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Research Article

Responses of *Colchicum speciosum* L. populations to conventional and nano-fertilizers of nitrogen through changes in morphological and biochemical attributes

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Abstract

There is an increasing interest in the use of nano-fertilizer as an alternative to traditional fertilizers, in order to increase the secondary metabolites in medicinal plants. The present study was conducted to observe the changes in morphological and biochemical properties of three populations of *Colchicum speciosum* L., from Iran (Filband, Sangdarka, and Kelerd), upon conventional (1.1 and 2.2 mg L^{-1}) and nano-N (1.5 and 3 mg L^{-1}) fertilizers based on completely randomized design (CRD). The anthocyanin and phenolic contents of leaves and corms were determined with a spectrophotometer and corm colchicine was extracted by high-performance liquid chromatography (HPLC). The results represented different responses of morphological and biochemical attributes to populations due to their different origins. Filband with higher altitude showed increased leaf area, plant length, corn weight and diameter when treated with N fertilizers. Increased total phenolic component (TPC) in leaves and corm and also leaf total flavonoids component (TFC) were reported in Filband upon N fertilizers. All fertilizers led to decreased leaf anthocyanin when N fertilizers were applied. A noticeable increase in colchicine was obtained in Kelerd and Sangdarka when plants were treated with 1.5 mg L^{-1} nano-N fertilizer. Heat map analysis of corm anthocyanin, leaf anthocyanin, leaf TPC, leaf TPC, and colchicine amount showed higher variability under the treatments. According to principal component analysis (PCA), corm TPC, leaf TFC, corm TFC, leaf area, and corm weight were specific traits of Kelerd. In conclusion, different responses of morphological and biochemical traits were reported between populations, and N fertilizers exerted diverse effects on these traits. The population from higher altitudes showed higher phenolic content and lower anthocyanin accumulation under conventional and nano-N fertilizers.

Keywords: colchicine; corm size; heat map; nanomaterials; phenolic content

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Introduction

Medicinal plants contain a wide range of bioactive compounds, commonly as secondary metabolites, which are more effective than primary metabolites to enhance plant capability to survive and overcome local challenges (Vázquez-León *et al.*, 2017). These plants are valuable resources for new drugs discovery due to high potential in producing the secondary metabolites (Dar *et al.*, 2017). Today, the due to increasing demand on medicinal plants, their harvesting is the global concern (Van Wyk *et al.*, 2018), particularly when plants are harvested under improper practices (Chapagain *et al.*, 2021). It not only leads to the extinction of species generations but also endangers the biodiversity of the region and the world (Chapagain *et al.*, 2021). Therefore, it is necessary to protect plants inside and outside the habitat, which requires botanical knowledge in terms of phytochemical attributes of plant species.

Colchicum speciosum L., belonging to the Colchicaceae family, is a perennial plant with underground corms or rhizomes and hypoginus flowers. Among the 14 species of genus *Colchicum* in this family, nine species have been distributed in the temperate area of Northern Iran. *C. speciosum* is a medicinal plant, which naturally grows in Iran, Turkey, and the Caucasus. It is widely used to treat osteoarthritis, gout, cancer, inflammations, and jaundice. *C. speciosum* are rich in phenolic compounds, namely vanillin, vanillic acid, coumaric acid, caffeic acid, and lutein (Çankaya *et al.*, 2019). Colchicine is the main alkaloid of this species that has various medicinal properties such as strong anti-inflammatory and being widely exerted to treat gout (Senizza *et al.*, 2020).

Nitrogen (N) is a vital nutrient in the plant life cycle and is found in a wide range of organic compounds such as amino acids, proteins, nucleic acids, and chlorophyll (Barker and Pilbeam, 2015). The N deficiency possesses biochemical and physiological defects that eventually leads to reduced cell division and disruption of photosynthesis. Nitrogen fertilizers as well as harvest time are the main factors that influence the accumulation of secondary metabolites, namely phenols, flavonoids, etc. (Cojocaru *et al.*, 2020).

In the age of climate change, global agricultural systems face numerous challenges in achieving food security. Nanotechnology is a useful tool to strengthen high quality and quantity products. Nanofertilizers can provide or more nutrients for plants, which in turn results in increased yield of plants (Liu and Lal, 2015). Nanomaterials due and these nanoscale compounds can easily pass through the pores and walls of root cells. Also, by passing through plasmodesmata, nanoparticles can be easily transferred from one cell to another one and increase crop yield. Compared to conventional and synthetic fertilizers, nanofertilizers can release the nutrient slowly and also alleviate the leaching of the substance. In addition, nanomaterials use more ion channels in plasma membranes for adsorption relative to synthetic fertilizers (Hasanuzzaman *et al.*, 2020). Nitrogen plays an important role in the biosynthesis and accumulation of alkaloids in plants.

Recently there is an increasing interest on the use of nanomaterials to increase growth and active ingredients of medicinal plants. The noticeable improvement of N nanofertilizers in improving plant growth through biochemical attributes have been reported on *Origanum syriacum* (Hamed *et al*., 2018), *Solanum tuberosum* (Abd El-Azeim *et al*., 2020), and *Fragaria* × *ananassa* Duch (Cvelbar *et al*., 2021). However, little information is about the effects of N nanoferilizers on *C. speciosum*. Therefore, the purposes of present study were (1) to investigate the effects of conventional and nanofertilizers of nitrogen on plant growth (leaf arena plant length, corm weigh, and corm size), and (2) to determine the changes of phenolic compounds, anthocyanins, and colchicine upon N fertilizers in three populations of *C. speciosum*.

Materials and Methods

Plant materials and site description

The *C. speciosum* plants in the flowering phase were collected from November of 2017 in their natural habitats in north of Iran (Table 1), and their corms were dried at shade conditions. The corms were cultivated at 50*30 cm pots. The conventional and nano-N fertilizers were purchased from Green and Khazra Company, respectively. The soil samples were also taken from the experimental farm and the chemical and physical properties of the soil were investigated (Table 2). The standard laboratories methods were used to analyse soil. Soil pH was measured by an EC meter. Phosphorous (P) and potassium (K) were measured by Olsen and flame photometer methods. Ca, Mg, Fe, and Cu were determined by atomic absorption spectroscopy (Perkin-Elmer 2340) (Chatzistathis *et al*., 2017). The plants were harvested after flowering at September 2019 to examine morphological and phytochemical analyses. For phenolic and alkaloid extraction, the samples were dried in room temperate at shade.

Table 1. Site properties of *Colchicum speciosum* L. populations

Population	Altitude (m asl)	Latitude	Longitude	Site type
Sangdarka	850	61° 26′ 28″	40° 23′ 02″	Forest
Kelerd	483	62° 26′ 33″	40° 14′ 55″	Forest
Filband	2141	63° 56′ 53″	40° 01′ 21″	Rangeland

Table 2. Soil properties of *Colchicum speciosum* L. sites

Population	EC (dS/m)	OM (%)	P (ppm)	K (ppm)	Ca (ppm)	Mg (ppm)	Zn (ppm)	Fe (ppm)
Filband	1.62	11.22	20.5	501	3370	220	4	83.2
Sagdarka	1.03	8.37	3.3	277	6840	690	2.5	56.4
Kelerd	0.73	6.67	94.8	1295	7760	490	4.9	63

Experiment design and treatment

The pot experiment was conducted in factorial based on completely randomized design (CRD) with nine replicates to study the effects of N fertilizers on different populations of *C. speciosum*. The N fertilizers were applied in five levels as conventional (1.1, and 2.2 mg L^{-1}) and nano form (1.5 and 3 mg L^{-1}) at two times during growth period as soil application; first time at June 20(in vegetative phase) and the second time at September 2 (before flowering time).

Morphological measurement

Leaf area was determined using a Leaf Area meter. Plant length were measured by a ruler. A digital caliper and scale were used to determine corm diameter and com weight respectively.

Total phenolic content (TPC)

The TPC was measured with Folin-Ciocalteu as a reagent and gallic acid (GA) as a standard. For this, 2 g of leaf sample was homogenized with 8 ml of 80% ethanol and centrifuged at 12000 × g for 20 min. After that, 0.5 mL of supernatant was transferred into 15 mL Falcons. Then 500 µl Folin-Ciocalteu was added to mixture and after 2 min, 1 mL sodium carbonate (7%) was added to the reaction mixture and the final volume was reached to 6 mL using distilled water. The mixture was remained in a 30 ° C (dark condition) bath for 90 min. The absorbance of the samples was measured in a 725 nm wavelength with a spectrophotometer (McDonald *et al*., 2001).

Total flavonoid content (TFC)

The amount of TFC was measured by aluminum chloride colorimetric method. In this method, 0.5 mL of the extract solution with mixed with 1.5 ml of 95% ethanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. After keeping the samples at room temperature for 30 min, the adsorption of the mixture was read at 415 nm. The quercetin standard was used to draw the curve (Chang *et al.*, 2002).

Anthocyanin measurement

According to the method of Nogués and Baker (2000), 1 g of fresh tissue was mixed in 10 ml of acidic methanol (99: 1) (hydrochloric acid: methanol, v/v) and refrigerated for 24 h. The mixture was then centrifuged and the supernatant was read at 530 nm with a spectrophotometer. Finally, equation of A = £ bc was applied to calculate the anthocyanin content with the extinction coefficient of $\varepsilon = 33000 cm^2 Mo^{l-}1$.

Colchicine extraction

To extract colchicine extraction, 0.5 g corm materials were mixed with 25 ml of oil ether and shaken twice for 60 min. After smoothing the mixture, solid materials were dried in air and then shaken with 10 ml of dichloromethane at room temperature for 30 min. Then, 10% ammonium solution (0.5 ml) was added to the mixture and shaken vigorously for 10 min. Subsequently, the solution was smoothed after 30 min. The residue was washed twice with 10 ml of dichloromethane and then mix with the filtered solution. The organic phase was evaporated and dried and then dissolved in 1 ml of 70% ethanol. To detect colchicine in the extract, the concentration was determined by high performance liquid chromatography (HPLC).

Identification and quantitative determination of the colchicine in the extracts were carried out by an Agilent brand 1260 model HPLC apparatus. The chromatographic system was equipped with an autosampler, a quaternary pump, a column compartment and a UV–VIS detector. Chromatographic analysis was carried out using a single-column isocratic reverse phase method. Separation was performed by ACE 5 C-18 column (250 mm × 4.6 mm id, 5 μm particle size). During the mobile phase 450 mL of 6.8 g/L solution of potassium dihydrogen phosphate and 530 mL of methanol were mixed. After the temperature of the mix had cooled down to room temperature, the volume of the mix was completed to 1000 mL with methanol. The pH of the final solution was adjusted to 5.5 with dilute phosphoric acid and filtered through 0.45 μm Millipore filters. The flow rates the mobile phase was 1.0 mL/min and the injection volume was 20 μL. The column temperature was kept at 30 °C and detection was carried out at 254 nm.

Data analysis

All data was statistically analyzed by SAS software in three replicates. The value of treatments was compared by Duncan's multiple range tests. The data were statistically investigated at 5% probability level. Details of grouping of three population treated plants by morphological and biochemical properties were studied by constructing a heatmap plot by R studio package.

Results

Leaf area and plant length

Leaf area was significantly ($P \leq 0.05$) changed in the populations and upon N fertilizers. In the non-fertilizer treatment (control), Sangdarka population showed the highest leaf area (48.7 cm^2) and followed by Kelerd and Filband to be 35.3 and 4.3 cm^2, respectively. The populations showed different responses of leaf area to N fertilizers. For Filband and Kelerd, nano-fertilizers especially 3 mg L^{-1} represented higher leaf area compared with control. However, Sangdarka revealed lower leaf area when supplied with N fertilizers. Nano-N fertilizers were more effective on the leaf area of Filband plants relative to other populations, where nano-N

1.5 and 3 mg L^{-1} led to 2.4 and 4.6-fold increases of leaf area (Table 3). Plant length increased with N fertilizer, and the largest plants were obtained in Kelerd population upon 1.1 mg L^{-1} N of to be 37.9 cm. By exerting N fertilizers, the deference of pant length between populations decreased compared with control plants (Table 3). Therefore, our results showed that populations have different response of leaf area and plant length to N fertilizers.

Table 3. Morphological traits of of *Colchicum speciosum* L. populations under conventional (N) and nano-N fertilizers

Population	N fertilizer	Leaf area	Plant length	Corm weight	Corm diameter
Filband	Control	4.3 ± 0.3^{d}	8.8 ± 0.58^{g}	3.7 ± 0.43^{cd}	0.43 ± 0.07^{d}
	N 1.1	5.3 ± 1.66^{d}	11.1 ± 1.25^{fg}	2.67 ± 0.67^{d}	0.43 ± 0.33^{d}
	N 1.2	7.9 ± 3.76^{cd}	$15.8\pm1.53^{e-g}$	$5.93\pm1.21^{a-c}$	0.93 ± 0.68^{cd}
	Nano-N 1.5	10.6 ± 0.63^{cd}	8.8 ± 0.19^{g}	$6.17\pm0.77^{a-c}$	1.07 ± 0.24^{cd}
	Nano-N 3	$20\pm1.07^{b-d}$	10.1 ± 2.53^{fg}	$6.47\pm0.39^{a-c}$	$1.93\pm0.24^{b-d}$
Kelerd	Control	$33.5\pm4.41^{a-c}$	$22.5\pm0.38^{c-e}$	8.3 ± 0.14^{a}	3.8 ± 0.1^{ab}
	N 1.1	37.5 ± 1.61^{ab}	37.9 ± 1.32^{a}	7.5 ± 0.5^{ab}	$3.17\pm0.55^{a-c}$
	N 1.2	$23.8\pm0.75^{a-d}$	$22.6\pm1.4^{c-e}$	$4.5\pm1.21^{b-d}$	$1.93\pm1.3^{b-d}$
	Nano-N 1.5	$34.3\pm4.3^{a-c}$	$24.8\pm1.75^{b-e}$	$6.07\pm0.57^{a-c}$	$2.43\pm0.24^{a-d}$
	Nano-N 3	42 ± 2.02^{ab}	$25.3\pm6.52^{b-e}$	7.2 ± 1.1^{ab}	3.7 ± 0.43^{ab}
Sangdarka	Control	48.7 ± 3.18^{a}	$26.2\pm3.22^{b-d}$	$5.33\pm1.03^{a-d}$	$1.4\pm0.22^{b-d}$
	N 1.1	$33.3\pm1.73^{a-c}$	33.7 ± 2.2^{ab}	$5.8\pm0.46^{a-c}$	$2.6\pm0.25^{a-d}$
	N 1.2	46.8 ± 1.51^{ab}	$29.5\pm2.4^{a-c}$	2.63 ± 0.05^{d}	0.37 ± 1.39^{d}
	Nano-N 1.5	$34.1\pm1.17^{a-c}$	$19.2\pm0.51^{d-f}$	3.43 ± 1.1^{cd}	$1.4\pm0.73^{b-d}$
	Nano-N 3	$32.3\pm5.12^{a-c}$	$19.3\pm3.63^{d-f}$	$6.4\pm0.34^{a-c}$	4.43 ± 0.03^{a}

Values are means ± standard error (SE) of nine replicates (n= 9). Different letters show statistically significant differences among treatments at $P\leq0.05$.

Corm weight and corm diameter

The populations showed different reposes of corm weight and diameter to N fertilizers. Filband population showed increased corm weight, particularly when the plants were nourished with 3 mg L^{-1} nano-N. However, although Kelerd population decreased in 1.1 mg L^{-1} of N and also 1.5 mg L^{-1} of nano-N, higher concentration of nano-N (3 mg L^{-1}) caused increased corm weight by 20% as compared to control. As described for corm weight, corm diameter also showed different reactions between populations upon N fertilizers. The noticeable increase of corm diameter was observed for Filband and Sangdarka when nano-N 3 mg L^{-1} was applied. Compared with control, 3 mg L^{-1} nano-N enhanced corm diameter as 4.6 and 3.1 folds for Filband and Sangdarka, respectively. Interestingly, all N fertilizers except 3 mg L^{-1} nano-N decreased corm diameter of Kelerd population (Table 3). Hence, the trend of changes for Filband and Sangdarka was similar upon N fertilization.

Total phenolic content (TPC)

TPC in both leaves and corms were significantly ($P\leq0.05$) different between *C. speciosum* populations upon N fertilizers. The increased leaf TPC for Filband was reported when plants were treated with the conventional and nano fertilizers. In contrast, N fertilizers resulted in decreased leaf TPC for Kelerd and Sangdarka populations. The 1.7, 2.7, 2.6, and 2.3-fod increases of leaf TPC were obtained for Filband population after nourishing by 1.1, 2.2, 1.5, and 3 mg L^{-1} of N fertilizers, respectively. The minimum leaf TPC for Kelerd and Sangdarka were observed upon 3 mg L^{-1} nano-N as 21 and 14.5 mg GA g^{-1} DW, respectively, while the maximum leaf TPC was recorded in control plants (Figure 1a). Like leaf TPC, corm TPC in Filband increased with N fertilizers, in which its maximum amount was obtained upon 2.2 mg L^{-1} of conventional N fertilizer (Figure 1b).

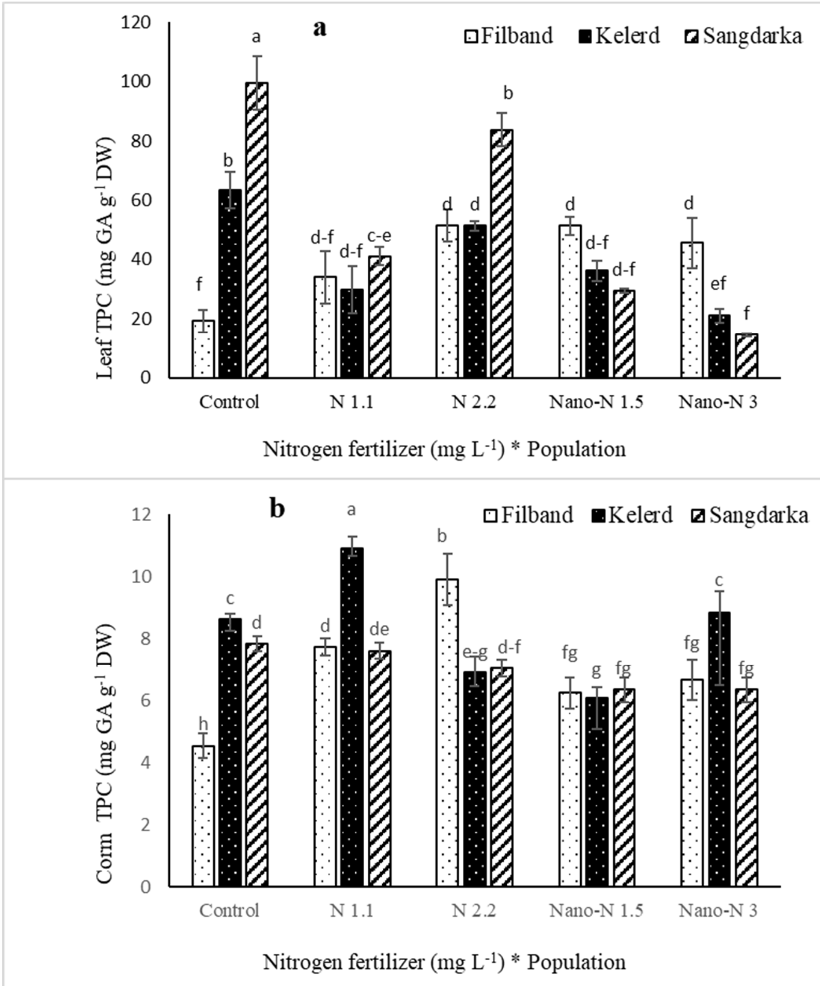

Figure 1. Total phenolic content (TPC) in leaves (a) and corms (b) of *Colchicum speciosum L.* populations under conventional (N) and nano-N fertilizers. Values are means ± standard error (SE) of nine replicates (n= 9). Different letters show statistically significant differences among treatments at $P \leq 0.05$. (N1.1, N2.2, Nano- N1.5 and Nano- N3 were explained in Table 3)

Total flavonoid content (TFC)

The *C. speciosum* populations showed different responses of leaf and corm TFC under N fertilizers. Totally, leaf TFC increased in all populations when plants treated with nano-N. This increase for Filband was higher than other pollutions, where the maximum leaf TFC was observed in plants nourished by 1.5 mg L^{-1} nano-N to be 3.3-fold raise compared with control. The conventional fertilizer led to decreased or unchanged TFC in *C. speciosum* leaves. Unlike Filabnd and Kelerd, Sangdarka represented the minimum changes under conventional and nano-N fertilizers (Figure 2a). The TFC in corms represented irregular trend between the populations. Generally, N fertilizers led to significant reduction in corm TFC for Filband and Kelerd. For Sangdarka, TFC decreased upon N fertilizer over control except 1.5 mg L^{-1} nano-N. Totally, the TFC in corms was lower than that reported in leaves. Accrediting, N fertilizers increased leaf TFC, but decreased corm TFC (Figure 2b).

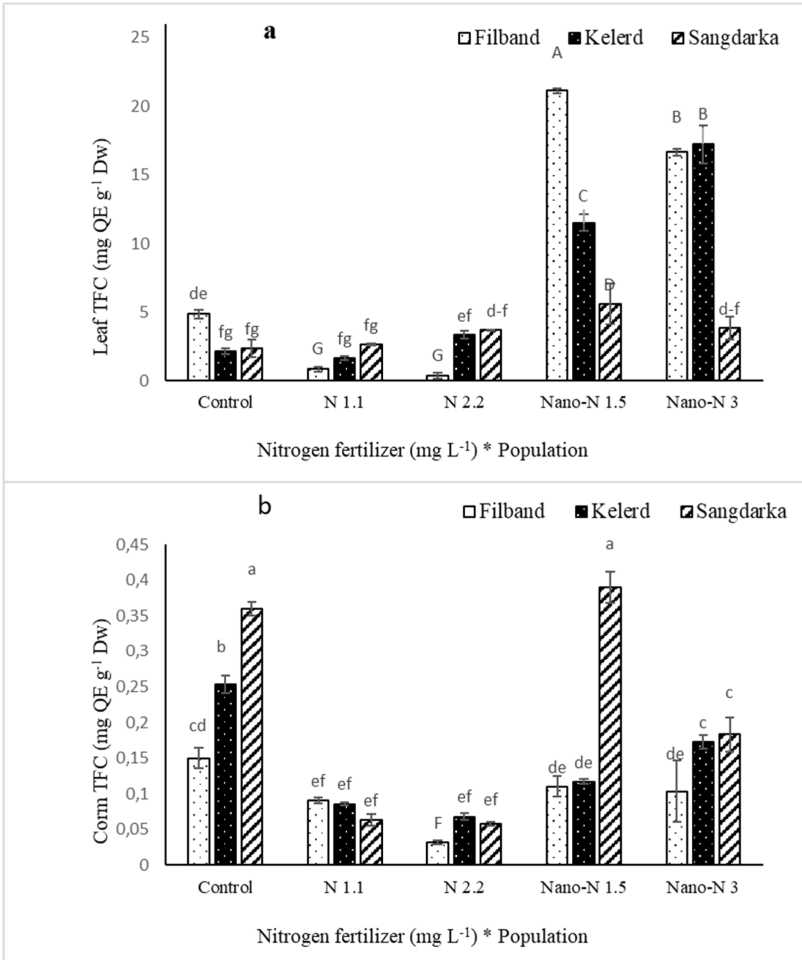

Figure 2. Total flavonoid content (TFC) in leaves (a) and corms (b) of *Colchicum speciosum L.* populations under conventional (N) and nano-N fertilizers. Values are means ± standard error (SE) of nine replicates (n= 9). Different letters show statistically significant differences among treatments at $P \leq 0.05$. (N1.1, N2.2, Nano- N1.5 and Nano- N3 were explained in Table 3)

Anthocyanin content

The *C. speciosum* populations represented different responses of anthocyanin content to N fertilizers. All fertilizers led to decreased leaf anthocyanin when N fertilizers were applied. The 1.1, 2.2, 1.5, and 3 mg L-1 of N fertilizers decreased leaf anthocyanin content of Filband population by 35, 49, 40, and 31%, respectively, as compared with control. This trend was also observed for other populations (Figure 3a). Sangdarka showed no significant change of corm anthocyanin upon N fertilizers, the noticeable reduction of this trait was observed in Filaband plants over control. It ranged from 0.046 mg g^{-1} in Filband upon 1.5 mg L^{-1} nano-N to 0.399 mg g^{-1} in Filband under non-N application (Figure 3b).

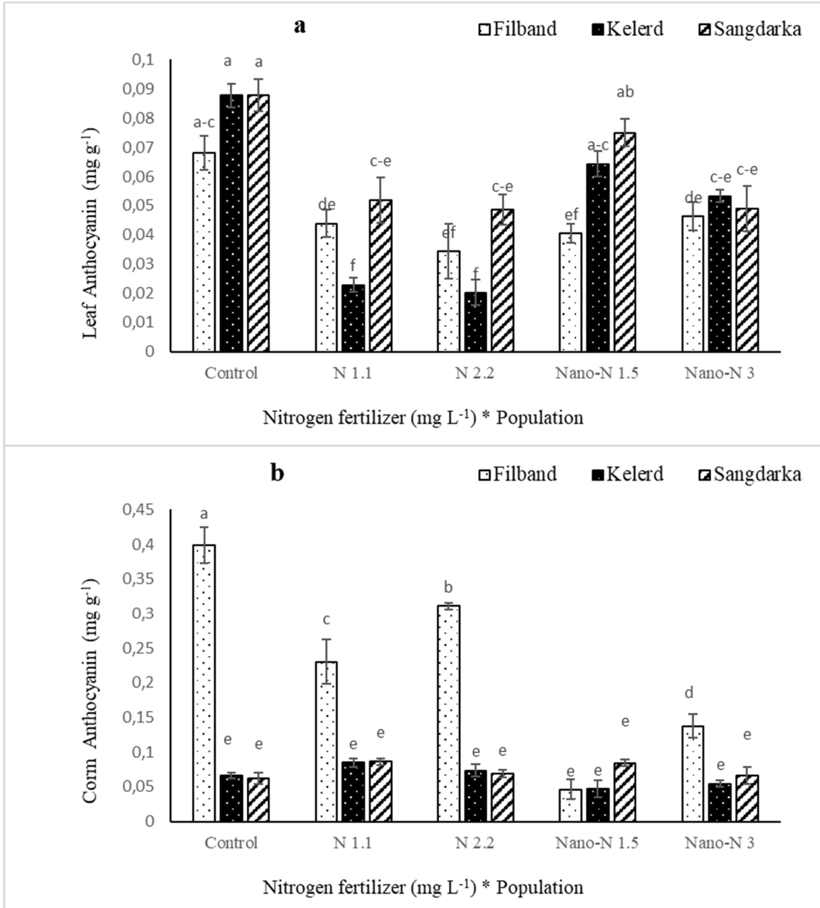

Figure 3. Anthocyanin content in leaves (a) and corms (b) of *Colchicum speciosum L.* populations under conventional (N) and nano-N fertilizers. Values are means ± standard error (SE) of nine replicates (n= 9). Different letters show statistically significant differences among treatments at $P \leq 0.05$. (N1.1, N2.2, Nano-N1.5 and Nano- N3 were explained in Table 3)

Colchicine content

The results of HPLC represented different corm colchicine amounts between the populations when treated with N fertilizers. The 1.5 mg L^{-1} nano-N resulted a significant increase of colchicine when plants treated with 1.5 mg L^{-1} nano-N fertilizer. Compared with control, 1.5 mg L^{-1} nano-N exerted the 3.8 and 2.4-fold increases for corm colchicine of Kelerd and Sangdarka, respectively (Figure 4).

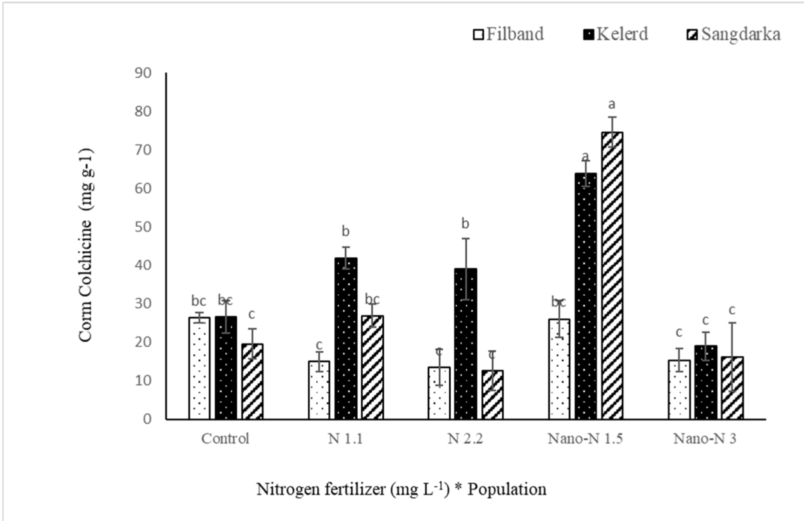

Figure 4. Colchicine content in corms of *Colchicum speciosum L.* populations under conventional (N) and nano-N fertilizers. Values are means ± standard error (SE) of nine replicates (n= 9). Different letters show statistically significant differences among treatments at $P \leq 0.05$. (N1.1, N2.2, Nano- N1.5 and Nano- N3 were explained in Table 3)

Heat map

According to heat map analysis, the distribution and variation of traits upon the treatments are shown by colour from blue (the lowest value) to red (the highest value). In this regard, corm anthocyanin, leaf anthocyanin, leaf TPC, leaf TPC, and colchicine showed the higher variability under the treatments. However, morphological traits (leaf area, plant height, corm diameter, and corm weight) and corm TPC showed lower variability among the treatments. For treatments analysis, 1.5 mg L^{-1} nano-N in all populations was identified in a distinguished cluster. Therefore, this level of N fertilizer is completely differed from others (Figure 5).

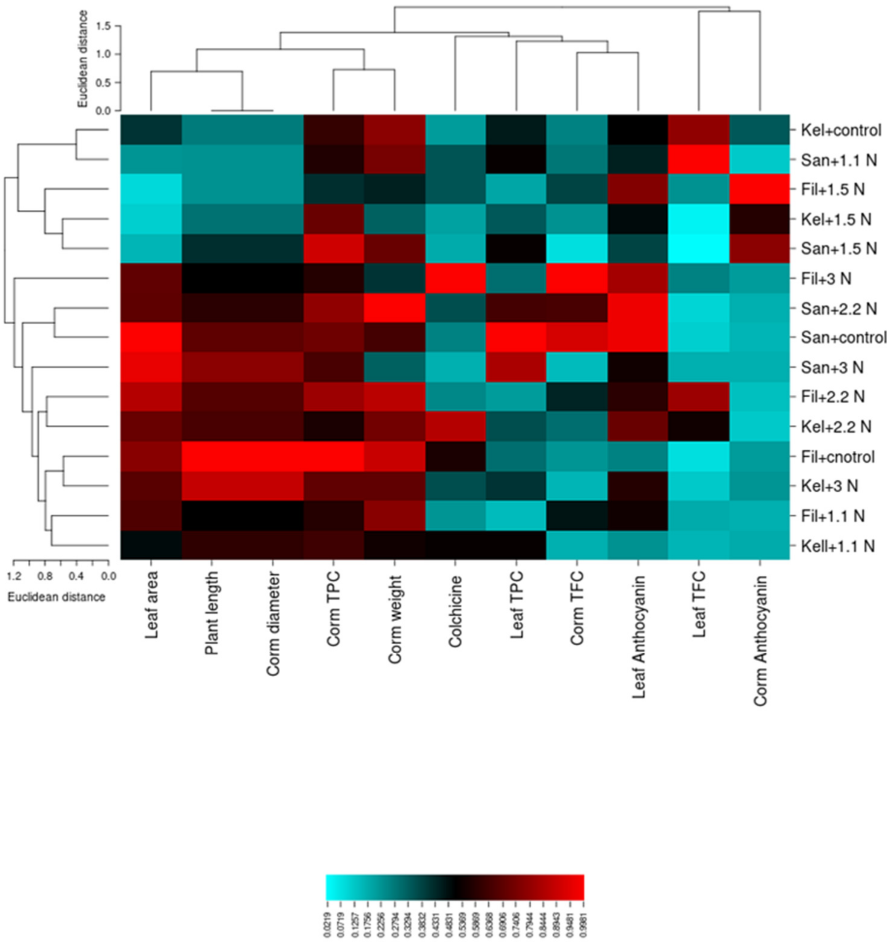

Figure 5. Heat map analysis of *Colchicum speciosum* L. populations under different concentration of nitrogen fertilizers

Principal component analysis (PCA)

According to PCA, Filband and Sangraka populations were described by F2, while Kelerd was specified by F1. Corm TPC, leaf TFC, corm TFC, leaf area, and corm weight were described by F1, while leaf TPC, colchicine, plant length, and corm diameter were determined by F2. These results showed Kelerd has a significant role in distinguishing the clusters according to morphological and biochemical traits of *C. speciosum* populations (Figure 6).

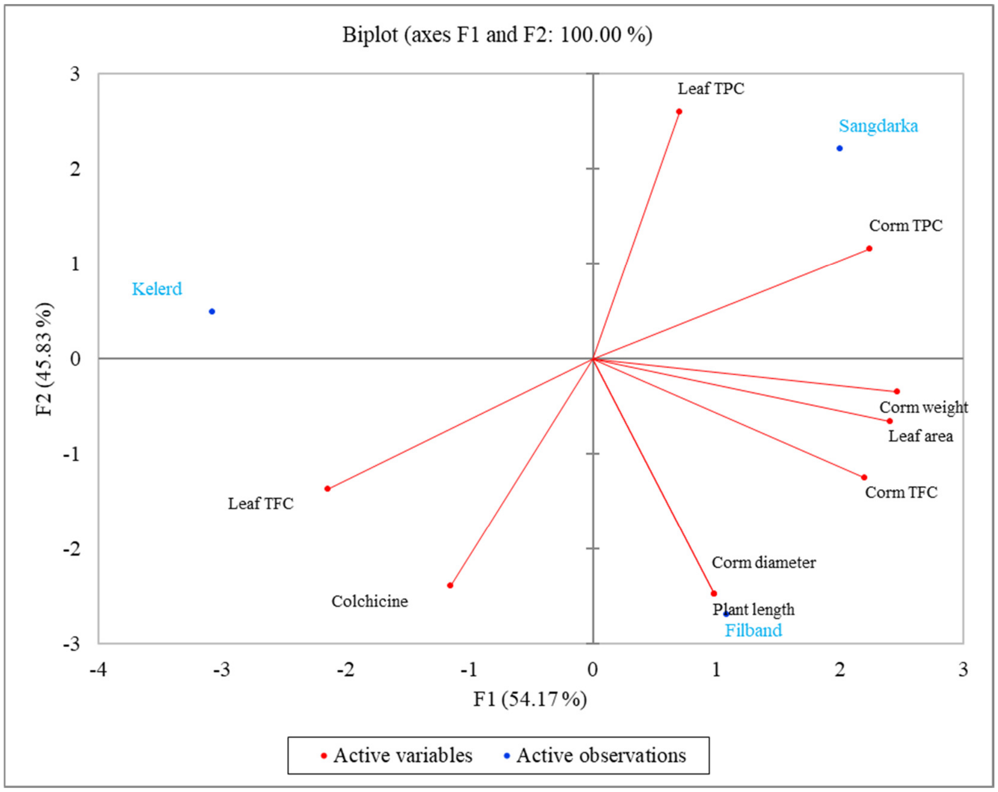

Figure 6. PCA of *Colchicum speciosum* L. populations under different concentration of nitrogen fertilizers

Discussion

The investigation of plant morphology is the most low-cost method on identification the differences between populations (Turner *et al.*, 2018). In this work, the noticeable changes of morphological traits among mint water populations. Heat map analysis showed the minimum variability of morphological traits between populations. The results of PCA showed corn diameter and plant length as remarkable traits in describing the conditions of Filaband. Therefore, these traits are selected as a useful tool on identification of *C. speciosum* populations under different geological conditions. Filband due to high altitude (2141 m asl.) represented the different responses of their morphological traits when compared with other populations with lower altitudes. Similar to our results, the diversity in morphological traits of different populations have been reported on *Melissa officinalis* L. (Pouyanfar *et al.*, 2018), *Geranium dissectum* (Bozchaloyi *et al.*, 2017), *Perilla frutescens* (Ghimire *et al.*, 2017), and *Salvia fruticosa* (Leontaritou *et al.*, 2020). AS previously reported by Pouyanfar *et al.* (2018), altitude is an important factor on creating the various populations of a plant species. Elevation gradient can change the morphological characteristic in plants to adapt the environmental variations like sunlight rate, wind speed, and soil properties (Tsozué *et al.*, 2019). Nitrogen is a critical element for the plant, because it is one of the main elements of chlorophylls and amino acids. It also plays a significant role in energy transfer compounds such as adenosine triphosphate (ATP). In line with the results of Filband, Fang *et al.* (2019) showed low levels of N fertilizer possess increases in leaf area index, plant height, and stem diameter of the increased of *Fagopyrum esculentum,* while high levels of N fertilizers decreased the value of the morphological properties.

The populations showed different responses to TPC and TFC accumulation. Previously, the high antioxidant capacity of *Colchicum triphyllum* was reported due to the noticeable phenolic compounds in coms and leaves (Senizza *et al.*, 2020). TFC of Filband was higher than others, which it can be due to the higher altitude of Filband natural site. The increased phenolic compounds by raising the altitude have been reported in *Thalictrum foliolosum* (Pandey *et al.*, 2018) and *Valeriana jatamansi* (Jugran *et al.*, 2016). Phytochemical characteristics are the main targets in plants, which can be altered under different geological locations (Hayat *et al.*, 2020). The variability in phenolic contents between populations have been reported by *Epilobium angustifolium* (Monschein *et al.,* 2015*) Thymus capitatus* (Jaouadi *et al.*, 2018), which can be due to site conditions such as soil, microclimate, and altitude. Phenolic compounds have different responses to conventional and nano-N fertilizers. The difference is related to the balance of carbon to nitrogen, which the plant needs this balance to produce phenols (Payyavula *et al.*, 2015). Regarding the negative effects of nitrogen fertilizer on the amount of phenols, it can be said that this negative effect is due to competition for phenylalanine, which can be used in the synthesis of phenols or used in protein synthesis (Barros and Dixon, 2020). It has been reported that there is competition between protein synthesis and phenylpropanoid for the use of phenylalanine precursors, which allows high ratio of protein synthesis that inhibits the access to phenylalanine or phenol biosynthesis (Payyavula *et al.*, 2015). Similarly, decreased phenolic content in *Cabernet Sauvignon* fruits have been reported after treating the plants with N fertilizer (Gutiérrez-Gamboa *et al.*, 2017). Nitrogen can modulate the biosynthesis of secondary metabolites like flavonoids and phenols. The negative correlation between phenolic content and photosynthesis (Zheng *et al.*, 2017) confirms the reduction of TPC and TFC upon N fertilizers. the decreased N fertilizer can be correlated to Therefore, based on site conditions (altitude, soil, etc.) and N fertilizers, the phenolic content could able to present different amounts.

All levels of conventional and nano-N led to decreased anthocyanin content in leave of all populations and corm of Filband; however, Sangdarka and kelerd showed no significant change of corm anthocyanin upon N fertilizers. Totally, anthocyanin in Filband was higher than other population, which can be due the difference in the sites, particularly the altitude. Ibrahim et al. demonstrated that enhanced N fertilization can reduce the anthocyanin content *L. pumila* leaves. Previous studies have shown that high nitrogen can reduce the production of secondary metabolites due to reduced phenyl alanine lyase (PAL) activity which was correlated with low C/N ratio, photosynthetic rates and total nonstructural carbohydrate (TNC) (Ibrahim *et al.*, 2011). Anthocyanins are natural bioactive phenolic compounds with major families of natural pigments A wide range of anthocyanins have been identified in nature, and they are produced by plants to attract insects to flowers for pollination and for the protection of plant cells against UV radiation damage (Salehi *et al.*, 2021). To confirm the results, Ferreyra *et al.* (2021) represented that plants at high latitudes can acclimate to increased anthocyanin against UV radiation damage. N fertilizers led to decreased anthocyanin content in leaves. Unlike our results, an increased anthocyanin content in *Cabernet Sauvignon* was reported by Cheng *et al.* (2022). Wu *et al.* (2013) found that organic N fertilizers had a greater effect on the accumulation of anthocyanins as compared with conventional N fertilizer. The difference on anthocyanin accumulation is related to the balance in C/N ratio. Based on the theory of interaction between plant growth and defense and competition for limited resources, which can affect the biosynthesis of secondary metabolites (Wu *et al.*, 2013). In addition, anthocyanins are considered as a component of antioxidant defense system for plants, and it suggests that N fertilizers can modulate the stressful conditions for plants and thereby results is deceased anthocyanin content. Hence the different amounts of anthocyanins can be obtained in different sites and also upon different N fertilizers.

Colchicine, the main alkaloid of *colchicum* species, possesses strong therapeutic properties on a wide range of pathogenic bacteria, fungi, etc. (Huczyński *et al.*, 2015). Although the populations represented no remarkable difference in control treatment, the levels of conventional and nano-N fertilizer exerted the changes of this alkaloid. The previous studies showed that high amounts of colchicine can be obtained by *colchicum* species (Bayrak *et al.*, 2019; Çankaya *et al.*, 2019). Sangdarka and Kelerd plants showed the higher colchicine in their corms when nourished by 1.5 mg L^{-1} nano-N. Nanomaterials due to their small size and high effective surface can easily platrate in plant cells and make changes in biochemical processes in plants (Afshari *et al*., 2021). Afshari *et al*. (2021) showed nano silicon increased essential oil compositions and phenolic compounds of *Coriandrum sativum* L.. Al-Fayyad *et al*. (2002) showed the increased colchicine in corms of *Colchicum tunicatum* plants under NPK fertilizers. The increased colchicine has been reported by slight amount of N fertilizer, while the high amounts led to decreased colchicine (Pandurangan and Philomina, 2010). Plant density and site features can affect colchicine amount, and if more than five plants are planted together, the plant becomes thinner to avoid competition and reduces its seed dry weight (Pandurangan and Philomina, 2010). In the present study, more than five plants were planted together, which it can be the reason to reduce the amount of colchicine in Filband population.

Conclusions

The results of the present study demonstrated the changes in morphological and biochemical properties of *C. speciosum* populations under N fertilizers. Totally, the populations due to different properties of their sites represented various reactions to N fertilizers. The population with higher altitude showed higher phenolic content and lower anthocyanin accumulation under conventional and nano-N fertilizes. In addition, the enhanced growth by determining leaf area and plant length was reported by N fertilizers. Although most traits showed no significant difference between connectional and nano-N fertilizers, leaf TPC and corm colchicine represented higher amount when plants treated with nanomaterials. Therefore, further investigations on medicinal plants in different sites are required to find the correlations between N fertilizers and geographical attributes.

Authors' Contributions (please add!)

MP and ZN conceived the experiment and designed the research. They also analysed and interpreted the data. MBN performed the experiments. MP and MBN wrote the manuscript. HA and SA contributed to preparing reagents, materials, analysis tools or data.

All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

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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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