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Isolation, Identification and Characterization of Microbes Associated with Leaves of Roadside Tree Species under Different Pollution Levels

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ABSTRACT

Air pollution is a significant issue in urban areas today. Plants play a crucial role in absorbing and adsorbing air pollutants, making them the first line of defense. Prolonged exposure to pollution leads to changes in various aspects of plants, including their appearance, functions, biochemistry and even the microorganisms that live on their leaves. In this study, researchers compared the phyllosphere (the microbial community on leaves) of eight common roadside trees between a control and polluted site. They observed that exposure to air pollution alters both the plants and the microbes associated with them. Interestingly, the microbial count was highest at the less polluted site (13.50 CFU/ml for bacteria and 3.44 CFU/ml for fungi) compared to the polluted site (2.50 CFU/ml for bacteria and 2.44 CFU/ml for fungi). Trees in polluted areas showed an abundance of specific bacteria such as *Shigellae*, *Enterobacter* and *Streptococcus* and fungi like *Absidia*, *Mucor*, *Phialophora* and *Aspergillus*. Additionally, these trees exhibited increased production of secondary metabolites such as phenols and ascorbic acid. Among the different species studied, *Acacia auriculiformis* showed the highest relative abundance of these characteristics. These findings underscore how changes in air quality affect the phyllosphere of plants. They provide valuable insights into how plants adapt to polluted environments and suggest that the phyllosphere could be a cost-effective resource for bioremediation of air pollution in urban settings.

Keywords : Air pollution, Phyllosphere, Secondary metabolites, Urban setting, Roadside trees

VEHICLE emissions are a significant contributor to air pollution in urban areas. The levels of air pollutants such as particulate matter, nitrogen oxides, sulfur dioxide and volatile organic compounds have reached alarming levels (Archibald *et al.*, 2017). In India, for instance, people are exposed to approximately 90 µg/m³ of PM 2.5 daily, well above the national standard *i.e.,* 60 µg/m³ (Balakrishnan *et al.*, 2017). The World Health Organization has identified air pollution as the largest environmental health threat globally (WHO, 2016). In India alone, air pollution is responsible for around 600,000 premature deaths annually (Ghude *et al.*, 2016). Continuous exposure to pollutants, especially along roadways, is causing a rise in health problems. Planting vegetation along side roads can effectively serve as a surface to capture air pollutants. Plant leaves, which cover approximately 4×10^8 km² of the earth's surface (Kembel *et al.*, 2014), along with the micro organisms on their surfaces (known as phyllosphere), play a crucial role in enhancing air pollution tolerance. Air pollutants get adsorbor /absorb to plant leaves, microflora associated with their aerial parts known as phyllosphere (Last 1955) contributes towards plants air pollution tolerance and reported to be able to biodegrade or transform pollutants into less toxic molecules (Last, 1955; Mueller *et al.*, 1996 and Ma *et al.*, 2016). It is an important aspect to have a reliable economic way out for future air pollution-related increasing problems. In the polluted environment, changes in plant's morphological, physiological and biochemical parameters are associated with a change in phyllosphere composition. Even different tree/ plant species vary in their phyllosphere composition (Redford *et al.*, 2010). Micro organisms such as bacteria, fungi and algae that inhabit leaves are crucial for plant health and ecosystem functioning (White *et al.*, 2013). The microbiome of the plant phyllosphere has been shown to play an important role in the adaptation of the plant host to different environmental stressors by enhancing tolerance to heat, cold, drought and salinity (Whipps *et al.,* 2008; Kembel *et al.,* 2014; Martirosyan & Steinberger, 2014; Agler *et al*., 2016 and Saleem *et al*., 2017). These microorganisms form complex communities that can influence leaf function, nutrient cycling and plant disease resistance (Selosse *et al*., 2017). For example, certain bacteria can fix nitrogen, enhancing soil fertility, while fungi such as mycorrhizae form symbiotic relationships with plant roots, aiding in nutrient absorption (Jones *et al*., 2009). Additionally, some fungi and algae can help protect leaves from pathogens by outcompeting harmful microbes (Anderson *et al*., 2009). Overall, these micro organisms contribute to the dynamic balance of plant ecosystems and the overall health of vegetation. With prolonged exposure to the polluted environment, some plants and so their phyllosphere acquire tolerance and thus, it becomes necessary to find out microflora associated with trees in different localities so that trees can be smartly used in landscaping either for phylloremediation (tolerant trees and their leaf associated microflora) or as biomonitor (susceptible trees) of air pollution. But the potential of trees and their leaf associated microbes for air remediation and their distribution in polluted and non-polluted phyllosphere is still unexplored. Microorganisms play a crucial role in enhancing plant tolerance to pollution, helping to mitigate the impact of contaminants on ecosystems. For instance, rhizobacteria such as *Pseudomonas putida* and *Bacillus cereus* can improve plant resilience to heavy metals like lead and cadmium by either detoxifying these metals or stimulating the plant's own stress response mechanisms (Khan *et al*., 2019). Similarly, arbuscular mycorrhizal fungi like *Glomus intraradices* are beneficial in contaminated soils, where they enhance plant growth by improving nutrient uptake and reducing metal toxicity (Alvarado *et al*., 2019). Endophytic fungi, such as *Fusarium oxysporum*, assist plants in tolerating organic pollutants, including pesticides and hydrocarbons, by degrading these substances or aiding in their removal (Davies *et al*., 2012). In the context of air pollution, algae like *Chlorella vulgaris* are used in biofilters to absorb airborne pollutants, including carbon dioxide and particulate matter (Xu *et al*., 2018). Lastly, nitrogen-fixing bacteria such as *Rhizobium* species maintain plant growth and soil health in polluted environments by fixing atmospheric nitrogen, which is crucial for plant nutrition (Garcia *et al*., 2018). These microorganisms not only support plant health but also contribute to broader environmental remediation efforts.

From the information provided, it's evident that roadside greenery serves as a natural buffer against air pollution from vehicle emissions, absorbing pollutants directly through their leaves. However, there's a notable absence of detailed studies on how roadside trees in India, particularly in Ludhiana, Punjab, contribute to phylloremediation the process by which plants and their associated microbes mitigate air pollution. To address this gap, the current study aims to conduct preliminary research focused on isolating and identifying bacteria and fungi found on the leaf surfaces of selected roadside trees in Ludhiana, Punjab. This initial step is crucial for identifying microbes that may play a role in enhancing plant tolerance to pollution.

MATERIAL AND METHODS

Study Area

Ludhiana city (30°-34' and 31°-01' and N to75°-18' and 76°-20' E latitude) has spread over an area of 159.37 km^2 having population of 1.86 million in 2019 with growth rate of 1.48 per cent with a height of 247 m above the sea level. Eight most common roadside trees (*Acacia auriculiformis*, *Alstonia scholaris*, *Cassia fistula*, *Cassia siamea*, *Chukrasia tabularis*, *Dalbergia sissoo*, *Heterophragma adenophyllum* and *Putranjiva roxburghii*) were selected at control /less polluted site (Punjab Agricultural University, Ludhiana) and Polluted site (NH-5). As reported by many earlier studies that particle concentrations will be highest closest to than 100-250 meters from the highway (De Winter *et al*., 2018; Ridem, 2019 and Rattigan *et al*., 2020). The samples were collected 100 m away from roadside from interior area of the campus which was treated as control site. Due to rapid industrialization and urbanization in Ludhiana, air pollution levels is reported quite high which makes it a suitable place for air pollution related studies (Rajesh Kumar *et al*., 2010 and Goel *et al*., 2021).

Sample Collection

Trees of uniform age, size and canopy spread were carefully selected to ensure consistency across the study. The research encompasses eight common roadside ornamental trees, each treatment replicated three times at both control and polluted sites. Leaf samples were collected from selected trees from control (100 m away from main road) and polluted site (next to road) at mid canopy height (1 to 2 m). These samples were immediately placed in ice-filled containers and stored in plastic zip-lock bags. Microbiological analysis commenced within 8 hours of sampling. The isolation of microbes followed a two-step process as outlined by Dickinson (1971). Firstly, the leaves were aseptically shredded into small pieces within a laminar flow hood. Approximately one gram of leaf sample was transferred to peptone water, which served as an enrichment medium. The suspension was then incubated under shaking conditions for 2 hours to detach all microorganisms from the leaf surfaces. After enrichment, the microbial suspension was serially diluted and spread onto specific media: nutrient agar (NA) for bacteria and glucose yeast extract (GYE) for fungi. Incubation followed at 37°C for 24 hours for NA plates and at 25°C for 3-5 days for GYE plates to facilitate optimal growth. Subsequently, the plates were examined for various parameters including total plate count, relative abundance (adapted from Walag and Canencia, 2016), colony characteristics, Gram staining as per Bergey's Manual and biochemical tests such as catalase, oxidase, nitrate reductase, starch hydrolysis, citrate utilization, gelatin liquefaction, methyl red and Voges-Proskauer reactions (Cheesbrough, 2006; Aneja, 2006; Olutiola *et al*., 2000 and Hunter & Barnett, 2000). These steps were part of the phenotypic characterization to identify and study

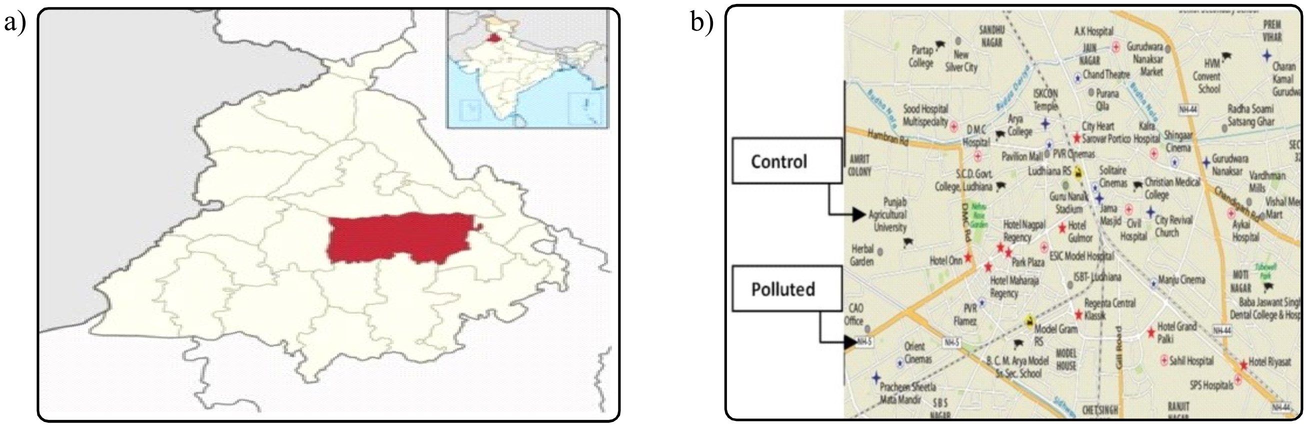


Fig. 1 : Map showing study area; a) Ludhiana District; b) Locations PAU campus (Control), NH-5 (Polluted)

the microbial communities associated with the leaf surfaces of roadside trees in Ludhiana, Punjab.

The identification of fungi involved morphological and microscopic analysis, which included examining the type of spores and conidia. This examination was conducted after staining with cotton blue lactophenol, following the method described by Hunter and Barnett (2000).

Statistical Analysis

The data were analyzed using a factorial Completely Randomized Design. Mean comparisons were conducted using Tukey's test at a significance level of 5 per cent, employing SAS software version 9.2 on a computer.

RESULTS AND DISCUSSION

Microbial colony forming units (CFU/ml) from bacterial and fungal cultures were assessed at both sites: Control and Polluted (Devakumar *et al.*, 2018) (Fig. 2). CFU counts were higher at the control location compared to the polluted site. Abdelfattax *et al.,* 2015; Venkatachalam *et al.,* 2016; Bhattacharyya *et al.*, 2017; Alsohaili & Bali-hasal, 2018 and Ray *et al.*, 2019. At control site, bacteria's were found dominating as compare to fungi (Thamchaipenet *et al.*, 2010 and Sanchez-Lopez *et al.*, 2018). A total of six bacterial isolates were purified from leaves of selected roadside trees at both control and polluted locations, based on their distinctive visual characteristics. These isolates were subsequently characterized by their colony morphology, which varies among bacterial genera.

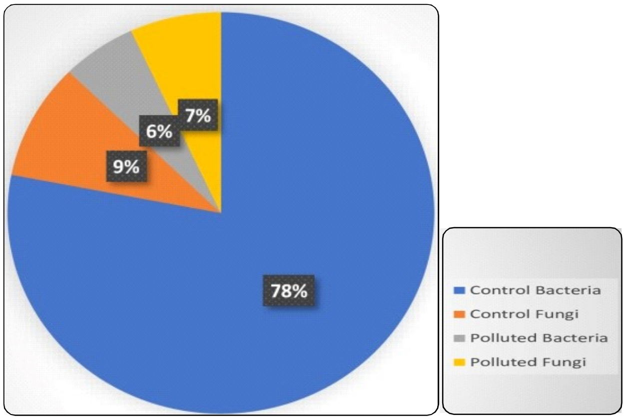


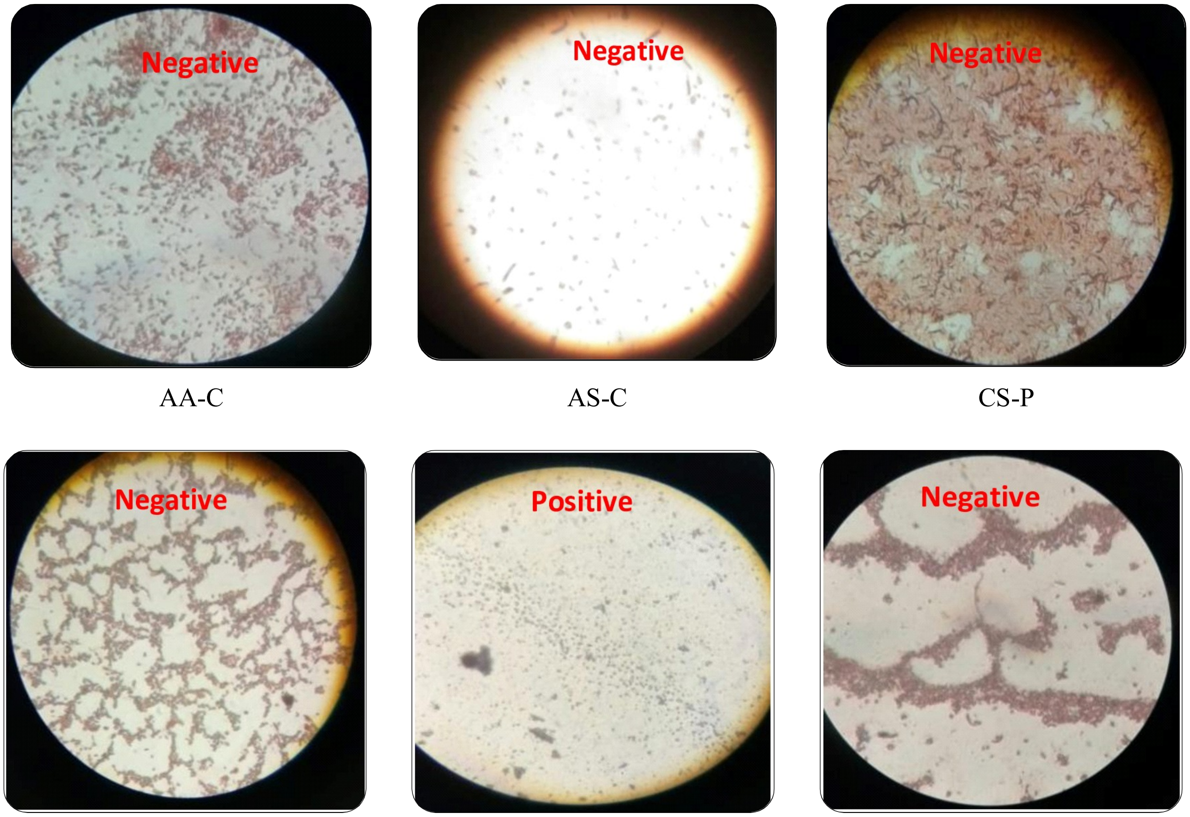
Fig. 2 : Plate count of bacteria isolated from leaves of roadside trees from control and polluted locations

TABLE 1

Morphological and biochemical characteristics of bacterial isolated from leaves of roadside trees from control and polluted locations

Sample	Colour	Optical Property	Texture	Shape/ Colony	Elevation	Appearance	Gram's test	Starch	Citrate	Oxidase	Catalase	Nitrate reductase	MR test	VP test	Gelatin	Expected genera
AA-C	Creamish	Opaque	Smooth	Cocci/ Undulate	Flat	Dull	-	+	+	+	-	-	+	-	+	*Yersinia*
AS-C	Creamish	Translucent	Smooth	Short Rods /Undulate	Flat	Shiny	-	+	+	+	-	-	+	-	-	*klebsiella*
CS-P	White	Opaque	Smooth	Bacillus/ Round	Flat	Dull	-	+	-	+	-	+	+	-	+	*Shigellae*
CT-C	Whitish Cream	Opaque	Smooth	Cocci/ Undulate	Flat	Dull	-	+	+	+	-	-	-	-	-	*Enterobacter*
DS-P2	Creamish	Opaque	Smooth	Cocci/ Undulate	Flat	Shiny	+	+	-	+	-	-	-	-	+	*Streptococcus*
PR-P	Creamish	Opaque	Smooth	Cocci/ Undulate	Flat	Dull	-	+	+	+	-	-	-	-	-	*Enterobacter*

AA-C: *Acacia auriculiformis* (Control); AS-C: *Alstoniascholaris* (Control); CS-P: *Cassia siamea* (Polluted);
CT-C: *Chukrasia tabularis* (Control); DS-P: *Dalbergia sissoo* (Polluted); PR-P: *Putranjiva roxburghii* (Pollution)



AA-C AS-C CS-P

CT-C DS-P PR-P

AA : *Acacia auriculiformis* (Control); AS-C : *Alstonia scholaris* (Control); CS-P : *Cassia siamea* (Polluted);
CT-C : *Chukrasia tabularis* (Control); DS-P : *Dalbergia sissoo* (Polluted); PR-P : *Putranjiva roxburghii* (Polluted)

Fig. 3 : Microscopic examination of Gram's Test on isolated pure bacterial cultures from control and polluted locations

Various morphological and biochemical traits of these isolates were analyzed and are summarized in Table 1 and Fig. 3.

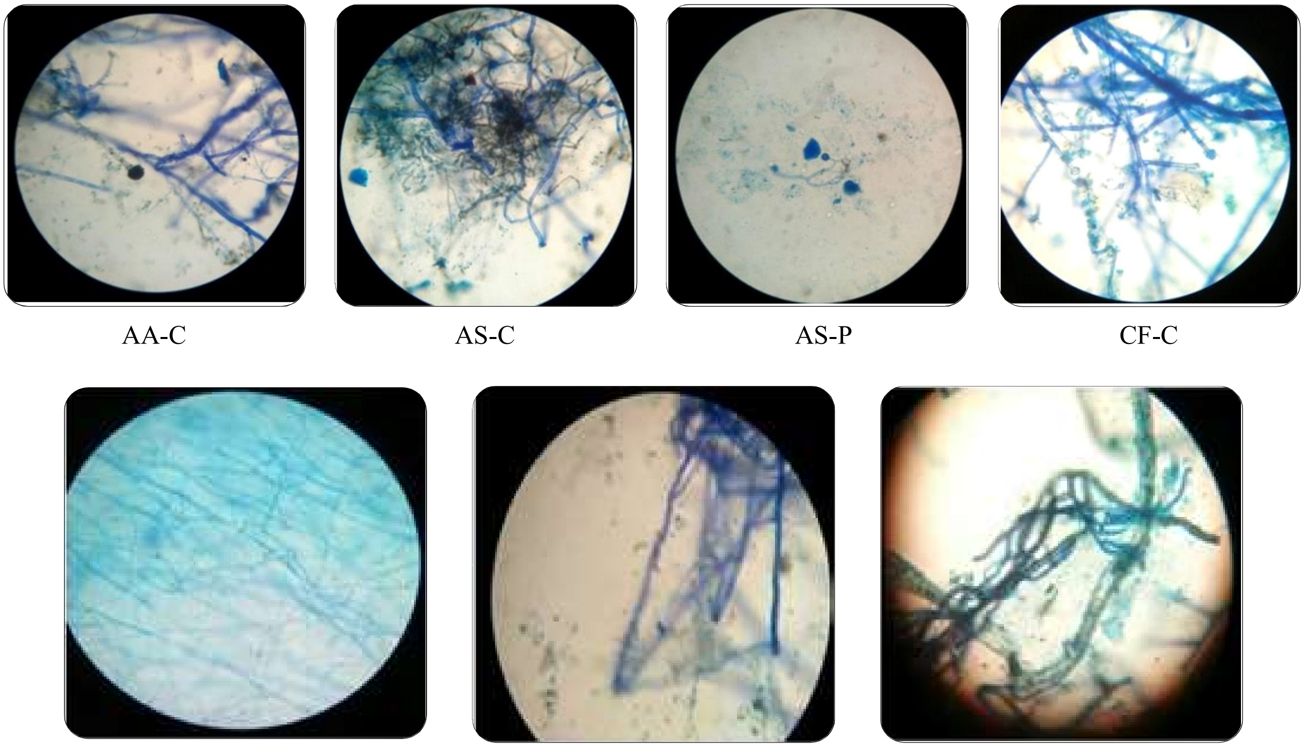
The study involving selected roadside tree species, seven fungal cultures were chosen for detailed microscopic examination based on their distinctive appearance. Morphological characterization was conducted on these fungal isolates purified from leaves collected at both control and polluted sites. Table 2 and Fig. 4, present various macroscopic and microscopic features observed in these fungal isolates. Characteristics such as colony morphology, spore type, hyphal structure, septation and branching patterns were assessed to identify the fungal isolates up to the genus level, following the methodology described by Wang *et al*. (2016). Fig. 5, depict the levels of phenol and ascorbic acid content in eight ornamental roadside trees across two locations. The content was consistently higher at the polluted location compared to the control site for all trees examined.

Our study investigated the effects of air pollution on leaf-associated microbial communities across eight tree species, revealing significant impacts on both microbial abundance and plant biochemical responses. At the control site, where pollution levels were lower, trees showed significantly higher colony-forming unit (CFU) counts, suggesting a more favorable environment for microbial growth. In contrast, the polluted site, characterized by elevated particulate matter, showed a marked reduction in microbial populations (Joshi, 2007 and Chaudhuri, 2017) Notably, specific bacterial genera such as *Shigella* and *Enterobacter* along with fungal genera like *Absidia*, *Mucor*, *Phialophora* and *Aspergillus* were more abundant at the polluted location (Kang *et al*.,

TABLE 2
Morphological characteristics of fungal isolated from leaves of roadside trees from the control and polluted locations

Source of culture	Microscopic features	Macroscopic features	Expected genera
AA-C	Sparsely septate broad hyphae	Cottony growth	*Rhizopus*
AS-C	Waxy bent broad mycelia with intercalating spores	The growth rate of colonies slow, texture velvety, with black colour	*Cladosporium*
AS-P	Small pear-shaped sporangia	Wooly grey colour fast-growing colonies	*Absidia*
CF-C	aseptate hyphae with sporangiospores	Cottony growth	*Rhizopus*
CS-P1	Ribbon like aseptate hyphae with right-angle branching	Fluffy cottony growth	*Mucor*
CS-P2	Branched coenocytic mycelium	Black velvety growth	*Phialophora*
CT-P	Broad septate hyphae with acute angle branching	Grayish green colour colonies	*Aspergillus*

AA-C: *Acacia auriculiformis*(control); AS-C: *Alstonia scholaris* (control); AS-P: *Alstonia scholaris* (Polluted); CF-C: *Cassia fistula* (Control); CS-P: *Cassia siamea* (Polluted); CT-P: *Chukrasia tabularis* (Polluted)

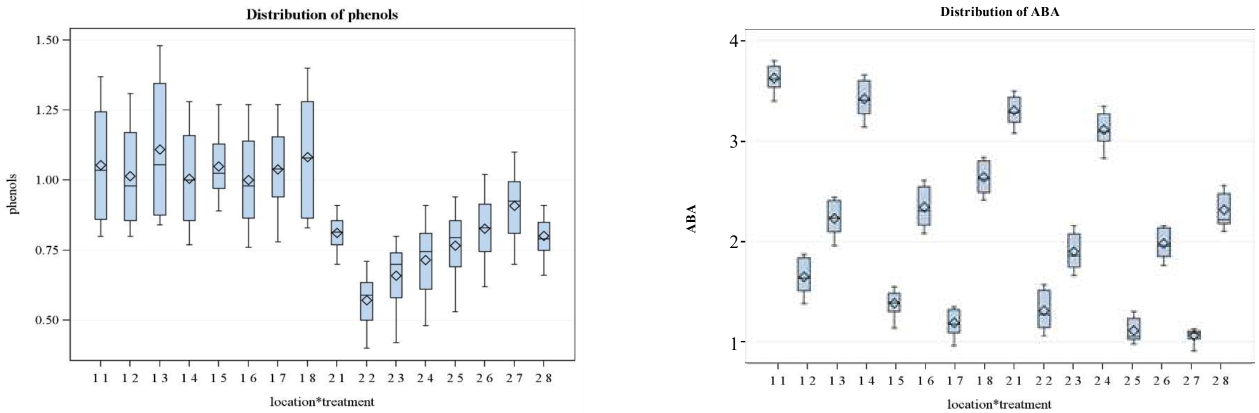


AA-C AS-C AS-P CF-C

CS-P1 CS-P2 CT-P

AA-C : *Acacia auriculiformis* (control); AS-C : *Alstonia scholaris* (control); *Alstonia scholaris* (Polluted);
CF-C : *Cassia fistula* (Control); CS-P : *Cassia siamea* (Polluted); CT-P : *Chukrasia tabularis* (Polluted)

Fig. 4 : Microscopic Examination of isolated pure fungal cultures from control and polluted locations



Treatments : 1 : *Acacia auriculiformis,* 2 : *Alstonia scholaris,* 3 : *Chukrasia tabularis,* 4 : *Cassia fistula, 5* : *Cassia siamea, 6* : *Dalbergia sissoo,* 7 : *Heterophragma adenophyllum,* 8 : *Putranjiva roxburghii*; Location : 1 : Polluted; 2 : Control

Fig. 5 : Variation in phenol and Ascorbic acid (ABA) content of selected roadside tree species growing at two different locations in Ludhiana city

2016; Bharti *et al*., 2012 and Li *et al*., 2018). These genera include opportunistic and pathogenic species, suggesting that pollution can create niches for harmful microbes while reducing populations of more beneficial ones.

Secondary metabolite production also provided insights into plant responses to pollution. Consistent with previous studies (Chandawat *et al*., 2014 and Alhesnawi *et al*., 2018), trees at polluted sites exhibited higher levels of total phenols and ascorbic acid. This increase is likely a defensive response to elevated oxidative stress resulting from pollution, reflecting the plants' attempts to mitigate environmental stressors through biochemical means.

These findings have significant implications for urban ecosystem management. The altered microbial dynamics and increased secondary metabolite production underscore the importance of understanding how air quality affects both plant health and microbial communities. The presence of metal-tolerant Enterobacter at polluted sites suggests a potential role in bioremediation, utilizing metal pollutants for growth. This insight could guide strategies for pollution management through targeted microbial interventions.

Moreover, the study highlights the need for incorporating these insights into urban green space design. By selecting tree species with high resilience to pollution and beneficial microbial interactions, urban planners can enhance both environmental quality and aesthetic value. Effective green infrastructure, including parks, green belts and green walls, can improve air quality and support plant health.

Ultimately, understanding the interactions between plants, microbes and pollutants provides a foundation for developing sustainable urban environments. These insights are crucial for advancing strategies that integrate ecological and environmental considerations into urban planning and management practices, aiming to create resilient and healthy urban ecosystems.

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