.0)	0 (0.0)	4(8.0)	2 (4.0)	0 (0.0)
ALM	1 (2.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (2.0)	0 (0.0)
ANG	5 (10.0)	2 (4.0)	0 (0.0)	0 (0.0)	1 (2.0)	2 (4.0)
ETF	2 (4.0)	1 (2.0)	0 (0.0)	0 (0.0)	1 (2.0)	0 (0.0)
FAJ	5 (10.0)	2 (4.0)	1 (2.0)	1 (2.0)	1 (2.0)	0 (0.0)
MORE	10 (20.0)	2 (4.0)	0 (0.0)	6 (12.0)	3 (6.0)	0 (0.0)
PG	2 (4.0)	0 (0.0)	0 (0.0)	1 (2.0)	1(2.0)	0 (0.0)
TOTAL	**32 (64.0)**	**8 (16.0)**	**1 (2.0)**	**12 (24.0)**	**10 (20.0)**	**2 (4.0)**

KEY: AKT = Akintola, ALM = Alumni, ANG = Angola, ETF = Education Trust Fund, FAJ = Fajuyi, MOR = Moremi, PG = Post Graduate Hall

Table 3 shows the prevalence of Candida on Food Contact Surfaces clearly showed that Candida is highly prevalent on surfaces coming in contact with food plates, 54%; pots, 14%; spoons, 10%; and knives, 4%. This indicates that surfaces directly associated with food preparation and consumption are focal points for Candida infections. Furthermore, co-infections were commonly observed to occur on food-contact surfaces: in the PG, 28.6% of rooms were positive for both Candida albicans and Candida glabrata. The abundance of several Candida species on surfaces that are in contact with food reflects the possibility of cross-contamination between species. Table 3 gives the exact distribution of Candida species along the food contact surfaces; notable co-infections were observed in different halls.

Table 3. Prevalence of *Candida species* on various food contact surfaces in Obafemi Awolowo University

		POSITIVE FOOD CONTACT SURFACES									
Hall	**Positive Rooms (%)**	**Plates (%)**	**Pots (%)**	**Spoons (%)**	**Grater (%)**	**Spatulas (%)**	**Forks (%)**	**Knives (%)**	**Cups (%)**	**Bowls (%)**	**Trays (%)**
AKT	6 (12.0)	5 (10.0)	0 (0.0)	1 (2.0)	1 (2.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (2.0)	0 (0.0)	1 (2.0)
ALM	1 (2.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (2.0)	0 (0.0)	1 (2.0)	0 (0.0)	0 (0.0)	0 (0.0)
ANG	4 (8.0)	2 (4.0)	2 (4.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (2.0)	0 (0.0)
ETF	5 (10.0)	2 (4.0)	1 (2.0)	2 (4.0)	1 (2.0)	0 (0.0)	1 (2.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
FAJ	4 (8.0)	4 (8.0)	1 (2.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
MORE	13 (26.0)	9 (18.0)	2 (4.0)	2 (4.0)	0 (0.0)	0 (0.0)	1 (2.0)	1 (2.0)	0 (0.0)	0 (0.0)	0 (0.0)
PG	5 (10.0)	5 (10.0)	1 (2.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Total	**38 (76.0)**	**27 (54.0)**	**7 (14.0)**	**5 (10.0)**	**2 (4.0)**	**1 (2.0)**	**2 (4.0)**	**2 (4.0)**	**1 (2.0)**	**1 (2.0)**	**1 (2.0)**

KEY: AKT = Akintola, ALM = Alumni, ANG = Angola, ETF = Education Trust Fund, FAJ = Fajuyi, MOR = Moremi, PG =Post Graduate Hall
Spatulas**:** $X^2 = 50.000$, *p = 0.000*; K

Table 4 shows Candida species distribution across selected residence halls in the Obafemi Awolowo University. The result shows that 76% of rooms where samples were collected from the halls tested positive for the presence of Candida. *Candida glabrata* was the most prevalent species of Candida, predominant in 28% of rooms of the halls of residence, with 35.7% recorded in Moremi Hall showing the highest prevalence.

Candida glabrata was the most common species in the study (28%), followed by Candida tropicalis (6%), and Candida albicans (6%). Co-infection was prevalent, particularly on surfaces that came into touch with food. For instance, co-infection between Candida albicans and Candida glabrata was found in 28.6% of rooms, mostly in the Postgraduate Hall (PG). According to Table 4, Moremi Hall had the highest prevalence of C. glabrata (35.7%), followed by Akintola Hall (28.6%) and PG Hall (16.7%). The study employed chi-square analysis to see whether there was a significant correlation between the kind of surface and the prevalence of Candida species. According to the findings, some surfaces—like knives and spatulas—exhibited statistically significant correlations with the presence of Candida species (X2 = 0.000, p = 0.000, and X2 = 25.818, p = 0.000, respectively) as shown in Table 4. This emphasizes the significance of specific kitchen tools in spreading Candida infection.

Table 4. Distribution of *Candida species* on various food contact surfaces in Obafemi Awolowo University

HALL	No. of Positive Rooms (%)	*C. alb (%)*	*C. glab (%)*	*C. trop (%)*	*C. Krus (%)*	*C.alb & C. glab* (%)	*C. glab & C.krus* (%)	*C. alb & C. trop* (%)	*C. glab & C. trop* (%)	*C. alb, C. glab and C. trop* (%)	*C. glab, C. trop and C krus* (%)
AKT	6 (12.0)	1(14.3)	1 (14.3)	0 (0.0)	1(14.3)	1(14.3)	1 (14.3)	0 (0.0)	0 (0.0)	1 (14.3)	0 (0.0)
ALM	1 (2.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)
ANG	4 (8.0)	1(40.0)	2 (40.0)	1 (20.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
ETF	5 (10.0)	0 (0.0)	2 (33.3)	0 (0.0)	1(16.7)	1(16.7)	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)
FAJ	4 (8.0)	0 (0.0)	4 (40.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
MORE	13 (26.0)	1(33.3)	5 (35.7)	1 (7.1)	0 (0.0)	1 (7.1)	1 (7.1)	1 (7.1)	1(7.1)	0 (0.0)	2 (14.3)
PG	5 (10.0)	0 (0.0)	0 (0.0)	1 (14.3)	0 (0.0)	2(28.6)	2 (28.6)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
TOTAL	**38 (76.0)**	**3 (6.0)**	**14 (28.0)**	**3 (6.0)**	**2 (4.0)**	**5(10.0)**	**4 (8.0)**	**1 (2.0)**	**3 (6.0)**	**1 (2.0)**	**2 (4.0)**

KEY: AKT = Akintola, ALM = Alumni, ANG = Angola, ETF = Education Trust Fund, FAJ = Fajuyi, MOR = Moremi, PG = Post Graduate Hall.
Key: *C. alb = Candida albican*, *C. glab = Candida glabrata*, *C. trop = Candida tropicalis*, *C. krus = Candida krusei*

Table 5, shows the distribution of Candida on Non-Food Contact Surfaces, highlighting species specificity on different surfaces. At a 50% contamination rate in Moremi Hall, Candida glabrata was the most prevalent species on non-food contact surfaces. Other surfaces with notable contamination included floors at 6% and gas cookers at 10%. The most polluted hall was Moremi Hall, which had 20%, while Akintola Hall had 14%. This difference might be because of different cleaning practices, occupancy, or hygiene expectations along the corridors. Moreover, 42% of rooms tested positive for Candida glabrata, showing dominance across non-food contact surfaces. These results imply that even surfaces that are not used directly for food preparation nevertheless pose a serious possibility of contamination.

Table 5: Distribution of Candida species on various Non-food contact surfaces in Obafemi Awolowo University

HALL	Number of positive rooms (%)	*Candida albican* (%)	*Candida. glabrate* (%)	*Candida tropicalis* (%)	*Candida krusei* (%)	*Candida albican & Candida glabrata* (%)
AKT	7 (14.0)	0 (0.0)	2 (9.5)	1 (33.3)	1 (14.3)	1 (14.3)
ALM	1 (2.0)	0 (0.0)	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)
ANG	5 (10.0)	0 (0.0)	3 (60.0)	1 (20.0)	0 (0.0)	1 (20.0)
ETF	2 (4.0)	0 (0.0)	2 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)
FAJ	5 (10.0)	1 (10.0)	4 (40.0)	0 (0.0)	0 (0.0)	0 (0.0)
MORE	10 (20.0)	0(0.0)	7 (50.0)	1 (7.1)	2 (14.3)	0 (0.0)
PG	2 (4.0)	0 (0.0)	2 (28.6)	0 (0.0)	0 (0.0)	0 (0.0)
TOTAL	**32 (64.0)**	**1 (2.0)**	**21 (42.0)**	**3 (6.0)**	**5 (10.0)**	**2 (4.0)**

KEY: AKT= Akintola, ALM= Alumni, ANG= Angola, ETF= Education Trust Fund, FAJ= Fajuyi, MOR= Moremi, PG=Post Graduate Hall

Discussion

The study findings, as obtained from the surfaces that come into contact with food and non-food in the residence halls of students at Obafemi Awolowo University in Ile-Ife, Nigeria, reveal a very high frequency of Candida species. With an 86% total contamination rate, these results have pointed out the importance of fungal contamination in shared living environments where a variety of hygiene practices and shared amenities provide the perfect conditions for microbial growth. Especially for the immunocompromised students, the high prevalence of Candida species, particularly on surfaces that are frequently in contact with food, underlines the potential public health risks in these settings. In our study, the prevalence of non-food contact surfaces, such as floors and tabletops, was relatively lower (64%) compared to the prevalence of food contact surfaces (76%). This is in agreement with the work of Belizario et al. (2021), which also reported high levels of fungal contamination in indoor non-clinical environments. However, the high prevalence of Candida on surfaces not involved in food contact shows that contamination is not just a problem in places where food is prepared. These findings align with various other studies that have shown how fungal species can successfully colonize a wide variety of surfaces even on those not necessarily handled by the handler of food (Cristina et al. 2023).

The distribution showed that Candida glabrata was the most prevalent species from food and non-food contact surfaces, closely followed by C. albicans and C. tropicalis. This distribution agrees with the results of other investigations that have found C. glabrata to be a common contaminant in environmental samples (Pappas et al. 2018). The complexity of fungal contamination in these environments is further demonstrated by the presence of multiple Candida species, including co-infections. Co-infections can increase the chances of infection by promoting the spread of fungal species among students, particularly on surfaces that are exposed to food. This is probably because of a difference in cleaning practices, room usage, or hygiene habits in Moremi Hall. Generally, cleanliness for Candida contamination reduction and sticking to cleaning schedules are very important, as this study has established. Some environmental and behavioral factors, though linked to fungal species proliferation in communal living spaces, also need further study.

The current research also highlights the need to improve sanitary standards in dorms where students reside. Preventing the spread of Candida and other microorganisms requires regular cleaning and disinfection of surfaces, especially those coming into contact with food such as plates, pots, and utensils. Students should also be made aware of the risks of fungal infections and the importance of maintaining personal hygiene, especially among immunocompromised individuals. There are several limitations with this study, despite it offering quite useful information about Candida contamination in student dorms. This study was only conducted for one university, thus limiting general applicability in other settings. Further studies are needed to investigate the incidence of Candida in student dorms in different geographical regions and institutes. The effect of environmental conditions such as Fungal growth is favoured by moisture and aeration. Future studies can also be done on the pathogenicity of the isolated isolates, focusing on virulence factors or resistance to antifungals. This study provides valuable information on the occurrence of Candida species in student residence halls and emphasizes the need for better hygiene procedures and routine monitoring to prevent fungal contamination and protect students' health. Understanding the dynamics of fungal contamination in shared living spaces could help public health concerns regarding opportunistic infections.

Conclusions

This study considers the prevalence of Candida species on food and non-food contact surfaces within residence halls of students in Obafemi Awolowo University in Ile-Ife, Nigeria. The results from this investigation indicate that in all rooms investigated, a high prevalence of Candida contamination was recorded (86%), with a contamination rate slightly higher in food contact surfaces compared to non-food contact surfaces, being 76% and 64%, respectively. Many species are found to co-infect surfaces, especially in areas where food comes into contact with the surface. Candida glabrata was the most common species, followed by Candida albicans and Candida tropicalis. The high contamination rates in this study point out the potential health risks that Candida infections can pose, especially to people with weakened immune systems. The hygiene standards in the students' hostels should be improved, particularly in terms of washing and sanitizing the frequently used surfaces like kitchenware, pots, and plates. These settings need to be periodically assessed to prevent the spread of Candida and to avert potential infections.

This study recommends enhanced public health education for students, especially regarding personal hygiene and the risks of fungal infection, considering the high prevalence of Candida species both on surfaces that come into contact with food and those that do not. Future studies should be directed at ascertaining the causes of fungal contamination in shared living areas, including the part played by environmental factors such as moisture and ventilation. Further studies are needed to determine the pathogenic potential of the isolated Candida strains and their resistance to antifungal drugs in order to understand the implications of these findings for students' health. In conclusion, this study points out the necessity of effective intervention methods to minimize fungal infection transmission in communal living environments and contributes to the growing literature on Candida contamination outside clinical settings.

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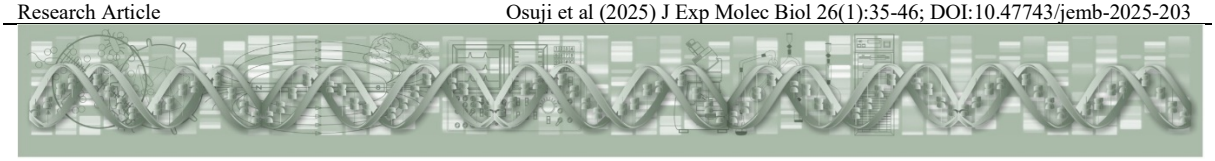
+ Authors declare no conflict of Interest

+ Authors contribution: Olaniran, O = Perform experimental and statistical analysis of the manuscript; Adewoyin, A. A = wrote the manuscript; Awoniyi, S.O, Bidmus, A.B and Boriwaye, O.C = collect the Samples.



NUTRITIONAL AND PHYSICOCHEMICAL CHARACTERIZATION OF SELECTED DROUGHT TOLERANT NIGERIAN LOCAL RICE CULTIVARS

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Abstract

Drought-tolerant rice cultivars with inherent good quality traits are important in ensuring adequate and nutritious food for humans, particularly in sub-Saharan African regions like Nigeria. Thus, for the attainment of effective nutrition security in a climate-changing world, it is expedient to scientifically characterize, identify, and develop good quality rice cultivars with intrinsic drought tolerance potentials. In this study, four potentially drought-tolerant rice cultivars were collected from local rice farmers in some Northern parts of Nigeria. They were first subjected to preliminary drought tolerance validation, then physicochemical, proximate, mineral, and transgene diagnostic analyses. The proximate analysis results showed that the cultivars have good protein content particularly "Nassarawa-Lafia" (10.20±0.00%) while "Jigawa-Mafa" has the highest fiber and magnesium content of 1.81±0.01% and 31.80±0.04% respectively. Mineral content analysis results also revealed cultivars like "Jigawa-Mafa" and "Nassarawa-Lafia" as high potassium enriched up to 118.72±0.41mg/100g and 117.06±0.91mg/100g. The molecular diagnostics showed no transgene presence in all the samples. These findings highlight the good nutritional qualities of the cultivars and their potential health benefits. This calls for a more in-depth search of the expansive local genetic pool for crops with promising abilities for cultivation and subsequent breeding programs to address the increasing food and nutrition challenges.
Keywords: cultivars, drought, nutrition, quality, rice

Introduction

The influence of unfavorable environmental conditions on plants leads to various biotic and abiotic stresses that affect their growth, development, and yields (Yadav et al. 2020). Drought is one of such condition and it adversely reduces rice productivity by causing abscission and senescence in the plant. This in turn impacts negatively on food and nutrition security as rice is a major staple food across the globe (Ndjiondjop et al. 2018).

Rice (*Oryza sativa* L.), is highly valued as one of the world's most important food crops for its rich dietary and calorific values (Sen et al. 2020). It is a rich staple food of choice for a large section of the global population especially in developing countries like Nigeria, due to its pleasant taste and ease of cooking. It supplies the body with a high amount of energy and digestible carbohydrates. With its protein quality and appreciable amounts of minerals and vitamins that play key body functional and regulatory roles, rice meals are adequate food for

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meeting and sustaining significant human nutritional needs (Mohidem et al. 2022). However, its high-water demand makes it very susceptible to drought, which is a major abiotic threat to plants in sub-Saharan Africa. Rice production has increasingly been exposed to unpredictable and devastating losses caused by drought stress due to the prevailing climate change conditions (Dar et al. 2020). Cultivation of some modern and improved rice cultivars with good quality potential has most times ended in huge losses because of the effect of unexpected drought conditions (Giri et al. 2020). Also, cultivars with good drought tolerance but little quality traits, on the other hand, are short of the 'adequate and nutritious' purpose of food consumption. The quality of rice is generally taken from two major aspects, viz; the physical quality which is mostly based on the appearance, and the second aspect, the nutritional content (Custodio et al. 2019). The physical outlook is a major determinant factor in the preliminary assessment of rice quality and represents the first quality evaluation of rice especially from the appearance point of view. This focal point of quality evaluation is a measure of the grain color, aroma, size, shape, and breakability (Custodio et al. 2019). The overall quality of rice is a key factor in its acceptability, economic significance, nutritional, and health benefits.

In Nigeria, over the past years, there has been a remarkable increase in rice production due to new Government policies that banned importation to encourage local production. Over five million metric tons of local rice is now produced in the country annually (Statista 2022). Regardless of this boom in production, there is also an astronomical rise in the cost of rice and Nigerians still crave for imported rice with higher qualities. This is majorly because of the problem of the production-to-consumption imbalance of rice, compounded by the cultivation of cultivars that fall short of adequate nutrition qualities and stress survival abilities. As a result of this, smuggling and racketeering of foreign rice from other countries like Thailand, China, and India becomes a bubbling side business.

Thus, this great demand for high-quality, drought-tolerant Nigerian rice cultivars requires rapid and more natural approaches in rice research programs (Danbaba et al. 2020). This is quite necessary for cultivation and subsequent breeding programs to meet the increasing demands of an expanding population size in Nigeria. Although different studies have been carried out on drought tolerance and nutritional analysis of some rice cultivars in Nigeria separately, not much has been reported for combined drought tolerance, physicochemical quality traits, and transgene diagnostic analyses.

This study is therefore devised to evaluate key nutritional and physicochemical characteristics of selected drought-tolerant indigenous rice cultivars devoid of genetic modification.

Materials and Methods

Preliminary Drought Tolerance Investigation

To confirm their drought tolerance using the preliminary seedling validation method, 50g of each rice cultivar seed specimens collected from major rice-growing states in the Northern part of Nigeria were taken to the Biotechnology Advanced Research Center in Sheda Science and Technology Complex. Lab codes (Samples 1-4) were assigned to them. Sample 1 denotes “Nassarawa-Lafia”, Sample 2; “Niger-Jerusalem” while Sample 3 and 4 denote “Kebbi-Jirani”, and *“*Jigawa-Mafa” cultivars respectively. These cultivars are local drought-tolerant varieties of rice cultivated by the local farmers in Kebbi, Nassarawa, Jigawa, and Niger states of Nigeria, hence their names. Polyethylene glycol (PEG-6000) was used at a high concentration of 20% on the seedlings to verify their tolerance to drought by creating a high level of potential water dearth according to the method previously described by Susilawati et al. (2022). Twenty grams (20 g) of the collected rice seed specimens from each of the cultivars were pulverized and subjected to physicochemical, mineral content, and transgene detection analyses using proximate, atomic absorption spectrophotometry (AAS) and molecular diagnostic analyses.

Physical Analysis

Samples from the four cultivars were subjected to the various physical analyses as follows:

Chalkiness index

The chalkiness indices were determined using twenty (20) dehulled rice grains from each sample on a lightbox and visually examining their chalkiness appearance. The percentage chalkiness of each sample was calculated by taking the average of the 20 values. The method as previously described by Dela-Cruz (2000) was used to express the chalkiness levels as; 0 (no chalkiness), 1 (less than 10% chalkiness), 5 (10 - 20% chalkiness), and 9 (more than 20% chalkiness).

Color

The visual method was used to physically identify the grain colors as shown in Figure 2 while the Lovibond system was used to corroborate it. Lovibond color system for visually determining and comparing colors using the Lovibond comparator scale according to the method previously described by Hadi et al. (2021).

Aroma

Following the method previously described by AOAC (2013) using pulverized samples in 1.7% KOH solution and incubated at 60ºC for 10 min., the aroma evaluation was carried out.

Grain Dimension (Length and Width)

Grain dimensions were determined using the general Vernier Caliper by randomly taking 20 grains from each sample and measuring their lengths and widths according to the method previously described by Ilia et al. (2023).

Grain kernel weight

This was determined using 1000 kernels from each sample and weighing separately to determine bulk Kernels' weight according to the method previously described by Singh et al. (2003).

Breakability

The breakability ratio was determined using the method previously described by Khosravi et al. (2011) which involved calculating the percentage of the broken grains after subjecting them to a Unimax 1010 shaker (Heidolph, Germany) at moderate speed.

Proximate Composition Analysis

The rice samples were properly sorted to remove all extraneous matters, then dehulled, pulverized, sieved, and weighed to collect 100 g of fine powdered samples for each cultivar. The proximate composition analytical process was used for evaluating moisture, crude proteins, crude fiber, crude lipid, crude ash, and carbohydrate contents in triplicates to determine the nutritional contents of the four different cultivar samples.

Crude Protein Content Determination

The crude protein contents of the rice samples were determined using the digestion and distillation method as previously outlined by Oko et al. (2012), in which the crude protein contents of samples are derived by measuring their total nitrogen contents and multiplying by a definite factor (6.25).

Moisture Content Determination

Moisture content was measured using the Standard Official Methods of Analysis as previously described by AOAC (1990) which involved drying the samples to a constant weight at 100°C and calculating the moisture after the weight losses of the dried rice samples.

Fiber Content Determination

The crude fiber content was determined using the protocols previously described by AOAC (1990), which involves hydrolyzing with petroleum ether.

Lipid Content Determination

The total crude lipid content in the rice sample was determined using Soxhlet extraction for 4 hours with methanol and ethanol according to the method previously described by Eromosele and Eromosele (1994).

Carbohydrate Content Determination

The percentage carbohydrate content was deduced by calculating the difference between the sum of other constituents and 100 as previously described by Onyeike et al. (1995).

Mineral Contents Determination

The Potassium, magnesium, Iron, Zinc, and Calcium contents of the four different cultivar samples were determined using flame Atomic Absorption Spectrophotometry (AAS) protocols according to the method previously described by Kouassi et al. (2013). Two grams (2 g) of pulverized sample from each cultivar was used for the analysis. This involved sample digestion using a combined mixture of tetraoxonitrate (v) acid and perchloric acid in a 4:1, v/v ratio and solubilization with distilled water prior to measurements with AAS.

Molecular Analyses

The molecular diagnostic analysis for the presence of transgene was carried out by extracting the DNA of the samples from the four different cultivars and amplifying them with the common rice transgene primers (Cry 1Ab and VIP3) along with positive and negative controls. Extraction of DNA from the leaves of the specimen samples was carried out with Zymo Research Plant DNA extraction kit according to the manufacturer's protocol (Zymo Research 2016) using 150 mg of leaf samples from each cultivar. The PCR amplification was carried out for transgene detection by VIP3/Cry 1Ab primer amplification according to the method described by Safaei et al. (2019).

Statistical Analysis

The samples were analyzed in triplicates and the generated data was evaluated using descriptive (means and standard deviation) and inferential statistics (one-way Analysis of Variance) using SPSS software (Version 20, SPSS Inc. Chicago, USA). The results were analyzed by applying the Duncan testing algorithm and expressed as mean±SD and variations considered statistically significant when $P< 0.05$.

Results and discussions

The drought tolerance preliminary validation results of the selected rice cultivars' seedlings with PEG-6000 were shown in Figure 1 while the statistical analysis using tables are shown in Table 1 (A, B, and C). The grain physical quality analyses were shown in Figure 2 and Table 2 while the proximate composition analyses were depicted in Table 3. Mineral content and molecular analyses results were presented in Table 4 and Figure 3 respectively.

Table 1.A: Drought Tolerance Preliminary Validation Indices 1

S/N	Germination Percentage (%)		Plant Height (mm)		Root Length (mm)	
Samples	**Control**	**Drought**	**Control**	**Drought**	**Control**	**Drought**
Sample 1	100.00±0.00b	85.00±1.00a	13.00±0.50a	11.00±0.00a	12.30±0.10b	17.00±.00d
Sample 2	100.00±0.00b	96.00±1.00c	14.00±1.00ab	14.07±0.06c	13.70±0.00d	16.70±0.17c
Sample 3	95.00±1.00a	92.00±2.00b	15.00±.00b	14.70±0.20d	10.27±0.06a	14.03±0.06a
Sample 4	100.00±0.00b	98.00±0.00c	14.73±0.35b	13.70±0.10b	13.30±0.10c	15.30±0.10b

*Each value is the mean ± standard deviation of three replicates. Means in the same column followed by the same letter are not significantly different at $p \geq 0.05$.

Table 1.B: Drought Tolerance Preliminary Validation Indices 2

S/N	Plant Length (mm)		Fresh Weight (g)		Root-shoot Ratio	
Samples	**Control**	**Drought**	**Control**	**Drought**	**Control**	**Drought**
Sample 1	25.30±0.20a	28.00±0.00a	0.09±0.00a	0.07±0.00a	0.92±0.010c	1.55±0.02c
Sample 2	27.57±0.21b	30.70±0.10d	0.17±0.00d	0.17±0.01d	0.98±0.01d	1.99±0.03d
Sample 3	25.30±0.00a	28.70±0.00b	0.12±0.00b	0.11±0.00b	0.69±0.01a	0.95±0.00a
Sample 4	28.00±0.00c	29.00±0.00c	0.14±0.00c	0.12±0.00c	0.90±0.00b	1.12±0.01b

*Each value is the mean ± standard deviation of three replicates. Means in the same column followed by the same letter are not significantly different at $p \geq 0.05$

Table 1.C: Summary of Drought Tolerance Preliminary Validation Indices

Samples	PSI (%)	RLI (%)	PLI (%)	DTI (%)
Sample 1	84.00±0.01a	138.20±0.02d	110.70±0.02b	85.00±0.01c
Sample 2	100.00±0.00d	121.90 ±0.01b	111.20±0.01c	87.50±0.02d
Sample 3	98.01±0.01c	135.90±0.02c	113.40±0.03d	84.60±0.01b
Sample 4	93.40 ±0.01b	115.00 ±0.01a	103.70±0.03a	83.30±0.02a

*Each value is the mean ± standard deviation of three replicates. Means in the same column followed by the same letter are not significantly different at $p \geq 0.05$.

Key:

1. **G%** = N/n×100 (where n is the number of germinated seeds at the end of experiment, N is the total number of the seeds)
2. **Drought Tolerance Index (DTI) =** $\frac{\text{G\% under drought}}{\text{G\% under control}}$ x100
3. **Plant Height Stress Index (PHSI in %) =** $\frac{\text{Plant height of plant under drought stress}}{\text{Plant height of control plants}}$ x 100
4. **Root Length Drought Stress Index (RLSI in %) =** $\frac{\text{Root length of stressed plant}}{\text{Root length of control plants}\boldsymbol{dx}}$ x 100

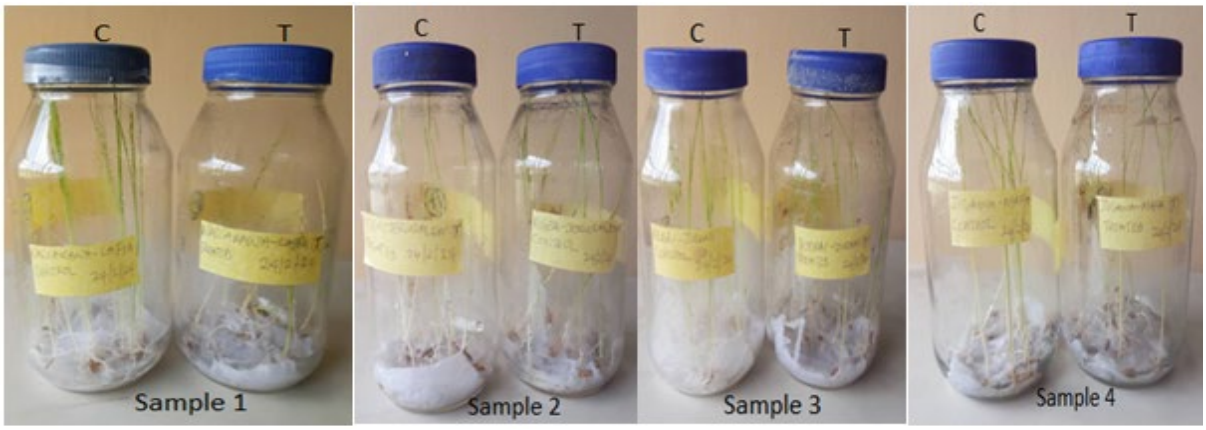

Figure 1. Preliminary Drought Validation of The Cultivars using 20% PEG-6000: C= Control, T= Treatment

Compared with the controls, the treated cultivars exhibited substantial resilience to the subjected drought stress treatment as shown in Figure 1 and Table 1(A, B, and C) which is a validation of their drought-tolerance ability.

Table 2. Physical Analyses

Samples	Color	Chalkiness	Aroma	Length (mm)	Width (mm)	Weight (g)	Breakability (%)
Sample 1	L-B	0	0	5.52±0.11a	2.24±0.04a	22.10±0.22b	6.16±0.06a
Sample 2	W	1	0	6.4±0.32b	2.41±0.02a	30.35±0.12d	9.48±0.10b
Sample 3	W	1	1	6.60±0.82b	2.27±0.01b	26.78±0.05c	11.13±0.02c
Sample 4	L-B	0	1	5.82±0.17a	2.53±0.02c	20.39±0.21a	13.48±0.12d

*Each value is the mean ± standard deviation of three replicates. Means in the same column followed by the same letter are not significantly different at $p \geq 0.05$; L-B = Light Brown; W= White; 0= none; 1= less than 10%

The four selected cultivars showed varying physical characteristics. "Niger-Jerusalem" and "Kebbi-Jirani" cultivars are somewhat whitish in color while "Nassarawa-Lafia*"* and "Jigawa-Mafa" are light-brownish in color respectively, as shown in Figure 2 below. The analysis of variance showed significant differences in the measured physical parameters at $P < 0.05$ among some of the cultivars as depicted with letters in the same column as shown in Table 2. From their Length and width values, "Niger-Jerusalem" and "Kebbi-Jirani" cultivars can be described as long grains while "Nassarawa-Lafia" and "Jigawa-Mafa" belong to the medium grain category in agreement with Nádvorníková et al. (2018). The breakability indices of the four cultivars ranging between 6.20 to 13.3% are a measure of their resistance to physical pressure and indication of good evacuation, nutritional, cooking, and storage qualities in line with Ashizume et al. (2005).

Figure 2. Physical Appearance Analysis of the Samples

Table 3. Proximate Analyses

Samples	Moisture (%)	Ash (%)	Fibre (%)	Lipid (%)	Protein (%)	Carbohydrate (%)
Sample 1	10.10±0.04a	1.10±0.02b	0.44±0.01c	1.38±0.02c	10.20±0.00a	76.89±0.04a
Sample 2	11.01±0.02b	0.82±0.02a	0.18±0.01a	1.35±0.02c	7.44±0.00a	79.20±0.05d
Sample 3	11.10±0.05c	0.82±0.04a	0.20±0.00b	1.18±0.00a	8.04±0.00a	77.05±0.02b
Sample 4	11.58±0.01d	1.39±0.00c	1.81±0.01d	1.27±0.02b	8.13±0.00a	77.43±0.02c

*Each value is the mean ± standard deviation of three replicates. Means in the same column followed by the same letter are not significantly different at $p \geq 0.05$

The analysis of variance of the proximate parameters showed significant differences at $P < 0.05$ among some of the cultivars as depicted with letters in the same column as shown in Table 3. The high carbohydrate content across the four cultivars is an affirmation of their primary role as a major energy source and mirrors other research findings like that of Oko and Onyekwere (2010) and Fukagawa and Ziska (2019). The crude fiber content which ranged between 0.44% to 1.81% showed variations across the 4 cultivars with "Niger-Jerusalem" having the least value

while "Jigawa-Mafa" had the highest value. Dietary fiber plays a regulatory role in carbohydrate absorption via hydrolytic enzyme action inhibition which helps to reduce glycemic index and type 2 diabetes risk factors (Makki et al. 2018, Mao et al. 2021). Fiber contents of food enhance their laxative effect in the gut and decrease the incidence of constipation. Good fiber content in the "Jigawa-Mafa" cultivar is a good indication of its potential healthy bowel and metabolic functions.

The results of crude protein contents of the four cultivars (7.44% to 10.20%) are quite indicative that they are important candidates for nutrition security particularly the "Nassarawa-Lafia" cultivar. Protein which plays a crucial role in the growth, development, and replenishment of worn-out tissues was notably high in some cultivars affirming the nutritional quality of the cultivars. Rice protein is of high biological value because of its hypo allergenicity and balanced profile of amino acids like threonine, leucine, and phenylalanine (Jayaprakash et al. 2022). It also has a high lysine content in comparison to most other cereals (Eggum et al. 1993).

The amounts of crude lipids present in the various rice cultivars were relatively low, in slight contrast with Arowora et al. (2021) but comparable to the results of Obembe et al. (2022). These variations could be attributed to genetic and edaphic factors. Nonetheless, the lipid content of rice is almost void of cholesterol (Silver 2017) due to their predominant constituents of unsaturated fatty acids like linoleic acid and palmitic acids which are known as essential fatty acids (Juliano and Goddard 1986). The moisture contents of the cultivars which ranged between 10.01% to 11.10% were on the lower ebb and good for long-term storage, in agreement with Oko et al. (2012). Moisture contents significantly affect the shelf-life of grains as well as their milling characteristics and taste (Ebuehi and Oyewole 2007, Zheng and Lan 2007, Yasothai 2020). While the ash content of rice is relatively small compared to lipid, protein, and carbohydrate, it is quite a significant factor in evaluating the mineral content and quality of rice. The four cultivars have good ash contents ranging from 0.81% to 1.39%.

Table 4. Mineral Content Analyses

Samples	Potassium (mg/kg)	Magnesium (mg/kg)	Iron(mg/kg)	Calcium(mg/kg)	Zinc(mg/kg)
Sample 1	118.72±0.41d	24.76±028c	2.22±0.04b	15.14±0.03a	1.51±0.03c
Sample 2	113.65±0.20b	23.09±0.01b	5.23±0.0 2d	18.16±0.13c	1.35±0.03b
Sample 3	110.10±0.26 a	22.64±0.13a	4.63±0.04c	19.20±0.01d	1.10±0.00a
Sample 4	117.06±0. 91c	31.80±0.04d	2.15±0.01a	14.40±0.06b	1.98±0.05d

*Each value is the mean ± standard deviation of three replicates. Means in the same column followed by the same letter are not significantly different at $p \geq 0.05$

The calcium, zinc magnesium, and iron contents of rice are of very important health value (Weyh et al. 2022). The four cultivars have good ash contents ranging from 0.81% to 1.39%. The iron and calcium contents of "Niger-Jerusalem" and "Kebi-Jirani" cultivars were considerably higher than the other two, in line with Priya et al. (2019), probably due to genetic and edaphic factors. "Jigawa-Mafa" cultivar on the other hand exhibited the highest magnesium content followed by "Nassarawa-Lafia" which makes them good for people predisposed to metabolic disorders. Magnesium is an important mineral that plays a vital role in the regulation of blood pressure and sodium balance in the body. It helps in lowering the risk of metabolic syndrome; a critical indicator of cardiovascular disease like heart attacks (Kass et al. 2012). The varying levels of five essential minerals analyzed depicted desirable values which are very crucial for improving micronutrient activities like growth, blood pressure, metabolism and the general wellbeing of human life.

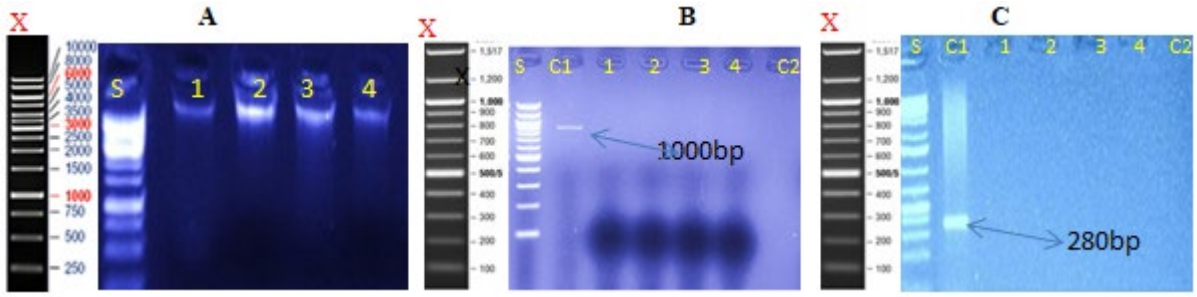

Figure 3. A. DNA Extraction, **B.** PCR Amplification using VIP 3 and, **C.** PCR Amplification using Cry Ab Primers

Key: X= Reference ladder chart, S= Step ladder, C1= +ve Control, C2= -ve control, 1-4 = sample 1 to sample 4.

The molecular diagnostic analysis conducted to check for transgene presence was to ascertain any sign of transgenic modification in the selected cultivars (Yang et al. 2010). Figure 3A showed the DNA extraction results of the samples while Figures 3B and 3C presented the molecular diagnostic analysis using PCR method for transgene detection. The results indicated the absence of any possible transgene, particularly the Cry 1Ab and Vip3 genes which are the most used transgenes in transgenic rice production (Xu et al. 2018).

The varying levels of five essential minerals analyzed depicted desirable values that are very crucial for improving micronutrient activities like growth, blood pressure, metabolism, and the general well-being of human life. Thus, the different nutritional contents of these selected four rice cultivars are not only culinary but medicinal as they play critical roles in reducing predisposing risk factors of many diseases thereby maintaining good health and preventing ailments. Rice meals enrich the body's nutritional value as they provide vitality and strength as well as help to regulate metabolic processes and remove toxic metabolites (Malabadi et al. 2022). Also, the hypoallergenic nature of rice protein which gives it an edge over other plant proteins and some animal sources is yet another value addition of rice cultivars with good protein contents.

Conclusions

The increasing demands for rice cultivars with good quality traits and high drought tolerance indices necessitate the need for more research focus in this area. The proximate analysis results showed that the cultivars have good nutritional qualities while the drought tolerance validation analyses reflected their substantial drought-tolerance abilities. The cultivars exhibited relatively high protein contents particularly “Nassarawa-Lafia” as well as good fiber and magnesium content as seen in the “Kebbi-Jirani” cultivar. Mineral content analysis results also revealed high potassium-containing cultivars like “Niger-Jerusalem” and “Nassarawa-Lafia”. The physicochemical and molecular analyses showed the natural physical qualities of the cultivars devoid of genetic modification. Thus, the cultivars demonstrated a blend of physicochemical and nutritional qualities which are key determinant factors in consumer acceptability, and nutritional and economic values of rice. These findings highlight the inherent good nutritional qualities of the cultivars, their potential health benefits, acceptability, and promising ability in helping to address food and nutrition challenges, especially in climate-changed and hunger-prone regions. This research therefore provides a foundation for more in-depth searching of our expansive local genetic pool for solutions to food and nutrition insecurity challenges. It also recommends future breeding approaches aimed at incorporating these cultivars into broader programs to enhance their availability, possible improvement for adoption among farmers, and commercialization.

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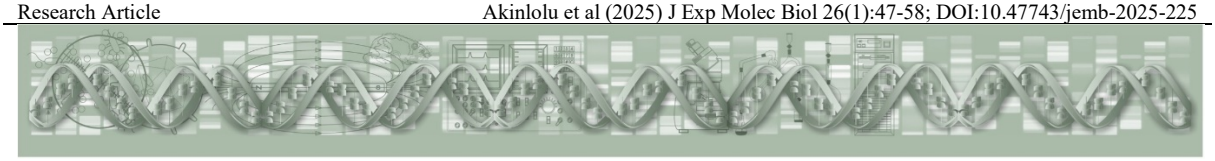

NEUROPROTECTIVE POTENTIALS OF EXTRACTS FROM *MORINGA OLEIFERA* AND *MUSA SAPIENTUM* AGAINST CADMIUM CHLORIDE-INDUCED NEUROTOXICITY IN RATS CEREBRI

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Abstract

Cadmium (Cd)-exposure in humans causes nervous system dysfunctions. In rats, Cd-exposure resulted in increases of nitric oxide and lipid peroxidation levels in the hippocampus. This study evaluated the neuroprotective potential of active drug compounds extracted from *Moringa oleifera* leaves (MO11) and *Musa sapientum* suckers (MS06) in cadmium chloride ($CdCl_2$)-induced neurotoxicity in rats. Adult male Wistar rats totalling 24 in number, were grouped randomly into six with four rats per group. Group 1 served as the control. An intraperitoneal single-dose of $CdCl_2$ was administered to rats of Groups 2 to 4 and 6 on Day 1. MO11-dose, MO11+MS06-doses, and Doxorubicin-dose were respectively administered to rats of Groups 3, 4, and 6 for post-treatment of $CdCl_2$-induced neurotoxicity. Rats of Group 5 were administered Olive Oil-dose (vehicle) for 17 days. Tissue concentrations of catalase, superoxide dismutase, cyclo-oxygenase-2 and cytochrome P450 in rats' cerebri were determined using ELISA. Statistical analyses ($p \leq 0.05$) of data were conducted using the Mann-Whitney U Test. Results showed increased catalase levels, similar superoxide dismutase levels, decreased cytochrome P450 levels and decreased cyclo-oxygenase-2 levels in rats of Groups 3, 4, and 6 in comparison with Group 2. The tested extracts impacted some levels of neuroprotection, neuroregeneration, antioxidant and anticancer capacities against neurotoxicity caused by $CdCl_2$ exposure.

Keywords: Cadmium, *Moringa oleifera, Musa sapientum,* neuroprotection, neurotoxicity

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Introduction

The World Health Organization classified cadmium (Cd) as one of ten (10) unsafe chemicals for human health (Andjelkovic et al. 2019). The International Agency for Research on Cancer and the National Toxicology Program further classified Cd as a carcinogen (Huff et al. 2007). Exposures to Cd caused functional anomalies, including hepatotoxicity and inflammation (Andjelkovic et al. 2019), and neurotoxicity (Schad et al. 2003, Batool et al. 2019).

Cd-exposure in humans causes nervous system dysfunctions producing symptoms such as developments of Parkinsonian-like symptoms and Alzheimer's disease, decreased cognitive functions, impaired learning capacity, headache, vertigo, poor vasomotor functioning, olfactory dysfunction, poor equilibrium, poor balance co-ordination, and peripheral neuropathy (Schad et al. 2003). In children, increased levels in total Cd-exposure caused decreased visual motor capacity, mental retardation, and dyslexia (Schad et al. 2003). In rats, Cd-exposure resulted in increases of nitric oxide and lipid peroxidation levels in the hippocampus (Lamtai et al. 2018). It will therefore, be of scientific relevance to develop herbal extracts and drug candidates that can prevent or mitigate the functional anomalies of the nervous system caused by exposures to Cd.

Musa sapientum (MS) and *Moringa oleifera* (MO), which are readily available plants in Nigeria, have been confirmed in many studies to have possible ethnomedicinal and therapeutic potentials (Akinlolu et al. 2021). Our research team previously fractionated MOF6 from MO leaves using column chromatography techniques (Omotoso et al. 2018). Omotoso et al. (2018) reported that MOF6 conferred significant neuroprotection and antioxidant potential against cuprizone-induced cerebellar damage in rats. Akinlolu et al. (2020) also reported that MOF6 conferred a degree of neuroprotection which ameliorated disruptions acetylcholinesterase levels in neurotoxicity caused by exposures to sodium arsenite in rats. In rats, MOF6 conferred a degree of anti-proliferation, anti-drug resistance capability, and hepatoprotection against hepatotoxicity caused by exposures to 7,12-Dimethylbenz[a]anthracene (Akinlolu et al. 2021). Furthermore, Akinlolu et al. (2021) disclosed that MSF1, which was fractionated from MS suckers using column chromatography techniques, conferred a degree of anti-drug resistance capacity, anti-proliferation, and hepatoprotection against hepatotoxicity caused by exposures to 7,12-Dimethylbenz[a]anthracene in rats.

The clear understanding of how exposures to Cd result in neurotoxicity is yet to be well deduced. However, studies suggested that Cd-induced neurotoxicity probably results from elevated dysfunctions of neurotransmitters and oxidative stress, interactions with heavy metals, i.e., cobalt and zinc, with accompanied epigenetic effects, as well as estrogen-like effect (Schad et al. 2003, Batool et al. 2019). Generally, Cd occurs as a bivalent, positively charged molecule, usually coupled to another element, for example, cadmium chloride ($CdCl_2$) (Andjelkovic et al. 2019). Thus, in order to understand how exposures to Cd result in neurotoxicity, and to test neuroprotective capacities of plant' extracts, this study evaluated the effects of MO11 (extracted from *Moringa oleifera* leaves) and MS06 (extracted from *Musa sapientum* suckers) in cadmium chloride ($CdCl_2$)-produced neurotoxicity in rats.

Materials and Methods

Ethical considerations

The ethical acceptance number for this study as obtained from the University of Ilorin's Ethical Review Committee is UERC/ASN/2018/1161. Thereafter, experimental procedures of this study were conducted in accordance with the internationally accepted principles for laboratory animal use and care.

Allocation of Herbarium Identification Numbers (HIN)

Leaves of MO and suckers of MS were freshly cut and collected from different plantations in Ilorin, the capital city of Kwara State in the North Central region of Nigeria. The MO leaves and MS suckers were authenticated at the Department of Botany of the University of Ilorin. Thereafter, the MO leaves were allotted HIN: UILH/001/1249, while the MS suckers were allotted HIN: UILH/002/1182.

Antioxidant and cytotoxic capacities of MO and MS extracts

The amended 2,2-diphenyl-1-picrylhydrazyl technique outlined by Chaves et al. (2020) was employed to evaluate the antioxidant activities of MO and MS extracts. In addition, the antimicrobial and cytotoxic potentials of MO and MS extracts were determined via evaluations of the cytotoxic effects of each extract against the growths of *Salmonella typhimurium* and *Escherichia coli* using the methods earlier outlined by Elisha et al. (2017).

Isolations of MO11 from MO leaves and MS06 from MS suckers

In this study, following a series of column chromatography and liquid chromatography-mass spectrometry methods accompanied with evaluations of antioxidant and antimicrobial cytotoxicity effects of plants' extracts, MO11 was isolated as the main drug compound from MO leaves while MS06 was isolated as the main drug compound from MS suckers (Akinlolu et al. 2023, Ameen and Akinlolu, 2023).

Animals

Twenty-four (24) adult male Wistar rats, which were two months old and of average weight of 155 g, were procured from a rat breeding center at Badagry in Lagos state of Nigeria. All rats were acculturated for a week, and thereafter grouped randomly into six with four rats per group and kept in standard laboratory conditions. Consequently, the body weights of rats in grams were computed every day.

Design of experimental procedures

Olive Oil was used as the vehicle to dissolve MO11 and MS06. Physiological saline (only) was administered to rats of Control Group 1 for 17 Days (Days 1-17). For the induction of $CdCl_2$-toxicity, the dose of 1.5 mg/kg body weight of $CdCl_2$ which was previously employed for induction of $CdCl_2$-induced testicular toxicity in rats was used in this study (Kawaguchi et al. 2005, Akinlolu et al. 2023). Similarly, the doses of MS06 extract, MO11 extract and Doxorubicin used in the present study were the same as doses employed in our previous studies, which compared the cytoprotective capacities of MO11 and MS06 extracts in $CdCl_2$-induced toxicity in comparison with Doxorubicin in rats (Akinlolu et al. 2023, Ameen et al. 2023).

1.5 mg/kg body weight of $CdCl_2$ (single dose) was given via intraperitoneal administration to each rat of Experimental Groups 2-4 and 6 (Sigma-Aldrich, Japan Co.) on Day 1. No further post-treatment was given to rats of Group 2 (Negative Control) all through Days 1-17. Consequently, 15 mg/kg body weight of MO11 was orally administered to each rat of Group 3 for 17 Days (Days 1-17) for the treatment of $CdCl_2$-induced toxicity. Similarly, the added doses of 15 mg/kg body weight of MO11 and 7 mg/kg body weight of MS06 was orally administered to each rat of Group 4 for 17 Days (Days 1-17) for the treatment of $CdCl_2$-induced toxicity. The rats of Group 5 were not exposed to administration of $CdCl_2$ throughout the experimental procedure. However, 1 ml/kg body weight of Olive Oil (vehicle) was orally administered to each rat of Group 5 for 17 Days (Days 1-17). Furthermore, single dose of 3.35 mg/kg body weight of the standard anticancer drug (Doxorubicin) was given via intravenous administration to each rat of Group 6 (Positive Control) for the treatment of $CdCl_2$-induced toxicity.

At the end of experimental procedures, each rat was sacrificed by cervical dislocation without anesthesia because concentrations of biomarkers to be evaluated in the present study may be endogenously modified by anesthetic agents demanding post-experimental control of confounding factors (Ogunwobi et al. 2012, Omotoso et al. 2018).

Tissue-biochemical analyses of levels of Catalase (CAT) and Superoxide dismutase (SOD) in rat cerebrum

Tissue-biochemical analyses of levels of CAT and SOD in rat cerebrum were evaluated using standard spectrometric methods of Sinha (1972), and Misra and Fridovich (1972), respectively, as modified by Akinlolu et al. (2013).

Tissue-ELISA analyses of Cyclo-oxygenase-2 (COX-2) and P450 in rat cerebrum

Tissue-ELISA analyses of levels of COX-2 and P450 in rat cerebrum using ELISA technique as described by Akinlolu et al. (2023). The ELISA kits for COX-2 and P450 were manufactured by CUSABIO Technology LLC, Houston, USA. Absorbance read at the wavelength of 450 nm using the AgileReader™ ELISA plate reader.

Analyses of computed data

The concentration of each of CAT, SOD, COX-2, and P450 was presented as arithmetic means ± standard deviation. The statistical comparison of the level of each biomarker between two groups was conducted using the Mann-Whitney U test (Wilcoxon-Mann-Whitney Test, 2016). Mann-Whitney U test was chosen because the total number of rats (sample size = 24) employed in this study is not up to 30. 95% confidence interval with $p \leq 0.05$ was used to determine statistical significant difference.

Results and discussions

1. Concentrations of CAT in the cerebrum of rats

There were statistically non-significant lower levels of CAT in rats of the $CdCl_2$-only treated Group 2, when compared with the normal saline-treated Control Group 1 ($p = 1.00$) (Figure 1). In addition, results showed significantly higher levels of CAT in the $CdCl_2$-exposed + MO11 + MS06 post-treated Group 4 ($p < 0.001$), the Olive Oil-only treated Group 5 ($p = 0.03$) and the $CdCl_2$-exposed + Doxorubicin post-treated Group 6 ($p < 0.001$), when compared with Group 2. An increase in CAT levels was also observed in the $CdCl_2$-exposed + MO11 post-treated Group 3, when compared to Group 2, although statistically non-significant ($p = 0.21$).

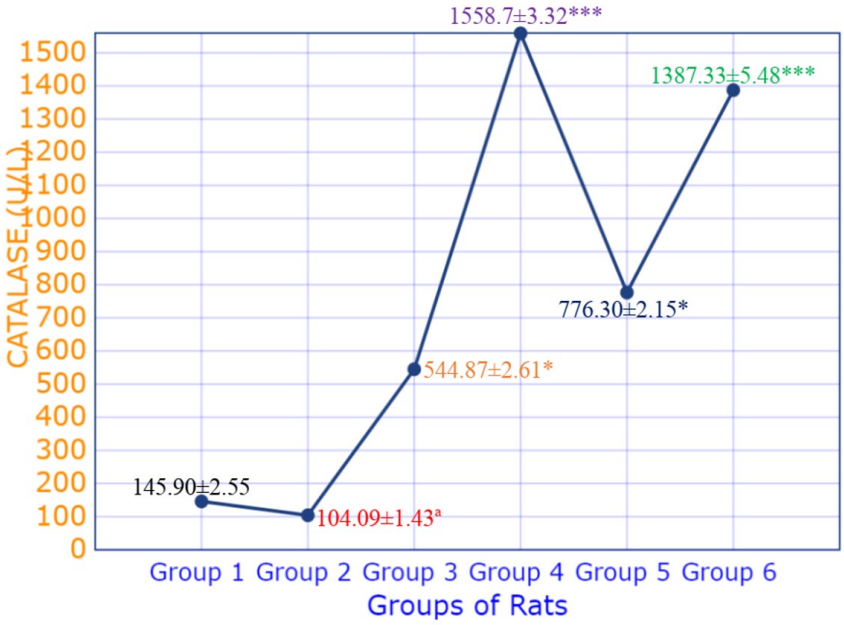

Figure 1. Concentrations (mean±SD) of Catalase (U/L) in cerebrum of rats
a - significant difference compared with the normal saline-treated control Group 1 at $p \leq 0.05$
*$p \leq 0.05$ and ***$p < 0.001$ - significant difference compared with CdCl2-only treated Group 2

In our previous study by Akinlolu et al. (2022), we reported that $CdCl_2$-induced neurotoxicity caused an abnormal increase in the population of chromatolytic cells with accompanied neurodegeneration of the prefrontal cortices of rats of Group 2 exposed only to $CdCl_2$ without further treatment. Contrariwise, there were decreased populations of chromatolytic cells accompanied with evident neuroregeneration in the prefrontal cortices of rats exposed to $CdCl_2$ but further post-treated with MO11, MO11 + MS06, as well as Doxorubicin (Akinlolu et al. 2022). These findings suggest that MO11, MS06, and Doxorubicin conferred neuroprotection against $CdCl_2$-induced neurotoxicity, which resulted in gradual amelioration and reversal of $CdCl_2$-induced neurodegeneration and chromatolysis in less than three weeks (Akinlolu et al. 2022).

CAT and SOD are antioxidant enzymes, which catalyse the conversion of hydrogen peroxide to water and molecular oxygen, and break down potentially harmful oxygen molecules in cells (Akinlolu et al. 2013, Younus et al. 2018). CAT and SOD, therefore, protect against tissue damage by scavenging free radicals and reversing the effects of oxidative stress. CAT is a peroxisomal marker enzyme and the role of brain CAT in ethanol oxidation as well as in central nervous system disorders due to hereditary peroxisomal diseases such as Zellweger syndrome has been reported (Schad et al. 2003). There was marked cytoplasmic staining of CAT mRNA in a multitude of neurons in the rat brain using tyramine/CARD (catalyzed reporter deposition)-enhanced nonradioactive *in situ* hybridization protocol (Schad et al. 2003). Hence, evaluation of CAT levels in the cerebrum is of interest in neuroregenerative studies.

The decreased CAT level in the $CdCl_2$-only treated Group 2, in comparison with the normal saline-only treated Control Group 1 (Figure 1) confirmed $CdCl_2$-induction of oxidative stress and reduction of antioxidant enzymes levels. This observation is in agreement with the observations of earlier studies which reported Cd-induced oxidative stress with accompanied increased levels of nitric oxide and lipid peroxidation (Lamtai et al. 2018), but decreased CAT levels (Elkhadragy et al. 2018). However, post-treatments of $CdCl_2$-induced cerebral oxidative stress with MO11, MS06 and Doxorubicin showed higher cerebral CAT levels in the $CdCl_2$-exposed + MO11 post-treated Group 3, the $CdCl_2$-exposed+ MS06 post-treated Group 4, the Olive Oil-treated Group 5, and the Doxorubicin-treated Group 6, when compared with the normal saline-treated Group 1 (Figure 1), confirming the pro-antioxidant potential of the extracts, Olive Oil and Doxorubicin.

Similarly, post-treatments with MO11, MS06, and Doxorubicin resulted in significant elevated levels of CAT in the cerebrum of rats of Groups 3, 4, and 6 respectively, when compared with Group 2 (Figure 1), confirming their antioxidant, neuroprotective and neuroregenerative potentials.

2. Concentrations of SOD in the cerebrum of rats

Results showed similar levels of SOD in rats of the $CdCl_2$-only treated Group 2, in comparison with the normal saline-treated Control Group 1 ($p = 0.19$), as detailed in Figure 2. No statistically significant changes in SOD levels were found in the CdCl2-exposed + MO11 post-treated Group 3 ($p = 0.17$), the $CdCl_2$-exposed + MO11 + MS06 post-treated Group 4 ($p = 0.28$), the Olive Oil-only treated Group 5 ($p = 0.16$) and the $CdCl_2$-exposed + Doxorubicin post-treated Group 6 ($p = 0.10$), when compared to Group 2.

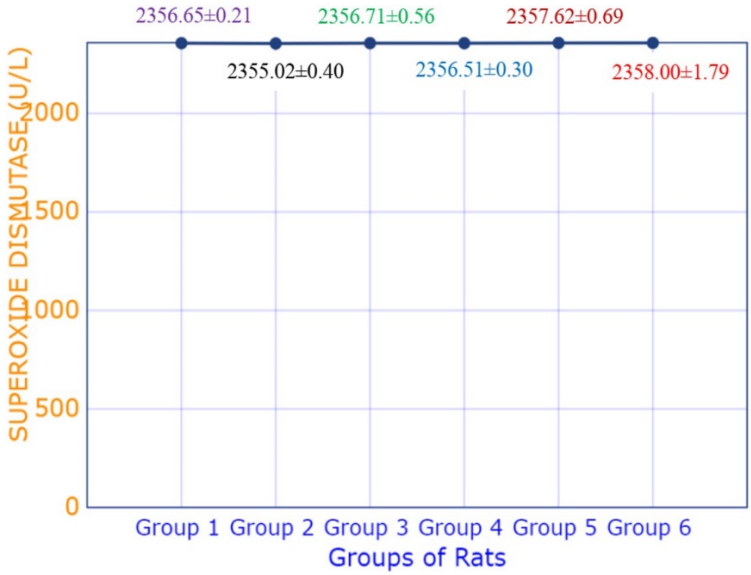

Figure 2. Concentrations (mean±SD) of Superoxide dismutase (U/L) in cerebrum of rats
Significant difference - $p \leq 0.05$

Endogenous expressions of SOD in different regions of the brain such as the cortex, the hippocampus, the hypothalamus, the brainstem, and the cerebellum (Ramanathan et al. 2002), and the whole brain (Otitoju et al. 2008 and Rao et al. 2021) were reported in rats. The findings of the present study showed similar SOD concentrations in the cerebrum of rats of Groups 1–6 (Figure 2). These findings suggest that exposure to $CdCl_2$ followed with post-treatments with MO11, MS06, and Doxorubicin had no significant effects on SOD levels within the 18 days of experimental procedures.

3. Concentrations of COX-2 in the cerebrum of rats

Statistical analyses showed significant higher levels of COX-2 in rats of the $CdCl_2$-only treated Group 2, in comparison with the normal saline-treated Control Group 1 ($p < 0.001$), as detailed in Figure 3. COX-2 levels were found significantly decreased in the CdCl2-exposed + MO11 post-treated Group 3 ($p < 0.001$), the $CdCl_2$-exposed + MO11 + MS06 post-treated Group 4 ($p < 0.001$), the Olive Oil-only treated Group 5 ($p < 0.001$) and the $CdCl_2$-exposed + Doxorubicin post-treated Group 6 ($p < 0.001$), when compared to Group 2.

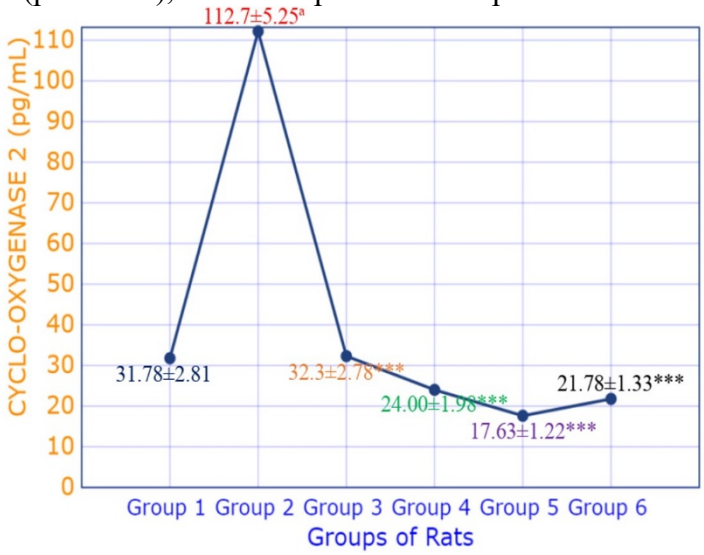

Figure 3. Concentrations (mean±SD) of Cyclo-oxygenase-2 (pg/mL) in cerebrum of rats
a - significant difference compared with the normal saline-treated control Group 1 at $P \leq 0.05$
***$P < 0.001$ - significant difference compared with CdCl2-only treated Group 2

COX-2 is the main cyclo-oxygenase isoform in the brain. Kawaguchi et al. 2005 reported COX-2 expression in hippocampal CA3, dentate gyrus, and cerebral cortex, emphasizing the relevance of evaluating COX-2-levels in the rat brain. Increased COX-2 activity results in increased oxidative stress and increased release of prostaglandins with accompanied injurious effects (Kawaguchi et al. 2005). Therefore, increased COX-2 levels results in increased oxidative stress, causing induction of inflammation, apoptosis, and carcinogenesis (Ogunwobi et al. 2012, Nørregaard et al. 2015).

The higher level of COX-2 in the $CdCl_2$-only treated Group 2, in comparison with the normal saline-only treated Control Group 1 (Figure 3) confirmed $CdCl_2$-induction of oxidative stress and promotion of inflammation, neuronal cell death and carcinogenesis. This finding is in agreement with those of Liu et al. 2009 and Junior et al. 2020, which reported Cd-induced increase in COX-2 levels with associated inflammation, apoptosis and carcinogenesis. In contrast, our results showed similar COX-2 levels in Groups 1 and 3, but lower COX-2 levels in Groups 4-6, when compared with the normal saline-treated Group 1 (Figure 3), confirming neuroprotective potential of the extracts, Olive Oil, and Doxorubicin. These findings also suggest that MO11, MS06, and Doxorubicin possess neuroprotective, neuroregenerative, anti-inflammatory, and anticancer potentials, which resulted in significant reduction of COX-2 levels in the cerebrum of rats of Groups 3, 4, and 6 respectively, in comparison with Group 2.

4. Concentrations of P450 in the cerebrum of rats

Statistical analyses showed significant higher levels of P450 in rats of the $CdCl_2$-only treated Group 2, in comparison with the normal saline-treated Control Group 1 ($p < 0.001$), as detailed in Figure 4. P450 levels were found significantly decreased in the $CdCl_2$-exposed + MO11 post-treated Group 3 ($p < 0.001$), the $CdCl_2$-exposed + MO11 + MS06 post-treated Group 4 ($P < 0.001$) and the Olive Oil-only treated Group 5 ($p < 0.001$) in comparison with Group 2.

P450 levels were also found decreased in rats of the $CdCl_2$-exposed + Doxorubicin post-treated Group 6 compared to the $CdCl_2$-only treated Group 2, although statistically non-significant ($p = 0.34$).

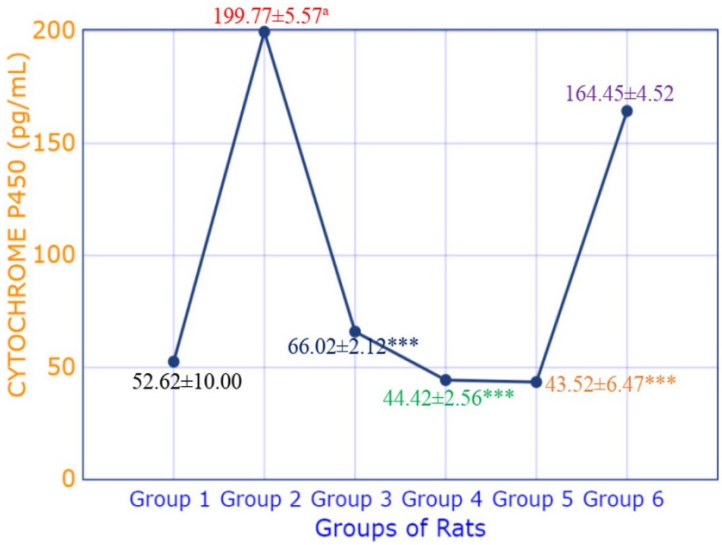

Figure 4. Concentrations (mean±SD) of Cytochrome P450 (pg/mL) in cerebrum of rats
a - significant difference compared with the normal saline-treated control Group 1 at $p \leq 0.05$
***$p < 0.001$ - significant difference compared with CdCl2-only treated Group 2

P450s are monooxygenases which oxidize xenobiotics, fatty acids, and steroids promoting the water-solubility and ejection of foreign agents. Hence, P450 regulates several processes such as homeostasis, drugs clearance, drugs detoxification, detoxification of xenobiotics,

metabolisms of vitamin D and cells, cholesterols synthesis, and synthesis of hormones (Rodriguez-Antona and Ingelman-Sundberg 2006, Mahmoudi et al. 2018). P450 mediates the activation/inactivation of carcinogens and anticancer drugs; therefore, P450 is of relevance in cancer therapy (Manikandan et al. 2018). The liver is the major site of xenobiotic metabolism and detoxification, and the brain P450 is very low, constituting about 0.5%-2% (Hedlund et al. 2001) or 0.2%-0.5% (Wang et al. 2013) of hepatic P450. Hence, the brain P450 does not appear significantly involved in regulatory roles of pharmacokinetics of the body's hormones and drugs. The brain P450 rather regulates brain cholesterol homeostasis, retinoids elimination, and levels of endogenous GABA receptor agonists (Hedlund et al. 2001, Wojciech et al. 2021). Wojciech et al. 2021 reported expression of different forms of P450 in the frontal cortex, thalamus, hypothalamus, striatum and hippocampus of rat brain. Therefore, the profiling of brain P450 in neurotoxicology becomes very important in understanding the mechanism of action of the neurotoxin and in the design of appropriate chemotherapy.

The higher P450 level in the $CdCl_2$-only treated Group 2, compared to the normal saline-only treated Control Group confirmed induction of increased P450 level. This observation is similar to those of Bhattacharyya et al. 2014, which reported that increased P450 levels are associated with increased oxidative stress via oxygen activation. Hence, the observed increased P450 levels could have resulted from $CdCl_2$-induced increased oxidative stress and decreased CAT levels in rats of the $CdCl_2$-only treated Group 2. Contrari-wise, our results showed similar or lower P450 levels in Groups 3–5, when compared with Group 1. However, results showed higher P450 level in Group 6 compared to Group 1. These findings imply that the extracts and Olive Oil have pro-P450 potential.

The increased P450 levels in the cerebrum of rats in this study is in contrast with previously reported significant decreases in liver P450 levels in Cd-induced hepatotoxicity in hamsters (Sripanidkulchai et al. 2005), and significant decreases in testicular P450 levels in Cd-induced testicular damage in rats (Alkhedaide et al. 2016). These differences could have been due to low brain P450 content versus high liver P450 content. In addition, the reason for the Cd-induced increased brain P450 levels versus Cd-induced liver and testicular decreased P450 levels could have been due to the shielding effect of the protective components of the brain resulting in increased P450 levels to aid clearance of $CdCl_2$ brain content.

Results showed significant reduction of P450 levels in the cerebrum of rats of Groups 3 and 4 respectively, when compared with Group 2 (Figure 4) confirming the neuroprotective and neuroregenerative potentials of MO11 and MS06. Contrari-wise, results of higher P450 levels in Group 6 compared with Group 2 suggest that Doxorubicin possesses lower neuroprotective and neuroregenerative potential, when compared with MO11 and MS06.

Conclusions

Overall, the findings of the present study suggest that post-treatments of $CdCl_2$-induced neurotoxicity with MO11 (extracted from *Moringa oleifera* leaves) and MS06 (extracted from *Musa sapientum* suckers), and Doxorubicin caused significant elevations of CAT concentrations, but significantly decreased COX-2 levels in rat brains. In addition, post-treatments of $CdCl_2$-induced neurotoxicity with MO11 and MS06 caused significantly decreased levels of P450; however post-treatments with Doxorubicin resulted in non-significant decreased levels of P450 in rat brains. These findings suggest that MO11 and MS06 impacted some levels of neuroprotection against $CdCl_2$-induced neurotoxicity, oxidative stress, and promotions of inflammation, apoptosis and carcinogenesis, when compared with Doxorubicin. Thus, MO11 and MS06 are recommended for further evaluations as possible drug agents for the treatments of neurodegenerative diseases and disorders of the central nervous system.

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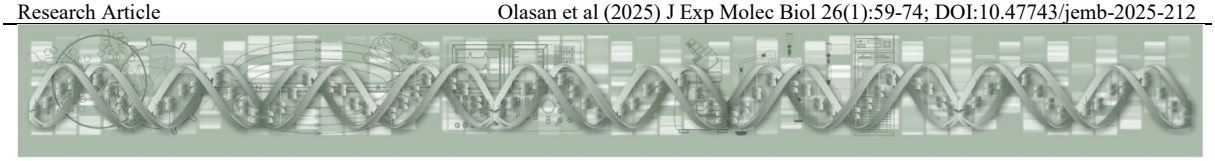

ANALYSIS OF GENETIC POLYMORPHISM FOR SCAB RESISTANT COWPEA VARIETIES USING SSR AND SNP MARKERS

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Abstract

Cowpea scab disease, caused by *Sphaceloma* sp., significantly reduces yield. This study screened nine cowpea varieties for resistance to scab using both phenotypic and molecular markers (SSR and SNP). The varieties were artificially inoculated, and disease incidence and severity were recorded. Genetic analysis with SSR markers revealed polymorphisms between resistant and susceptible varieties. Results showed significant genetic diversity among cowpea varieties, with IT99K-573-1-1 and TVx-3236 being resistant, while FUAMPEA-4 and UAM-09-130-20-4 were susceptible. SSR markers CP 29/30, CLM 0348, and CP 67/68 were the most informative in tracking resistance.

Keywords: Cowpea, Scab Resistance, SSR Markers, SNP Markers, Genetic Polymorphism, Disease Resistance

Introduction

Cowpea [*Vigna unguiculata* (L.) Walp.] is an indigenous leafy vegetable and a grain legume widely grown in the semiarid areas of sub-Saharan Africa (Da Silva et al. 2019). The importance of cowpea in this region stems from its drought tolerance and ability to grow under water stress conditions (Carvalho et al. 2017), and its crucial role in ensuring food security and supporting the livelihoods of millions of smallholder farmers who depend on it for their economic and nutritional well-being (Bolarinwa 2022). The nutritional value of cowpea stems from its high protein content (25%) (Ogbonnaya et al. 2003, Mekonnen et al. 2022), which plays a considerable role in balancing the predominantly carbohydrate-based nutrition of the rural population in the West Africa subregion (Krasova-Wade et al. 2006, Singh et al. 2022). Cowpea is also an integral part of a sustainable agriculture and land use system (Ogbonnaya et al. 2003) and an essential component of traditional intercropping systems (Singh 2002). Integration of cowpea in cropping systems promotes buildup of soil organic matter and carbon and nitrogen fixation and ultimately improves soil fertility physical characteristics such as the water infiltration and retention capacity (Sánchez-Navarro et al. 2019a,b).

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Cowpea is grown on about 14, 911, 307 million hectares worldwide, with an annual grain production of about 8, 986, 191.25 million tons (FAO 2021). Nigeria produced about 3, 628, 612.65 million tons of cowpea, making her the world largest producer; followed by Niger (2, 661, 882.93 million tons), Burkina Faso (705, 768.3 tons) and Kenya (250, 260 tons) (FAO 2021). In Nigeria, cowpea is predominately grown in the drier northern parts of the country; however, advances in crop development have opened opportunities for its production in the wetter agroecologies (Nwofia et al. 2006), with the north-central guinea savanna zone contributing 29% production in 2020 (NAERLS 2020).

Although the West African sub-region accounts for over 95% of world cowpea production (Samireddypalle et al. 2017), its production has largely been due to increase in land mass rather than productivity per unit area. Studies have shown that the yield from farmers' fields is very low (500 kg/ha) compared to that obtained in the USA (2000 kg/ha) and in Australia (2200 kg/ha) (Quin 1997). The low yield is attributed to the effect of several biotic and abiotic factors (Omoigui et al. 2007). However, biotic stresses like cowpea scab disease, caused by *Sphaceloma* sp. (Emechebe 2014), threaten cowpea production. The disease affects all above-ground parts of the cowpea plant, including leaves, stems, pod and severe infections can lead to significant yield losses (Afutu et al. 2016). Molecular markers have emerged as effective and reliable tools for the genetic analysis of plant traits such as disease resistance (Sharma and Sharma 2020). Genetic analysis offers a powerful approach for characterizing scab resistant and susceptible varieties, towards the development of resistant varieties using molecular tools (Meuwissen et al. 2016). Identifying resistant varieties through phenotypic and molecular approaches such as the use Simple Sequence Repeats (SSR) and Single Nucleotide Polymorphisms (SNP) marker systems is crucial for breeding programs aimed at improving resistance and ensuring sustainable cowpea production.

Materials and Methods

Field Screening of cowpea for Reaction to Scab Infection

Plant Materials, Experimental Design and Location

Nine cowpea varieties (FUAMPEA-4, Gujarat Cowpea-3, Gujarat Cowpea-5, Gujarat Cowpea-6, IT99K-573-1-1, Pant Lobia-4, Pant Lobia-1, TVx-3236, and UAM-09-130-20-4) were used. They were obtained from the cowpea breeding program of the Molecular Biology Laboratory, Joseph Sarwuan Tarka University Makurdi, from the Germplasm of the Stress Tolerant Orphan Legume (STOL) project comprising Germplasm from Nigeria and India.

The field experiment was conducted at the Teaching and Research Farm of the Joseph Sarwuan Tarka University of Agriculture, Makurdi, following a randomized complete block design with three replications.The field was laid out in plots according to the experimental design. Each plot consisted of a single row, 4 m long, and seeds were sown at an intra-row spacing of 25 cm, resulting into 16 hills and 32 plants per plot.

Cultural Practices

At sowing, Pendimethalin, a pre-emergence herbicide was applied to subdue weeds until crop establishment. The herbicides were applied at a dilution of 150 mls per 20 litre knapsack sprayer.

Nitrogen, Phosphorus and Potassium were applied in the form of NPK 15:15:15 fertilizer at the rate of 100kg/ha. This is the equivalent of 0.075kg (75g) per plot, applied by side placement as a single dose at one week (7 days) after planting (7 DAP). Weeding was done manually and it was carried out for all plots at 3 weeks (21 days) after planting and subsequently as needed to keep the field free from weeds till maturity. Cypermethrine + Dimethoate was sprayed at the rate of 50 g a.i/ ha to control insect pests.

Disease Inoculation

Cowpea plants were inoculated with *Sphaceloma* sp. at 14 days post-planting, and disease incidence and severity were recorded at intervals. The Spore suspension for disease inoculation was prepared at the Crop and Environmental protection Laboratory as described by Afutu et al. (2016). The concentration of spores in the solution was determined using a hemocytometer, and adjusted to 10^5 spores/mL for the inoculation.

Cowpea plants were artificially inoculated with the scab disease by spraying the spore suspension of *Sphaceloma* sp. onto the plants at the flower initiation stage. The inoculum was applied to the plants' canopy with a hand-held sprayer until runoff at 14 DAP. After inoculation, water spray was applied to plants in the evening to maintain high humidity for disease development.

Data Collection and Statistical Analysis

Observations were made a plot by plot basis, on the incidence and severity of scab infection. Disease incidence and severity were assessed at 14 days post-inoculation (DPI) and then every 7 days after, up to 28 DPI.

According to Afutu et al. (2016), the symptoms of cowpea scab disease caused by *Sphaceloma* spp. include:

1. Small, circular, dark brown to black spots or lesions on the leaves, stems, and pods.
2. Lesions may merge to form larger, irregularly shaped spots.
3. Spots may have a reddish-brown border and a grayish center.
4. Severe infections can lead to defoliation, reduced pod formation, and lower yields.

Disease incidence was measured as the percentage of plants showing symptoms of scab infection (Gerstman, 2015) as shown below:

Incidence (%) = (Number of infected plants / Total number of plants) × 100

Disease severity was determined using a rating scale of 1 to 10, with 1 indicating minimal infection and 10 indicating severe infection (Gerstman 2015). The disease severity scale is as follows:

0: No symptoms
1: 1-10% infection
2: 11-20% infection
3: 21-30% infection
4: 31-40% infection
5: 41-50% infection
6: 51-60% infection
7: 61-70% infection
8: 71-80% infection
9: 81-90% infection
10: 91-100% infection (plant death)

Genetic Analysis

DNA Extraction

Using disease incidence and severity score, Scab resistant and scab susceptible cowpea varieties were identified. For the Genetic analysis, DNA was extracted from young, healthy trifoliate leaves of 7 days old plants of Scab resistant and scab susceptible cowpea varieties using the cetyltrimethylammonium bromide (CTAB) method (Xin and Chen 2012) at 7 DAP. The method was adapted with slight modification; leaf samples were collected from each cowpea variety in silica gel placed in small appropriately labelled ziploc bags for drying to a crispy state (suitable for grinding). The quality and quantity of the extracted DNA were assessed using agarose gel electrophoresis. Gels were prepared as described by (Sambrook and Russell 2001).

PCR Amplification.
Polymerase chain reaction (PCR) was performed using 13 Simple Sequence Repeat (SSR) markers to screen for polymorphism between the resistant and susceptible varieties. Each PCR reaction contained 20 ng of genomic DNA, 10 µL of PCR master mix, 1 µL of forward and reverse primers, and nuclease-free water to a final volume of 20 µL. Polymerase Chain Reaction (PCR) was carried out in a Biorad Thermal cycler under the following thermal cycler conditions for PCR reaction. 35 cycles of denaturation (95ºC) for 30 seconds, annealing (55ºC) for 30 seconds and extension (72ºC) depending on the product size.

Gel Electrophoresis
The amplified products were separated on a 2% agarose gel stained with ethidium bromide. The gels were run at 100 volts for 1 hour and visualized under ultraviolet light. Gel images were captured using a Digital camera. Band patterns were observed and amplification of single DNA band was scored as 1, amplification of multiple bands was scored as 2 and no band was scored as 0. This scoring generated the genotypic (molecular) data for genetic analysis.

Results and discussions

Table 1 shows that 9 cowpea varieties evaluated for their reaction to scab infection showed varied reactions in terms of disease incidence and severity measurement. The variety FUAMPEA-4 and UAM-09-130-20-4 showed the highest incidence at 71.67%, while IT99K-573-1-1 and TVx-3236 exhibited the lowest incidence at 1%. All the cowpea varieties recorded disease severity scores greater than 5, except for IT99K-573-1-1 and TVx-3236. The variety UAM-09-130-20-4 had the highest severity score of 6.7, followed by Pant Lobia-1 with a score of 6.2, while IT99K-573-1-1 and TVx-3236 showed the lowest severity score of 1.

Table 1. Mean Disease Incidence and Severity Response of 9 Cowpea varieties to Scab infection

Variety	Mean Incidence (%)	Mean Severity (1-10)
FUAMPEA-4	71.67	5.67
UAM-09-130-20-4	71.67	6.67
Gujarat Cowpea-3	53.33	5.33
Gujarat Cowpea-5	15.00	6.00
Gujarat Cowpea-6	13.67	5.50
Pant Lobia-4	11.67	5.67
Pant Lobia-1	6.67	6.17
IT99K-573-1-1	1	1
TVx-3236	1	1
Standard Error (SE)	1.41	0.15

This result indicates that FUAMPEA-4 and UAM-09-130-20-4 were susceptible to scab caused by *Sphaceloma* sp. while IT99K-573-1-1 and TVx-3236 were resistant. In similar studies, variations in susceptibility to cowpea scab caused by *Sphaceloma* sp. have been observed, with some varieties demonstrating higher levels of resistance. This aligns with the findings of Jorem et al. (2023), who reported that disease severity and incidence in cowpea vary depending on the genetic makeup of the varieties tested, environmental conditions, and the virulence of the pathogen.

Genetic resistance is a critical factor in managing scab disease. Research by Emechebe and Florini (1997) noted that certain cowpea genotypes express resistance to a range of pathogens,

including *Sphaceloma* sp., responsible for scab disease. Their work supports the idea that resistant varieties like IT99K-573-1-1 and TVx-3236 are crucial for integrated disease management strategies, as noted by Amayo et al. (2014). and are also essential for breeding programs aimed at improving cowpea resilience. They emphasized the role of breeding programs in developing scab-resistant cowpea varieties as a sustainable approach to disease control.

The variation in disease incidence in this study may be attributed to host-pathogen interactions, which play a significant role in determining the outcome of infection. A study by Jorem et al. (2023) explored how cowpea's genetic diversity influences its interaction with pathogens, leading to varying levels of disease resistance.

The severity of scab infection has direct implications for cowpea yield, as observed in this study. High disease severity can reduce plant vigor and yield. A study by Mbong et al. (2012) highlighted the negative impact of scab disease on yield performance in susceptible varieties, stressing the importance of early detection and the use of resistant cultivars.

Plates 1, 2 and 3 show the agarose gel images of 13 screened Simple Sequence Repeats (SSR) Marker employed in the DNA amplification for polymorphism between scab resistant cowpea varieties (**IT99K-573-1-1 and TVx-3236**) and susceptible cowpea varieties (**UAM-09-130-20-4 and FUAMPEA-4**). The primers showed varying degrees of genetic polymorphism depending on the DNA of the cowpea varieties amplified and the SSR primer used. All primers produced visible bands except in CP15/16, CLM1190 and CLM1182. DNAs of scab resistant and susceptible varieties were well resolved in primers CLM059, CP85/86, CP29/30, CLM0348 and CP 67/68.

Table 2 presents the banding pattern of 13 SSR primers employed to identify polymorphism between scab resistant and susceptible varieties of cowpea. Presence of bands were indicated by 1 or 2 to represent single or double bands respectively while absence of band was indicated as zero (0). Double bands were produced by CP 29/30, CLM 0348 and CP 67/68 in IT99K-573-1-1(scab resistant variety). Double bands were produced by CLM 057, CP 85/86, CP 29/30, CLM 0348 and CP 67/68 in FUAMPEA-4 (scab susceptible variety). Other banding patterns occurred singly where present. Nine (9) primers produced bands in the four varieties studied representing 69.2% of the primers.

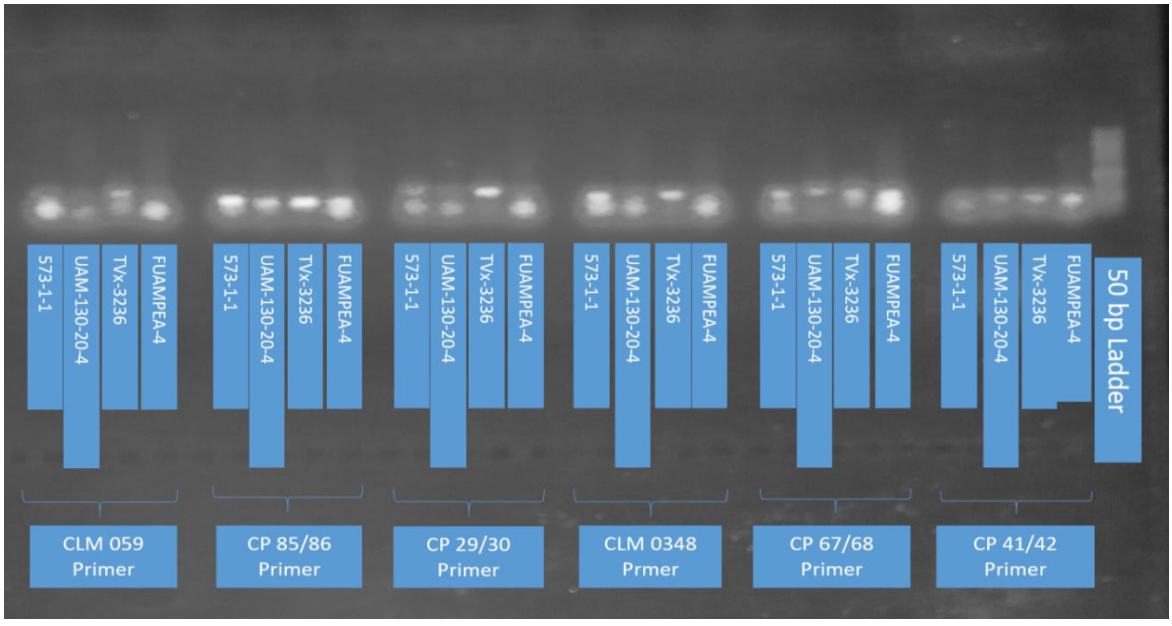

Plate 1. Agarose gel image showing screening of SSR markers (SET 21) for polymorphism between Scab resistant cowpea varieties (IT99K-573-1-1 and TVx-3236) and susceptible cowpea varieties (UAM-09-130-20-4 and FUAMPEA-4)

Each group of four represents screening with a single marker as labelled. Lane 1 and 3 in each group is DNA from resistant parents IT99K-573-1-1 and TVx-3236 respectively, while Lane 2 and 4 is DNA from susceptible parents UAM-09-130-20-4 and FUAMPEA-4 respectively.

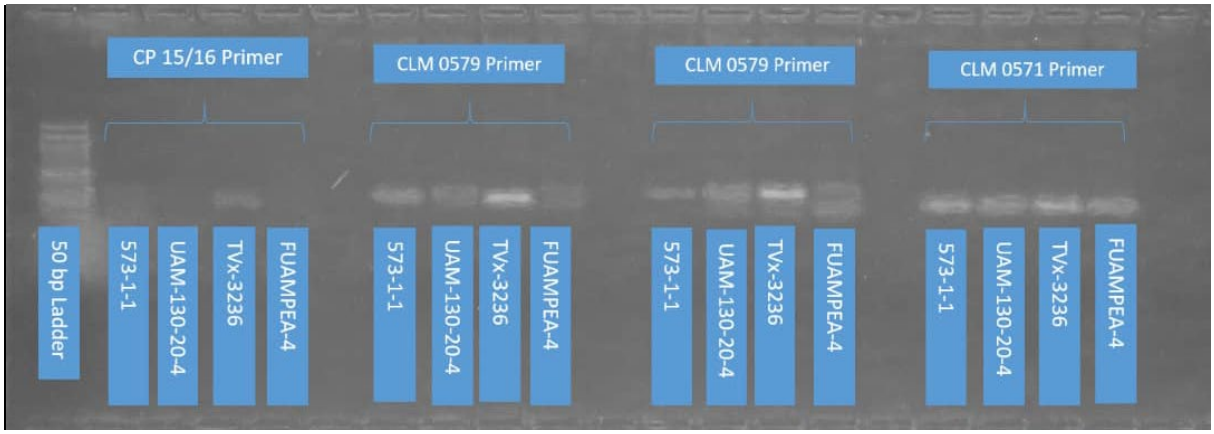

Plate 2. Agarose gel image showing screening of SSR markers (SET 2) for polymorphism between Scab resistant cowpea varieties (IT99K-573-1-1 and TVx-3236) and susceptible cowpea varieties (UAM-09-130-20-4 and FUAMPEA-4)
Each group of four represents screening with a single marker as labelled. Lane 1 and 3 in each group is DNA from resistant parents IT99K-573-1-1 and TVx-3236 respectively, while Lane 2 and 4 is DNA from susceptible parents UAM-09-130-20-4 and FUAMPEA-4 respectively.

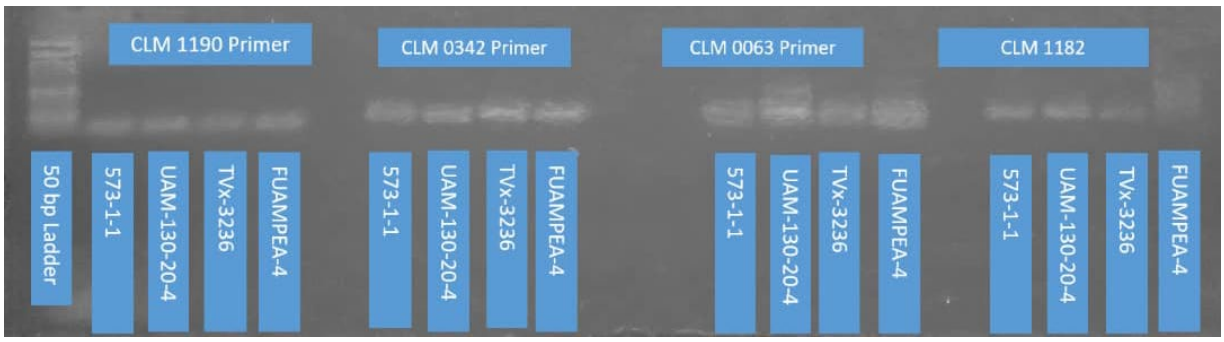

Plate 3. Agarose gel image showing screening of SSR markers (SET 3) for polymorphism between Scab resistant cowpea varieties (IT99K-573-1-1 and TVx-3236) and susceptible cowpea varieties (UAM-09-130-20-4 and FUAMPEA-4)
Each group of four represents screening with a single marker as labelled. Lane 1 and 3 in each group is DNA from resistant parents IT99K-573-1-1 and TVx-3236 respectively, while Lane 2 and 4 is DNA from susceptible parents UAM-09-130-20-4 and FUAMPEA-4 respectively.

Band analysis in IT99K-573-1-1 variety (resistant type) showed 13 bands representing 100% of the total primers employed in the DNA amplification. There were 3 double bands (23.1%) and 9 single bands (69.2%) as shown in Figure 1. UAM-09-130-20-4 (susceptible variety) had 10 bands (76.9%) and all were single bands as shown in Figure 2. In TVx-3236 variety (resistant type), there were 13 (100%) bands grouped into 1 double band (7.7%) and 12 single bands (92.3%) as shown in Figure 3. In FUAMPEA-4 (susceptible variety), there were 11 (84.6%) bands grouped into 5 double bands (38.5%) and 6 single bands (46.2%) as shown in Figure 4.

Table 2. Band Pattern of SSR Primers to Identify Polymorphism Between Crab Resistant and Susceptible Varieties of Cowpea

Primer	Forward / Reverse sequence	IT99K-573-1-1	UAM09-130-20-4	TVx-3236	FUAMPEA-4
CP-15/16	GTAGGGAGTTGGCCACGATA CAACCGATGTAAAAAGTGGACA	0	0	1	0
CLM 0579	CCTAAGCTTTTCTCCAACTCCA CAAGAAGGAGGCGAAGACTG	1	0	1	2
CLM 0571	GATTTGTTTGGTTTCCTTAAG GGTTGATCTTGGAGGCATTTT	1	1	1	1
CLM 1190	GTCAAAGCAATGGACTAA TGAATTTGATACACACACTACT	1	1	1	1
CLM 059	AAACTGACACTTGAACACGA CTCATGCAGAGTTCAAGATC	1	0	2	1
CP 85/86	GATCACCTCCCACACCTCAG TAGCAGTTTCCCACCAGCTT	1	1	1	2
CP 29/30	AATGACCCACAAAGCAAAGT TTGGCCCAAAATATCACACA	2	1	1	2
CLM 0348	GCTTTGCATGTGGATTTCCT GGGGAGAATGAAACTAAAGTAATGTT	2	1	1	2
CP 67/68	GATGCTGGTGCTTGTATGGA TAATTTCTACGCAAGGGAGAGAG	2	1	1	2
CP 41/42	ACCTGCATTGCCTCATATCC GCTGATTCGGCTTGTTCTTC	1	1	1	1
CLM 0342	GATCCAACATTTCCTGTGTCTC GGAGCACCCGACAAGCCCCT	1	1	1	1
CLM 0063	ACTTCGCACACAGATCCAAC AATTGCCGGCTTTCCCATTG	1	1	1	1
CLM 1182	TTCAGACAGCATAGCTCCCA GGCCGTATCAAGGATGAACA	1	1	1	0

The markers CLM059, CP85/86, CP29/30, CLM0348, and CP67/68 were able to distinguish between the resistant and susceptible varieties clearly, showing good resolution on the agarose gel. SSR markers are highly effective for revealing genetic diversity due to their co-dominant nature and ability to detect even small differences in the DNA of different genotypes. The presence of polymorphism in this study screened markers indicates genetic variability between resistant and susceptible cowpea varieties. This is consistent with findings by Diouf and Hilu (2005), who demonstrated that SSR markers are reliable for detecting genetic differences among cowpea genotypes, especially in relation to disease resistance traits.

These markers showing genetic polymorphisms not only reveal diversity but are also essential for identifying loci associated with scab resistance. SSR markers linked to resistance traits in cowpea have been well documented in other studies, such as the work of Omo-Ikerodah et al. (2008) who used AFLP and SSR cowpea linkage map to identify QTLs for resistance to flower bud thrips. Similarly, Gioi et al. (2012) used SSR markers to identify and validate a QTL for cowpea yellow mosaic virus (CYMV) resistance in cowpea. According to Asare et al. (2010) SSR markers can effectively differentiate between resistant and susceptible genotypes, making them valuable for marker-assisted selection (MAS) in breeding programs.

Three markers CP15/16, CLM1190, and CLM1182 which did not produce visible bands, may suggest the absence of the specific loci they amplify in the cowpea varieties tested or inadequate primer annealing due to sequence mismatches. This kind of issues can also occur in PCR amplification when there are high levels of sequence variation or when primers are not well-suited to the varieties in question. As noted by Fatokun et al. (1993), such issues can arise when designing primers for highly polymorphic regions, especially in genetically diverse populations.

The successful resolution of DNA from resistant and susceptible varieties with the markers CLM059, CP85/86, CP29/30, CLM0348, and CP67/68 suggests that these primers are linked to regions of the cowpea genome associated with scab resistance. This agrees with Omoigui et al. (2019) who used SSR markers to identify genomic regions associated with *Cercospora* disease resistance in cowpea.

The identified polymorphisms between resistant and susceptible varieties using SSR markers can be leveraged in breeding programs aimed at enhancing scab resistance in cowpea. Similar studies have demonstrated the utility of SSR markers for developing improved varieties with resistance to fungal diseases. Boukar et al. (2016, 2019) emphasized the role of SSR markers in breeding strategies for cowpea, especially for traits like disease resistance, drought tolerance, and yield improvement.

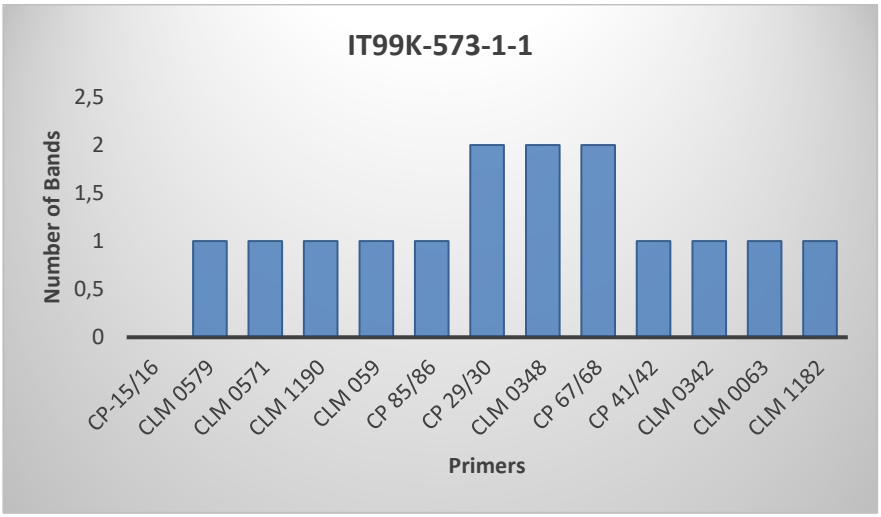

Figure 1. Band analysis in IT99K-573-1-1 Variety

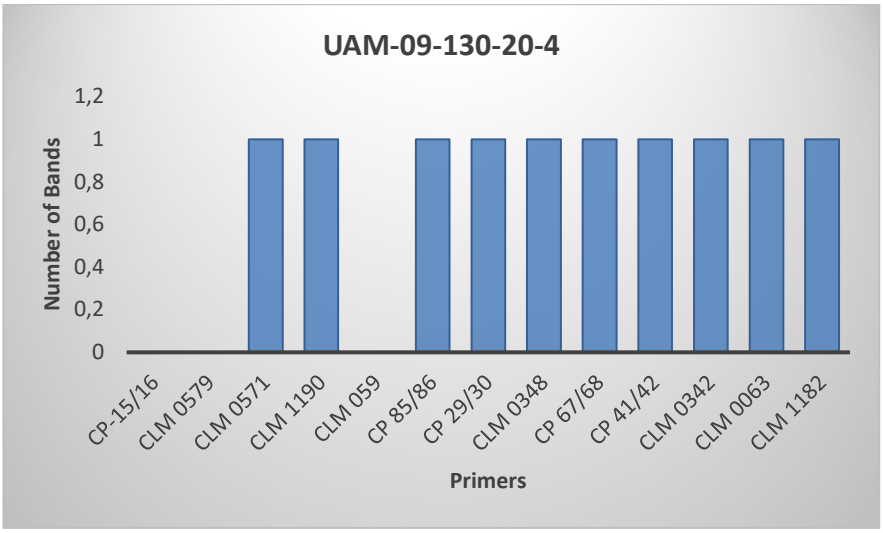

Figure 2. Band analysis in UAM-09-130-20-4 Variety

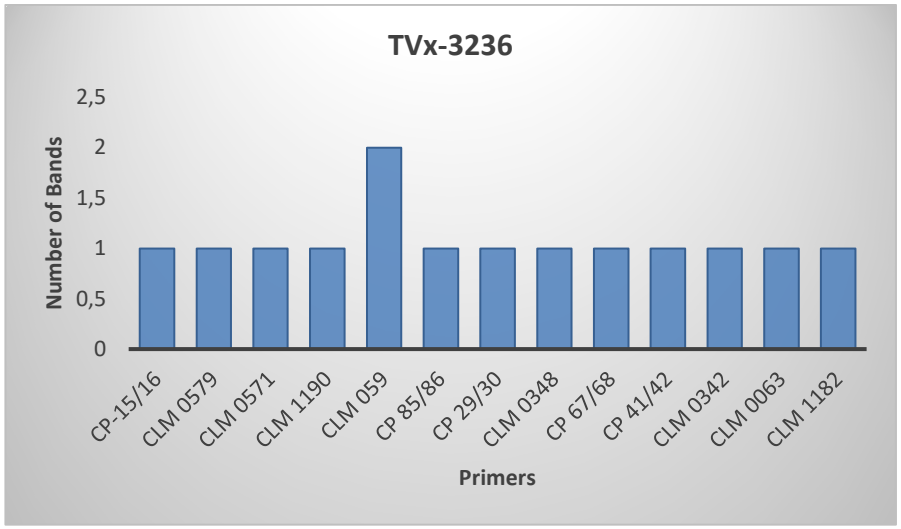

Figure 3. Band analysis in TVx-3236 Variety

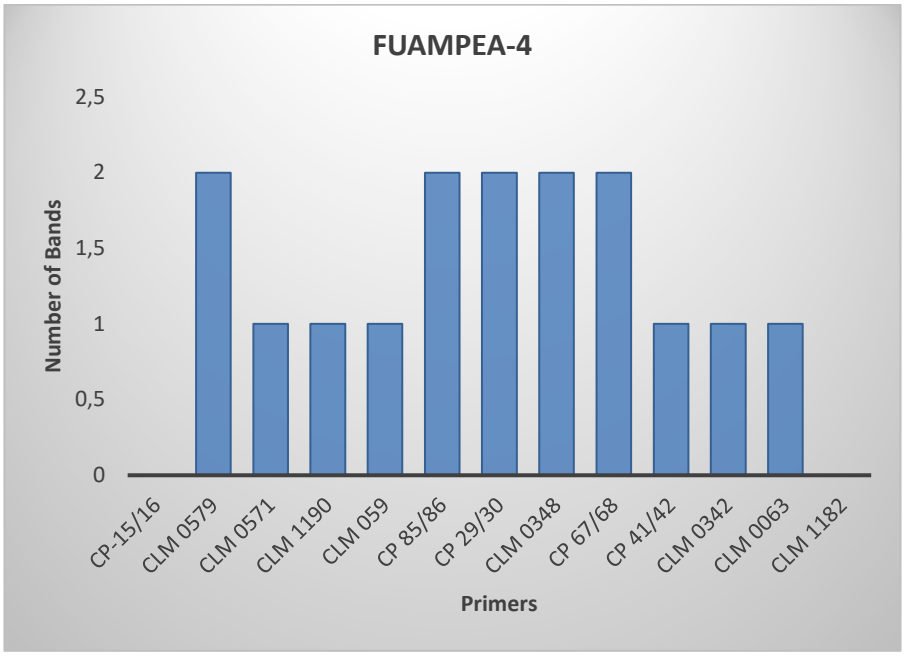

Figure 4. Band analysis in FUAMPEA-4 Variety

Primers were clustered on the basis of the DNA amplification results as shown in the dendrogram (Figure 5). Genetic distance ranged from 1.00 to 3.00 with similarity coefficient of 66.7 to 0.00 respectively. There were two clusters. Cluster 1 comprised a group of primers (CLM0571, CLM1190, CP41/42, CLM0342 and CLM0063) that produced single bands and appeared in all the four varieties of cowpea. The clustering of primers CLM0571, CLM1190, CP41/42, CLM0342, and CLM0063 in Cluster 1, which produced single bands across all four cowpea varieties, indicates that these primers are likely amplifying conserved regions in the cowpea genome. This suggests that these loci are not strongly associated with scab resistance or susceptibility, but rather represent general genetic similarity across varieties. As seen in studies by Timko et al. (2007), such primers often amplify housekeeping genes or other highly conserved sequences in cowpea. Divergent clustering pattern was observed in CP15/16 that produced a lone band in TVx-3236 resistant variety and in CLM059 that produced bands in all varieties except the UAM09-130-20-4 (susceptible variety). These observations suggest these primers target loci more specific to resistance traits. CP15/16 may be linked to a genetic locus

contributing to resistance in TVx-3236. According to Chen et al. (2004), certain SSR markers were specific to disease resistance genes. Cluster 2 comprised primers that produced 2 double and 2 single bands in the four varieties.

Figure 6 shows the dendogram of the four varieties of cowpea. Clustering pattern was divergent and independent of scab resistance or susceptibility status of the varieties. Coefficient of similarity ranged from 23.6 to 86.7 with genetic distance of 1.53 to 0.27 respectively. Results showed that IT99K-573-1-1 (scab resistant) and FUAMPEA-4 (scab susceptible) varieties shared closer genetic similarity than other varieties. The close genetic similarity between IT99K-573-1-1 (scab resistant) and FUAMPEA-4 (scab susceptible) indicates that despite their phenotypic difference in disease resistance, they may share similar genetic backgrounds. Similar results were reported by Price and Cishahayo (1986), where genetically similar cowpea varieties exhibited varying degrees of resistance to different pathogens, underscoring the complexity of disease resistance mechanisms. UAM09-130-20-4 (susceptible) was genetically divergent from IT99K-573-1-1 and FUAMPEA-4, suggesting it may possess unique genetic traits not shared with the other varieties. This could imply that UAM09-130-20-4 either lacks the resistance-associated alleles present in other varieties or carries susceptibility loci. This is consistent with the finding of Omo-Ikerodah et al. (2008), who identified divergent susceptible cowpea genotypes using SSR markers and linked them to specific susceptibility traits.

The most divergent and farthest in genetic distance among the four varieties was TVx-3236 (resistant type). This suggests that TVx-3236 may possess unique resistance mechanisms or genetic backgrounds. Its distinctiveness aligns with the divergent patterns seen in CP15/16, which was specific to this variety. This result echoes the finding of Asare et al. (2010), who reported that highly divergent cowpea varieties often carry unique alleles associated with specific disease resistance traits.

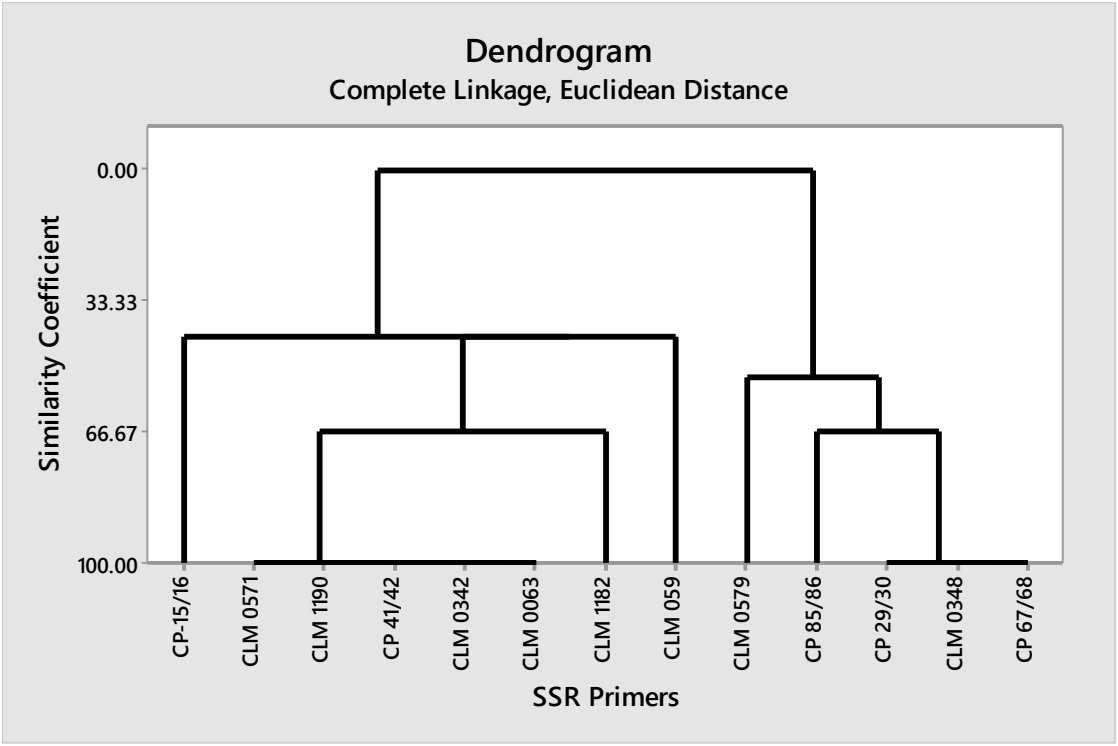

Figure 5. Dendrogram of SSR Primers

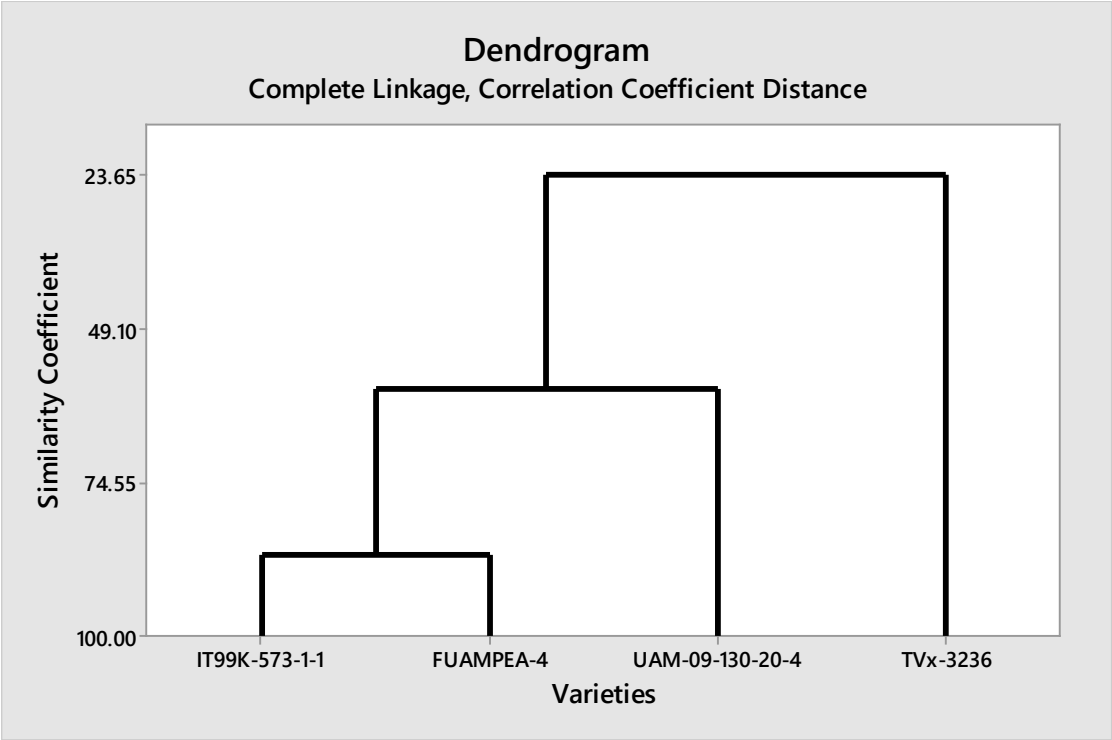

Figure 6. Dendrogram of Cowpea Varieties

Table 2 gives information on the total number of bands and relative polymorphic bands (RPB) of the SSR primers. The 13 SSR primers produced 55 bands. Number of bands per primer ranged from 1 in CP-15/16 to 8 bands. Primers CP 29/30, CLM 0348 and CP 67/68 produced 8 bands each with RPB of 7.6%. This group was followed by CP 85/86 primer that produced 5 bands with RPB of 6.3%. Varietal polymorphism was higher in the scab susceptible varieties than the resistant varieties in the following order: FUAMPEA-4 (27%), UAM09-130-20-4 (26%), IT99K-573-1-1 (24%) and TVx-3236 (23%).

Table 3 presents indices of polymorphism of SSR primers. Heterozygosity of primers (H) ranged from 0.26 in CLM 1182 to 0.62. Primers CLM 0579, CP 29/30, CLM 0348 and CP 67/68 had the highest H value (0.62) and Polymorphic Information Content (PIC) of 0.55. The highest Marker Index (MI) was found in CLM 0579 primer (MI =0.73). Effective Multiplex Ratio (EMR) of primers was highest in CP 29/30, CLM 0348 and CP 67/68 with value of 3.0 while Resolution Power (RP) was between 23.5 and 25.5 among the 13 primers.

Table 2. Frequency and Percentage of Polymorphism among SSR Primers

Primer	Total number of bands	Frequency of polymorphism	% Polymorphism
CP-15/16	1	0.01	1.3
CLM 0579	4	0.05	5.1
CLM 0571	4	0.05	5.1
CLM 1190	4	0.05	5.1
CLM 059	4	0.05	5.1
CP 85/86	5	0.06	6.3
CP 29/30	6	0.08	7.6
CLM 0348	6	0.08	7.6
CP 67/68	6	0.08	7.6

CP 41/42	4	0.05	5.1
CLM 0342	4	0.05	5.1
CLM 0063	4	0.05	5.1
CLM 1182	3	0.04	3.8
	55		

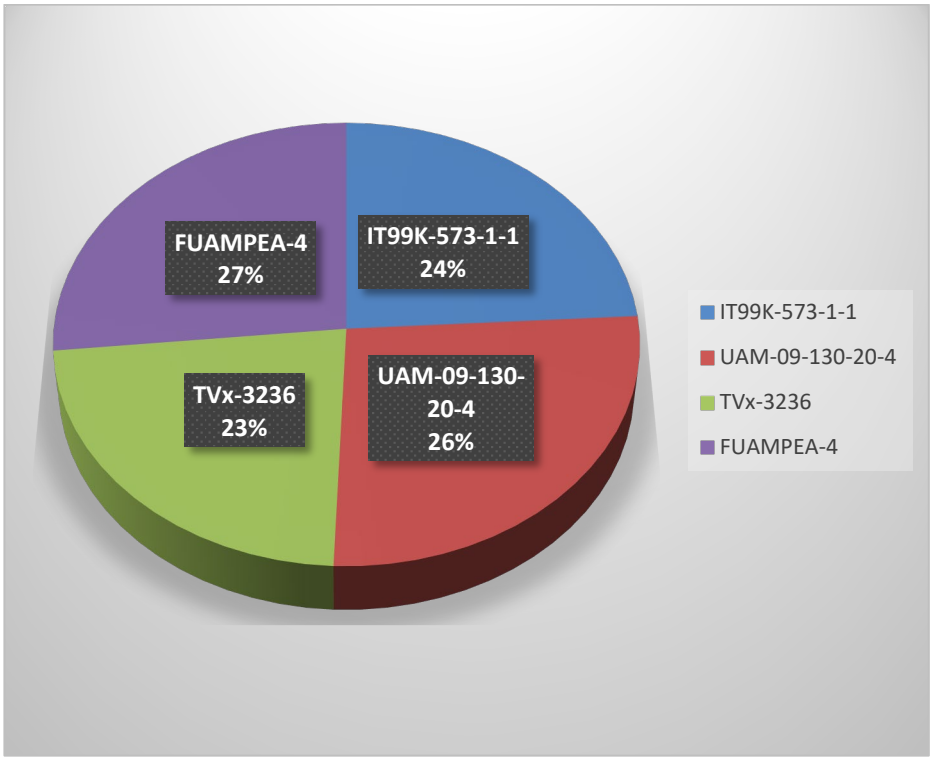

Figure 7. Percentage Polymorphism among Four Cowpea Varieties

Table 3. Polymorphic Indices of SSR Primers

Primer	H value	PIC	EMR	MI	RP
CP-15/16	0.50	0.38	0.50	0.19	25.5
CLM 0579	0.62	0.55	1.33	0.73	23.5
CLM 0571	0.32	0.27	0.5	0.13	25.5
CLM 1190	0.32	0.27	0.5	0.13	25.5
CLM 059	0.50	0.38	0.5	0.19	25.5
CP 85/86	0.54	0.47	1.33	0.62	23.5
CP 29/30	0.62	0.55	3.0	1.64	23.5
CLM 0348	0.62	0.55	3.0	1.64	23.5
CP 67/68	0.62	0.55	3.0	1.64	23.5
CP 41/42	0.32	0.27	0.5	0.13	25.5

CLM 0342	0.32	0.27	0.5	0.13	25.5
CLM 0063	0.32	0.27	0.5	0.13	25.5
CLM 1182	0.26	0.22	0.5	0.11	25.5

H= Heterozygosity of primers
PIC= Polymorphic Information Content of primers
EMR= Effective Multiplex Ratio of primers
M1= Marker Index
RP= Resolution Power

The indices of polymorphism in our study highlight the efficiency and informativeness of the SSR markers used to distinguish between scab-resistant and susceptible cowpea varieties. Primers with high heterozygosity, PIC, MI, EMR, and RP, such as CLM0579, CP29/30, CLM0348, and CP67/68, are particularly valuable for genetic studies aimed at improving resistance traits through marker-assisted selection (MAS). Studies like that of Li et al. (2011) have shown that SSR markers with high heterozygosity are more effective in identifying polymorphisms between different genotypes, making them useful for genetic mapping and breeding programs. PIC values above 0.5 are generally considered highly informative, as noted by Botstein et al. (1980), which suggests that these markers are reliable for studying genetic diversity. Higher MI values signify greater utility in distinguishing genotypes, as supported by studies such as Powell et al. (1996), which emphasize the importance of using markers with high MI for comprehensive genetic analysis. According to Varshney et al. (2007), high EMR values are advantageous in large-scale genotyping and breeding programs where high-throughput marker efficiency is required. According to Chesnokov and Artemyeva (2015) RP is a key indicator of a marker's discriminatory power. High RP values suggest a marker's strong ability to differentiate between closely related genotypes, making these markers particularly useful in genetic diversity studies.
Thus our findings show that the SSR markers used in this study are useful tools for detecting genetic diversity and potentially identifying loci linked to scab resistance. The high PIC and MI values further reinforce the utility of these markers for breeding programs, as they offer robust polymorphism and high discriminatory power. Additionally, the high RP values ensure that these markers can effectively resolve differences among cowpea genotypes, which is crucial for developing resistant varieties and improving overall crop resilience.

Conclusions

The study demonstrated significant genetic variability in cowpea scab resistance. Characterization with SSR Markers revealed genetic variability among the cowpea varieties screened expressed in the form of genetic polymorphism. SSR Markers CLM 0579, CP 29/30, CP 67/68 and CLM 0348 were the most informative markers in discriminating among the cowpea varieties at the molecular level. CLM 0571, CLM 1190, CLM 0342, CLM 0063 and CP 41/42 were monomorphic between cowpea varieties. CLM 0579, CP 29/30, CLM 0348 and CP 67/68 showed consistent polymorphism and band pattern between scab resistant and susceptible cowpea varieties indicating their suitability to track scab resistance in cowpea. SSR markers CP 29/30, CLM 0348, and CP 67/68 are useful for identifying resistant varieties, and IT99K-573-1-1 and TVx-3236 can serve as parental lines in breeding programs. In addition, the polymorphism observed in the SSR markers between resistant and susceptible cowpea varieties suggests that these markers could be linked to loci controlling scab resistance. These findings provide valuable insights for cowpea improvement programs aimed at enhancing scab

resistance. This insights will be crucial for breeding efforts aimed at improving cowpea resistance to scab and other diseases. Further studies could focus on mapping these markers to specific QTLs for marker-assisted selection, improving the efficiency of breeding programs targeting disease resistance.

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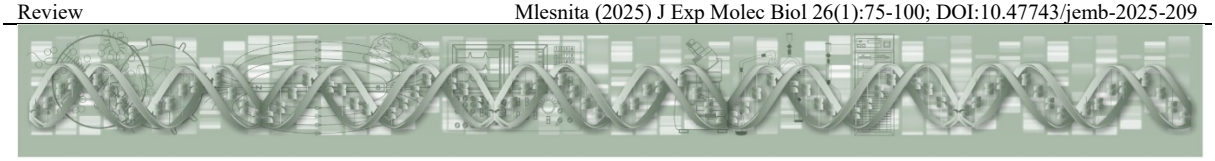

TACKLING THE SOIL MICROBIOME – CHALLENGES AND OPORTUNITIES

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Abstract

The health of the terrestrial ecosystems is directly dependent on the microbial composition that fulfills essential functions, such as sustaining plant growth, nutrient cycling and carbon sequestration. The study of the soil microbiome has gained popularity in the last decades due to its significant impact on the health of the environment and its inhabitants. This review explores the diversity and functions of soil microbial communities, with a particular focus on microbial dark matter, a subset of organisms that cannot be cultured through classical microbiological techniques. The evolution of DNA extraction methods and sequencing technologies coupled with the transition from amplicon sequencing to metagenome-assembled genomes (MAGs) and continuously developing bioinformatic pipelines has led to the discovery of novel microbial taxa, community networks, metabolic pathways and potentially useful molecules. Soil microbiome research is gaining momentum in Romania, as a big part of studies try to assess the impact of agricultural practices on the environment. Designing sustainable agricultural practices and implementing them with the goal of preserving the heterogeneity of the microbiome contributes significantly to the resilience of ecosystems, preserving the health of the environment, as well as the well-being of its residents.

Keywords: soil microbiome, microbial dark matter, metagenomics, DNA extraction, Next Generation Sequencing, amplicon sequencing, metagenome-assembled genomes

Introduction - The soil microbiome and us

In the past few decades, there has been growing recognition for the vital links between the ecosystem's health and that of plants, animals and humans. Themed under the umbrella of the term "OneHealth", the fitness of the aforementioned concepts reflects a worldwide objective driven by the concerning trends that the environment is subjected to, including climate change, emergent antimicrobial resistance, and diseases, as well as challenges related to ensuring food safety and security (Nadeu et al. 2023). Primarily, the soil acts as a nutrient storage and supplier, a fertile agricultural soil being able to sustain the production of qualitative food for animals and humans, all in a high yield. The nutrient content and its variations in the last two decades mirrors the global trend of the increasing need for higher quantities of feed along with the rise in population. The intensification of agriculture leading to a decline in the organic matter impairs the storage of the nutrients, their recycling into plant-available forms and their atmospheric and water distribution. The subsequent actions and decisions taken to attain the continuously-increasing food demand are the main reasons that lead to soil devaluation and successive deterioration of ecosystems (Brevik et al. 2020). The association between the ecosystem and their inhabitants is finely linked by the colonizing microbial communities. Within a high array

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of global ecosystems, microbes and especially prokaryotes dominate every habitat they inhabit through a high genetic and metabolic diversity, the soil harboring the most complex microbial communities out of all the environments (Nadeu et al. 2023). The terms "microbiome" and "microbiota" are intertwined, being used to describe microbial communities formed out of prokaryotes, fungi, viruses, algae, and protozoans that populate a specific habitat. The microbiome also includes the associations formed in these cooperative structures, within and outside the community, adding the encompassing environmental conditions (Marchesi and Ravel 2015). Microbiome research has rapidly grown in the last decades, especially following discoveries linking a dysregulated human gut microbiota to various gastrointestinal diseases (Fan and Pedersen 2021). The human microbiome is directly influenced by dietary habits. Consequently, the quality of the diet is linked to the agricultural practices, which are, in turn, dependent on multiple factors alongside the soil health. A healthy soil is described as a substrate capable of sustaining the productivity of plants and animals as well as with promoting their health. At the same time, this substrate has the capacity to manage the quality of the water and air, having a major contribution against climate change. And as everything is connected into the One Health concept, the health of the soil is largely sustained by the diverse accompanying microbiota (Banerjee and van der Heijden 2023). The soil microbiome is linked to numerous functions, aiding in bioremediation, discovery of antimicrobial substances, and sustainability and security of food systems, all of these having implications for the human health (Brevik et al. 2020).

Amidst the introduction of new molecular biology methods, the knowledge regarding various microbial communities has significantly increased in the last three decades. The advancement of -omics research encompassed the exploration of the complete genetic makeup of the microbiota in a culture independent fashion. This branch of study is also known as "metagenomics", term being oftentimes interchangeably and improperly used with "microbiome" (Marchesi and Ravel 2015). Comparative to the human microbiome research, the study of the soil bacterial communities hasn't been of much interest to the academic community (Stulberg et al. 2016). The interest for this ecological niche has surged over the last decade as there has been a dramatic rise in literature regarding the microbiome of the soil, the quality and health of soil being directly linked to the agricultural system and thus, all being dependent to the indigenous microbiota (Clarke et al. 2020, Hermans et al. 2023).

The composition of the microbiome and its functions

The composite microbes of the soil microbiota are represented primarily by bacteria and fungi, being followed by archaea, protists and viruses (Bar-On et al. 2018), distinguishing different habitats based on the diversity and distribution of species. Examples of such soil regions are the bulk soil and the rhizosphere (Xiong and Lu 2022). Among all known microbiomes, the soil microbiota is the most complex, with bacterial, fungal and archaeal species being the key players through their high metabolic diversity necessary to survive different environments (Fierer 2017). The study of the soil microbiome has been burdened by the limiting inability to culture most of the microorganisms. In consequence, culture-independent techniques emerged as a solution to explore the full extent of microbiota's diversity (He et al. 2008).

The microbial abundance of soil is high, being often reported that a single gram can contain billions of microorganisms representative of up to tens of thousands of species (Raynaud and Nunan 2014, Fierer 2017). Bacterial species are highly abundant in soil, comprising 70-90% of the total biomass, with fungi being subsequent, whereas the abundance of archaeal species insignificantly higher in extreme environments (Wang X. et al. 2024). In variable abundance, the bacterial representatives belong to the phyla *Pseudomonadota, Actinobacteria, Acidobacteriota, Verrucomicrobiota, Bacterioidota, Planctomycetes, Chloroflexi,* and

*Firmicutes.*When it comes to fungi, the relative abundance picture is represented in big part by species from the phylum *Basidiomycota,* the rest being completed by *Ascomycota* and *Zygomycota* species (Fierer 2017, Delgado-Baquerizo et al. 2018, Labouyrie et al. 2023). The culturable bacterial fraction is diverse, being constituted of a significant number (over 88% out of the entire bacterial division) of *Pseudomonadota* species, with A*ctinobacteria, Firmicutes* and *Bacterioidetes* following (Nikolaki and Tsiamis 2013). Whitin a soil sample, the culturable fraction is dominated by the *Arthrobacter* genus (He et al. 2008). On the other hand, rare microbial species, although present in relatively small numbers, contribute to more than 65% of the diversity within the entire community (Xiong and Lu 2022). Scattered in between abundant microbial populations, with great dependence to the niche populated, there is an unculturable microbial fraction that plays a huge role in maintaining the balance of the entire community. Recognized as the "microbial dark matter", its role is well-known in the stability of the microbiome (Ma et al. 2023). The great heterogeneity and interactions between different taxa support the resilience and productivity of the ecosystem. This diversity is seen also on a functional level, with the majority of microbial strains performing important environmental functions, while a small fraction act as pathogens (Banerjee and van der Heijden 2023).

As an essential component of soil composition, the vast diversity of microbial taxa mediates important and essential functions for the ecosystem, having either direct or indirect impact on the environment and its inhabitants. Specifically, the microbiota is capable of sequestering and storing carbon from the environment, , playing a big role in mitigating the greenhouse gasses and their effect (Dubey et al. 2019, Tao et al. 2023). The microbial diversity aids in the degradation of soil organic matter, an essential step in the cycle of nutrients in the environment, and by oxidizing organic residues left behind by plants and animals, nutrients are made available for the growing plants (Anthony et al. 2020). Fungal and bacterial species, primarily from the phylum *Actinomycetes* and the *Bacillus* genus target mostly proteins, making nitrogen available for other species (Bhatti et al. 2017, Nicolás et al. 2019, Gómez-Brandón et al. 2020, Rana Chhetri et al. 2022). The impact of bacteria on plant health and growth is significant as they interact with plant roots and aid the formation of beneficial relationships with growth promoting rhizobacteria, mycorrhizal fungi, and other microorganisms. Growth promoting microorganisms are represented by rhizobacterial species or mycorrhizal fungi found in the rhizosphere, root tissue or are integrated into the nodules of plants, that interact with the microbiome, either synergically or antagonistically, promoting plant growth through nutritional and hormonal balance regulation, aiding in nutrient eased solubilization and uptake along with providing resistance against pathogens. Under the influence of stress-inducing factors such as high salinity, heavy metal contamination, drought, and flooding, rhizhobacterial strains were seen to protect and promote the growth of the plants either alone or in synergy with mycorrhizal fungi. Mycorrhizal fungi facilitate water absorption and nutrient uptake, being estimated that around 80% of phosphorus is supplied to plants by them. Because of their localization and their potential in agriculture, research regarding the inoculation of growth-promoting microorganisms is of interest at the moment, as this approach could improve crop productivity and quality in a more sustainable way (Nadeem et al. 2014, Lopes et al. 2021). Nitrogen-fixing bacteria, such as species from genera *Achromobacter, Anabena, Azotobacter, Azospirillum, Rhizobium, Bradyrhizobium, Beijerinckia, Clostridium, Frankia, Klebsiella,* and *Nostoc* (Lopes et al. 2021) along with mycorrhizal fungi as *Funelliformes* sp., *Gigaspora* sp., and *Rhizophagus* sp. (formerly known as the genus *Glomus*) (Chalk et al. 2006) are featured as key players in maintaining soil fertility and sustaining terrestrial ecosystems, inoculi of one or more of these species being actively tested (Nadeem et al. 2014). All these microorganism associations highlight the intricate relationships between the soil microbiome and plants (Banerjee and van der Heijden 2023). Along with nitrogen-fixing bacteria, other species capable of fixing or producing derivatives out of phosphorus (*Arhtorbacter* sp., *Bacillus* sp., *Burkholderia* sp.,

Penicillium sp., *Pseudomonas* sp., *Serratia* sp., *Aspergillus* sp., *Achromobacter* sp., *Agrobacterium* sp., *Erwinia* sp., *Micrococcus* sp., *Rhizobium* sp.), sulfur (*Bacillus* sp.) and iron (*Azobacter* sp., *Bacillus* sp., *Fusarium* sp., *Pseudomonas* sp., *Serratia* sp., *Streptomyces* sp., *Burkholderia* sp., *Enterobacter* sp., *Grimotella* sp.) (Lopes et al. 2021, Banerjee and van der Heijden 2023) have a direct role in the biogeochemical cycling of macro- and microelements. It is estimated that soil bacteria accounts for the bioavailability of 18 essential elements out of 29 elements necessary for maintaining plant health (Brevik et al. 2020, Banerjee and van der Heijden 2023).

Another function mediated by the soil microbiome involves conferring resistance to aboveground pests, a concept that is gaining interest in the agricultural field (Pineda et al. 2017, Pineda et al. 2020). Noteworthy to highlight, by aiding in the formation of soil aggregates, the microbiome maintains the soil structure, preventing its erosion and protecting the associations between the root system of the plants and the soil as a nutritive substrate (Bergmann et al. 2016, Angst et al. 2021).

In the last decade researchers have investigated the impact of heavy metal soil contamination, severe pollution, and the effect of climate change on the normal microbiota. Contamination with heavy metals negatively influences the structure of the microbiome, with descending relative abundance for species from phyla *Nitrospirae, Bacterioidia* and *Verrucomicrobia* (Li et al. 2020). Moreover, the relative abundance and species variability are impacted by elevated levels of aluminum, variable carbon-to-nitrogen ratios, available phosphorus, and pH levels (Hermans et al. 2017). Plastic pollution affects the soil microbiome's composition, abundance and functions by altering the water and carbon availability (Lear et al. 2021). Pesticide usage causes a decrease in the microbial population and diversity, and as a consequence, affects the nutrient cycling by the mycorrhizal fungi. Nonetheless, human actions affect the soil mainly through urbanization, unsustainable agricultural practices and intense cropping. A disrupted soil microbiome can affect the soil health and associated functions, with alterations in the microbiome potentially acting as a bioindicator of such conditions. Despite the significance and need of new pollution bioindicators, research is still in early stages (Banerjee and van der Heijden 2023). The various soil microbiome functions along with its disrupting factors are depicted in Figure 1.

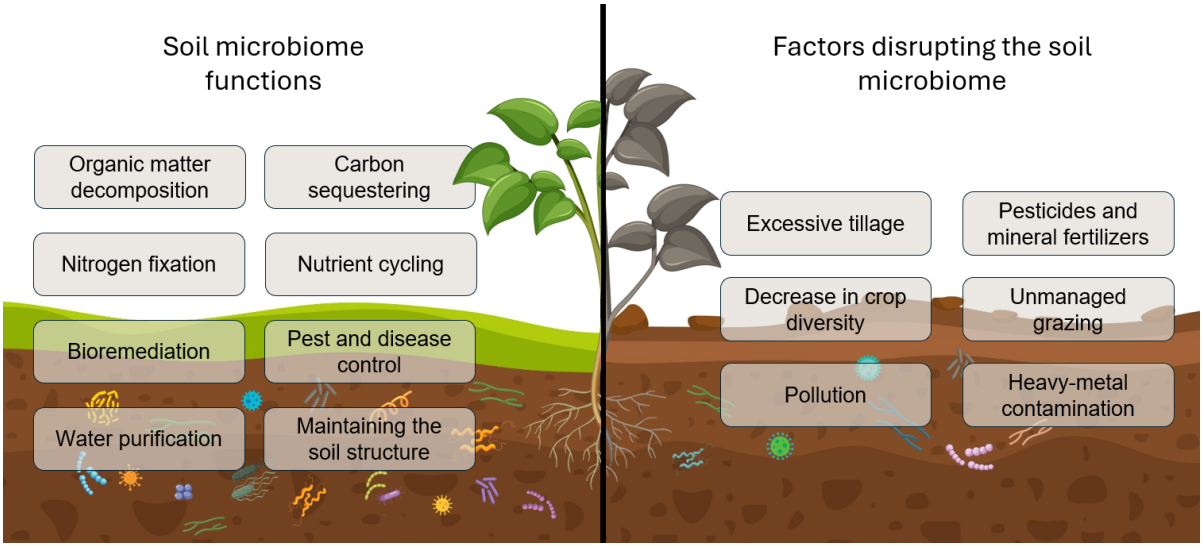

Figure 1. Functions of the normal soil microbiota (left) and factors influencing negatively the soil microbiota diversity (right). The microbiome is a major contributor to the health of the soil, being associated with the normal growth and development of plants. (*Figure created using vectors from* www.vecteezy.com)

A recent study compared the soil microbial variations and microbial biomass from three sites used for urban leisure, traffic and urban agriculture. The researchers observed that the agricultural site had the lowest biodiversity of them all but high fungal richness, whereas the leisure site represented a stable setting for the development of specialized microbial communities and microbial plant symbionts (Christel et al. 2023).
Understanding the significance of a healthy soil and the interdependence of humans and microbial communities might lead us towards a cleaner environment that promotes sustainable agriculture and stable ecosystems.

If it's too small, it doesn't mean it's not powerful: the microbial dark matter

The study of microorganisms found in the environmental niches primarily focused on isolating and characterizing them from a pure culture. The DNA sequencing methods described in 1977 changed the whole perspective on taxonomical classification of bacteria, transitioning from the field of microbiology to that of molecular biology. Phylogenetical studies based on sequencing followed shortly as the gene encoding the small subunit of the ribosome was described as a feasible taxonomic marker (Woese and Fox 1977, Woese et al. 1990, Nikolaki and Tsiamis 2013). Although the first bacterial genome was successfully sequenced in 1995, it didn't take long for researchers to try to characterize a bacterial community (Land et al. 2015). Other marker genes taken into consideration in metagenomic studies are the internal transcribed spacer (ITS) region for distinguishing fungal species and the 18S and 23S rRNA for other eukaryotes that compose the microbiota (Pérez-Cobas et al. 2020, Nam et al. 2023). It was a great surprise to find out that the already described soil microbiome through culture techniques accounted for 1% of the total microbiota found in the environment. The great unculturable microbial fraction, recognized as the "microbial dark matter" is being represented in big part by archaea and bacteria (Solden et al. 2016, Jiao et al. 2021, Ma et al. 2023). This reservoir of newly identified species was described later as a new clade, appointed the name of Candidate Phyla Radiation (Hug et al. 2016, Jiao et al. 2021). The identification of previously uncharacterized microbes presents a potential resolution to emerging medical and biotechnological challenges. Given that the majority of antimicrobial substances discovered in the "Golden Age" were of microbial origin, the diverse and numerous species present within microbial dark matter became an exciting subject to pursuit in context of combating the antimicrobial resistance phenotypes (Ma et al. 2023). Other noteworthy potential applications are represented by the bioremediation capacity from soil and water, generation of biofuels and agricultural fertilizers as well as the synthesis of disease markers (Nikolaki and Tsiamis 2013). The considerate complexity and heterogeneity of the soil microbial dark matter presents numerous challenges in the investigation of this ecological community. A number equal to one million is estimated to represent the unknown species (Zha et al. 2022). Studying these novel organisms require considerable computational resources along with bioinformatic tools capable to mine through the data, a significant obstacle being the absence of reference genomes in databases.

How is soil microbiota affected by current agricultural practices

Because the health of the soil relies on the constituent microbiota, external factors that have a negative impact on the microbial communities interfere with the soil's ability to sustain the well-being of plants, animals and humans while also contributing to a cleaner environment. As pollution and environmental changes are taking their chance to hinder the soil's microbiome functions, the conventional agricultural practices pose a harmful influence on the long term sustainability of food production (Food and Agriculture Organization United Nations 2022,

Hermans et al. 2023, Nadeu et al. 2023). Practices such as excessive tillage, usage of antimicrobial substances, synthetic fertilizers and pesticides with excessive grazing lead to loss of biodiversity and homogenizes the microbial community of the soil (as seen in Figure 1). Consequently, this leads to soil erosion and compaction as well as pesticide contamination, all with a bad prognostic for the future of food (Banerjee and van der Heijden 2023, Hermans et al. 2023). Trying to preserve the health of the soil and the high yield of crops, regenerative agriculture approaches have been described in recent years taking into consideration the need of enhancing crop resilience to environmental stresses. In opposition to traditional agriculture, the sustainable agriculture movement is represented by practices such as reduced tillage with low or no usage of mineral fertilizers and pesticides. For protecting the biodiversity, the recommendations follow that there should be crop rotation practices between fields with diversified plants cultivated as well as managing the grazing of livestock towards quick recoveries of skimmed soil patches (Hermans et al. 2023).
Multiple projects have been amended in the last decades in the hope of saving the environment, and by this, the soil microbiota that contributes greatly to the agricultural sector. Understanding and tackling the potential that the soil microbiome holds are essential for optimizing agricultural practices and enhancing crop resistance to environmental stresses in a sustainable manner (Nadeu et al. 2023).

The soil microbiota of Romania – What we know up until now

Research on soil microbial diversity is currently gaining momentum in Romania. A multitude of studies have set the stage for uncovering the microbial complexity of the soil, with a primary focus on its implications for sustainable agriculture and the preservation of environmental diversity. A great part of research conducted on the Romanian soil microbiome take culturing or metataxonomic approaches, bacterial strains being the primary focus of these studies. Numerous studies tried to describe the extremophile species from Romania, from either soil, sediments, karst or water from habitats defined by severe conditions that don't allow the survival of most organisms (Andrei et al. 2017, Sarbu et al. 2018, Chiciudean et al. 2022, Bogdan et al. 2023, Szekeres et al. 2023). A map displaying the geographical coordinates associated to the soil microbial studies conducted in Romania is represented in Figure 2.

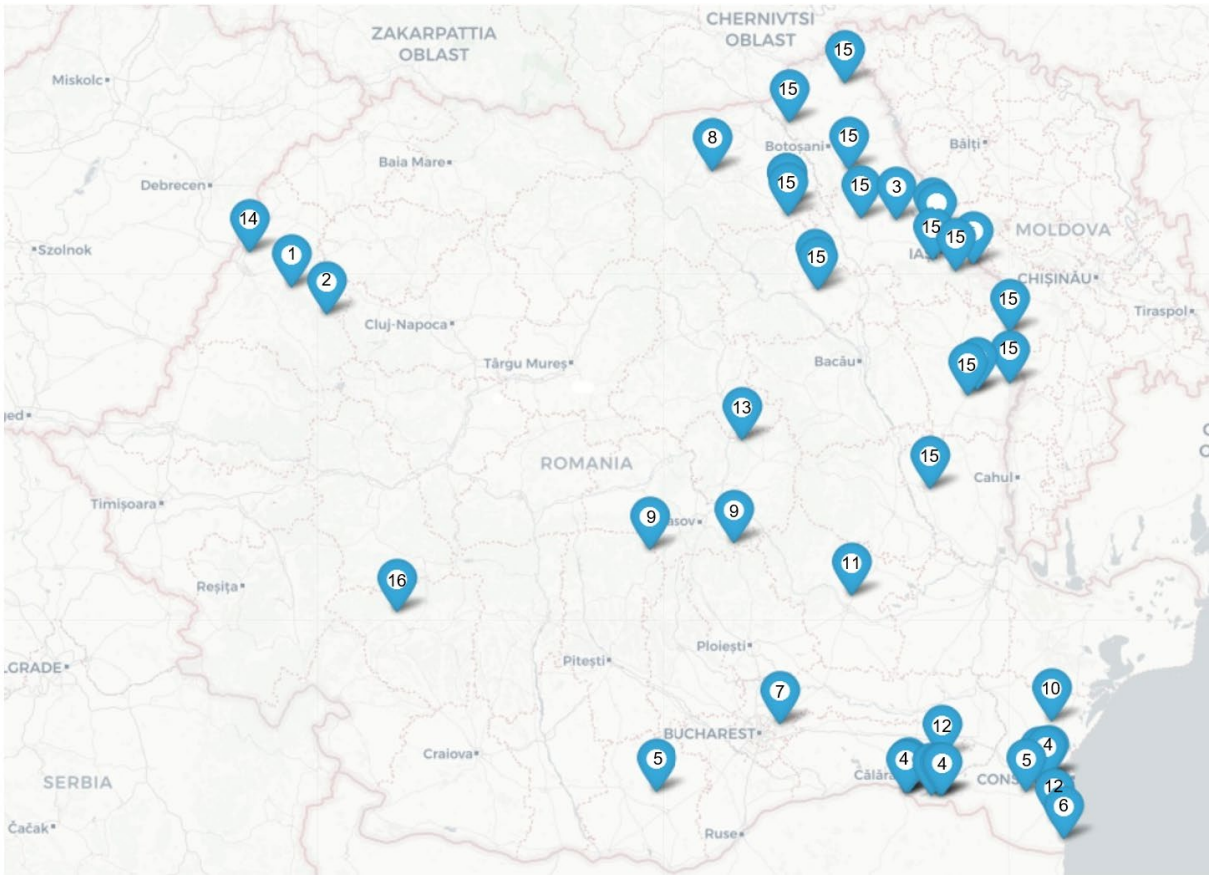

Figure 2. Soil microbial studies conducted in Romania between the years 2005-2024. Legend: **1 -** Onet et al. 2024**; 2** - Bogdan et al. 2023; **3** - Gafencu et al. 2023; **4 -** Steiner et al. 2023**; 5 -** Ghiță et al. 2022; **6 -** Chiciudean et al. 2022**; 7** - Dușa et al. 2022; **8 -** Choma et al. 2021**; 9 -** Dinca et al. 2021**; 10** - Matei et al. 2020; **11 -** Toader et al. 2019; **12** - Ditu et al. 2018; **13** - Sarbu et al. 2018; **14 -** Onet et al. 2019**; 15** - Ulea et al. 2017; **16** - Gornoavă et al. 2005

For instance, the samples examined from the Sulfur Cave were characterized by the presence of *Mycobacteria* sp., *Ferroplasmaceae* sp., *Acidithiobacillus* sp., and *Metallibacterium* sp. with the first taxon being the most abundant (Sarbu et al. 2018). The diversity of taxons from the soil samples collected in the Leșu cave is represented by taxons primarily from the phyla *Pseudomponadota, Verrucomicrobio, Actinomycetota, Acidobacteriota, Patescibacteria, Nitrospirota*. The central difference between the different collection sites was the abundance. Even though all the samples contained species from the mentioned phyla, their abundance was different throughout all the collection sites (Bogdan et al. 2023).

The rhizosphere bacterial communities of five rare plant species (*Adonis vernalis, Opopanax chironium, Asphodeline lutea, Paeonia tenuifolia, Potentilla emilii-popii*) were investigated using a mass spectrometry approach. With a focus on the cultivable fraction of the rhizosphere microbiota, the findings indicate that the genera variation among samples was not high. Species from genera such as *Bacillus, Pantoea, Serratia, Pseudomonas* were present in almost all of the samples analysed, these microorganisms having a function in mediating the plant growth. The outlook of the research states that the discovery of beneficial strains along with microbial indicators showcasing the health of the plant might be useful in conservation approaches (Ditu et al. 2018).

Soil pollution is majorly affecting the health of the crops along with their yield. Ulea et al. (2017) studied the impact of the agricultural practices and the seasonal variability on different soil types from Moldavia region. They took into consideration the abundance and composition of bacterial strain as indicators for the health of the soil. Compared on a temporal scale from

May to September, the highest bacterial abundance was registered in spring, whilst the lowest was registered in autumn. The agricultural practices directly influenced the microbial community abundance and dynamics as an undisturbed forest soil presented the highest bacterial count, whereas a vineyard soil which was subjected to a set of conventional agricultural practices presented the lowest bacterial count. Concluding, the authors state that the dynamics and changes in the structure of the soil bacterial population contribute to a better management of the agricultural habits, leaning towards a healthier future for the environment and promoting sustainable food production (Ulea et al. 2017).
The majority of studies were based on cultural methods for the identification of bacterial species. Consequently, the full picture of the whole studied soil microbiome hasn't been painted yet, as the unculturable fraction accounts for 99% of the whole microbiome. The study of soil microbial communities could contribute to developing more sustainable agricultural practices, leading to a healthier environment for the future generations. Food obtained through green and eco-friendly practices not only reduces the impact of agriculture but also improves the nutritional quality, promoting a long-term sustainable system for the food production.

DNA extraction – the essence of a metagenomics protocol

Studying the complex microbial communities present in a specific niche has opened doors to new insights into their ecological interactions, metabolic capacities, and evolutionary processes. The conventional culturing methods can't give an answer to all the questions that arise from a microbial network as they can't entirely portray its composition. With the advent of molecular biology methods, the study of taxa by amplicon sequencing and the field of metagenomics emerged answering a considerate number of questions. Metagenomic studies generate large quantities of data and even larger challenges to take into consideration, all in exchange for creating an almost perfect microbial picture on the canvas of the ecological niche and environmental changes.
A basic metagenomics protocol is described by the acquisition of the sample from the environment, extraction of the nucleic acids and their processing, sequencing, and analysis of the obtained data. The central step of the pre-sequencing stage consists of the nucleic acid isolation, step influencing both the quality and quantity of DNA for successive analysis. A lot of attention has been invested in soil DNA extraction methods, primarily due to the particularities of each technique and the varying outcomes in dependence to each environmental sample taken into analysis. Characteristics of a DNA extraction protocol from soil samples have been extensively reviewed by Wydro (2022). The DNA extraction from soil samples can be done through indirect or direct approaches. The indirect isolation of nucleic acids involves the separation of the cells from the soil sample, followed by their lysis. As eukaryotic cells are excluded, the separated organisms are represented by bacteria and archaea. Even though high amounts of DNA are extracted, this becomes a disadvantage for downstream analyses. Another impediment of this approach is the inability to study eukaryotic sequences and their interactions with prokaryotes. Direct isolation of DNA from soil implies the processing of the whole sample, the cells present in the soil matrix being lysed. This approach is beneficial for obtaining high yields and allowing the analysis of a high number of microorganisms (Wydro 2022).
A sum of factors that may influence the quantity and quality of the extracted DNA from soil samples include the organic content and type of the soil, the lysis method, the samples size, its transport and storage until downstream processing (Wydro 2022). The outcomes of a metagenomic protocol may also be influenced by the batch effect or the limited number of replicates taken into analysis (Child et al. 2024 Preprint). Soil contains a high number of impurities. Of interest are the humic acids that can co-precipitate and inhibit the DNA extraction process, consequently resulting in the failure of the PCR reaction. The physical, chemical and

enzymatic lysis techniques employed in extracting the DNA from environmental samples are key determinants of the microbial diversity recovered (Wydro 2022). For example, metagenomic studies encounter challenges with the sample preparation process, as it could impact the number of lysed cells, mostly affected being the fungal species (Child et al. 2024 Preprint). Papers comparing different protocols, commercial kits or laboratory developed methods have emerged, each test being ran on different types of soil samples, trying to assess the best kits in regard of DNA yield, purity and impact on downstream analysis (Plassart et al. 2012, Santos et al. 2015, Tanase et al. 2015, Child et al. 2024 Preprint, Jensen et al. 2024). Table 1 provides a summary of DNA extraction methods and kits reported and compared in the literature from the last two decades with emphasis to the soil types taken into analysis.

Table 1. Comparison of commercial DNA extraction kits and laboratory-developed methods used for soil microbial community analysis, as reported in the literature over the past two decades. It summarizes the DNA yields and purity ratios obtained, with reference to the specific soil types tested

Commercial kit / method	DNA yield	A_{260}/A_{280} A_{260}/A_{230}	Soils tested on	Reference
DNeasy® PowerSoil® Pro Kit (Qiagen)	60 ± 21 ng/mg	N/A N/A	Arable, pasture, woodland, healthy soil	Child et al. 2024 Preprint
	0.5 - 68-8 ng/µl	0.75 - 5.31 0.01 - 0.4	Martian soil, mars stimulant soil	Wang et al. 2024
DNeasy® 96 PowerSoil® Pro QIAcube® HT Kit (Qiagen)	0.16 - 4.20 µg	1.79 - 2.88 0.95 - 2.15	Beach sand, clay, organic, sand, sand-clay	Jensen et al. 2024
QIAamp DNA Stool Mini KitTM (Qiagen)	4.7 - 54.7 ng/µl	N/A N/A	Compost, soil, mangrove sediment, decaying coffee pulp	Guillén-Navarro et al. 2015
ExtroSpin® Soil Kit (Lvjia Agro-tech Co., Ltd)	0.3 - 0.5 ¼g/g soil	1.69-1.82 0.08-0.19	Paddy soil, clayey soil	Li et al. 2014
FastDNA™ SPIN Kit for Soil (MP BioMedicals)	32 ± 17 ng/mg soil	N/A N/A	Arable, pasture, woodland, healthy soil	Child et al. 2024 Preprint
	1914.6-20333.33 ng	1.26-1.87 0.06-0.35	Woodland	Bollmann-Giolai et al. 2020
	2.1 ug/g soil	1.9 ± 0.2 N/A	Permafrost	Vishnivetskaya et al. 2014
	3.51 ± 0.03 µg/g soil	1.50 - 1.62 N/A	Garden soil, sewage sludge, lake soil, compost	Devi et al. 2015
	8.39 - 9.33 ng/µl	2.47 - 2.7 0.001	Martian soil, mars stimulant soil	Wang et al. 2024
	1.45-2.26 ¼ g/g soil	1.74-1.84 1.23-1.52	Paddy soil, clayey soil	Li et al. 2014

Commercial kit / method	DNA yield	A_{260}/A_{280} A_{260}/A_{230}	Soils tested on	Reference
FastDNA™-96 Soil Microbe DNA extraction Kit (MP BioMedicals)	0.02 - 2.91 µg	2.08 - 3.17 0.18 - 1.95	Beach sand, clay, organic, sand, sand-clay	Jensen et al. 2024
HiPurA soil DNA isolation kit (Himedia)	3.52 µg/ g soil	N/A N/A	Agricultural fields	Tanveer et al. 2016
Modified HiPurA soil DNA isolation kit	7.35 µg/ g soil	N/A N/A	Agricultural fields	Tanveer et al. 2016
ISOIL for Beads Beating kit (Nippon Gene)	1.02 - 2.15 ¼g/g soil	1.77-1.92 1.17-1.32	Paddy soil, clayey soil	Li et al. 2014
MagBeads FastDNA™ Kit for Soil (MP BioMedicals)	38 ± 20 ng/mg	N/A N/A	Arable, pasture, woodland, healthy soil	Child et al. 2024 Preprint
Meta-G-NomeTM DNA Isolation Kit (Epicentre Biotechnologies)	0.06 µg/g soil	1.7 ± 0.02 N/A	Permafrost	Vishnivetskaya et al. 2014
Power Lyzer™ PowerSoil® DNA Isolation Kit (Qiagen, formerly MOBIO)	8.7–47.5 µg/ g soil	1.8-1.9 1.5-2.1	Grassland, arable	Santos et al. 2015
	0-1203.33 ng	2.02-2.12 0.82-1.77	Woodland	Bollmann-Giolai et al. 2020
	0.9 µg/g soil	> 2.00 N/A	Permafrost	Vishnivetskaya et al. 2014
	2.5-3.5 ng/µl	N/A N/A	Beach sand	Gallard-Gongora et al. 2022
	2.47–6.96 ± 1.56 µg/g soil	1.13–1.64 1.28–1.58	Agricultural yellow loess soil	Kathiravan et al. 2015
PowerMax Soil™ (Qiagen)	0.8-0.9 ng/µl	N/A N/A	Beach sand	Gallard-Gongora et al. 2022
SPINeasy® DNA Pro Kit for Soil (MP BioMedicals)	40 ± 12 ng/mg	N/A N/A	Arable, pasture, woodland, healthy soil	Child et al. 2024 Preprint
Soil DNA Isolation Kit (NorgenBiotech)	1.08± 0.18 µg/ g soil	2.31± 0.17 0.29± 0.12	Rich humic acid and clay content soil polluted with kerosene	Tanase et al. 2015
Soil DNA extraction kit (MACHEREY-NAGEL)	14 µg/µl	2.2 0.86	Loam	Basim et al. 2020

Commercial kit / method	DNA yield	A_{260}/A_{280} A_{260}/A_{230}	Soils tested on	Reference
Soil Master DNA extraction kit (Epicentre)	0.79 µg/ml	1.32 1.21	Rhizospheric soil	Fatima et al. 2014
Zymo Research Quick-DNA Fecal/Soil Microbe Miniprep Kit (Zymo Research)	12 ± 16 ng/mg	N/A N/A	Arable, pasture, woodland, healthy soil	Child et al. 2024 Preprint
ZymoBIOMICS1 96 MagBead DNA Kit (Zymo Research)	0.03 - 1.08 µg	1.38 - 1.68 0.03 - 0.07	Beach sand, clay, organic, sand, sand-clay	Jensen et al. 2024
ZR Soil Microbe DNA Miniprep™ (Zymo Research)	11.5 - 62.5 ng/µl	N/A N/A	Compost, soil, mangrove sediment, decaying coffee pulp	Guillén-Navarro et al. 2015
ISO-11063 Standard Method	3.87± 0.23 µg / g soil	N/A N/A	Crop soil, forest soil, grassland	Plassart et al. 2012
ISOm	19.03± 2.22 µg/g soil	N/A N/A	Crop soil, forest soil, grassland	Plassart et al. 2012
	21.5–43.4 µg/ g soil	1.5± 0.010 1.6-1.8	Grassland, arable	Santos et al. 2015
GnS-GII	26.26±2.20 µg/ g soil	N/A N/A	Crop soil, forest soil, grassland	Plassart et al. 2012
	8.2–49.7 µg/ g soil	1.6-1.7 1.5-1.6	Grassland, arable	Santos et al. 2015
Tanase et al. 2015 modified GnS-GII	40±6.16 µg/ g soil	1.55±0.05 0.56±0.05	Rich humic acid and clay content soil polluted with kerosene	Tanase et al. 2015
S	49.38±9,8 µg/ g soil	1.52± 0.02 0.69± 0.02		
SP	75.70±9.4 µg/ g soil	0.74± 0.02 0.38± 0.08		
S-CTAB	25.58±8.62 µg/ g soil	1.56± 0.02 0.62± 0.02		
SDE	468-2913.33 ng	1.29-1.45 0.60 - 0.87	Woodland	Bollmann-Giolai et al. 2020
PEG/NaCl method	0.73 µg/ml	1.26 1.12	Rhizospheric soil	Fatima et al. 2014
Mannitol-PBS-PEG/NaCl method	2.2 µg/ml	1.81 1.84	Rhizospheric soil	Fatima et al. 2014
Mannitol-PBS-PEG method	2.36 µg/ml	1.84 1.93		
Mannitol-PBS-CTAB	2.67 µg/ml	1.85 2.07		
Phenol-chloroform	7.5 - 125.0 ng/µl	N/A N/A	Compost, soil, mangrove	Guillén-Navarro et al. 2015

Commercial kit / method	DNA yield	A_{260}/A_{280} A_{260}/A_{230}	Soils tested on	Reference
Enzymatic lysis	7.5 - 75 ng/µl	N/A N/A	sediment, decaying coffee pulp	
Lysozyme method	12.5 - 100 ng/µl	N/A N/A		
Modified enzymatic lysis	0 - 100 ng/µl	N/A N/A		
Protocol A	10 µg/µl	1.9 2.4	Loam	Basim et al. 2020 (Basim et al. 2020)
Protocol B	14 µg/µl	1.6 0.65		
Protocol D	135 µg/µl	2 2.2		
Manual method	232.42 µg/g soil	N/A N/A	Agricultural fields	Tanveer et al. 2016
Slurry method	8.6-8.7 ng/µl	N/A N/A	Beach sand	Gallard-Gongora et al. 2022
Tsai and Olson 1991 method	3.38 ± 0.05 µg/ g soil	1.33 - 1.48 N/A	Garden soil, sewage sludge, lake soil, compost	Devi et al. 2015
	7.55 ± 0.73 µg/g soil	1.18 ± 0.015 0.82 ± 0.035	Garden soil, domestic and cellulose waste dumping sites, sewage contaminated site	Verma et al. 2017
Yeates et al. 1998 method	3.42 ± 0.04 µg /g soil	1.40 - 1.56 N/A	Garden soil, sewage sludge, lake soil, compost	Devi et al. 2015
Modified Yeates et al. 1998 method	5.87 ± 0.04 µg/g soil	1.72 - 1.82 N/A		
Modified Yeates et al. 1998 method	23.62± 4.65 µg/g soil	1.23± 0.06 0.92± 0.04	Agricultural yellow loess soil	Kathiravan et al. 2015
Zhou et al. 1996 method	1.29 ± 0.02 µg/ g soil	1.14 - 1.29 N/A	Garden soil, sewage sludge, lake soil, compost	Devi et al. 2015
	19.1±1.74 µg/g soil	1.25±0.03 0.94±0.04	Garden soil, domestic and cellulose waste dumping sites, sewage contaminated site	Verma et al. 2017
Siddhapura et al. 2010 method	8.51 ± 0.93 µg/g soil	1.34±0.03 1.25±0.03	Garden soil, domestic and cellulose waste dumping sites, sewage contaminated site	Verma et al. 2017
Singh et al. 2014 method	1.33 ± 0.16 µg/ g soil	1.02±0.01 1.00±0.01		
Verma et al. 2017 method	15.55±0.80 µg/ g soil	1.74±0.03 1.70±0.02		

Commercial kit / method	DNA yield	A_{260}/A_{280} A_{260}/A_{230}	Soils tested on	Reference
Verma and Satyanarayana 2011 method	11.23 ± 1.04 μg/ g soil	1.48 ± 0.030 1.32 ± 0.055		
Volossiouk et al. 1995 method	9.36 ± 0.60 μg/ g soil	1.11±0.02 0.85±0.05		
Bürgmann et al. 2001 method	33.8 ± 2.71 μg/g soil	1.27± 0.03 0.86± 0.02	Agricultural yellow loess soil	Kathiravan et al. 2015
Kathiravan et al. 2015 method	42.48± 5.59 μg/g soil	1.24–1.43 0.52-0.96	Agricultural yellow loess soil	Kathiravan et al. 2015
Porteous et al. 1994 method	9.31 - 15.89 ± 1.34 μg/g soil	1.04± 0.02 0.80± 0.01		

In 2012, a standardized method for extracting microbial DNA was published under the name "ISO-11063: Soil quality - Methods to directly extract DNA from soil". Although this method could be used to isolate bacterial DNA from soil samples, the other microbial species from the soil such as archaea and fungi were overlooked. Thus, diverse approaches were explored with much greater success in describing all the constituents of the soil microbiota (Plassart et al. 2012, Terrat et al. 2012, Terrat et al. 2015). By testing different protocols to discover the best ones when it comes to capture a snapshot of the soil microbiome, different standard-derived, developed in laboratory methods emerged. Two methods that became popular because of the results obtained were GnS-GII and ISOm. The ISOm standard is a method derived from the last-mentioned international standard that implies the usage of FastPrep® bead-beating (MP BioMedicals, USA). Compared with the GnS-GII method that involves the use of the same mechanical lysis step and being time consuming, it is more lightweight, meaning it could be routinely applied when working with a big batch of samples. The DNA obtained from using each method varies in quantity and quality, being much greater than using the standard ISO protocol. The authors concluded that the ISOm methods was the best option to use in extracting DNA for metagenomic studies, as the GnS-GII method introduced heterogeneity in the bacterial composition (Plassart et al. 2012, Terrat et al. 2015). The soil homogenization process was described as the most significant step to have an impact on the procedure efficiency (Plassart et al. 2012). Despite that applying the FastPrep® bead-beating in the last-mentioned protocols provided a higher DNA yield than the standard method, the results differed in between methods, with the greatest variations between soil types being registered when working with the GnS-GII protocol. This method had the highest distinguishing capacity between the soil types, being able to assess the heterogeneity of the microbial community accurately even though the yield was not the expected one (Terrat et al. 2012).

As the years passed, the methodology was advancing as the soil microbiome field was gaining popularity. Commercially available kits assess a variety of isolation methods to achieve high DNA yields, purity and integrity of nucleic acids while maintaining a high throughput and reproducibility. Various studies have compared different DNA extraction protocols for metagenomics analysis, across diverse soil sample types, such as agricultural, polluted, forest, and many more. These comparisons have highlighted the importance of selecting an optimal DNA extraction method to ensure accurate microbial community profiling and functional information retrieved (Tanase et al. 2015, Child et al. 2024 Preprint). GnS-GII was compared

with other modified methods, such as S and S-CTAB. Interestingly, when tested on humic and kerosene-polluted soil samples, the S and S-CTAB exhibited superior performance or the results were equal to the ones obtained by applying the GnS-GII method, the DNA yield and purity being suitable for consecutive analyses. The highest DNA yield was obtained through the SP method, being almost two-fold higher than the yield obtained from the GnS-GII, although the purity was the lowest. Taking into consideration the higher DNA yield and proportionately equal purity when compared with the GnS-GII method, the authors concluded that the S method could be a great alternative when studying humic and clay soils (Tanase et al. 2015). The GnS-GII and ISOm methods were compared with the Power Lyzer™ PowerSoil® DNA Isolation Kit (MoBio Laboratories, Carlsbad, California) to assess their capacity to extract the protist DNA from grassland and arable soil samples. Although the GnS-GII and the ISOm had good yields of extracted DNA, the MoBio isolation kit had the best yield and purity, with reasonable cell-breaking capability and great abundance recovery ability, aspects important for describing the small fraction of soil protists, an important component of the microbiome (Santos et al. 2015).

In a very recent study, researchers compared the extraction capacity of five different kits for isolating DNA from soil samples taken from a pasture, an arable field, a dry healthy soil, and one collected from woodland. Some of the main differences between the samples was the pH of the environment along with the organic composition from the substrate. The authors compared the kits based on the characteristics of the extracted DNA: yield, purity, integrity, the impact on the read length based on the contrast between DNA length and read length, taxonomic classification rates based on DIAMOND aligned reads, and the effect of soil composition on the last-mentioned aspects. The analysis of the tested kits is summarized in Table 2.

Table 2. Comparison between different soil DNA extraction kits. The analyzed samples were representative of a pasture and arable field (neutral soil), dry healthy soil and woodland soil (acidic soil). Comparison between these kits could be interpreted from the graphical descriptors: ↑ - the best results, ● – average results, ↓ - the least favorable results (Child et al. 2024 Preprint)

Kit name	DNA yield	DNA purity	DNA integrity	Average DNA length	Average read length	Decrease in average read length	Impact on taxonomic classif.
FastDNA™ SPIN Kit for Soil	●	↓	↑	↑	↓	High decrease	●
SPINeasy® DNA Pro Kit for Soil	●	●	↑	↑	↑	High decrease	↑
MagBeads FastDNA™ Kit for Soil	●	↓	↑	↑	↓	High decrease	●
DNeasy® PowerSoil® Pro Kit	↑	↑	↑	↓	↑	Low decrease	↑
Zymo Research Quick-DNA Fecal/Soil Microbe MiniPrepTM Kit	↓	●	●	↓	●	Low decrease	↑

Based on their assessment, the authors determined that the optimal DNA extraction kit for soil samples is the DNeasy® PowerSoil® Pro Kit (Qiagen, UK), given its superior DNA yield, purity, and integrity. The decrease in read length that seems to normalize the performance of other kits with relatively average scores in the mentioned aspects is low for the DNA extracted

using this kit. When it comes to the fungal communities, the Zymo Research Quick-DNA Fecal/Soil Microbe MiniPrepTM Kit (Cambridge Bioscience, UK) showed the lowest percentage of reads whereas the other kits closely followed with higher number of reads, with FastDNA™ SPIN Kit (MP BioMedicals, UK) for Soil, and MagBeads FastDNA™ Kit for Soil (MP BioMedicals, UK) leading the ranking. Average results could be seen for the SPINeasy® DNA Pro Kit for Soil (MP BioMedicals, UK), with an average yield and a high decrease in read length (Child et al. 2024 Preprint).

Sequencing technologies and their impact in revealing the soil microbial dark matter

The concept of microbial ecology is described by the relationship that forms inside a microbial community and outside of it, in regard to the interaction of the microbiota with the environment. Revealing the phylogenetic diversity of a sample can be tackled through metataxonomic or metabarcoding approaches, and uncovering the complex associations from soil samples has witnessed remarkable progress with the advent of metagenomics. By directly studying the genetic material of a microbial community with the aid of cutting-edge next generation sequencing (NGS) technologies and continuously evolving bioinformatic pipelines, the field of metagenomics has seen a great development in the last decade. The great advantage that metagenomics offers in uncovering the complexity of the microbiome resides in the ability to study the unculturable fraction of the microbial population, the soil' microbial dark matter.
The history of microbiome studies encompasses multiple time-stamps, all overlapping on the evolution of sequencing technologies. In the early days, the pioneering technology used to describe microbial communities was Sanger sequencing. At the time, newly described phylogenetic markers, mainly ribosomal genes, were sequenced, making possible the discovery of microbial diversity from different samples. This approach has later been termed as metataxonomics. Sanger sequencing technology implies the use of terminator nucleotides, yielding a maximum of 96 reads averaging 650 base pairs per run. The emergence of the high throughput, parallel sample sequencing technologies of the second generation achieved greater sample yields at lower costs than the first sequencing generation. Four technologies contoured this period, with Illumina sequencing passing the test of time. The first technology employed was the 454-sequencing platform. This determined the nucleotide sequence through the detection of a signal obtained in the DNA polymerization reaction. The luminous signal was determined by the released pyrophosphate. Compared with Sanger sequencing, the advantage of this technology was represented by higher yields at lower prices, but with shorter reads averaging 250 nucleotides. Reads determined with the G5 FLX Pyrosequencer could be used to assemble small genomes, such as bacterial and viral ones. This is mainly due to the quality and the contiguity of genomic data (Nikolaki and Tsiamis 2013). The primary drawbacks identified in the quality of the sequences obtained were the inaccurate insertions and deletions determined by long homopolymeric regions. Acquired by Roche in 2007, the pyrosequencing technology can't be used anymore as the related reagents and platforms were discontinued less than a decade ago (Escobar-Zepeda et al. 2015). Formerly known as Solexa, the Illumina platforms were the second to emerge. Employing dye-labelled reversible terminators in DNA polymerization though bridge-PCR on a glass surface, this technology is feasible for shotgun metagenomics for the high throughput and high quality. Even though the small read lengths (<150 nucleotides) seem to constitute a drawback, the error rates less than 1% and the small running costs along with the advanced bioinformatic tools developed to process the reads conquered the field (Quince et al. 2017). Another short-read sequence technologies that have been developed in the last two decades are represented by the SOLiD platform that uses the ligation of fluorescently labelled di-base probes and the Ion Torrent platform that detects the signal emitted by the protons released during DNA polymerization. The error rates of these two

technologies range from <0.06% to <1.78% for the Ion Torrent platform. The output yields and the running costs are not comparable to the Illumina platforms, this being two of the reasons Illumina gained popularity (Nikolaki and Tsiamis 2013, Escobar-Zepeda et al. 2015).

The principal challenge associated with the fragment sequences obtained through short-read sequencing is represented by the accurate assembly of genomes, as the coverage of the sequence fails to accurately represent the genome. The third generation of sequencing technologies highlights the significance of the long reads, which enables the sequencing of whole genomes. Pacific Biosciences (PacBio) and Oxford Nanopore Technologies (ONT) developed sequencing platforms employing new sequencing procedures. PacBio platforms are characterized by the single molecule real time (SMRT) sequencing technology. Recent advancements employ a circularized DNA strand with hairpin adapters that act as primers for a polymerase. Upon binding to the polymerase, the DNA is loaded in a chamber termed zero-mode waveguide. As the polymerase incorporates fluorescently labelled nucleotides, a distinct signal is detected, allowing to differentiation of the nucleotide sequence. ONT platforms employ a fixed nanopore that allows a single strand of DNA or RNA to pass through it. As the nucleotide strand translocates the nanopore, the ionic flow is altered, with variations in the recorded charges translating into the nucleotide sequence. These new principles output sequences averaging in lengths over 10 kilobases. This represents the first advantage of long read sequencing over short read sequencing: the ability to generate fewer reads with wider coverage. Another advantage is the identification of structural variants along with assessing epigenetic modifications. Comparable to sequencing short DNA fragments, the sequencing of lengthy DNA strands represents a challenge for the third generation of sequencers concerning error rates. Recent advancements within these technologies has seen great progress with increasingly higher sequencing accuracies (Kim et al. 2024).

As sequencing technologies evolved, a series of advantages and drawbacks have emerged when uncovering the soil microbiome. NGS technologies facilitate a thorough evaluation of the microbial diversity, advancements in these technologies allowing the identification of novel microbial taxa, as well as uncovering the unculturable fraction of the microbiome through deep sequencing of environmental samples. By directly sequencing the microbial community from an environmental sample, NGS bypasses the need for culturing microorganisms such as bacteria and fungi. Through the application of whole genome sequencing, previously unidentified genes and biochemical pathways have been uncovered. Sequencing technologies such as ONT make gene expression analysis possible, as this method is feasible for RNA sequencing. As NGS platforms continue to evolve, tackling the microbiome becomes more affordable, accompanied by a reduction in sequencing time. On the other hand, due to the high yields and huge volumes of data generated, costly computational resources are required to analyze the metagenomic data, as well as bioinformatic pipelines, and basic programing expertise. Sequence quality might be subjected to artifacts associated with error rates of sequencing platforms or the extraction methods used. When multiple samples are multiplexed, a metagenomic study might be influenced by the batch effect which can negatively impact the analytical outcomes. The discovery of new microorganism may also be impacted by the lack of reference genomes in public databases (Garg et al. 2024). Two frequently used sequencing platforms in metagenomics, namely Illumina and ONT, although they facilitate the comprehensive characterization of microbial communities, they exhibit differences in accuracy and taxonomic resolution. More specifically, Illumina MiSeq provides superior accuracy as the MinION sequencer offers longer reads at a lower initial cost (Stevens et al. 2023).

Recent studies have introduced innovative approaches like culturomics-based metagenomics, aimed to enhance the recovery of both taxonomic and functional diversity in desert soils, capturing previously missed diversity and enabling the identification of novel bacterial candidates. The culturomics-based metagenomics approach combines the cultivation of the

samples under multiple culture conditions, followed by 16S amplicon sequencing and shotgun sequencing. This approach resulted in an increase in the number of amplicon sequence variants (ASVs) and qualitatively metagenome-assembled genomes (MAGs). Despite this, the relative abundance and the functional pathways present in the *in situ* environment have not been properly represented. The integration of multi-omics approaches in metagenomic studies represents a promising future approach in recovering the untapped microbial dark matter. (Li et al. 2023).

Altogether, metagenomic analyses managed to uncover a big piece of the soil microbiome puzzle, but the whole picture is not even half complete. Even though databases were populated with sequences of new taxa obtained at reasonable costs, advances in the field are still sought as the interaction network of these microorganisms could describe the applicative potential of the microbiome. A metagenomic analysis cannot describe by itself all the particularities of the functionally active community as the sequenced DNA is composed out of relic DNA of dead or metabolically inactive species or by DNA trapped in biofilms. In dependence to external factors, such as climatic conditions following season's change, varying nutrient quantities and even the spatial separation in soil aggregates, metagenomics miss on the metabolic versatility of the microbiome dependent on the exterior and the microbiome's interactions within- and outside of it. By cumulating and contextualizing the genomic data with those obtained from metatranscriptomic, metaproteomic, metabolomic analyses, as well as the effect of the external-conditioning factors, obtaining a more comprehensive description of the microbiome's potential could be attained. Metatranscriptomics describes the activity of the microbial community and their adaptations while metaproteomics includes the post-translational modifications of proteins which could aid in the discovery of novel species when compared to genome bins. However, modelling detailed interaction networks of the soil microorganisms in regard to their active metabolic pathways which include signaling metabolites or synergic/agonistic interactions between the members of the community is what the future hopes to hold in its approaches (Jansson and Hofmockel 2018). Specifically, culturomics approaches could aid in describing species interactions with a higher resolution but are hindered by the lack of growth particularities (Liu et al. 2022).

This way, multiple approaches are being taken at the moment to characterize the interaction network of the soil microbiome. Research on the mangrove sediments to assess the microbial community assembly using a genome scale metabolic modelling-based approach and network analysis from MAGs and metatranscriptomic data concluded that over half of the assembled species had a high potential of metabolic interactions. Still, from the entire community taken into study, over 98% of the microorganism pairs were not seen to interact with one another through sharing metabolites. However, five small groups of microorganisms were seen to interact divergently into successfully carrying out metabolic functions (Du et al. 2022). Adaptation to drought stress response on the rhizosphere microbiome was studied using MAGs and metatranscriptomic data for an agricultural site. Researchers observed that in drought conditions, the microbiome is enriched in bacterial groups such as *Actinobacteria*, possessing traits for carbohydrate metabolism and iron transport. When disrupting the iron homeostasis, the drought adapted microbes were affected, and in turn, the plant's ability to withstand the stress as well (Xu et al. 2021).

From amplicon sequencing to metagenome-assembled genomes (MAGs)

Studying the genetic material from environmental samples has taken different approaches along the way, as briefly described in the last section. The terminology used in the field tries to differentiate the genome microbiome studies, taking into consideration both the sequencing material and intended outcome. In this context, the concepts of metataxonomics (which

involves the sequencing of phylogenetic markers) and metagenomics (which involves the sequencing of the whole genetic material of a sample) describe two independent approaches to take in studying environmental samples. Owing to their highly conserved and hypervariable regions, phylogenetic marker-based taxonomy became the easiest way to classify organisms, achieved by amplifying shorter regions of these genes, and subsequently sequencing these amplicons. This approach has been known in the field as amplicon sequencing. On the other hand, shotgun or long-read sequencing of an environmental sample describe an authentic metagenomics approach, the whole DNA content of a sample being taken into consideration. The main advantages of sequencing the whole genome lie in its capacity to uncover new functional genes, metabolisms and obtaining draft genomes of uncultured organisms, which encompass members of the microbial dark matter - element that cannot be achieved at the same scale by sequencing taxonomic markers. Additionally, another great advantage of sequencing the whole genome is avoiding the PCR biases that might appear when amplifying marker genes (Pérez-Cobas et al. 2020, Nam et al. 2023). Metagenomics data is processed into metagenome-assembled genomes (MAGs), a further refinement of metagenomic approaches. The reconstruction of MAGs has aided for the uncovering of bacterial diversity, especially discovering the microbial dark matter (Quince et al. 2017).

Amplicon sequencing takes into consideration the sequences of targeted amplified phylogenetic markers. 16S rRNA is specific for the identification of prokaryotes and identifying eukaryotes has resided in the sequencing of 18S, 26S or ITS. These marker genes are characterized by hypervariable regions that allow the classification of taxons down to species level (Pérez-Cobas et al. 2020, Nam et al. 2023).

NGS made available the evolution of metagenomics by making possible the description of the full diversity of complex microbial communities through deep-sequencing. This transition provides a more comprehensive understanding of the microbiome. Metagenomic data is processed in the scope of constructing a representative picture through the MAGs. A basic bioinformatic pipeline for the construction and analysis of MAGs consists of quality control of sequenced reads, genome reconstruction through assembly and binning, high-resolution taxonomic and functional prediction, and data visualization. A vast array of bioinformatic tools and databases currently used in genomic reconstruction and following analysis have been reviewed by Wajid et al. (2022). Even though the computational resources needed to conduct such analyses are considerably pricey, they provide greater insight into the complete picture of a microbiome (Nam et al. 2023).

Large-scale excavation efforts have reconstructed metagenome-assembled genome bins, revealing a vast number of unknown species-level genome bins that significantly expand the microbial diversity and functional landscape of the soil. Ma and colleagues (2023) tackled the soil's microbial dark matter from 3304 metagenome data. After reconstructing over 40,000 metagenome-assembled genome bins, they identified 21,077 species-level genome bins, out of which, almost 80% were unidentified species-level genome bins. The authors identified many unknown genes that need further analysis, as well as a great number of potential biosynthetic gene clusters that might code for useful secondary metabolites. Associations between viruses and hosts was described by a numerous range of viruses that infect different bacterial hosts, with prophages taken as the best predictor of these associations. Last but not least, they analyzed the "immune system" of the microbial community, discovering over 8500 CRISPR-Cas genes, the soil microbiome portraying a large resource of Cas proteins (Ma et al. 2023).

In another study, Singh et al. (2023) constructed MAGs from the International Space Station, their analysis revealing insights into microbial metabolic and antimicrobial potential, as well as the network interactions within the community. By undertaking a metagenome-to-phenome approach, two bacterial and one fungal novel species were also discovered. The authors conclude that the reconstructed genomes contribute to our understanding of microbial life in

microgravity and low-dose irradiation when compared to the microorganism's evolution on Earth (Singh et al. 2023).

Conclusions

The soil microbiome is a central component of the environment, maintaining ecosystem functions and supporting agricultural productivity. Composed in big part by bacteria and fungi, the varying abundance and diversity of species characterizes different soil types and supports numerous soil functions such as carbon sequestration, organic matter decomposition, nutrient cycling, bioremediation, aggregate formation, pest and disease control along with many others. The soil microbiome's composition and functions are dependent to numerous factors, with agricultural practices, such as excessive tillage, use of pesticides and mineral fertilizers, significantly influencing and potentially disrupting these microbial communities. Observing this interdependence, sustainable agricultural practices are created and started being implemented in the field, aiming to preserve the health of the soil and quality of food, as a final objective. The development that the field of metagenomics has seen in the last years shows promising future approaches for describing the complexity of the microbiome, as well as identifying novel microbial species and new metabolic pathways with application in medicine and biotechnology. Soil microbial dark matter presents as a huge reservoir of such pathways that have not been described and new metabolites that might have bioactive potential (Ma et al. 2023). Rise in antimicrobial resistance genes determined by wastewater containing antibiotics or animal waste is due to increase because of the global demand for food production and pollution. Although the ecological stress leads to formation in soil bacteria of compounds similar to antimicrobials, their discovery is still much slower than the emergence of resistance phenotypes (Brevik et al. 2020). At the same time, discovery of novel antimicrobials, probiotics, biocontrol agents is hampered by the incapacity to cultivate many of these microorganisms (Fierer 2017, Liu et al. 2022). To understand the functional potential of interesting but yet-uncultured microorganisms, developing cultivating methods to isolate these species is a priority now. -omics data helped in broadening the knowledge about new microorganisms and their theoretic potential, but they cannot confirm that what is gene-encoded also functions as hypothesized. Thus, culturing microorganisms overpowers metagenomic analysis as it facilitates the study of biochemical and physiological traits under different, but controlled growth conditions. Advancements in using metagenomic data are made for selective isolation and cultivation; where possible, growth traits are deduced. However, metagenomic data quality still relies on DNA extraction methods, element that can represent a drawback in deducting such traits (Liu et al. 2022).

Efficient DNA extraction methods remain decisive for accurate downstream microbial analysis by sequencing. Comparison between different nucleic acid extraction methods, either applied from commercially available kits or lab developed has been an intriguing subject of discussion. Soil contains many inorganic and organic substances, along with PCR-inhibitors that can affect the extraction process and downstream metagenomic analyses. Thus, because of soil's nature, extracted DNA yield and quality vary, even when using the same method. Even though the metagenomics of the soil becomes more and more of interest in the actual context of trying to conserve biodiversity and acquire food security, integrating a cost-effective method is even harder. Lab-developed protocols appear to achieve the results of the DNA extraction kits but their efficacy and bias has not been properly described in the majority of cases by using an extraction control made out of a known quantity of bacterial cells.

Combined short- and long- read sequencing approaches or culturomics based metagenomics are just some of the latest procedures used to obtain metagenome-assembled genomes of a high quality. Along with the continuously updating bioinformatic pipelines and databases which

hope to facilitate new discoveries through data mining, the field of soil metagenomics promises notable discoveries in agriculture, pharmacology, ecosystem preservation – being valuable for the OneHealth concept.

Code availability: The Python (v3.12.4) script used for mapping the coordinates on the Romania map can be accessed at the following address: https://github.com/AndaMM/Map_coordinates_in_Ro.

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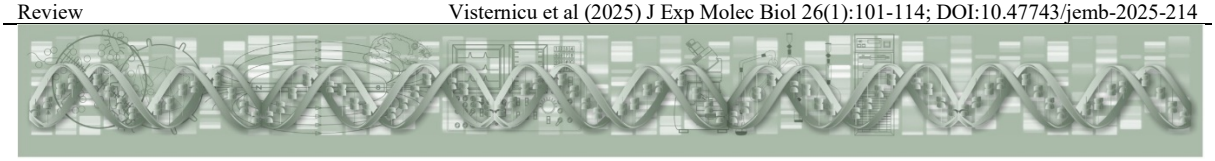

EVALUATION OF THE THERAPEUTIC EFFECTS OF *SALVIA ROSMARINUS* ON NERVOUS SYSTEM DISORDERS

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Abstract

Rosemary (*Salvia rosmarinus* Spenn) is an aromatic plant that has been used for centuries in traditional medicine for its remarkable therapeutic properties. Rosemary contains bioactive compounds with antioxidant, anti-inflammatory, and neuroprotective actions, and it is recognized for its beneficial effects on mental and cognitive health. This review explores the therapeutic potential of rosemary in alleviating nervous system disorders such as Alzheimer's disease (AD), major depressive disorder (MDD), epilepsy, Parkinson's disease (PD), addiction, and attention-deficit hyperactivity disorder (ADHD). Preclinical and clinical research suggests that rosemary extracts, through their active compounds, may enhance cognitive function, protect neurons from oxidative stress, and modulate neurotransmitters involved in cognitive and emotional processes. The findings indicate that rosemary could be a promising complementary therapy in managing nervous system disorders, offering significant benefits for mental and neurological health. However, further studies are needed to fully understand the efficacy and safety of long-term use.

Keywords: *Salvia rosmarinus*, nervous system disorders, neuroprotective effect, memory, ADHD, Alzheimer, Parkinson, epilepsy MDD

Introduction

Nervous system disorders, which involve abnormalities in the functioning or structure of the central or peripheral nervous system (Rahbardar and Hosseinzadeh 2020), have become a major public health issue, contributing to increased morbidity and mortality rates (Teixeira 2024). In recent years, researchers have increasingly focused on medicinal plants, recognized as natural sources for treating various conditions. These plants provide a renewable source of compounds, offering an almost unlimited array of new and complex chemical structures (Andrade et al. 2018). In this context, rosemary, a well-known medicinal plant, is widely used for improving memory deficits and neurodegenerative disorders (Oresanya and Orhan 2024).

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Salvia rosmarinus, an aromatic plant with thin, needle-like leaves, belongs to the Lamiaceae family (de Macedo et al. 2020), known for its multiple therapeutic properties (De Oliveira et al. 2019). Although originally from the Mediterranean region (Rahbardar and Hosseinzadeh 2020), rosemary is found worldwide (De Oliveira et al. 2019). Rosemary leaves are commonly used as a culinary spice, but the plant also has a long history of medicinal use. In traditional medicine, it has been valued for its stimulating and mild analgesic effects, being used to alleviate headaches, improve circulation, reduce inflammation, and combat physical and mental fatigue (Rašković et al. 2014). In folk medicine, it was used as an analgesic, antispasmodic, and treatment for migraines, emotional disorders, depression, and insomnia (Rahbardar and Hosseinzadeh 2020). Due to its antimicrobial (Andrade et al. 2018), anti-inflammatory, antioxidant, anticancer (Li Pomi et al. 2023), and neuroprotective (Faridzadeh et al. 2022) properties, rosemary has become the subject of extensive research, with numerous studies confirming its benefits (Singleman and Holtzman 2014; Rahbardar and Hosseinzadeh 2020).
This study aims to evaluate the therapeutic effects of *Salvia rosmarinus* on nervous system disorders, focusing particularly on AD, MDD, epilepsy, PD, addiction, and ADHD. The study will identify the mechanisms through which the active compounds in rosemary influence cognitive function, reduce oxidative stress at the neuronal level, and regulate neurotransmitters involved in order to evaluate the effectiveness of the plant as a complementary therapy in managing these disorders.

Materials and Methods

This review is based on scientific articles accessed from recognized online databases. Articles written in English were included, using relevant keywords to facilitate the searches. The papers used for this analysis come from the period between 2009 and 2024, and the databases used were: PubMed, Google Scholar, and ScienceDirect. To select and correlate information from the numerous articles found, keywords related to the health benefits of rosemary were used, with a special focus on its positive effects on nervous system disorders. Following the bibliographic analysis, we found that scientific literature uses several names for rosemary, including *'Salvia rosmarinus*,' *'Rosmarinus officinalis*,' and 'rosemary.' These terms are commonly found in scientific studies, peer-reviewed papers, and research, reflecting both linguistic variations and the historical and cultural context of studies on rosemary, highlighting its importance in botany, pharmacology, and gastronomy.
The term (rosemary [Title/Abstract] is the most frequently used and recognized in the scientific literature, with significant results in PUBMED (n=2876), GOOGLE SCHOLAR (n=19800), and SCIENCE DIRECT (n=18638), due to its versatility. Searches using (*Salvia rosmarinus* [Title/Abstract]) in PUBMED (n=1606), GOOGLE SCHOLAR (n=8320), and SCIENCE DIRECT (n=2155), and (*Rosmarinus officinalis* [Title/Abstract]) in PUBMED (n=1606), GOOGLE SCHOLAR (n=3840), and SCIENCE DIRECT (n=5646) generated relevant results (see Figure 1). This terminological diversity can influence how research and interpretations are conducted in studies, emphasizing the importance of considering all names when investigating specialized literature.

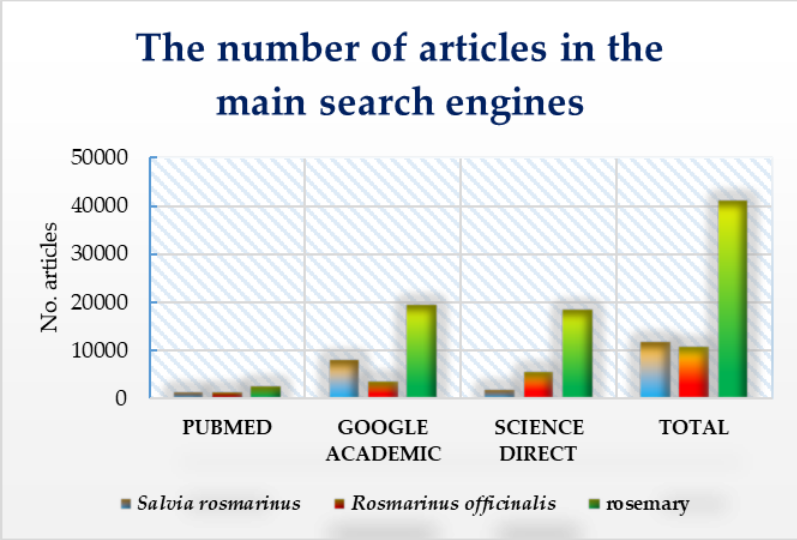

Figure 1. Graphical presentation of the total number of articles found using the main search engines

Results and discussions

To conduct a comprehensive and systematic search, we used the following keywords: ((*Salvia rosmarinus* [Title/Abstract]) AND (memory [Title/Abstract])) AND ((*Salvia rosmarinus* [Title/Abstract]) AND (nervous system disorders [Title/Abstract])) AND ((*Salvia rosmarinus*) [Title/Abstract]) AND (neuroprotective effect [Title/Abstract])) AND ((*Salvia rosmarinus* [Title/Abstract]) AND (ADHD [Title/Abstract])) and combinations thereof.
Inclusion criteria focused on studies published up to September 2024 in English that evaluate the health benefits of rosemary and its therapeutic effects on nervous system disorders, particularly Alzheimer's disease, major depressive disorder, epilepsy, Parkinson's disease, addiction, and attention-deficit hyperactivity disorder. The following exclusion criteria were applied: case reports, letters, abstracts, expert opinions and comments, conference abstracts, books, book chapters, unpublished results, and non-English papers.
Out of the 5.180 initial reports collected through electronic search, 54 articles were included. Due to the heterogeneity of the studies, narrative synthesis was deemed the most appropriate approach. The benefits of rosemary and its therapeutic effects on nervous system disorders were extensively addressed in numerous scientific articles accessed through specific searches. The results of these searches relate to the benefits of rosemary in various fields, particularly highlighting its impact on neurological health.

Medicinal properties of rosemary

Numerous phytochemical studies have demonstrated that the essential oils extracted from rosemary contain terpenoids, flavonoids, and alkaloids, which confer medicinal properties. The most active components identified include diterpenes, triterpenes, and phenolic acids, among which are rosmarinic acid, carnosic acid, rosmanol, carnosol, ursolic acid, and betulinic acid (De Oliveira et al. 2019; Rahbardar and Hosseinzadeh 2020). Triterpenic acids, such as ursolic, oleanolic, and micromeric acids have been shown to be the most effective in reducing inflammation. In addition to crude extracts, rosemary essential oil, which contains compounds such as β-pinene, 1,8-cineole, borneol, camphor, limonene, and verbenone, can be used for topical applications (Micić et al. 2021). The main compounds of rosemary essential oil are α-pinene (14.2–21.4%), 1,8-cineole (3.3–28.3%), and camphor (1.6–25.3%). Variations in the chemical composition of essential oil in different studies have been attributed to differences in

varieties, geographic origins, harvest seasons, environmental conditions, and sampling and extraction methods (de Macedo et al. 2020, Hashemi et al. 2023).

The pharmacological effects of rosemary include reducing inflammation, controlling high blood pressure, managing diabetes, alleviating bronchial asthma, treating peptic ulcers, preventing atherosclerosis, controlling hypercholesterolemia and oxidative stress, exhibiting antiviral action, reducing lipid peroxidation in the heart and brain, as well as combating physical and mental fatigue, in addition to lowering blood sugar levels (De Oliveira et al. 2019). Table 1 presents a detailed analysis of the general therapeutic effects of rosemary extracts and essential oils, including their specific compounds, highlighting their impact on health in various therapeutic contexts, and the neuroprotective power of rosemary will be included in Table 2. Rosemary essential oil is also recognized for its antibacterial activity, having the ability to inhibit the growth of pathogenic bacteria. According to research conducted by Saleh et al. (2022) and Hashemi et al. (2023), essential oil can be effectively used in health product formulations, playing a significant role in combating bacterial infections (Saleh et al. 2022; Hashemi et al. 2023). Additionally, rosemary essential oil exhibits antifungal activity, demonstrating increased sensitivity to fungal infections. This aspect extends its applicability in antifungal treatments (Neves et al. 2018).

Table 1. Pharmacological effects of phytocompounds from *Salvia rosmarinus* reported in the literature

Phytocompounds	***Pharmacological effect***	***Results***	***References***
Rosemary essential oil	Antibacterial activity	Inhibits the growth of bacteria	(Saleh et al. 2022, Hashemi et al. 2023)
	Antifungal activity	Inhibits the growth of fungi	(Neves et al. 2018)
	Wound healing	Accelerated wound healing in both diabetic and non-diabetic animals	(Umasankar et al. 2012)
Rosemary extract	Alopecia	A positive effect in hair growth	(Murata et al. 2013)
Caffeic acid	Antibacterial	Inhibits the growth of gram-negative and gram-positive bacteria	(Kim et al. 2018)
	Antioxidants	Reduces oxidative stress	(Liu et al. 2018)
Carnosic acid	Antitumor	Inhibits the growth of melanoma cells and significantly arrests the cell cycle.	(Lin et al. 2018)
Rosmarinic acid	Anti-inflammatory activity	Reduces biomarkers	(Patil et al. 2019)
		Significant reduction of skin lesions	(Lee et al. 2017)

Carnosic acid and carnosol	Antioxidant activity	Reduces cytochrome c and scavenge hydroxyl radicals	(Murata et al. 2013)
Rosmarinic acid	Anticarcinogenic (skin cancer) activity	Oral administration completely prevented the formation of skin tumors	(Khwaza et al. 2018)
Oleanolic acid	Antivirals	Prevented the entry of the virus by inhibiting the binding of the influenza virus hemagglutinin protein to host cells.	(Khwaza et al. 2018)
α-pinene	Antimicrobial	Antimicrobial capacity against tested species	(Ložienė et al. 2018)

Another remarkable benefit of rosemary essential oil is its ability to accelerate the wound healing process. A study has shown that this oil can improve wound healing in both diabetic animals, which are prone to complications, and non-diabetic animals (Umasankar et al. 2012). This property makes it valuable in regenerative medicine and dermatology. Rosemary extract has also been studied for its effects on hair growth, showing promising results in treatments for alopecia (Murata et al. 2013). This aspect makes rosemary an interesting choice in cosmetic and dermatological products. The extract used in these studies is a hydroalcoholic extract, which is known for its bioactive compounds beneficial in various cosmetic formulations. Caffeic acid also has strong antibacterial effects, inhibiting the growth of both gram-negative and gram-positive bacteria, according to research conducted by Kim et al. (2018). Additionally, caffeic acid is suitable for use in dietary supplements and health care products because it is an effective antioxidant that reduces oxidative stress (Kim et al. 2018, Liu et al. 2018).

Carnosic acid stands out for its antitumor properties, particularly its ability to inhibit the growth of melanoma cells, as highlighted by Lin et al. (2018). This activity positions it as a potential therapeutic agent in cancer treatments. Meanwhile, rosmarinic acid has been found to exhibit anti-inflammatory effects properties, reducing inflammatory biomarkers, suggesting its applicability in managing inflammatory conditions (Patil et al. 2019). Carnosol, another bioactive compound, aids in reducing of skin lesions, positively impacting skin health (Lee et al. 2017). Moreover, both carnosic acid and carnosol have demonstrated antioxidant activity, capable of reducing cytochrome c and capturing hydroxyl radicals, indicating a significant role in cellular protection (Aruoma et al. 1992).

According to Sharmila and Manoharan (2012), rosmarinic acid has anticancer effects, preventing the formation of skin tumors through oral administration. This suggests that rosmarinic acid could be a viable option in cancer prevention.

Finally, oleanolic acid exhibits antiviral properties, demonstrating the ability to prevent the influenza virus into host cells by inhibiting the binding of the hemagglutinin protein, according to the study by Khwaza et al. (2018). Furthermore, α-pinene exhibits significant antimicrobial properties against various species, suggesting its use in hygiene product formulations and natural preservatives (Ložienė et al. 2018). These findings underline the therapeutic potential of rosemary and its compounds, highlighting the need for further studies to explore its applications in medicine and cosmetology.

Moreover, rosemary has been traditionally used as an infusion in the treatment of several diseases, especially against neuropsychiatric disorders, including anxiety, depression, and cognitive disorders. Compounds in rosemary have been associated with reduced anxiety levels and alleviation of depressive symptoms, thus contributing to an overall sense of well-being. Additionally, the consumption of rosemary may improve memory and attention, potentially being useful in cases of dementia or AD. Its pleasant aroma may also help alleviate stress and headaches, such as migraines (Achour et al. 2022). Numerous studies confirm that rosemary has beneficial effects on memory, anxiety, depression, and insomnia. Memory can be improved through its inhibitory effect on acetylcholinesterase in the brain (Nematolahi et al. 2018).

Therapeutic effects of rosemary on nervous system disorders

Mental health issues are becoming increasingly prevalent worldwide, representing a significant challenge for society. In this context, various strategies have been developed to improve mental health, including a growing interest in scientific studies investigating the use of aromatic and medicinal plants as complementary and alternative treatment methods (Sasaki et al. 2013).

Alzheimer's disease is a complex condition characterized by interactions between genetic and environmental risk factors. The most common symptoms include memory loss, deterioration of speech function, and decline in intellectual abilities (Al-Tawarah et al. 2023). The main pathological characteristic of this disease consists of the progressive accumulation of beta-amyloid plaques (Aβ) and neurofibrillary tangles (Malik et al. 2022). The progression of cognitive decline in AD can also be influenced by other factors, such as neuroinflammation and oxidative stress, with the primary source of reactive oxygen species (ROS) being the electron transport chain in the inner mitochondrial membrane (Fernandes et al. 2022).

Although there is no effective remedy for this condition (Capatina et al. 2020), antidepressants used for AD patients fail to significantly alleviate symptoms (Malik et al. 2022). The most used medication for cognitive improvement is methylphenidate (Wenthur 2016). In recent years, natural compounds of plant origin have begun to be considered a promising alternative in the treatment of AD. The pharmacological properties of these compounds are due to their unique structures, allowing them to interact with key enzymes, receptors, antioxidant systems, transcription factors, and cytokines (Malik et al. 2022).

Recent studies suggest that rosemary has beneficial effects on AD, being rich in phenolic compounds and terpenoids that confer antioxidant, antidepressant, and anti-inflammatory activity (Ayaz et al. 2017, Malik et al. 2022). The efficacy of rosemary in the context of dementia and AD has been supported by in vivo research, such as that conducted by Ozarowski et al. (2013), which demonstrated that rosemary extract had a significant impact on long-term memory and cognitive responses in rats. This improvement was associated with the inhibition of acetylcholinesterase activity, an enzyme involved in the degradation of the neurotransmitter acetylcholine, and the stimulation of butyrylcholinesterase (BuChE) in the brains of the rats. These findings suggest that rosemary may play a crucial role in modulating cognitive function, with the potential to support treatments for neurodegenerative conditions (Ozarowski et al. 2013).

Major depressive disorder represents a severe mental health condition characterized by sleep disturbances, suicidal tendencies, lack of energy (Guo et al., 2018), depressed mood, and anxiety (Azizi et al. 2022). This multifactorial disorder is associated with changes in serum cytokine levels, and animal studies have highlighted a link between MDD and various inflammatory pathways, including the activation of tumor necrosis factor (Pferschy-Wenzig et al. 2022). Only one-third of patients with depression respond favorably to antidepressant treatments. This variability in clinical response, along with the risks of side effects and the slow onset of action, poses significant challenges in medical practice, prompting researchers to seek new alternatives for the treatment of depression (Azizi et al. 2022).

Carnosol, a principal active compound found in rosemary, is recognized for its strong antioxidant and anti-inflammatory properties. Due to these characteristics, carnosol has become a subject of interest in neuroprotective research (Faridzadeh et al. 2022). A study conducted by Kim et al. (2006) examined the protective effects of carnosol against rotenone-induced neurotoxicity, a chemical used to model PD. This neurotoxicity affects dopaminergic neurons, which are essential for the normal functioning of the nervous system (Kim et al. 2006).

Epilepsy is a neurological disorder characterized by chronic and persistent neuronal activity resulting from a reduced seizure threshold in the central nervous system (Ayaz et al. 2017). Overactivation of glutamate receptors causes seizures, which can lead to neuronal death. Glutamate plays an essential role in cognitive functions, including learning, memory, and synaptic plasticity; however, increased concentration and overactivation of its receptors contribute to neurodegeneration in the central nervous system (Rahbardar and Hosseinzadeh 2020). Approximately 20-30% of patients with epilepsy experience seizures that cannot be controlled by currently available medications (Schmidt 2009). Assorted studies have demonstrated the effectiveness of rosemary extract in treating induced seizures, highlighting that rosmarinic acid counteracts the effects of hypoxia and ischemia, improving mobility, spatial memory, and cognitive functions (Li et al. 2020, Faridzadeh et al. 2022).

Parkinson's disease is a neurological condition that causes difficulties in maintaining balance, walking, and coordination (Faridzadeh et al. 2022). This idiopathic degenerative disorder of the central nervous system primarily affects the elderly and is characterized by the degeneration of dopaminergic neurons in the substantia nigra of the brain, leading to motor symptoms such as tremors, rigidity, and bradykinesia. In advanced stages, cognitive problems often occur, frequently associated with dementia. The initial treatment for PD includes levodopa (L-DOPA) and dopamine agonists; however, their effectiveness decreases as the disease progresses, and long-term use can lead to motor complications, dyskinesia, and drug-induced toxicity (Stoker and Barker 2020).

The benefits of rosemary in the context of PD are underscored by the neuroprotective action of its bioactive compounds, such as carnosol, eugenol, and luteolin. Carnosol has demonstrated the ability to activate cellular signaling pathways, increase glutathione synthesis (a crucial antioxidant), and improve cellular viability by inhibiting apoptosis. Eugenol has had positive effects on neuronal viability and dopamine release, thus contributing to the alleviation of symptoms associated with PD (Kosmopoulou et al. 2024). Luteolin is associated with reducing oxidative stress and neurotoxicity, protecting neurons from damage. Additionally, compounds like 1,8-cineole and α-pinene have demonstrated antioxidant effects, contributing to neuronal protection by inhibiting the accumulation of ROS. Ursolic acid has also shown neuroprotective potential by improving mitochondrial function. Thus, rosemary may play a crucial role in preventing and managing PD by protecting neurons from damage and supporting brain health (Kosmopoulou et al. 2024).

Addiction is a psychological and physical condition in which a person develops a compulsive need to consume a certain substance or engage in a particular behavior, despite the negative effects it may have on their health and life in general (Heilig et al. 2021). In recent years, substance addiction, and alcohol consumption have become well-documented global issues (Krendl and Perry 2023), with recognition that vulnerability to dependence varies significantly from person to person. These individual differences can be attributed to both genetic and environmental factors, although the influence of each of these factors may vary (Nishizawa and Ikeda 2015). A study involving 81 patients demonstrated that rosemary (8-16 capsules per day, containing 300 mg of dried rosemary leaves) could be used as an herbal remedy to alleviate withdrawal symptoms in the treatment of opioid addiction, and in the case of other opioids as well (Solhi et al. 2013). Additionally, aromatherapy with rosemary oil may offer certain benefits in managing addiction, but it does not represent a complete solution for treating it.

Rosemary essential oil is recognized for its stimulating properties on the brain, its ability to enhance concentration, and its potential to improve well-being, which can indirectly help manage some symptoms associated with addiction (Skipper and Birkmayer 2018).

Attention-deficit/hyperactivity disorder is a commonly encountered neuropsychiatric condition that generates significant difficulties and dysfunctions throughout life. Recent research has provided new insights into the evolution of this disorder, identifying childhood risk factors that may influence the remission or persistence of ADHD in adulthood (López-Martín et al. 2024). However, despite advances in understanding the biological mechanisms of the disorder, the diagnosis of ADHD remains clinical, based on behavioral symptoms such as inattention, impulsivity, and hyperactivity (Zalsman and Shilton 2016; Leffa et al. 2022). One study assessed the effects of administering 75 mg/kg/day of rosemary for 4 weeks to young rats with rotenone-induced ADHD. Rotenone increased impulsivity, oxidative stress, inflammation, and apoptosis; however, rosemary mitigated these effects, improving locomotor activity, recognition index, and reducing oxidative stress and inflammation (Abdelrazik et al. 2023). Rosemary has been shown to improve cognitive performance in both healthy animals and those with cognitive deficiencies. This suggests a positive effect on memory and cognition, although results may vary depending on species, type of extract, and treatment duration.

Recent studies underscore the potential of rosemary in supporting cognitive and emotional health, also highlighting the importance of administering the correct dose to prevent adverse effects (Table 2). Nematolahi et al. (2018) analyzed the impact of administering rosemary extract to 68 students, with an average age of 22.9 years. Participants received a dose of 500 mg twice daily for one month. Rosemary powder, made from dried aerial parts that were encapsulated in 500 mg doses. The rosemary used had a total phenolic content of 20.1 ± 0.12 mg gallic acid/g dry weight. The results showed significant improvements in memory as well as a reduction in anxiety and depression, contributing to better sleep quality. These findings highlight the benefits of rosemary on cognitive and emotional health, particularly among young people (Nematolahi et al. 2018).

Additionally, Achour et al. (2021) investigated the effects of consuming rosemary tea by administering 5 g of dried rosemary infused in 100 ml of hot water daily for 10 days. The results showed promising anxiolytic and antidepressant effects, including an increase in BDNF levels, an important biomarker associated with depression (Achour et al. 2022). Furthermore, Filiptsova et al. (2017) conducted a study on the effects of sprayed rosemary essential oil, involving 53 adolescents aged 13 to 15 years. The results clearly demonstrated that aromatherapy with rosemary essential oil led to improvements in short-term memory (Filiptsova et al. 2017).

Table 2. Efficacy of rosemary in improving cognitive function and emotional state

Administration	*Participant*	*Age*	*Administration*	*Time*	*Results*	*References*
Rosemary	68	22.9 ± 1.7 years	500 mg 2 times/day	1 month	Stimulates memory	(Nematolahi et al. 2018)
Rosemary tea	22	20 - 50 years	5 g of dried rosemary in 100 ml of boiled water once/day	10 days	Reduces anxiety and depression and improves sleep quality	(Achour et al. 2022)
Rosemary essential oil was sprayed	53	13-15 years	-	-	Promising anxiolytic and/or antidepressa nt effects as	(Filiptsova et al. 2017)

					it increases BDNF levels	
Rosemary essential oil aromatherapy	28	86,1 ± 6,9 years	0.08 ml in the morning	28 days	Positive effect on short-term human memory	(Jimbo et al. 2009)
In the form of dried leaf powder	28	Mean age, 75 years	750 mg	7 days	Aromatherapy may have some potential to improve cognitive function, particularly in AD	(Pengelly et al. 2012)
			6000 mg	7 days	Statistically significant beneficial effect compared to placebo	
Rosemary leaves	81	20-50 years	8-16 capsules/day (300 g dry leaves)	14 days	Significant impact effect	(Solhi et al. 2013)

AD- Alzheimer's disease; BDNF - brain-derived neurotrophic factor

Another study conducted by Jimbo et al. (2010) examined the effects of aromatherapy with rosemary essential oil on cognitive function among 28 elderly participants. A dose of 0.08 ml of oil was administered daily in the morning for 28 days. The results indicated that aromatherapy could improve cognitive function, especially in patients with AD (Jimbo et al. 2009). Additionally, research conducted by Pengelly et al. (2012) investigated the effects of rosemary in the form of dried leaf powder on 28 elderly individuals with an average age of 75 years. Participants received 750 mg of rosemary in 7 days, and the results showed a significant beneficial effect on cognitive functions compared to a placebo group. In contrast, the administration of a higher dose of 6000 mg had a significantly negative impact on cognitive function, suggesting that excessive use of rosemary can be harmful (Pengelly et al. 2012).

Conclusions

Due to its remarkable properties, rosemary can be used in the treatment of the nervous system, including anxiety and depression. After analyzing the literature, it is concluded that rosemary has a significant potential to improve cognitive functions, demonstrating efficacy in healthy animals' models as well as those with cognitive impairments. Research suggests a positive impact of rosemary on memory and cognition, thus supporting its use as an adjunct in treatments for cognitive disorders, including AD. However, the available studies show considerable variability, indicating the need for further research to identify the specific factors influencing the efficacy of rosemary, considering the various doses, treatment durations, and species analyzed.

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