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RESEARCH

EXPLORING MORPHOLOGICAL DETERMINANTS AND PHYTOCHEMICAL VARIATIONS IN *PLUMBAGO ZEYLANICA* L. ACROSS FOUR DISTRICTS OF WEST BENGAL

Sourav Mukherjee and Sudipta Roy*

Department of Botany, University of Kalyani, Kalyani, Nadia, 741235, West Bengal, INDIA
Email:dr.sudiptaroy@gmail.com

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Abstract: *Plumbago zeylanica* L. (Family: Plumbaginaceae) is known for its immense therapeutic potential. This study aims to find a relationship between the morphological characters and phytochemical contents of naturally grown four accessions of *P. zeylanica* from different districts of West Bengal. Different plant descriptors were studied for their morphological variations. The phytochemical contents from various plant parts demonstrated the superiority of the root. Morphological parameters and root phytochemicals revealed a strong correlation between total tannin content (TTCR) and leaf characters and between total phenolic content (TPCR) with stem diameter and petiole base width. The plumbagin content correlated with root fresh weight, petiole base width, and salt gland index. The studied environmental parameters showed an influence on the morphological and phytochemical spectrum. Principal component and cluster analysis distinguished the four accessions to identify an elite chemotype of *P. zeylanica*.

Keywords: Correlation, Morphology, Multivariate analysis, Phytochemical, *Plumbago zeylanica*

INTRODUCTION

Plumbago zeylanica L., popularly known as 'Chitrak' in Ayurvedic medicine, is a perennial shrub distributed in tropical and subtropical regions of the World. It is commonly found as a naturally grown plant in Madhya Pradesh, Uttar Pradesh, Haryana, Chhattisgarh, Punjab, Bihar, West Bengal, and Southern India. This plant exhibits many bioactivities, including astringent, expectorant, cytotoxic, antibacterial, antifungal, anthelmintic, anti-inflammatory, antihyperglycemic, antidiabetic, anticancer, and anti-allergic properties (Mandavkar & Jalalpure, 2011; Roy *et al*., 2022). The plant parts have been traditionally used to treat various ailments, such as scabies, liver problems, rheumatism, paralysis, and diarrhoea, and to stimulate the central nervous system (Shukla *et al.*, 2020). These therapeutic abilities are attributed to a diverse array of bioactive constituents, including naphthoquinones, bi-naphthoquinones, coumarins, alkaloids, flavonoids, tannins, simple phenols, and triterpenes, found in the plant. The well-studied bioactive compound from *Plumbago* is plumbagin, a naphthoquinone, implicated in the bioactivities exerted by the plant. Plumbagin has also been reported to inhibit the growth of various proliferative cancerous cell lines, signifying its pharmacological importance (Shukla *et al*., 2020).

The requirement for *P. zeylanica* plant parts has increased due to their pharmacological importance and to be used as traditional medicine. Until now, naturally grown wild plants have primarily catered to the demand for *Plumbago* in herbal industries. However, the plant's availability in nature is shrinking due to overexploitation and erosion in wild vegetation (Chaplot *et al*., 2006). For sustainable long-term use of the plant, it is essential to recognize the genetic variability and intraspecific similarity. Although plant genetic diversity occurs during the evolutionary process, environmental parameters play an important role in intraspecific variability (Roy *et al*., 2022). Along with genetic diversity studies and morphological variations, phytochemical content determination is another useful method, particularly for medicinal plants, for effectively identifying the variabilities in different plant populations of a species (Nsuala *et al.*, 2017). The phytochemical spectrum of a plant type depends on its genetic makeup and the environmental factors where it grows. On the other hand, morphological traits play an important role in plant taxonomy, and the various descriptors serve as precise identifiers of the organism. Variations in phytochemical contents in *P. zeylanica* were studied by a few studies (Shukla *et al*., 2020; Roy *et al*., 2022), but a relationship between morphological and phytochemical variables is unattended.

Therefore, the study aimed to find a relationship between the morphological traits and the phytochemical contents of *P. zeylanica*, selecting four accessions from different districts of the Lower Gangetic plains of West Bengaland considering the environmental parameters. A multivariate PCA

*Corresponding Author

analysis delineated the relationships between the morphological, phytochemical, and environmental parameters. Furthermore, cluster analysis eventually grouped the different accessions, showing their mutual relationship. The study is significant in finding an elite chemotype of *P. zeylanica* with the help of its morphological characters, which may be useful for its conservation and propagation.

MATERIALS AND METHODS

Plant material:

P. zeylanica plants, grown in wild conditions, were collected from different localities of four districts in the Gangetic plains (Damodar River basin: Bankura 23.466295°N 87.185752°E and Bardhaman-Purba 23.635672°N 88.124548°E; Hooghly River basin: Hooghly 23.035514°N 88.15776°E and Nadia 22.988894°N 88.447128°E) of West Bengal, India (Figure 1) as four accessions and named according to their respective collection sites. The mature plants were sampled after cleaning (10 plants per locality), and morphological parameters were recorded. The plant parts (leaf, stem, root, flower) were separated and transported to the laboratory for phytochemical and microscopical analyses. The plants from four localities were identified with the help of a taxonomic manual and further authenticated by the Botanical Survey of India (BSI), Kolkata. The representative herbarium specimens were deposited at the BSI, Kolkata (KU/SM-02).

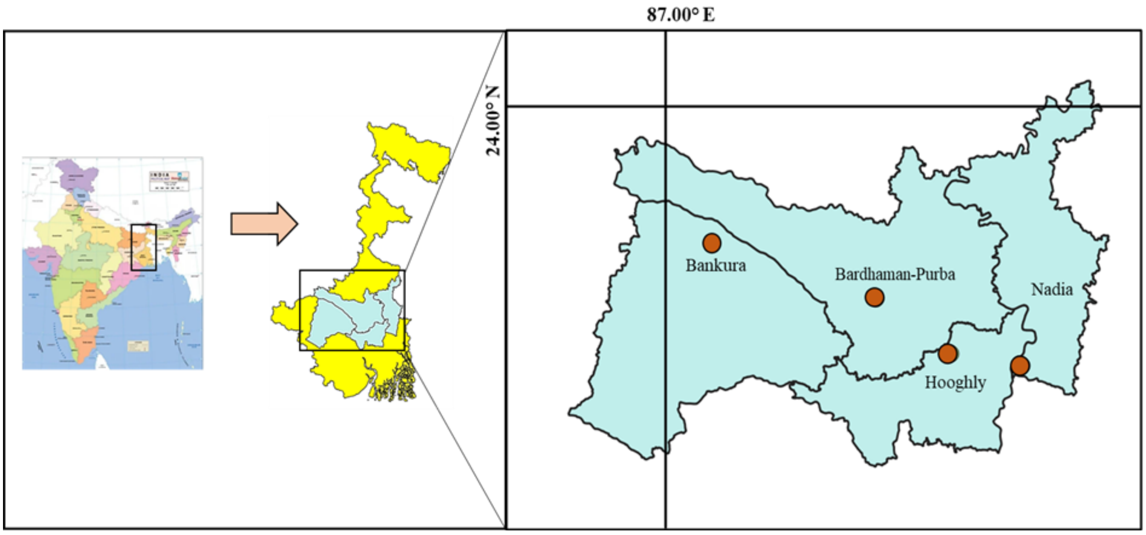

Figure 1: Collection sites of *P. zeylanica* accessions from four districts of West Bengal. The map was drawn in QGIS version 3.34.1.

Morphological characterization of the plant:

The phenotypic characteristics of the plants from each accession were studied following the guidelines provided by the International Plant Genetic Resource Institute in their descriptor list of different species of economic significance (Khadivi-Khub&Barazandeh, 2015). In this study, twenty phenotypic traits of *P. zeylanica* were studied, comprising sixteen vegetative and four reproductive characters. The leaf characteristics were examined with the fully mature leaves (from the 5 - 9th node from the apex). The traits like lengths of internode, petiole, leaf blade, and leaf blade width (the widest part at the middle) were measured using a centimeter scale. The stem diameter and lengths of the petiole base, bract, flower, corolla tube, and calyx were measured using a millimeter scale. The leaf area (cm^2) was calculated using graph paper. For microscopic (Olympus, India) determination of stomatal and salt gland index, the following formula was used -

$$\text{Stomatal/salt gland index} = \frac{\text{No. of stomata/salt gland per unit area}}{\text{No. of } \frac{\text{stomata}}{\text{salt}} \text{ gland} + \text{No. of epidermal cell}} \times 100$$

To observe the salt gland and stomata at higher magnification, scanning electron microscopy (SEM) was used following a standard protocol (Naidoo & Naidoo, 1998). Briefly, plant parts were fixed in 2.5% glutaraldehyde for 4 h at 4° C. The fixed materials were washed in phosphate buffer solution (PBS; pH- 7.4) before dehydration with ethanol. The dehydrated materials were dried and placed on coverslips, which were eventually placed on a stab and coated with gold particles following the protocols of the scanning electron microscopy facility at Kalyani University. The images were captured in a Zeiss EVO LS10 scanning microscope.

Preparation of extract from *P. zeylanica* plant parts:

The plant parts (root, stem, and leaf) were shade-dried for three weeks, and the shade-dried samples were ground with a mixer grinder (Bajaj Rex, 750W) to make a powdery mass. The powdered samples (50 g) of root, stem, and leaf were extracted with methanol (95 %; sample: solvent ratio- 1:10) at room temperature (RT; 30±2° C) for 48 h with mild shaking (45 rpm). Methanolic extracts were filtered with filter paper (Whatman® filter paper, Grade 1) and allowed to dry under reduced pressure in a rotary evaporator (Büchi, Switzerland) at 40° C. The resulting crude extracts were stored at 4° C for further use.

Phytochemical estimation of the extracts:
The determinations of total phenolic contents (TPC), total flavonoid contents (TFC), and total tannin contents (TTC) were done using standardized protocols regularly used in our laboratory (Ojha *et al*., 2018)

For TPC, the extracts (500 µl; stock 1mg/ml) were mixed with double-distilled water (ddH_2O; 500 µl), Folin Ciocalteu (FC) reagent (1 ml), and 700 mM sodium carbonate (2 ml), incubated in the dark for 15 min, and the absorbances recorded at 765 nm. The contents were determined using a calibration curve (r^2= 0.995) of gallic acid and expressed as milligram gallic acid equivalent per gram plant part (mg GAE/g).

The TFC in the samples (500 µl; stock 1mg/ml) was estimated by successive addition of ddH_2O (1.5 ml), sodium nitrite (5%; 200 µl), aluminium chloride (10%; 200 µl), sodium hydroxide (1N; 600 µl); absorbance was measured at 510 nm. The calibration curve (r^2= 0.986) of quercetin was used to quantify TFC and expressed as milligram quercetin equivalent per gram plant part (mg QE/g).

The TTC was determined by combining the extract samples (500 µl; stock 1mg/ml) with ddH_2O (7.5 ml), ferric chloride (0.1M; 500 µl), and potassium ferricyanide (8 mM; 500 µl), incubated for 10 min at dark, and measured at 720 nm. A calibration curve of gallic acid (r^2= 0.997) was used for quantifying TTC and expressed as mg GAE/g plant part.

The plumbagin (PLG) estimation in the plant parts content was performed using the method of Israni et al. (Israni *et al*., 2010) with minor modifications. The extracts (500 µl; 2mg/ml stock) were mixed with ddH_2O (500 µl), alcoholic KOH (10%; 1ml), and absolute ethanol (1ml) and incubated for 2 min at RT (30 ± 2 °C). The characteristic magenta-pink colour was measured at 520 nm. The PLG content was quantified from a calibration curve of plumbagin (r^2= 0.989) and expressed as mg plumbagin equivalent per gram plant part (mg PLG/g).

Environmental parameters at collection sites:
The localities' meteorological data, such as average temperature, rainfall, and humidity, of four accessions were collected from July to October (2019 to 2021) using the India Meteorological Department (IMD) web server.

Soil samples were collected from five spots surrounding the plant population at 20-40 cm depth in each locality. Different samples from each site were combined, blended, air-dried, and kept at RT (30±2°C) for laboratory analyses. The soil suspensions (10 gm soil sample in 25 ml ddH_2O) were used to determine soil pH (using a pH meter), while the EC was measured using a conductivity meter with the clear supernatants of the dissolved soil samples. The OC of the soil samples was estimated using a standard method (Walkley & Black,1934). Soil macronutrients like available nitrogen (N) were determined by the alkaline permanganate method, available phosphorus (P) following Bray's method, available potassium (K) content by flame photometric method (Maity&Das,2007), micronutrients like iron (Fe), copper (Cu), zinc (Zn) and manganese (Mn) by diethylenetriaminepentaacetic acid (DTPA) reagent extracted soil solutions using atomic absorption spectrophotometry (PinAAcle 900 series AA), following the protocols of American Society of Agronomy (Lindsay & Norvell, 1978).

Statistical analysis:
Descriptive statistics were performed by Jamovi software (www.jamovi.org) version 2.2.5. The coefficient of variation (CV%) was determined by using the [CV= (SD/mean) x 100] formula. One-way analyses of variance (ANOVA) followed by Tukey's multiple comparison tests ($p < 0.05$) using IBM SPSS statistics 25 software were employed to compare the result of polyphenolic contents of each plant part of different districts. Individual paired t-tests were applied only to the root extracts from four districts to compare their significant differences. The obtained data were grouped for statistical analysis. The Pearson correlation coefficient and PCA among the variables were determined using R studio (https://support--rstudio-com.netlify.app/products/rstudio/download/). The Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster analysis was done using PAST software.

RESULTS AND DISCUSSION

Phenotypic plasticity and descriptive statistics of *P. zeylanica* among four accessions:
The collected *P. zeylanica* accessions from four districts of the lower Gangetic plains were undershrub with an upright or scandent habit (Figure 2A, B). The leaves are simple, alternate, ovate-elliptical, with a pointed tip and a heart-shaped base, acute-acuminate, petiolate, petiole base mostly auriculate, with stomata and characteristic salt-secreting glands (Figure 2C, D). The leaf bases of *P. zeylanica* are auriculate, mostly in young leaves, as ear-like appendages. The stomata of *P. zeylanica* are amphistomatic and anisocytic, and they are present more on the abaxial surface (Figure 2D, E). *P. zeylanica* is found in mesophytic habitats, though it possesses features reminiscent of a halophyte (Sudhakaran, 2019). The plant consists of sparsely

distributed salt glands on both leaf surfaces, comprising eight cells, with the inner four functioning as secretory cells and the outer four known as accessory cells (Figure 2D, F). Flowers are pentamerous, arranged in spike-like clusters, bracteate, calyx elongated, prominently ribbed, persistent, gamopetalous, with five sticky lobes containing numerous trichomes, glandular trichomes with mucilaginous head on a stalk, non-glandular trichomes short, with stiff stalk (Figure 2G, H, I). The corolla tube is long, and the corolla lobes obovate to obtuse (Figure 2G). *P. zeylanica* has an extensive tap root system with secondary roots (Figure 2J). The morphological variations of twenty traits in individual accessions are represented in Table 1, as such characteristics are important in studying the morphological diversity of the plant. Five of these, such as internode length (IL), leaf blade ratio (LR), primary root length (RL), root fresh weight (RFW), and root dry weight (RDW), represented non-significant ($p > 0.05$) variations among the accessions. The characters like plant height (PH), stem diameter (SD), petiole length (PL), petiole base width (PbW), and salt gland adaxial surface (SGAd) showed significant differences ($p < 0.05$) in Bardhaman -Purba than others, however, non-significant variations ($p > 0.05$) existed among the remaining three. The traits like leaf area (LA), stomatal index abaxial surface (SIAb), salt gland abaxial surface (SGAb), flower length (FL), and corolla tube length (CtL) showed significant differences in the accession of Bankura. The population of Nadia differed significantly ($p < 0.05$) from the others in leaf blade length (LL) and calyx length (CL).

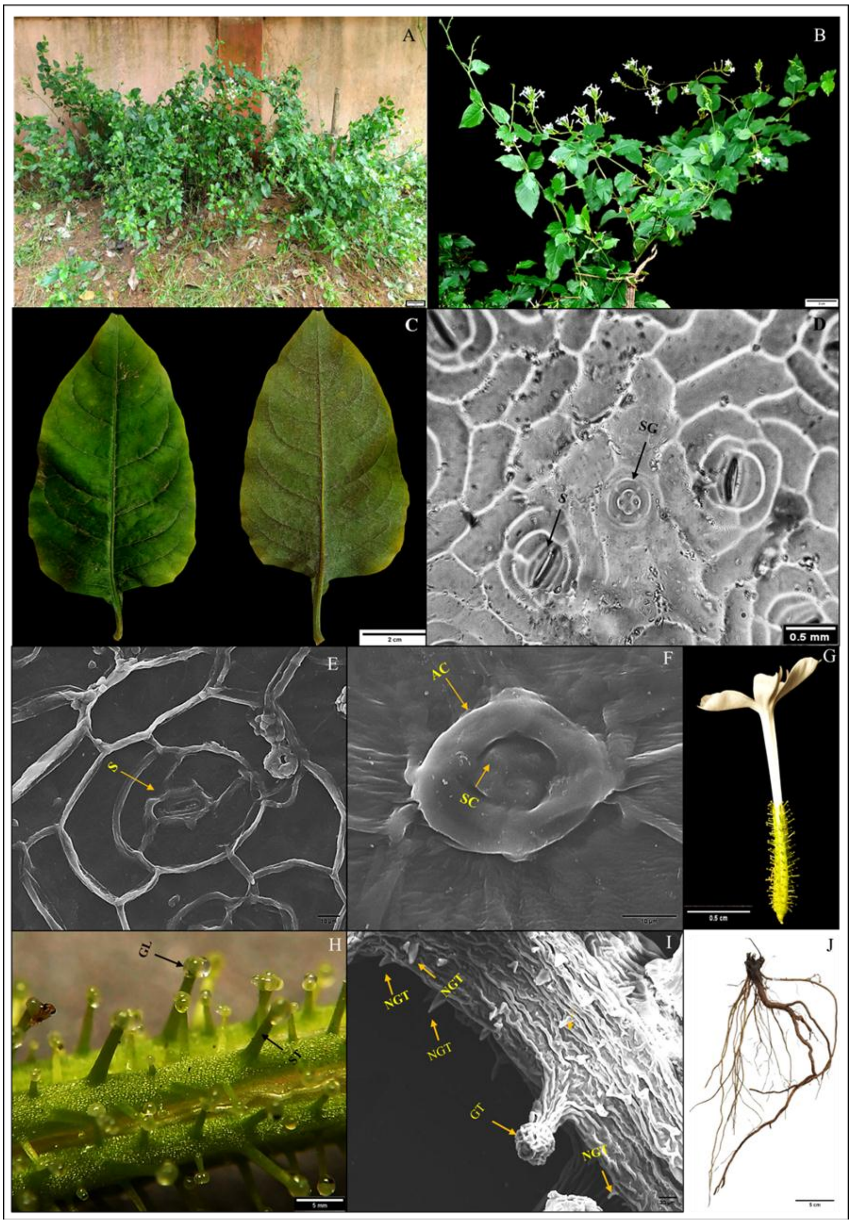

Figure 2: Morphological characteristics of *P. zeylanica*. **(A)** entire plant, **(B)** flowering twig, **(C)** leaf (adaxial and abaxial), **(D)** light microscopic imageof the leaf showing salt gland and stomata, **(E, F)** scanning electron

microscopic (SEM) images of the stomata and salt gland respectively, **(G)**singleflower,**(H)** calyx (enlarged) showing sticky gland,**(I)** SEM image of the glands on calyx,**(J)** Root. [S - stomata; SG- salt gland; AC- accessory cell; SC- secretory cell; GL- gland; ST- stalk; GT- glandular trichome; NGT- non-glandular trichome].

Table 1. Comparison of morphological traits of *P. zeylanica*accessions from four districts of West Bengal.

Traits	Bankura	Nadia	Hooghly	Bardhaman-Purba	Mean
Plant height (m)	1.50±0.08^{b}	1.47±0.13^{b}	1.44±0.16^{b}	2.6±0.22^{a}	1.75
Stem diameter (mm)	28.2±2.92^{ab}	27±1.92^{ab}	19.4±2.01^{b}	32±3.30^{a}	26.65
Internode length (cm)	8.18±0.42^{a}	6.60±0.42^{a}	5.98±0.73^{a}	7.07±0.96^{a}	6.96
Leaf area (cm^2)	48.3±5.87^{a}	27.75±2.57^{b}	35.95±2.24^{ab}	28.2±2.97^{b}	35.05
Leaf blade: length (cm)	9.26±0.47^{b}	12.38±0.36^{a}	10.84±0.52^{ab}	7.29±0.34^{c}	9.94
Leaf blade: width (cm)	5.56±0.22^{b}	6.99±0.13^{a}	7.25±0.43^{a}	5.06±0.34^{b}	6.21
Leaf blade: ratio length/width	1.67±0.04^{a}	1.77±0.03^{a}	1.50±0.07^{a}	1.47±0.11^{a}	1.60
Petiole length (cm)	2.39±0.18^{ab}	1.72±0.14^{b}	2.01±0.14^{b}	2.82±0.13^{a}	2.23
Petiole base width (mm)	5.4±0.85^{b}	3.5±0.49^{b}	4.7±0.46^{b}	8±0.63^{a}	5.40
Stomatal index (Adaxial)	10.45±0.43^{a}	7.68±0.43^{b}	5.66±0.23^{c}	23.77±1.69^{a}	7.78
Stomatal index (Abaxial)	17.47±0.96^{b}	19.57±0.54^{ab}	23.60±0.47^{a}	7.34±0.28^{b}	21.10
Salt gland (Adaxial)	1.03±0.10^{ab}	0.82±0.05^{b}	0.79±0.06^{b}	1.23±0.05^{a}	0.97
Salt gland (Abaxial)	2.09±0.12^{a}	0.88±0.14^{c}	1.53±0.06^{b}	1.66±0.15^{ab}	1.54
Flower length (mm)	30.8±0.77^{a}	26.6±0.61^{b}	24.8±0.23^{bc}	23.3±1.21^{c}	26.35
Corolla tube length (mm)	21.8±0.33^{a}	18.9±0.30^{b}	16.7±0.44^{b}	17.1±0.96^{b}	18.62
Calyx length (mm)	9±0.49^{c}	12.8±0.18^{a}	11.5±0.20^{ab}	10.5±0.49^{bc}	11.07
Bract length (mm)	5.2±0.23^{a}	3±0.24^{b}	4.7±0.41^{a}	3.9±0.38^{ab}	4.20
Primary root length (cm)	34.68±3.47^{a}	30.98±1.68^{a}	39.34±5.91^{a}	53.14±9.40^{a}	39.53
Root fresh weight (gm)	12.71±1.12^{a}	8.14±0.61^{a}	8.24±0.68^{a}	11.41±1.58^{a}	10.12
Root dry weight (gm)	5.13±0.54^{a}	3.43±0.21^{a}	3.40±0.27^{a}	4.20±0.59^{a}	4.07

Different alphabets represent significant differences ($p < 0.05$) among accessions.

The descriptive statistics of twenty morphological characters of *P. zeylanica* in four different accessions are presented with their mean values and the extent of variations (Table 2). The coefficient of variation (CV) varied from 12.99% (FL) to 41.11% (petiole base width, PbW). Among the traits, fourteen exhibited CV values greater than 20%, representing substantial variability. Like the PbW (41.11%), a relatively higher CV was also observed in RL (40.51%), PH (35.43%), LA (34.29%), SGAb (34.22%), and RFW (31.78%). The traits with lower CV were found in FL (12.99%), followed by LR (13.13%), CtL (13.17%), CL (13.69%), SIAb(17.25%), and leaf blade width (LW; 19.00%).

Table 2. Descriptive statistics of twenty morphological traits showing variations in four *P. zeylanica* accessions.

Traits	Abbreviation	Min	Median	Max	Mean	Sd	CV %
Plant height (m)	PH	1.07	1.61	3.35	1.75	0.62	35.43
Stem diameter (mm)	SD	14	26.0	46	26.6	7.60	28.57
Internode length (cm)	IL	3.43	7.12	9.75	6.96	1.75	25.14
Leaf area (cm^2)	LA	18.3	34.5	60.3	35.0	12.0	34.29
Leaf blade length (cm)	LL	6.10	10.0	13.0	9.94	2.17	21.83
Leaf blade width (cm)	LW	4.10	6.30	8.80	6.21	1.18	19.00
Leaf blade ratio (length/width)	LR	1.26	1.65	1.95	1.60	0.21	13.13
Petiole length (cm)	PL	1.30	2.25	3.10	2.23	0.55	24.66
Petiole base width (mm)	PbW	2.00	4.75	10.0	5.40	2.22	41.11
Stomatal index (Adaxial)	SIAd	4.89	7.29	11.7	7.78	1.94	24.93
Stomatal index (Abaxial)	SIAb	14.5	20.9	27.2	21.1	3.64	17.25
Salt gland (Adaxial)	SGAd	0.58	0.95	1.43	0.97	0.24	24.74
Salt gland (Abaxial)	SGAb	0.49	1.52	2.51	1.54	0.52	34.22
Flower length (mm)	FL	19.0	25.8	33.0	26.4	3.43	12.99
Corolla tube length (mm)	CtL	14.0	18.8	23.0	18.6	2.45	13.17
Calyx length (mm)	CL	8.00	11.3	13.0	11.1	1.52	13.69
Bract length (mm)	BL	2.00	4.00	6.00	4.20	1.14	27.14
Primary root length (cm)	RL	17.5	36.4	73.4	39.5	16.0	40.51
Root fresh weight (gm)	RFW	6	9.28	15.4	10.1	3.20	31.78
Root dry weight (gm)	RDW	2.04	3.96	6.60	4.07	1.22	29.98

The CV is essential for determining a morphological trait's ability to differentiate different populations. The morphological character with a high CV reflects higher variation and discriminates more among different groups. Hence, such characters may act as reliable markers for characterizing different populations. The traits with low CV are more homogeneous, exhibiting relative stability among populations in these traits (Khadivi-Khub & Barazandeh, 2015).

Plant height and leaf characteristics directly impact morphological diversity and productivity. As the leaves contribute to the significant photosynthetic efficacy of plants, the larger leaves can contribute to higher biomass, which is critical for an increase in the overall yield of different metabolites. In the studied accessions, PH and SD range from 1.07 m to 3.35 m and 14 mm to 46 mm, respectively. Comparing the four accessions, the highest mean values for these traits were 2.6 m and 32 mm, respectively, observed in Bardhaman -Purba. On leaf characters, LA varied considerably between 18.3 - 60.3 cm^2, while LL and LW variations ranged from 6.10 - 13.0 cm and 4.10 - 8.80 cm. The PL and PbW showed considerable plasticity, ranging from 1.30-3.10 cm and 2.00-10.00 mm, respectively. Among four accessions, the maximum mean values of these two traits were found in Bardhaman-Purba. The stomatal index is a crucial morphological trait influenced by climatic factors and the soil moisture regime (Liu *et al.*, 2024). Although the SIAb (CV-17.25%) is more than the adaxial (CV- 24.93%), the variations were low in both cases. Although the four accessions were collected from the two river basins of lower Gangetic plains, the climatic factors were nearly identical, which may account for such low variability in the stomatal index. Due to the presence of salt glands, the plants effectively evade salt stress and are known as exo-recretohalophytes (Sudhakaran, 2019). The salt glands are also implicated as the growth indicator of the plant (Yuan*et al.*, 2016). Between the two leaf surfaces, the variations in salt gland index were higher in the abaxial surface for all four accessions. The plasticity in floral characters in the studied accessions is less, evidenced by the low CV of FL (12.99%), CtL (13.17%), CL (13.69%), and BL (27.14%). *P. zeylanica*harbours a variety of bioactive phytochemicals in its root (Shukla *et al.*, 2020). Although the root parameters showed high variance in bulk population, variations between the accessions were non-significant, reflecting the consistency of root parameters.

Phytochemical estimation of the extracts:

Different phytochemicals of *P. zeylanica*, like polyphenolics (total phenolics-TPC, total flavonoid-TFC, total tannin-TTC), and plumbagin-PLG, exhibited higher contents in roots than in leaves and stems with significant differences (Figure 3). As the results were consistent in all the studied areas, the variabilities of these parameters in four regions were further studied only with root phytochemicals. Paired t-tests concerning root phytochemicals of different accessions determined significant differences in TPC and PLG among the four districts (Figure 3).

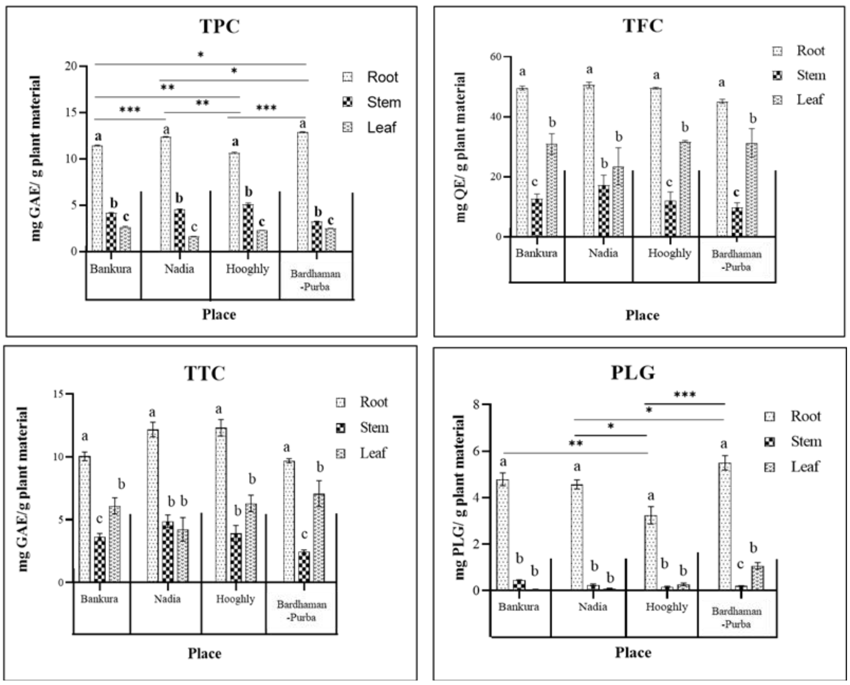

Figure 3: Determination of total phenolic content (TPC), total flavonoid content (TFC), total tannin content (TTC), and plumbagin content (PLG) of different plant parts of *P. zeylanica* accessions from four districts of West Bengal.

Different alphabets represent significant differences ($p<0.05$) among the plant parts. The asterisk(s)represents the significance level between the root phytochemicals by paired t-test (*-p<0.05, **-p<0.01, ***-p<0.001).

Knowledge of the phytochemical composition of different plant parts is an important criterion for collecting medicinal plants from natural sources. Our study exhibited that the root contained the highest TPC, TFC, TTC, and plumbagin content compared to leaves and stems. Various studies determined phytochemicals from individual plant parts, but a comparative account of different plant parts is limited (Zeng *et al*., 2024). In general, polyphenolics are more prevalent in aerial parts of plants; however, in some instances, higher metabolite contents in roots were observed due to more accumulation of flavonoids, chlorogenic acid derivatives, and naphthoquinones (Munakata *et al*., 2019). The naphthoquinone plumbagin, a medicinally important bioactive metabolite, is of common occurrence in *P. zeylanica* roots (Shukla *et al*., 2020). The presence of plumbagin is also identified in other plant parts but in lesser quantities. In contrast to our findings, a study on *P. zeylanica* plant parts observed higher amounts of TPC and TFC in the stem (Zeng *et al*., 2024).

The total phenolic contents in the root (TPCR) showed significant differences ($p < 0.05$) among the studied accessions (Table 3). The maximum TPCR (mg GAE/g) was observed in Bardhaman-Purba (12.88±0.03), followed by Nadia (12.39±0.02), Bankura (11.45±0.04) and Hooghly (10.65±0.11) district with a CV value of 8.41%. In total flavonoid of root (TFCR; mg QE/g), Nadia showed the highest amount (50.59±0.98), while Bankura (49.59±0.64), Hooghly (49.58±0.29), and Bardhaman-Purba (45.11±0.69) possessed relatively lower contents. However, the variations among the four districts were non-significant ($p > 0.05$), which was also determined by the low CV (5.03%). The total tannin contents of root (TTCR) among the different accessions were also non-significant ($p > 0.05$), with the highest value (mg GAE/g) in Hooghly (12.30±0.65), followed by Nadia (12.18±0.58), Bankura (10.04±0.34), and Bardhaman-Purba (9.69±0.17). The CV of TTCR was calculated as 12.43%. The root plumbagin (PLGR) contents (mg PLG/g) in the four districts showed significant differences ($p<0.05$) among them. Also, the CV (20.84%) was relatively high in PLGR among all the studied phytochemicals. The highest PLGR was determined at Bardhaman-Purba (5.50±0.30), followed by Bankura (4.79±0.27), Nadia (4.57±0.19), and Hooghly (3.24±0.37).

Table 3. Phytochemical estimation of *P. zeylanica*root extracts and their variations among the accessions from four districts.

Phytochemical	Bankura	Nadia	Hooghly	Bardhaman-Purba	Mean	CV (%)
TPCR	11.45 ± 0.04^{c}	12.39 ± 0.02^{b}	10.65 ± 0.11^{d}	12.88 ± 0.03^{a}	11.8	8.41
TFCR	49.59 ± 0.64^{a}	50.59 ± 0.98^{a}	49.58 ± 0.29^{a}	45.11 ± 0.69^{a}	48.7	5.03
TTCR	10.04 ± 0.34^{a}	12.18 ± 0.58^{a}	12.30 ± 0.65^{a}	9.69 ± 0.17^{a}	11.1	12.43
PLGR	4.79 ± 0.27^{ab}	4.57 ± 0.19^{b}	3.24 ± 0.37^{c}	5.50 ± 0.30^{a}	4.53	20.84

Different alphabets represent significant differences ($p < 0.05$) calculated by Tukey's post-hoc test.
TPCR (mg GAE/g): root total phenolic content; TFCR (mg QE/g): root total flavonoid content; TTCR(mg GAE/g): root total tannin content; PLGR(mg PLG/g): root plumbagin content.

The lower CV of the parameters highlighted that the observed phytochemicals did not significantly vary in the total populations of the four areas. However, ANOVA analyses among the different accessions showed the superiority of Bardhaman-Purba in TPCR and higher PLGR content in both Bardhaman-Purba and Bankura with non-significant ($p > 0.05$) variations. Such variations among populations can be attributed to the varied environmental factors at different locales. The higher amounts of plumbagin in the population of Bankura were previously reported (Shukla *et al*., 2020). The report also inferred the influence of different agroclimatic conditions on plumbagin content (Shukla *et al*., 2020). Our study revealed that Bardhaman-Purba has the highest plumbagin content, with no significant difference from Bankura. Notably, these two districts belong to the Damodar basin area and fall within similar agro-climatic zones, which may contribute to their higher plumbagin content.

Correlation between the morphological traits and root phytochemical contents:

The correlations between root phytochemicals and morphological characters are more pertinent in this study (Figure 4). The TPCR positively correlated significantly with PbW ($r= 0.45$; $p< 0.05$) and SD ($r= 0.48$; $p< 0.05$), implying that selecting thicker stems and more extended petiole bases may help in choosing plant populations with higher phenolic contents. The TTCR exhibited significant positive correlations with LL ($r= 0.59$; $p< 0.01$) and LW ($r= 0.59$; $p< 0.01$), with longer leaves tending to have higher tannin concentrations in their roots. In contrast, negative correlations between TTCR and SD ($r= -0.46$; $p< 0.05$), PL ($r= -0.56$; $p< 0.01$), PbW ($r= -0.55$; $p< 0.05$), SIAb ($r= -0.52$; $p< 0.05$), salt gland index at both adaxial and abaxial surfaces (r= -0.50, -0.54 respectively; p< 0.05) were observed. The plumbagin content (PLGR), an important bioactive compound of *P. zeylanica*, was interrelated with RFW (r= 0.60; p< 0.01), the PbW (r= 0.53; p< 0.05), and salt gland index at the abaxial surface (r= 0.45; p< 0.05), revealing significant positive correlations. On the other hand, PLGR showed a significant negative correlation with the LL (r= -0.60; p< 0.01).

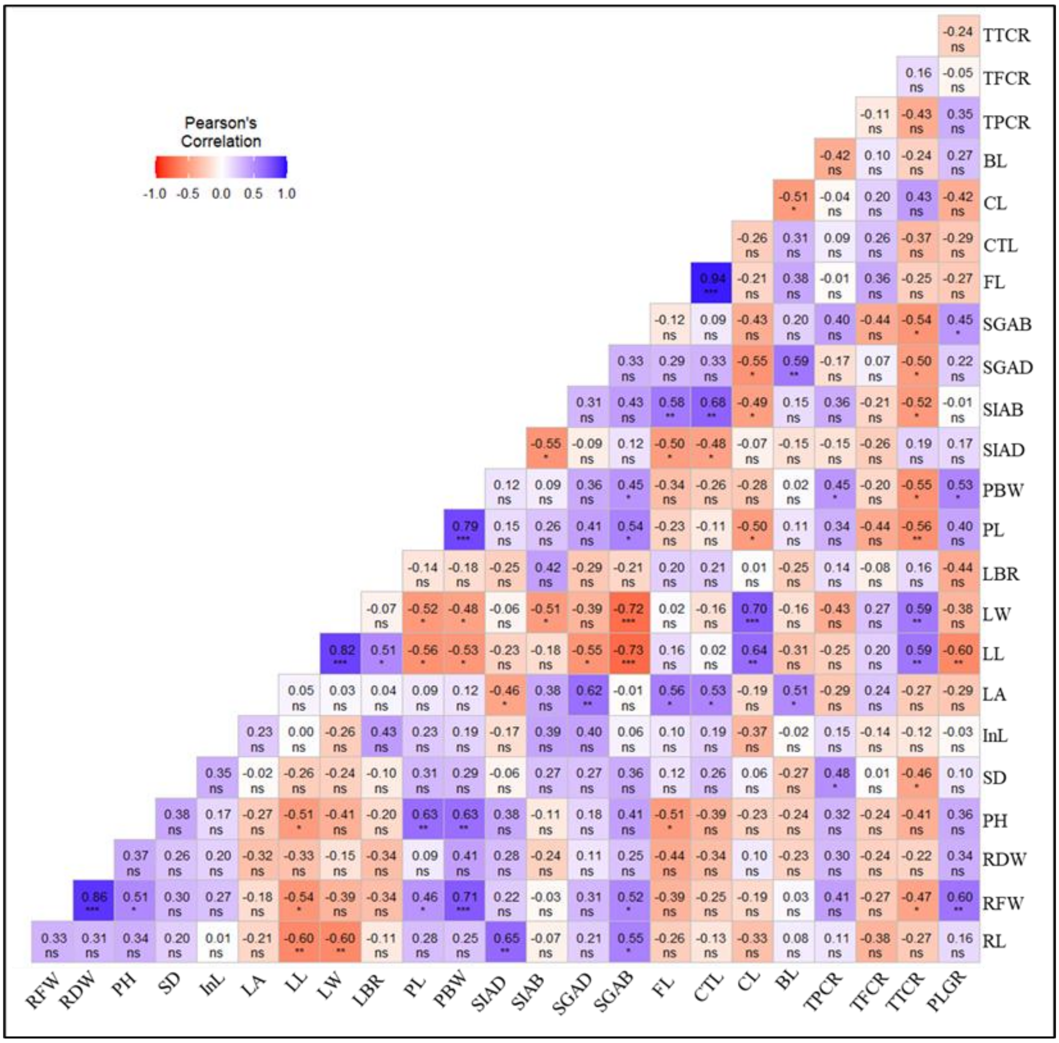

Figure 4: Correlation coefficients between the morphological parameters and root phytochemical contents of four populations of *P. zeylanica*.

Asterisks (*, **, ***) indicate significance levels at p < 0.01, 0.05, and 0.1, respectively; ns- not significant.

A plant's growing condition significantly impacts its metabolite composition (Yang *et al.*, 2018). As the studied accessions of *P. zeylanica* were collected from different localities, climatic and edaphic factors can contribute to morphological and phytochemical variations. The correlation study helps to identify the morphological parameter(s) as selection criteria for a particular phytochemical. The use of morphological characters for selecting plant populations with higher polyphenolics was studied in *Adenostemma* sp. (Nurlela *et al.*, 2022) and *Trigonella* sp. (Riasat *et al.*, 2018). Plumbagin content in *P. zeylanica* roots is usually low, and due to its growing demand in herbal industries, identification of better chemotypes is a priority for a plant collector. The study highlighted that a broader petiole base, higher salt gland index, and more root fresh weight could be the markers for finding *P. zeylanica* plants with higher plumbagin content. The correlation studies of morphological traits with artemisinin and essential oil were demonstrated for selecting better genotypes of *Artemisia annua* (Paul *et al.*,2011).

Environmental determinants of the four localities: Thirteen environmental parameters, including edaphic and meteorological (Table 4), were studied to measure the ecological variations in the four *P. zeylanica* accessions. Among the three physiochemical properties of soil, the pH was slightly acidic (mean 6.24) in all the samples, with no significant differences. A relatively lower CV (7.08%) also substantiates the narrow pH range in all the studied areas. On the other hand, the soil electrical conductivity (EC) demonstrated significant variations in Bardhaman-Purba than others and showed high variability among the samples by revealing a higher CV (81.17%) value. However, the variations among Bankura, Hooghly, and Nadia were non-significant ($p > 0.05$). The organic carbon (OC; %) was highest in Bardhaman-Purba (1.26±0.03), followed by Hooghly (0.87±0.02), Nadia (0.54±0.02), and Bankura (0.48±0.01), showing significant differences among them, and the large variability was also documented by the higher CV (37.47%). The soil pH in the four localities was near neutral, ranging between 6.16 and 6.94, which is suitable for the optimum growth of the plants since most nutrients in the soil are soluble in this range, making them available to the plants. The electric conductivity (EC) represents the total soluble salt concentration of the soil, and the values in the present study at four locales showed optimal for vegetation. Organic carbon (OC) holds water and nutrients in the soil, increasing soil aggregation and texture and is crucial for nutrient cycling (Zhou *et al.*, 2020). Compared to the four accessions, Bardhaman-Purba manifested better soil physicochemical properties.

Table 4. Comparison of the ecological parameters (soil and meteorological) at the collection sites of four accessions of *P. zeylanica*.

Ecological parameter	Bankura	Nadia	Hooghly	Bardhaman-Purba	Mean	CV (%)
pH	6.8±0.23[a]	6.94±0.15[a]	6.33±0.19[a]	6.16±0.09[a]	6.24	7.08
EC (dS/m)	0.16±0.00[b]	0.1±0.21[b]	0.42±0.01[b]	1.1±0.04[a]	0.53	81.17
OC (%)	0.48±0.01[d]	0.54±0.02[c]	0.87±0.02[b]	1.26±0.03[a]	0.76	37.47
N (kg hc^{-1})	94.08±0.57[d]	128.58±0.46[b]	163.07±0.77[a]	106.62±0.32[c]	123	21.87
P (kg hc^{-1})	9.3±0.17[c]	10.86±0.33[c]	102.14±1.75[a]	18.70±0.63[b]	33.8	114.20
K (kg hc^{-1})	237.77±0.75[b]	161.95±0.86[c]	924.56±1.29[a]	225.12±1.15[b]	375	80.27
Zn (kg hc^{-1})	3.47±0.19[c]	3.337±0.24[c]	6.9±0.19[b]	10.16±0.16[a]	5.8	45.52
Fe (kg hc^{-1})	29.61±0.51[b]	26.62±0.76[c]	68.99±0.14[a]	27.32±0.50[c]	37.1	47.98
Cu (kg hc^{-1})	1.012±0.03[d]	3.07±0.09[c]	6.06±0.16[b]	5.24±0.20[a]	3.67	51.50
Mn (kg hc^{-1})	19.60±0.58[a]	19.29±0.33[a]	15.66±0.61[b]	13.18±0.31[b]	15.8	17.97
Temp (°C)	29.43±0.17[a]	28.73±0.12[a]	28.83±0.12[a]	28.9±0.14[a]	29	1.24
Rainfall (mm)	806.67±7.20[b]	823.33±7.20[ab]	843.33±7.20[ab]	853.33±9.81[a]	834	2.64
Humidity (%)	79±0.47[a]	80±0.47[a]	80±0.47[a]	81±0.47[a]	80	1.44

Different alphabets represent significant differences ($p < 0.05$) among the accessions represented by their districts. EC: electric conductivity; OC: organic carbon; N: nitrogen; P: phosphorus; K: potassium; Zn: zinc; Fe: iron; Cu: copper; Mn: manganese; Temp: temperature.

The macronutrient contents, such as nitrogen (N), phosphorus (P), and potassium (K), were highest in Hooghly, significantly different from other districts. The CV of N content was moderate (21.87%), ranging from the highest value at Hooghly (163.07) to the least at Bankura (94.08). However, the variation in P content was maximum (CV-114.20%), ranging between 9.3 (Bankura) to 102.14 (Hooghly). The variation in K content was also high (CV-80.27%), with its maximum at Hooghly (924.56) to the minimum at Nadia (161.95).

Zinc (Zn) and copper (Cu) were highest in Bardhaman-Purba in the micronutrients, while iron (Fe) was highest in Hooghly. They differed significantly ($p < 0.05$) from other districts. Bankura and Nadia's manganese (Mn) content significantly ($p < 0.05$) varied from the other two districts. The Zn, Fe, and Cu contents showed substantial variations in the obtained data reflected by their higher CV values (45.52%. 47.98%, and 51.50%, respectively). The Mn contents were uniform in different studied areas, with a modest CV value of 17.97%. Soil macronutrients likeN and P are essential components of cellular macromolecules. At the same time, K is necessary for many enzymatic activities as a cofactor and also participates in ionic balance and osmotic regulation. The studied soil micronutrients, such as Cu, Fe, Zn, and Mn, play vital roles in plant growth, development, and metabolism (Kumar *et al.*, 2021).

The meteorological parameters (temperature, rainfall, and humidity) were uniform in the four localities, as depicted by their low CV values. The variations in temperature and humidity among the four districts were non-significant ($p > 0.05$), with an average of 29ºC and 80%, respectively. The rainfall was lowest in Bankura, with significant differences ($p < 0.05$) from other districts. The climatic factors not only help in plant growth and development but also contribute to the phytochemical diversity of the plant (Kumar *et al.*, 2017). However, the results showed uniformity in the studied climatic factors in four localities, implying their minor role in morphological and phytochemical diversity.

Multivariate data analyses based on the morphological traits, ecological parameters, and root phytochemical contents:

Principal component analysis (PCA)

A PCA biplot was made based on PC1 and PC2, contributing 52.7% of total variations, of which PC1 constituted 27.6% and PC2 25.1% (Figure 5). Although not significant enough, PC1 explained the major variations of SIAb, FL, CtL, and Mn, while PC2 accounted for humidity, PH, PL, PbW, SGAb, PLGR, EC, and OC. The plot also distinguished the four districts based on the contributing variances and overall correlations of morphological, phytochemical, and environmental parameters. Among the four, the Hooghly district was found on the right half of PC1 with contributions from macronutrients like K, P, and N and the micronutrient Fe. The contributing factors for Nadia were TFCR and LL, positioning at the midway of PC1. The Bankura district was distantly placed from Hooghly and Nadia at the left half of PC1. The district's main contributions were from FL, CtL, and SIAb. On the other hand, the contributions of Bardhaman-Purba were from PLGR, PbW, PH, and EC and were placed far away from the three districts at the upper half of the plot.

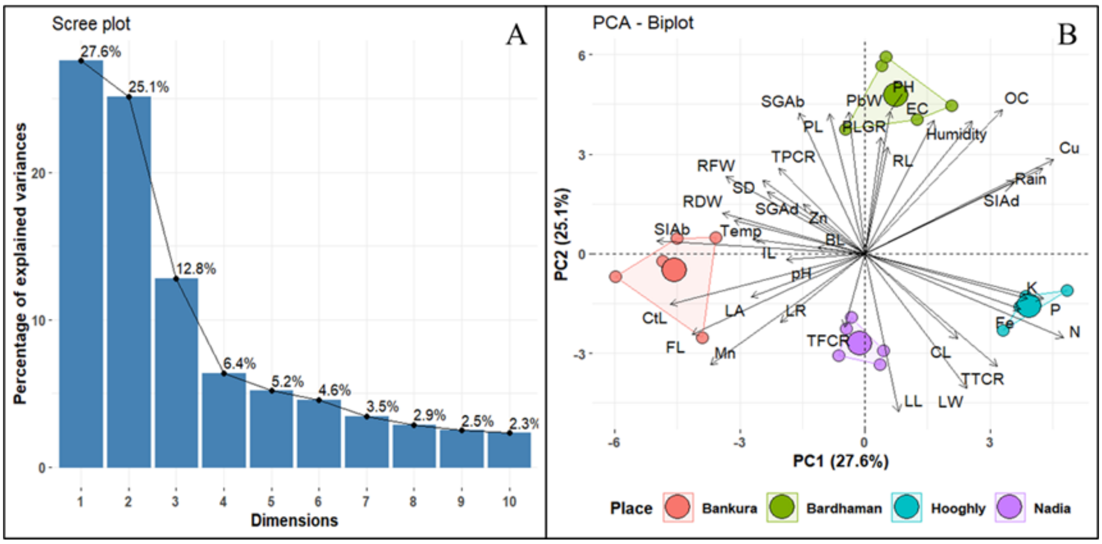

Figure 5: Principal component analysis (PCA) based on the morphological traits, root phytochemical contents and ecological parameters. A: Scree plot representingeigenvalues and principal components accounting for their variances. B: Biplot depicting the distribution and relationshipsofthe studied parameters.

PCA analysis revealed a negative correlation between the TPCR and macronutrients, which was substantiated by the studies that reported that the abundance of nitrogen inhibits the accumulation of phenolic compounds by altering the activity of phenolic acid biosynthetic enzymes (Chishaki & Horiguchi, 1997). The phenol content in the leaves of *Fagus sylvatica* and *Picea abies* increased when grown on soils with low nitrogen content (Kolstad *et al.*, 2016). On the other hand, soil N, P, K, and Fe content showed a significant positive correlation with root total tannin content, consistent with previous work (Chabel*iet al.*, 2008). The present study demonstrated a significant correlation between Zn and the root plumbagin content, as evidenced by the variances of PC2. Zn is essential for plants' vegetative and reproductive growth. Applying Zn as foliar spray enhanced essential oil contents in *Satureja khuzistanica* (Mumivand *et al.*, 2021). Increased phytochemicals in *Brassica oleracea* were also noticed with higher Zn content (Barrameda-Medina *et al.*, 2017). As shown in the plot (Figure 5), plant height and petiole characteristics like length, base width, and primary root length correlate with plumbagin content in the present study. Meanwhile, stem diameter, fresh and dry root weight, and salt glands interrelate with the TPCR contents, and the TTCR content is associated with foliar morphology, such as length and width. These morphological characters are potential markers for identifying a specific phytochemical in *P. zeylanica* and are influenced by edaphic factors.

UPGMA Cluster analysis

The dendrogram was obtained by combining morphological, phytochemical, and environmental traits. The representation categorized the four accessions into two major groups based on Euclidean distances. Group I comprises Bardhaman-Purba, separated from the rest at a metric distance of 9.5, forming a distinct group. At a metric distance of 9.0, the second group (Group II) divided Bankura as the first subgroup, and the second subgroup was further subdivided into Nadia and Hooghly at a metric distance of 7.3.

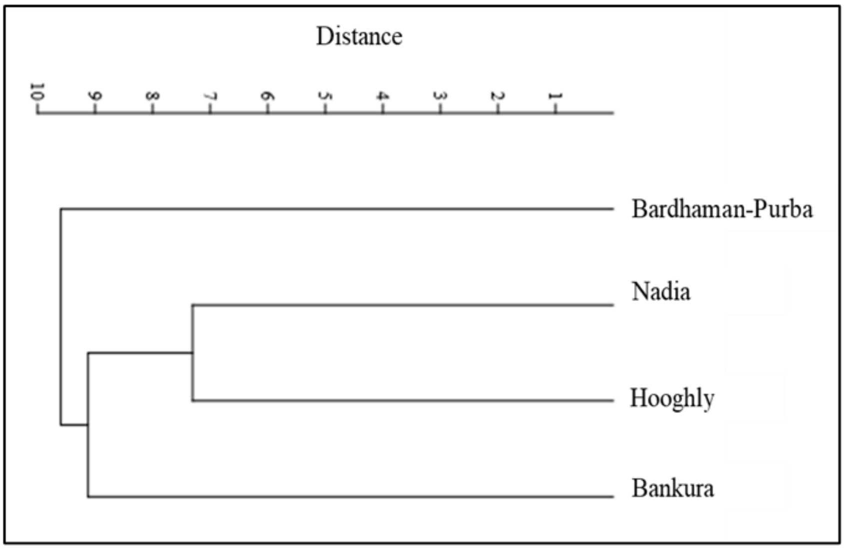

Figure 6: Dendrogram by UPGMA hierarchical cluster analysis based on morphological traits, root phytochemical contents, and ecological parameters of four accessions of *P. zeylanica*.

The cluster analyses show that Bardhaman-Purba is a distinct group but closer to Bankura than Hooghly and Nadia. These two localities belong to the Damodar basin, and their similar agro-climatic parameters may influence their morphology and phytochemical contents. Notably, the plumbagin content is higher in both Bardhaman-Purba and Bankura. A previousstudy demonstrated maximum plumbagin yield in the accession of Bankura among thirteen studied *P. zeylanica* accessions (Shukla *et al*., 2020). The present study focused on morphological, environmental, and phytochemical characteristics of naturally grown *P. zeylanica* from different localities of Gangetic plains of West Bengal, and this may lead to finding a superior plant chemotype.

CONCLUSION

The four accessions of *P. zeylanica* collected from four districts of the Lower Gangetic plains of West Bengal showed substantial variations in morphological characters and phytochemical contents. Based on twenty morphological and four phytochemical parameters, a correlation study determined the relationship between TPCR with stem diameter and petiole base width, TTCR with leaf morphologies, and root plumbagin content with root fresh weight, petiole base width, and salt gland index. The environmental spectrum in these four localities revealed that edaphic factors impacted the plant's morphologies and metabolite contents. Considering all these relationships, PCA discriminated the four accessions as distinct groups based on their effect on variances of different parameters. Furthermore, cluster analysis grouped the accessions from four districts, which may be useful in selecting a better chemotype of naturally grown *P. zeylanica* at Lower Gangetic plains.

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