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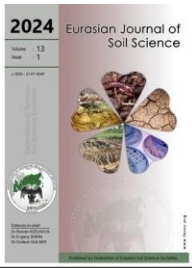

Tea plantation shade tree leaf influences the susceptibility of rhizosphere microbial consortium: A comprehensive study on their leaf extract cross tolerance

Arindam Ghosh [a], Arnab Nag [a], Sukanya Acharyya [a], Sumedha Saha [a], Soumya Majumder [a], Sourav Chakraborty [a,b], Malay Bhattacharya [a,*]

[a] Molecular Biology and Tissue Culture Laboratory, Department of Tea Science, University of North Bengal. Rajarammohanpur, Siliguri, Darjeeling- 734013, India

[b] Post Graduate Department of Botany, Darjeeling Government College, Darjeeling, West Bengal, 734101, India

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Author(s)

A.Ghosh

A.Nag

S.Acharyya

S.Saha

S.Majumder

S.Chakraborty

M.Bhattacharya*

* Corresponding author

Abstract

Leguminous shade trees are ubiquitous parts of tea plantations of the Terai region. However, their shed leaves might have an effect on the soil microflora under those shade trees, so it is important to find out how leaf litter affect the soil microflora. Isolation of soil microbial consortia followed by downstream experiments were conducted to observe the tolerance/susceptible pattern of those soil microflora against the fallen leaves. Sample from under *Albizia odoratissima* has higher organic carbon, organic matter and nitrogen content but the same property was found to be low in the sample collected under *Melia azedarach*. Isolation of consortia was done on nutrient agar. *In vitro* tolerance assay was conducted to find out the tolerance pattern against leaf extracts, heavy metal salts, pesticides, antibiotics and antifungals. Heavy metals salts like Arsenic trioxide (AS_2O_3) and Cupric chloride ($CuCl_2$); and pesticides like Thiamethoxam; Spiromesifen; Phorate etc. showed no inhibition against all the isolated consortia. Co-Trimoxazole and Augmentin have not showed any inhibition except consortia under *Derris robusta*, whereas no antifungals but Itraconazole had an impact over all the consortia. Shade trees, being a crucial member of the tea plantations, cannot be removed but replacement of these with other species could be a probable option, besides this limited use of chemical pesticides and fertilizers should be taken into consideration strictly to restrain the microbial population in tea garden soil. So, this study has disclosed the acceptability of each and every shade tree used in this region.

Keywords: Shade tree, tea plantation, rhizosphere, soil microbes, tolerance.

Introduction

The Tea plant [*Camellia sinensis* (L.) Kuntze], is cultivated for the production of tea. Tea is the 2nd most consumed beverage worldwide and boasts an aromatic flavor as well as beneficial to the drinker. The Tea industry is one of the oldest agro-based industries in India and it is greatly valued for its economic significance as it contributes 1% of the GDP of the country (Sharma et al., 2019). Tea plantations generally employ shade trees to protect their bushes from scorching sunlight. They also help in the betterment of the soil nutrient profile by hosting symbiotic nitrogen-fixing microorganisms and a range of nutrients being added through the degradation of leaf litter from these trees (Ghosh et al., 2021; Ghosh et al., 2022). The shade trees along with tea bushes create a microclimate that improves soil microbial population with an increase in soil organic carbon and moisture (Ghosh et al., 2022). Organic matter supplementing soil microbes is involved in several ecological, biodegradable cycles (Malviya et al., 2021) and mitigates heavy metal toxicity (Pandey et al., 2019). Soil rhizosphere contains diverse microbes, of which plant growth promoting ones improve soil fertility.

To meet the rising demand for tea, the use of fertility management techniques, high yielding clones, longer pruning cycle etc. have been introduced. The microflora hosted by the soil in these gardens may be subject to

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a variety of factors including but not limited to exposure to the phytochemical effects of leaf litters, pesticides, antibiotics etc., resulting in the acquisition of tolerance to said irritants. So, in order to find out whether or not these factors are affecting the soil microflora we explored their antibiotic, antifungal, heavy metal and pesticide tolerance abilities. As this region lies under the Himalayan foothills, anthropogenic activities and the use of chemical fertilizers and pesticides are to be expected.

Shade tree leaf litter is a pool of secondary metabolites that may promote or restrict soil microbes (Saha et al., 2022). Shade trees vary in secondary metabolite content, so there must be differential antagonism toward soil microbes. Not much work has been done on this particular issue. Though the inhibitory effect of tea plantation shade tree leaf extract on phosphate solubilizing bacteria (PSB) has been studied by Saha et al. (2022), where it was found that metabolites like 1-heptanol, 2-propyl-, neophytadiene, phytol and squalene from shade trees like *Albizia odoratissima*, *Albizia chinensis*, *Albizia procera* exhibited antimicrobial activity as they showed *in vitro* inhibition zones in PSB culture collected from tea gardens. But much in this context remains to be explored.

So, this research intended to isolate microbial consortia from the soil of the tea plantation of Terai region and explore their tolerance ability against leaf litters, pesticides, heavy metal salts as well as various antibiotic and antifungal agents. Thus, this study was aimed to gain an understanding of the growing resistance ability of microflora and their adaptability that may ultimately help in maintaining soil fertility which is an indispensable part of proper plant growth and development.

Material and Methods

Collection of soil samples

Seven Shade trees viz. *Albizia odoratissima* (L.f.) Benth. (AO), *Albizia chinensis* (Osbeck)Merr. (AC), *Acacia lenticularis* Benth. (AL), *Dalbergia sissoo* Roxb. (DS), *Derris robusta* (DC.) Benth. (DR), *Leucaena leucocephala* (Lam.) de Wit (LL) and *Melia azedarach* L. (MA) were selected and considered for the present study. Vacant land (VL) as well as shade tree free (WST) areas were used as control plots for the study. All the collection sites were from NBU tea plantations (26.713006 N, longitude 88.3480252 E). Soil samples were collected from the cover area of the shade tree by following the protocol of Saha et al. (2020) with slight modifications. Soil sampling was done early in the morning after the pre-monsoon showers.

Soil analysis

Soil parameters like pH, organic carbon (OC), organic matter (OM) content, total nitrogen, available phosphorus, potassium and Sulphur contents were evaluated from the collected sample. Before analysis soil samples were air-dried and sieved (Mukherjee et al., 2020; Saha et al., 2020).

Isolation of Consortia

Soil microbial consortia were isolated by mixing 500mg of soil sample in 1 ml of sterile distilled water. 500µl of the soil solution was added to the pre-autoclaved nutrient broth and was incubated overnight at 37º C. The isolates were preserved at 4ºC for downstream experiments (Saha et al., 2021).

Effect of shade tree leaf extract on the growth of Rhizopsheric Microbial Consortia (RMC)

Antimicrobial study was conducted with shade tree leaf extracts on the isolated consortia. Pour plate inoculation followed by well diffusion addition of leaf extracts of AO, AC, AL, DS, DR, LL and MA was employed for the study (Ghosh et al., 2021), where 200µl of cultures (RMC) were inoculated and 100µl (250 mg/ml) of leaf extracts were added in the wells of the semisolid agar. The Petri dishes were incubated at 37ºC for 24 hours.

Heavy Metal tolerance assay

To determine the heavy metal tolerance test, we followed the disc diffusion method proposed by Acharyya et al. (2021) with slide modification, where we took five different heavy metal salts like $CuCl_2$, As_2O_3, $Pb(NO_3)_2$, $CdCl_2$ and $HgCl_2$with the concentration of 2.5 mg/ml. To test the isolated culture, we prepared semi-solid nutrient media with 100µl of culture being spread throughout via the spread plate technique. After that, the disc was dipped in different heavy metal salts and placed on the petri dish.

Antibiotic and antifungal tolerance assay of Consortia

In vitro, antibacterial tolerance assay was conducted by following the protocol of Saha et al. (2020) against the selected isolated culture by using antibiotic discs. 200µl of isolated culture was pour plated in nutrient agar media. After the hardening of the agar, common antibiotic hexa-discs of Bacitracin (B10), Chloramphenicol (C30), Penicillin-G (P10), Polymixin B (PB300), Gentamicin (Gen10), Neomycin (N30), Cefotaxime (CTX30), Augmentin (AMC30), Erythromycin (E10), Ofloxacin (OF5) and Co-Trimoxazole (COT25), of HiMedia

(Catalogue No. HX032 and HX038) were were aseptically placed on top of the agar and then incubated these plates at 35°C for 24 hours. After 24 hours, inhibition zones around the discs were recorded.

To assess the tolerance activity of the fungal population in the soil, we followed the protocol of Saha et al. (2020), where 200µl of culture sample was inoculated in the plate with the pour plate technique. And six common antifungals like Amphotericin-B (AP100), Clotrimazole (CC10), Fluconazole (FLC25), Itraconazole (IT10), Ketoconazole (KT10) and Nystatin (NS100) from HiMedia (Catalogue No HX104) were used against consortia and incubated for 96 hours at 35°C. After incubation, the inhibition zones around the discs were recorded.

Pesticide tolerance assay

To determine their pesticide tolerance ability, we followed the protocol of Saha et al. (2020), where nine common pesticides like Emamectin benzoate, Fipronil, Phorate, Deltamethrin, Flubendiamide, Spiromesifen, Thiamethoxam, Fenazaquin, Quinalphos being usually applied in the tea plantation to get rid of pests were chosen. Pour plate and well diffusion method was followed to assess this test, where 200 µl of isolated culture was poured in petri dish with 2.8% nutrient agar media. Different concentrations like 2.5, 5, 10 and 20 mg/ml of the nine pesticides were chosen whereas the recommended value was 2.5mg/ml, and 100 µl of each concentrated different pesticide were added in the wells of the petri dishes followed by incubation of the plates at 30ºC for 48 hours.

Results

Soil analysis

pH and EC

Changes in soil pH mean a tenfold change in the amount of acidity or alkalinity. Tea Board of India proposed that the pH of the soil of tea plantations should be between 4.5-5.5 (Mukherjee et al., 2020). In this study, we found that the pH of the soil collected from underneath the tea plantation shade trees range between 4.5 to 6.1. Maximum soil pH was recorded in MA (6.1) while lowest pH was recorded in DR (4.5).

Electrical conductivity (EC), typically used to enumerate the charged particles present in particular soil samples, was also found to vary between plant species. The maximum EC (172 µS/cm) was found in the soil sample of DR plant species and the lowest EC (103.3 µS/cm) was detected in the soil of MA plant species (Table 1).

Table 1. Results of soil physicochemical properties

	pH	EC, µS/cm	OC,%	OM,%	Total N, %	Available K, mg/kg	Available S, mg/kg	Available P, mg/kg
AC	5.21±0.29	124.00±6.24	6.66±0.44	11.45±0.76	0.57±0.04	49.00±1.73	24.00±6.07	52.33±9.50
AO	4.89±0.07	145.00±2.65	10.37±1.31	17.84±2.25	0.89±0.11	42.67±5.86	16.27±2.16	37.33±9.71
AL	4.73±0.07	132.33±17.24	9.34±2.63	16.07±4.52	0.80±0.23	31.33±2.52	16.67±2.54	29.00±11.27
DS	4.88±0.12	143.33±32.65	7.70±1.16	13.24±1.99	0.66±0.10	41.00±3.61	19.80±2.25	24.33±6.66
DR	4.58±0.17	172.00±4.58	7.19±0.37	12.36±0.64	0.62±0.03	30.33±3.51	17.30±2.93	11.33±4.04
LL	5.31±0.10	145.00±11.27	6.93±0.36	11.93±0.63	0.60±0.03	59.33±6.51	18.97±4.41	40.67±15.01
MA	6.12±0.19	103.33±21.50	5.97±0.72	10.27±1.24	0.51±0.06	60.67±8.08	17.93±5.09	21.67±10.69
VL	4.86±0.07	111.67±1.53	6.59±0.59	11.33±1.02	0.57±0.05	44.00±2.65	17.30±4.03	0.67±1.15
NS WST	5.24±0.05	132.67±4.04	7.15±0.36	12.29±0.62	0.61±0.03	50.67±10.79	19.37±1.44	20.33±2.89

Albizia chinensis (AC), *Albizia odoratissima* (AO), *Acacia lenticularis* (AL), *Dalbergia sissoo* (DS), *Derris robusta* (DR), *Leucaena leucocephala* (LL), *Melia azedarach* (MA), Vacant land (VL) and WST (Without shade tree)

Organic carbon and Organic matter

Soil organic matter is an aggregation of all organic components present in the soil including plant and animal matter excluding mineral carbon. Organic matter (OM) makes up just 2-10% of most of the soil's mass. Organic carbon (OC) content should be 1-2% as recommended by the Tea Board of India. In this study, the average organic carbon was found to be about 7.73%. Whereas maximum organic content was recorded at 10.37% for AO and the minimum was found in MA (5.96%).

Moreover, non-shaded areas (WST) showed a moderate level of organic matter and organic carbon content due to the presence of shaded tea leaf litter on it while the vacant land (VL) portrayed a low trend in OC and OM content compared to others. Interestingly, MA resulted in the lowest OC and OM values in its cover region where possible explanation can be found only after future comparative nutrient analysis on shed leaves in particular.

Nitrogen, Phosphorous, Potassium and Sulphur

Nitrogen, phosphorous, potassium and sulfur are the macronutrients for plants that help plants regulate essential physiological processes, growth and development etc. (Mukherjee et al., 2020). In our study, we found that 0.665% of nitrogen (N) was present in analyzed samples which were ideal. The highest nitrogen content was recorded in AO (0.892%) and the minimum in MA (0.513%).

Phosphorous (P) is present in the soil as phosphate (P_2O_5) and the ideal level recommended by the Tea Board of India is 10 to 20 mg/kg. But in our study, we found the highest phosphate content was in AC with an amount of 52.34 mg/kg and the lowest was detected in DR. Surprisingly, in vacant land (VL) phosphate level was very low (0.67 mg/kg).

The optimum level of potassium (K) as potash in soil recommended by the Tea Board of India is 60 to 80 mg/kg. Our results revealed that the average potassium level was lower than the optimum. However, the maximum potash level was found in MA (60.67 mg/kg) and the minimum potash level was recorded in DR (30.34 mg/kg). Whereas soils from non-shaded areas (WST) and vacant land (VL) showed an ideal level of potash i.e., 50.67 mg/kg and 44 mg/kg respectively.

Sulphur (S) is present in the soil as sulphate (SO_4) and the average sulphate level was recorded to be 18.71 mg/kg. The maximum value was recorded in AC (24 mg/kg) and the lowest was recorded in AO (16.27). Table 1 indicates that non-shaded soil or soil without shade trees (WST) contained a huge amount of sulphate compared to other samples.

Effect of shade tree leaf extract on the growth of Rhizospheric Microbial Consortia (RMC)

Aqueous extracts of shade tree leaf from AO, AC, DS, DR, Alen, LL and MA showed variable inhibition zones against consortia isolated from soil under different shade trees of tea gardens including vacant land and shade tree free areas (Table 2 and Figure 1). In this study, we found that leaf extracts of DR showed an inhibitory effect on six consortia collected from under six regions whereas DS, AO and AC showed an inhibitory effect on consortia of five areas respectively. In most of the cases, consortia showed resistance towards leaf extract with a percentage of 53.96%, whereas, 46.03% of consortia were found to be susceptible. All the consortia from under each shade tree except DS (negligible inhibition zone 6mm), VL and WST, have shown resistance against their respective leaf extracts. This might be due to either the developed resistance of microbes present in soil which has been exposed to leaf litter year after year or it can be assumed that the leaf extracts do not possess any kind of antimicrobial compound. Most of the maximum inhibition zones were found on the consortia collected from under LL, meaning the consortia under this tree were susceptible to all the leaf extracts except its own leaf. Apart from the LL consortia, leaving behind the consortia from LL, the highest inhibition zones were seen on the consortia of AC and AO, created by the extract of DS (17mm and 15mm respectively), followed by MA, VL which was inhibited by AO leaf extract 15mm respectively. So, leaf extracts of DS and AO possess some antimicrobial compounds, due to which some microbes of the consortia are susceptible.

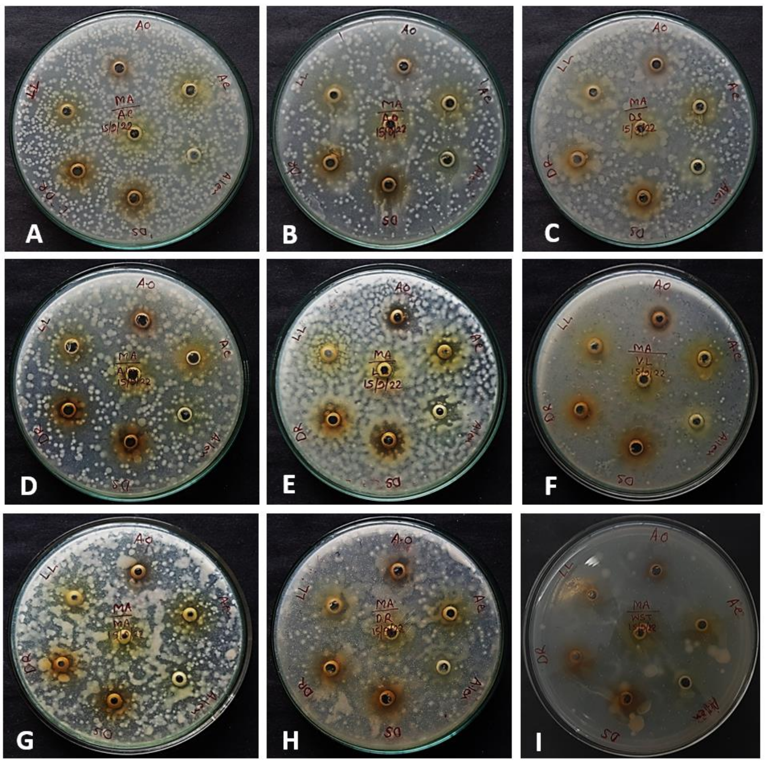

Figure 1. Resistance and susceptibility of consortia against different shade tree leaves extract where (A-I) represents the Consortia culture collected from under the AC, AO, DS, AL, LL, VL, MA, DR, WST respectively

Table 2. Inhibition zone (mm) created by leaf extracts on different consortia

	AC	AO	AL	DS	DR	LL	MA	VL	WST
AC	0	6	0	6	6	16	0	6	0
AO	0	0	0	6	0	18	15	15	13
AL	0	6	0	6	0	21	0	6	0
DS	17	15	7	6	0	18	0	0	0
DR	7	7	14	6	0	7	0	6	13
LL	0	0	0	0	0	0	0	0	0
MA	0	0	6	6	0	14	0	0	0

Albizia chinensis (AC), *Albizia odoratissima* (AO), *Acacia lenticularis* (AL), *Dalbergia sissoo* (DS), *Derris robusta* (DR), *Leucaena leucocephala* (LL), *Melia azedarach* (MA), Vacant land (VL) and WST (Without shade tree)

Heavy metal tolerance assay

Contamination occurring from metals like mercury (Hg), arsenic (As), and cadmium (Cd) etc. poses a serious toxic threat to all living organisms as it can cause cancer in humans when present in small quantities (Acharyya et al., 2021). These traumatic and stressful surroundings often encourage microbial populations to adapt and gain tolerance toward the metals. As this region is under the Himalayan range, it is prone to exposure to elevated levels of heavy metals which encourage microbes to build tolerance towards them. Therefore, we intended to study the heavy metal tolerance ability of the consortium presence in this region. The inhibition zones formed by selected heavy metals salt on isolated consortia from the tea plantations are shown in Figure 2 and Figure 3. Inhibition zones created by As_2O_3, $CdCl_2$, $CuCl_2$, and Pb $(NO_3)_2$ were less but in case of $HgCl_2$, the inhibition zones were much higher compared to other heavy metal salts (Table 3). Almost all the consortia have shown tolerance or are gaining tolerance against Lead nitrate, Arsenic trioxide and Cupric chloride, whereas the salts of cadmium and mercury produced inhibition zones against all the consortia.

Table 3. Inhibition zones (mm) exhibited by heavy metals on different consortia Colour code: 0 =Tolerant (Red), 6-9= mildly tolerant (Yellow), 10-17= moderately susceptible (Blue), 18 and above= Susceptible (green))

	Lead nitrate $Pb(NO_3)_2$	Arsenic trioxide AS_2O_3	Cadmium chloride $CdCl_2$	Cupric chloride $CuCl_2$	Mercuric chloride $HgCl_2$
AC	14	0	17	0	25
AO	0	0	12	0	25
AL	0	0	9	0	13
DS	6	0	7	0	20
DR	6	0	20	6	16
LL	0	0	10	0	15
MA	0	6	6	0	10
VL	6	6	10	0	31
WST	0	0	11	0	32

Albizia chinensis (AC), *Albizia odoratissima* (AO), *Acacia lenticularis* (AL), *Dalbergia sissoo* (DS), *Derris robusta* (DR), *Leucaena leucocephala* (LL), *Melia azedarach* (MA), Vacant land (VL) and WST (Without shade tree)

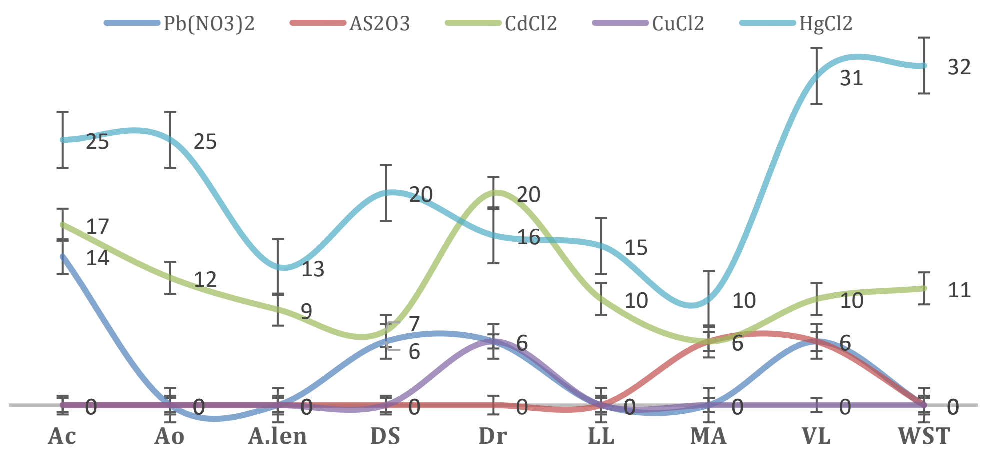

Figure 2. Graphical representation of resistance and susceptibility pattern of consortia against five heavy metal salts (x axis representing different consortia and y axis representing inhibition zones)

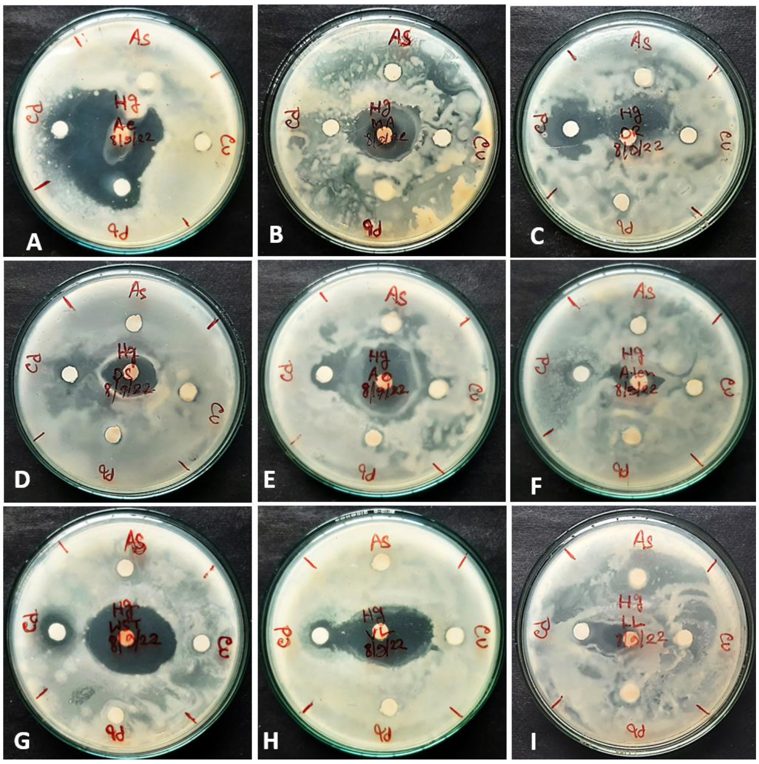

Figure 3. Resistance and susceptibility of consortia culture against five different heavy metal salts, where (A-I) represents the Consortia, culture collected from under the different areas of AC; MA; DR; DS; AO; AL; WST; VL; LL respectively

Antibiotic tolerance assay

Bacteria and fungi are the main biotic components of the microflora of soil consortia. Since its discovery, antibiotics have been used extensively. These antibiotics tend to accumulate in soil and water, eliciting an effect on beneficial microorganisms harbored in these regions (Saha et al., 2020). In recent years, antibiotics have become very handy for treatment against various bacterial infections, be it as medication for humans to livestock rearing. The use of antibiotics in agricultural fields has also gained momentum as a means to rid the fields of unwanted, harmful bacterial invasions. Improper knowledge of dosage can often lead to a disastrous end. With the development in the field of science, synthetic antibiotics have become a convenient choice. The secondary metabolites from the leaves and pods, often contain antimicrobial (antibacterial or antifungal) compounds. Hence, this study was carried out to understand the behaviour of the isolates in the face of some common antibiotics. Eleven different antibiotics were used for this study.

Studies revealed that different antibiotics act differently on consortia. Results of the antibiotic tolerance assay have been expressed in Table 4 and Figure 4. In Table 4 of the nine samples which had been tested against eleven different antibiotics indicated that 35.35 % of samples were tolerant against above mentioned antibiotics, while 22.22 % of samples were susceptible to those antibiotics. Besides these, 16.16 % of samples exhibited mild tolerance and 26.26 % of samples showed moderate tolerance levels. Among the different shade tree cover soil microbes, AC, MA and LL showed tolerance against maximum antibiotics. On the contrary, DR showed a more or less susceptible nature against all used antibiotics.

Table 4. Inhibition zone (cm) of different samples tested against different antibiotics (Colour code: 0 =Tolerant (Red), 0.1-1= mildly tolerant (Yellow), 1.1-2= moderately susceptible (Blue), 2 and above= Susceptible (green))

	AC	AO	AL	DS	DR	LL	MA	VL	WST
Chloramphenicol	0.5	1.3	0.9	0.6	1.6	0.7	0.8	0.8	0.4
Ofloxacin	2.3	2.5	2.6	2.4	2	2.5	2.6	2.4	2.2
Cefotaxime	1	2.5	2.6	1.5	2.5	2	0	0.9	1.6
Erythromycin	0	2.5	2.5	0.8	2	0	0	0.9	0
Co-Trimoxazole	0	0	0	0	1.6	0	0	0	0
Augmentin	0	0	0	0	2.3	0	0	0	0
Bacitracin	0	0	1.1	0	0.9	0	1	0	1
polymyxin B	1.1	1.3	1.2	1	1.5	1.3	1.2	1.2	0.9
Gentamicin	0.8	2.5	1.2	2.3	1.5	1	1.5	0.8	1.8
Neomycin	0	2.4	2	2	0	1.3	1	0.8	2.2
Penicillin G	0	0	1.3	0	0	0	0	0	1.1

Albizia chinensis (AC), *Albizia odoratissima* (AO), *Acacia lenticularis* (AL), *Dalbergia sissoo* (DS), *Derris robusta* (DR), *Leucaena leucocephala* (LL), *Melia azedarach* (MA), Vacant land (VL) and Without shade tree (WST)

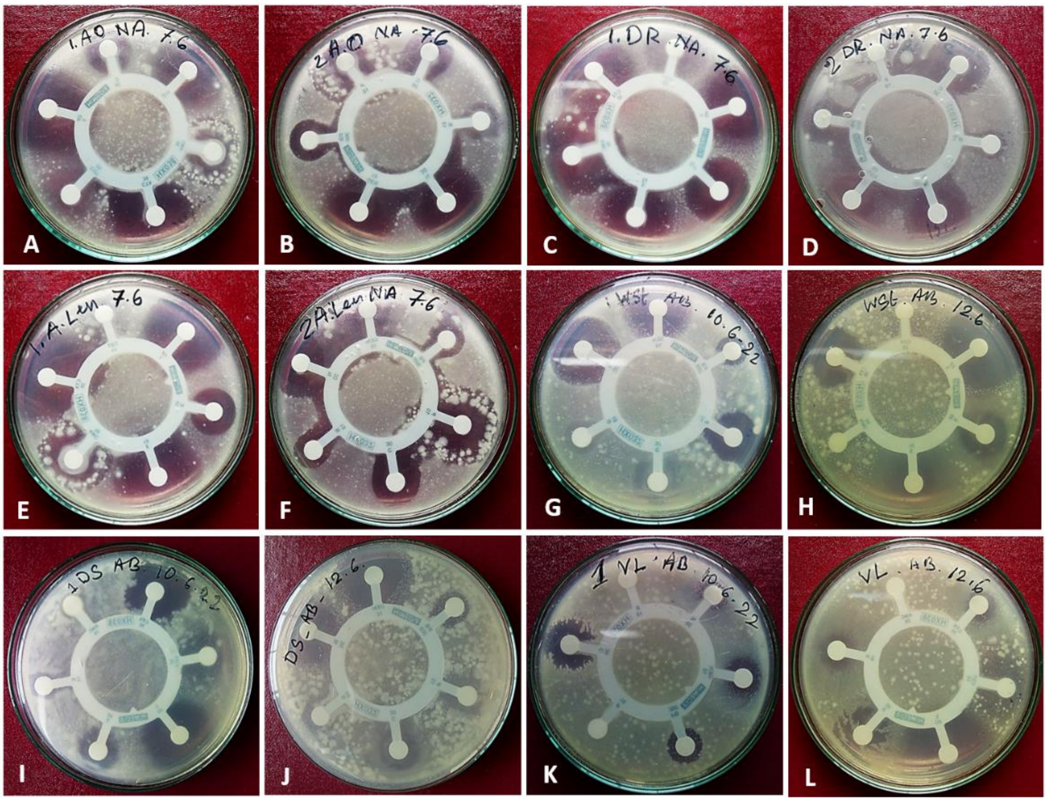

Figure 4. Antibiotic susceptibility and resistance of different consortia where (A-L) represent the consortia culture of under from AO; DR; AL; WST; DS; VL respectively

Co-Trimoxazole and Augmentin exhibited the lowest efficacy in inhibiting the *in vitro* growth of the bacterial populations isolated from the soil samples under the cover of all the shade trees selected for this study, except for DR, which showed considerable susceptibility as seen from the graph Figure 4. On the other hand, Ofloxacin showed the highest efficacy in terms of inhibition zone against the bacterial populations isolated from the cover region of all the shade trees.

Out of the eleven antibiotics tested OF5 (2.388±0.19 cm) showed average maximum and COT25 (0.177 ± 0.53 cm) showed minimum inhibition zone. So, it can be considered that COT25 was the least effective and OF5 was the most effective for the isolated consortia. The increasing order of tolerance observed in our study is COT25; AMC30; P10; B10; C30; E10; PB300; N30; Gen10; CTX30; OF5 (Table 4).

Antifungal tolerance assay

Fungal invasions are also a great threat to plants. Thus, needless to say, the use of antifungals in agriculture is an inevitable solution that people reach for to get rid of fungal infections/fungi. With the application of antifungals, there comes a possibility of developing antifungal tolerance by the fungal populations. Also, the antimicrobial components present in the dropped plant parts may play a role too just as in the case of antibiotics mentioned above. Therefore, the study of antifungal tolerance by the isolates has become an absolute necessity. Antifungals used in the assay have different target patterns for different fungi present in the consortia. The data for the antifungal assay are represented in Table 5 and Figure 5 concerning their tolerance ability. The data represents the tolerance pattern of the fungal population isolated from the soil samples of the respective shade trees. Fungicides having azole groups are the most widely used antifungals against fungal plant pathogens (Saha et al., 2020). In this study, we have used four antifungals viz. CC10, FLC25, IT10 and KT10 have azole groups. All the samples have full resistance against the five antifungals i.e., Amphotericin-B (AP100), Clotrimazole (CC10), Fluconazole (FLC25), Ketoconazole (KT10), and Nystatin (NS 100) i.e., they have not shown any inhibitory effect in the consortia. Itraconazole (IT10), on the other hand, has shown an inhibitory effect in all the consortia, wherein the sample of AC, MA and VL, it showed a full inhibition zone of 3.5cm each, followed by 3cm; 2.4cm; 2.3cm in AL, WST and DS respectively and 2.2 cm in three samples of AO; DR and LL.

Pesticide tolerance assay

Based on the application of pesticides in tea plantations, the nine most regularly used pesticides were selected and *in vitro* tolerance assay was done to find out the level of tolerance in isolated consortia. Over a period of time, chemical pesticides in tea plantations accumulate in an area of soil where microbial diversity is at its maximum level. In our study, our consortia showed variable levels of tolerance against different concentrations of each pesticide (Table 6 and Figure 6). Four pesticides viz. Spiromesifen; Thiamethoxam, Phorate and Flubendiamide showed no inhibition zone in any of the consortia at the studied concentrations. So, consortia isolated from underneath all the shade trees, vacant land and without shade trees are tolerant to all concentrations of pesticides. All the pesticides, except Fenazaquin, showed inhibition zones only above 5mg/ml concentrations. So, this concludes that all the consortia from tea plantations are highly tolerant against these pesticides (excluding Fenazaquin) at these concentrations. Fenazaquin showed a gradual increase in inhibition zone with an increase in concentration against consortia of AO, AL and WST, whereas against the consortia of AC and DS, this pesticide showed a density gradient increase in inhibition zones in all the higher concentrations but no inhibition zone was found at the lowest concentration. Deltamethrin also showed a gradual increase in inhibition zones in all the higher concentrations against the consortia collected from under AC, AO and DS but with the concentration of 2.5mg/ml, there were zero inhibition zones.

Table 5. Inhibition zone (cm) of different samples tested against different antifungals (Colour code: 0=Tolerant (Red), 2.1-3= moderately susceptible (Blue), 3.5 and above= Susceptible (green))

	AC	AO	AL	DS	DR	LL	MA	VL	WST
Amphotericin B	0	0	0	0	0	0	0	0	0
Clotrimazole	0	0	0	0	0	0	0	0	0
Fluconazole	0	0	0	0	0	0	0	0	0
Itraconazole	3.5	2.2	3.0	2.3	2.2	2.2	3.5	3.5	2.4
Ketoconazole	0	0	0	0	0	0	0	0	0
Nystatin	0	0	0	0	0	0	0	0	0

Albizia chinensis (AC), *Albizia odoratissima* (AO), *Acacia lenticularis* (AL), *Dalbergia sissoo* (DS), *Derris robusta* (DR), *Leucaena leucocephala* (LL), *Melia azedarach* (MA), Vacant land (VL) and Without shade tree (WST)

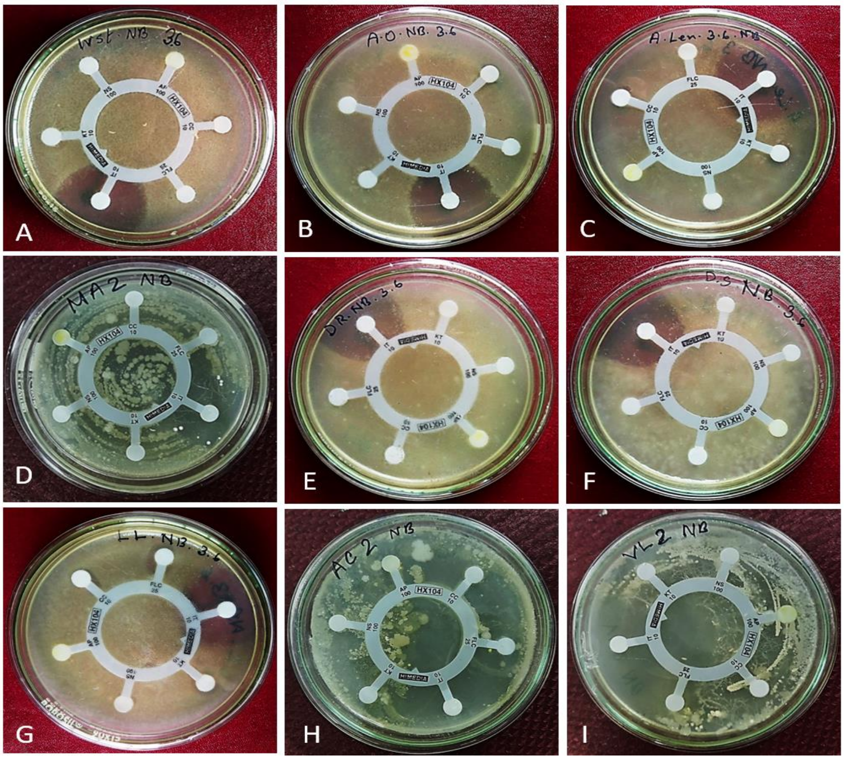

Figure 5. Tolerance and susceptibility of consortia against six different Antifungal agents where (A-I) represents the consortia culture under from WST, AO, AL, MA, DR, DS, LL, AC, VL respectively

Discussion

In this study we have found that all the soil are found to be acidic the possible reason behind this because that maximum shade trees belong to the Fabaceae family that characteristically shed their leaves once a year and contribute to the accumulation of a massive amount of organic matter and simultaneously increases organic carbon and nitrogen content in the soil. Being a non-leguminous plant, MA shade tree cannot form root nodules; thus, nitrogen content was also low in its cover soil. In the presence of a good amount of mineral nutrition and organic matter, microorganisms thrive and produce humic acid in the soil consequently decreasing the pH. Table 1 reveals that the MA shade tree contributes the least amount of organic matter to the soil. Furthermore, the shed leaves of MA may contain some chemical compounds that can inhibit the growth of microorganisms, thereby limiting the pH drop that would otherwise have resulted from the profuse growth of microbes as previously discussed.

The maximum quantity of phosphate also came from leaf litter and organic matter. Leaves of certain shade tree species like AC, LL, AO and AL may provide a good amount of phosphorus (Table 1) which leads the phosphate solubilizing microorganisms to digest the phosphorus as substrate to produce an available form of phosphate for plant uptake, while, DR (11.33) and DS (24.33) might have some antagonistic effect (antibacterial effect) on these microorganisms as the results suggest. Recently, Ghosh et al. (2021) reported the antibacterial activities of these leaves against microorganisms. Table 1 shows that where electrical conductivity is higher, consequently potassium level is lower, it is because of the leaching of potassium ions into subsoil. Lack of nutrient management system and fertilizer application in tea gardens could be the reason behind unfamiliar results (unlike other nutrient parameters) or the high amount of sulphate present in non-shade areas or without shade tree regions.

Based on our heavy metal tolerance assay, it can be presumed that the isolated consortia are tolerant or slightly tolerant towards metals like Pb, As and Cu but susceptible towards Cd and Hg. The tolerance property of these consortia towards heavy metal salts like As_2O_3, $CuCl_2$, and $Pb(NO_3)_2$ is a serious concern as it may have been stimulated by the persistence of these salts in soil.

Table 6. Inhibition zones (mm) exhibited by concentrations of pesticide on different consortia (Colour code: 0= Tolerant (Red), 10-12= moderately susceptible (Yellow), 13 and above= Susceptible (green).)

Pesticides	Time	Concentration	AC	AO	DS	DR	Al	LL	MA	VL	WST
Emamectin benzoate	x	2.5mg/ml	0	0	0	0	0	0	0	0	0
	2x	5mg/ml	0	0	13	0	0	0	0	0	0
	4x	10mg/ml	0	11	16	0	0	0	0	0	0
	8x	20mg/ml	0	12	17	0	15	0	0	0	0
Fipronil+Imidacloprid	x	2.5mg/ml	0	0	0	0	0	0	0	0	0
	2x	5mg/ml	0	0	0	0	0	0	0	0	0
	4x	10mg/ml	0	11	0	0	0	0	0	0	0
	8x	20mg/ml	0	13	0	11	12	12	0	0	0
Spiromesifen	x	2.5mg/ml	0	0	0	0	0	0	0	0	0
	2x	5mg/ml	0	0	0	0	0	0	0	0	0
	4x	10mg/ml	0	0	0	0	0	0	0	0	0
	8x	20mg/ml	0	0	0	0	0	0	0	0	0
Thiamethoxam	x	2.5mg/ml	0	0	0	0	0	0	0	0	0
	2x	5mg/ml	0	0	0	0	0	0	0	0	0
	4x	10mg/ml	0	0	0	0	0	0	0	0	0
	8x	20mg/ml	0	0	0	0	0	0	0	0	0
Deltamethrin	x	2.5mg/ml	0	0	0	0	0	0	0	0	0
	2x	5mg/ml	12	11	11	0	0	0	0	0	0
	4x	10mg/ml	16	13	12	0	14	0	0	0	0
	8x	20mg/ml	16	14	15	14	16	0	0	0	0
Phorate	x	2.5mg/ml	0	0	0	0	0	0	0	0	0
	2x	5mg/ml	0	0	0	0	0	0	0	0	0
	4x	10mg/ml	0	0	0	0	0	0	0	0	0
	8x	20mg/ml	0	0	0	0	0	0	0	0	0
Flubendiamide	x	2.5mg/ml	0	0	0	0	0	0	0	0	0
	2x	5mg/ml	0	0	0	0	0	0	0	0	0
	4x	10mg/ml	0	0	0	0	0	0	0	0	0
	8x	20mg/ml	0	0	0	0	0	0	0	0	0
Quinalphos	x	2.5mg/ml	0	0	0	0	0	0	0	0	0
	2x	5mg/ml	0	10	0	0	0	0	0	0	0
	4x	10mg/ml	10	11	0	0	11	0	0	0	0
	8x	20mg/ml	11	12	0	0	11	0	0	0	0
Fenazaquin	x	2.5mg/ml	0	11	0	0	11	0	0	0	11
	2x	5mg/ml	11	13	11	0	13	0	0	0	12
	4x	10mg/ml	12	14	12	0	13	0	0	0	15
	8x	20mg/ml	14	14	14	0	15	0	0	0	17

Albizia chinensis (AC), *Albizia odoratissima* (AO), *Acacia lenticularis* (AL), *Dalbergia sissoo* (DS), *Derris robusta* (DR), *Leucaena leucocephala* (LL), *Melia azedarach* (MA), Vacant land (VL) and Without shade tree (WST)

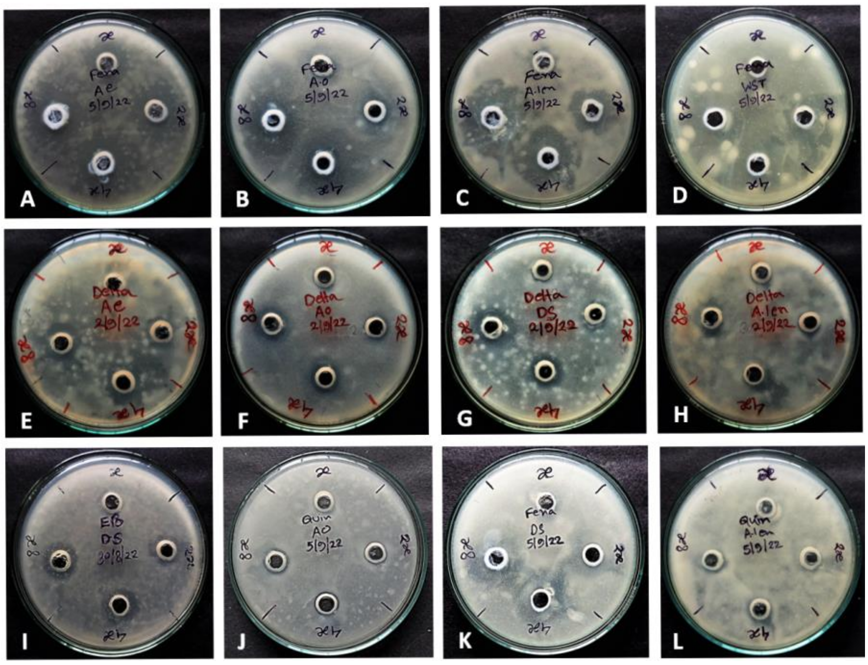

Figure 6. Resistance and susceptibility of consortia culture against different pesticides with different concentrations where (A-L) represents the Consortia culture collected from under the different shade trees AC; AO, AL; WST; DS

The exploitation of antibiotics/antifungals in agricultural fields can lead to the eradication of the useful bacterial/fungal populations that inhabit the soil and thereby have an effect on the nutrient uptake processes of plants. There also lies the risk of developing antibiotic/antifungal tolerance by the bacterial/fungal population. Initially, these compounds were isolated from the secondary metabolites produced by microorganisms such as bacteria and fungi. In this antibiotic tolerance assay, though a few antibiotics were found to be ineffective against some of the microbes, most of them elicited a considerable range of susceptibility from the microbes. Their pattern of tolerance is mainly based on the respective shade tree cover region from where the soil sample was collected, as we can see in case of DR.

On an average farmers use antibiotics in their agricultural fields with the intention of getting rid of the harmful bacteria that may affect the plants (Saha et al., 2020). But while doing this, it also increases the chances of developing antibiotic resistant microbes. This could be the possible reason for the acquisition of tolerance by some of the bacterial populations. Another reason for such tolerance may have to do with antimicrobial components detected in the shed parts of the trees. Photographs of some plates have been attached below (Figure 4).

In the antifungal assay, a resistance pattern towards all the antifungals except IT10 has been found which may be a result of the unsupervised overuse of fungicides in tea plantations or another plausible reason may be the possible co-selection of resistance genes under selective pressure of survival related to other elements like heavy metals (Sun et al., 2021). Hence, we could conclude that IT10 is probably not used as much or use of this is close to nil in this tea plantation.

Pesticides are very toxic and harmful to the ecosystem as they accumulate in soil. It brings changes in soil microflora by killing and reducing the microflora count or diversity or may even modify tolerance towards the pesticides for resisting pesticides. Pesticides have a half-life and the half-life of Phorate is more than Emamectin Benzoate and the mixture of Fipronil and Imidacloprid. Phorate is slightly soluble in water when kept over time and 2g of this is added to the soil during the plantation of plantlets and is not spread further in later years. Prolonged use of cultural practices like the use of chemical pesticides could be the reason for tolerance observed in the microbial population.

Probable candidates of leaf extracts for growth inhibition validates by GC-MS

To find out the probable reason for getting inhibition zone in the different cultures of consortia by the shade tree leaf extracts, we have done Gas chromatography and mass spectrometry analysis. In our GC-MS analysis, we found a total of 74 compounds in the leaf extracts of seven shade trees having microbial properties, where 11 compounds were from each extract of AO, DS, AL and MA comprising 14.86%, 13 compounds from DR adding up to 17.56%, 9 in AC at 12.16%; and 8 compounds in LL of 10.81% (Table 7).

Phytol, an acyclic diterpene, has an antimicrobial effect against *Pseudomonas aeruginosa*, *Maphomina phaseolina* and triterpene compound squalene also has antimicrobial activity (Saha et al., 2022). Both of these compounds have been detected in all the leaf extracts (Table 7). Neophytadiene, another antimicrobial component has been found in all the extracts except MA (Non-leguminous). 1-Heptanol, 2-propyl-; .gamma. -

Linolenic acid, methyl ester; Cyclopentadecanone, 2-hydroxy-; Dehydroabietylamine; Longifolene; Longiborneol (activity against *Candida albicans, Trichophyton rubrum, Aspergillus fumigatus*, *Staphylococcus aureus*); 9,12-Octadecadienoic acid, methyl ester was only found in AO and are reported to have antimicrobial property, only found in AO, whereas in the extracts of AC, 13-Hexyloxacyclotridec-10-en-2-one; Dehydroabietic acid and its TMS derivative; Ethanone, 1-phenyl-; and Menthol were unique. Tetradecane and 9-octadecenal, (z)- were only found in DS. Four reportedly antimicrobial compounds, (2e)-3,7,11,15-tetramethyl-2-hexadecene; 9-Octadecenoic acid, methyl ester, (E)-; Hexadecanoic acid; and Methyl abieta-8,11,13-trien-18-oate were only distinguished in DR. beta- amyrin; Octadecane and Methyl commate d with 22.58% area were detected in AL. 2-methyloctacosane is the only compound found in MA, reported to have antimicrobial properties. In the leaf extract of LL, no particular compounds were found to be unique but two compounds Phenol, 2,4-bis(1,1-dimethylethyl)-, phosphite (3:1) and vitamin E with antimicrobial activity were found with a high percentage of 20.75 and 12.88 peak area respectively. Antimicrobial compounds worth 17.56% were found in the leaf of DR. In the consortia of DR, we found almost no inhibition. With a percentage of 10.81 in the leaf extracts of LL, it showed the lowest number of antimicrobial compounds (Figure 7). The consortium of LL was found to be susceptible except to the extract of LL. Thus, it can be said that leaf litter do have an impact on the growth of microflora and can restrict the beneficial microorganisms of soil.

Table 7. GC-MS detected compounds with peak area percentage and antimicrobial activity

Compounds with antimicrobial activity	**GC-MS (Peak area %)**							**References**
	AO	**AC**	**DS**	**LL**	**DR**	**AL**	**MA**	
1-Heptanol, 2-propyl-	3.69	8.7						Saha et al., 2022
13-Hexyloxacyclotridec-10-en-2-one		1.34						Saha et al., 2022
Beta-Amyrin						8.64		Elfadil et al., 2015
Gamma. -Linolenic acid, methyl ester	1.4							Saha et al., 2022
Cyclopentadecanone, 2-hydroxy-	0.61							Saha et al., 2022
Dehydroabietic acid and its TMS derivative		1.42						Saha et al., 2022
Dehydroabietylamine	0.81							Saha et al., 2022
Ethanone, 1-phenyl-		1.27						Saha et al., 2022
Neophytadiene	4.94	1.27	6.67	2.29	10.30	5.71		Singh et al., 2012
Phytol	5.64	1.77	29.48	0.52	23.46	7.15	47.14	Islam et al., 2018
Squalene	5.59	8.7	7.80	5.99	3.95	14.73	1.42	Rautela et al., 2017
Longifolene	1.04							Saha et al., 2022
Longiborneol	3.14							Saha et al., 2022
1,2-Benzenedicarboxylic acid, diethyl ester	2.55	1.45						Premjanu and Jaynthy, 2014
9,12-Octadecadienoic acid, methyl ester	2.13							Saha *et al.*, 2022
Heptadecane			1.29		0.85		2.54	Adeyemi et al., 2017
Tetradecane			1.80					Nambi and Raju, 2017
Dodecane, 4,6-dimethyl-			1.22	1.07	0.72	1.34	1.85	Añides et al., 2019
Heneicosane			5.51	0.75	1.62	2.38	2.17	Vanitha et al., 2020
Eicosane			8.75	2.61	1.62	0.87	5.84	Octarya et al., 2021
9-octadecenal, (z)-			1.69					Subavathy and Thilaga, 2016
Gamma.-sitosterol			6.12				7.89	Akpuaka et al., 2013
Methyl Commate b			10.86			3.76		Arora and Kumar, 2017
Menthol		1.80						Freires et al., 2015
Phenol, 2,4-bis(1,1-dimethylethyl)-, phosphite (3:1)				20.75			2.32	Tyagi et al., 2021
Trimethylsilyl 2,6-bis[(trimethylsilyl)oxy] benzoate					0.22		0.33	Ajala et al., 2020
(2e)-3,7,11,15-tetramethyl-2-hexadecene					0.37			Mickymaray and Alturaiki, 2018
9-Octadecenoic acid, methyl ester, (E)-					1.90			Muflihunna et al., 2021
Hexadecanoic acid					0.63			Hameed et al., 2015
Methyl abieta-8,11,13-trien-18-oate					0.42			Burčová et al., 2018
Octadecane						0.65		Adeyemi et al., 2017
Methyl commate d						22.58		Bihana et al., 2018
2-methyloctacosane							0.80	Pelo et al., 2021
Vitamin E				12.88	9.08	15.61	2.94	Hartmann et al., 2020
% Of total antimicrobial compounds detected through GC-MS	31.54	27.72	81.19	46.86	55.14	83.42	75.24	

Albizia chinensis (AC), *Albizia odoratissima* (AO), *Acacia lenticularis* (AL), *Dalbergia sissoo* (DS), *Derris robusta* (DR), *Leucaena leucocephala* (LL), *Melia azedarach* (MA)

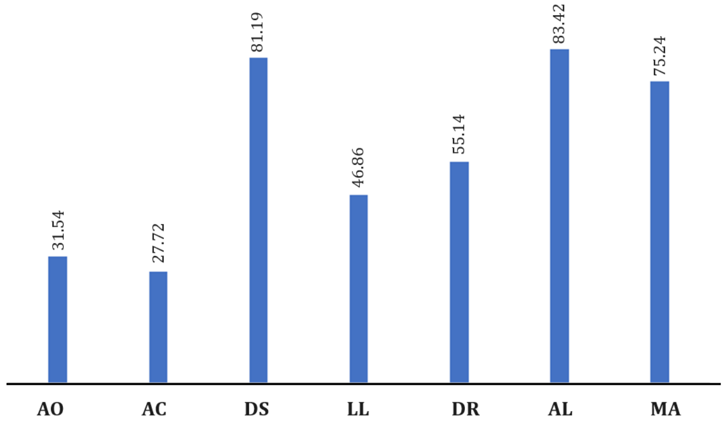

Figure 7. Percentage of total reported antimicrobial compounds detected by GC-MS in each extract

Conclusion

This research was conducted to investigate the effects of shed leaves on the soil microflora; as well as, to analyze important parameters like soil physicochemical properties, antibiotic, antifungal, heavy metal and pesticide tolerance abilities to characterize the tea garden soil covered by different shade trees. Variations in the results have been detected among soil samples (under shade trees, non-shade tree areas and vacant land) and after comparative study bring about an interesting conclusion. Shed leaf litter from these deciduous trees surely adds a huge amount of organic matter to the soil of tea gardens. Leguminous shade trees can also increase nitrogen fixation, but due to the antimicrobial effects of leaf litter or otherwise, the soil microflora can be adversely affected which is detrimental to the soil of tea plantations. However, as these shade trees are an indispensable part of the tea plantations in the plains of North Bengal they cannot be removed from the plantation. Replacement of these shade plants with other shade trees could be a probable option although further studies are needed as well. Besides these, other factors should be given thorough consideration to maintain a thriving and diverse system of beneficial microflora in tea garden soil; such as limited use of chemical pesticides and fertilizers. So, the results of this study give us a detailed understanding of the continued use of each and every shade tree in this region to inform our judgment about the overall impact of the studied shade trees on these plantations

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