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Microbial Diversity of Bacterial Species (*Rhizobium and Azospirillum*) under Different Agroforestry Land Use Systems

Priya [a++], S.B. Agrawal [a#], Vishnu Solanki [a†], Ashish Kumar [a†], B.S. Dwivedi [a†], Ajay Kumar Shah [a‡*], Kamal Kishor Patel [a++] and Sanjay Singh Jatav [a++]

[a] College of Agriculture, JNKVV, Jabalpur, Madhya Pradesh, India.

Authors' contributions

This work was carried out in collaboration among all authors. Author Priya carried out the experimental during the research period, Author SBA given guidance for above investigation. Authors VS, AK and BSD supporting for laboratory guidance and valuable suggestion for the research after that authors AKS and KKP were supported in data collection, soil testing analysis and lot of moral supported for research. All authors read and approved the final manuscript.

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[++] *Ph.D. Scholar;*
[#] *Professor;*
[†] *Assistant Professor;*
[‡] *Guest Faculty;*
**Corresponding author: Email: shahajay.1209@hotmail.com;*

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ABSTRACT

This investigation is associated with the microbial diversity of different agroforestry systems. The study interpreted the changes of bacterial population during the duration of the experiments. This study was analyzed by CRBD (Complete Randomized Block Design) with 5 treatments (*i.e.* agroforestry systems) and 4 replications. The research was conducted on cropping systems at JNKVV, Jabalpur, and the Forest Research Farm during the Rabi season of 2021-22 and 2022-23. Soil collection was carried out at a depth of Rhizosphere soil (0 to 15 cm) in different agroforestry systems; later on, the soil was tested through the use of serial dilution methods. The bacterial population influence to the decomposition of leaf litter and straw material in the soil.The result revealed that the *Rhizobium* species population was found in maximum T_1-*D. sissoo*- wheat (66.28 and 66.96 X 10^7 cfu g^{-1}) in the respective years of 2021-22 and 2022-23, followed by the sequence of population of *Rhizobium* spp. had T_3 (*M. pinnata*-Wheat) > T_2 (*G. arborea*-Mustard) > T_5 (*M. indica*-linseed) > T_4 (*A. nilotica-wheat)* estimated under agroforestry systems. The *Azospirillum* species population increases year to year under the agroforestry system. the population sequence under agroforestry system had T_1 (*D. sissoo*-wheat) >T_3 (*M. pinnata*-wheat) > T_2 (*G. arborea*-mustard) > T_5 (*M. indica*-linseed) > T_4 (*A. nilotica-wheat)* obtained under agroforestry systems. The overall conclusion of this investigation is that the bacterial population is obtained under maximum in the *D. sissoo* with wheat-based agroforestry systems.

Keywords: Azospirillum spp; Rhizobium spp; agroforestry system; microbial etc.

ABBREVIATIONS

D. sissoo : Dalbergia sissoo
M. pinnata : Millettia pinnata
G. arborea : Gmelina arborea
A. nilotica : Acacia nilotica

1. INTRODUCTION

Soil microbial communities are the diverse communities of microorganisms that inhabit soil, including bacteria, fungi, algae, etc. These microbes play a vital role in soil health and function by influencing nutrient cycling, greenhouse gas emissions, and other processes. Soil microbes are essential for plant growth, nutrient mineralization, and the stability of agricultural systems. Various systems are included in the category of agroforestry in umbrella. Agroforestry systems include roadside planting systems, shelterbelts, pastures, and reforestation. In temperate zones, cropping systems have become increasingly popular because they can maintain or increase productivity while being more environmentally friendly than conventional agricultural systems (Foley et al., 2005). The microbial activity in soil help the decomposition of orgainc material in the soil (Hoorman and Islam, 2010). This investigation carried out due to estimation of population in agroforestry systems soil with different age of time. This spatial proximity of trees and crops allows for a variety of interspecific interactions in these systems, such as competition for resources and complementary (microbial activity) use of resources (Jose et al., 2004). Plant health and productivity and nutrient cycling are strongly influenced by soil organisms, particularly microorganisms (Berendsen et al., 2012). In recent decades, many soil organisms have been studied in temperate agroforestry systems, ranging from soil macro-fauna (Cardinael et al., 2019) against microorganisms (Guillot et al., 2021). Also the impact of agroforestry systems on soil fauna and their functions has been extensively studied recently (Marsden et al., 2020), Agroforestry systems soil microbial populace sizes, and that this useful impact can expand steadily into the crop rows (Jaramillo et al., 2013; Sridhar & Bagyaraj, 2017). Additionally, fungi can also additionally advantage greater than micro-organism, as numerous research indicated a boom with inside the fungi micro-organism ratio. Agroforestry structures that have to be taken into consideration while analyzing soil micro-organisms (Singh et al., 2023; Maurya et al., 2012; Coelho et al., 2025). This problem to find out the microbial biomes have in different agroforestry systems and it role play for the leaf litter decomposition problem in agroforestry systems based agriculture.

2. MATERIALS AND METHODS

The experimental trial was carried out in the agroforestry field of the Forestry Research Farm,

Department of Forestry, College of Agriculture, JNKVV, Jabalpur. These studies were conducted during *the Rabi* season of 2021-22 and 2022-23. The systems soil collected in surface soil (0 to 15 cm depth) during the investigation year used different land used systems. Tree age was found under different agroforestry systems, *i.e., D. sissoo* (24-25 years), *G. arborea* (6-7 years), *M. piñata* (14-15 years), *A. nilotica* (24-25 years), and M. indica (4-5 years). The soil sample was packed in tightly in polythene; after that, the estimation of bacterial population through serial dilution methods. The bacterial population was counted manually and multiplied with dilution factor after that, the statically analysis done by Gomez and Gomez (1984) with 5 treatments (i.e. Agroforestry field soil) and 4 replication.

2.1 Serial Dilution Method for *Rhizobium*

Soil samples were collected periodically for microbial study and processed for serial dilution by suspending 1 g of soil sample in 9 ml sterilized water in a test tube and shaking it thoroughly, which resulted in a 10^{-1} dilution. Subsequent serial dilutions were made up to 10^{-9} dilution for plating purposes.

2.1.1 Plating (pour plate method)

Plating in sterilised Petri plates was done by taking 1 ml each of 10^{-6} to 10^{-9} dilutions as required for rhizobia counts in rhizospheric soil treatment-wise. Plating was performed in triplicate for each dilution. The platings were done as per the composition of growth media for the respective microorganism, *viz.* YEMA (Rhizobium). The serial dilutions obtained from soil samples collected at the initial and harvest stages of the chickpea crop were used for plating, adopting the pour plate method because more surface area is covered as the sample is spread throughout the media than in other plate methods. Soil dilution (10^{-6} to 10^{-9}) aliquots of 1 ml were taken in a Petri plate; to this, 15 ml of the melted medium was poured within the aseptic environment of the laminar air flow chamber. After pouring the growth medium, plates were rotated gently clockwise and anticlockwise to mix the soil dilution with the medium. After solidification of the medium, the plates were incubated upside down at 27±2 °C for 3-6 days. The colonies were counted with specific growth characteristics (Table 1).

Table 1. Composition media used for population counts of *Rhizobium* and *Azospirillum* species

Reagents	YEMA (*Rhizobium*)	Azospirillum Spp.
$CaCO_3$	1 g	-
$MgSO_4.7\ H_2O$	0.2 g	-
$MgSO_4$	-	0.2 g
$MnSO_4$	-	-
$MnSO_4.7\ H_2O$	-	0.01 g
$CaCl_2$	-	0.02 g
KOH (for pH)	-	4.5 g
NH_4Cl	-	1.0 g
$FeSO_4$ $FeSO_47H_2O$	-	0.01 g
NaCl	0.1 g	0.1 g
KCl	-	-
$C_6H_{12}O_6$	-	-
Yeast Ext.	1 g	0.02 g
Agar	20 g	20 g
K_2HPO_4	0.5 g	0.5 g
Malic Acid	-	5.0 g
Mannitol	10 g	-
Bromothymol blue 5% solution	-	2.0 ml
pH	-	6.6-7.0
Distilled Water	1000 ml	1000 ml

2.2 Serial Dilation Method for *Azospirillum* spp.

A sequential method was used to estimate the soil microbial population. Soil samples were collected regularly according to the planned microbial research program. Serial dilutions were made by suspending 10 g of soil sample in 90 ml of sterile water in a bottle. The suspension was effectively disrupted, which was a ratio of 10^{-1}. The next series was increased to 10^{-1} to determine the target. Plating (pour plate methods): The aliquots of 1 ml each of 10^{-2} to 10^{-8} dilutions were poured into sterilised petri plates to determine the rhizobial population count in soil; each dilution was plated in triplicate. The composition of growth medium for respective microorganisms is tabulated in Table 1. The pour plate method was used to plate serial dilutions obtained from soil samples collected after the harvest of wheat, mustard, and linseed. In a petri plate, a 1 ml aliquot of soil dilution (10^{-2} to 10^{-8}) was taken, and 15 ml of sterilised melting medium was poured to evenly mix the soil dilution with the medium. Once the medium had solidified, the plates were incubated upside down at 28±2 ^{0}C for 3-7 days.

$$Viable\ Cells\ (Cfug^{-1}\ Soil) = \frac{Number\ of\ colonies}{Oven\ dry\ weight\ of\ soil\ (1g)} \times Dilution\ factor$$

3. RESULTS AND DISCUSSION

3.1 Population of *Rhizobium* spp

The data about the population of *Rhizobium spp. in* the surface soil (0 to 15 cm depth) during the investigation years 2021-22 and 2022-23 under different land use systems are presented in Table 2 and Fig. 1.

The population of *Rhizobium* spp. in the surface soil (0 to 15 cm depth) in 2021-22 ranged from 33.36 to 66.28 cfu g^{-1} with an average of 49.25. cfu g^{-1}. T_1- *D. sissoo*-wheat exhibited a significantly maximum response with 66.28, which was 50.33% more than that of T_4 – *A. nilotica*-wheat (33.36 cfu g^{-1}). This was followed by the response of T_3 - *M. Pinnata* wheat with 65.23 cfu g^{-1}.

The population of *Rhizobium* spp. in the surface soil (0 to 15 cm depth) at 2022-23 ranged from 34.08 to 66.96 with an average of 50.31 cfu g^{-1}. T_1- *D. sissoo*—wheat exhibited a significantly maximum response with 66.96 cfu g^{-1}. Which was 50.89% more than that of T_4 – *A. nilotica*-wheat (34.08 cfu g^{-1}). This was followed by the response of T_3 - *M. Pinnata* wheat (34.08 cfu g^{-1}).

On the other hand, pooled data shows a similar pattern followed. Where the maximum was found in T_1 (66.62 cfu g^{-1}), it was significantly superior to T_2 (42.07 cfu g^{-1}), T_3 (33.72 cfu g^{-1}), and T_5 (40.78 cfu g^{-1}) and at par with T_5 (65.72 cfu g^{-1}). Data on differences between years to year were found to be non-significantly different under different agroforestry systems. The data was similar to that noted by Marco et al., (2023), i.e., the diversity of root-nodulating rhizobia, morphology, and formation of legumes, and augmented by Janati et al. (2021); moreover, like Rhizobium and Bradyrhizobium, they are associated with leguminous plants.

3.2 Population of *Azospirillum spp* (*cfu g^{-1} soil)*

The data about the population of *Azospirillum spp. in* the surface soil (0 to 15 cm depth) during the investigation years 2021-22 and 2022-23 under different land use systems are presented in Table 3 and Fig. 2.

The population of *Azospirillum spp. in* the surface soil (0 to 15 cm depth) in 2021-22 ranged from 5.32 log cfu (2.08 x 10^5 cfu g^{-1}) to 9.38 log cfu (2.29 x 10^9 cfu g^{-1}) with an average of 7.40 log cfu (2.53 x 10^6 cfu g^{-1}). T_1- *D. sissoo*-wheat exhibited maximum response with 9.38 log cfu (2.39 x 10^9 cfu g^{-1}) which was 56.72% more than that of T_4 – *A. nilotica*-wheat 5.32 log cfu (2.08 x 10^5 cfu g^{-1}). This was followed by the response of T_3 - *M. pinnata* wheat with 8.16 log cfu (1.45 x 10^8 cfu g^{-1}).

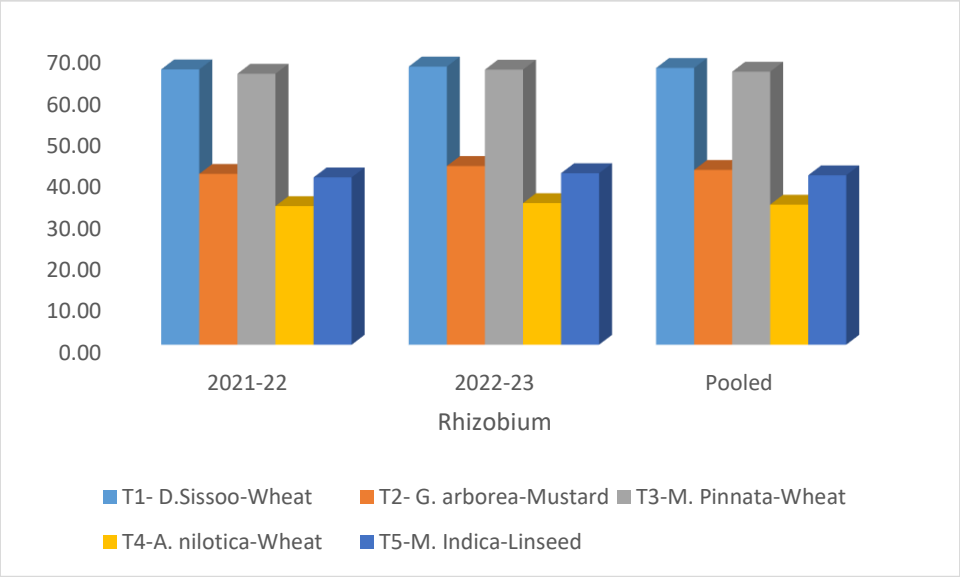

Fig. 1. Rhizobium spp (X 10^7 cfu g^{-1} soil) in rhizosphere soil under different land used systems

Table 2. Rhizobium spp (X 10 ^{7} cfu g^{-1} soil) in soil under different land used systems

Treatments	2021-22	2022-23	Pooled
T_1- *D.sissoo*-wheat	**66.28**	**66.96**	**66.62**
T_2- *G. arborea*-Mustard	41.12	43.02	42.07
T_3-*M.pinnata*-wheat	65.23	66.22	65.72
T_4-*A. nilotica*-wheat	**33.36**	**34.08**	**33.72**
T_5-*M. Indica*-linseed	40.27	41.28	40.78
Mean	**49.25**	**50.31**	**49.78**
Sem±	2.88	4.12	2.25
CD	10.70	15.34	8.83
SEm±(Year)	1.59	**SEm±(YXT)**	3.56
CD(Year)	6.24	**CD(YXT)**	13.96

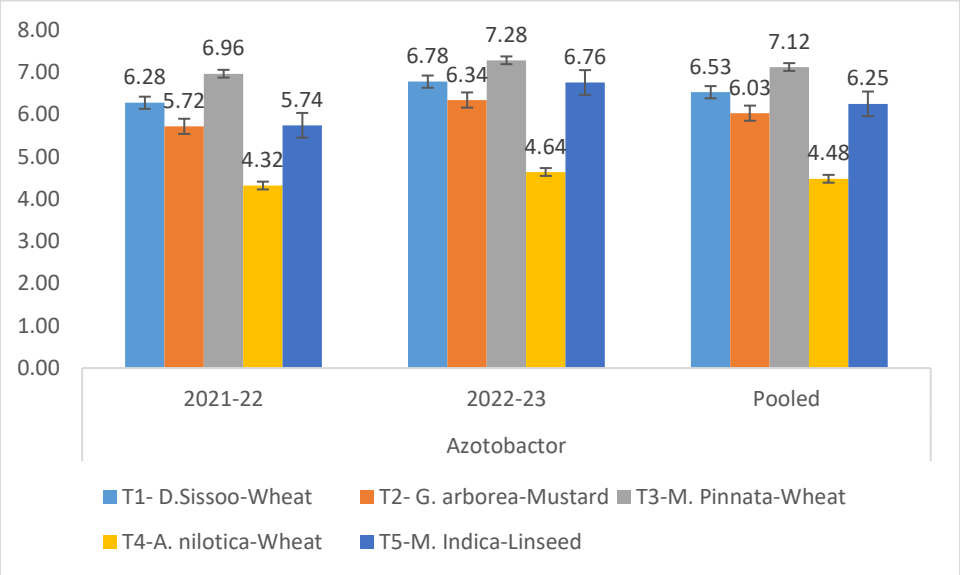

Fig. 2. *Azospirillum* spp (cfu g^{-1} soil) in rhizosphere soil under different land used systems

Table 3. *Azospirillum spp* (cfu g^{-1} soil) under different land used systems

Treatments	2021-22	2022-23	Pooled
T_1- *D.Sissoo*-Wheat	**9.38 (2.39X10^{9})**	**9.47 (2.97X10^{9})**	**9.42 (2.64X10^{9})**
T_2- *G. arborea*-Mustard	7.22 (1.65X10^{7})	7.84 (6.92X10^{7})	7.53 (3.38X10^{7})
T_3-*M. Pinnata*-Wheat	8.16 (1.45X10^{8})	8.48 (3.02X10^{8})	8.32 (2.10X10^{7})
T_4-*A. nilotica*-Wheat	**5.32 (2.08X10^{5})**	**5.64 (4.34X10^{5})**	**5.48 (3.00X10^{5})**
T_5-*M. Indica*-Linseed	6.94 (8.76X10^{6})	7.96 (9.02X10^{7})	5.48 (2.81X10^{7})
Mean	7.40 (2.53X10^{7})	7.88 (7.51X10^{7})	7.64 (4.36X10^{7})
Sem±	0.45	0.52	0.31
CD	1.66	1.95	1.21
SEm±(Year)	0.22	**SEm±(YXT)**	0.49
CD(Year)	0.85	**CD(YXT)**	1.91

The *Azospirillum spp.* population in the surface soil (0 to 15 cm depth) in 2022-23 ranged from 5.64 log cfu (4.34 x 10^{5} cfu g^{-1}) to 9.47 log cfu (2.97 x 10^{9} cfu g^{-1}) with an average of 7.88 log cfu (7.51 x 10^{7} cfu g^{-1}). T_1- *D. sissoo*-wheat exhibited maximum response with 9.47 log cfu (2.97 x 10^{9} cfu g^{-1}) which was 56.71% higher than that of T_4 – *A. nilotica*-wheat 5.64 log cfu (4.34 x 10^{5} cfu g^{-1}). This was followed by the response of T_3 - *M. Pinnata* wheat with 8.48 log cfu (3.02 x 10^{8} cfu g^{-1}).

The *Azospirillum spp.* population in the surface soil (0 to 15 cm depth) pooled in varied from 5.48

log cfu (2.81 x 10^7 cfu g^{-1}) to 9.42 log cfu (9.42 x 10^9 cfu g^{-1}) with an average of 7.64 log cfu (4.36 x 10^7 cfu g^{-1}). 2021-22 exhibited maximum response with 9.42 log cfu (2.64 x 10^9 cfu g^{-1}) which was 58.17% higher than that of T_4 – *A. nilotica*-wheat 5.48 log cfu (3.00 x 10^5 cfu g^{-1}). This was followed by the response of T_3 - *M. pinnata* wheat with 8.32 log cfu (2.10 x 10^7 cfu g^{-1}). The free-living bacteria include cyanobacteria (blue-green algae), *Azotobacter*, *Azolla, Azospirillum*, *Agrobacterium, Clostridium, Gluconobacter, Flavobacterium,* and *Herbaspirillum.* They are habitually associated with nonlegumes. Mott et al., 2007. *Azospirillum* species and Frankia are associated with cereal grasses and certain dicotyledonous species reported by Çetiz and Memon 2021; and Marco et al., 2023.

4. CONCLUSION

Thus, the agroforestry system increases the bacterial population (Azospirillum and Rhizobium spp.) with duration, but there is not a significant variation in the time of investigation between years. The result was estimated that the soil bacterial population in aspect to Rhizobium spp. was found in T_1-*D. sissoo*-wheat (tree crop combination)-based system, followed by T_3-*M. pinnata*-wheat-based system, and the list population estimated in rhizosphere soil T_4-*A. nilotica*-wheat. Furthermore, in the case of Azospirillum spp. populations, a similar trend was estimated in the 2021-22 and 2022-23 seasons, as well as in the statistically pooled analysis data. This investigation was more effective for the agroforestry research, those are crops taken with tree components.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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