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Short Communication: Metabolites of *Albizia* inhibit in vitro growth of phosphate solubilizing microbial consortia isolated from tea garden soil of Darjeeling hills, India

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Abstract. *Saha S, Ghosh A, Acharyya S, Bhattacharya M. 2022. Short Communication: Metabolites of* Albizia *inhibit* in vitro *growth of phosphate solubilizing microbial consortia isolated from tea garden soil of Darjeeling hills, India. Biodiversitas 23: 2865-2870.* Phosphate solubilizing microorganisms (PSM) play a crucial role in maintaining the nutritional status and fertility of the soil. PSMs help in solubilizing the metallic phosphate salts of soil into available phosphate anions for easy uptake by plants. However, this beneficial group of microorganisms also face different challenges for survival from its immediate surroundings. This study was carried out to assess the effect of shade tree litters on the PSM consortia isolated from different tea garden soils of Darjeeling hills, since shade trees are an integral part of tea plantations. *Albizia odoratissima*, *Albizia chinensis* and *Albizia procera*, widely used in tea gardens as shade trees were selected. GC-MS analysis was carried out to detect the metabolites produced by the litters. Twenty-three compounds exhibiting antimicrobial activities were detected. Major peak was found in *A. odoratissima*, followed by *A. chinensis* and least in *A. procera*. Compounds like 1-heptanol, 2-propyl-, neophytadiene, phytol and squalene were common in all three extracts and are considered to provided antimicrobial activity to *Albizia*. A proportional relation has been observed between magnitude of inhibition zones and peak area percentage in *Albizia* spp. PSM isolates from shade tree gardens were observed to be more tolerant toward the leaf extracts.

Keywords: *Albizia*, antimicrobial, GC-MS, PSM, shade tree, tolerance

INTRODUCTION

Plant health largely depends on the macronutrients (NPK) present in the soil of which phosphorus (P) is the second most essential element required in the field. It plays an indispensable role in the proper growth, reproduction and functioning of the plant. Nutrients play a key role in metabolic processes like photosynthesis, energy transfer and storage, signal transduction, nitrogen fixation in legumes, maintaining crop quality and resistance to plant diseases (Khan et al. 2014) and is a vital component of elongation and proliferation of roots (Wang 2021). Phosphorus is also an absolute essential element of many coenzymes, phosphoproteins and phospholipids, and forms a part of the genetic material of the plant, *i.e.*, DNA (Ozanne 1980). Thus, soil turns out to be the main reservoir for uptake of phosphorus by the plants. In fact, it has been seen that the total phosphorus content in the soil is much more than a plant usually needs (Malboobi et al. 2009). It is estimated that around 400-1200 mg kg^{-1} of phosphorus, which is around 0.05% (w/w), is present in soil, though only 0.1% (approx. 1ppm) of it is available to the plants (Rodríguez and Fraga 1999). This is because the plant cells take up P in inorganic anionic form *i.e.*, in orthophosphate ($H_2PO_4^-$ and HPO_4^{2-}) depending upon the soil pH (Beever and Burns 1981; Mahdi et al. 2011). But majority of the phosphorus in soil is immobilized by metallic elements such as Ca, Al and Fe to form calcium, aluminum and iron phosphates which are almost insoluble or completely insoluble in water. Therefore, these insoluble phosphate salts are then rendered unavailable for absorption by the plants (Walpola and Yoon 2012). These accumulated insoluble phosphates present in the soil are estimated to be adequate for plant growth with maximum yield for over a hundred years worldwide if mobilized (Walpola and Yoon 2012). In this scenario, phosphate solubilizing microorganisms (PSMs) have been reported to play a central role in the solubilization and mineralization of insoluble phosphates to anionic forms for facilitating plant uptake. PSM includes both bacteria and fungi that cooperatively form an efficient technique in solubilization process (Khan et al. 2007). Thus, proper exploitation of PSMs can trigger a productive yet cost-effective technology to increase the yield manifolds.

But, in an ecosystem, PSM faces several abiotic and biotic stresses and needs to compromise its ability or existence. Such a stressed condition prevails in the tea plantation ecosystem. In a human-influenced ecosystem of tea plantations of lower altitudes and plains, shade trees are an inseparable companion to the tea bushes. Shade trees are considered an indispensable component since they provide shade to tea bushes and prevent them from the direct

scorching sunlight (Ghosh et al. 2020). Being leguminous plants, they provide soluble nitrogen salts to the plants growing around by fixation of atmospheric nitrogen. Moreover, deciduous leguminous plants shed their leaves in winter and the dry leaves decompose in the soil. Plant parts are treasures of antibiotic molecules and secondary metabolites, so they must have some role in influencing the growth of soil microbes. Some antibacterial compounds from the members of Fabaceae have already been reported. But there is no report on the effect of shade tree litter on soil PSMs isolated from tea plantation soil. So, this work was conducted to insight into the influence of *Albizia* shade tree leaf litter on PSM growth.

MATERIALS AND METHODS

Collection of soil, physicochemical analysis and isolation of PSM

Protocol of Saha et al. (2021) was considered for collection of topsoil samples from tea gardens (three with shade trees and three without shade trees) located at the Terai and hilly terrain of Darjeeling district followed by isolation of PSM consortia. Soil samples (20 sites of each garden) were collected randomly from the shaded region of *Albizia* canopy and unshaded region, followed by mixing to make the working sample. Soil physicochemical analysis (pH, EC, OC, OM, nitrogen and phosphorus) were conducted following Saha et al. 2021. PSMs were isolated from those six working samples. For isolation, 500 mg of soil sample was added to sterile distilled water and the contents were vortexed thoroughly. 500 µL of the extract was added to one-fourth strength of autoclaved Pikovskaya's media and incubated at 30ºC for 48 hours.

Collection of shade tree litter and preparation of extracts

Leaf litters from *Albizia odoratissima* (L.f.) Benth. (AO), *Albizia chinensis* (Osbeck) Merr. (AC) and *Albizia procera* (Roxb.) Benth. (AP), were collected from tea plantations followed by separation of leaf by handpicking (Figure 1). The shade tree litters were air-dried and 0.6 g of mechanically crushed leaves were extracted with distilled water for 48 hours. Water was removed by gently heating over a rotary evaporator and the un-evaporated remains were dissolved in DMSO (0.3 gmL^{-1}).

Effect of extracts from shade tree litter on PSM growth

The effects of extracts were studied using well diffusion method of Saha et al. (2020). 100 µL of the PSM isolates were pour plated in autoclaved Pikovskaya's agar media. Wells were bored using properly sterilized cork borer and 100 µL of each extract was added to each well of the plate followed by incubation at 30ºC for 48 hours. Formations of inhibition zones around the wells, if any, were noted.

GC-MS of *Albizia*

For GC-MS analysis extract of three *Albizia* spp. (AO, AC and AP) was evaporated at low temperature and dissolved in methanol (25 mg mL^{-1}). 1 µL of extract CJLE was injected in split mode in the instrument (GCMS-QP2010 Plus, Shimadzu Co., Japan). The injection temperature was 260°C and the interface temperature was set to 270°C. The Ion Source temperature was adjusted to 230°C. Helium was used as carrier gas. The total flow rate was 16.3 mL min-1 and the column flow rate was 1.21 mL min-1. Mass spectra were recorded at 5 scan sec-1 with a scanning range of 40-650 m z-1. Quantification of compounds was done based on their peak areas (Majumder et al. 2020; Chakraborty et al. 2021).

RESULTS AND DISCUSSION

Soil physicochemical analysis and authentication of PSM

Physicochemical analysis of the soil samples collected from shaded and unshaded region depicted variable results (Table 1). pH of soil collected from shaded region were less than unshaded region, while organic carbon (OC), organic matter (OM), N and P of shaded region were higher in quantity than the unshaded region. Low pH of shade garden soil may possibly be due to accumulation of humic acid as reported by Ghosh et al. 2022. OC, OM, N and P richness may be due to nutrient recycling from leaf litter. The isolates in Pikovskaya's media were tested for authentication following Dias et al. 2009. The formation of transparent halo around the colonies confirmed that the isolates have phosphate solubilizing ability. Halo forming colonies were considered for performing downstream experiments.

Effect of extracts from shade tree litter on PSM growth

Extracts of shade tree leaf litters from AO, AC and AP showed variable inhibition zones against phosphate solubilizing consortia isolated from soil of tea gardens without shade trees (Figure 2). Among the leaf litter extracts, the PSMs demonstrated maximum inhibition towards the leaf extracts of AO (2.9±0.2361) followed by AC (2.2±0.3642) and AP (1.8±0.4672) (Figure 3). Interestingly there was no visible inhibitory effect on PSM consortia isolated from soil with shade trees.

Table 1. Physicochemical properties of soil sample collected from shaded and non-shaded region

	Sample	pH	EC (µS/cm)	OC (%)	OM (%)	N (%)	P (ppm)
With shade	2	3.93	375	6.31	10.85	0.57	30
	5	3.78	417	5.41	9.30	0.48	36
	10	3.87	367	5.92	10.18	0.54	17
Without	6	4.05	490	4.45	7.65	0.39	12
shade	9	4.14	317	4.01	6.89	0.37	6
	13	4.21	510	3.98	6.84	0.36	16

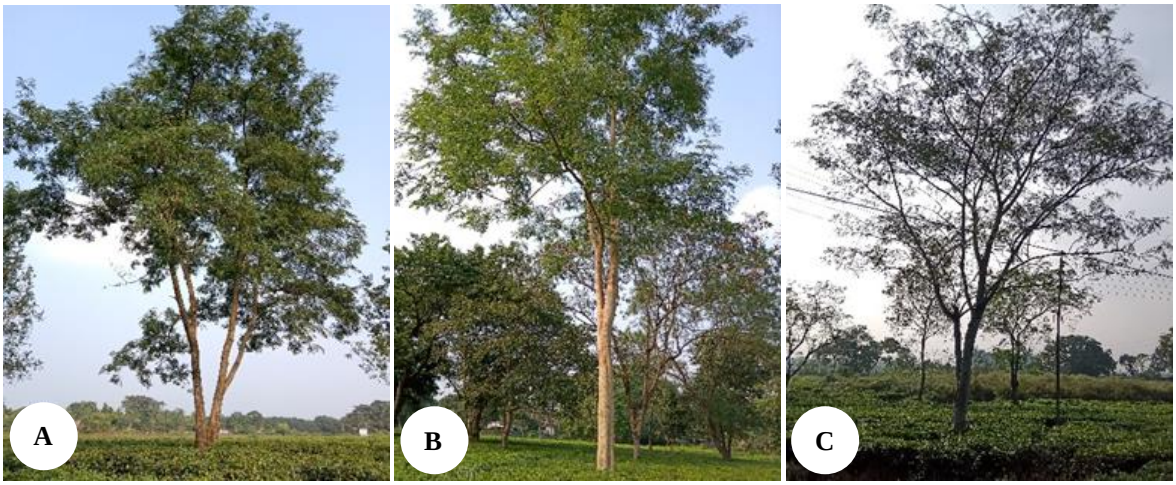

Figure 1. A. *Albizia odoratissima* (L.f.) Benth. B. *Albizia procera* (Roxb.) Benth. C. *Albizia chinensis* (Osbeck) Merr

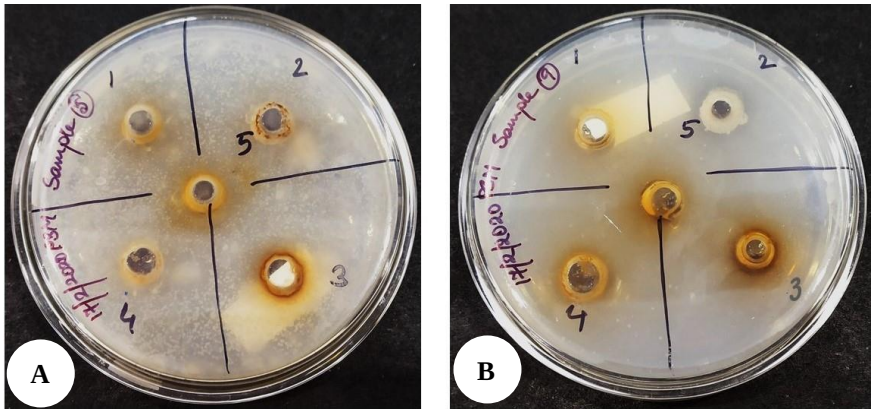

Figure 2. Plate A and B show minimum and maximum inhibition by the PSM isolates of shaded and non-shaded tea gardens against the leaf extracts of AC, AP and AO marked as 1, 2 and 3 respectively

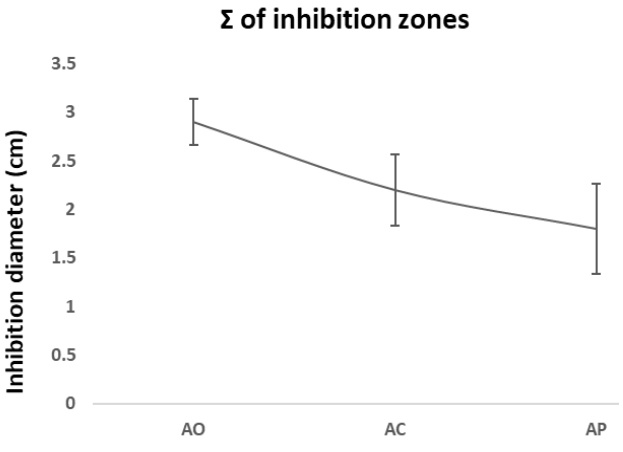

Figure 3. Graphical representation of summative zones of inhibition by leaf litter extracts on growth of PSM isolated from tea garden soil without shade trees (average of three replicates)

GC-MS of *Albizia*

GC-MS was conducted to detect the antimicrobial candidates conferring growth-inhibiting properties to the *Albizia* leaf litter extracts. The results of GC-MS reveal that twenty-three compounds (Table 2) exhibiting antimicrobial, antibacterial, antifungal or antibiotic activities were detected. AO, AC and AP contained twelve, nine and thirteen compounds respectively, though peak area coverage percentage was highest in AO (32.08%), followed by AC (26.6%) and least in AP (22.37%). Four bioactive compounds (1-Heptanol, 2-propyl-; neophytadiene; phytol and squalene) were common in all the three plant extracts and cover 12.3%, 6.16% and 12.98% area in extracts of AO, AC and AP respectively. These four compounds showed over four percent coverage in peak area in either of the extracts (Figure 4-6).

The GC-MS analysis of the extracts of *Albizia* spp. Revealed the presence of many antimicrobial compounds of which four bioactive compounds were common in all the three species. Among the four common bioactive compounds 1-heptanol, 2-propyl- (2-propyl-1 heptanol) is an oxo-alcohol (branched fatty alcohol) biosynthesized by pathway initiating in the chloroplast (https://www.genome.jp/kegg/). This compound detected in *Aloe vera* (Lakshmi and Rajalakshmi, 2011) was reported as antimicrobial (Ghosh et al. 2021).

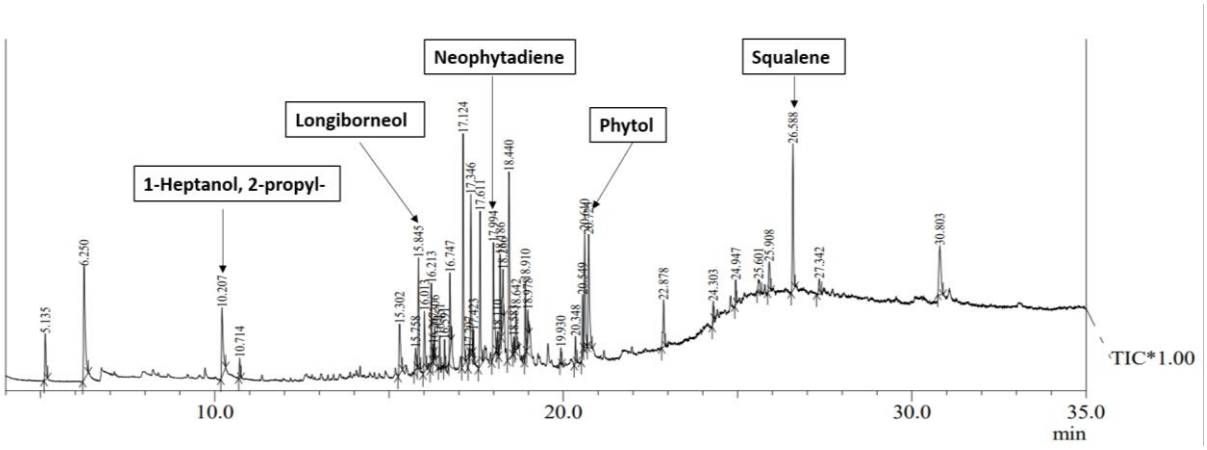

Figure 1. Chromatogram of major antibacterial and antifungal compounds from AO detected in GC-MS

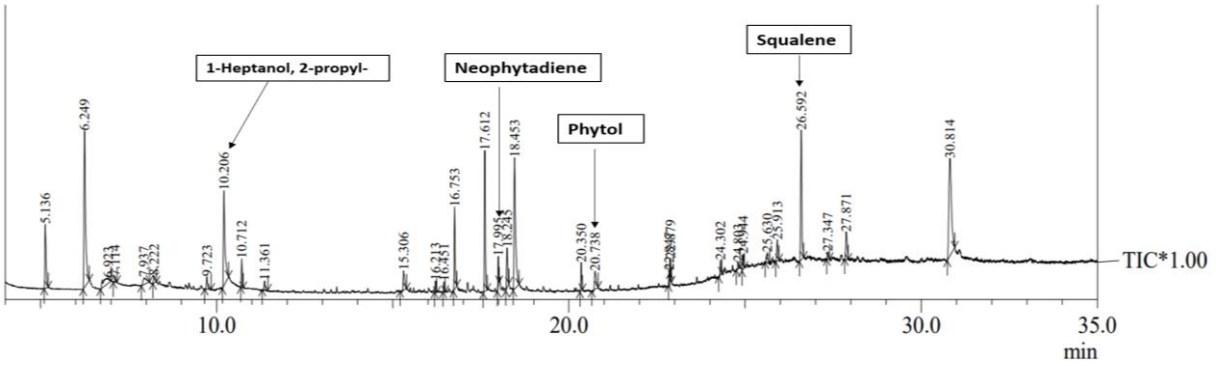

Figure 2. Chromatogram of major antibacterial and antifungal compounds from AC detected in GC-MS

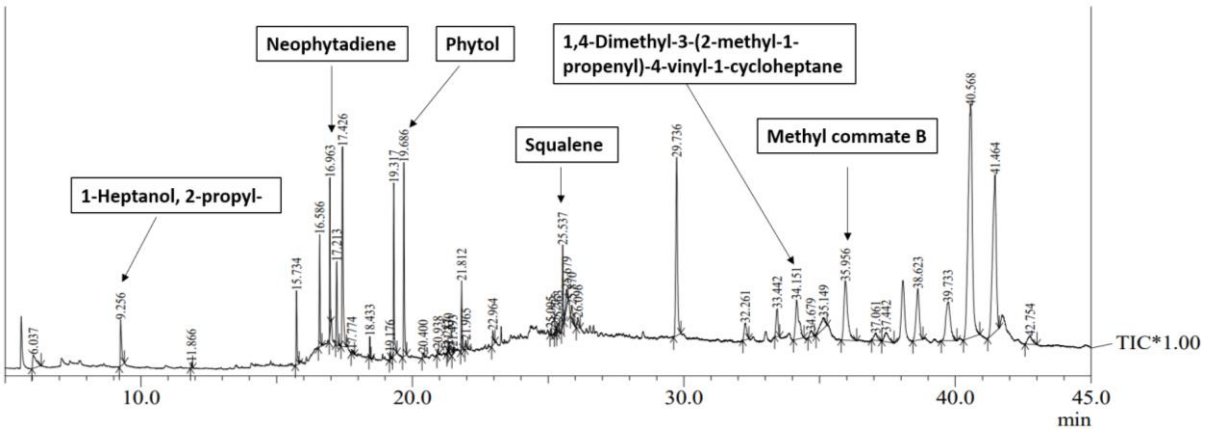

Figure 3. Chromatogram of major antibacterial and antifungal compounds from AP detected in GC-MS

Phytol (3,7,11,15-tetramethyl-2-hexadecen-1-ol-), a constituent of chlorophyll is an acyclic diterpene long-chain primary fatty alcohol. Chemically it is hexadec-2-en-1-ol with alkylation across 3,7,11 and 15 'C' positions having previous report as a surface disinfectant (Ghaneian et al. 2015), antibacterial agent against *Pseudomonas aeruginosa* (Lee et al. 2016) and antifungal restricting the pathogen fungi *Macrophomina phaseolina* (Banaras et al. 2017). Squalene is an antibacterial (Ghosh et al. 2021) triterpenoid moiety of 2,6,10,15,19,23-hexamethyltetracosane constituting of six double bonds at the 2-, 6-, 10-, 14-, 18- and 22- 'C' positions with (all-E)-configuration. It has previously been reported in olive oil, soybean oil, rice, grape seed oil, peanut, corn, and amaranth (Lozano-Grande et al. 2018). Methyl commate B (Methylursolate) is a triterpene glycoside biosynthesized in plant cytosol from squalene 2,3-oxide through steroid saponin biosynthetic pathway (Majumder et al. 2020). It shows antibacterial

activity against *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Candida albicans* (Fatima et al. 2017). Besides these four compounds methyl commate B unique in AP covered a peak area of 5.59%. These five compounds are considered prime contributors for conferring antimicrobial activity to *Albizia*. of these antimicrobial metabolites neophytadiene, phytol, squalene and methyl commate B are terpenoids.

Sixteen antimicrobial compounds were unique in either of the *Albizia* extracts. Six compounds (.gamma.-Linolenic acid, methyl ester; cyclopentadecanone, 2-hydroxy-; dehydroabietylamine; 1,4-methanoazulene,decahydro-4,8,8trimet or longifolene; 1,4-methanoazulen-9-ol, decahydro-1,5,5,8a- or longiborneol and 9,12-octadecadienoic acid, methyl ester) in AO covering an area of 9.13%, two compounds (dehydroabietic acid and its TMS derivative and ethanone, 1-phenyl-) in AC covering an area of 2.69 and eight compounds (9-octadecenamide, (Z)-; .beta.-Amyrin; 4,4,6a,6b,8a,11,11,14b-Octamethyl-1,4,4a,5,6,6a,6b,7,8,8a,9 or .beta-amyrone; methyl commate B; pentafluoropropionic acid, heptadecyl ester; phenol; stigmasterol and 1,4-dimethyl-3-(2-methyl-1-propenyl)-4-vinyl-1-cycloheptane) in AP covering an area of 12.88% were unique in their respective extracts. Besides them, 1,4-methanoazulen-9-ol, decahydro-1,5,5,8a- or longiborneol (3.14%); 1,2benzenedicarboxylc acid, diethyl ester (2.55%) and 9,12-octadecadienoic acid, methyl ester (2.13%) in AO and 1,4-dimethyl-3-(2-methyl-1-propenyl)-4-vinyl-1-cycloheptane (3.33%) in AP showed moderate presence. Longiborneol shows antifungal activities against *Candida albicans*, *Cryptococcus neoformans*, *Trichophyton rubrum*, *Aspergillus fumigatus*, *Pycricularia oryzae* (Guo et al. 2008) and antibacterial potential against *Staphylococcus aureus* (Demetzos et al. 2002). 1,2benzenedicarboxylc acid, diethyl ester is antibacterial and antifungal (Ghosh et al. 2021). 9,12-octadecadienoic acid, methyl ester is antibacterial (Ghosh et al. 2021) and antifungal against phytopathogenic fungus *Macrophomina phaseolina* (Banaras et al. 2017). 1,4-dimethyl-3-(2-methyl-1-propenyl)-4-vinyl-1-cycloheptane shows antimicrobial property (Ghosh et al. 2021). These sixteen compounds also have some inhibitory activity for PSM growth. They have reported antibacterial, antimicrobial and antifungal activities. Antibacterial activity has been reported in 9-octadecenamide, (Z)- ; beta–amyrin); .gamma. -Linolenic acid, methyl ester; dehydroabietylamine; longifolene; 1,2-benzenedicarboxylic acid, diethyl ester (Ghosh et al. 2021); cyclopentadecanone, 2-hydroxy- (Oyedoh et al. 2020); dehydroabietic acid and its TMS derivative (Fukui et al. 1978); pentafluoropropionic acid, heptadecyl ester (Pandey et al. 2011); phenol (Nwosu et al. 2022); stigmasterol (Vida et al. 2012); Antimicrobial property was observed in 13-hexyloxacyclotridec-10-en-2-one (Malayaman et al. 2019); longifolene; 1,2-benzenedicarboxylic acid, diethyl ester; 1,4-dimethyl-3-(2-methyl-1-propenyl)-4-vinyl-1-cycloheptene (Ghosh et al. 2021). Activities against fungal isolates were reported in beta–amyrin (Ghosh et al. 2021); dehydroabietic acid and its TMS derivative (Feio et al. 1999); dehydroabietylamine; 13-hexyloxacyclotridec-10-en-2-one; ethanone, 1-phenyl-; 1,2-benzenedicarboxylic acid, diethyl ester (Ghosh et al. 2021). So, all these compounds have individually or synergistically expressed growth-inhibiting activity on PSM consortia.

The extracts of *Albizia* leaf litter inhibited the growth of PSM consortia isolates from the tea garden soil of Darjeeling because of antimicrobial compounds present in them. The inhibition zones observed on the isolates of shade tree gardens were much less as compared to the non-shade tree gardens. In our previous report on PSM consortia isolates isolated from different tea plantation soils of Darjeeling by Saha et al. 2021, it was observed that prolonged use of pesticides in tea plantations has induced tolerance towards some pesticides. Therefore, we assume that PSM isolates of shade tree gardens must also have acquired some tolerance over the antimicrobial candidates of *Albizia* thus showing no or less inhibition zones. A proportional relation has been observed between the magnitude of inhibition zones and summation of peak area percentage in *Albizia* spp.

So, it may be logical to say that PSM consortia must have gained tolerance against one or synergistic effect of secondary metabolites present in *Albizia* leaf litter. This lack of inhibition zone in PSM consortia i.e., gain in tolerance ability of PSM consortia isolated from soil of shaded tea gardens may be a blessing to tea plantation soil microflora as the consortia of PSM is inevitable for solubilizing insoluble phosphates present in the soil.

Table 2. Characterization of leaf extracts of *Albizia odoratissima*, *Albizia chinensis* and *Albizia procera* over the presence of different antimicrobial compounds

Compounds with antibacterial and antifungal activity	**GC-MS (Peak area %)**		
	AO	**AC**	**AP**
1-Heptanol, 2-propyl-	3.69	8.7	1.54
9-Octadecenamide, (Z)-			0.25
13-Hexyloxacyclotridec-10-en-2-one		1.34	0.1
beta-Amyrin			0.45
beta-Amyrone			0.63
. gamma. -Linolenic acid, methyl ester	1.4		
Cyclopentadecanone, 2-hydroxy-	0.61		
Dehydroabietic acid and its TMS derivative		1.42	
Dehydroabietylamine	0.81		
Ethanone, 1-phenyl-		1.27	
Methyl commate B			5.59
Neophytadiene	4.94	1.27	2.26
Pentafluoropropionic acid, heptadecyl ester			0.4
Phenol			1.17
Phytol	5.64	1.77	3.94
Squalene	5.59	8.7	1.65
Stigmasterol			1.06
Longifolene	1.04		
Longiborneol	3.14		
1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester	0.62	0.68	
1,2-Benzenedicarboxylic acid, diethyl ester	2.55	1.45	
1,4-Dimethyl-3-(2-methyl-1-propenyl)-4-vinyl-1-cycloheptane			3.33
9,12-Octadecadienoic acid, methyl ester	2.13		

In conclusion, tea bush-shade tree association has several positive roles to play in the tea plantation ecosystem. This research work with the aim to study shade tree litter-PSM interaction observed deleterious effect of shade trees on the growth of PSM consortia isolated from soil of unshaded tea plantations. Our results highlight tolerance of PSM isolates from shade tree gardens towards *Albizia* leaf extracts; so, investigating PSM with tolerance towards shade tree antimicrobials can be focused for future research.

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