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In vitro regeneration and phytochemical evaluation of pippali (*Piper longum*) for conservation and therapeutic applications

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ABSTRACT

The present study was carried out during 2021–2022 at College of Agriculture, Odisha University of Agriculture and Technology, Bhubaneswar, Odisha with two varieties of *Pippali* (*Piper longum* L.) to establish a standardized protocol for *in vitro* regeneration, mass multiplication and biochemical characterization in a climber and a local creeper variety. An efficient and reproducible procedure for clonal multiplication via callus regeneration was successfully developed. Murashige and Skoog (MS) medium supplemented with 2 mg/L BAP and 0.5 mg/L K in induced optimal shoot induction, producing 4–5 shoots/1 cm node cutting. Organogenic calli (120 mg/explant) with an 80% induction frequency were obtained from the basal portions of *in vitro*-grown plantlets on MS medium supplemented with the same concentration of BAP and Kin. The combination of 2 mg/L BAP and 0.5 mg/L IBA yielded the highest number of shoots and promoted spontaneous rooting on the same medium. A high survival rate (95%) was observed following two weeks of hardening. The creeper variety demonstrated steady growth in height, leaf production and fruit yield, while the climber exhibited delayed but robust fruit set. Comparative biochemical profiling showed that the climber variety had higher protein content in both fruit and leaf tissues compared to the creeper, along with significant differences in glycine and phenol levels. High-performance thin-layer chromatography (HPTLC) analysis revealed that the highest piperine content (100, 260.4 AU, 2.62%) was present in the fruit extract of the creeper variety using ethanol, while the lowest piperine content (5,816.4 AU, 0.15%) was detected in the leaf extract of the creeper variety using methanol. Infrared (IR) absorption analysis identified functional groups such as alcohols, amides, alkanes and carboxylic acids. Notable peaks were observed at 3,343.39/cm and 1,253.02/cm in the leaves of the climber variety, and at 3,340/cm and 2,118.46/cm in the leaves of the creeper variety. These findings highlight the potential of pippali as a valuable source of bioactive compounds with significant pharmaceutical applications.

Keywords: Chemo-profiling, *In vitro* regeneration, Medicinal plant, Pippali

Piper longum L. (2n = 24 to 2n = 96), commonly known as long pepper or pippali, is a perennial flowering vine belonging to the Piperaceae family and the Piperales order (Biswas *et al.* 2022, Phan *et al.* 2024). Pippali requires hot and humid conditions (temperatures between 30–40°C in the summer and 4–10°C in the winter, with an average rainfall of 2,000–3,500 mm with 60% humidity), and with partial shade (ideally around 20–25%), for optimal growth (Thapa *et al.* 2024). It is adaptable to various soil types including laterite, black cotton and virgin soils, although well-drained, organic-rich soils are preferred with *p*H between 5.5–8.5 (Wu *et al.* 2024). The plant's stems are pubescent, with simple ovate leaves and unisexual cylindrical inflorescences opposite to the leaves (Sharma *et al.* 2024). The fruits of pippali are used for their culinary and medicinal uses (Bachu *et al.* 2023, Phurailatpam *et al.* 2024). However, the genome of long pepper has been sequenced, which is of 447.7 Mb, consisting of 89,204 scaffolds (Mathew and Valsalan 2024), which could help in identifying its diverse pharmacological properties including anti-asthmatic, anti-inflammatory, anti-cancer and anti-microbial activities (Jo *et al.* 2024, Joshi *et al*. 2024).

The natural populations of *Piper longum* face significant threats due to overharvesting for its medicinal value, habitat destruction from agricultural expansion and deforestation, and limited natural regeneration capacity (Wan *et al.* 2023). These pressures have led to a decline in its wild populations, raising concerns about its long-term availability and genetic diversity (Afroz *et al.* 2024). In response to these challenges, conservation efforts and sustainable utilization strategies are imperative to safeguard this valuable plant species (Darro and Khan 2023). However, establishing *in vitro* regeneration

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protocol of pippali would not only facilitate its conservation but also provide a sustainable source of plant material for further research and commercial cultivation (Afroz *et al.* 2024). Moreover, by quantifying the levels of bioactive compounds, such as piperine, alkaloids, and flavonoids, by phytochemical analysis, can evaluate the pharmacological properties of *in vitro* propagated pippali plants (Ly *et al.* 2024). Therefore, this study aimed to establish a robust protocol for the *in vitro* regeneration of pippali using nodal segments as explants, and to conduct a comprehensive phytochemical analysis of the regenerated plants for identifying and quantifying key bioactive compounds.

MATERIALS AND METHODS

Plant materials, explants source, nutrient media, and growth regulators: The present study was carried out during 2021–2022 at College of Agriculture, Odisha University of Agriculture and Technology, Bhubaneswar (20°15'52.24"N, 85°48'43.27" E), Odisha, for morpho-biochemical evaluation and *in vitro* regeneration using basal salts along with different plant growth regulators, at different concentrations (Murashige and Skoog 1962). Two different varieties, viz. creeper variety (UP1) and climber variety (UP2) of pippali were collected from Medicinal Plants Knowledge Centre, Patrapada, Bhubaneswar, Odisha (20.2429641N, 85.7622415E).

In vitro *culture and plant regeneration*: In this study, nodes (1–2 cm) from field-grown plants were thoroughly washed and surface sterilized using a series of treatments including carbendazim, ethanol, Tween20, and mercuric chloride. The sterilized nodes were trimmed and cultured on Murashige and Skoog (MS) medium with various concentrations of growth hormones like 6-Benzylaminopurine (BAP), kinetin (kin), and IAA for different purposes such as shoot elongation, callus induction, and rooting. The culture medium included sucrose and myoinositol, adjusted to a *p*H of 5.6–5.8, and solidified with agar. The media were autoclaved, and thermo-labile compounds were added post-autoclaving under sterile conditions. The cultures were maintained at 25 ± 2°C with a 16 h photoperiod and light intensity provided by fluorescent lamps. Explants were sub-cultured every 2–3 weeks (Thapa *et al.* 2024). For shoot initiation, nodes were inoculated on MS medium with BAP and kin. Subsequent subcultures were made every 25–30 days. Regenerated shoots were excised, elongated on media with BAP and kin, and then transferred to rooting media with BAP and IAA. Well-rooted plantlets were acclimatized by washing off agar and transferring to plastic containers with sterilized vermiculite, followed by planting in pots with soil, sand and farmyard manure (Afroz *et al.* 2024).

Standards of secondary metabolites and biochemical parameters assessment: Pipperine was purchased from Sigma chemicals. Organic solvent methanol and ethnanol procured from Spectrochem, Merck company was used for Soxhelet-solvent extraction of phytochemicals. The HPTLC silica gel 60 F254 plates (Aluminium backed, layer thickness 200 pm) used for phytochemical analysis and HPTLC plates were procured from Merck company (Yeotkar *et al.* 2010). Protein estimation was conducted by Lowry's method (Nabi *et al.* 2013), amino acid was estimated by Ninhydrin method (Shukla and Kashaw 2018), and phenolic analysis was conducted by a standard method (Jahan *et al.* 2014).

HPTLC and FTIR analysis: The leaves and fruits of the plant samples were shade-dried, ground, and extracted with methanol in a Soxhlet apparatus for 8–10 h, yielding a dark residue that was dissolved in methanol for HPTLC analysis of piperine (Yeotkar *et al.* 2010). Stock solutions of piperine (1 mg/ml) were prepared in methanol and ethanol for TLC analysis, using aluminum plates coated with HPTLC silica gel 60 F254. Samples were applied via a Linomat IV under nitrogen gas, and the solvent system for separation was ethyl acetate (8:2 v/v). After developing the plates, they were treated with a staining mixture and heated to visualize piperine spots. Quantitative analysis was performed using a Camag TLC Scanner at 254 nm and 366 nm, with Rf values calculated based on the distance traveled by the solute and solvent (Burma and Chakrabortty 1955). Calibration curves were created using various volumes of standard solutions, and data analysis was performed to identify significant differences (Langenberg *et al*. 2023).

RESULTS AND DISCUSSION

In vitro *regeneration*: In the present study, Fig. 1 visually documented the *in vitro* propagation of pippali, showcasing stages from initial explant preparation to the successful establishment of hardened plants in pots. Moreover, the Supplementary Table 1 summarized the effects of different treatments on root regeneration in plants, measured across several parameters. The treatments vary by the type and concentration of growth regulators added to Murashige and Skoog (MS) medium. Treatment T_1, with 1.0 mg/L BAP, resulted in 43.34% shoot response, with 3–4 roots regenerating after 21 days and 7–8 roots after 42 days. Increasing BAP to 2.0 mg/L in T_2 improved the response to 46.67%, with 3–4 roots after 21 days and 9–10 roots after 42 days. Treatment T_3, with 0.5 mg/L IBA, showed a lower response rate of 33.33%, with only 1–2 roots after 21 days and 4–5 roots after 42 days. Combining 1.0 mg/L BAP with 0.5 mg/L IBA in T_4 increased the response to 50.12%, resulting in 4–5 roots after 21 days and 8–9 roots after 42 days. Treatment T_5, which included 1.5 mg/L BAP and 0.5 mg/L IBA, had a further enhanced response of 54.67%, with 5–6 roots after 21 days and 10-12 roots after 42 days. The highest response was observed with T_6, containing 2.0 mg/L BAP and 0.5 mg/L IBA, achieving 66.67% shoot response, 7–8 roots after 21 days, and 12–14 roots after 42 days. The mean response across all treatments was 49.22%, with a critical difference of 0.07 at P=0.05.

In vitro regeneration demonstrates a clear correlation between the concentration of auxin (IBA) and cytokinin (BAP) and the efficiency of root regeneration in plants. The optimal combination of these growth regulators proved to be crucial for maximizing root number and shoot response.

Table 1 Comparison of the secondary metabolite content of various pippali varieties' extracted samples

Plant part	Plant variety	Dry sample weight (g)	Dry crude weight (mg)	Solvent	Peak	Start position (Rf)	Start height (AU)	Max position (Rf)	Max height (AU)	Max%	End position (Rf)	End height (AU)	Area (AU)	Area %	Piperine content (%)
Standard				Methanol	1	0.54	28.1	0.64	715.3	86.34	0.72	37.0	38182.3	100	1
Leaf	UP2 (climber)	5	510	Methanol	1	0.53	192.2	0.56	370.2	13.73	0.57	347.4	8812.7	14.81	0.23
Leaf	UP1 (creeper)	5	525	Methanol	1	0.56	216.1	0.57	332.0	18.82	0.58	236.8	5816.4	12.67	0.15
Fruit	UP1 (creeper)	5	610	Methanol	1	0.57	301.4	0.61	713.7	44.79	0.71	35.6	48800.6	62.50	1.27
Fruit	UP1 (creeper)	5	595	Ethanol	1	0.47	114.0	0.62	712.8	39.73	0.78	139.2	100260.4	68.21	2.62
Leaf	UP2 (climber)	5	505	Ethanol	1	0.53	267.5	0.60	529.2	15.18	0.61	519.5	25233.5	16.40	0.66

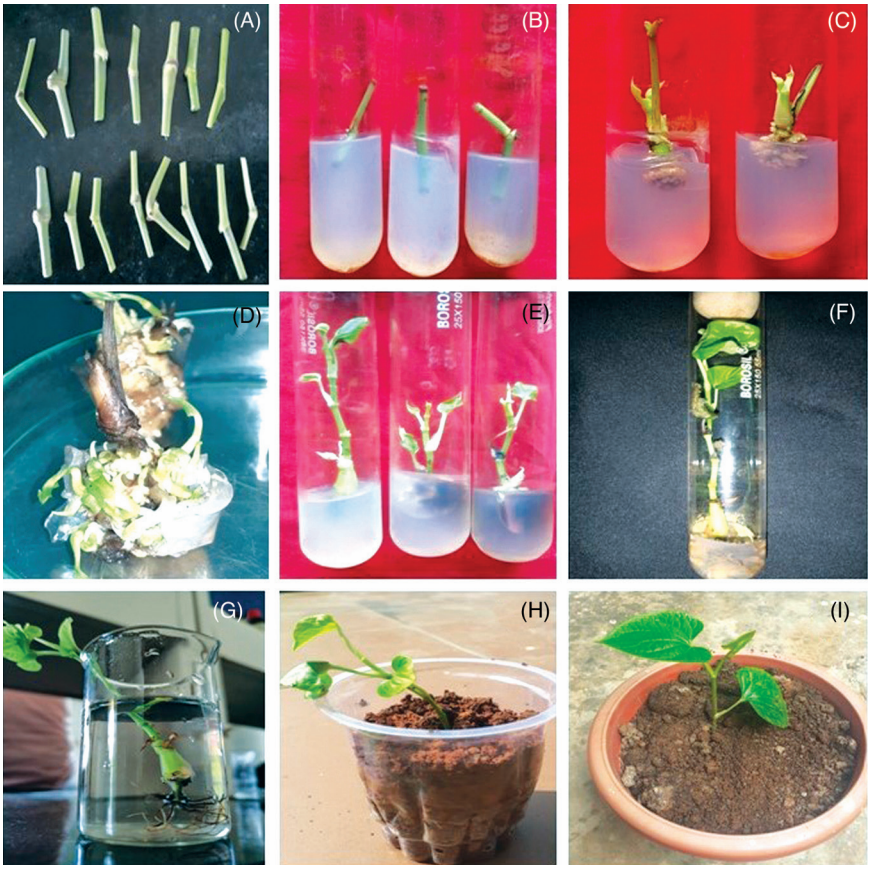

Fig. 1 *In vitro* culture and pippali plant regeneration. (A) Node cuttings; (B) Inoculation of node cuttings; (C) Explant after 14 days; (D) Regeneratnts development; (E) Callus in regeneration media with well-developed shoot; (F) Root development; (G) Well-developed shoot along with healthy roots kept in water before hardening; (H) Well-developed shoot along with healthy roots pre-hardened in plastic cup; (I) Hardened plant in pot after 2 weeks.

These findings aligned with previous studies highlighting the synergistic effects of auxins and cytokinins on plant growth and development, in which an efficient regeneration protocol for pippali was established using MS medium supplemented with various growth regulators (Padhan 2015). Further research could focus on elucidating the underlying molecular mechanisms to refine the application of these growth regulators for improved *Piper longum* propagation efficiency (Dat *et al.* 2024).

Morphological and biochemical characterization: The number of leaves grew from 12–47 over the same period, while the number of fruits increased from 2–9. Climber (UP2) had a shorter initial height of 24.3 cm, which grew to 67.2 cm after 90 days (Supplementary Table 2). The number of leaves for climber (UP2) started at 9 and rose to 51. Interestingly, this variety had no fruits at 30 days but produced 14 fruits by 90 days. This comparison showed that while both varieties experienced substantial growth in height and leaves, the climber (UP2) had at a later stage more significant fruit production compared to the creeper (UP1). Moreover, for the creeper variety, the fruit contains 7.85 mg of protein, 1.71 mg of glycine, and 0.95 mg of phenol/100 mg, while the leaf had higher values with 15.15 mg of protein, 2.02 mg of glycine, and 1.04 mg of phenol/1000 mg. The climber variety showed a slightly higher protein content in both its fruit and leaf, with 8.01 mg of protein in the fruit and 16.22 mg in the leaf. Glycine and phenol contents in the climber variety were notable, with the fruit containing 1.58 mg of glycine and 0.92 mg of phenol/1000 mg, and the leaf having 1.85 mg of glycine and 1.01 mg of phenol/1000 mg.

These findings indicated the importance of variety selection for specific agronomic and biochemical traits (Deka *et al.* 2024). The climber variety's higher protein, glycine and phenol contents in both fruit and leaf compared to the creeper variety may be attributed to genetic differences that enhance metabolic activity or nutrient allocation. These variations could also result from environmental factors such as vertical growth habits, which may provide better light exposure and resource acquisition, influencing biochemical composition (Deka *et al.* 2024, Wu *et al.* 2024). However, the morphological and biochemical characterization of the creeper and climber varieties of *Piper longum* highlighted distinct growth patterns and biochemical profiles, which have important implications for their cultivation, pharmacological properties, and applications in traditional and modern medicine (Wan *et al.* 2023). The variations in phytochemical composition, particularly in terms of piperine content and other bioactive compounds, underscore the need for targeted research to fully understand and utilize the medicinal potential of these two varieties of pippali (Wu *et al.* 2024).

HPTLC profile: The HPTLC profile was developed using a toluene: ethyl acetate: acetic acid solvent system under controlled conditions, with peaks scanned at 254 nm to measure Rf values and quantify secondary metabolites in area units (AU). Result shown in (Supplementary Fig. 1 and 2) indicated that all the reference standard and sample constituents were separated clearly on silica gel 60 F254 TLC plates. The Rf values of the bands for reference standards is, Piperine @Rf 0.54 (Table 1). It was also observed that maximum content of piperine (2.62%) was recorded in fruit extract of creeper variety in ethanol solvent (100260.4 AU), followed by fruit extract of creeper variety (1.27%) in methanol solvent (48800.6 AU), leaf extract of climber variety (0.66%) in ethanol solvent (25233.5 AU), leaf extract of climber variety (0.23%) in methanol solvent (8812.7 AU), and low content of piperine was recorded in leaf extract of creeper variety (0.15%) in methanol solvent (5816.4 AU).

The results indicate a higher piperine concentration in fruit extracts compared to leaf extracts, with the creeper variety exhibiting a notably higher piperine content than the climber variety across both fruit and leaf samples. The higher piperine concentration in fruit extracts compared to leaf extracts may be due to its role in protecting seeds and aiding in fruit defense mechanisms. The creeper variety's notably higher piperine content could stem from genetic factors or environmental conditions favoring secondary metabolite production in its growth habit (Rajopadhye *et al.* 2011). These findings suggest potential variations in the bioactive compound composition between these two varieties, warranting further investigation into other

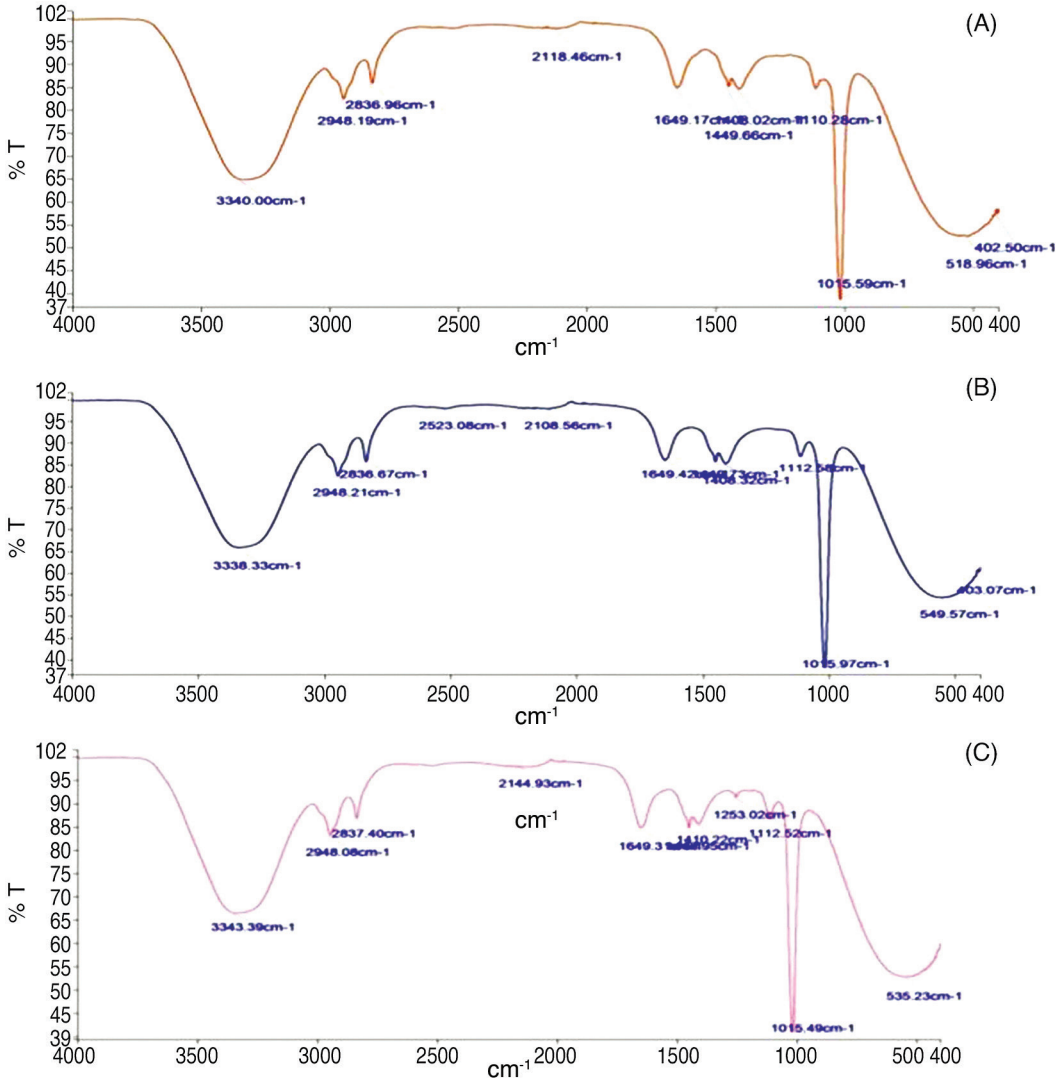

Fig. 2 FTIR analysis of different samples of pippali. (A) Leaves of climber variety; (B) Leaves of creeper variety; (C) Fruits of climber variety.

Table 2 Estimation of functional groups present in pippali

Sample	Peak No	Absorption	Class of compounds	Intensity	Bonds	Y (%T)
Leaves of climber variety of pippali	1	3343.39	Alcohol; Amides; Amine; Carboxylic acid	Strong, broad; Weak-medium; Weak; Strong, broad	O-H stretch; N-H symmetric stretch; N-H stretch O-H stretch	66.55
	2	2948.08	Alkanes and Alkyls; Carboxylic acid	Strong; Strong, broad;	C-H stretch; O-H stretch	83.35
	3	2837.4	Carboxylic acid	Strong, broad	O-H stretch	87.04
	4	1649.31	Alkenes; Amines; Amides	Very weak-medium; Weak-medium; Strong, broad; Medium-strong;	C=C stretch; N-H bend; C=O stretch; N=H bend;	85
	5	1253.02	Esters; Ethers; Alkyl halides	Strong-very strong; Medium-strong; Very strong	O=C-O-C stretch; =C-O-C asymmetric stretch; C-F stretch	91.5
	6	1112.52	Ethers; Alkyl halides	Strong; Very strong	C-O-C stretch; C-F stretch	87.27
	7	1015.49	Alkyl halides	Very strong	C-F stretch	40.94
	8	535.23	Alkyl halides	Strong	C-Br stretch	52.75
Leaves of creeper variety of pippali	1	3340	Alcohol; Amides; Amine; Carboxylic acid	Strong, broad; Weak-medium; Weak; Strong, broad	O-H stretch; N-H symmetric stretch; N-H stretch; O-H stretch	64.89
	2	2948.19	Alkanes and Alkyls; Carboxylic acid	Strong; Strong, broad;	C-H stretch; O-H stretch	82.63
	3	2836.96	Carboxylic acid	Strong, broad	O-H stretch	86.07
	4	2118.46	Alkynes	Medium	C=-C stretch;	97.97
	5	1649.17	Alkenes; Amines; Amides	Very weak-medium; Weak-medium; Strong, broad; Medium-strong;	C=C stretch; N-H bend; C=O stretch; N=H bend;	85.05
	6	1110.28	Ethers; Alkyl halides	Strong; Very strong	C-O-C stretch; C-F stretch	84.91
	7	1015.59	Alkyl halides	Very strong	C-F stretch	38.6
	8	518.96	Alkyl halides	strong	C-Br stretch	52.51
	9	402.5	Alkyl halides	strong	C-I stretch	57.42
Fruit of climber variety of pippali	1	3338.33	Alcohol; Amides; Amine; Carboxylic acid	Strong, broad; Weak-medium; Weak; Strong, broad	O-H stretch; N-H symmetric stretch; N-H stretch; O-H stretch	65.96
	2	2948.21	Alkanes and Alkyls; Carboxylic acid	Strong; Strong, broad;	C-H stretch; O-H stretch	82.57
	3	2836.67	Carboxylic acid	Strong, broad	O-H stretch	85.91
	4	2523.08	Carboxylic acid	Strong, broad	O-H stretch	98.19
	5	2108.56	Alkynes	Medium	C=-C stretch;	98.12
	6	1649.42	Alkenes; Amines; Amides	Very weak-medium; Weak-medium; Strong, broad; Medium-strong;	C=C stretch; N-H bend; C=O stretch; N=H bend;	86.21
	7	1112.58	Ethers; Alkyl halides	Strong; Very strong	C-O-C stretch; C-F stretch	87.11
	8	1015.97	Alkyl halides	Very strong	C-F stretch	38.12
	9	549.57	Alkyl halides	strong	C-Br stretch	54.31
	10	403.67	Alkyl halides	strong	C-I stretch	60.34

phytochemicals for a comparative analysis (Rajopadhye *et al*. 2011). However, HPTLC analysis has proven to be a reliable method for the qualitative and quantitative analysis of *Piper longum*, ensuring the accurate identification and quality control of its key bioactive compounds (Hamrapurkar *et al.* 2011).

FTIR analysis: It suggested that, for the leaves of the *climber* variety, eight peaks were identified, showing a range of compound classes such as alcohols, amides, alkanes and carboxylic acids (Fig. 2). Notable peaks included a strong-broad absorption at 3343.39/cm related to O-H and N-H stretches, and a peak at 1253.02/cm associated with esters and ethers. The creeper variety's leaves exhibited similar compound classes with peaks at 3340/cm (O-H and N-H stretches), 2118.46/cm (alkynes), and notable peaks for ethers and alkyl halides. For the climber variety's fruit, the absorption patterns were comparable, with strong-broad absorptions at 3338.33/cm and 2523.08/cm for alcohols, amides and carboxylic acids, and a significant peak at 2108.56/cm indicating alkynes. Across all samples, the absorption intensity percentages (Y) varied, indicating differences in the concentration and presence of specific functional groups in the different parts and varieties of pippali (Table 2).

The presence of characteristic absorption bands corresponding to alcohols, amides, alkanes, carboxylic acids, ethers and alkyl halides suggested a complex mixture of compounds (Jacob *et al.* 2012). While both climber and creeper varieties exhibited similar functional groups, variations in peak intensities indicated differences in compound concentrations. The identification of piperine as a major constituent in the fruit extract of the creeper variety, as confirmed by HPTLC, is corroborated by the FTIR spectra. These findings underscore the phytochemical diversity within pippali and provide a foundation for further targeted analysis of specific compounds of interest (Huang *et al.* 2020). Moreover, FTIR analysis of pippali can be effectively interpreted using the chemical composition information provided by chromatographic techniques (Yadav *et al.* 2019). The characteristic absorption bands of monoterpenes, sesquiterpenes and alkaloids such as piperine and piperlongumine can be used to identify and quantify these compounds in various parts of the plant (Janaranjani *et al.* 2024).

This study comprehensively investigated the morphological, biochemical and phytochemical characteristics of creeper and climber long pepper varieties. Distinct growth patterns, biochemical profiles and phytochemical compositions were observed between the two varieties. By optimizing the concentration of auxins (IBA) and cytokinins (BAP), the research highlights an effective protocol for enhancing root and shoot development *in vitro*. It is also observed that, the creeper variety, for instance, shows a higher piperine content compared to the climber. These insights into the distinct biochemical compositions of each variety have implications for their cultivation and medicinal use. Moreover, comprehensive phytochemical profiling coupled with pharmacological studies is essential to unlock the full potential of pippali as a source of bioactive compounds for therapeutic applications.

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