

Determination of genetic diversity, sinigrin contents, and elicitors-induced enhancement of sinigrin in *Nasturtium officinale* L.

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Abstract: *Nasturtium officinale* L. is a plant of significant medicinal importance. The effect of climatic variations on its genetic makeup and biochemistry has not been studied in Pakistan. Present study was conducted to evaluate genetic diversity and variations in sinigrin contents, an important anticancer metabolite, in the leaves of *N. officinale* collected from different districts of Pakistan including Muzaffarabad, Chilas, Jaglot, Dir, Swat, Abbottabad, and Haripur. Furthermore, the effects of exogenous abscisic acid (ABA), salicylic acid (SA), and sodium nitroprusside (SNP) on the contents of sinigrin and expression of 2-oxoacid-dependent dioxygenase (GSL-OH) gene (TR14312) were also studied. Randomly amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) markers were used for fingerprinting and determination of genetic relationship. Plant from Dir showed dissimilarity and maximum genetic diversity with those collected from other districts, while plants from Abbottabad were grouped with Haripur, and the plants from Chilas were grouped with Jaglot. The polymorphism information content (PIC) which measures the quality of polymorphism ranged from 0.03 to 0.41. The resolving power (Rp) of markers ranged from 0 to 2.0. A strong and quite linear relationship was observed between the resolving power (Rp) of a primer and its ability to distinguish genotypes. High-performance liquid chromatography was carried out to quantify sinigrin contents. Plant from Dir depicted the highest levels of sinigrin (71.12 µM/g) followed by Muzaffarabad (64.86 µM/g), Abbottabad (56.83 µM/g), and Haripur (58.18 µM/g), while plants from Swat and Chilas showed lower values. The plants exogenously treated with elicitors (ABA, SA, and SNP) accumulated more sinigrin than the control plants, similarly exogenous applications especially ABA also upregulated the expression of a gene involved in glucosinolates synthesis. Our findings revealed that *N. officinale* growing in different localities exhibited genetic and biochemical diversity, and accumulation of phytochemical was positively affected by the application of exogenous elicitors.

Key words: Abscisic acid, glucosinolates, genetic diversity, *N. officinale*, salicylic acid

1. Introduction

Nasturtium officinale L. is a semi aquatic plant species with the common name watercress and is a member of family *Brassicaceae* (Googoollee et al., 2020). It is perennial plant mostly found in Asia and Europe, where people consume it as vegetable (Lakoba et al., 2020). *N. officinale* is a good source of vitamin A, B₁, B₂, C, iodine, iron, folic acid, protein, calcium, and sulphur-containing compounds. It has been reported as herbal medicine for diabetes, asthma, inflammation, and to purify blood (Pandey et al., 2018). It also acts as a potent inhibitor of cancer mainly due to the presence of glucosinolates (Munday and Munday, 2004). Glucosinolates are water-soluble, heat-stable

compounds and consist of a side chain derived from amino acids and glucose (Agerbirk and Olsen, 2012). Based on the side chain, glucosinolates can be classified into three main groups: 1) aliphatic, 2) aromatic aryl, and 3) aromatic indole glucosinolates (Wittstock and Halkier, 2002). Sinigrin (2-propenyl glucosinolate) is present as a major glucosinolate in cruciferous vegetables and is also a precursor of the anticancer compound allyl isothiocyanate (Cartea and Velasco, 2008). As constituents of food and feed, glucosinolates are the precursor for a range of bioactive compounds (thiocyanates, isothiocyanates, epithionitriles, and nitriles) that have been acknowledged for their characteristic benefits to plant defense and nutraceuticals

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for consumers. Glucosinolates are converted to phenethyl isothiocyanates after sudden exposure to stress conditions by an enzyme myrosinase (Van et al., 2009).

For therapeutic activity and phytochemical composition of medicinal plants, stress, by means of various elicitors, is an important factor. "Elicitor is defined as a substance when applied in small quantity to a living system induces or improves the biosynthesis of specific metabolites that play an important role in the adaptation of plants to a stressful condition" (Radman et al., 2003). Elicitors are divided into two types abiotic and biotic based on their nature. Osmotic stress is an abiotic physical elicitor (Vasconsuelo and Boland, 2007) and is one of the important environmental stresses that can change the biochemical and physiological properties of plants and enhance the production of secondary metabolites in plant tissues (Zobayed et al., 2007). Salicylic acid (SA) and Jasmonic acid (JA) are the most studied plant hormonal abiotic elicitors (Sembdner et al., 1993). Thiruvengadam et al. (2016) reported that SA, JA and abscisic acid (ABA) have increased the glucosinolates contents and biological activities in turnip plants. Another abiotic elicitor nitric oxide (NO) is a diffusible gaseous free radical (Laspina et al., 2005) and is involved in the signaling process within plants (Wendehenne et al., 2001) and plays a central role in adaptive responses to environmental stresses (Kazemi et al., 2010). Treatment with 100 μ M NO in the form of sodium nitroprusside (SNP) increases the proline and protein content in watercress tissues to some extent (Namdjoyan and Kermanian, 2013).

In the Brassicaceae family, glucosinolates biosynthesis involves three steps namely, elongation of the amino acid side chain, core structure formation, and secondary modifications of side chains that involve several genes (Zang et al., 2009). These glucosinolates biosynthetic genes are induced by the exogenous elicitors in plants (Yi et al., 2016). 2-oxoacid-dependent dioxygenase (GSL-OH) gene (TR14312) is involved in aliphatic glucosinolates side-chain modification, which is an essential step in glucosinolates biosynthesis (Harun et al., 2020). In Brassica crops, exogenous SA treatment has been reported to stimulate the biosynthesis and accumulation of all three types of glucosinolates: aromatic, indole, and aliphatic glucosinolates (Mikkelsen et al., 2003). GSL-OH (Bol033373) gene expression is related to accumulation of aliphatic glucosinolates by exogenous SA treatments in kale leaves (Yi et al., 2016).

In the evolutionary process, environmental changes that bring plants under stress conditions play a key role in bringing genetic diversity within populations (Huang et al., 2016). The populations adjust to varying environments by means of genetic changes. It might be possible that with more variations, some individuals in a population will acquire changes of alleles that are apt for the environment,

and, with those alleles, individuals are more likely to live on to produce offspring (Bhandari et al., 2017). Various genetic markers are used to find out the genetic diversity among different plant species, these include inter simple sequence repeats (ISSR), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), and microsatellite markers (Fernandez et al., 2002). Polymorphic information content (PIC) is the popular scientific method for the calculation of genetic diversity through a widely-acceptable formula to measure the information content of molecular markers (Yaldiz et al., 2018). Depending on the number of detectable bands and the distribution of their frequency, PIC determines the value of a marker for detecting polymorphism within a population (Wei et al., 2014).

There exists a huge genetic diversity in different species of Brassicaceae family. Singh et al. (2018) analyzed genetic diversity in nineteen species of Brassicaceae family belonging to eight different genera by using genic-SSR, which showed 80.67% polymorphism. Another study conducted by Sheridan et al. (2001) on commercial populations of allied Brassicaceae inferred and *N. officinale* revealed 6%–35% polymorphism in different populations within southern England; however, they found 40% polymorphism between watercress of southern England with Japanese watercress. The genetic diversity and biochemical analysis of watercress has not yet been studied in Pakistan.

Watercress is a naturally growing plant in fresh-water bodies such as springs and streams at diverse localities (Vinten and Bowden-Smith, 2020). Due to occurrence at different altitudes with varied climatic and edaphic conditions, there might be great variations in the genetic properties and nutritional composition of this plant that have rarely been explored yet. Therefore, the current study was focused on the genetic diversity evaluation, sinigrin concentration, and expression of GSL-OH gene in watercress collected from different climatic zones of Pakistan. In addition, watercress was further treated with various exogenous elicitors to assess their impact on the expression of the GSL-OH gene and the concentration of sinigrin.

2. Materials and methods

2.1 .Plant collection

Leaves of *N. officinale* were collected in spring 2018, from seven different districts of Khyber Pakhtunkhwa (KPK), Gilgit Baltistan (GB), and Azad Jammu & Kashmir (AJK), Pakistan (Figure 1). The climatic conditions, altitude, soil texture, and anthropogenic activities are variable albeit at different levels (Table 1). Plant specimens were identified by an expert taxonomist and subsequently confirmed by Flora of Pakistan (Ali and Qaiser, 1995–2008).

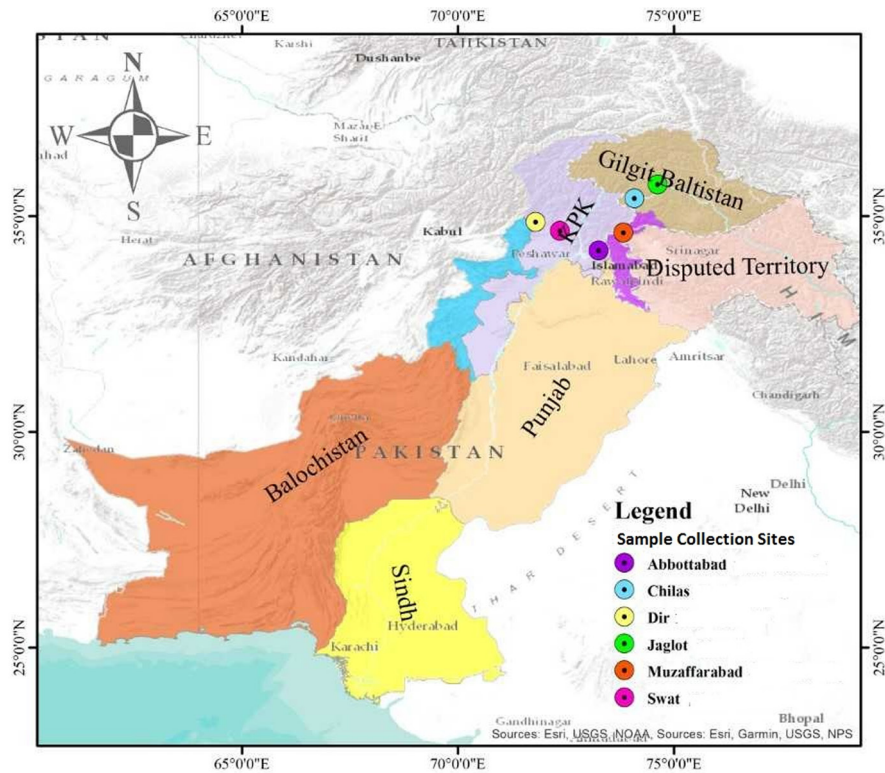


Figure 1. Geographical map shows plant collection sites, i.e., KPK, GB, AJK.

Table 1. Altitude of selected districts, climate pattern (2018) and physical properties of soil of collected *N. officinale* for genetic and phytochemical analysis.

Districts	Altitude	AT (°C)		RH (%)		ARF (mm)		PPS
		Min	Max	Min	Max	Min	Max	
Muzaffarabad	1200	4	32	20	74	16	501	Sandy loam
Swat	970	0	29	30	62	29	399	Sandy
Jaglot	1988	-9	20	28	70	20	88	Sandy clay loam
Diamer	1265	-25	16	38	67	3	71	Silt loam
Dir	1420	-4	33	15	52	12	60	Loam
Abbottabad	1256	2	34	24	65	16	501	Silt clay loam
Haripur	520	9	39	22	54	3	225	Silt clay

AT: average temperature, RH: relative humidity, ARF: average rainfall, PPS: physical properties of soil.

2.2. Genomic DNA extraction

Collected plants were grown in pots containing natural soil under open environment in nursery field at COMSATS-Abbottabad (34° 11.9631' N, 73° 14.8483' E). Fresh plant samples were used for DNA extraction, following the method as explained earlier (Kim and Hamada, 2005). In short, *N. officinale* leaves (1 g) were ground by using

mortar and pistol, and prewarmed (65 °C) extraction buffer (2% CTAB, 100 mM Tris (pH 8.0), 20 mM EDTA, 1.4 M NaCl, 0.2% Beta mercaptoethanol) was added. The sample was mixed with chloroform: isoamyl alcohol (24:1, v/v) in equal amount in 15 mL falcon tube and centrifuged at 4 °C for 15 min at 11,000 rpm. The supernatant was transferred to a new falcon tube. An equal volume of

isopropanol was mixed with the collected supernatant and was incubated for 30 min at -20°C . After that, the centrifugation was carried out at 4°C for 15 min at 11000 rpm. The pellet obtained was washed with ethanol (70%), air-dried, and resuspended in 75 μL autoclaved deionized water. The DNA was visualized by UV light after running it on agarose gel (1%). The purity and the concentration of the DNA were determined by Irmeco spectrophotometer (Germany).

2.3. Polymerase chain reaction (PCR)

The PCR amplification of RAPD and ISSR markers was carried out by following the previously described protocol (Williams et al., 1990). For ISSR markers, the primers were adopted from literature (Garg and Sharma, 2015) and obtained from Macrogen (South Korea). The primers for RAPD markers were used from Eurofins 10mer kit (USA) (Table 2). Reaction mixture 10 μL containing

5 μL of Thermo Fisher Scientific PCR 2x master mix (USA), 2 μL DNA, 2 μL primer and 1 μL HPLC grade water (Sigma–Aldrich), was used. An Applied Biosystems (USA) thermocycler was used for PCR with the following conditions: a preliminary denaturation of 5 min at 94°C , then 40 cycles of 1 min denaturation at 94°C , 1 min annealing at 38°C , 2 min extension at 72°C and a final extension of 10 min at 72°C ; for RAPDs and for ISSR primers, annealing temperature was 34°C . PCR product was analyzed on 8% polyacrylamide gel in tris-borate EDTA (TBE) buffer by running at 120 V for 120 min. The gel was stained with ethidium bromide and Cleaver Scientific UV gel documentation system (UK) was used for visualization of DNA bands on the gel.

2.4. Data analysis and construction of phylogenetic tree

Data were analyzed by the previously described protocol (Liu et al., 2020). Amplification profiles generated by ISSR

Table 2. Profile of eighteen ISSR and RAPD primers used for molecular characterization and polymorphism detection in samples of seven different districts.

Marker	Sequence 5'-3'	TNB	NPB	P%	PIC	Rp
RAPDs						
OPH-05	CAAGGGCAGA	10	3	30	0.106	1.685
OPB-20	GGACCCCTTAC	6	2	33	0.109	1.476
B-04	TGCGCCCTTC	13	9	69	0.226	1.494
B-05	GGTGACGCAG	5	1	20	0.082	1.714
B-07	TGGGGGACTC	7	2	28	0.069	1.51
B-08	GACCAATGCG	7	4	57	0.128	1.306
B-09	GGACTGGAGT	8	1	12	0.031	1.964
Average Values		8	3.14	35.57	0.107	1.59
ISSRs						
ISSR-01	AGAGAGAGAGAGAGAGT	11	6	54	0.230	1.48
ISSR-02	GAGAGAGAGAGAGAGAT	12	2	16	0.408	1.714
ISSR-03	CTCTCTCTCTCTCTCTT	17	4	23	0.067	1.831
ISSR-04	TCTCTCTCTCTCTCTCC	7	7	0	0	2
ISSR-05	ACACACACACACACACT	6	3	50	0.123	1.619
ISSR-06	ACACACACACACACACG	5	3	60	0.163	1.028
ISSR-07	ACACACACACACACACC	16	6	37.5	0.108	1.571
ISSR-08	ACCACCACCACCACCACC	13	7	53	0.184	1.428
ISSR-09	GACGACGACGC	7	3	42	0.209	1.795
ISSR-10	ACACACACACACACA-CACACACACACACACTG	6	4	66	0.122	1.571
ISSR-11	CTCTCTCTCTCTCTCTGC	11	1	9	0.022	1.974
Average Values		10.09	4.18	37.32	0.116	1.64

TNB: total number of bands, NPB: number of polymorphic bands, P: percentage of polymorphic bands, PIC: polymorphic information content, Rp: resolving power.

and RAPD primers were utilized for genetic diversity analysis based upon the presence (1) or absence (0) of all bands of plants. The band, which was present in some samples and absent from the others was counted as polymorphic, while the band present in all samples was counted as monomorphic. For phylogenetic tree construction, binary matrix was generated for both the markers by using the data. Jaccard's similarity coefficient was used to compute pair-wise genetic similarity. An independent similarity matrix for RAPD and ISSR markers was generated. The dendrograms were made by using the online UPGMA tool (<http://genomes.urv.cat/UPGMA/>) and analyzed by iTOL (Letunic and Bork, 2021).

2.5. Polymorphism information content (PIC) and resolving power (Rp)

The PIC value of ISSR and RAPD primers was evaluated by formula (Roldan-Ruiz et al. 2000)

$$PIC = 2f(1 - f)$$

Where, F represents the frequency of the bands present in the studied plants and (1 - F) represents the frequency of the absent bands. Resolving Power (Rp) of a primer is,

$$Rp = \sum Ib$$

where Ib (band informativeness) takes the value of $1 - [2(0.5 - p)]$, p is the proportion of the seven plants analyzed containing bands (Prevost and Wilkinson, 1999).

2.6. Extraction of sinigrin

Sinigrin extraction from plant samples was carried out using the procedure as explained earlier (Girgin and El, 2015). Briefly, leaves of the plant were washed with distilled water, covered with a paper bag, shade dried, and ground to a fine powder by using mortar and pistol. Plant material (25 mg) was taken in Eppendorf tube and extracted three times with 800 µL 70% methanol. Eppendorf tube was placed in water bath with preheated temperature (80 °C) for 10 min and centrifuged at 12000 rpm (Germany). Supernatant was collected and placed in incubator at 37 °C for 48 hours to reduce the volume to 150 µL, then 600 µL Barium acetate (Sigma Aldrich) (0.4 M) was added and increased the volume to 1 mL with high performance liquid chromatography (HPLC) grade water (Sigma Aldrich). The sample was incubated for 30 min at room temperature and centrifuged for 10 min at 16000 rpm. The supernatant was collected and mixed with HPLC grade water until the mixture became 1 mL. The sample was filtered through Thermo Fisher Scientific syringe filters having pore size 0.45 µm (USA) and utilized for HPLC analysis.

2.7. Quantification of sinigrin

Extracted sinigrin was analyzed quantitatively through HPLC by using previously described protocol with modifications (Jia et al., 2009). The HPLC system comprised Cecil low pressure quaternary gradient pump Adept CE 4104, UV detector Adept CE 4200

(Cecil, UK). The system was controlled by power stream chromatography manager version 4.2. Separation was achieved using gradient elution : 0–2 min: 0–1% B, 2–20 min: 1–50% B, 20–24 min: 50–100% B, 24–26 min: 100% B, 26–27 min: 100–1% B, and 27–35 min: 1–0% B at a flow rate of 1 mL/min with mobile phase A (0.1 M ammonium acetate buffer pH = 5.5) and B (Acetonitrile and Purified water having ratio 40: 60 v/v) over Agela analytical column 250 mm × 4.6 mm, 5 µm, packing C18 (Waters, USA). The column was equilibrated at ambient condition for 35 min and the samples and standard (n = 3) were injected using Adept Cecil CE 4800-100 auto sampler. Detection was performed at 229 nm and the quantity of sinigrin was calculated in each sample from the peak area of the sample compared with the calibration graph of standard solution ranging from 10 µg/ml to 100 µg/ml. Identification of the components was also carried out from retention time against the standard used. The retention time of sinigrin was about 9.5 min and the total run time was 35 min.

2.8. Exogenous application of ABA, SA, and SNP on plants

Exogenous elicitors were applied by following the previously described protocol by Thiruvengadam et al. (2016) with modifications. Plants of *N. officinale* from Abbottabad district were grown in pots containing natural soil under an open environment in nursery field of COMSATS- Abbottabad (34° 11.9631' N, 73° 14.8483' E). The plants of equal height and visibly same health were utilized for exogenous application after 4 weeks of growth. ABA, SA and SNP were applied in a concentration of 50 µM and 100 µM through a foliar spray. The foliar spray was repeated after 24 h, and control plants were sprayed with distilled water only. Two days post foliar application sampling of leaves was performed for real time PCR analysis. The experiment was replicated for three times.

2.9. Quantitative realtime PCR analysis

Real time PCR was performed by the protocol of Yi et al. (2016). The leave from control and elicitors treated plants were collected and flash frozen in liquid nitrogen. Leaf material was washed with distilled water, finely ground, and homogenized. Total RNA was extracted from fresh leaf material by using Gene JET plant RNA purification kit (Thermo Scientific, USA). After checking the quality of total RNA by Irmeco UV-Vis spectrophotometer (Germany), cDNA was synthesized by using cDNA synthesis kit (Thermo Scientific, USA) by following the manufacturer provided protocol. To investigate the expression of 2-oxoacid-dependent dioxygenase (GSL-OH) (TR14312) gene (Zang et al., 2009) in control and elicitors treated plants, real-time PCR was performed. The primers (F: 5' CGTGGAGCATAGAGTTTGTG 3' R: 5' CACAGAGTTTCCATCGAG 3') were obtained from

Macrogen (Korea). The quantitative PCR was carried out by Bio-Rad MiniOpticon™ real time PCR detection system (USA). Reaction was performed in a total volume of 50 µL containing 5 µL cDNA, 10 µL Bioline cyber green (Canada) and 10 µL gene specific forward and reverse primer pair in triplicates. The following PCR program was used for all PCR reactions: 95 °C for 12 min, followed by 39 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 40 s. The gene expression level was calculated by 2⁻(ΔCt) method by using the housekeeping gene *Actin* as a reference.

2.10. Statistical analysis

For quantification of glucosinolates contents, extraction was carried out in triplicate from the plants of seven different districts of Pakistan and from each treatment of ABA, SA, and SNP and the control plants. Values of samples from selected districts and each of three treatments in triplicates were averaged and represented as means with standard deviations (SD) by using Microsoft 365°. Tukey HSD test in combination with one-way ANOVA analysis was used to evaluate significant differences at a significance level <0.05 (Bhatt and Debnath, 2021).

3. Results

3.1. ISSR and RAPD amplification

Out of 14 ISSR primers, 11 primers generated 111 scorable bands, of those, 46 were polymorphic, while 3 primers gave no readable bands. ISSR-03 showed maximum number of bands (17), while ISSR-06 showed the minimum number of bands (05). The number of polymorphic bands for ISSR primers ranged from 1 (ISSR-11) to 7 (ISSR-04, ISSR-08) with an average of 4.18. The percentage of polymorphism ranged from 0 % (ISSR-04) to 66% (ISSR-10) with an average polymorphism of 37.32% (Table 2).

A total of 37 RAPD primers was utilized to evaluate the polymorphism through PCR. In the PCR analysis, 24 RAPD primers showed no amplification; whereas, 7 primers generated 22 polymorphic bands, and 6 primers generated unreadable band patterns (data not included). Amplification of the 7 RAPD primers gave a total of 56 scorable bands, B-05 showed maximum number of bands (13) while B-07 showed minimum bands (5). The number of polymorphic bands for RAPD primers ranged from 1 (B-04, B-07) to 9 (B-05) with an average of 3.14. Percentage of polymorphism ranged from 12% (B-09) to 69% (B-04) with an average polymorphism of 35.57% (Table 2).

3.2. Polymorphism information content (PIC) and resolving power (Rp) for ISSRs and RAPDs

The range of PIC value was calculated for ISSR and RAPD primers to measure bands diversity. PIC values ranged from 0.00 for ISSR-04 to 0.23 for ISSR-01. The Rp for eleven ISSR primers ranged from 1.028 for ISSR-06 to 2.0 for ISSR-04 (Table 2). Band's size ranged from 100 to

1000+ bps, and numbers of average bands per primer were 10.1 (Figure 2a-d).

Values of PIC for RAPD primers vary from 0.031 to 0.226 for primers B-09 and B-04, respectively. The Rp values obtained with RAPDs were 1.30 and 1.96 for B-08 and B-09 primers, respectively (Table 2). Band's size ranged from 100 to 1000+ base pairs (Figure 2e-h). The frequencies of alleles amplified with eleven ISSR primers are elaborated in Figure 3a. The frequencies of bands obtained from plants of seven diverse habitats with seven RAPD primers are elaborated in Figure 3b. PIC values obtained for RAPD and ISSR markers indicate the variability among the studied plants, while Rp shows the discriminating power of the primers.

3.3. Dendrogram obtained with ISSRs and RAPDs

Dendrogram obtained with ISSR primers produced two branches: branch-I constituted of plants from district Dir, which serves as out group, while branch-II further divided into two subbranches; sub branch-I constituted of *N. officinale* plants from district Muzaffarabad and sub branch-II further divided into 2 rows. Row-1 represents *N. officinale* plants from Swat district, which is placed closer to Muzaffarabad and showed similarity with each other. Row-II constituted two subgroups, subgroup-1 includes closely related samples with maximum similarity from Jaglot and Chilas, and subgroup-II includes samples from Abbottabad and Haripur (Figure 4a). In the case of ISSR primers, Jaccard's similarity index revealed 95% similarity between plants from Chilas and Jaglot, while plants from Abbottabad showed 93% similarity with plants from Haripur. Plants from Muzaffarabad showed 85% similarity with the plants from Swat, while the plants from Dir were dissimilar with all other plants (Table S1), while the Jaccard's distance coefficient showed 0%–23% variation among different samples (Table S2).

Dendrogram obtained with RAPD primers produced two branches: branch-I constituted *N. officinale* plants from district Dir, which is dissimilar with all other plants, while branch-II further divided into two subbranches; subbranch-I constituted of *N. officinale* plants from district Muzaffarabad and subbranch-II further divided into 2 rows. Row-1 represented *N. officinale* plants from Abbottabad and row-II represents plants from Haripur. Abbottabad and Haripur samples showed similarity to each other. Row-II further constituted of 2 subgroups. Subgroup-1 constituted of samples from district Jaglot and subgroup-II constituted of closely related samples from district Chilas and Swat, which showed resemblance with each other (Figure 4b). In case of RAPD primers, plants from Chilas showed 95% similarity with plants from Swat and plants from Abbottabad district showed 86% similarity with plants from Haripur. Samples from Muzaffarabad and Abbottabad showed 80% similarity with each other. Plants

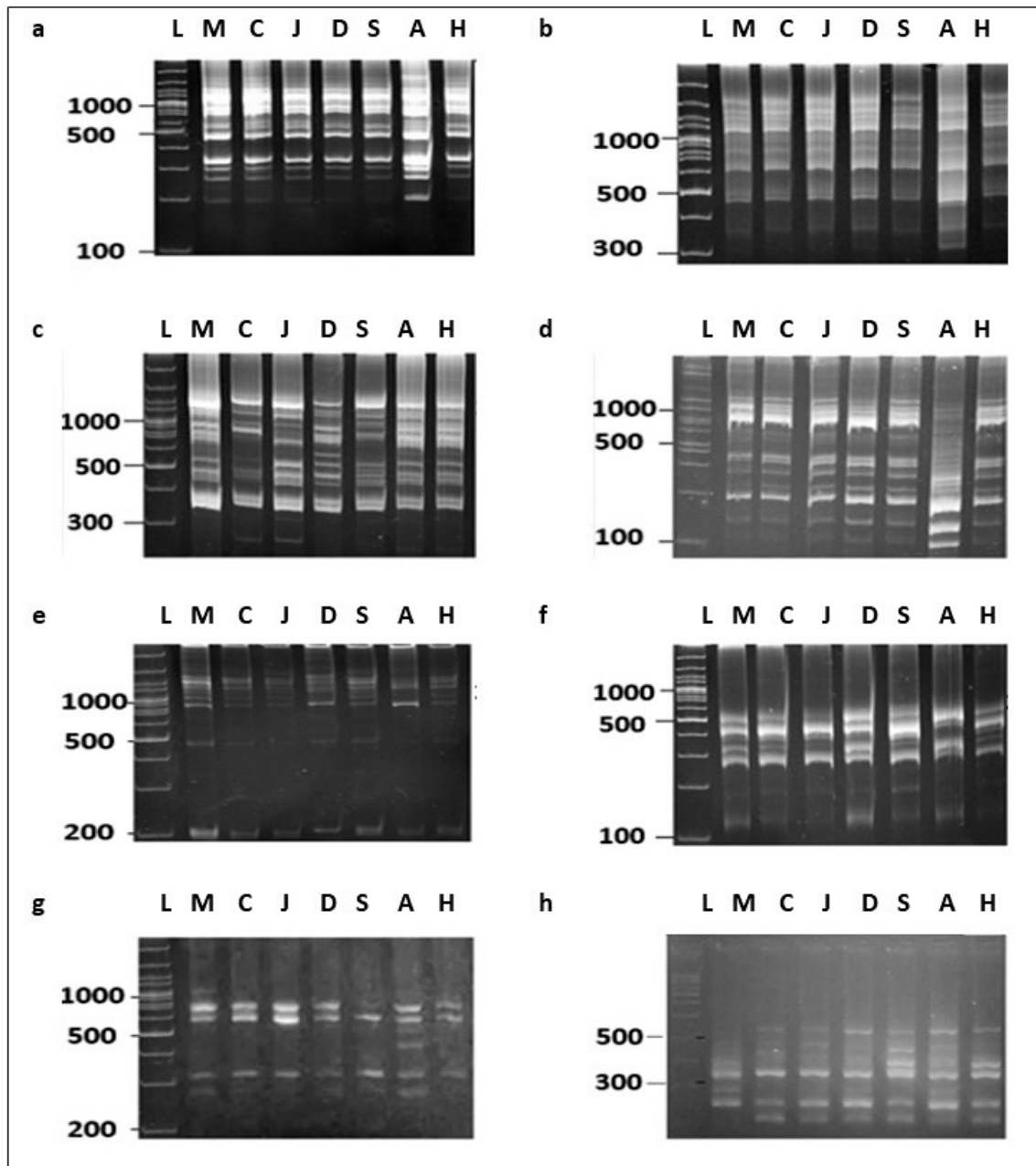


Figure 2. ISSR and RAPD profile showing the genetic polymorphism in *N. officinale*. (a, b, c, d), detected with ISSR primer number 03, 04, 07, 11, respectively. (e, f, g, h) detected with RAPD primer number 07, 08, 05, 20, respectively. Lane 1- 8, sample profile of *N. officinale* collected from seven districts, i.e., 1 Muzaffarabad, 2 Chilas, 3 Jaglot, 4 Dir, 5 Swat, 6 Abbottabad, 7 Haripur, 8 L = 1000bp+ ladder.

from Dir were placed out to the clad (Table S3), while the Jaccard's distance coefficient for the samples showed 0%–30% variations among different samples (Table S4).

3.4. Quantification of sinigrin in *N. officinale* collected from different localities

The sinigrin was quantified in the *N. officinale* leaves collected from various localities of Pakistan through

HPLC. The plants collected from Dir showed highest level of sinigrin (71.12 $\mu\text{M/g}$ of DW) followed by plants collected from Muzaffarabad (64.86 $\mu\text{M/g}$ of DW). *N. officinale* collected from Abbottabad, Haripur, Chilas, and Swat accumulated 56.83 $\mu\text{M/g}$, 58.18 $\mu\text{M/g}$, 47.39 $\mu\text{M/g}$ and 41.08 $\mu\text{M/g}$ of sinigrin, respectively. The statistical analysis demonstrated that there was a significant

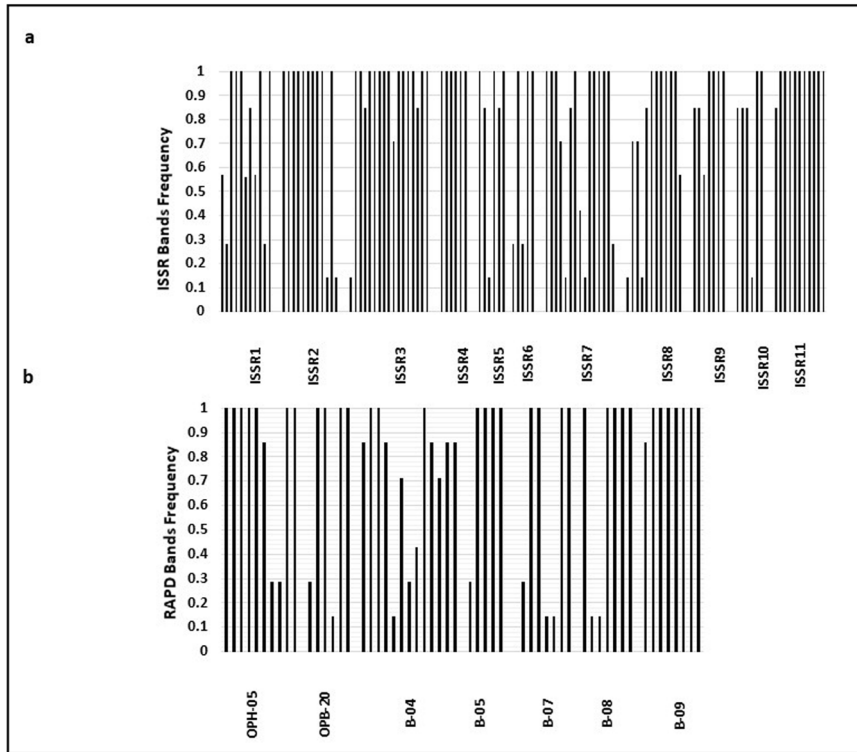


Figure 3. Allele's frequency spectra of a) ISSR, b) RAPD markers in seven *N. officinale* accessions.

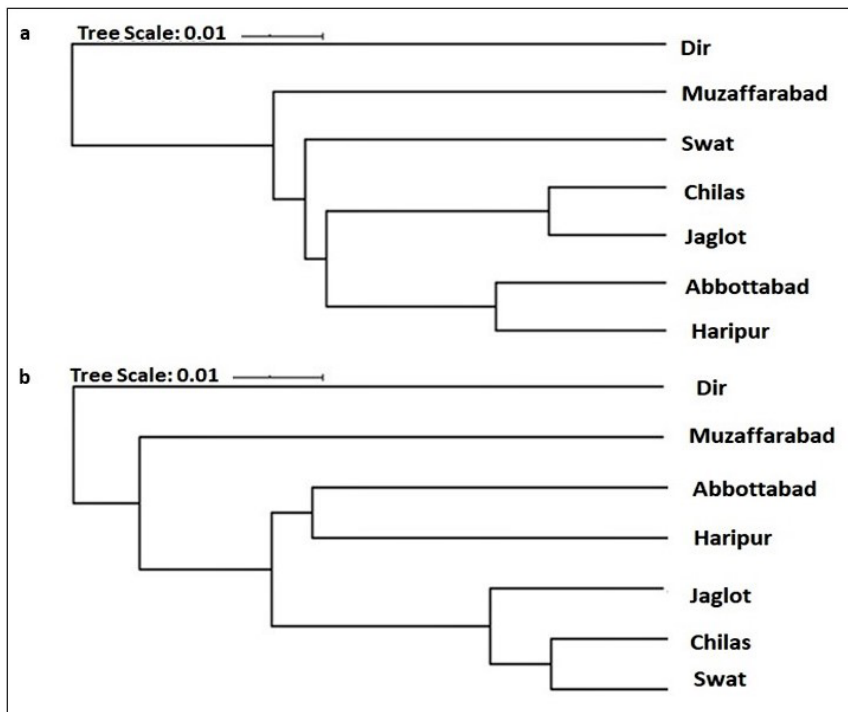


Figure 4. Dendrograms (UPGMA) showing clustering of *N. officinale* plants from Dir, Muzaffarabad, Swat, Chilas, Jaglot, Abbottabad, Haripur districts based on the PCR markers: a) ISSR, b) RAPD.

difference among the sinigrin contents of plants collected from different districts (Figure 5).

3.5. Exogenous application of elicitors affected accumulation of sinigrin in *N. officinale*

To evaluate the effect of different exogenously applied elicitors on sinigrin contents, plants from Abbottabad district were treated with ABA, SA, and SNP. The plants treated with ABA 50 μM accumulated significantly higher sinigrin contents (80.25 $\mu\text{M/g}$ of DW) than all other treated plants, followed by ABA 100 μM treated plants (77.25 $\mu\text{M/g}$ of DW). The SA 50 μM treated plants depicted 71.32 $\mu\text{M/g}$ of DW sinigrin, while the level of sinigrin was slightly attenuated in plants treated with SA100 μM as compared to control. The plants treated with SNP 50 μM and SNP 100 μM , depicted 66.5 $\mu\text{M/g}$ of DW and 66.37 $\mu\text{M/g}$ of DW sinigrin contents, respectively, which were significantly higher than nontreated control plants (Figure 6).

3.6. Effect of application of ABA, SA and SNP on expression of GSL-OH gene involved in glucosinolates synthesis

The effect of ABA, SA, and SNP treatments on the expression of GSL-OH gene involved in glucosinolates biosynthesis in *N. officinale* plants was studied. All three treatments altered expression of the gene. Expression of the gene was more upregulated by 50 μM ABA, SNP, and SA treatments as compared to 100 μM treatments. Particularly, ABA 50

μM , showed the strong induction of GSL-OH gene, i.e., 12.9-folds. Plants treated with SNP and SA 50 μM showed 1.4- and 1.2-fold changes in expression of GSL-OH gene, respectively as compared to control plants. Contrary to 50 μM treatment of elicitors, application of 100 μM ABA and SNP could change the expression up to 1.2 and 1.1-fold, respectively. Interestingly the expression of the gene was dropped to 0.61-fold upon treatment with SA 100 μM (Figure 7).

4. Discussion

In present study, we reported the genetic diversity, quantification of sinigrin contents, effect of exogenous application of ABA, SA, and SNP on sinigrin and GSL-OH gene expression in *N. officinale* plants collected from Khyber Pakhtunkhwa (Haripur, Abbottabad, Dir & Swat), Gilgit Baltistan (Chilas & Jaglot) and Kashmir (Muzaffarabad) regions of Pakistan.

In this study, the average polymorphism is 37.32% and 35.57% for ISSR and RAPD, respectively. However, these values were found lower than those reported by Ahmed, (2012) in canola (ISSR: 85%, RAPD: 82.6%), Havlíčková et al. (2014) in *Brassica napus* L. (ISSR: 90.6%) and Maraş-Vanlıoğlu et al. (2020) in some species of *Brassicaceae* family (ISSR: 88.75%). The average PIC value of the RAPD markers in this study was 0.11 and that of ISSR is 0.12. The PIC value of these markers is lower than those reported by Maraş-Vanlıoğlu (2020) in brassica species (ISSR: 0.33),

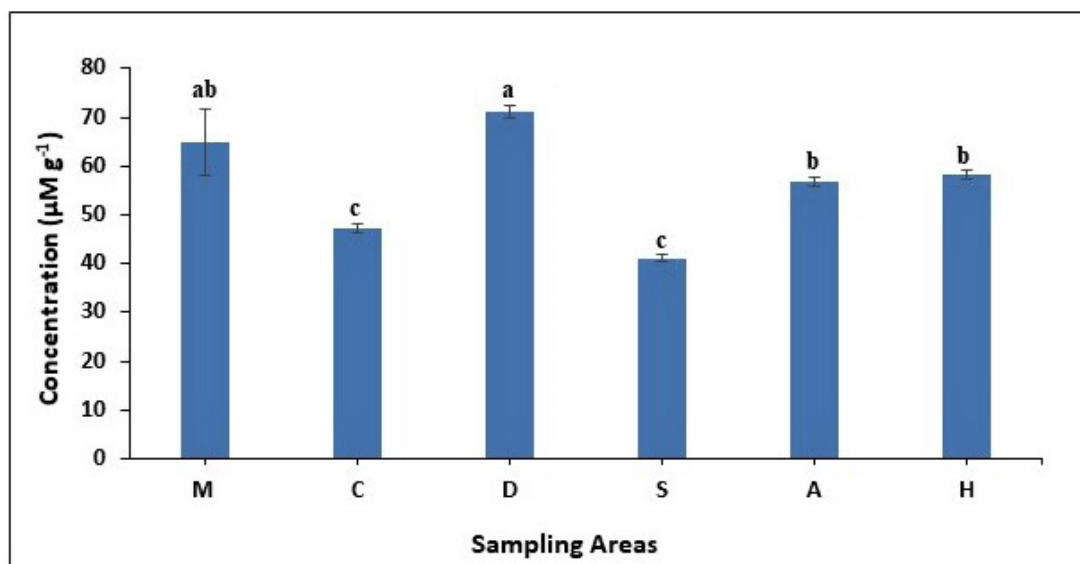


Figure 5. Concentration of sinigrin in *N. officinale* leaves from different geographical areas with abbreviations: Muzaffarabad (M), Chilas (C), Dir (D), Swat (S), Abbottabad (A) Haripur (H) determined by HPLC. Data are represented as an average of triplicates. Mean ($\pm\text{SE}$) was calculated from three replicates for each treatment. Bars with distinct letters are significantly different at $p < 0.05$.

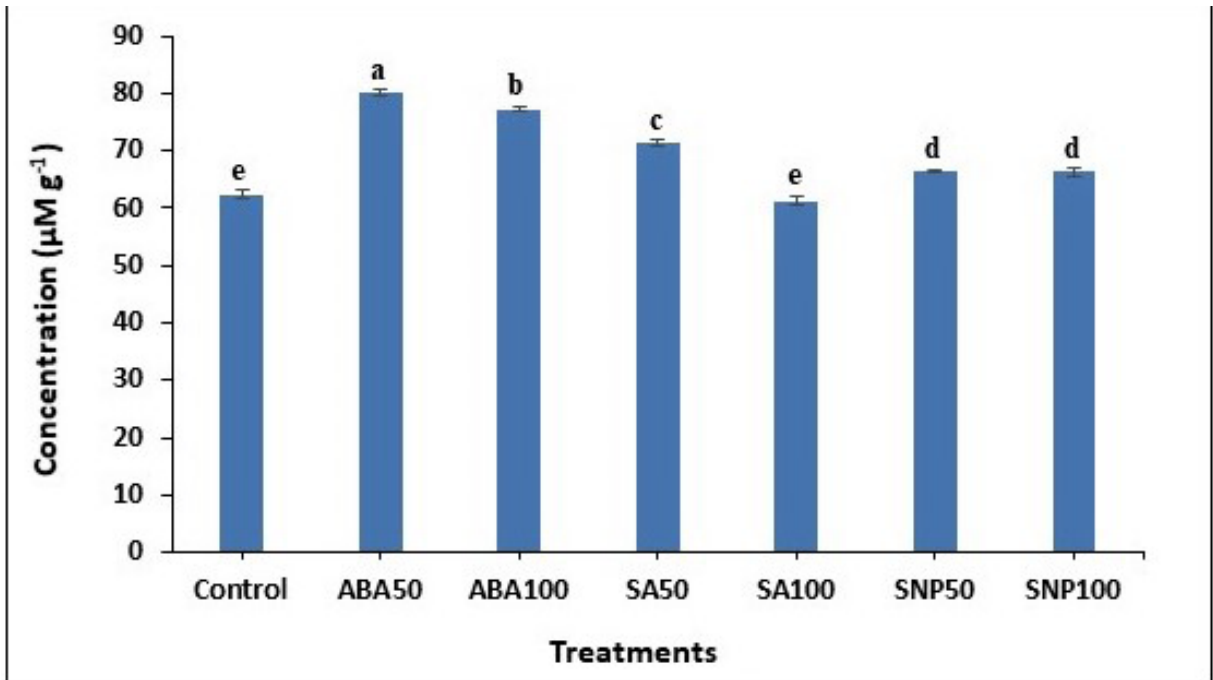


Figure 6. Effect of different levels of exogenous abscisic acid (ABA), salicylic acid (SA) and sodium nitroprusside (SNP) on sinigrin levels in *N. officinale*. (1) Control without ABA SA and SNP (2) ABA 50 μM, (3) ABA 100 μM, (4) SA 50 μM, (5) SA 100 μM, (6) SNP 50 μM, and (7) SNP 100 μM. Mean (±SE) was calculated from three replicates for each treatment. Bars with distinct letters are significantly different at $p \leq 0.05$ applying the T test.

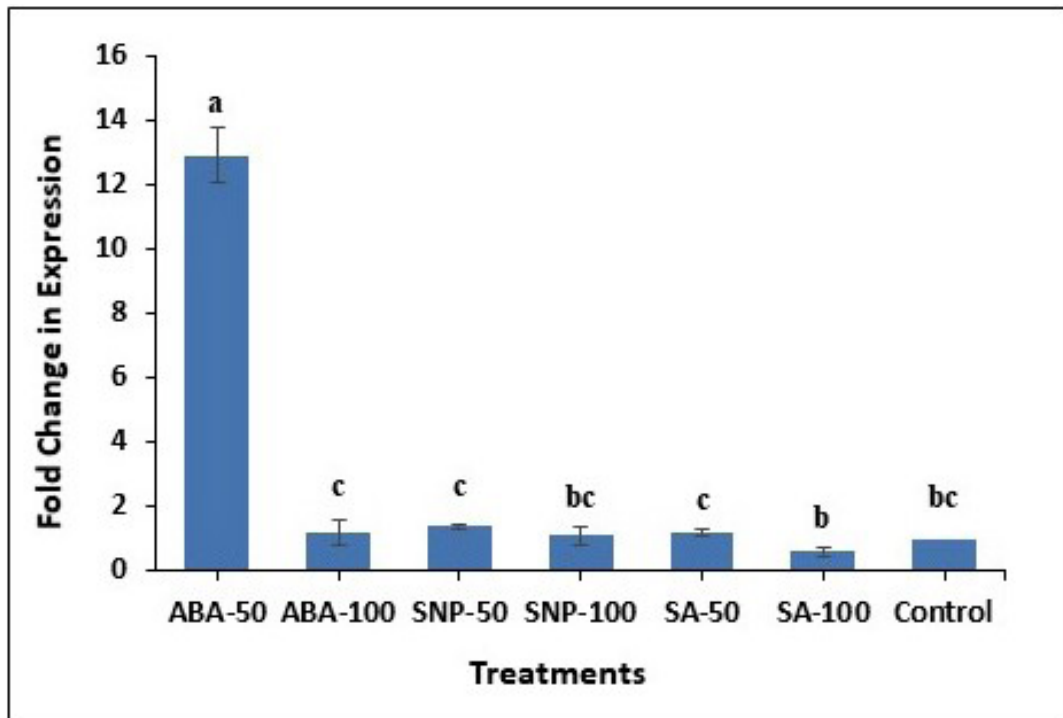


Figure 7. Effect of different levels of exogenous abscisic acid (ABA), salicylic acid (SA) and sodium nitroprusside (SNP) on expression of glucosinolates biosynthesis gene GSL-OH in *N. officinale* 1) Control without ABA, SA, and SNP (2) ABA 50 μM, (3) ABA 100 μM, (4) SA 50 μM, (5) SA 100 μM, (6) SNP 50 μM and (7) SNP 100 μM. Mean (±SE) was calculated from three replicates for each treatment. Bars with distinct letters are significantly different at $p < 0.05$.

Zhu et al. (2018) in *Brassica oleracea* var. *botrytis* (ISSR: 0.316), and Singh et al. (2013) in Indian mustard (RAPD: 0.8169), while, higher PIC values than those were reported by Raza et al. (2020) in different brassica species (RAPD: 0.088). Furthermore, the average Rp value was 1.59 and 1.63 for RAPD and ISSR, respectively. The Rp value obtained in this study is lower than the one previously reported by Maraş-Vanlıoğlu, (2020) in brassica species (ISSR: 8.29) and Gupta et al. (2014) in Indian mustard (RAPD: 6.89). These differences between the various studies may be due to the populations used and the nature and number of molecular markers (El Harfi et al., 2021). *N. officinale* produces many seeds that could disperse by wind and running water, as this plant grows along with water sources. Certainly, there is a possibility of genetic material exchange among the plants of districts in proximity (Schofield et al., 2018). Divergence in the genetic makeup of Dir's plants from all other districts can be attributed to distance in locality, difference in physical properties of soil, climatic conditions, altitude, and absence of common water channels (Escudero et al., 2003; Ohsawa and Ide, 2008; Chen et al., 2009; Adhikari et al., 2012; Govindaraj et al., 2015). Dendrogram obtained with ISSRs produced slightly variant clustering pattern as compared to RAPDs, which may be explained by genome portion covered by each kind of marker, their distribution all over the genome and the extent of the DNA target, which is examined by specific assay. Also, low reproducibility of RAPDs could be another possible reason (Karp et al., 1997). However, along with difference in dendrograms of RAPD and ISSR markers, these have more in common, like Dir is outgrouped in both, and plants from Chilas and Jaglot were close to each other, while plants from Abbottabad and Haripur showed maximum similarity. As sample size could make positive effect on genetic variation, larger the size of the samples the more reliable the results will be (Leimu et al., 2006); thus, smaller number of the available samples is the limitation of the study due to specific habitat of watercress plant (Cockram and Mackay, 2018).

The concentration of sinigrin in this study is varied among plants collected from different districts, corresponding with those reported by Santolamazza-Carbone et al., (2014), who found the variability in the concentration of sinigrin in the *Brassica oleracea* var. *acephala* plants collected from different localities. However, biochemical variations are certainly due to the genotypic diversity, but, in Pakistan, this plant is found in diverse localities, and different environmental conditions may also affect the synthesis of sinigrin (Amelung et al., 1999).

Medicinally and commercially important phytochemical's synthesis can be improved by exogenous elicitors treatment, an eco-friendly, simple, and

commercially viable method for improving the quality of *N. officinale* plants (Amer et al., 2021). Brassica plants such as watercress contain important phytochemicals which can be enhanced exogenously by the application of numerous elicitors (Fritz et al., 2010). HPLC analysis showed variable amount of sinigrin in treated *N. officinale* leaves with 50 μ M and 100 μ M ABA, SA and SNP. Previously, Thiruvengadam et al. (2016) reported the enhanced production of sinigrin at 100 μ M concentration of ABA and SA treatments in *Brassica rapa* ssp. *Rapa*. However, in this study, the contents of sinigrin in the leaves were remarkably higher when treated with 50 μ M concentration of elicitors (ABA: 80.25 μ M/g of DW, SA: 71.32 μ M/g of DW, SNP: 66.5 μ M/g of DW) as compared to 100 μ M (Figure 6). The difference in the sinigrin profile of treated plants is due to genetic variation (Giamoustaris and Mithen, 1996).

qPCR was performed to find out the effect of elicitors on the expression level of a GSL-OH gene, mandatory at final stage of glucosinolate biosynthesis in most plants (Zang et al., 2009). The qPCR results showed that enhanced gene expression in turn has induced the biosynthesis of phytochemicals in *N. officinale* leaves. Expression pattern was significantly increased by 50 μ M concentration of all elicitors (ABA: 12.9-fold, SNP: 1.4-fold, SA: 1.2-fold) as compared to 100 μ M (ABA: 1.2-fold, SNP: 1.1-fold, SA: 0.6-fold). Particularly, ABA 50 μ M strongly induce GSL-OH gene in leaves of the plant i.e., 12.9-fold (Figure 7), consistent with a reported study by Liang et al (2013) in which contents of caffeic acid, rosmarinic acid and salvianolic acid were significantly increased from 0.37, 3.66 and 7.39 mg/g to 1.96, 7.45 and 26.3 mg/g by 50 μ M ABA treatment in *Salvia miltiorrhiza* hairy roots as compared to 150 μ M and 200 μ M. Another study by Mousavi and Shabani (2019) reported that ABA in different concentrations (0, 5, 25, 50 and 100 μ M) was used in the shoots of *Melissa officinalis* L. An increased concentration of rosmarinic acid within 7 days of ABA application was observed and its maximum was detected in the shoots treated with 50 μ M ABA. This may be due to the reason that in some plant leaves contain high amount of ABA as the amount of ABA varies among different parts and exogenous application of elicitors increase de novo biosynthesis of ABA in the plants at specific suitable concentration (Xiong and Zhu, 2003). Furthermore, under non-stressful conditions, ABA in plant cells is maintained at low levels required for normal plant growth, which can be restored to the normal level by exogenous ABA that causes highest induction (Finkelstein and Rock, 2002).

Overall, this study described novel knowledge about existence of genetic diversity and biochemical variations in *N. officinal* in Pakistan. We observed that treated plants exhibited increased sinigrin contents and gene expression was also upregulated, especially by ABA (50 μ M) that

have never been reported in previous studies. Therefore, the present study demonstrated that exogenous elicitors' application could bring changes in the expression of genes involve in phytochemical biosynthetic pathways that ultimately bring changes in the phytochemical composition of *N. officinal* plants.

5. Conclusion

In conclusion, the genetic and biochemical analysis showed variability in the studied samples of *N. officinale*, and samples from district Dir were found distinct in genotype from all other samples and exhibited the highest concentration of sinigrin, which can also be increased by the

application of exogenous elicitors. Based on these findings, plants from Dir are recommended for commercial-scale cultivation and production of sinigrin, which has a role in the treatment of various diseases including cancer.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Supplementary Tables.**Table S1.** Matrix based on pair wise similarity between populations using ISSRs.

	Muzaffarabad	Diamer	Jaglot	Dir	Swat	Abbottabad	Haripur
Muzaffarabad	1	0.878	0.857	0.823	0.850	0.840	0.875
Diamer		1	0.958	0.79	0.871	0.861	0.917
Jaglot			1	0.77	0.87	0.842	0.896
Dir				1	0.765	0.808	0.823
Swat					1	0.853	0.888
Abbottabad						1	0.937

Table S2. Matrix based on pair wise difference between populations using ISSRs.

	Muzaffarabad	Diamer	Jaglot	Dir	Swat	Abbottabad	Haripur
Muzaffarabad	0	0.122	0.143	0.177	0.150	0.160	0.125
Diamer		0	0.042	0.210	0.129	0.139	0.083
Jaglot			0	0.23	0.13	0.158	0.104
Dir				0	0.235	0.192	0.177
Swat					0	0.147	0.112
Abbottabad						0	0.063
Haripur							0

Table S3. Matrix based on pair wise similarity between populations using RAPDs.

	Muzaffarabad	Diamer	Jaglot	Dir	Swat	Abbottabad	Haripur
Muzaffarabad	1	0.787	0.783	0.698	0.787	0.800	0.809
Diamer		1	0.953	0.769	0.955	0.840	0.851
Jaglot			1	0.765	0.909	0.837	0.848
Dir				1	0.769	0.815	0.788
Swat					1	0.84	0.851
Abbottabad						1	0.86
Haripur							1

Table S4. Matrix based on pair wise difference between populations using RAPDs.

	Muzaffarabad	Diamer	Jaglot	Dir	Swat	Abbottabad	Haripur
Muzaffarabad	0	0.213	0.217	0.302	0.213	0.200	0.191
Diamer		0	0.047	0.231	0.045	0.160	0.149
Jaglot			0	0.235	0.091	0.163	0.152
Dir				0	0.231	0.185	0.212
Swat					0	0.16	0.149
Abbottabad						0	0.14
Haripur							0