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Research Article

Evaluation of gas chromatography-mass spectrometry analysis and yield attributing traits of caffeine treated *Trigonella corniculata* L.: A medicinally important herb

Neha NAAZ[1*], Sana CHOUDHARY[1a], Nidhi SHARMA[1], Nazarul HASAN[1], Najla A. AL SHAYE[2b], Diaa ABD EL MONEIM[3c]

[1]*Aligarh Muslim University, Department of Botany, Cytogenetics and Plant Breeding Laboratory, Aligarh, India; nehanaaz440@gmail.com (*corresponding author); sana.12amu@gmail.com; nidhisharma7517@gmail.com; nazarulpasha143@gmail.com*
[2]*Princess Nourah bint Abdulrahman University, College of Science, Department of Biology, Riyadh, Saudi Arabia; naaalshaye@pnu.edu.sa*
[3]*Arish University, Faculty of Environmental Agricultural Sciences, Department of Plant Production (Genetic Branch), El-Arish, Egypt; dabdelmoniem@aru.edu.eg*
[a,b,c]*These authors contributed equally to the work*

Abstract

Trigonella corniculata L. (Kasuri methi), a medicinal plant from the Fabaceae family, with exceptional culinary value, nutritional importance, and therapeutic properties that offer valuable curative benefits. Mutation breeding is highly regarded by plant breeders as an effective method of enhancing crop productivity and achieving sustainable crop production. Genetic diversity serves as the foundation for plant breeding programs, compelling the introduction of mutations to enhance variability. This study employed caffeine treatment of five concentrations (0.2%, 0.4%, 0.6%, 0.8% and 1.0%) on *T. corniculata* (var. 'Pusa kasuri') seeds, resulting in significant effect ($p<0.05$) on the morpho-physiological and quantitative parameters. Various statistical methods, including Pearson correlation analysis and principal component analysis (PCA) unveiled correlations between plant characteristics. A strong positive correlation (0.99) emerged between clusters per plant and seed yield. Principal component analysis revealed that the first two out of twelve principal components contributed to 90% of the variation, indicating genotypic diversity. The correlation between clusters per plant, pods per cluster, and seed yield, as indicated by PCA and Pearson's correlation heatmap analysis, affirms high-yield potential. Methanolic extracts of *T. corniculata* were subjected to gas chromatography-mass spectrometry (GC-MS) analysis, revealing 17 major phytocompounds known for their pharmacological activities (antioxidant, antimicrobial, anti-inflammatory, etc.). This study has the potential to pave the way for developing novel herbal remedies for various diseases using *T. corniculata*, potentially resulting in the formulation of new medications.

Keywords: bioactive compounds; gas chromatography-mass spectrometry (GC-MS); genetic diversity; medicinal plant; yield potential

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Introduction

Plant breeding is a specialized field dedicated to the targeted and continuous development of novel plant varieties. It leverages the genetic variation within plant species to create improved varieties by combining desirable traits (Holme and Gregersen, 2019). Mutation breeding is a crucial component of modern plant breeding, involving the creation of new varieties through induced mutagenesis. A core advantage of mutation breeding lies in its capacity to enhance specific characteristics of a variety without altering the genetic background (Khan *et al*., 2009). Assessing the genetic diversity of germplasm is crucial for identifying genotypes with higher diversity and better performance in specific conditions (Naaz *et al*., 2024a). Induced mutagenesis is a valuable tool for pinpointing key regulatory genes and molecular mechanisms, serving as a promising strategy for producing novel varieties with enhanced agronomic traits, including higher stress tolerance (both biotic and abiotic stress) and bio-fortification. The purpose of induced mutations is to increase the frequency of beneficial variants for selection in plant breeding (Oladosu *et al*., 2016). Two primary approaches, irradiation and chemical mutagens, are used for inducing mutations in plants (Leitao, 2011). Irradiation through X-rays and gamma radiation induces substantial chromosome deletions and point mutations, while chemical mutagens such as NaN_3, EMS, and MNU mainly cause single base substitutions (transitive, i.e., from G/C to A/T). Chemical mutagens offer the advantage of generating mutant populations with a high density of mutations, simplifying the screening process for specific mutations. Induced mutagenesis is also being applied to enhance medicinal plants due to their high demand. Often, seeds are used for chemical mutagenesis. Promising results have been observed using caffeine, a purine alkaloid, to improve agronomic traits. Caffeine's mutagenic effect is attributed to its interaction with DNA, altering its physical properties such as denaturation temperature and increasing the rate of spontaneous mutations (Szarejko *et al*., 2017). Due to its purine nature, caffeine holds mutagenic potential and can synergistically induce chromosomal aberrations in mammalian cells (Truta *et al*., 2007). The mutagenic impact of caffeine has been demonstrated in various crops, including *Capsicum annum* (Aslam *et al*., 2017), *Lens culinaris* (Yousuf *et al*., 2023) and *Trigonella* (Naaz *et al*., 2023).

Trigonella corniculata L. belongs to a genus comprising about 135 species within the Leguminosae family. Among these species, *T. corniculata* holds significance as a high-yield crop extensively cultivated in the Punjab region, particularly in Kasur. Methi from Kasur is renowned for its fragrance throughout India and also serves as a geographical indicator of Kasur (Pakistan) as "Kasuri Methi" (Erum *et al*., 2011). Globally recognized as "Kasuri Methi," this plant holds a prominent position as a traditional and highly valued medicinal herb. Its reputation has been established over numerous decades due to its exceptional culinary value, nutritional significance, and therapeutic properties with curative benefits (Jiang *et al*., 2017). Cultivated Fenugreek (*Trigonella corniculata* L.) is an herbaceous, bushy, slow-growing annual spice crop primarily grown for its dry herbage. Its leaflets are oblong-wedge-shaped, measuring 1-4 cm in length, 8-35 mm in width, with notched tips. Flowers form in clusters of 8-20 on peduncles measuring 1.5-6 cm. Flower stalks are 3 mm long, with a 3-4 mm sepal cup and nearly equal sepals, shorter or equal to the tube length. The yellow flowers are 6-7 mm long, with wings shorter than the keel. The pods exhibit a sickle-shaped form, ranging 1.2-2.0 cm in length, and containing 4 to 8 seeds. Its fresh tender leaves and pods are consumed as fried vegetables, offering rich iron, calcium, protein, and vitamin content. Additionally, fenugreek leaves are abundant in vitamins, including 2.34 mg of carotene, 0.04 mg of thiamine, 0.31 mg of riboflavin, 0.8 mg of nicotinic acid, and 52.0 mg of vitamin C per 100 g of the edible portion (Tayade *et al*., 2021). The primary nutritional constituents in *T. corniculata* seeds encompass carbohydrates (45-60%), protein (30-40%) rich in lysine and tryptophan, ash (3-4%), and a lipid content (7.5%) (Kang *et al*., 2013; Campia *et al*., 2017). Fenugreek is a remarkable medicinal herb with the potential to alleviate various ailments (Abhani and Raut, 2016). Its seeds and leaves are abundant in nutritional content and are extensively used for their anti-diabetic, anti-oxidant, anti-microbial, and anti-inflammatory properties, as well as for treating cancer (Chau *et al*., 2004). Furthermore, the significance of fenugreek as a seed spice is steadily growing, with its seeds being increasingly exported to the global market (Rana *et al*., 2015).

Medicinal plants possess a diverse array of bioactive compounds with the potential to offer antimicrobial, anticancer, anti-inflammatory, and antioxidant effects. Traditional plant-based remedies often utilize crude extracts, which comprise complex combinations of various phytochemicals, to address both chronic and infectious ailments (Sahoo and Manchikant, 2013). In India, from ancient time, different parts of medicinal plants (~ 80,000 species) have been employed as traditional medicines within various systems of Indian medicine to treat a range of diseases (Pandey *et al*., 2013). Developing effective screening methods is crucial for identifying novel chemicals and ensuring quality control (Melongane *et al*., 2017). The extraction and categorization of these bioactive compounds have led to the creation of specialized medicines with high-activity profiles (Yadav *et al*., 2017). Gas chromatography-mass spectrometry (GC-MS) has recently been adopted to identify various bioactive constituents within medicinal plants (Fan *et al*., 2018). GC-MS is a reliable approach for identifying a range of chemicals in plant extracts such as alkaloids, flavonoids, organic acids, amino acids, etc (Razack *et al*., 2018). Numerous studies have been conducted on various crops such as *Amomum nilgiricum* (Konappa *et al*., 2020), *Parkia timoriana* (Ralte *et al*., 2022) and *Piper betle* (Madhumita *et al*., 2019). Therefore, the present study is focused on exploring genetic variability through chemical mutagenesis and identifying bioactive compounds within the methanolic extract of *Trigonella corniculata* seeds through GC–MS analyses.

Materials and Methods

Certified seeds of *Trigonella corniculata* L. (var. 'Pusa Kasuri') were procured from Indian Agricultural Research Institute (IARI), New Delhi, India. Uniform seeds containing 10% moisture content were soaked in water for 12 hours to enhance mutagen's efficacy. A stock solution of caffeine (1% v/v) (manufactured by Sigma Aldrich, Mumbai, India) was prepared at a pH 7.0 and this pH was maintained through buffer tablets. Subsequently, the seeds were treated with 0.2, 0.4, 0.6, 0.8 and 1.0% caffeine concentrations for 9 hours with intermittent shaking at room temperature 25±2 °C. Following the treatment, the seeds underwent thorough washing under running tap water for 30 mins to remove any excess mutagen. The 100 untreated and treated seeds from each concentration were sown in five replicates of 20 seeds each in 12-inch earthen pots. This was carried out within the net house of the Department of Botany, Aligarh Muslim University, Aligarh, India during the rabi season in the year 2019-20, in order to raise the M_1 generation. The experimental site, Aligarh is characterized by its semi-arid, subtropical climate, with temperatures ranging from 28-45 °C during summer and 5-11 °C during winter. The region experiences an annual rainfall of approximately 800 mm, mainly occurring from late June to early October, resulting in increased humidity. The soil is sandy loam and alkaline in nature.

The primary objective of this experiment was to evaluate the impact of caffeine on the studied parameters within the M_1 generation. Subsequently, the obtained results were precisely screened and analyzed.

Morphological and quantitative characteristics

Morphological variations, such as plant height, growth habit, cotyledonary leaf characteristics (shape and color), vegetative leaf characteristics (shape and color), flower position, pod shape and seed variations were meticulously observed and evaluated. For quantitative traits assessment, data were collected by analyzing samples from fifteen plants within each replication, and the outcomes were presented as means. Plant height was determined by measuring the distance from the plant's base to its highest point or apex. The number of fertile branches per plant was evaluated by tallying the branches at the maturity stage. Counting the pods from successive harvests facilitated the calculation of pods per plant. To determine seeds per pod, the seeds within randomly selected pods from the fifteen plants were counted. Seed weight was determined by weighing 1000 seeds taken from a randomly selected set of fifteen plants. Furthermore, by weighing all the seeds harvested from the fifteen randomly selected plants, the overall plant yield was evaluated.

Physiological parameters

Fresh leaves (1 g) were ground and subsequently mixed with 20 ml of 80% acetone. The resulting mixture was homogenized and then subjected to centrifugation at 5000 rpm for 10 minutes. The resulting supernatant was adjusted to a final volume of 100 ml using an 80% acetone solution in a volumetric flask. Absorbance readings were taken at 645 and 663 nm for chlorophyll, and at 480 and 510 nm for carotenoids, using a blank as reference. The quantification of chlorophyll content in the leaf extracts followed the equation proposed by (Arnon, 1949), while the carotenoid content was determined using the formula developed by (Kirk and Allen, 1965).

$$\text{Total chlorophyll content (mg/g)} = [20.2\,(OD_{645}) + 8.02\,(OD_{663})]\frac{V}{1000 \times w}$$

$$\text{Carotenoid content (mg/g)} = [7.6\,(OD_{480}) - 1.49\,(OD_{510})]\frac{V}{1000 \times d \times w}$$

OD_{645}, OD_{663}, OD_{480}, OD_{510} are the optical densities by 645, 663, 480 and 510 nm respectively; V, Volume of an extract; W, the mass of leaf tissues; d, the length of light path (1.4 cm).

Proline estimation

The measurement of free proline was performed using a spectrophotometric method based on the procedure outlined by (Bates *et al*., 1973). Fresh leaves (500 mg) were extracted using 10 ml of a 3% aqueous solution of sulphosalicylic acid, and the resulting homogenate was then filtered. To 2 ml of the filtrate in a glass test tube, 2 ml of glacial acetic acid and 2 ml of acid ninhydrin were added. The mixture was heated in a boiling water bath for an hour and then cooled in an ice bath. Following this, 4 ml of toluene was introduced into the reaction mixture and vigorously stirred for 20-30 seconds. The absorbance of the resultant toluene-containing chromophore was measured at 520 nm.

Cytological study

The flower buds of 30-day-old plants, randomly selected from both control plants and those subjected to chemical treatments, were harvested. These buds were then fixed in Carnoy's solution (a mixture of ethanol, pure chloroform, and acetic acid in a 6:3:1 ratio) for 24 hours. Afterward, they were transferred to a 70% alcohol solution. Anthers were squashed onto slides with 2% acetocarmine, covered with a cover slip, and then examined under a light microscope to observe chromosomal behavior. For further chromosome analysis, permanent slides were made using the NBA-GAA series. These slides were then mounted in Canada balsam and subjected to drying at a temperature of 45 °C.

Preparation of methanolic extract

For the investigation, 5 g of seeds from both the control group and the mutants were ground to a powder using a mortar and pestle. The powdered seeds were subsequently immersed in 10 ml of methanol and left to soak for 24 hours. Following this, the methanolic extract of the seeds was subjected to centrifugation at 10000 rpm for a duration of 15 minutes. The resulting pellet was removed, and the supernatant was filtered through Whatman filter paper No.1. The filtered extract was gathered and stored in vials for subsequent analysis.

GC-MS analysis

Gas chromatography-mass spectrometry (GC-MS) analysis of the methanolic extract was conducted using a GC-MS-TQ8050 NX (Shimadzu Corporation, Kyoto, Japan) at the central instrumentation laboratory of the Central University of Punjab, Bathinda, India. The split injector temperature was set to 250

°C with a split ratio of 5. The temperature of the oven was gradually increased from 0 to 40 °C, followed by a further increase to 220 °C, during which hold periods of three and five minutes were observed, respectively. Subsequently, the temperature was raised to 250 °C and maintained for 5 minutes. Helium was employed as the carrier gas, flowing consistently at a rate of 1 mL min^{-1}. The ion source temperature was set at 230 °C, and the interface temperature at 250 °C, with a mass scan range spanning from 40 to 800 amu. The flow rate through the column was maintained at 1.00 mL min^{-1}, while the oven temperature was set at 40 °C. For each sample, a volume of 1 µL was loaded and injected. To analyze the bioactive compounds in the methanolic extract, the NIST17R library and NIST17M2 library were utilized, utilizing their m/z ratios and retention times as the basis for identification.

Statistical analysis

The collected data from the study were subjected to statistical analysis through RStudio software. Treatment differences were determined using one-way analysis of variance (ANOVA), followed by the Duncan Multiple Range Test (with a significance level of $p \leq 0.05$) to compare means across treatments. Graphs were generated using GraphPad Prism 9. Furthermore, for a comprehensive analysis, techniques including principal component analysis (PCA), and Pearson correlation analysis were employed. Both GraphPad Prism 9 and RStudio were utilized to plot these analyses, aiding in the visualization, and understanding of relationships among variables.

Results and Discussion

Morphological and quantitative characteristics

Different concentrations of caffeine led to diverse morphological variations, primarily affecting features such as cotyledons, leaves, plant height and structure, flowers, pods, and seeds. The detectable changes caused by caffeine-induced mutagenesis in the fenugreek are visually represented in Figure 1 (A-F). The variations can be categorized into morphological differences like cotyledon's shape, size and orientation, shape, size, lamina and color of leaves, plant height and habit, flowers per cluster, shape of pods, and seed shape (Table 1). Control seedlings had two opposite, entire, green, oblanceolate cotyledonary leaves of equal size (Figure 1Aa). Following caffeine treatment, cotyledonary leaf variations were observed at 0.4%, where both cotyledonary leaves were oriented towards one axis (Figure 1Ab), and at 0.8%, tips of both leaves were notched (Figure 1Ac). The control vegetative leaf was compound, alternate, trifoliate, with normal green, entire, and comprised three small obovate to oblong leaflets (Figure 1Ba). The following leaf variations were observed: curly leaflets with wavy margin and thickness at 0.4% (Figure 1Bb), acute leaflets at 0.4% (Figure 1Bc), two normal leaflets and another bifurcated leaflet at 0.6% (Figure 1Bd), interveinal chlorosis in some parts of the leaflets at 0.8% (Figure 1Be) and slightly xantha variant at 1.0% (Figure 1Bf). Regarding plant height and growth pattern, control plants exhibited normal height and branching, resulting in a moderate yield (Figure 1Ca). Variations included a tall bushy variant with high yield at 0.2% (Figure 1Cb) and a normal single-branched variant with low yield at 0.8% (Figure 1Cc). Other variations included the number of flowers per cluster (Figure 2A), pods variations such as a greater number of pods per cluster, thin and broad pods (Figure 2B), and seed variations including large bold and small round shaped seeds (Figure 2C). These morphological variations have proven advantageous in mapping studies and contribute to the understanding of crop evolution (Gaur and Gour, 2003). Variations, including plant height, growth pattern, leaf morphology, and pod variation, were considered as monogenic recessive.

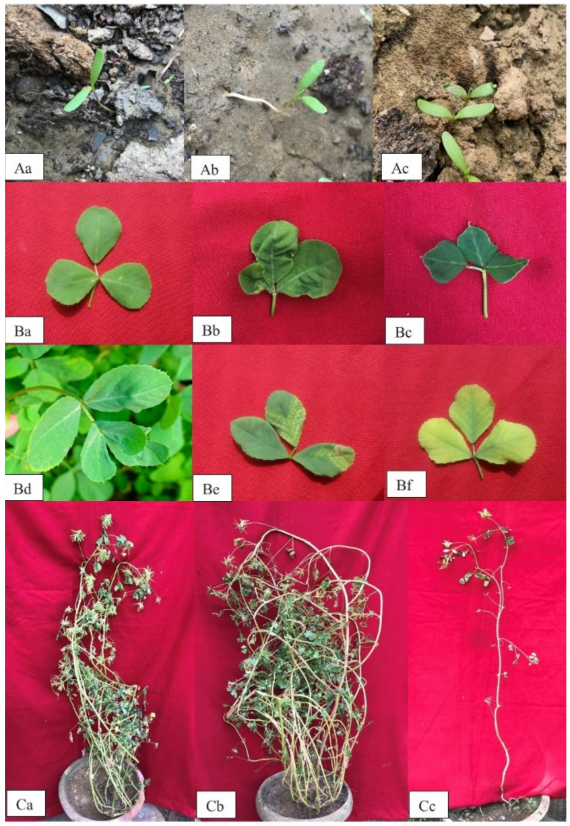


Figure 1. Morphological variations induced by caffeine treatments in *T. corniculata*: A) cotyledons; B) leaf; C) plant height and habit

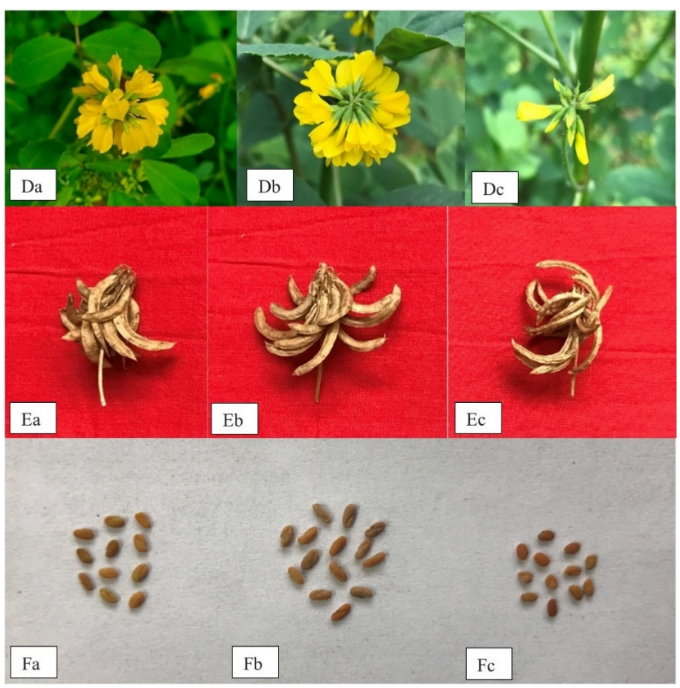


Figure 2. Morphological variations induced by caffeine treatments in *T. corniculata*: A) flower; B) pod; C) seed

Similar tall and dwarf variants were observed in fenugreek (Hassan *et al*., 2018; Naaz *et al*., 2023). The production of phytohormones like gibberellic acid and brassinosteroids is controlled by numerous genes, and a mutation within these genes leads to changes in plant height (Mitchum *et al*., 2006). Variations in leaf morphology have been reported in *Trigonella foenum graecum* (Choudhary *et al*., 2012; Hassan *et al*., 2018; Naaz *et al*., 2020; Naaz *et al*., 2023). The leaf anomalies detected in the mutagenized populations could be attributed to disturbances in internal growth regulators, like indole-3-acetic acid (IAA), which might be stimulated by mutagen activity or could arise from the primary or secondary effects of free radicals induced by the mutagens (Tao *et al*., 2002; Islam *et al*., 2019). Seed variations could be ascribed to the mutagen-triggered disturbance of one or a few genes associated with these traits. Similar variations have been reported in crops such as linseed (Jahan *et al*., 2020) and lentil (Sharma *et al*., 2022) (Table 1).

Table 1. Various phenotypic variations observed in the mutagenized population of *T. corniculata*

No.	Characters	Sub-characters	Description
1.	Plant height		Tall, semi-dwarf and dwarf
2.	Branching habit		Few branching, profuse branching, no branching
3.	Leaf	Leaf shape	Trifoliate, elongate, notched, rounded, ovate
		Lamina margin	Tripartite, serrulate, curly surface with wavy margin.
		Leaf color	Usually light green, dark-green, marginal, and inter-veinal chlorosis, xantha
4.	Pod	Pod shape	Mostly Sickle-shaped, broad sickle shaped; narrow sickle shaped.
5.	Seed	Seed shape	Mostly cuboid, elongated, small-round

The unexpected results of quantitative traits, such as plant height, number of fertile branches per plant, number of clusters per plant, number of pods per cluster, number of seeds per pod, 1000 seeds weight, and seed yield, leaned towards improvements in crop yield, as shown in the Figure 3. The maximum significant increase in plant height (88.00 cm, at $p<0.05$) was recorded at the 0.2% concentration, whereas there was a significant yet minimal decrease (75.40 cm, at $p<0.05$) at the 1.0% concentration level compared to the control plant's height (80.60 cm). A significant increase in fertile branches per plant was observed at 7.11 and decreased to 5.87 (at $p<0.05$) in 0.1% and 1.0% concentration, respectively. The maximum increase in clusters/plant and pods/cluster values was 36.55-27.26 and 24.46-17.34 in 0.1-1.0% concentrations, respectively and was significant (at $p<0.05$), while the minimum was found in 1.0% and 0.8% compared to control. The 0.1% concentration shows maximum mean values in both seeds per pod and seed weight and a minimum in 1.0% concentration, which were significant (at $p<0.05$). Maximum 1000-seed weight (g) was observed in 0.1% (1.98, at $p<0.05$), while seed yield per plant (g) was observed at 0.1% concentration (at $p<0.05$), while both were minimum at 1.0% as compared to that of the control plant. In this context, it was noted that the average plant height exhibited a significant increase in comparison to the control at lower and moderate mutagen concentrations. Nonetheless, with the increase of mutagenic treatments, there was a trend of decreasing plant height. Decreased plant height has been previously acknowledged in various crops, including *Trigonella foenum-graecum* (Hassan *et al*., 2018) and *Capsicum annum* (Hasan *et al*., 2022a). The reduction in plant height could be attributed to changes in ascorbic acid, meristematic tissues, and physiological disturbances (Dhamayanthi and Reddy, 2000). The results of mean number of branches per plant align with earlier studies in fenugreek (Hassan *et al*., 2018; Naaz *et al*., 2020; Naaz *et al*., 2023), in urdbean (Goyal *et al*., 2022) and in lentil (Sharma *et al*., 2022). The rise in fertile branches per plant among populations treated with lower mutagen doses can be attributed to the decreased synthesis of strigolactones. In addition, various research studies have indicated a significant rise in pod per plant in many crops, such as in cowpea (Opoku Gyamfi *et al*., 2022), in lentil (Laskar and Khan, 2017), and in faba bean (Khursheed *et al*., 2019). The mean number of pods per plant, as well as the total seed yield per plant are interrelated with one another. As the number of pods per plant rose, it had a direct impact on the overall seed production. Consequently, a higher quantity of pods and seeds led to a subsequent increase in the overall yield per plant. Here, higher concentrations of mutagen had inhibitory effects on the yield, whereas lower and intermediate concentrations showed stimulatory effects on the seed yield. These results align with earlier studies (Amin *et al*., 2019; Hasan *et al*., 2020; Naaz *et al*., 2024a). This decrease in yield might be attributed to disruptions in meiosis, alterations in normal microspore and megaspore frequency, physiological disturbances, chromosomal damage, disturbed spindle formation, restricted pairing, prolonged DNA synthesis, and high pollen sterility.

Physiological parameters:

The caffeine treatment had a substantial impact on the levels of chlorophyll and carotenoids. The results indicated that at the lowest concentration of caffeine (0.2% and 0.4%), chlorophyll content increased significantly (2.89 mg g^{-1} and 2.63 mg g^{-1}, at $p<0.05$) respectively, in comparison to the control (2.11 mg g^{-1}), as demonstrated in the Figure 4. Similar to chlorophyll, the carotenoid levels also exhibited an increase compared to the control. The highest significant carotenoid content of 0.77 mg g^{-1} ($p<0.05$) was recorded under the 0.2% treatment of caffeine. However, increased doses of caffeine resulted in a remarkable decline in chlorophyll and carotenoid content. These outcomes align with previous studies on *Trigonella* (Kavina *et al*., 2020; Naaz *et al*., 2023) and *Capsicum annum* (Hasan *et al*., 2020), providing further evidence that mutagenic treatments influence photosynthetic processes. The rise in overall chlorophyll and carotenoid contents within the mutants was primarily linked to higher levels of chlorophyll-a and β-carotene (Tomlekova *et al*., 2009). The increased activity of chlorophyllase, an enzyme responsible for the breakdown of chlorophyll, could play a role in the decrease of chlorophyll content (Reddy and Vora, 1986). The activation of carotenoid biosynthesis genes resulting from mutagenic treatments could potentially enhance resistance to diverse climate-induced stresses, including drought and salinity. Additionally, carotenoids possess antioxidant properties that reduce oxidative stress in the body (Fiedor and Burda, 2014).

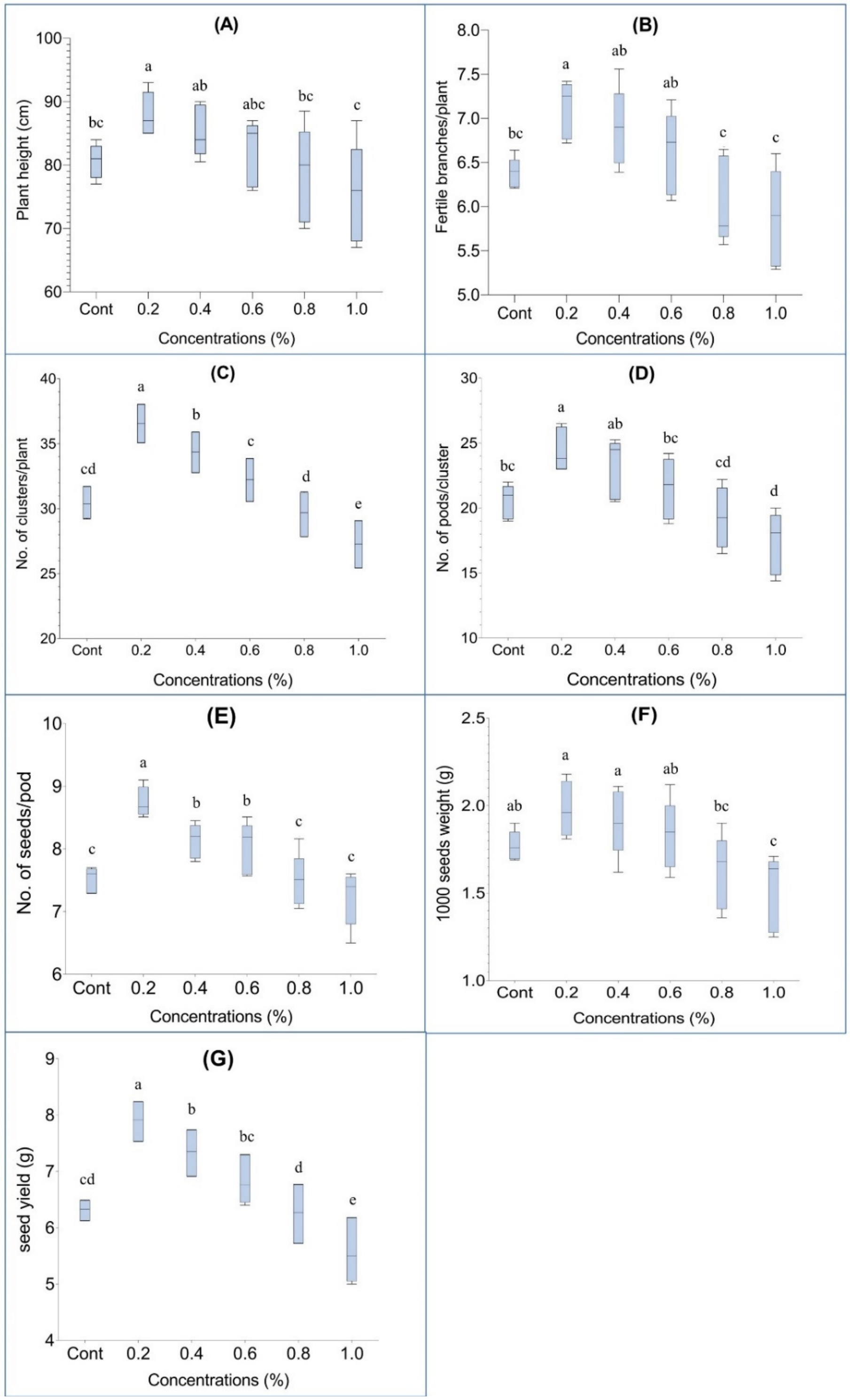


Figure 3. Effect of caffeine treatment on A) plant height, B) fertile branches per plant, C) number of clusters per plant, D) number of pods per cluster, E) number of seeds per pod, F) 1000 seeds weight and G) seed yield of *T. corniculata*

Data is presented as mean ± SE of five replicates (n=5) of each treatment. Error bars with different lowercase letters are significant at 5% level of significance, tested by Duncan Multiple Range Test (DMRT)

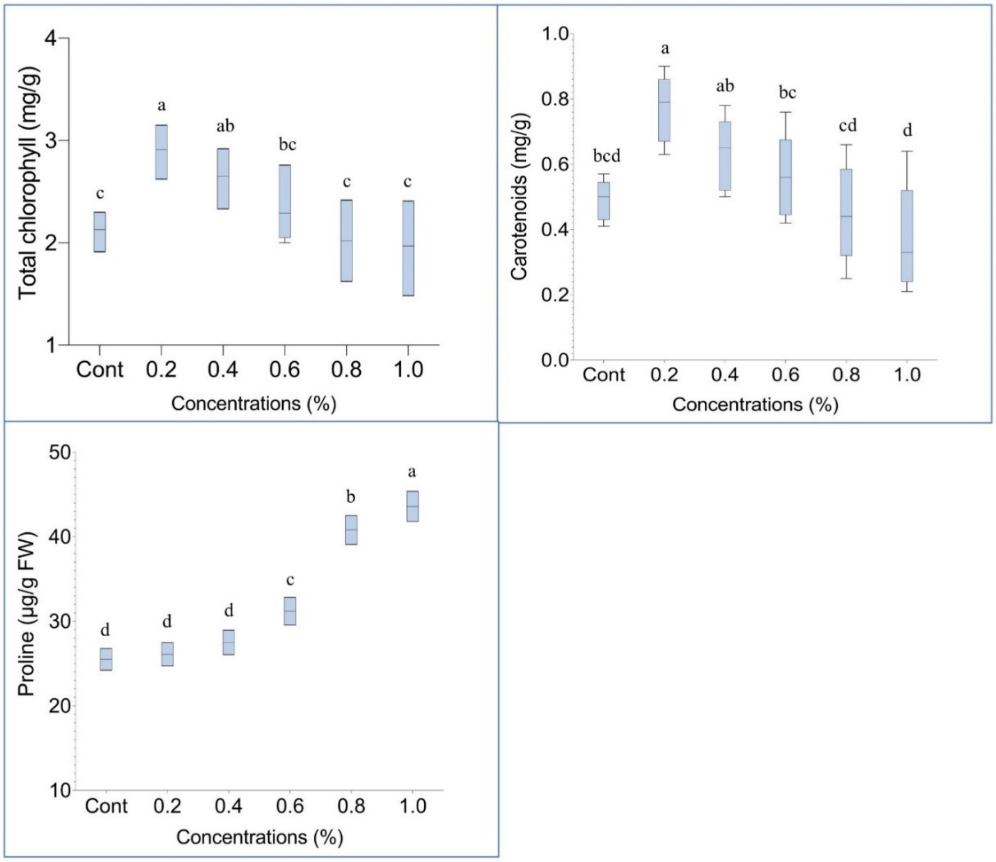


Figure 4. Effect of caffeine treatment on: total chlorophyll content, carotenoids and proline content of *T. corniculata*

Data is presented as mean ± SE of five replicates (n=5) of each treatment. Error bars with different lowercase letters are significant at 5% level of significance, tested by Duncan Multiple Range Test

Proline estimation

The proline content exhibited a consistent upward trend as the concentrations of caffeine increased in comparison to the control plants (Figure 4). Notably, when subjected to higher caffeine treatments, there was a substantial rise in proline content. The proline content exhibited a significant increase with 43.59 µg g^{-1} FW ($p<0.05$) at the 1.0% concentration, as compared to the control (25.5 µg g^{-1} FW). This suggests that the treatment with higher levels of caffeine led to a pronounced enhancement in proline accumulation, indicating a potential response to the treatment's effects. Proline is essential for regulating cell functions, protecting cell membranes, and ensuring the stability of biological macromolecules (Nadar and Rathod, 2020). These results correspond with findings in *Coriandrum sativum* (Kumar and Pandey, 2019), and *Capsicum annum* (Hasan *et al*., 2022b), underscoring the significance of proline in enhancing stress tolerance and facilitating osmoregulation.

Cytological study

For evaluating chromosomal abnormalities in chemically treated fenugreek plants, pollen cells were analyzed using a compound microscope. Normal meiotic division with eight perfect bivalents (2n=16) at metaphase, anaphase, and telophase was observed in the control plants (Figure 5a, 5b). Various chromosomal abnormalities, such as sticky anaphase I with three laggards (Figure 5c), univalents (Figure 5d), stray bivalents at metaphase I (Figure 5e), and disturbed polarity (Figure 5f), etc. were identified during the cytological study.

The frequency of these chromosomal abnormalities in the mutagen-treated plants differed according to the mutagen concentration (Table 2). The highest frequency of chromosomal abnormalities was observed at a 1.0% concentration, showing a dose dependent relationship. The total percentage of abnormal pollen mother cells (PMCs) ranged from 2.53% to 21.64% in the plants. The presence of laggards, as observed in this study, is attributed to the appearance of abnormal spindle fibres that fail to bind and transport respective chromosomes to the polar region during meiosis, leading to the formation of laggards (Tarar and Dnyansagar, 1980). Chromosomal stickiness, arising from nucleic acid depolymerization or incomplete dissociation of nucleoproteins, induces alterations in chromosomal arrangement following exposure to mutagens. The presence of stray chromosomes during metaphase can be linked to spindle dysfunction and clumping of chromosomes (Bhat *et al*., 2007). These results align with earlier investigations carried out on diverse plant species like *Trigonella foenum-graecum* (Choudhary *et al*., 2012; Naaz *et al*., 2024a), *Capsicum annum* (Hasan *et al*., 2022a; Hasan *et al*., 2022b), and *Lens culinaris* (Sharma *et al*., 2022).

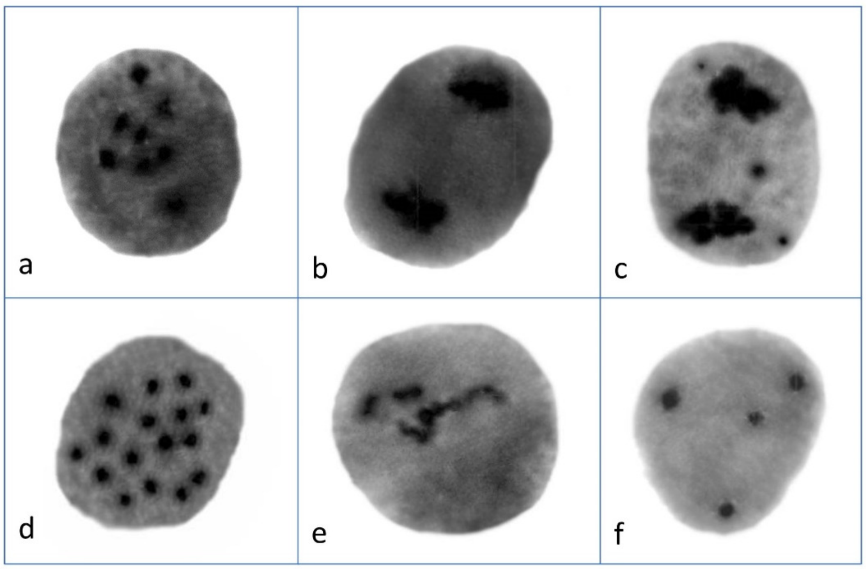


Figure 5. Chromosomal abnormalities: a) diakinesis of prophase I; b) anaphase I; c) sticky anaphase I with three laggards; d) univalent; e) stray bivalents at metaphase I; f) disturbed polarity induced by caffeine treatment in *T. corniculata*

Table 2. Frequency of chromosomal abnormalities induced by caffeine in *T. corniculata*

Treatments (caffeine %)		***Trigonella corniculata***					
		Control	**0.2%**	**0.4%**	**0.6%**	**0.8%**	**1.0%**
Total no. of PMCs observed			277	271	281	260	268
Prophase-I (Diakinesis)	Univalents	-		1	2	2	3
	Multivalents	-		1	1	3	4
	% of abnormal PMCs	-	0.00	0.74	1.07	1.92	2.61
Metaphase-I/II	Univalents	-		1	1	2	4
	Multivalents	-		1	2	3	3
	Precocious movement	-	1		2	3	5
	Stray chromosome	-		1	1	1	3
	Stickiness	-	1	1	2	3	3
	% of abnormal PMCs	-	0.72	1.48	2.85	4.62	6.72

Anaphase-I/II	Laggards	-		1	2	3	4
	Bridges	-		1	1	2	4
	Unequal separation	-	1	1	2	3	2
	% of abnormal PMCs	-	0.36	1.11	1.78	3.08	3.73
Telophase-I/II	Laggards	-		1	2	4	2
	Bridges	-	1	2	1	3	3
	Unequal separation	-	1	1	2	3	5
	Micro nucleate	-		1	3	3	4
	Multi nucleate	-			2	2	4
	Disturbed polarity	-	1	1	2	2	2
	Cytomixis	-	1	1	2	2	3
	% of abnormal PMCs	-	1.44	2.58	4.98	7.31	8.58
Total no. of abnormal PMCs observed		-	7	16	30	44	58
Total % of abnormal PMCs observed		-	**2.53**	**5.90**	**10.68**	**16.92**	**21.64**

Pearson correlation analysis

Pearson correlation coefficients were computed to evaluate the correlations between the plant parameters. The results were then visually represented as a heatmap in the Figure 6. The Pearson correlation coefficients, which provide insights into the relationships between pairs of 12 parameters, displayed a range of values spanning from -0.74 to 0.99. This indicates that the parameters exhibited diverse levels of correlation, with some showing strong positive associations (close to 1) or strong negative associations (close to -1), while others displayed weaker or negligible correlations (closer to 0). Significant positive correlations were observed, with a coefficient of 0.99, between clusters per plant and seed yield. Similarly, a strong positive correlation was found between total chlorophyll and chlorophyll b. The variation in these coefficients signifies the complexity and diversity of interactions among the studied parameters. In the heatmap, the darker red shades signified strong positive correlations among the parameters, whereas the blue hues indicated negative correlations. The main contributors to high yield potential were clusters per plant, pods per cluster and seed yield. The presence of more clusters per plant, a higher number of pods per cluster, and subsequently, a substantial seed yields collectively contribute to a plant's ability to achieve a high yield potential. These results agree with Naaz *et al*. (2024a).

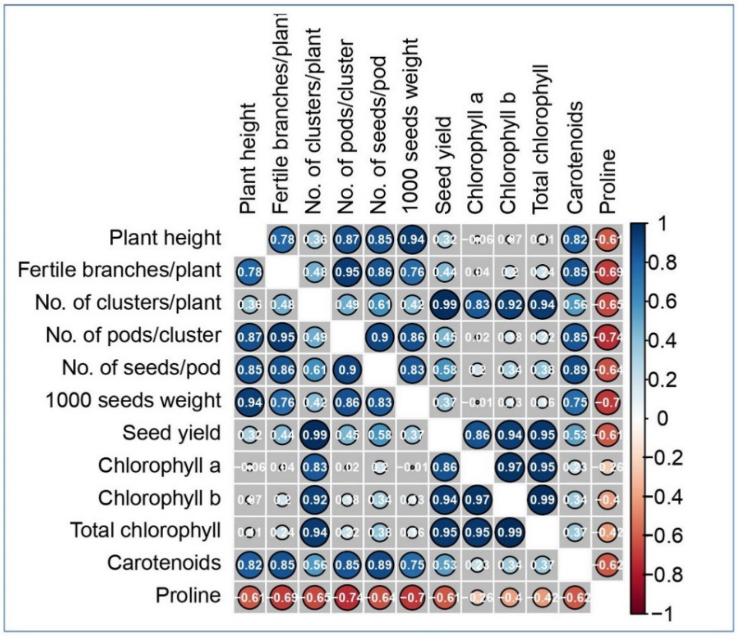


Figure 6. Pearson correlation coefficient between the plant parameters and visually represented as heatmap

PCA biplot analysis

Principal Component Analysis (PCA) was conducted to assess the relationships between various characteristics. The principal component analysis revealed that out of the twelve PCs, the first two PCs accounted for up to 90% of the variation, with eigen value greater than one contributing to genotypic diversity. PC1 contributed to 60.86% of the variation, primarily driven by traits like cluster per plant (0.866), pods per plant (0.836), seeds per pod (0.889), seed yield (0.842) and carotenoids (0.856), exhibiting higher positive factor loading values (Table 3). The trait proline (− 0.799) with a lower negative factor loading value also contributed to the variability of PC1. This suggests that genotypes with higher cluster per plant, pods per plant, seeds per pod, seed yield, and carotenoids align with this component. The second principal component accounted for 29.48% of the variation, with cluster per plant (0.481) and seed yield (0.522) showing positive factor loading values, and plant height (− 0.577) exhibiting a lower negative factor loading value contributed to the variability of this component. The genotypes falling under this component tend to have higher cluster per plant, seed yield, chlorophyll a, chlorophyll b, total chlorophyll, and proline content. The biplot diagram illustrated the distribution of genotypes based on traits (Figure 7). Genotypes with proline were placed in the first quadrant, those with chlorophyll a, chlorophyll b, total chlorophyll, seed yield, and clusters per plant were in the second quadrant. Genotypes related to plant height, branches per plant, pods per cluster, seeds per pod, 1000 seed weight, and carotenoids were distributed over the third quadrant, while the fourth quadrant contained genotypes that did not align with either of these characteristics. The Figure 8 presented the distribution of variance, both as individual and cumulative percentages across twelve principal components. The scree plot depicted the relationship between eigenvalues and principal components, explaining the percentage of variation (Figure 9). The PCA can facilitate the work of plant breeders in determining the required number of plants to evaluate and the traits that guide the selection of high yielding genotypes. Additionally, it provides an opportunity to utilize appropriate germplasm to enhance specific plant attributes during crop improvement efforts. In this study, plant yield had the most significant influence on genetic variability, highlighting its importance in selecting high-yielding genotypes. The cumulative variance exceeding 90% across the initial two principal components underscores that the traits along these axes substantially impact the overall phenotype of the accession, thus being useful for selection purposes.

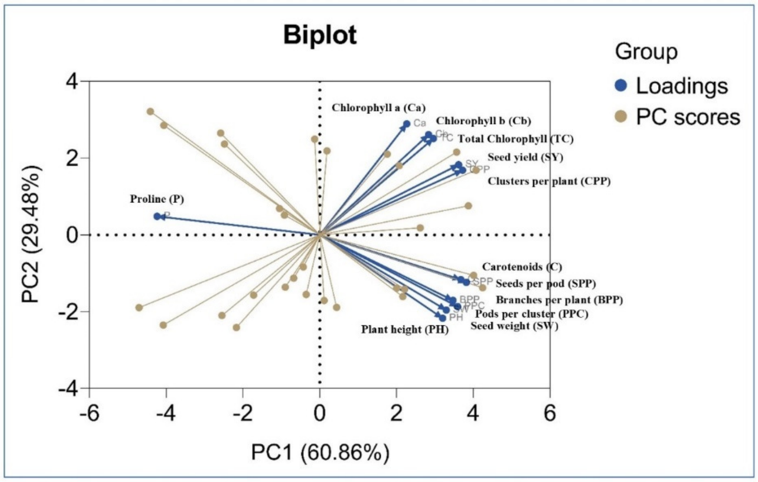


Figure 7. Biplot diagram of the quantitative parameters (plant height (PH), branches per plant (BPP), cluster per plant (CPP), pods per cluster (PPC), seeds per pod (SPP), seed weight (SW), seed yield (SY)) and physiological parameters (Chlorophyll a (Ca), Chlorophyll b (Cb), total chlorophyll (TC), carotenoids (C) and proline (P) of *T. corniculata*

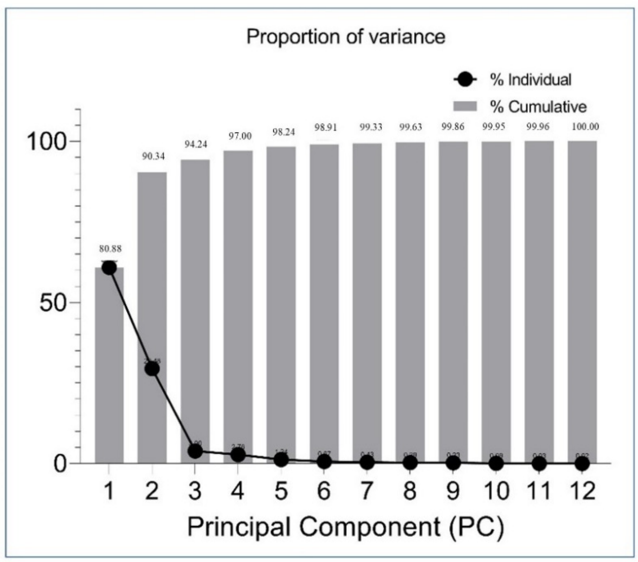


Figure 8. Proportion of variance presented both as individual and cumulative percentages across twelve principal components

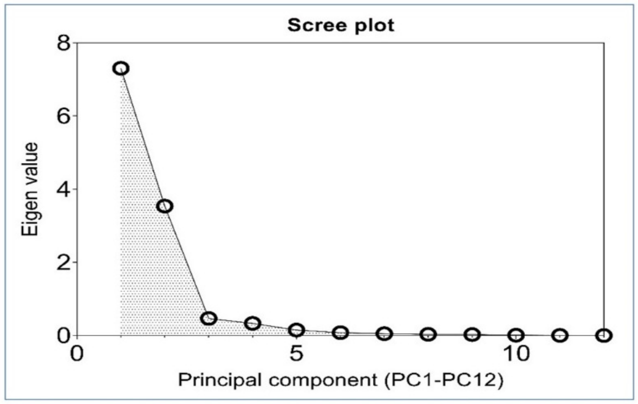


Figure 9. The scree plot depicts relationship between eigenvalues and principal components

Table 3. Eigen values, percent variability and cumulative percentage of *T. corniculata*

	PC1	PC2
Statistical variables		
Eigen value	7.303	3.537
Percent variability	60.86%	29.48%
Cumulative percentage	60.86%	90.34%
Traits		
Plant height (PH)	0.745	-0.577
Branches per plant (BPP)	0.807	-0.453
Clusters per plant (CPP)	0.866	0.481
Pods per cluster (PPC)	0.835	-0.496
Seeds per pod (SPP)	0.888	-0.329
1000 seeds weight (SW)	0.767	-0.520
Seed yield (SY)	0.841	0.521
Chlorophyll a (Ca)	0.527	0.826
Chlorophyll b (Cb)	0.659	0.746
Total chlorophyll (TC)	0.689	0.716
Carotenoids (C)	0.855	-0.309
Proline (P)	-0.799	0.137

GC-MS analysis

The chemical structure and composition of the extracts can be used to identify the biological potential of medicinal plant extracts. The analysis of extracts obtained from *Trigonella* seeds unveiled a diverse range of phytochemicals. The methanolic extract contained significant phytochemical elements, including trihydroxy alcohol, ketones, thiophene, esters, fatty acids, amines, fatty amides, etc. In the GC–MS chromatogram of methanolic extract of the control and the variants, a total of 40-45 peaks in control and some variants while in the remaining 25-30 peaks were identified as bioactive compounds (Figure 10). This identification was based on comparing their peak retention time and peak area (%) to those of known compounds listed in the National Institute of Standards and Technology (NIST) library (Table 4). In particular, the composition of major phytocompounds found in the methanol extracts is displayed in Table 5, along with their class, molecular formula and weight, compound ID, pharmacological activities, and chemical structure.

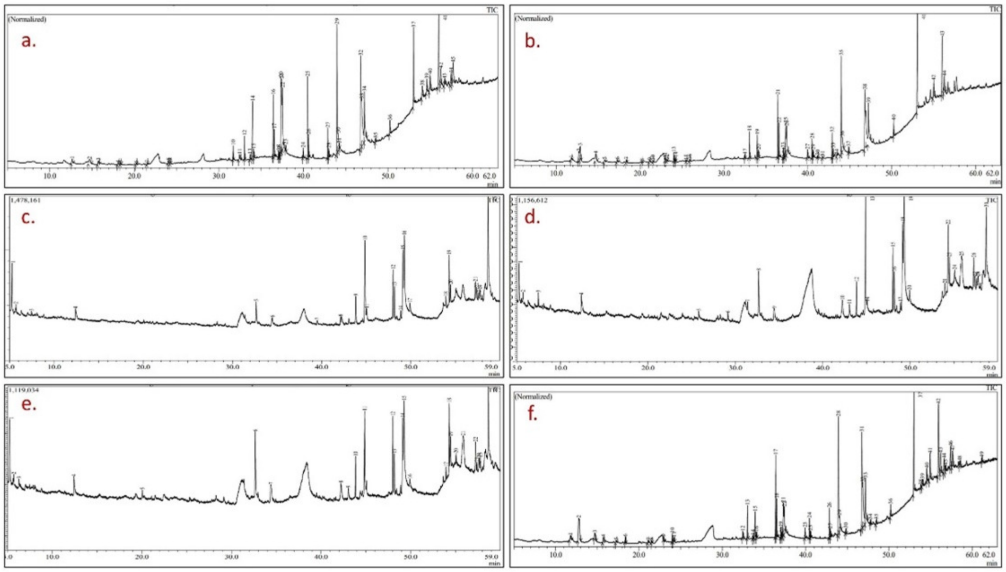


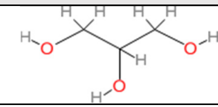
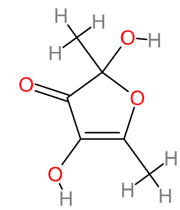
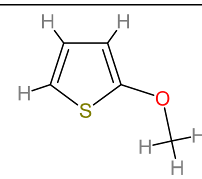
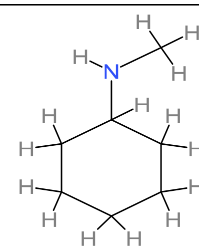
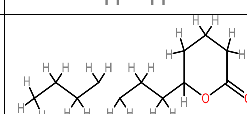
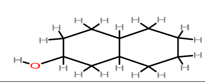
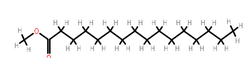
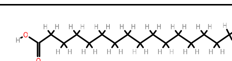
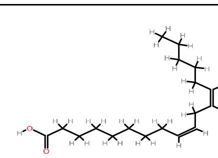
Figure 10. GC-MS chromatogram of the seed methanolic extract: a) control; b) 0.2% caffeine; c) 0.4% caffeine; d) 0.6% caffeine; e)0.8% caffeine; f) 1.0% caffeine of *T. corniculata*

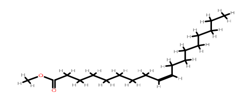
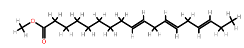
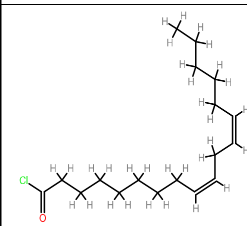
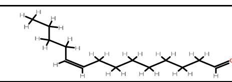
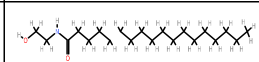
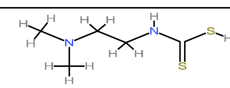
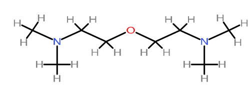
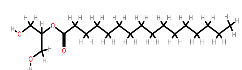
Table 4. Retention time and area percent (%) of major phytocompounds identified from the methanolic extracts of caffeine treatments of *T. corniculata*

Phytocompounds	Control		0.2% caff		0.4% caff		0.6% caff		0.8% caff		1.0% caff	
	R. Time	Area %	R. Time	Area %	R. Time	Area %	R. Time	Area %	R. Time	Area %	R. Time	Area %
Glycerin	-	-	-	-	5.267	6.32	5.267	3.41	5.246	6.49	-	-
2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan	-	-	-	-	12.404	2.51	12.391	2.16	12.411	2.83	-	-
2-Methoxythiophene	12.642	0.61	12.687	1.74	-	-	-	-	-	-	-	-
Cyclohexanamine, N-methyl-	-	-	12.848	3.09	-	-	-	-	-	-	12.771	5.21
.delta.-Dodecalactone	14.710	0.99	14.720	1.99	-	-	-	-	-	-	14.712	1.14
2-Naphthalenol, decahydro-	24.021	0.16	24.027	0.76	32.664	2.48	32.656	3.36	32.655	6.07	24.002	0.57
Hexadecanoic acid, methyl ester	33.043	1.19	33.047	2.16	43.844	2.45	43.848	2.10	43.845	2.85	33.024	1.91
n-Hexadecanoic acid	34.003	5.11	33.959	2.49	44.876	11.63	44.886	11.69	44.862	7.40	33.943	2.13
9,12-Octadecadienoic acid (Z,Z)-, methy	36.426	3.32	36.428	5.28	48.019	5.12	48.016	4.39	48.010	5.98	36.404	4.92
9-Octadecenoic acid, methyl ester, (E)-	-	-	-	-	48.183	4.14	48.182	3.64	48.180	3.92	-	-
9,12,15-Octadecatrienoic acid, methyl e	-	-	36.548	3.13	-	-	-	-	-	-	-	-
9,12-Octadecadienoyl chloride, (Z,Z)-	36.553	1.66	-	-	-	-	-	-	49.276	12.41	36.528	2.45
9,12-Octadecadienoic acid (Z,Z)-	37.415	5.23	-	-	49.136	12.47	49.150	11.21	49.121	9.25	37.123	0.27
13-Hexyloxacyclotridec-10-en-2-one	-	-	37.350	4.81	-	-	-	-	-	-	37.328	3.52
9-Tetradecenal, (Z)-	-	-	-	-	49.297	16.40	-	-	-	-	37.439	3.24
cis-9-Hexadecenal	37.515	5.00	37.461	4.44	-	-	-	-	-	-	-	-
Octadecanamide, N-(2-hydroxyethyl)-	40.621	11.95	40.498	2.40	54.476	7.29	54.475	7.38	54.464	8.77	40.472	1.21
Carbamic acid, 2-(dimethylamino)ethyl	42.997	2.38	42.896	3.93	-	-	-	-	-	-	42.967	3.22

Bis(2-(Dimethylamino)ethyl) ether	-	-	43.427	0.16	57.396	3.52	57.404	3.50	57.390	3.35	-	-
Hexadecanoic acid, 2-hydroxy-1-(hydro	44.107	11.55	44.093	14.49	58.683	14.26	58.685	7.15	58.675	7.56	44.070	11.67

Table 5. Major phytocompounds identified from the methanolic extracts of *Trigonella corniculata*

Phyto compounds	Class of compounds	M.F.	M.W. (g/mol)	Pubchem CID	IUPAC name	Activities	references	Molecular structure
Glycerin	trihydroxyalcohol	$C_3H_8O_3$	92.09	753	propane-1,2,3-triol	Antiherpetic and antiviral	(Sasaki *et al*., 2022)	
2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan	Ketone	$C_6H_8O_4$	144.12	538757	2,4-dihydroxy-2,5-dimethylfuran-3-one	Antioxidant	(Sakika *et al*., 2022)	
2-Methoxythiophene	Thiophene	C_5H_6OS	114.17	85610	2-methoxythiophene	Antifungal	(Zhou *et al*., 2022)	
Cyclohexanamine, N-methyl-	Aliphatic amine	$C_7H_{15}N$	113.2	7514	N-methylcyclohexanamine	No activity reported	-	
.delta.-Dodecalactone	Ester	$C_{12}H_{22}O_2$	198.3	12844	6-heptyloxan-2-one	No activity reported	-	
2-Naphthalenol, decahydro-	-	$C_{10}H_{18}O$	154.25	13216	1,2,3,4,4a,5,6,7,8,8a-decahydronaphthalen-2-ol	Antineoplastic, anti-infectives and antioxidant	(Wei *et al*., 2011)	
Hexadecanoic acid, methyl ester (Methyl palmitate)	Fatty acid esters	$C_{17}H_{34}O_2$	270.5	8181	methyl hexadecanoate	Antimicrobial, antioxidant, hypocholesterolemic, pesticide, lubricant, antiandrogenic and hemolytic 5-alpha-reductase inhibitor	(Shaaban *et al*., 2021)	
n-Hexadecanoic acid (Palmitic acid)	Saturated fatty acid	$C_{16}H_{32}O_2$	256.42	985	hexadecanoic acid	Antibacterial and antifungal	(Aparna *et al*., 2012)	
9,12-Octadecadienoic acid (Z,Z)-, methy (Linoleic Acid)	omega-6 fatty acid (unsaturated)	$C_{18}H_{32}O_2$	280.4	5280450	(9Z,12Z)-octadeca-9,12-dienoic acid	Antioxidant, anticancer, antimicrobial, Anti-inflammatory, hypocholesterolemic, antiarthritic, anticoronary,	(Dhar Dubey *et al*., 2019; Ghavam *et al*., 2021; Adnan *et al*., 2019)	

						antieczemic and antiacne.		
9-Octadecenoic acid, methyl ester, (E)- (Methyl elaidate)	Fatty acid	$C_{19}H_{36}O_2$	296.5	5280590	methyl (E)-octadec-9-enoate	Antioxidant, Antibacterial and antifungal	(Santos *et al*., 2023; Jalalvand *et al*., 2019)	
9,12,15-Octadecatrienoic acid, methyl e (Methyl elaidolinolenate)	Fatty acid	$C_{19}H_{32}O_2$	292.5	5367462	methyl (9E,12E,15E)-octadeca-9,12,15-trienoate	antimicrobial	(Jain *et al*., 2022)	
9,12-Octadecadienoyl chloride, (Z,Z)- (Linoleoyl Chloride)	Fatty acid	$C_{18}H_{31}ClO$	298.9	9817754	(9Z,12Z)-octadeca-9,12-dienoyl chloride	Antioxidant, antibacterial, anti-nociceptive, anti-inflammatory, larvicidal and repellent effects	(Kannaiyan *et al*., 2019; Ghosh *et al*., 2021; Yakubu *et al*., 2021)	
9-Tetradecenal, (Z)-	-	$C_{14}H_{26}O$	210.36	5364471	(Z)-tetradec-9-enal	No activity reported	-	
Octadecanamide, N-(2-hydroxyethyl)-	Fatty amides	$C_{20}H_{41}NO_2$	327.5	27902	N-(2-hydroxyethyl)octadecanamide	Anti inflammatory	(Kosiakova *et al*., 2021)	
Carbamic acid, 2-(dimethylamino)ethyl	Amines	$C_5H_{12}N_2O_2$	132.16	48131	2-(dimethylamino)ethyl carbamate	No activity reported	-	
Bis(2-(Dimethylamino)ethyl) ether	Amines	$C_8H_{20}N_2O$	160.26	18204	2-[2-(dimethylamino)ethoxy]-N,N-dimethylethanamine	Anti tumor	(Palmer *et al*., 1988)	
Hexadecanoic acid, 2-hydroxy-1-(hydro (2-Palmitoylglycerol)	Lipids	$C_{19}H_{38}O_4$	330.5	123409	1,3-dihydroxypropan-2-yl hexadecanoate	Antiinfective, Anti-inflammatory and Antiprotozoal	(Adnan *et al*., 2019)	

The major phytocompounds in the methanol seed extract of *T. corniculata* were found to be propane-1,2,3-triol; 2,4-dihydroxy-2,5-dimethylfuran-3-one; 2-Methoxythiophene; N-methylcyclohexanamine; 6-heptyloxan-2-one, 1,2,3,4,4a,5,6,7,8,8a-decahydronaphthalen-2-ol; methyl hexadecanoate; n-Hexadecanoic acid; (9Z,12Z)-octadeca-9,12-dienoic acid; methyl (E)-octadec-9-enoate; methyl (9E,12E,15E)-octadeca-9,12,15-trienoate; (9Z,12Z)-octadeca-9,12-dienoyl chloride; (Z)-tetradec-9-enal; N-(2-hydroxyethyl)Octadecanamide; 2-(dimethylamino)ethyl carbamate; 2-[2-(dimethylamino)ethoxy]-N,N-dimethylethanamine; 1,3-dihydroxypropan-2-yl hexadecanoate. This study detected significant compounds including linoleic acid, palmitic acid, stearamide, elaidic acid, and dodecanoic acid, etc., known for their value in pharmaceutical, medicinal, and industrial applications. Previous studies have also conducted GC-MS analyses of fenugreek seeds, revealing numerous phytochemicals with health benefits (Ashraf *et al*., 2019; Qadir *et al*., 2022; Alabi *et al*., 2023). The compounds such as 2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan; 2-Naphthalenol, decahydro-; methyl palmitate; Linoleic Acid; methyl elaidate; and Linoleoyl Chloride antioxidant potentials. The compounds Glycerin; 2-Methoxythiophene; 2-Naphthalenol, decahydro-; methyl palmitate; Palmitic acid; Linoleic Acid; methyl elaidate; Methyl elaidolinolenate; Linoleoyl Chloride; and 2-Palmitoylglycerol have shown to possess significant antimicrobial potentials. Linoleic acid has exhibited potent anti-cancer properties, especially in treating breast cancer (Iqbal *et al*., 2017). Hexadecanoic acid, classified as an ester, shows a range of activities including antioxidative, hypocholesterolemic, pesticidal, lubricating, antiandrogenic, flavor-enhancing, and a hemolytic 5-alpha-reductase inhibitor (Shaheed *et al*., 2019). The compounds identified belong to a diverse range of chemical groups, and a substantial portion of these compounds has been confirmed to possess important biological activities (Ruthiran and Selvaraj, 2017; Naaz *et al*., 2024b). The significant chemical compounds identified within various extracts are believed to be components of the plants' defense mechanisms. These compounds can be categorized as protective elements present in the plant, often referred to as 'phytoanticipins' and 'phytoprotectants' (Salehi *et al*., 2019). Such compounds are of particular interest due to their potential therapeutic applications in treating various diseases and promoting overall health.

Conclusions

The study showcased notable enhancements in both morpho-physiological and quantitative characteristics of fenugreek through caffeine treatment. The most significant improvements across these traits were observed in the 0.2% caffeine treatment. The strong correlation between clusters per plant, pods per clusters, and seed yield, as elucidated through PCA and Pearson's correlation heatmap analysis, underpins the potential for achieving high yields. The present study was also focused on identification of various bioactive compounds from the extracts of different caffeine treatments in *T. corniculata* by GC-MS analysis. These compounds possess various therapeutic and pharmacological benefits. The research also highlighted the antioxidant, antimicrobial and anti-inflammatory potentials of these compounds. Notably, the presence of a wide spectrum of bioactive compounds validates the traditional usage of these plant parts by practitioners for treating various ailments. The plant exhibits promising potential for developing reliable and effective drugs against a variety of diseases. Further exploration of its bioactivity and subsequent clinical trials are imperative steps for unveiling new drug formulations.

Authors' Contributions

N.N.: Conceptualisation, methodology, formal analysis, investigation, data curation, writing—original draft & editing, statistical methods. S.C.: Conceptualisation, methodology, investigation, supervision, writing—review & editing. N.S.: Methodology, investigation, writing—review & editing. N.H.: Methodology, investigation, writing—review & editing. N.A.: Funding acquisition, writing—review & editing. D.A.: Writing—review & editing. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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