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Assessment of Plant-Pathogenic Fungi in Anthill Soils

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ABSTRACT: This study aimed at assessing the presence of plant pathogenic fungi in soil samples collected from the immediate vicinities of anthill mounds and adjacent areas. Soil samples were collected from anthill mound and their corresponding adjacent soils, from four sites located in Ekosodin and Ugbowo respectively with both locations sited in Benin City, Nigeria. Enumeration, characterization and identification of fungal isolates were done using standard mycological methods. Selected physical and chemical properties of the soil samples such as total nitrogen and pH were evaluated using appropriate procedures. The expressed phenotypic pathogenicity of the fungal isolates was assessed using relevant techniques. There was a significant difference in the number of pathogenic fungi present in anthill soil when compared to adjacent soil. Fungal isolates observed in all soil samples included; *Penicillium citrinum, Trichoderma* sp. and *Penicillium chrysogenum.* All the soil samples were acidic with pH values ranging from 5.20 to 6.80 and all the soil samples were also sandy with % sand particle values varying from 91.00 to 96.00. Although anthill soil can be utilized as a soil amendment, there is a need to screen out pathogenic fungi from anthill soil in other to enhance its use as soil amendment.

Keywords: Food safety, Soil engineer, Soil microbiome, Soil security, Synthetic fertilizer

Introduction

Judicious soil management has been described as a key component of sustainable agriculture and is also known to offer a valuable lever for climate regulation as well as a route for protecting ecological services and processes in general (Yin *et al.*, 2022). In light of the observation that about 40% of the soils in Africa as well as approximately 33% of the universal soils are currently degraded (Oldeman, 1992, Gomiero, 2016), special attention focusing on the restoration of dilapidated soils and the conservation of soil health is crucial (Hou *et al.*, 2020). Soil fertility reduction is impacted by several factors such as cropping systems, irrational local and international incentive policies, biological and physical degradation, nutrient deficit, incorrect crop assortments, poverty, land dilapidation, pests, and diseases (Takele *et al.*, 2015; Javed *et al.*, 2022). Soil fertility reduction results in low crop production, output and starvation (Amoo and Babalola, 2019). To make up for soil fertility loss, many research studies have advised farmers to use soils engineered by bioturbators (Ezeaku *et al.*, 2015;Enagbonma *et al.*, 2021; Enagbonma and Babalola, 2022). These recommendations are based on the rich nutrients accrued in engineered soils (Enagbonma and Babalola, 2019; Apori *et al.*, 2020; Adebajo *et al.*, 2021). Lately, in Zambia (Chisanga *et al.*, 2019), Zimbabwe (Nyamangara and Nyagumbo, 2011) and Sierra Leone (Turay *et al.*, 2022), local farmers have been advised to grow crops on top of anthill soils. This recommendation when applied, will decrease the full dependence on synthetic pesticides or fertilizers by agriculturalists and then lessen the adverse effect of too much and persistent use of chemical fertilizers on the surroundings (Amoo and Babalola, 2019). While the use of anthill soil in sub-tropical and tropical regions is

gaining ground, it is pertinent to ascertain if utilizing anthill soil could be a way of introducing pathogenic organisms like fungi to plants.

Fungi are one of the principal causative agents of plant diseases (Chen *et al.*, 2019). To colonize plants and cause disease, pathogenic fungi use various tactics like deploying an excess of virulence factors or interfering with major plant defense (Sharma, 2021). Some fungi eradicate their hosts by secreting poisons to destroy plant tissue and feed on dead material, while others inhabit the living tissue therefore utilizing effector molecules to subdue plant cell death and influence plant metabolism in favor of the pathogen (Doehlemann *et al.*, 2017, Enagbonma *et al.*, 2023).

In their agroecological domains, plants are frequently confronted with fungal communications that influence pathogenic outcomes (Chauhan *et al.*, 2019). Such fungal encounters of host plants necessitate a significant reprogramming of the small RNAs (Chauhan *et al.*, 2019). Infections of plant tissues by pathogenic fungi can lead to yield losses as high as 50 to 75 % (Nazarov *et al.*, 2020). For instance, *Fusarium* species generally infect plants irrespective of developmental stage (Al Masri *et al.*, 2017). The growth of *Fusarium* and the accumulation of their mycotoxins can negatively impact grain quality (Goertz *et al.*, 2010). The intake of contaminated plant material causes a grave threat to animal and man health (Reddy *et al.*, 2010). To establish if utilizing anthill soil could be a way of introducing pathogenic organisms to plants, this study was developed to determine the types and numbers of pathogenic fungi present in anthill soils. This data can help to devise means to control the proliferation of potential plant-pathogenic fungi that could be present anthill soils, which would in turn avert the pollution of agricultural crops with detrimental mycotoxins.

Materials and methods

Study locations and soil sampling: Soil samples were collected from the anthill mounds and adjacent soils located at Ekosodin (Lat. 6° 23' 42″ North, Long. 5° 36' 49″ East) and Ugbowo (Lat. 6° 23' 45″ North, Long. 5° 36' 54″ East), Benin City, Edo State, Nigeria. A total of eight soil samples (i.e. four from Ekosodin (A1a–d) and four from Ugbowo (A2a–d) were collected from anthill soils at a depth of 0 to 15 cm, a depth where mainstream of ants and microbial activities are seen (Chisanga *et al.*, 2020) by using a soil coil. For comparative purposes, eight adjacent soil samples (i.e. four from Ekosodin (S1a–d) and four from Ugbowo (S2a–d)) which were 10 metres away from the anthills were collected at a depth of 0 to 15 cm. The 10 m distance between the anthill and adjacent soils was chosen because anthills were absent from these regions (Enagbonma *et al.*, 2021). The collected soil samples in the fields were preserved temporarily *via* cooler boxes filled with ice blocks and subsequently conveyed to the microbiological laboratory at the University of Benin that same day for the isolation of bioagents and physicochemical analysis.

Physical and chemical analysis of soil samples: The soil physical and chemical properties of the anthill and adjacent soils were estimated using standard analytical methods formerly described by Wakung'oli *et al.* (2020) and Enagbonma and Babalola (2022). The total nitrogen content was evaluated by using the Kjeldhal technique while the reading of the exchangeable potassium (K) was done with the flame photometer. The pH of the soil samples was measured with a pH meter in clean water in a 2.5:1 water: soil ratio. An atomic absorption spectrophotometer (AAS) was used in reading exchangeable calcium (Ca) and magnesium (Mg) found in the extracts (at pH 7.0) obtained from one mole of ammonium acetate. Available phosphorus (P) was measured with a spectrophotometer while organic carbon was evaluated using the method previously described by Okoduwa *et al.* (2022).

Preparation of potato dextrose agar (PDA), serial dilution and enumeration of fungi colony from the anthill and adjacent soil samples: PDA medium (39g) was dissolved in 1000ml of distilled water in a conical flask then closed with a cork stopper. The suspension was first dissolved completely by shaking and then sterilized by autoclaving at 121°C for 15 min. The medium was allowed to cool then dispensed aseptically into sterile petri dishes. The Petri dishes were thereafter covered and allowed to solidify. Ten thousand-fold serial dilution of the samples were prepared aseptically in sterile physiological saline. An aliquot of 0.1 ml was inoculated using the pour plating technique. Potato Dextrose Agar (PDA) was supplemented with chloramphenicol for fungi. Plates were cultured at 28 ± 2°C for 48 h. The number of colony forming unit per milliliter (cfu/g) was calculated based on the formula below that was previously used by Rodriguez-Tudela *et al.* (2008):

$$cfu/g = \frac{number\ of\ colonies\ x\ dilution\ fold/series}{volume\ of\ inoculum}$$

The colour and pigmentation of the fungal colonies present on the agar plates were observed after 48 hours. The shape, texture and size of the respective fungal colonies were also observed. Purification of unique discrete fungal cultures was attained *via* streak plate technique using freshly prepared PDA agar plates. Following successful pour plating, the inoculated PDA plates were incubated from 4 to 21 days at 28 ± 2°C. Then, the colonies that appeared on the medium were counted and the fungi were morphologically identified by observing their morphological features using the taxonomic literature (Dugan, 2006, Campbell and Johnson, 2013, Thiyam and Sharma, 2013, Nguyen *et al.*, 2023). Thereafter, pure cultures of fungal isolates were made from a single colony and characterized using cultural characteristics. According to the procedure outlined by Jeon and Ka (2015), the slide culture from pure cultured plates was created. Using a sterile inoculating wire loop, a smear of the isolate to be stained was prepared on a clean glass slide. Lactophenol blue was used to stain the smear, which was then viewed under a 40x microscope. On previously recognized cultivated PDA plates, the microscopic attributes of the respective fungal isolates were observed (Song *et al.*, 2019).

Pathogenicity tests on fungi isolates: The fungi isolated and identified with cultural, morphological characteristics *via* staining were further subjected to DNase and lipase tests to determine their pathogenic nature. To conduct the DNase test, 39 g of the DNase medium was initially dissolved in 1000 ml of distilled water in a conical flask and sealed with a cork. The suspension was thoroughly dissolved by shaking before being auto-cleaved at 121°C for 15 min and cooled to 45 °C to sterilize it. After allowing the medium to cool, it was aseptically poured into sterile Petri dishes. The test microorganism was added to DNase agar plate using a sterile loop, and the plates were then incubated at 37 °C for 48 h. The results were obtained by observing for DNAse hydrolysis around the streaked microbial colony on the agar plates; those that did were considered positive (+) and those that did not were negative (-). The lipase activity of the isolates was assayed on PDA plates and supplemented with 1 % Tween 80 (v/v). The density of this suspension was adjusted to 0.5 Mc Farland standard, which is the equivalent of 1.5×10^8 spores/ml. Samples were then incubated at 37 °C for 48 h after which the result was documented.

Statistical analysis: Analysis of data was carried out using Statistical package for social sciences (SPSS) package version 21.0, where the P values were determined by two-way analysis of variance (ANOVA), followed by the Duncan test. The replicated data were expressed as mean ± SD (standard deviation).

Results

Examination of nutrient properties from the anthill and adjacent soils: The assessment of the soil's physical and chemical properties indicated that the numerical values of P, Mg, OC, K, Ca, OM, TKM, and clay (excluding S2b and S2d) were higher in anthill soils when compared to the values obtained from adjacent soils. However, the amount of pH, sand, and silt in adjacent soil samples was higher than the values obtained from the anthill soils (Table 1).

Table 1: Soil properties assessment from anthill and adjacent soils

Property	A1a	A1b	A1c	A1d	S1a	S1b	S1c	S1d	A2a	A2b	A2c	A2d	S2a	S2b	S2c	S2d
pH	6.10	6.00	5.20	6.10	6.60	6.40	6.60	6.40	5.90	6.30	6.40	6.30	6.80	6.50	6.80	6.60
OC (%)	1.74	1.82	1.72	1.82	1.72	1.16	1.37	1.28	0.65	0.87	0.92	0.83	1.47	1.45	1.47	1.45
OM (%)	3.00	3.14	2.97	3.14	2.97	2.00	2.36	2.21	2.53	2.5	2.53	2.5	1.21	1.5	1.59	1.43
TKN (%)	2.16	1.78	1.56	2.38	1.22	1.14	1.22	1.78	2.13	2.36	2.13	2.36	1.28	1.94	1.22	1.17
P (mg/L)	23.15	48.78	22.63	48.78	22.63	20.27	28.17	31.46	36.54	43.11	36.54	43.11	18.63	9.54	14.93	17.62
Ca (mg/L)	0.35	0.32	0.27	0.32	0.27	0.25	0.22	0.18	0.28	0.37	0.28	0.37	0.12	0.18	0.13	0.14
K (mg/L)	6.32	6.12	5.63	6.12	5.63	5.48	4.36	5.27	5.18	5.23	5.18	5.23	3.11	3.28	2.87	2.93
Mg (mg/L)	0.97	0.88	0.97	0.94	0.95	0.78	0.82	0.78	0.93	0.82	0.93	0.93	0. 74	0.79	0.78	0.82
Sand (%)	94.00	92.00	95.00	93.00	96.00	93.00	96.00	96.00	94.00	91.00	93.00	92.00	95.00	92.00	94.00	95.00
Silt (%)	1.72	1.74	1.2	1.24	2.75	2.14	2.75	2.14	1.25	1.85	1.25	1.48	1.63	2.27	1.35	1.85
Clay (%)	4.28	6.26	3.8	2.76	1.25	4.86	1.25	4.86	3.37	6.73	5.68	3.52	4.75	6.15	4.75	6.15

A1 anthill from Ekosodin, A2 anthill from Ugbowo, S1 adjacent soils from Ekosodin, and S2 adjacent soils from Ugbowo, A1a–d and A2a–d represent anthill soils from Ekosodin and Ugbowo, correspondingly, whereas S1a – d and S2a – d represent adjacent soils from Ekosodin and Ugbowo, correspondingly.

Total fungal count and occurrence: In general, the average total fungal count (log10 (cfu/g) in anthill soils in both locations A1 and A2 were 12.50 and A2 = 10.00 respectively while the average total fungal count (log10 cfu/g) in adjacent soils were S1 = 39.75 and S2 = 5.50 respectively (Fig. 1). Fungal isolates cultured from the soil samples include; *Penicillium citrinum*, *Trichoderma* sp., and *Penicillium chrysogenum*. Meanwhile,

Fusarium sp. was only observed in anthill soils while *Aspergillus flavus* and *Aspergillus niger* were present only in adjacent soils (Table 2).

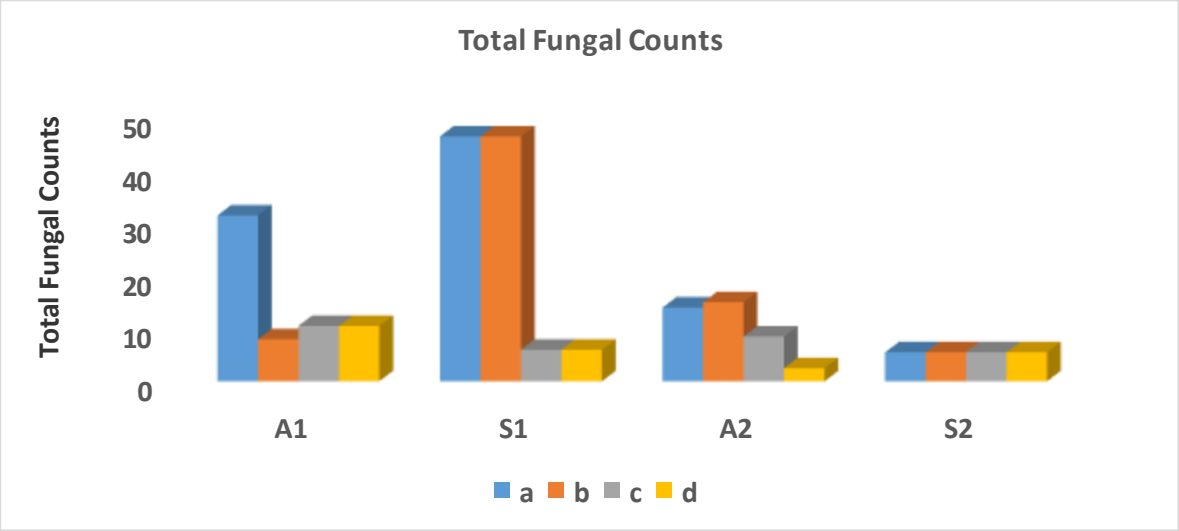

Fig. 1: Total fungal count in all soil samples. A1a–d and A2a–d represent anthill soils from Ekosodin and Ugbowo, correspondingly, whereas S1a – d and S2a – d represent adjacent soils from Ekosodin and Ugbowo, correspondingly.

Table 2: Fungal isolates observed from anthill and adjacent soil samples

Fungal	A1	S1	A2	S2
Aspergillus niger	-	+	-	+
Trichoderma sp	+	+	+	+
Penicillium chrysogenum	+	+	+	+
Fusarium sp	+	-	+	-
Penicillium citrinum	+	+	+	+
Aspergillus flavus	-	+	-	+

Keys: A1 anthill from Ekosodin, A2 anthill from Ugbowo, S1 adjacent soils from Ekosodin, and S2 adjacent soils from Ugbowo, + = present, - = absent

Pathogenicity test of fungi isolated from anthill and adjacent soils: The virulence attributes of the identified fungal isolates *via* lipase and DNAse test showed that *Trichoderma* sp., *Penicillium chrysogenum, Fusarium* sp. and *Penicillium citrinum* present in the anthill soils were positive (+) to lipase while *Penicillium chrysogenum* and *Penicillium citrinum* were negative (-) to DNase. Furthermore, fungal isolates recovered from the adjacent soil samples showed that *Trichoderma* sp. was positive to both lipase and DNAse test. It also revealed that *Penicillium chrysogenum* and *Penicillium citrinum* were only positive for lipase while *Aspergillus niger* and *Aspergillus flavus* were only positive for the DNase test (Table 3).

Table 3: Pathogenicity test of fungi isolates

Fungal isolates	Lipase	DNAse
Anthill soil		
Trichoderma sp	+	+
Penicillium chrysogenum	+	-
Fusarium sp	+	+
Penicillium citrinum	+	-
Adjacent soil		
Aspergillus niger	-	+
Trichoderma sp	+	+
Penicillium chrysogenum	+	-
Penicillium citrinum	+	-
Aspergillus flavus	-	+

Keys: Lipase: + = secretes lipase: virulence effects; -- = does not secrete lipase: no virulence effects.
DNAse: + = degrades DNAse: virulence effect present; -- = does not degrade or utilize DNAse: virulence effects absent

Discussion

The ecologically friendly plant growth-promoting potential and disease control approaches are vital in cultivating crops (Enagbonma and Babalola, 2019). The use of anthill soils for promoting crop yield is now gaining ground among indigenous farmers, particularly in tropical and subtropical regions (Petal *et al.*, 2003, Turay *et al.*, 2022). So, this study made a first-time effort to establish if utilizing anthill soil could be a way of introducing pathogenic organisms to plants. This result showed that the average total fungal count in anthill soils was significantly higher in A2 when related to the corresponding S2 however the reverse was the case in A1 versus S1 (Fig. 1). The distinction of fungal complexes of the anthill from the adjacent soils can be considered the nonappearance of the species *Fusarium sp.* in the adjacent soils and the absence of *Aspergillus flavus* and *Aspergillus niger* in anthill soils (Table 2). Fungi are known to be a diverse group of plant pathogens with a colossal effect on agriculture (Anand and Rajeshkumar, 2022). *Fusarium* sp., which was obviously missing in the adjacent soils, are widely spread and poison plants regardless of growing stage (Maurya *et al.*, 2019). The growth of the *Fusarium* fungi and the accumulation of their mycotoxins can reduce grain value (Abbas *et al.*, 2013). *Fusarium oxysporum* have been reported to infect the xylem tissue and is a well-known cause of *Fusarium* wilt (Zhang *et al.*, 2015, Gordon, 2017). It is known that *Penicillium citrinum* which was seen in anthill soil, produces a mycotoxin citrinin with strong nephrotoxic action (Heperkan *et al.*, 2009). Coutinho *et al.* (2020) reported that *Penicillium citrinum* was a novel phytopathogen of orange fruit. The virulence attribute of the identified fungal isolate *via* lipase and DNAse tests showed that *Trichoderma sp., Penicillium chrysogenum, Fusarium* sp. *and Penicillium citrinum* present in the anthill soils were positive (+) to lipase while *Penicillium chrysogenum* and *Penicillium citrinum* were negative (-) to DNase (Table 3). Anthill soil can be utilized as a potential source of soil amendment due to the amount of soil nutrients present in the soil matrix as supported by results of the physicochemical analysis presented in the current study (Table 1), which showed that the anthill soil samples harbored appreciable amounts of soil nutrients in relation to the examined nearby soils (Table 1).

In this research, it was observed that the anthill soil had higher clay in comparison to the adjacent soil, which possessed sandy characteristics. Organic matter (which was higher in anthill soils compared to the adjacent soils) is the most popular natural fertilizer used in farming (Enagbonma and Babalola, 2019). It is a rich reservoir of carbon and plays a key role in keeping the carbon dioxide balance in the environment. Golichenkov *et al.* (2019) reported that the long-term application of organic soil amendments can aid in the intensification of carbon sequestration in the soil and increased food safety. This trend could also collaborate responses provided by most farmers which indicated the benefits of utilizing anthill soil as soil amendment in crop production activities (Chisanga *et al.*, 2019). However, there is a need to screen out pathogenic fungi from anthill soil in other to enhance its usage as a soil amendment.

Conclusion

Insight into the activities of plant-pathogenic fungi in anthill soils, will make room for the proper screening of anthill soils by local farmers prior to the usage of these soil types as soil amendment. This research revealed the presence of some pathogenic fungi like *P. citrinum*, *P. chrysogenum* and *Fusarium* sp., which are capable of producing toxins that are harmful to plants. Although anthill soil is seen as a likely source of biofertilizers and biocontrol agents and a good source of soil amendment, there is need to screen out pathogenic microorganisms from anthill soil in other to enhance its use as soil amendment.

Authors' contributions

BJE and EEI conceived and designed the study. EFM and ODE carried out most of the laboratory work. BJE and EEI analyzed and interpreted the data. BJE and NOO helped in writing the original and final manuscript draft. All authors read and approved the final manuscript.

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Availability of data and materials

The authors declare that all relevant data supporting the findings of this study are included in this article.

Declarations Ethics approval and consent to participate

Not applicable.

Competing interests

The authors have no conflict of interest to declare.

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