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Exploratory analysis of agro-morphological characteristics in *Nigella sativa* L. plant genotypes to determine mutagen colchicine ameliorative/ non-ameliorative impacts

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This experimental study aimed to elucidate the optimal colchicine concentration for inducing polyploidy and to examine the morphological effects on *Nigella sativa* L. (family Ranunculaceae) plants recognized as 'Kalonji' in India. Here, seeds were exposed with different concentration of colchicine ranging from 0.025 to 0.4% with varying time duration (24–48 h). The agro-morphological attributes and chromosome counts of the putative polyploids were compared with control diploid plants, revealing significant differences. The ploidy level determined by chromosome counts revealed that 0.05–0.1% concentration of colchicine induced tetraploids within both plant genotypes for 24 h and 48 h. However, results based on agro-morphological trait correlation analysis revealed more significant association among yield traits at 0.1% concentration and the principal component analysis revealed that the maximum possible ameliorative effect of the colchicine dose was the lowest concentration (0.025% for a 48-hour exposure time) for the AN1 genotype; likewise, a 0.05% concentration established a more positive association in terms of growth and yield attributes for the AN20 genotype. This study demonstrated that low dosages (0.025% and 0.1%) strongly impact plant growth and yield, whereas higher dosages obliterate these positive effects and add destructive characteristics within plants which ultimately reduces yield.

Keywords *Nigella sativa*, Ranunculaceae, Colchicine, Polyploids, Chromosome count

Nigella sativa L. (Kalonji) is an important commercial minor seed spice crop that is cultivated in few regions of India. Its distribution expanded from the Mediterranean region through West Asia to the North Indian region. Many tropical and subtropical countries that cultivate *Nigella* are India, Bangladesh, Pakistan, Egypt, Iraq, Nepal, Saudi Arabia, Turkey, southern Europe, Syria and Sri Lanka[1]. India is the world's largest global producer of *Nigella*, even though its cultivation is limited to a few states, such as Punjab, Assam, Bengal, Bihar, Gangetic Plains, Himachal Pradesh, and Maharashtra[2]. The genus *Nigella* comprises approximately 20 species of annual herbs worldwide. However, the only species *Nigella sativa* is widely popular as an edible species consumed in the form of seeds and as oil worldwide. It has a very appealing primitive flower characterized by a colorful bluish white petalloid sepals and small suppressed petals. Its fruit is a capsule, which is the outcome of a superior semicarpous ovary that contains multiple black-colored seeds.

The seeds of *Nigella* species are aromatic, and their oil has a strong pungent odor, which is very peculiar to this plant. Along with its wild species found in India, *Nigella sativa* has been widely used as a condiment and spice since ancient times in pickles as well as in curry as a *panchforan* and also for the treatment of various diseases[3] and infections, such as asthma, bronchitis, headache, rheumatism, paralysis, inflammation, and hypertension, very traditionally[4]. It is an annual flowering herb that is a combination of various potential enduring properties, such as spice, condiment, seasoning, and flavoring agents. Its oil possesses numerous therapeutic properties[5], such as nutritional, healthical, neutraceutical, antioxidant[6], antimicrobial, anticancer, antidiabetic, antidepressant,

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antifungal, antitumor[7] and cardio-protective effects as well as radical scavenging properties[8,9]. Thymoquinone is a well-known volatile compound extracted from this plant[10]. Herbal medicines and natural remedies are currently getting priority rather than contemporary medicines that are synthesized artificially moving on the backfoot because of very adverse effects or ramifications after medication. Long-term use has led to the development of other chronic diseases to avoid these effects, and millions of people worldwide have gradually been attracted to and lured toward ayurvedic or herbal treatments[1]. It is appropriate to emphasize herbal and organic product studies, which can offer a healthy life without side effects, in contrast to chemical drugs. Spices that have one such ingredient and are loaded with therapeutic properties can be extensively effective against illness[11]. *Nigella* species are known for their unique features and several pharmacological properties. The climate has been abruptly and constantly changing in the 21st century[12]; therefore, to establish India as the world's new growth engine in the field of herbal pharmaceuticals, it is necessary to contribute beyond leaps and bounds to the global trade of spices.

India owns the crown of World's Spice Bowl[13], which is also distinguished as 'Land of Spices'[14]. To fulfill future needs, agricultural crop exports and supplies should be continuously emphasized. Genetic improvement of spice crops is a crucial step in breeding programs. An effective and efficient breeding technique is mandatory for plant germplasm improvement programs, among which polyploid production is desirable because it is not only easy but also cost effective for the production of new and improved cultivars[15].

The natural alkaloid colchicine is prominently used since 1930 to artificially induce polyploidy and mutagenic effects in various plant species for instance maize[16], watermelon[17], and *Lilium regale*[18] plants. 'Colchicine' is an active secondary metabolite component of the *Colchicum autumnale* (meadow saffron) plant that is utilized by plant breeders as a mutagen[19]. Polyploidization is a biotechnological technique mostly used to evolve an improved variety of plants with various agronomic characteristics, such as plant height, fruit size, and leaf length[20]. Very little work has been reported regarding therapeutic, medicinal and economically important plants as well as spice plants through breeding techniques[21,22]. *Nigella sativa* L. a spice crop with cargoes of medicinal properties is also among one of those spices which was meagerly studied on the basis of their ploidy levels along with their agro-morphological variation. For advanced genetic breeding techniques genetic variability analysis may offer sufficiently enough juncture to select traits for further crop enhancement through various plant breeding programs[23]. Therefore, assessing the genetic and statistical attributes of *Nigella sativa* L. genotype plants treated with different concentrations of colchicine for various durations could reveal significant and crucial information for breeding research. Genetic diversity could suggest the degree of differentiation among species as well as within species[24]. Hence, the present study is focused on determining the most effective colchicine concentration according to ploidy level and agro-morphological variation analysis of two genotypes of *Nigella* plants in the humid subtropical region of Varanasi, India.

Materials and methods

Sample collection

Seed samples of *Nigella sativa* L. two genotypes, AN-1 (Ajmer *Nigella*-1) and AN-20 (Ajmer *Nigella*-20), was accomplished from the National Research Centre on Seed Spices (NRCSS), Tabiji, Ajmer, Rajasthan, India, and stored in airtight containers for further study under ambient conditions to prevent moisture.

Experimental methodology

The study was performed during the winter (Rabi) crop season at the experimental site in the pots at coordinates 25°15×49.9′′N, 82°58×21.7′′E located in Varanasi, Uttar Pradesh, India. The alluvial dark brown soil was thoroughly mixed with coco-peat to maintain porosity and organic manure as a fertilizer and then transferred to 25×25 cm pots, which had a slightly basic pH of 7.4±0.1. The monthly meteorological data, i.e., minimum and maximum temperatures (°C), rainfall (mm) and relative humidity (%), (%) during the experimental period retrieved from the Indian Meteorological Division (IMD), BHU, and Varanasi located within the range of 1.5 km from the experimental site are presented in Table 1.

Colchicine treatment

At first, 1800 seeds of both genotypes (a total of 3600 seeds) were used for this experiment, and these were divided into 36 groups with 50 seeds each. In this experiment, different concentrations of colchicine (Sigma–

Year	Month	Temperature (°C)		Rainfall (mm)	Relative humidity (%)
		Min	Max		
2021	Nov	9	32	-	50–96
	Dec	4.8	28	33.2	53–100
2022	Jan	5.9	24.7	272.4	47–100
	Feb	6.5	30.5	708.6	28–97
	Mar	13	40.2	-	22–90
	Apr	19.6	45	-	04–84
	May	23.4	46	45	20–86

Table 1. The monthly meteorological data of Varanasi, India from November 2021–May 2022 temperature (°C): minimum (min), maximum (max), rainfall (mm), and range of relative humidity (%).

Aldrich) ranging from 0.025, 0.05, 0.1, 0.2, and 0.4% (w/v) were chosen, and groups of 50 seeds (in triplicate) were directly soaked in each concentration for 24 h as well as 48 h at room temperature (in a 5 ml vial tube). An LD_{50} concentration of 0.4% was recorded during the experiment[25]. This lethal dose significantly inhibited 50% of the population of *Nigella sativa* plant seeds from growing regardless of their genotype. However, one seed group of each genotype that was left untreated, and further exposed to distilled water were used as controls. After treatment, the seeds were thoroughly rinsed two to three times with distilled water, and then, petridish-sustaining colchicine-treated seeds were kept in the dark along with control plants to maintain uniformity during the experiment for germination to acclimatize and overcome chemical treatment. After 2 to 3 days, germination of the seeds of both genotypes started, and the seeds grew after one week, as shown in Fig. 1. Furthermore, approximately 25 germinated seeds of each and every treatment (in triplicate) were sown in pots under natural environmental conditions on 20–22 November 2021. The plants were irrigated with tap water as required to maintain moisture.

Ploidy level determination

Initially, ploidy level determination was performed via microscopy through the determination of mitotic chromosome counts within the root tip cells of germinated seeds and from stomatal analysis of abaxial surface of leaves. For the chromosome count analysis, approx. 25 seeds from each sample were kept for root germination. When the root tips had grown to a length of 1–2 cm, they were sliced and rinsed with distilled water two to three times. Furthermore, Carnoy's solution was used as a fixative i.e. ethanol: acetic acid at a 3:1 ratio for at least 24 h. After fixation, the fragmented root tips were preserved in 70% ethanol solution in a refrigerator at 4 °C until further use.

For slide preparation, the preserved root tips were omitted from the solution, and thoroughly rinsed 2–3 times with distilled water. Then the root tips were immersed in a 1 N HCl solution for approximately 15 min. and again rinsed for 2–3 times using distilled water. After that, for staining freshly prepared 2% (w/v) aceto-carmine solution was utilized. In that staining solution, root tips were soaked for approximately 10–20 min and heated, if required. Finally, after removing excess stain and debris, the stained root tips were kept on glass slides, squashed and focused at 45X or 100X under microscope (Magnüs MX21*i*LED) for chromosome count analysis and images were captured via a web camera (Winjoe 1.0).

Data collection

Approximately, after sixty days bud formation started in the plants, it was observed that AN-1 genotype plant buds emerged from soil ten days prior than AN-20 genotype plants. Thereafter, agro-morphological data collection began, five plants of each replicate from each and every treatment were randomly selected for measurement

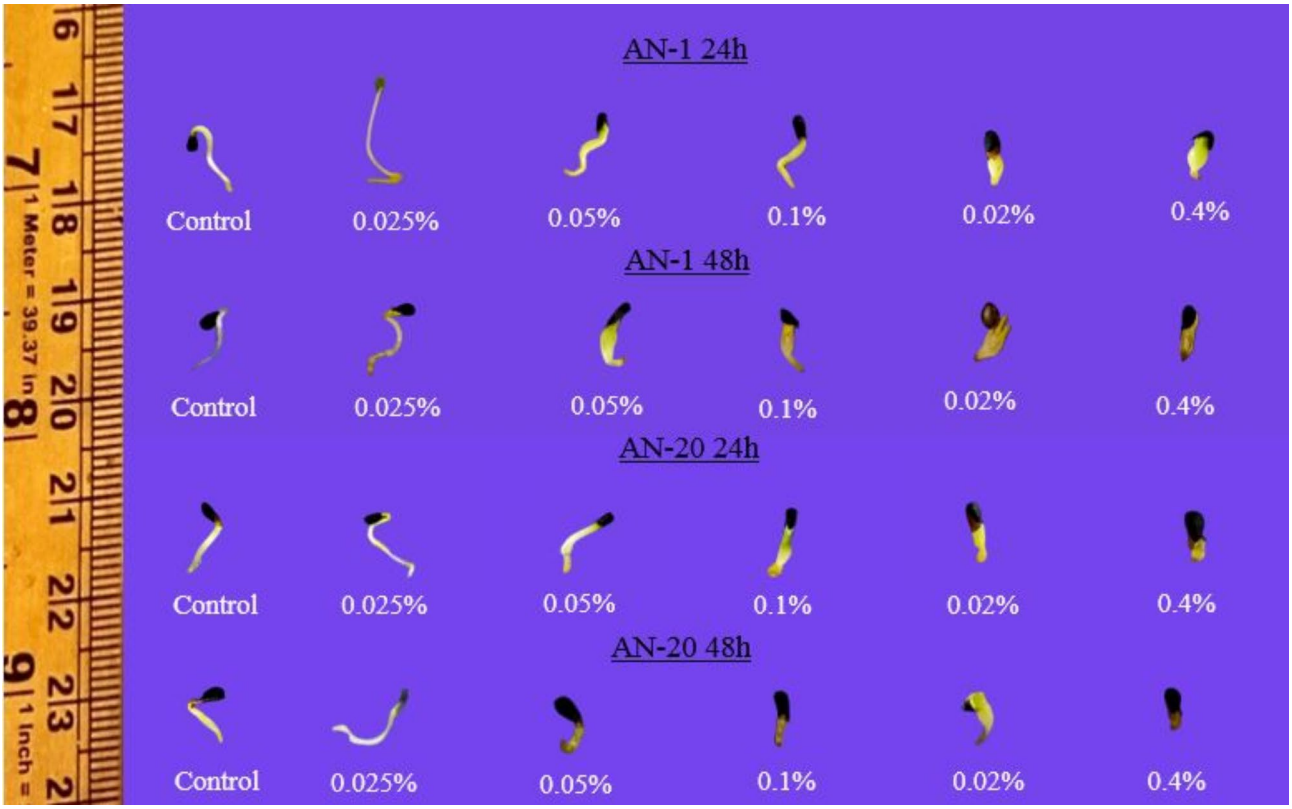

Fig. 1. *Nigella sativa* L. plant seeds treated with different concentrations of colchicine ranging from 0.025–0.4% for 24 and 48 hours' duration (along with control) after one week of treatment.

and calculations. The growth attributes at maturity (plant height, number of branches, leaf length, number of flowers, and number of capsules per plant) were recorded after ninety days of sowing, whereas yield attribute data (number of seeds per capsule, capsule weight, seed yield per capsule, seed yield per plant, total capsule weight per plant, biological yield, and seed yield per five plants) were calculated after harvest[17,26]. Among the five growth attributes, plant height and leaf length were measured through a measuring tape (in cm), whereas the remaining three and yield attributes were counted (in no.) or weighed (in g) as needed. The cultivar AN-1 was harvested on 26 April 2022 (150-155 day life span), while the other cultivar AN-20 was harvested after 10 days on 05 May 2022 (160-165 day life span).

Statistical analysis

The collected datasets of both genotypes were tested via the Shapiro–Wilk normality test to check for homogeneity in their twelve agro-morphometric traits and were found to be normally distributed and significant at the 95% level. Furthermore, normally distributed datasets were subjected to parametric tests such as one-way analysis of variance (ANOVA), Tukey's test, correlation analysis, and principal component analysis (PCA). Descriptive statistics (minimum, maximum, average, standard error), coefficient of variation (CV), Tukey's post hoc test, and correlation coefficient analysis were performed via IBM SPSS statistics software version 2020[27], whereas other statistical analyses and graphs, such as one-way ANOVA, principal component analyses and heatmap plots, were performed via R Studio version 2020 (library 'variability and ggplot2')[28] and Origin Pro Software version OriginPro 2024b (64-bit)[29] respectively.

Results

Morphological variation analysis and ploidy determination

Morphological variations were observed among the control and putative polyploids; the control plants possess thin and slender cotyledons, whereas the putative polyploid plant cotyledons were thick, small and bulky with sluggish germination rate (Fig. 2a and b). Significant changes were also observed in the leaves; the control plant leaves seemed thin and delicate, whereas leaves of the putative polyploid plants were comparatively flatter and thicker (Fig. 2c). Furthermore, flowers size did not vary significantly, but the petals were broader and more number of locules than the control diploid plants (Fig. 2d). However, at maturity the growth of tetraploid plants significantly differed from that of control diploid plants. The tetraploid plants were taller, with an increased number of branches and flowers (Fig. 2e).

Chromosome count analysis and stomatal analysis revealed that, compared with control diploid ($2n=12$) plants, putative tetraploid ($2n=24$) plants have significantly thicker chromosomes, broader and larger stomatal alignment in both genotypes (Fig. 3A and B).

After being treated with colchicine for 24–48 h time duration the seeds were analyzed manually and thereafter via microscopy. Where, it was observed that the survival rate declined with increase in concentration. However, the production of putative polyploids increased abruptly. Furthermore, the concentrations of 0.05% w/v and 0.1% w/v were responsible for inducing tetraploidy, while concentrations above this produced hexaploids, octaploids and even mixoploids at relatively higher rates (Table 2).

Variability in agro-morphometric traits w.r.t. concentrations of colchicine

The *Nigella sativa* L. genotypes AN1 and AN20, which are indigenous to India, were observed in this study. The effects of colchicine concentrations of 0.025, 0.05, 0.1, 0.2, and 0.4% (w/v) for 24 h and 48 h on the agro-morphological characteristics (growth and yield) were examined. The data for five growth attributes and seven yield attributes (Table 3) were recorded at maturity and at harvest[30]. For the phenotypic variability analysis among treated plants, one-way analysis of variance (ANOVA) was performed, which revealed significant variability ($P<0.05$) for all twelve morphometric traits[31]. The average values of each morphological trait, such as plant height (37.59 cm), number of branches (10.84), leaf length (9.37 cm), number of flowers (8.89), number of capsules per plant (5.67), number of seeds per capsule (64.71), capsule weight (0.17 g), seed yield per capsule (0.13 g), seed yield per plant (0.56 g), total capsule weight per plant (0.64 g), and biological yield (1.39 g), are mentioned in Table 2. Of these, the trait biological yield ranged from 0.49 to 2.63 g and had the maximum coefficient of variation, i.e., 42.21%, whereas the number of flowers per plant ranged from 5.60 to 12.40 and had the minimum coefficient of variation, 22.25% (Table 3).

A comparative analysis was performed for all twelve morphometric traits according to the criterion of different concentrations, and their average values with significant differences ($P<0.05$) based on Tukey's post hoc test are tabulated for genotype AN-1 in Table 4 and for genotype AN-20 in Table 5. The effects of exposure to different concentrations of colchicine for 24 h and 48 h time duration, shows significant variations among morphological traits in this study. One-way ANOVA—based significance levels from 0.001 to 0.05 are shown with corresponding signs. For the AN-1 genotype, plant height and number of flowers at 0.05% w/v significantly varied ($P<0.01$); however, the seed yield per plant, biological yield and seed yield per five plants possess significant differences ($P<0.05$) at 0.025% w/v concentration. Moreover, the variation in leaf length was significant ($P<0.01$) for most of the concentrations (Table 4).

Similarly, in the AN-20 genotype, leaf length significantly varied ($P<0.001$) for most of the concentrations. However, at 0.2% w/v the number of seeds per capsule, seed yield per plant along with seed yield per capsule significantly varied ($P<0.05$) (Table 5). The significant differences indicate that the exposure time 24 h and 48 h at different concentrations also considerably impact few morphological traits[32].

A comparison of yield-related attributes[33,34], number of seeds per capsule, biological yield and seed yield per five plants was also performed for all of the concentrations used, taking into consideration the duration of treatment, i.e., 24–48 h, for both genotypes (Fig. 4A—C). The histogram of each concentration is represented along with the standard error and significant differences for both genotypes, which are accordingly denoted

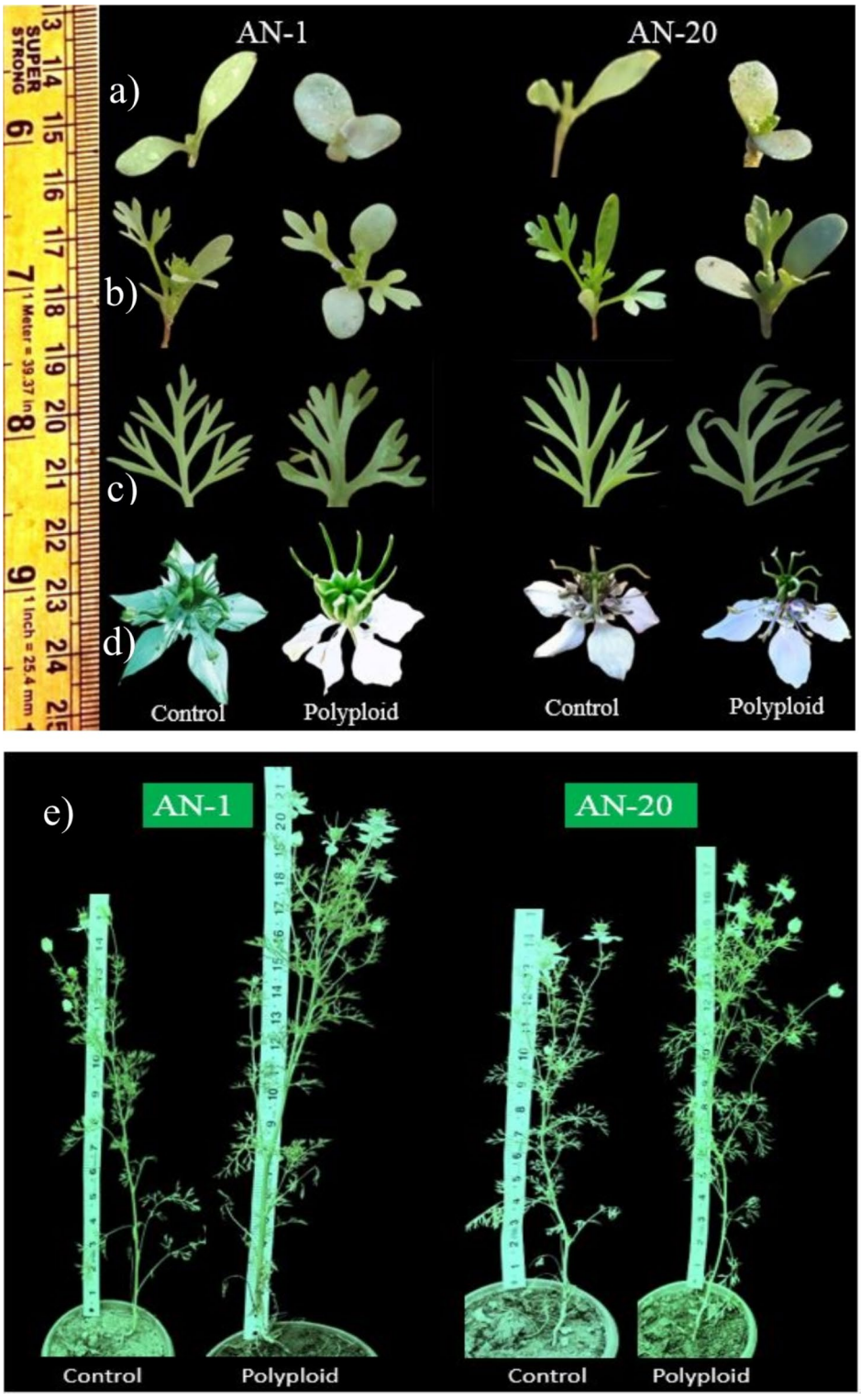

Fig. 2. Morphological variations among dicotyledons (a-b), leaves (c), flowers (d) and mature plants (e) recorded for both genotypes.

as AN1 (24–48 h), and AN20 (24–48 h). Significant variability among the data were calculated with Tukey's homogeneity post hoc test, and significant differences were annotated with different letters individually or in combination from a to d. As shown in Fig. 4A, the graph between the number of seeds per capsule and concentration, shows the highest value at 0.05% w/v for AN1 24 h, approximately 90 seeds; followed by AN1 48 h, approximately 85 seeds at 0.05 and 0.025% w/v; in contrast, at 0.4% w/v concentration, AN20 48 h has the minimum number of seeds per capsule, ranging from 30 to 35 seeds at 0.4% concentration. Figure 4B, the

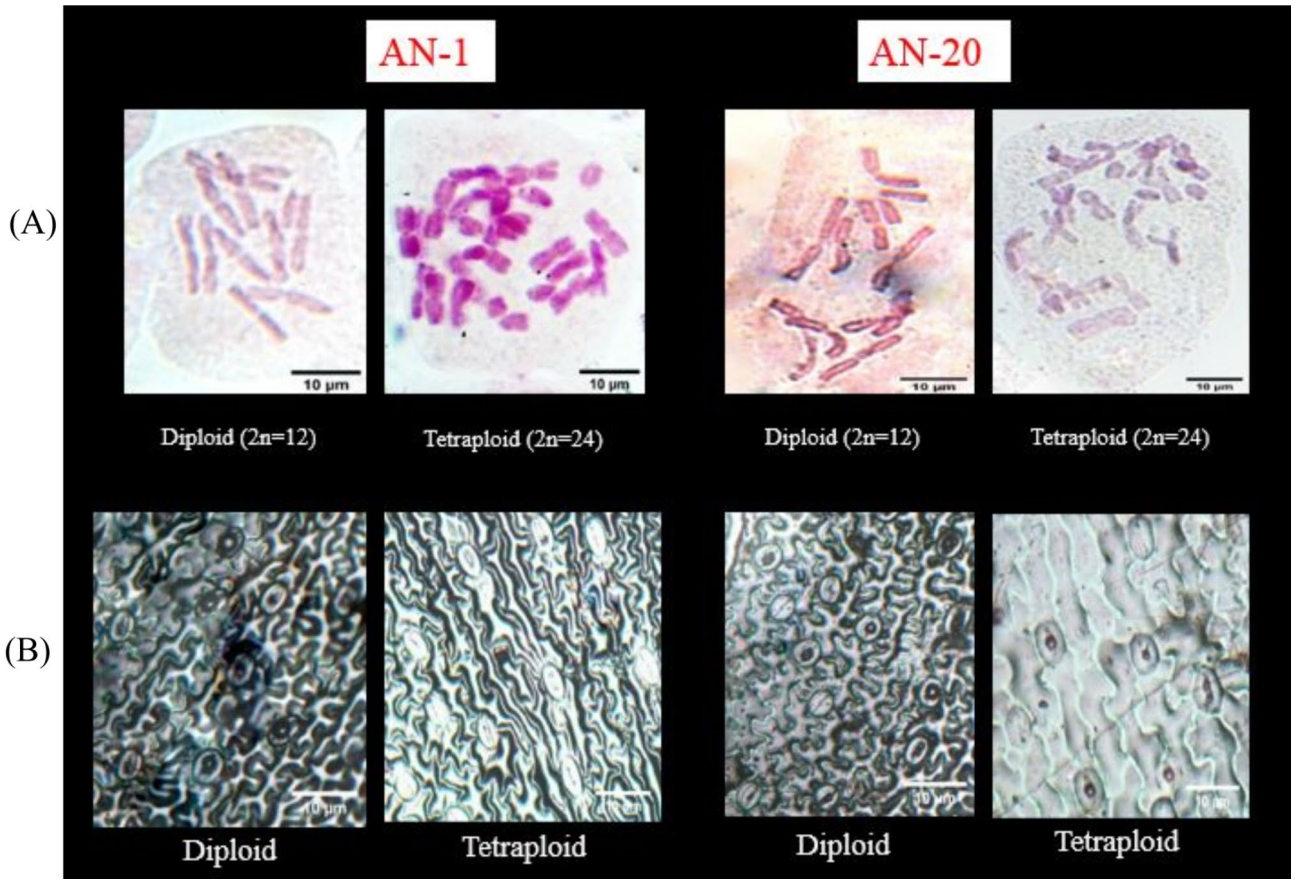

Fig. 3. Image showing the number of chromosomes in diploid and tetraploid plants (A) and the density with size of the stomata (B) for both genotypes.

Colchicine concentration %	Genotype	Exposure time duration (hour)	Survival Rate %	Diploid %	Tetraploid %	Mixoploid % (or ploidy > tetraploid)
0	G1	24	100	100	0	0
	G1	48	100	100	0	0
	G2	24	100	100	0	0
	G2	48	100	100	0	0
0.025	G1	24	100	90	5	0
	G1	48	100	82	11	0
	G2	24	100	90	4	0
	G2	48	100	81	10	0
0.05	G1	24	95	73	22	0
	G1	48	90	60	35	0
	G2	24	97	73	26	0
	G2	48	93	62	37	0
0.1	G1	24	82	0	70	5
	G1	48	70	0	81	10
	G2	24	85	0	77	2
	G2	48	75	0	85	7
0.2	G1	24	72	0	4	75
	G1	48	58	0	1	90
	G2	24	76	0	6	87
	G2	48	62	0	2	91
0.4	G1	24	55	0	5	90
	G1	48	50	0	0	96
	G2	24	58	0	4	93
	G2	48	52	0	0	98

Table 2. Diploid (%), tetraploid (%) and mixoploid (%), survival rates (%) of genotypes AN-1 (G1) and AN-20 (G2) according to colchicine treatment with an exposure time of 24–48 h.

Morphometric Traits (unit)	Abb. Codes	Minimum	Maximum	Mean ± SE	CV%	Sig.
Plant height (cm)	PH	18.76	51.10	37.59 ± 1.847	24.07	***
No. of branches	NB	5.60	16.20	10.84 ± 0.570	25.78	***
Leaf length (cm)	LL	4.32	13.20	9.37 ± 0.462	24.17	*
No. of flowers	NF	5.60	12.40	8.89 ± 0.403	22.25	***
No. of capsule per plant	NC	3.20	8.00	5.67 ± 0.308	26.59	***
No. of seeds per capsule	NSpC	34.00	92.40	64.71 ± 3.496	26.47	***
Capsule weight (g)	CW	0.07	0.23	0.17 ± 0.045	27.32	***
Seed yield per capsule (g)	SYpC	0.07	0.18	0.13 ± 0.006	25.46	***
Seed yield per plant (g)	SYpP	0.24	0.79	0.56 ± 0.034	30.05	***
Total capsules weight per plant (g)	CWpP	0.26	1.07	0.64 ± 0.050	38.63	***
Biological yield (g)	BY	0.49	2.63	1.39 ± 0.120	42.21	***
Seed yield per five plant (g)	SY/5P	0.73	2.73	1.73 ± 0.127	36.10	**

Table 3. Variability in all 12 morphometric traits among the AN1 and AN20 genotypes of *Nigella* showing-Abb. Codes, (abbreviations for traits); minimum, maximum, mean ± standard error, coefficient of variation (CV); sig, significant according to one-way ANOVA; *, significant at $P < 0.05$; **, significant at $P < 0.01$; ***, significant at $P < 0.001$.

biological yield and concentration graphs, shows the highest values (peaks) for AN1 48 h at 0.025, 0.05 and 0.1% w/v, ranging from 2.0 to 2.5 g in descending order, and the minimum values for AN1 24 h 0.5 g at 0.4% w/v. Figure 4C shows that the highest yield per five plants was 0.05% and 0.025% w/v, i.e., approximately 2.7 g, whereas, the lowest yields were obtained for AN20 24 h and AN20 48 h at 0.4% w/v, respectively, with values of approximately 0.7 g. The lower peaks for the entire yield attribute in the above graph plots were associated with the 0.4% w/v concentration genotype (Fig. 4A-C), whereas most of the highest histogram peaks were associated with the 0.025 or 0.05% w/v concentration genotype. These differences among the concentrations suggest that a low dose of colchicine may significantly improve plant growth and yield; in addition, these higher doses treatment negatively affects plant growth, ultimately leading to decreased yield[35].

Correlation analysis for each concentration

To determine the variability among genotypes, we performed correlation analysis coupled with two-tailed t tests; significance was defined as $P < 0.01$ and < 0.05, where the Pearson correlation coefficient (r) is the most common method for analyzing linear correlation between two variables[36]. It depicts the bonding and strength between two variables and how closely both are associated with each other. The correlation values among all morphometric traits ranged from r = -0.52 to 1 (Fig. 5a and f). The greatest positive significant correlation was found for biological yield and capsule weight per plant at the 0.1% w/v concentration ($r = 0.73$; $P < 0.01$), which revealed that an increase or decrease in capsule weight per plant significantly impacts the biological yield of plants[37,38]. In contrast, the highest negative significant correlation was reported between leaf length and treatment time (r = -0.52 to -0.87; $P < 0.01$), which indicated that leaf length significantly varied with varying exposure treatment time.

Principal component analysis

In this study, PCA was conducted to summarize the agro-morphological data of twelve attributes that were observed after treatment with different concentrations of colchicine for different durations (24 h and 48 h). The correlation matrix of PCA was considered to be greater than the covariance matrix for this study since variables possess multiple units of measurement[39]. Two-dimensional score plot of PC1 versus PC2 is illustrated in Fig. 6, and the corresponding values are shown in Table 6, which illustrates the cluster distribution of two genotypes exposed to different colchicine concentrations[40]. The genotype variables with significant positive effects lies in the positive quadrant of the PC1 and PC2 principal components[41]. Low and medium colchicine concentrations (i.e. 0.05 and 0.1% w/v respectively) for 24 h as well as 48 h of the AN1 genotype had positive values for both of these PCs. Whereas, lowest, low and medium concentrations (i.e. 0.025, 0.05 and 0.1% w/v respectively) for 24 h of AN20 genotype were reported positive[42]. In contrast, High48 G1 and Highest48 G1 (i.e. 0.2 and 0.4% w/v respectively) for the AN1 genotype treated with colchicine for 48 h had significantly negative values for both PCs. Similarly, Medium48 G2, High48 G2 and Highest48 G2 colchicine concentrations (i.e. 0.1, 0.2 and 0.4% w/v respectively) of the AN20 genotype were found negative for both of the principle components.

The lowest (0.025% w/v) and low (0.05% w/v) doses for both of the genotype are grouped in cluster 1. In contrast, the high and highest doses with non-improving effects are gathered in cluster 2 (Fig. 6). PC1 had the highest variance (84.43%), which indicated that the variance in the data were specifically due to PC1 (Table 6)[43]. The Lowest48 G1 (AN1 genotype treated with 0.025% w/v colchicine concentration for 48 h) was strongly correlated with PC1 followed by Lowest24 G1, Low24 G1 (AN1 genotype treated with 0.025 and 0.05% w/v concentration for 24 h); these results were consistent with those of the AN1 genotype with 0.025 and 0.05% w/v concentration of colchicine, while lower colchicine concentrations in AN20 genotype were also significantly positive but less than AN1 genotype. These findings indicate that lower concentrations of colchicine ameliorate both genotypes, similar to the findings of Singh et al. (2019)[44].

Traits	AN-1 genotype											
	0 (Control)		0.025%		0.05%		0.1%		0.2%		0.4%	
	Mean ± SE	Sig.	Mean ± SE	Sig.	Mean ± SE	Sig.	Mean ± SE	Sig.	Mean ± SE	Sig.	Mean ± SE	Sig.
PH	41.38 ± 0.92	bc	49.83 ± 0.99	a	45.59 ± 1.72	ab**	40.78 ± 1.11	bc	39.57 ± 1.21	c	27.05 ± 1.51	d
NB	13.00 ± 0.45	ab	15.50 ± 0.93	a	13.60 ± 0.58	a	10.40 ± 0.54	bc	9.40 ± 0.48	c**	7.90 ± 0.67	c
LL	9.27 ± 0.28	b*	11.47 ± 0.28	a	12.45 ± 0.34	a*	11.90 ± 0.43	a**	9.51 ± 0.61	b***	7.63 ± 0.66	b***
NF	9.10 ± 0.50	bc	11.50 ± 0.48	a	11 ± 0.70	ab**	8.40 ± 0.52	cd	7.20 ± 0.33	cd	6.60 ± 0.48	d
NC	6.70 ± 0.37	ab	08 ± 0.37	a	7.20 ± 0.42	ab	6.00 ± 0.42	bc	4.80 ± 0.25	c	3.20 ± 0.25	d
NSpC	74.50 ± 2.48	b	82.80 ± 3.97	ab	90 ± 2.89	a	74.30 ± 4.45	b	57.00 ± 4.44	c	38.00 ± 1.44	d
CW	0.17 ± 0.01	abc	0.21 ± 0.01	a	0.22 ± 0.02	a	0.21 ± 0.02	ab	0.15 ± 0.02	bc	0.12 ± 0.02	c
SYpC	0.14 ± 0.01	bc	0.18 ± 0.01	a	0.17 ± 0.01	ab	0.14 ± 0.01	bc	0.12 ± 0.01	c	0.08 ± 0.01	d*
SYpP	0.68 ± 0.02	ab	0.74 ± 0.03	a**	0.74 ± 0.04	a	0.62 ± 0.03	b	0.44 ± 0.02	c	0.27 ± 0.02	d**
CWpP	0.80 ± 0.05	ab	1.06 ± 0.08	a	0.83 ± 0.10	ab	0.73 ± 0.12	bc	0.47 ± 0.05	cd	0.29 ± 0.02	d
BY	1.89 ± 0.05	a	2.29 ± 0.21	a**	2.08 ± 0.21	a	1.71 ± 0.22	a	0.97 ± 0.10	b	0.55 ± 0.06	b
SY/5P	1.78 ± 0.09	bc**	2.62 ± 0.18	a*	2.57 ± 0.15	a	2.25 ± 0.16	ab	1.59 ± 0.09	cd	1.18 ± 0.13	d

Table 4. Average values ± SEs (standard errors) of 12 morphometric traits for the **AN-1** genotype evaluated after treatment with different concentrations of colchicine as percentages, i.e., 0 (control), 0.025%, 0.05%, 0.1%, 0.2%, and 0.4% and their significances based on Tukey's post hoc test letters (a-d) for the groups along with one-way ANOVA centered corresponding signs i.e. *** (significant at the 0.001 level), ** (significant at the 0.01 level) and * (significant at the 0.05 level). The abbreviations used for trait information are the same as those above in Table 3.

Traits	AN-20 genotype											
	0 (Control)		0.025%		0.05%		0.1%		0.2%		0.4%	
	Mean ± SE	Sig.	Mean ± SE	Sig.	Mean ± SE	Sig.	Mean ± SE	Sig.	Mean ± SE	Sig.	Mean ± SE	Sig.
PH	41.34 ± 1.42	ab	45.74 ± 1.10	a	42.40 ± 0.91	a	34.84 ± 1.86	b	27.94 ± 1.66	c*	20.47 ± 1.57	d
NB	10.60 ± 0.37	b	11.60 ± 0.64	ab	13.30 ± 0.47	a	11.20 ± 0.66	b	7.60 ± 0.40	c	6.00 ± 0.33	c
LL	7.87 ± 0.39	ab**	9.45 ± 0.50	a***	9.96 ± 0.41	a***	8.89 ± 0.68	a***	7.80 ± 0.41	ab**	6.26 ± 0.69	c***
NF	9.10 ± 0.28	b	10.80 ± 0.42	a	10.70 ± 0.42	ab	9.70 ± 0.49	ab	7.00 ± 0.45	c	5.60 ± 0.22	c
NC	5.90 ± 0.23	ab	7.40 ± 0.31	a	5.80 ± 0.42	b	5.30 ± 0.26	bc	4.30 ± 0.30	cd	3.50 ± 0.27	d
NSpC	61.90 ± 1.82	b	69.6 ± 1.93	ab	78.80 ± 2.28	a	67.20 ± 3.18	b	45.60 ± 2.46	c**	36.80 ± 1.87	c*
CW	0.16 ± 0.01	bc	0.22 ± 0.02	a	0.19 ± 0.02	ab	0.15 ± 0.01	bc	0.13 ± 0.02	cd	0.08 ± 0.01	d
SYpC	0.13 ± 0.00	b	0.16 ± 0.00	ab	0.16 ± 0.01	a	0.15 ± 0.01	ab	0.09 ± 0.01	c*	0.08 ± 0.01	c
SYpP	0.64 ± 0.02	a**	0.69 ± 0.02	a	0.64 ± 0.03	a	0.51 ± 0.02	b	0.43 ± 0.02	b*	0.27 ± 0.01	c
CWpP	0.71 ± 0.04	ab**	0.83 ± 0.03	a	0.66 ± 0.05	bc**	0.56 ± 0.03	c	0.42 ± 0.03	d	0.28 ± 0.02	e
BY	1.24 ± 0.08	bc*	1.74 ± 0.10	a	1.59 ± 0.10	ab	1.17 ± 0.15	bc	0.92 ± 0.11	cd	0.60 ± 0.05	d
SY/5P	1.86 ± 0.05	a	2.14 ± 0.14	a	1.97 ± 0.09	a	1.22 ± 0.14	b	0.88 ± 0.05	bc	0.73 ± 0.03	c

Table 5. Average values ± SEs (standard errors) of 12 morphometric traits for the **AN-20** genotype evaluated after treatment with different concentrations of colchicine as percentages, i.e., 0 (control), 0.025%, 0.05%, 0.1%, 0.2%, and 0.4% and their significances based on Tukey's post hoc test letters (a-d) for the groups along with one-way ANOVA centered corresponding signs i.e. *** (significant at the 0.001 level), ** (significant at the 0.01 level) and * (significant at the 0.05 level). The abbreviations used for trait information are the same as those above in Table 3.

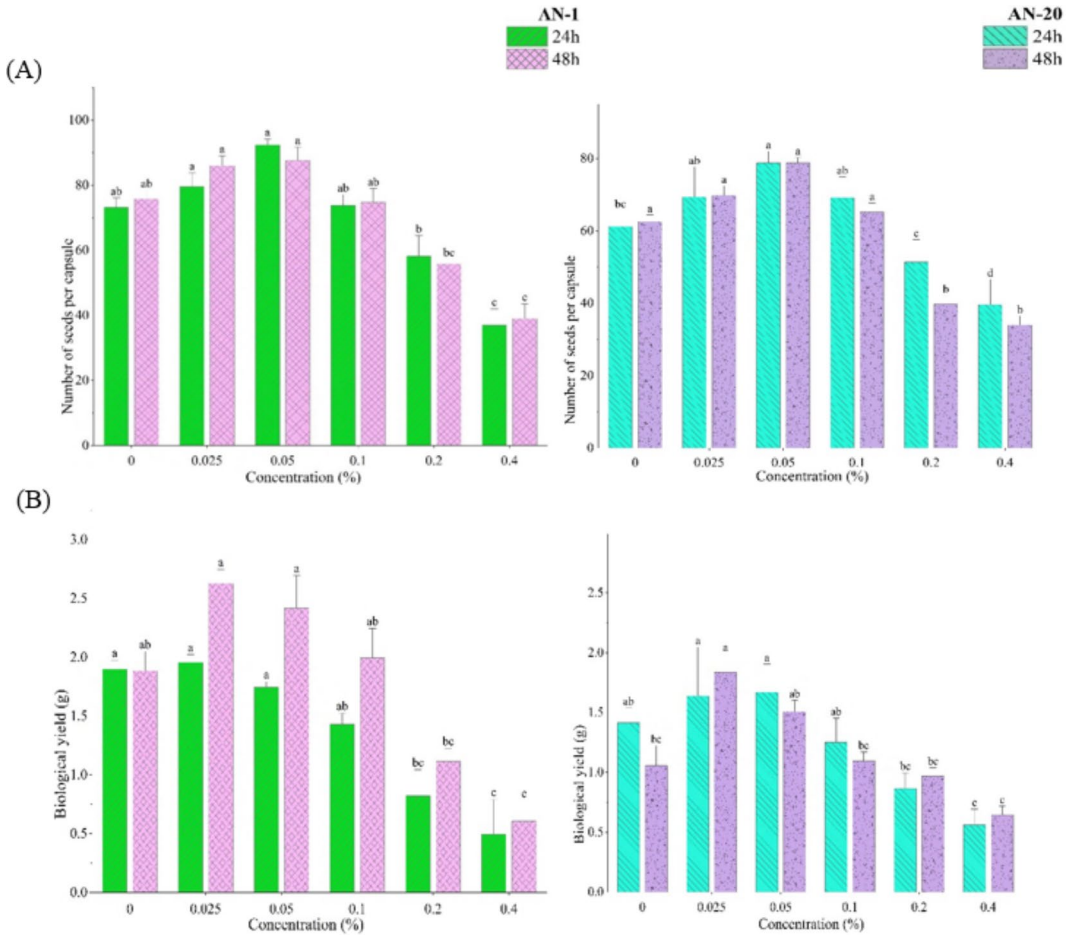

Fig. 4. Comparison of yield-related attributes of the AN1 and AN20 genotypes of *Nigella* on the basis of their duration of exposure to colchicine for 24 h (24 h) and 48 h (48 h) at different concentrations (0 (control), 0.025, 0.5, 0.1, 0.2 and 0.4%. (**A**) Number of seeds per capsule, (**B**) biological yield and (**C**) seed yield per five plants.

Discussion

In this study, the prominent and well-known spice *Nigella sativa* L. (Kalonji), which has played a significantly important role in the livelihoods of people, since the traditional era as medicine and as a seasoning and flavoring agent in cuisine, was analyzed and investigated. This *Nigella* species so far relentlessly consumed worldwide in day-to-day life. According to the *Food and Agriculture Organization of the United Nations* (FAO), India is the greatest contributor of spices and aromatic crops. As per FAOSTAT 2022 report approximately 2.8 million tons of spices and aromatic crops in 2022 were exported from India[45]. Considering the future demand, it is highly important to develop novel, improved, resistant and high-yielding varieties that could be commercialized and popularized widely among farmers in India to boost their economy, as 70% of the rural population of India still solely depends on agriculture for their livelihood. Since it has the potential to cure several ailments, although its standardized medicinal dosage can be a safe and better option as an organic herbal medicine alternative to recent contemporary medicines. Kalonji spice can be a prime spice, as can other spices, such as cumin and carom seeds, in terms of export, which could ultimately contribute to the wealth of India. As India is the largest producer, exporter and consumer of kalonji, despite this production being limited to only a few states. Considering this, ICAR-NRCSS, Ajmer, Rajasthan, India, is continuously focusing on spices and attempting to produce better plants. The two varieties AN1 and AN20 are resultant of this; among them, AN-1 is resistant to root rot and was released at the national level (2019-20), while AN-20 is a local variety at the state level (2014-15). The average life spans of plants at the above mentioned coordinates of Varanasi are a few days longer similar to earlier findings[46], in contrast to those reported earlier in Kant et al. (2009)[47], possibly due to regional climatic changes or due to mutagenic (colchicine) exposure for 24–48 h, which delays germination, flowering, fruiting and maturity processes compared with those of the control (without treatment) plants[48].

Both of these genotypes were exposed to the well-known plant-based alkaloid colchicine[49] to observe the impact of several concentrations of colchicine for different durations, 24–48 h. With the chromosome count analysis it was found that the colchicine concentration 0.05% as well as 0.1% are responsible for tetraploid plants (Table 2) and with the morphological study of plants also revealed that these were the concentrations which shows highest enhancement in growth characters and yield increment (Fig. 4). The highest coefficient of variance for biological yield, i.e., 42.21%, indicates that values are significantly dispersed around their mean

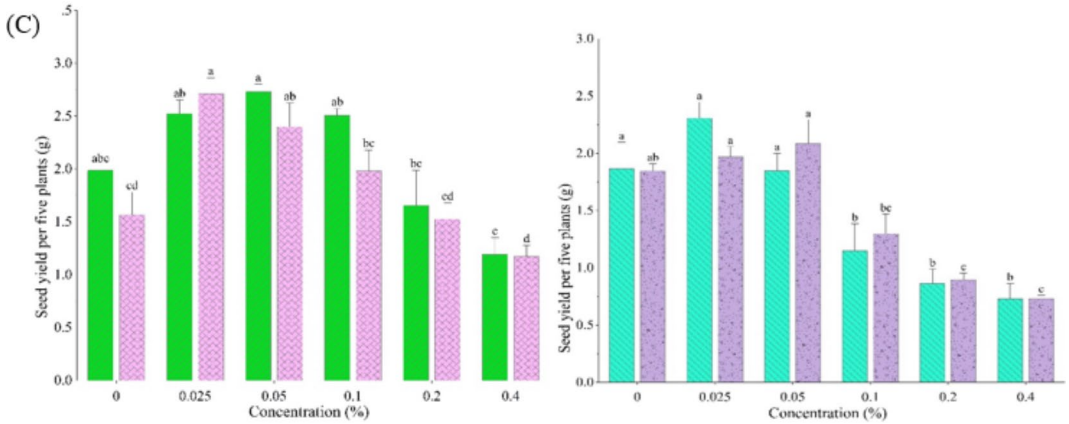

Figure 4. (continued)

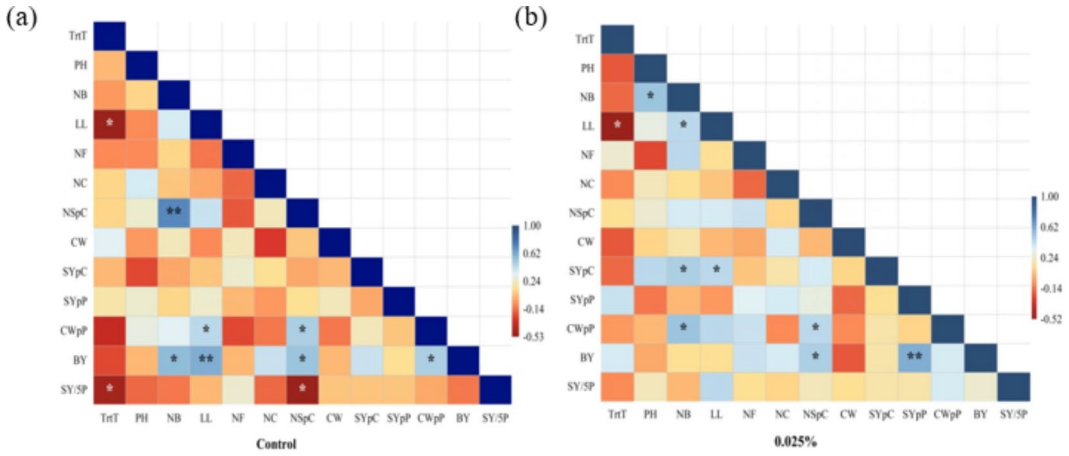

Fig. 5. Heatmap showing Pearson correlation for each concentration of colchicine used on *Nigella sativa* L. genotypes AN1 and AN20: **(a)** control, **(b)** 0.025%, **(c)** 0.05%, **(d)** 0.1%, **(e)** 0.2%, and **(f)** 0.4%. Values are significant at the $P < 0.01$ and 0.05 levels (2-tailed). Abbreviations: treatment time, TrtT; plant height, PH; number of branches, NB; leaf length, LL; number of flowers, NF; number of capsules per plant, NC; number of seeds per capsule, NSpC; capsule weight, CW; seed yield per capsule, SYpC; seed yield per plant, SYpP; total capsule weight per plant, CWpP; biological yield, BY; seed yield per five plants, SY/5P.

(Table 3), similar to the findings of studies of *Nigella* genotypes in Ethiopia[50]. The significance difference was found for leaf length for all concentrations tested in AN-1 as well as AN-20 (Tables 4 and 5). A similar finding was reported for triticale hybrids[51] and large cardamom cultivars (*Amomum subulatum*), where productivity was determined by elevation in the Sikkim Himalayas, where a significant difference was reported for different cultivars of cardamom[52].

Although colchicine is a plant-derived chemical, it can affect plants positively or negatively depending on its dosage. Thus far, the use of colchicine as a mutagen could improve or enhance yield by introducing polyploidy to chromosomes[53,54] at low dosages; however, despite being polyploid, plant growth and yield are limited to some extent when the dose increases. To determine the effective ameliorative concentration of colchicine, further exploratory analysis was performed in this study, which revealed a significant positive correlation coefficient[37,55] between the seed yield per plant and the biological yield at a 0.025% w/v concentration ($P < 0.01$) (Fig. 6b), and the mean plot seed yield per plant was also significantly high for both of the genotypes (Fig. 1D). Although the seed yield per plant at 0.025% w/v was also comparable to that at 0.05% and 0.1% w/v, the values at 0.05% w/v were not found significant; however, at 0.1% w/v, we reported significant results (Fig. 6c and d). A similar study of 49 genotypes of *Panicum virgatum* was reported for the assessment of drought tolerance[56,57].

To gather additional information and acquire additional knowledge, principal component analysis was subsequently performed. PCA has the potential to reduce the dimensionality of large datasets, and a correlation-based PCA matrix is utilized for different variables of multiple measures[40,43]. In this study, PC1 was found to be the most powerful since it was the only factor responsible for 84.43% of the variance in the data (Table 6). On this basis, in the score plot (Fig. 3), lower dosages were reported with the highest positive PC1 values and were clustered at one location, on the other hand, higher dosages with negative PC1 but positive and negative PC2

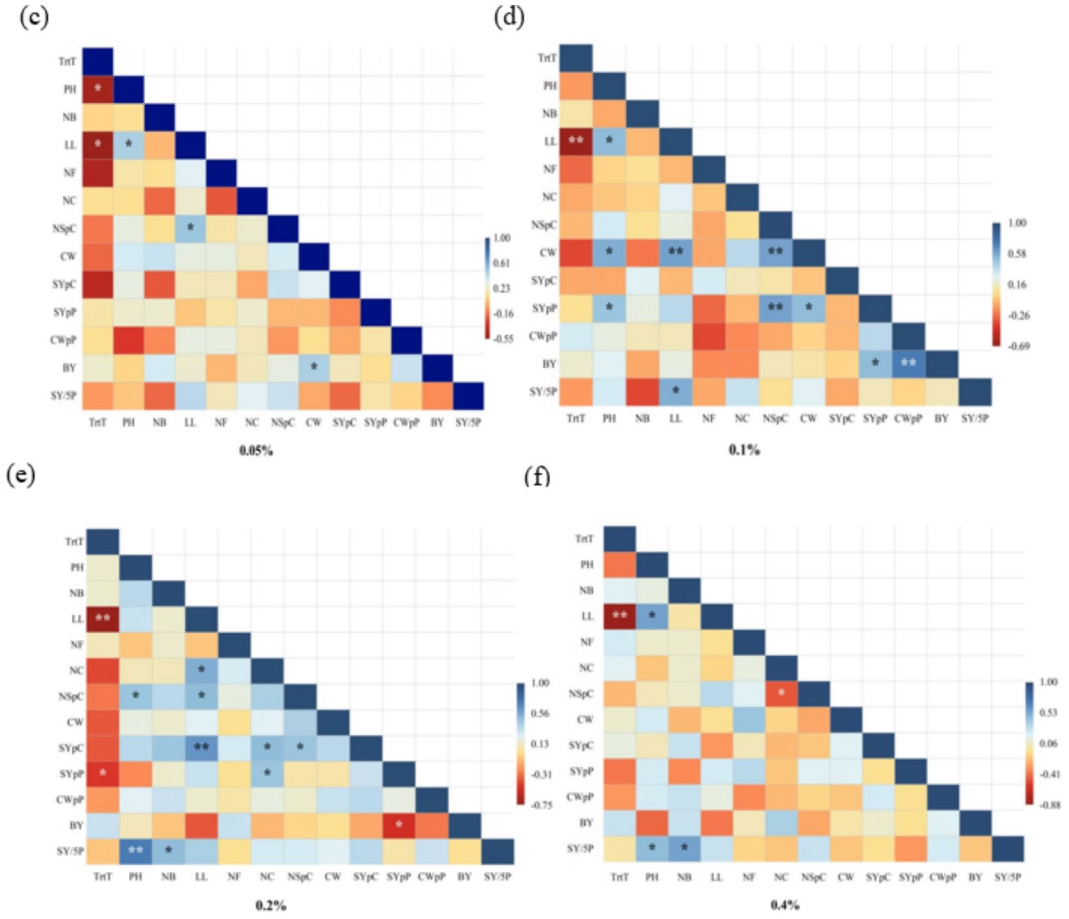

Figure 5. (continued)

were clustered in another group. Similarly, a score plot study was used for morpho-physiological evaluation of potato genotypes in response to drought stress[58] and in rice[59]. PC1 was an important component with almost all the morphological attribute contributions, and PC2 had the highest variance due to significant variation in leaf length (0.8), followed by capsule weight and seed yield per five plants (0.295, 0.211). In a previous study investigating water deficit tolerance in cotton[60,61] and in tomato genotypes[62,63], great variation was shown in the studied material under normal and water deficit conditions, thus supporting the present investigation. Genetic diversity depends on morpho-agronomic characteristics in the Ethiopteran species *Nigella sativa* L. who reported that principal components can strongly influence yield[24,64].

Conclusion

It is concluded that, under the environmental conditions of the subtropical region of Varanasi (Uttar Pradesh, India), the AN1 genotype results in far greater improvement than does the AN20 genotype of *Nigella*. The initial growth response after mutagenic colchicine treatment was observed for the AN1 genotype prior to the AN20 genotype. The preliminary growth of the plants was inhibited more potently in the AN20 genotype than in the AN1 genotype due to the mutagen colchicine, and in terms of the different concentration treatments, the higher concentrations of 0.2 and 0.4% w/v were able to prolong the initial growth period of the plants. Consequently, the growth of plants, primary branches and number of capsules also diminished in the AN20 genotype compared with the AN1 genotype. However, the growth and yield of the AN1 genotype plants improved overall. Treatment with lower dosages of colchicine, such as 0.025, 0.05 and 0.1% w/v, which possess putative tetraploid chromosomes had ameliorative or beneficial impacts on the morphology of the plants, as indicated by plant height, primary branches, and leaf length, which ultimately resulted in better seed yield per capsule as well as per plant, whereas higher dosages of colchicine, such as 0.2 and 0.4% w/v, that were responsible for inducing hexaploids, octaploids and even mixoploids led to non-ameliorative or negative impacts on the growth of the plants. Among both of the genotypes, the AN1 genotype plants were more likely to benefit from a lower dosage of the mutagen colchicine than was the AN20 genotype; thus, the AN1 genotype can be preferred over the AN20 genotype for further breeding programs. Principal component analysis (Fig. 6) and yield parameter plots (Fig. 4A-F) revealed that the lowest dose of colchicine for a longer time period, 48 h, which was denoted as the 'lowest48G1', had the greatest ameliorative effect might be due to heterochomatization or euchromatization occurrence which needed to be explored. This *Nigella* L. spice needs to be explored well and further research

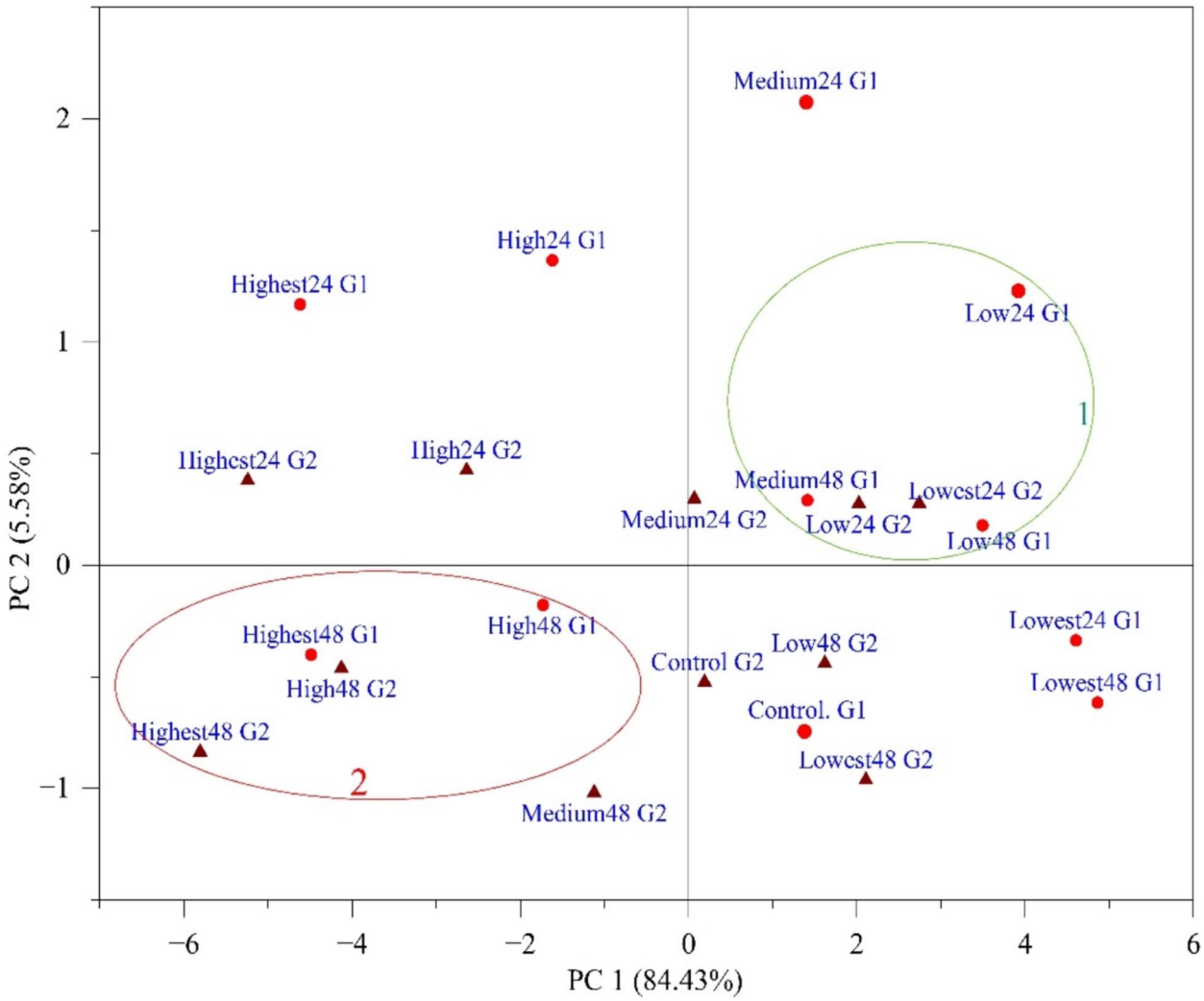

Fig. 6. Score plot showing two principal components, PC1, and PC2, of the AN1 (G1) and AN20 (G2) plant genotypes for the control, 0.025% (lowest), 0.05% (low), 0.1% (medium), 0.2% (high), 0.4% (highest) with varied time durations of 24 h and 48 h respectively.

should have carried out to produce more successful polyploids with the incorporation of other species that could further enhance their qualitative characters and seed yield.

(a).	Principal components	
Traits	PC1	PC2
Eigenvalue	10.132	0.669
% of Variance	84.43%	5.58%
Cumulative Variance	84.43%	90.01%
(b).	**Coefficients of extracted eigenvectors**	
PH	0.299	-0.053
NB	0.292	-0.230
LL	0.226	0.800
NF	0.287	-0.216
NC	0.302	-0.137
NSpC	0.302	0.074
CW	0.286	0.295
SYpC	0.297	-0.031
SYpP	0.299	-0.111
CWpP	0.286	-0.225
BY	0.288	-0.191
SY/5P	0.288	0.211

Table 6. PCA (principal component analysis) showing **(a).** Eigenvalue, percentage of variance and cumulative variance percent of three principal components; PC1, and PC2. **(b).** Coefficients of eigenvectors for the 12 morphometric traits in terms of three major principal components. Abbreviations: plant height, PH; number of branches, NB; leaf length, LL; number of flowers, NF; number of capsules per plant, NC; number of seeds per capsule, NSpC; capsule weight, CW; seed yield per capsule, SYpC; seed yield per plant, SYpP; total capsule weight per plant, CWpP; biological yield, BY; seed yield per five plants, SY/5P.

Data availability

The datasets analyzed during the current study available from the corresponding author on reasonable request.

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Author contributions
1st author i.e. SV, designed the experiment, collected the data, prepared all the figures & tables and also wrote the whole manuscript. All authors SV, MH and SK read and reviewed the whole manuscript.

Declarations

Competing interests
The authors declare no competing interests.

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