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ARTICLE

Evaluation of Tomato Hybrids for Resistance against Tomato Mosaic Virus (ToMV)

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ABSTRACT

Tomato mosaic virus (ToMV) drastically affects the tomato production worldwide. To deal with this problem, breeding of ToMV-resistant hybrids/varieties is the ultimate need and most successful approach. In wild tomato species, three dominant ToMV-resistant genes (*Tm-1*, *Tm-2* and *Tm-2*^{2}) were identified and the World Vegetable Center developed few fresh market tomato lines resistant to ToMV by the introgression of these genes. Recently at Nuclear Institute for Agriculture and Biology, Faisalabad, Pakistan a breeding programme was initiated to develop high yielding and ToMV tolerant hybrids using these lines. Current study was performed to screen elite F_1 hybrids carrying *Tm* gene along with their parents against ToMV using mechanical inoculation, confirmation of the virus using DAS-ELISA and marker assisted selection of hybrids. Out of 28 hybrids and 17 parent accessions/genotypes, eight hybrids and five accessions were found to be highly resistant and the virus was not detected in DAS-ELISA. Five hybrids were resistant, nine hybrids and four genotypes were tolerant, while the remaining six hybrids and eight genotypes were susceptible. For the confirmation of *Tm-2*^{2} gene, the tomato hybrids and their parents were subjected to molecular analysis using cleaved amplified polymorphic sequence (CAPS) primers. The result of CAPS markers for the confirmation of *Tm-2*^{2} gene was found consistent with phenotypic data of the inoculated tomato genotypes/hybrids. Higher phenolic content, total soluble proteins, better CAT and SOD activities were positively correlated with resistance. Screening results based on phenotype, biochemical and molecular marker data indicate that hybrids carrying *Tm-2*^{2} gene are good sources of resistance against ToMV.

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1. Introduction

Tomato (*Solanum lycopersicum L.*) is the second most produced and consumed vegetable in the world, but face several biotic stresses. Among 136 viruses infecting tomato [1], *Tomato Mosaic Virus* (ToMV) is one of the highly stable, contagious, cosmopolitan tobamovirus and is particularly problematic for greenhouse tomato production [2,3]. ToMV infected tomato plants show wrinkles, light green or yellow mottling, curved leaves, shoestring, stunted growth with irregular ripening of fruits [4,5]. To overcome ToMV problem in tomato, resistant variety is the most desirable and practical approach [2]. In wild tomato species, three dominant ToMV-resistant genes (*Tm-1*, *Tm-2* and *Tm-2²*) were identified [6] and have been used to incorporate resistance in cultivated tomato [7]. These resistant genes inhibit viral replication, hence increasing durability of crops [8]. The *Tm-1* gene was originally identified in *S. habrochaites* and is incompletely dominant gene, while both *Tm-2* and *Tm-2²* are dominant genes identified in *S. peruvianum* [7]. *Tm-1* gene is present on chromosome 2 [9], while genes *Tm-2* and *Tm-2²* are located on chromosome 9. Among these genes, *Tm-2²* is the most effective and durable *R* gene [10] and provides resistance against all the three known strains of ToMV (0, 1 and 2) [11]. *Tm-2²* confers resistance by recognizing ToMV movement proteins [12]. For *Tm* genes confirmation in tomato, several markers were developed and used [13,14]. DNA-based molecular markers linked to resistant genes are promising tools without recording phenotypic data and hence reduces the time and cost involved in conventional approaches [5]. Recently, for *Tm-2²* gene confirmation, an efficient and robust CAPS marker were reported by Panthee, D. R. et al. [5],which can successfully identify tomato genotypes carrying *Tm-2²* resistant gene [2]. The World Vegetable Center had developed few fresh market tomato lines resistant to ToMV by the introgression of *Tm-2²*. Our previous study confirmed the resistance level of these *Tm-2²* harboring accessions against Pakistani isolate of ToMV [2]. Based on our finding, we initiated a breeding programme at Nuclear Institute for Agriculture and Biology, Faisalabad, Pakistan (NIAB) to develop high yielding and ToMV tolerant tomato hybrids. In this study, we analyzed the elite F_1 hybrids developed through hybridization with *Tm-2²* parent accessions against ToMV using mechanical inoculation in insect-free glass-house. We further identified *Tm-2²* presence in resistant hybrids using molecular markers linked to this gene. In this study we also determine the biochemical alterations in selected six resistant and susceptible hybrids following ToMV inoculation.

2. Materials and Methods

2.1 Inoculation and Screening

The ToMV isolate (MG975645) used in this study was described in Ullah et al. [2] and maintained on susceptible tomato variety "Riogrande" in an insect-proof glasshouse. Plant material comprising five *S. lycopersicum* accessions (NB-324, NB-327, NB-328, NB-333, NB-336) harboring *Tm-2²* gene, 12 cultivated genotypes without *Tm-2²* gene and 28 hybrids developed at Nuclear Institute for Agriculture and Biology (NIAB) Faisalabad, Pakistan through hybridization of *Tm-2²* gene carrying accessions and cultivated genotypes. Nursery seedling of each genotype was raised in pots under an insect-proof glasshouse. One month post germination, plants were thinned into four plants per pot. At the age of six week, test plants were mechanically inoculated keeping variety "Nagina" as susceptible control. Top fully expanded leaves pre-dusted lightly with carborundom were inoculated with ToMV infected leaves sap (triturated in phosphate buffer; 0.01 M, pH 7) and subsequently washed after 10 min with distilled water. Plants were kept in good condition by adopting normal agronomic practices. Experimental unit was observed daily and data were recorded as the percentage of disease transmission, latent period and disease severity using the modified rating system given in Table 1 [15]. Leaf sample from each tomato genotype was collected and tested for ToMV presence using Double Antibody Sandwiched Enzyme-Linked ImmunoSorbant Assay (DAS-ELISA) [3]. For this purpose polyclonal antibodies (V061-K1, ACD, Inc.) were used according to the protocol of the manufacturer (ACD, Inc.). Results were assessed by visual observation and spectrophotometric absorbance at 405 nm. Samples were considered as positive if the optical density (OD) at 405 nm was at least twice of the negative control [9]. Total DNA from tomato hybrids and genotypes, plant samples were extracted using CTAB (Cetyltrimethylammonium Bromide) method. The extracted DNA was used as template in PCR using cleaved amplified polymorphic sequence primers (CAPS). Each 25 µL of PCR reaction mixture consist of 12.5 µL PCR Master Mix (enzynomics, Korea), 0.5 µL each *NCTm-019* 5′-AATTTGGGCATACTGACATC-3′ and 5′-GTTGCACACATTGGTTGTAG-3′ primers, 2 µL DNA template and 9.5 µL nuclease free water. Thermal profile was set for initial denaturation at 94 °C for 5 min, followed by 35 cycles (denaturation at 94 °C for 45 s, annealing at 54 °C for 1 m, extension at 72 °C for 1.5 min) and final extension at 72 °C for 10 min. PCR products were first separated on 1.5% agarose gel, stained with EtBr and DNA bands were observed using gel documentation system (UVIpro). After amplification

with CAPS primers, the obtained PCR products were restricted using HaeIII enzyme (Cat. No R0108S, New England Biolabs) at 37 °C for 1 hour. For enzyme digestion, 25 µL reaction mixture (8 µL PCR product, 2.5 µL of 10x enzyme buffer, 1 µL HaeIII enzyme and 13.5 µL distilled water) was prepared. Digested PCR products were separated on 2% agarose gel, stained with EtBr and observed using gel documentation system (UVIpro).

Table 1. Disease scale rating for mosaic disease in tomato caused by ToMV

Rating	Symptoms	Severity index	Disease reaction
0	No visible disease symptoms. Virus can't be detected in plant tissues via molecular techniques.	0	Highly resistant
1	Complete absence of symptoms but virus can be detected in plant tissues using moleculartechniques. OR Slight mosaic appearance or mottling and leaf deformity but no shoe stringing.	0.01-1.4	Resistant
2	Moderate mosaic appearance or mottling and leaf deformity followed by minor shoe stringing.	1.5-2.4	Tolerant
3	Severe mosaic or mottling. Leaf deformity, shoe-stringing, minor to medium stunting with minor flower shedding and minor reduction in fruit setting but marketable fruit setting.	2.5-3.4	Susceptible
4	Severe mosaic or mottling, leaf deformity, shoe-stringing, stunting with no or few unmarketable fruit setting.	3.5-4.0	Highly susceptible

2.2 Biochemical Parameters

To study the biochemical changes occurred as a result of ToMV infections in six resistant and susceptible tomato hybrids, leaf samples were ground in extraction buffer. After grinding, the mixture was brought to centrifugation at maximum speed (14,000 rpm) for 10 minutes. Following centrifugation, supernatant was removed and samples were stored at –20 °C to study various antioxidant and enzymatic activities.

Total phenolic contents (TPC) were estimated among virus-inoculated and non-inoculated plants as described by Ainsworth and Gillespie (2007) [22]. A standard curve was prepared using different concentration of gallic acid and a linear regression equation was calculated to determine TPC. Total soluble protein (TSP) contents were measured using Bradford's method [23]. For catalase (CAT) estimation, procedure described by Beers and Sizer [24] was followed. Superoxide dismutase (SOD) activity was estimated as described by Dixit et al. [25]. The level of lipid peroxidation was measured in terms of Malondialdehyde (MDA), a product of lipid peroxidation) [26]. Estimation of pigments like chlorophyll (Chl. *a* and *b*), total chlorophyll, lycopene and carotenoids was done following the method of Hameed et al. [27].

2.3 Statistical Analysis

Significance level of data was calculated by ANOVA and Tukey's (HSD) test at $p \leq 0.05$ by using XL-STAT software. Mean ± S.E values are shown in graphs with different alphabets that differ significantly from each other.

3. Results

3.1 Evaluation of Hybrids and Parents

ToMV was successfully transmitted to 28 hybrids and 17 parent accessions/genotypes belonging to *S. lycopersicum* using mechanical inoculation. ToMV infection in these hybrids and parent accessions/genotypes was confirmed based on their phenotpes and following DAS-ELISA. Eight hybrids (NBH-148, NBH-149, NBH-151, NBH-174, NBH-175, NBH-200, NBH-256 and NBH-260) and 5 accessions (NB-324, NB-327, NB-328, NB-333 and NB-336) were found to be highly resistant as no disease symptoms were observed on these hybrids/accessions till the end of experiment i.e., 90 days post inoculation (Tables 2 and 3). However, 5 hybrids (NBH-154, NBH-204, NBH-258, NBH-259 and NBH-261) showed a much greater degree of resistance (with slight mosaic appearance or mottling and leaf deformity) with the latent period (LP) ranging between 13-18 days, severity index (SI) value of 1.0 to 1.3 and low virus titer through ELISA (0.82 to 0.97) with no reduction in disease severity throughout the experiment (Table 2).

Nine hybrids (NBH-150, NBH-152, NBH-227, NBH-229, NBH-263, NBH-265, NBH-266, NBH-267 and NBH-268) and four genotypes (NB-8, NB-10, NB-279 and NB-299) were tolerant with SI value of 1.7 to 2.3, LP ranging between 14-18 days and low virus titer through ELISA (1.60 to 2.14) as compared to susceptible hybrids/accessions. Remaining six hybrids and eight genotypes were susceptible. ToMV symptoms started as mild mosaic or mottling and leaf deformity from 11 to 12 days post inoculation in all susceptible hybrids and genotypes. All these genotypes developed severe mosaic or mottling, and leaf deformity, shoe-stringing, minor to medium stunting at 30 days to post inoculation with SI ranging from 2.7 to 3.3 with virus titer ranging from 2.37 to 3.50 (A_{405nm} 1 h) (Tables 2 and 3).

Table 2. Tomato hybrids evaluated against ToMV in an insect-proofed glasshouse.

Sr. No	Hybrid	Pedigree	Habit	Latent period	Disease severity	Disease response	ELISA		PCR confirmation of Tm-2^2 (–/+)
							–/+	Values*	
1	NBH-147	NB-11×NB-327	D	11	3.0	Susceptible	+++	3.50	–
2	NBH-148	NB-11×NB-333	D	-	0.0	Highly resistant	–	0.57	+
3	NBH-149	NB-242×NB-327	D	-	0.0	Highly resistant	–	0.59	+
4	NBH-150	NB-242×NB-333	D	14	2.3	Tolerant	++	1.85	+
5	NBH-151	NB-8×NB-327	D	-	0.0	Highly resistant	–	0.59	+
6	NBH-152	NB-242×NB-327	D	14	2.0	Tolerant	++	1.90	+
7	NBH-154	NB-242×NB-333	D	17	1.3	Resistant	+	0.90	+
8	NBH-174	NB-328×NB-285	D	-	0.0	Highly resistant	–	0.61	+
9	NBH-175	NB-285×NB-328	D	-	0.0	Highly resistant	–	0.58	+
10	NBH-196	NB-216×NB-327	D	11	3.3	Susceptible	+++	3.36	–
11	NBH-200	NB-243×NB-327	D	-	0.0	Highly resistant	–	0.58	+
12	NBH-204	NB-279×NB-327	D	15	1.3	Resistant	+	0.82	+
13	NBH-227	NB-11×NB-336	D	14	2.3	Tolerant	++	1.87	+
14	NBH-228	NB-242×NB-336	D	12	3.0	Susceptible	++	2.18	–
15	NBH-229	NB-8×NB-336	D	17	2.3	Tolerant	++	1.96	+
16	NBH-255	PRN×NB-324	ID	12	3.0	Susceptible	+++	3.25	–
17	NBH-256	PRN×NB-333	ID	-	0.0	Highly resistant	–	0.60	+
18	NBH-257	PRN×NB-336	ID	12	3.0	Susceptible	+++	2.90	–
19	NBH-258	NB-10×NB-324	D	18	1.3	Resistant	+	0.93	+
20	NBH-259	NB-10×NB-333	D	17	1.0	Resistant	+	0.97	+
21	NBH-260	NB-10×NB-336	D	-	0.0	Highly resistant	–	0.57	+
22	NBH-261	NB-210×NB-324	D	13	1.3	Resistant	+	0.87	+
23	NBH-263	NB-210×NB-336	D	17	1.7	Tolerant	+++	2.14	+
24	NBH-265	NB-242×NB-32	D	16	2.3	Tolerant	++	2.09	+
25	NBH-266	NB-279×NB-324	D	16	2.3	Tolerant	++	2.20	+
26	NBH-267	NB-279×NB-333	D	15	2.3	Tolerant	+++	1.86	+
27	NBH-268	NB-14×NB-324	D	13	2.3	Tolerant	+++	1.94	+
28	NBH-281	NB-8×NB-324	D	12	2.7	Susceptible	+++	3.30	–

D= determinate; ID= indeterminate: * = ELISA absorbance values (A405 nm) after 1h: ELISA -/+ = - is absence of ToMV/ Tm-2^2 gene and + is presence of ToMV/ Tm-2^2 gene

Table 3. Tomato accessions/genotypes evaluation against ToMV using sap-inoculation under glasshouse.

Sr. No	NIAB code	Habit/ Source	Latent period	Severity index	Disease response	ELISA		PCR confirmation of Tm-2^2 (–/+)
						–/+	Values*	
1	NB-324	D/AVRDC	-	0	Highly resistant	+	0.83	+
2	NB-327	SD/AVRDC	-	0	Highly resistant	+	0.77	+
3	NB-328	SD/AVRDC	-	0	Highly resistant	+	0.87	+
4	NB-333	D/AVRDC	-	0	Highly resistant	+	0.86	+
5	NB-336	D/AVRDC	-	0	Highly resistant	+	0.76	+
6	NB-8	D/AARI	15	2.3	Tolerant	++	1.60	–
7	NB-10	D/Bulgaria	16	2.0	Tolerant	++	1.90	–
8	NB-11	ID/Bulgaria	15	3.0	Susceptible	+++	3.50	–
9	NB-210	D/Bulgaria	12	3.3	Susceptible	+++	2.89	–
10	NB-216	D/EFUP	13	2.3	Susceptible	+++	2.93	–
11	NB-242	D/TGRC	12	3.3	Susceptible	+++	3.49	–
12	NB-243	D/USA	12	3.3	Susceptible	+++	3.26	–
13	NB-260	D/AARI	13	3.3	Susceptible	+++	3.10	–
14	NB-279	D/GWP	18	2.3	Tolerant	++	1.84	–
15	New Yorker	D/TGRC	12	3.3	Susceptible	+++	2.37	–
16	NB-299	D/TGRC	16	2.3	Tolerant	++	1.88	–
17	PRN	ID/?	13	3.3	Susceptible	+++	2.90	–

* = ELISA absorbance values (A405nm) after 1h: ELISA -/+ = - is absence of ToMV/ Tm-2^2 gene and + is presence of ToMV/ Tm-2^2 gene; D= determinate; ID= indeterminate; SD= semi-determinate; AARI= Ayub Agricultural Research Institute, Faisalabad, Pakistan; TGRC= Tomato Genetic Resources Centre, United States of America; AVRDC= Asian Vegetable Research and Development Centre, Taiwan; EFUP= Establishment of facilitation unit for participatory vegetable seed and nursery production programme, Pakistan; GWP= Gujranwala Pakistan.

3.2 Molecular Analysis for the Identification of *Tm-2^2* Gene

After defining the resistance/susceptibility criteria in the hybrids and their respective parent genotypes through mechanical inoculation, tomato hybrids and their parent were subjected to molecular methods for further confirmation. The CAPS marker was used for the confirmation of *Tm-2^2* gene. CAPS markers efficiently differentiated the resistant hybrids and their parent from the susceptible ones. PCR products of 870 bp was successfully amplified for all the tomato hybrids and their parent tested against ToMV. However, when the CAPS primers amplified products of susceptible hybrids and their parent were digested with HaeIII restriction enzyme, it produced a single uncleaved intact band of 870 bp which showed the absence of *Tm*-2^2 gene in these hybrids and their parent. Conversely, when the highly resistant/resistant hybrids PCR products were restricted, it showed three different bands of 870 bp, 600 bp and 270 bp. These results shows the presence of *Tm-2^2* gene in these hybrids in heterozygous conditions. HaeIII restricted PCR product of highly resistant parent genotypes showed two bands of 600 bp and 270 bp, which confirmed the presence of *Tm-2^2* gene in these genotypes in homozygous conditions (Tables 2 and 3). Furthermore, result of CAPS markers digested with HaeIII was found consistent with phenotypic data of the inoculated tomato genotypes/ hybrids.

4. Biochemical Analysis

In the present study, the level of TPC in ToMV-inoculated plants of all the tested hybrids was differed significantly within highly resistant, resistant and susceptible classes (Table 4). TPC were higher in highly resistant hybrid NBH-149, while lower in susceptible hybrid NBH-268. TPC values for tomato hybrids viz., NBH-149, NBH-151, NBH-265, NBH-154, NBH-196 and NBH-268 after inoculation with ToMV were 925.0 µM/g.f.wt., 825.0 µM/g.f.wt., 397.5 µM/g.f.wt., 352.5 µM/g.f.wt., 246.0 µM/g.f.wt., and 165.0 µM/g.f.wt., respectively.

Amount of TSP in all the highly resistant, resistant and susceptible classes was differed significantly (Table 4). TSP content was more in resistant hybrids as compared to susceptible ones. Its amount was particularly high in hybrids NBH-154, NBH-149 and BH-265 than other hybrids. Moreover, significant differences were observed between highly resistant (NBH-149 and NBH-151) and susceptible hybrids (NBH-196 and NBH-268). TSP values for tomato hybrids viz., NBH-154, NBH-149, NBH-265, NBH-151, NBH-196 and NBH-268 after inoculation with ToMV were 153.3 mg/g.f.wt., 143.7 mg/g.f.wt., 135.5 mg/g.f.wt., 101.5 mg/g.f.wt., 79.3 mg/g.f.wt. and 47.0 mg/g.f.wt., respectively.

Hybrids tested for catalase activity showed significant differences among all the three classes: highly resistant, resistant and susceptible (Table 4). Level of catalase was higher in resistant hybrid NBH-265, whereas least in susceptible hybrid NBH-196. Catalase level was significantly different among the classes. In the resistant class, NBH-265 and NBH-154 were statistically different from each other. Also, in the susceptible ones, NBH-196 and NBH-268 were statistically different. Catalase values for tomato hybrids viz., NBH-265, BH-151, NBH-149, NBH-154, NBH-268 and NBH-196 after inoculation with ToMV were 572.5 units/g.f.wt., 430.0 units/g.f.wt., 385.0 units/g.f.wt., 285.5 units/g.f.wt., 235.0 units/g.f.wt. and 62.5 units/g.f.wt., respectively.

Hybrids subjected to superoxide dismutase activity showed that they were significantly different among classes but insignificant between classes (Table 4). Highest SOD activity was observed in NBH-151 (highly resistant) and least in NBH-268 (susceptible). However, results were insignificant between highly resistant, resistant and susceptible categories. SOD values for tomato hybrids viz., NBH-151, NBH-149, NBH-196, NBH-265, NBH-154 and NBH-268 after inoculation with ToMV were 293.75 units/g.f.wt., 247.64 units/g.f.wt., 224.19 units/g.f.wt., 206.82 units/g.f.wt., 193.05 units/g.f.wt. and 106.15 units/g.f.wt., respectively.

In the present study, level of malondialdehyde in ToMV-inoculated plants of all the tested hybrids was insignificant within highly resistant, resistant and susceptible classes (Table 4). Statistical trend was similar in all the members of hybrid categories except NBH-196 that showed slight significance in its behaviour. MDA content in NBH-196 recorded 330.38 µM/g f.wt. MDA level in other hybrids viz., NBH-268, NBH-151, NBH-149, NBH-154 and NBH-265 was 239.16 µM/g.f.wt., 218.48 µM/g.f.wt., 216.2 µM/g.f.wt., 182.0 µM/g.f.wt. and 176.29 µM/g.f.wt., respectively.

Chlorophyll *a* (Chl a) level in ToMV-inoculated plants of all the tested hybrids recorded insignificant differences among highly resistant, resistant and susceptible categories (Table 4). Chl *a* was higher in resistant hybrid NBH-154, whereas least in susceptible hybrid NBH-268. Chl *a* values for tomato hybrids *i.e.,* NBH-154, NBH-149, NBH-151, NBH-196, NBH-265 and NBH-268 after inoculation with ToMV were 520.28 µg/g.f.wt., 507.66 µg/g.f.wt., 507.55 µg/g.f.wt., 505.69 µg/g.f.wt., 501.12 µg/g.f.wt. and 497.52 µg/g.f.wt., respectively.

Level of Chl *b* recorded in tomato genotypes proved insignificant among the tested hybrids (Table 4). Chl *b* was higher in highly resistant hybrid NBH-149, whereas least in resistant hybrid NBH-154. However, the trend of resistant hybrids was generally different from highly resistant and susceptible hybrids. Chl *b* values for tomato hybrids *i.e.*, NBH-149, NBH-151, NBH-196, NBH-268, NBH-265 and NBH-154 after inoculation with ToMV were 614.64 µg/g.f.wt., 597.23 µg/g.f.wt., 592.5 µg/g.f.wt., 563.61 µg/g.f.wt., 528.74 µg/g.f.wt. and 492.96 µg/g.f.wt., respectively. After calculating chlorophyll *a* and *b* level in ToMV-inoculated hybrids, total chlorophyll was also determined in all classes (highly resistant, resistant and susceptible). Results were statistically insignificant with NBH-149 being highest in total chlorophyll content and NBH-154 being lowest. Chlorophyll (a+b) values for tomato hybrids in NBH-149, NBH-151, NBH-196, NBH-268, NBH-265 and NBH-154 after inoculation with ToMV were 1122.31 µg/g.f.wt., 1104.79 µg/g.f.wt., 1098.19 µg/g.f.wt., 1061.13 µg/g.f.wt., 1029.86 µg/g.f.wt. and 1013.23 µg/g.f.wt., respectively (Table 4).

Level of lycopene in ToMV-inoculated plants of all the tested hybrids recorded nonsignificant differences among highly resistant, resistant and susceptible classes (Table 4). Lycopene was higher in highly resistant hybrid NBH-149, whereas lower in resistant hybrid NBH-265. Lycopene values for tomato hybrids *i.e.*, NBH-149, NBH-151, NBH-268, NBH-196, NBH-154 and NBH-265 after inoculation with ToMV were 18.31 mg/g.f.wt., 17.72 mg/

Table 4. Summary of comparison of different biochemical parameters in tomato hybrids inoculated with ToMV.

Parameter (g.f.wt.)	Hybrids					
	NBH-149	NBH-151	NBH-265	NBH-154	NBH-196	NBH-268
TPC (µM)	925^{a}	825^{a}	397.5^{b}	352.5^{bc}	246^{cd}	165^{d}
TSP (mg)	143.7^{a}	101.5^{b}	135.5^{a}	153.3^{a}	79.3^{b}	47.0^{c}
Catalase (units)	385.0^{b}	430.0^{b}	572.5^{a}	285.5^{c}	62.5^{e}	235.0^{d}
SOD (units)	247.64^{ab}	293.75^{a}	206.82^{bc}	193.05^{c}	224.19^{bc}	106.15^{d}
MDA (µM)	216.2^{b}	218.5^{b}	176.3^{b}	182.0^{b}	330.4^{a}	239.2^{b}
Lycopene (mg)	18.31^{a}	17.72^{a}	15.82^{a}	16.11^{a}	16.36^{a}	16.40^{a}
Chl *a* (µg)	507.66^{a}	507.55^{a}	501.12^{a}	520.28^{a}	505.69^{a}	497.52^{a}
Chl *b* (µg)	614.64^{a}	597.23^{a}	528.74^{a}	492.96^{a}	592.50^{a}	563.61^{a}
Total chl. (µg)	1122.31^{a}	1104.79^{a}	1029.86^{a}	1013.23^{a}	1098.19^{a}	1061.13^{a}
Carotene (mg)	45.47^{a}	45.24^{a}	43.95^{a}	45.24^{a}	45.09^{a}	42.71^{a}

Mean sharing similar letters in the same box do not differ from each other at p ¼ 0.05.

g.f.wt., 16.4 mg/g.f.wt., 16.37 mg/g.f.wt., 16.11 mg/g.f.wt. and 15.82 mg/g.f.wt., respectively.

Carotene level in all the ToMV inoculated hybrids proved to be insignificant within highly resistant, resistant and susceptible groups. Highest carotene level in NBH-149 and lowest in NBH-268 were observed (Table 4). Carotene values for tomato hybrids in NBH-149, NBH-151, NBH-196, NBH-268, NBH-265 and NBH-154 after inoculation with ToMV were 45.47 mg/g.f.wt., 45.24 mg/g.f.wt., 45.24 mg/g.f.wt., 45.09 mg/g.f.wt., 43.95 mg/g.f.wt. and 42.71 mg/g.f.wt., respectively (Table 4).

5. Discussion

ToMV being a highly infectious, contagious and rapidly multiplying virus, significantly reduces tomato yield [23]. Several measures were reported to manage this disease but the development and use of resistant variety are the most practical approach [2]. We tested 28 tomato hybrids and 17 parent genotypes against ToMV following mechanical inoculation. Wide variations were observed in results, from highly resistant to susceptible ones. Of these, 20 hybrids and 12 parent genotypes displayed a range of phenotypic reaction to ToMV infection. Besides their phenotypic response, ToMV presence was confirmed using DAS-ELISA. Eight hybrids and five accessions were symptomless. However, on five hybrids slight mottling and leaf deformity symptoms were recorded and they were rated as resistant with low ELISA absorbance value (A405 nm value; 0.87 to 0.97) compared to highly susceptible genotypes. Nine hybrids and four genotypes were tolerant and showed moderate level of symptoms. Rest of the six hybrids and eight parent genotypes were susceptible. In resistant hybrids, delayed symptoms development and low virus titer was observed (based on ELISA absorbance value). In contrast, susceptible hybrids were severely infected and showed high virus titer in ELISA. Severe mosaic, mottling, stunting and leaf deformation were noticed on susceptible hybrids/genotypes after ToMV inoculation. ToMV can induce different symptoms depending on susceptibility of genotype infected [3]. Level of disease severity can serve as an indicator of resistance level of a plant species against a pathogen [19-23]. Resistant varieties contain low viral titer, while the susceptible genotypes accumulate high virus titer [25-27]. Low viral titer accumulates in highly resistant genotypes [26], while in susceptible genotypes severe symptoms developed and facilitated high viral titer [25-28].

In this study we further tested the tomato hybrids for the confirmation of *Tm-2*^{2} gene using CAPS primers. A PCR product of 870 bp was successfully amplified from all the tomato hybrids and genotypes tested against ToMV. However, when the CAPS primers (NCTm-019) amplified products of susceptible genotypes were digested with HaeIII enzyme, a single un-cleaved intact band of 870 bp was observed, which showed the absence of *Tm-2*^{2} gene. Conversely, when the highly resistant/resistant hybrids PCR products were restricted, they showed three different bands of 870 bp, 600 bp and 270 bp.

These results show the presence of *Tm-2²* gene in these hybrids in heterozygous conditions. Two bands of 600 bp and 270 bp were observed when PCR product of highly resistant parent accessions were restricted with HaeIII enzyme. Appearance of two bands after restriction confirms the presence of *Tm-2²* gene in these accessions in homozygous conditions. Furthermore, CAPS markers digested with HaeIII enzyme were found consistent with phenotypic data of the inoculated tomato genotypes/hybrids. All the inoculated plants of highly resistant tomato hybrids and lines harboring *Tm-2²* resistant gene were symptomless even at the end of the experiment. These genotypes were also negative for ToMV infection tested in DAS-ELISA. A number of markers have been reported for the confirmation of *Tm* genes in tomato [13,14]. However, a recently reported sequence-based CAPS marker (*NCTm-019)* is found more efficient, robust and specific to *Tm-2²* gene [5]. Moreover, this marker clearly differentiate ToMV resistant genotypes carrying *Tm-2²* from susceptible ones on molecular level [2]. The sequence-based markers are more efficient than markers from flanking region [5]. Our results further confirm that CAPS marker NCTm-019 is a reliable, robust marker and its results are consistent with phenotypic response of the tested genotypes.

In the present study we also investigated that how appearance of symptoms relates to biochemical alterations. Phenols are compounds with well-known antifungal, antibacterial and antiviral properties that occur in plants and play a vital role in defense by enhancing the mechanical strength of host cell walls by the synthesis of lignin and suberin, both of which are involved in the formation of physical barriers that block the spread of pathogens. High levels of phenols is correlated with increased resistance in plants [29]. Our results concerning the criterion proved that amount of phenols in highly resistant and moderately resistant hybrids were significantly higher as compared to the amount of phenols in the susceptible hybrids. A positive correlation exists among the host resistance, total phenols and increased enzyme activities. However, it was exact opposite among susceptible hybrids. The positive relation between phenols and increased resistance could be of great significance for identification of resistant hybrids during screening of large populations [30]. Another such example of positive correlation was found in grape leaves and black currants presented by Vagiri et al. [31].

Many plant-pathogen interactions have shown the involvement of proteins and its components in plant disease resistance. Stimulation of defense proteins make the plants resistant to pathogens. Usually, infected plants show high levels of proteins that may be because of activation of host defense system or pathogen attack mechanism [32]. In our study, variable trend was seen in the protein contents of healthy plants. Resistant genotypes have shown increased levels of proteins, whereas significant decrease was observed in susceptible hybrids. A possible clarification for this significant decrease after infection may be because of high level of susceptibility of these hybrids [20]. Low level of soluble proteins might be possibly due to decrease in synthesis of ribulose-1,5-bisphosphate carboxylase (RuBPC); a major soluble protein of leaf. Loss of leaf protein could be because of damaged chloroplast or inhibition of protein synthesis [33].

CAT is an oxygen-scavenger, which protects cells from the toxic effects of substrates (H_2O_2) during development, which could be fatal otherwise [33]. In our study, CAT activity significantly increased within moderately resistant cultivars, whereas a significant decrease was observed among the hybrids of susceptible class. Usually, the reduction of CAT increases resistance in plants against pathogenic attack as plants can maintain high concentrations of H_2O_2 [29]. Reduction in CAT activity could be a result of increased proteolysis. On the other hand, higher CAT levels may be linked with decrease in H_2O_2 level and in lipid peroxidation [35].

Superoxide dismutase is another scavenger enzyme that catalyzes the dismutation of superoxide radicals to active oxygen species. Enhanced SOD activities were observed in resistant hybrids, but variable trend was seen in susceptible class. SOD activity in one of the susceptible hybrid NBH-196 was significantly different (higher) from NBH-268. Summing up, resistant is associated with SOD activity. In another study performed on strawberry leaves that were infected with *Mycosphaerella fragariae*, confirmed that SOD was higher in resistant genotypes than the susceptible ones [34]. Research conducted by Lu et al., also confirmed that resistance is positively related to the increase in SOD activities [36].

Malondialdehyde (MDA) is an abundant aldehydic lipid breakdown product. MDA acts as a secondary messenger that up-regulates several genes in plants under stress conditions [37]. MDA produced during lipid peroxidation acts as an indicator to measure extent of cellular damage as a result of pathogenic infection. MDA levels determined were statistically insignificant (similar) among all the highly resistant, moderately resistant and susceptible classes except NBH-196 that showed an irregular increase in MDA levels. This represents that MDA content has nothing to do with resistance of plants. The same concept was also confirmed by Siddique et al., where MDA was statistically similar in all resistant and susceptible varieties [34].

The photosynthetic system (chlorophyll a + b) is the

physiological basis of plant growth and crop production. Any kind of environmental stress affecting photosynthetic system will directly affect plant growth. In our present study, chlorophyll (a and b) rates remain totally unaffected and their differences proved statistically insignificant. However, Chl *b* level recorded was higher than Chl *a* in the inoculated genotypes. This is due to the reason that Chl *b* is involved in the virus tolerance mechanism. Similar insignificant results were also presented by Siddique et al. [38].

Lycopene is a pigment that gives red or orange-red colour to fruits and vegetables. Lycopene acts a free-radical scavenger and have antioxidant properties [39]. Evaluation of lycopene content has shown to be statistically insignificant. However, lycopene levels were different among highly resistant, moderately resistant and susceptible classes. This suggests that there does not exist any correlation between lycopene levels and imparting resistance to plants.

Carotenes are accessory pigments in the light harvesting systems [40]. They are also concerned with photo-protection [41]. Carotene levels calculated for different hybrids shown variable trend. Highest carotene levels were recorded in highly resistant class. However, carotene content in susceptible class was higher than moderately resistant hybrid class. This also suggests that carotene level is not associated with implying resistance to plants.

6. Conclusions

ToMV is considered as a potential threat for tomato cultivation in green/glass-houses because of its contagious nature. In this study we identified tomato hybrids and accessions highly resistant against ToMV using multiple approaches. *Tm2*^{2} gene was successfully transferred to tomato hybrids which was further confirmed using molecular markers. This gene was found highly effective providing complete resistance against ToMV in tomato hybrids. Furthermore, CAPS marker (*NCTm-019)* is validated as efficient and robust marker associated with ToMV resistance in tomatoes. Results of this marker are highly consistent and reproducible with phenotypic data which suggest its potential use in tomato improvement using genome assisted breeding programme. Higher phenolic content, total soluble proteins, better CAT and SOD activities are positively correlated with resistance. On the other side, MDA, photosynthetic pigments (Chl *a* + *b*), lycopene and carotene are pigments essential in light harvesting processes but are not concerned with resistance of plants. The use of identified highly resistant hybrids will be beneficial to manage ToMV problem more efficiently. However, before the use of ToMV-resistant hybrids for general it is very important to check their horticultural characteristics along with market-preferred traits regarding fruit and quality features.

Conflict of Interest

There is no conflict of interest.

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ARTICLE

Screening and Analysis of Methanolic Leaf Extract of *Psorospermum febrifugum* (SPACH)

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ABSTRACT

Phytochemical components have been reported for various plants but very little information on *Psorospermum febrifugum* (SPACH). The presence of biocidal activity makes the spach of potential interest for the control of micro-organisms. Methanolic extract of the leaves of spach shows the various constituents (alkaloids, flavonoids, terpenoids, tannins, phenols, and steroids). Further investigation revealed phytoconstituents of methanolic leaf extract using gas chromatography-spectrophotometric techniques (GC-MS). Result of GC-MS analysis revealed the presence of eight (8) botanical pesticides with valuable biological activities. The GC-MS results revealed that eight (8) biocidal activities were present in spach namely: 1, 2, 3, 4- tetrachloronaphtalene, Permetrin-a, permetrin-b, cyfluthrin-b, cypermethrin-a, cypermethrin-c, and flumethrin-b. The result clearly shows that *Psorospermum febrifugum* hold phytocomponents species of botanical interest that could still be exploited.

1. Introduction

Biocidal pesticides are the naturally occurring secondary metabolites extracted from plant sources which can control and kill pests thus helping in agricultural pest management. Plants with bioactive compounds have been used to manage different crop pests with notable successes [1]. *Psorospermum febrifugum* is a flowering plant species in the genus *psorospermum.* It grows in Savannah areas and Africa, or appertain to ancestry Hypericaceae. *P. febrifugum* is being employed in the treatment of various illnesses in Africa especially fever, skin problem, leprosy, poison antidote and purgative [2]. Lamoerde [3] investigated the use of *P. febrifugum* extract in the treatment of human immune deficiency virus (HIV) infections in Uganda. The use of *Psorospermum febrifugum* to treat skin rash has also been documented [4]. Similarly, the use of roots bark expressrate has been reported for the treatment of syphilis [5]. Secondary metabolites derived from plant sources have varieties of biological activities, structural arrangements

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and properties [6].

The first report on *Psorospermum febrifugum* appeared in 1980 [7] where the xanthone: *psorospermin,* and a derivative of methyl *psorospermin chlorohydrins* were isolated from its ethanol root extract. Xanthones obtained from P. febrifugum are reported to be accountable for its antiviral as well as prevention of cancer activities in bone marrow and other blood - forming organs [8]. According to Tsaffack [9] emodin is capable of producing good result against antitumor agent, with ability of treatment to provide a beneficial effect [10] and lung cancers [11]. The chemical that has a medicinal effect on the body in this plant is reported to have a negative side effect on DNA [12]. However, other species obtained from the plant are said to be successful for antimicrobial agents [12]. In this study we aimed to investigate chemical components from its methanolic extract of P. febrifugum using GC-MS techniques.

2. Materials and Methods

Study Area

Benue state where the study was carried out is located on longitude and latitude (6°21′ - 8°10′ N and 7°44′ E - 9°55′ E) with total size of 30,955 km^2 and a population of 5,741,815 people. The state has 23 LGA. The state is bounded with Nasarawa to the north, Taraba to the east, Cross River to the south, Enugu to the south-West and kogi to the west. The population density per square kilometer was 138 persons (NBS, 2017). Benue state consists of twenty-three (23) Local Government Areas and with three major ethnic groups namely: Tiv, Idoma and Igede. Seventy- five percent (75%) of these ethnic groups were predominantly farmers. The vegetation cover is mostly made up of giant grass (elephant grass) and tree species like: *Vetellaria paradoxa*, *Parkia biglobosa, Prosopis africana, Vitex doniana, Khaya senegalensis*, *Psorospermum febrifugum* etc. Along the banks of the River Benue are found hydromorphic soils, which are fertile for several crops cultivation which has earned the State a nick-name: "The Food-Basket of the Nation".

3. Collection and Authentication of Plant Material

The plant (*P. febrifugum*) was collected from the wild in Mobile Police Barracks area in Makurdi Local Government, Benue State, Nigeria. Taxonomic identification of the plant sample was by a taxonomist Dr. Namadi Sunusi of Botany Department, Faculty of Science, Ahmadu Bello University Zaria (ABU) with Voucher Specimen number 0936. A specimen was deposited in Botany Department, Joseph Sarwuan Tarka University Makurdi Benue state (Former Federal University of Agriculture, Makurdi).

4. Preparation of Extracts

The leaves of *P. febrifugum* plant were rinsed with distilled de-ionized water in other to remove the adsorbed soil particles and contaminants such dust, soot and aerosols. The leaves were dried in a shade for a period of seven days at ambient temperature and thereafter ground into powder using silica crucible pestle mortar. The powdered sample was kept inside 100 mL McCartney bottles until needed for further analysis.

Figure 1. *Psorospermum febrifugum*

Extraction was done using Soxhlet extraction techniques as reported by Abah and Egwari [13] with slight amendement. Two hundred grams (200 g) of powdered plant material was weighed and placed in the extraction thimble and 400 mL of methanol (ME) added. This was refluxed at a temperature of 64.7 °C (boiling point of methanol). Excess solvent was removed to dryness to give a crude extract (0.3 g).

5. Preliminary Phytochemical Screening

Screening of methanolic leaves extract of *P. febrifugum* was carried out based on interprete of previous works [14-16]. The extract was tested for saponins, flavonoids, alkaloids, cardiac glycosides, terpenoids, phenols, tannins, steroids.

6. Column Chromatographic Separation

A column of 15 cm (length) × 1 cm (internal diameter) was packed first with glass wool and then with about 7.5 g of activated silica gel prepared in a slurry form in CH_3CN. About 5 g of anhydrous Na_2SO_4 was placed at the top of the column to absorb any water in the sample or the solvent. Prior-to elution was done with 15 mL of CH_3CN, without exposing the Na_2SO_4 layer to air, to avoid evaporation of the silica gel adsorbent. The strenuous extract

was passed through the column and allowed to sink below the Na_2SO_4 layer. Elution was done with 3 × 10 mL portions of CH_3CN. The eluate was collected, dried with anhydrous Na_2SO_4 and evaporated to dryness under a flow of analytical grade nitrogen (99.99%) for GC-MS analysis.

7. Construction of Calibration Curves

Original solutions of the Pyrethroids (1000 mgL^{-1}) (Allethrin, Bifenthrin, Cyfluthrin, Cypermethrin, Permethrin, Tetramethrin, Telfluthrin, Phenothrin, Deltamethrin Resomethrin) pesticides were prepared and diluted serially to make non- identical concentrations between 0.03 µg/L and 0.05 µg/L) of individual pesticides. Stock standard solutions were stored in amber coloured bottles at 4 °C in a refrigerator and working standard solutions were newly prepared prior to use. Original standard solutions of the pesticides passed through GC-MS under the set chromatographic conditions and average peak areas were marked against concentrations to obtain calibration curves of individual pesticides.

8. GC-MS Analysis

The dried eluates were rejuvenated with one (1) mL 2, 2, 4-trimethylpentane with the help of a Hamilton micro syringe, exactly1 µL of the extract was infused into the injection port of a gas chromatograph along with a mass spectrometer detector (GC-MS, Hewlett Packard 7890A series II). The column comprised of a DB-17 fused silica capillary column (30 m × 0.32 mm i.d. × 0.22 m film thickness). The temperatures of the injector was 250 °C while that of detector was 330 °C (held for 5 min), respectively. The hot air chamber temperature programme started from 60 °C (1 min) and continued at the rate of 20 °C/min to 150 °C and at 5 °C/minute to 280 °C held for 4 min. The injection was done on a splitless injector at 200 °C and the purge activation time was 30 s. The conveyer gas was helium at 30 mL/min; and the splitless flow rate was 19.6 mL/min. The run time was 30 min. The various phyto-constituents were identified by comparing the elution time of standard pesticides with those in the samples, while each pesticide was quantified by comparing the response factor and ion quantity of the pesticides in samples with those in standard. Chemstation software was used to achieve all this.

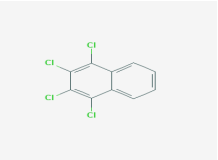
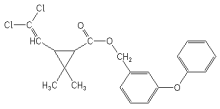
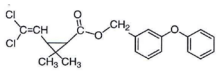
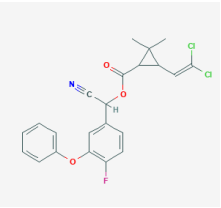
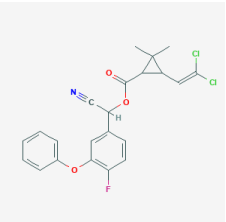
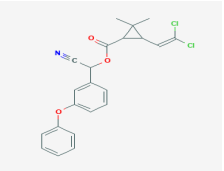
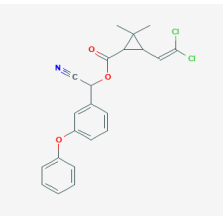
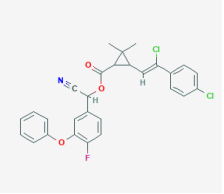
9. Results and Discussion

The results of the preliminary screening of *P. febrifugum* leaf methanolic extract are presented in Table 1.

Table 1. Preliminary Screening of *P. febfifugum* Leaf Methanolic Extract

S/no	Phytochemical	Test/Reagents	Observation	Remark
1	Saponins,	Frothing test.	The formation of an emulsion, is an indication of the presence of saponins	+
2	Flavonoids,	Dil.Ammonia and Conc H_2S0_4.	Yellowish coloration shows the presence of flavonoids.	+
3	Alkaloids	Draggen dorffs and Mayer;s reagent..	The presence of yellowish precipitate indicate the presence of alkaloids	+
4	Cardiac Glycosides	Kedde's Keller-Kiliani	The absence of the brown ring at the interface shows the absence of of deoxysugar properties of cardenolides.	-
5	Terpenoids,	Salkowski test.	The formation of reddish brown coloration at the interface shows the presence of terpenoids	+
	Phenol	Ferric chloric test	The violet or blue colouration shows the presence of phenol	+
	Tannins	Ferric chloride reagent test.	The formation of blue black colouration was observed for presence of tannins	
	Steroids	Salkowski's Liebermann- Burchard	The resultant mixture aid not change colour from violet to blue or green, an indication that steroid is not present -	_

Table 2. Phytoconstituents identified from methanolic leaf extract of *P. febrifugum* by GC-MS analysis

S/No	Name of compound	Retention time	Molecular formula	Molecular weight	Biological activity	Chemical structure
1.	1,2,3,4-Tetra Chloro-naphthalene	4.798	$C_{10}H_4Cl_4$	265.9 g/mol	Antimicrobial/toxic	
2	Permethrin-a	6.753	$C_{21}H_{20}Cl_20_3$	391.29 g/mol	Insecticide/poisonous	
3	Permethrin-b	76.753	$C_{21}H_{20}Cl_20_3$	391.29 g/mol	Insecticide/poisonous	
4	Cyfluthrin-b	9.887	$C_{22}H_{18}Cl_2FN0_3$	434.3 g/mol	Insecticide	
5	Cyfluthrin-d	11.897	$C_{22}H_{18}Cl_2FN0_3$	434.3 g/mol	Insecticide	
6	Cypermethrin -a	13.002	$C_{22}H_{19}Cl_2N0_2$	416.30 g/mol	Poisonous/toxic	
7	Cypermethrin-c	14.007	$C_{22}H_{19}Cl_2N0_2$	416.30 g/mol	Poisonous/toxic	
8	Flumetrin b	19.764	$C_{28}H_{22}Cl_2F0_2$	510.4 g/mol	Toxic/poisonous	

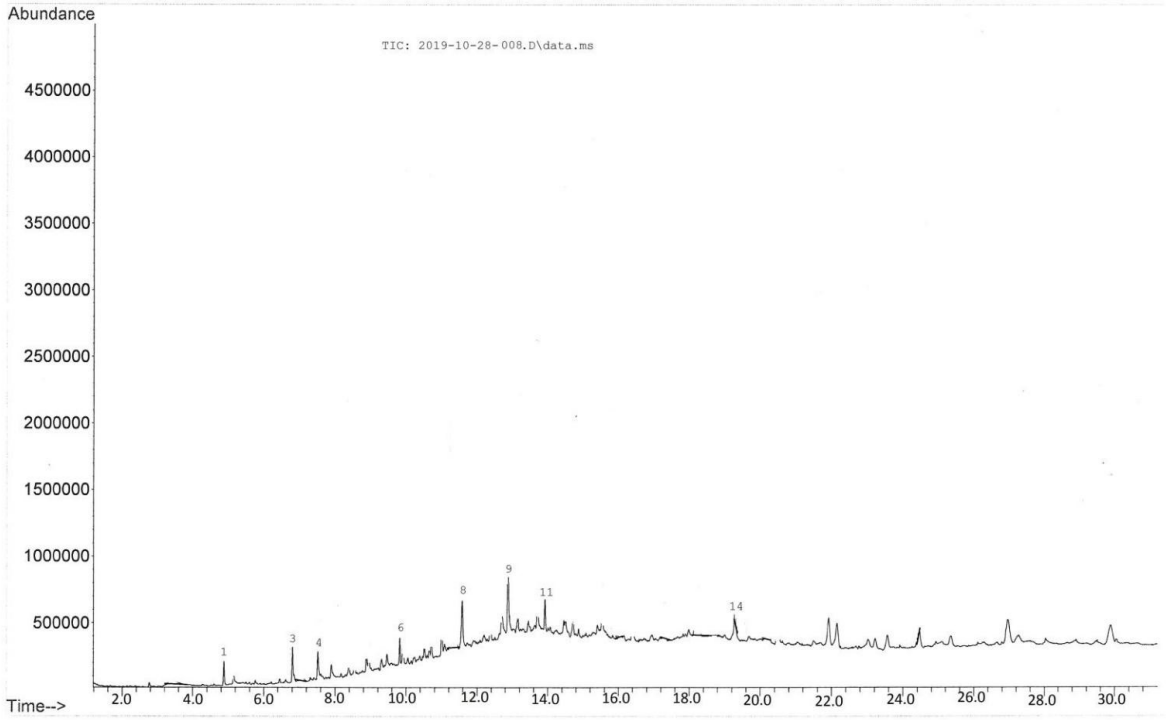


Figure 2. GC-MS Chromatogram of methanolic leaf extract of *P. febrifugum*

Preliminary phytochemical investigation on methanolic leaf extract of *P. febrifugum* revealed presence of species or constituents that have been reported to have antioxidant. These secondary metabolites include: alkaloids, flavonoids, tannin, phenols, saponins, terpenoids (Table 1). These are classes of plant secondary metabolites that are commonly present in plants [17-19]. A number of studies have focused on the biological activities of tannins, phenol, flavonoids and terpenoids which are antioxidants and free radical scavangers [20]. These bioactive secondary metabolites have been demonstrated to have prevented majority of cancers and diabetes, anti-inflammatory, antihepatotoxic, antitumor, antimicrobial and antifungal activities [21].

According to Saeed [22] Flavonoids have been implicated to be highly effective scavengers of most oxygenate molecules that are known for treatment of different diseases. Flavonoids have anti-oxidative and mucosal protective effect [23]. Vegetables with abundant flavonoids are useful on foods since they can be used to treat heart related diseases [24]. The bioavailability and, hence, constant dietary consumption of flavonoids has been documented to give pharmacologically important plasma concentrations in humans [25]. Similarly, research has reported the possible cardioprotective effects of flavonoids against ischemia reperfusion [26]. Saponins may switch on mucous membrane protective factors, while tannins lower the solubility of mucosa to chemical itching. On the other hand, saponins and tannins lower inflammation, exert astringent and protective action on the stomach mucosa, and curb excess acidity. Similarly, terpenoids and alkaloids have also been reported to have potent activity against gastric ulcers [27]. Alves-silva [28] has reported terpenoids to have effect on relax cardiovascular smooth muscle by inhibition of Ca^{2+} ions influx in vascular smooth muscle. The presence of these constituents in methanol fraction of *P. febrifugum* leaves possibly shows its numerous medicinal properties.

The result of GC-MS analysis of methanolic leaf extract of *P. febrifugum* led to identification of the following pesticides: 1,2,3,4-Tetra Chloronaphthalene, Permethrin-a, Permethrin-b, Cyfluthrin-b, Cyfluthrin-d, Cypermethrin-a, Cypermethrin-c, Flumetrin-b. Coleman [29] reported that Permethrin is a medication used in the management and treatment of scabies and pediculosis but although reports of toxicity exist when using permethrin as an insecticide, there are only a few adverse events associated with its topical use. Subramanya [30,31] reported Cyfluthrin and a synthetic cypermethrin pyrethroid insecticide which is sold as a mixture of isomers, and is highly toxic to fish, invertebrates, and insects while less toxic to humans. Flumethrin is effective against cattle ticks (*Boophilus* spp) and has been perfected as a collar combined with propoxur for the prevention of ticks and fleas in dogs [32]. Similarly, WHO [33] reported that a cohort study on workers exposed to chlorinated naphthalenes at a cable manufacturing plant found an excess of deaths from cirrhosis of the liver. From our

results, we conclude that the methanolic extract of *P. febrifugum is* a promising candidate as a botanical compound to control pest and diseases.

The results show that *Psorospermum febrifugum* contains various phytocomponents with potentials as botanical pesticides of interest. The compounds are saponins, flavonoids, alkaloids, terpenoids, phenols and tannins. Cardiac glycosides and steroids are however absent. Isolation of phytochemical constituents and subjecting them to biological activity will definitely yield fruitful results and open a new area for investigation of individual components for their botanical potency.

Conflict of Interest

There is no conflict of interest.

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ARTICLE

Increase of Trigonelline in *Trigonella persica* Plant under Drought Stress

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ABSTRACT

Trigonella persica is a valuable medicinal plant which comprises trigonelline that is secondary metabolite and important component in cosmetic and medicine. This research was conducted in order to evaluation the drought stress effect on growth parameters, root anatomical changes and trigonelline content in *T. persica*. Plants were grown under soil moisture corresponding to 100%, 75%, 50% and 25% field capacity for two weeks. The data showed that drought stress was significantly decreased fresh weight and dry weight of shoot and root. In addition, leaf area was declined due to drought stress. Interestingly, root length was enhanced by drought stress. Root microscopic study demonstrated that drought stress increased thickness of epidermal, endodermal, vascular bundle, central cylinder and parenchyma in *T. persica*. Drought stress caused a significant increment in alkaloid and trigonelline content in aerial parts and roots of *T. persica*. These results revealed that *T. persica* responded to drought stress by increasing the alkaloid and trigonelline, as well as the anatomical changes in root. Considering the importance of trigonline and alkaloids, this work may open prospects for production of the pharmaceutically valuable secondary metabolites thereby drought stress.

1. Introduction

Drought is one of the most important reasons limiting agricultural production, which extremely influences crop yield [1]. Metabolites play a vital role in plant growth and development. Metabolites are involved in energy storage, cell signaling, membrane construction, and whole plant source distribution under stress conditions. Drought stress change plant metabolism and metabolites thereby metabolic enzyme limitation, substrate scarcity, excess demand for particular combinations, or a combination of these and some other factors [2]. Production of the metabolites by the plants is considered an adaptive ability in coping on stress conditions [3].

There is no doubt that drought stress constantly augments the content of specific plant metabolites. However, growth is considerably declined in drought-stressed plants as well; thus, the decrease in biomass could result in the enhanced content of specialized plant metabolites [4,5]. In some recent reports, it had been shown that drought stress promoted production of secondary metabolites include

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phenols and terpenes in plants [6,7]. Alkaloids are the most diverse class among nitrogenous combinations. Alkaloids have numerous biological activities, and there are some drugs accessible on the market manufactured from natural plant alkaloids [8]. The content of total alkaloid in stems of *Dendrobium moniliforme* enhanced under drought stress [9]. Yahyazadeh et al. [10] showed that drought stress increased alkaloid content in *Chelidonium majus* L.

Plant roots, as organs that directly uptake water, play an important role in drought stress. Developed roots can aid plants to completely uptake and consume the stored water in the soil so that plants can live the drought period. Configuration of root system include root branches, root hair and root density can significantly influence the water shortage of plants [11]. Anatomical alterations may occur in roots under drought to maintain and adapt the plants to this stress. Root anatomical properties influence axial and radial water transport in roots, which would influence the efficiency of water absorption and distribution. Cortical characters and the existence of suberized cell layers may influence radial conductance while Xylem vessel characters (diameter, number,and area) influence axial water conductance [12]. Moderate drought enhanced aerenchyma development in root cortex but decreased the diameter and number of xylem vessels in the vascular cylinder in *Typha domingensis* [13].

The genus Trigonella is one of the largest genera of the tribe Trifoliatae in the family Fabaceae and sub-family Papilionaceae [14]. Leaves, stems and seeds of trigonella are consumed in various countries around the world for various goals such as decreasing blood sugar, lowering cholesterol level, anti-diabetic, anti-microbial, anti-cancer, etc. [15]. The pharmacological and biological properties of the trigonella are attributed to the variety of its components such as N-compounds, steroids, amino acids, polyphenolic constituents, and volatile constituents [16]. *Trigonella persica* Boiss is the only endemic species in Iran [17]. Trigoneline is an important metabolite in *T. persica*. Trigoneline or N-methyl nicotinic acid is a secondary metabolite derived from pyridine nucleotides [18]. Trigonelline is considered as a physiologically active constituent in plants which can cause the leaf movements and act as an osmoregulator and osmoprotectant in response to abiotic stresses [19].

Investigating the anatomical and architectural properties that contribute to rooting depth is necessary for prompting crop performance under drought stress. Thus, the aim of this work was to study the impacts of various levels of drought on the root anatomy of *T. persica*. The information will be useful for evaluation of root anatomy associated to drought tolerance and choice of important traits for drought tolerance. There are no data on how drought conditions influence on trigonelline accumulation in *T. persica*. Thus, the other aim of this work was to study how drought stress impacts the content of trigonelline in *T. persica*.

2. Materials and Methods

Plant growth condition and drought treatment

Seeds of *T. persica* were obtained from Semirom Agricultural Institute, Isfahan, Iran, and used for the experiments. The seeds of *T. persica* were sterilized by hypochlorite for 15 min and then rinsed with distilled water. The sterilized seeds were sown in plastic pots. The pots contained autoclaved soil. Soil properties were determined using XRF (PW 2404, Philips, and Netherland) the results of which are shown in Table 1.

Table 1. Physico-chemical properties of the experimental soil

Soil texture	pH	Zn (%)	Rb (%)	Ba (%)	Zr (%)	Sr (%)
Loam-clay	7.8	0.079	0.008	0.066	0.017	0.041

The pots were put in the Phytotron system (at temperature 25 °C and humidity 40%). Also, in this system, 16 h of light (combined of fluorescence and filament lamp) and 8 h of darkness were provided during the growth period (at a photon fluence rate of 3.35 μmol m^{-2} s^{-1}). After forty days, the pots were divided into four groups. There were three replicates per treatment and the plastic pots were arranged into completely randomized block design in a factorial arrangement. Forty day-old plants were irrigated to 100%, 75%, 50%, and 25% FC for two weeks. Irrigation was performed three times per week. Two weeks after water deficit, plants were collected for analyses in all the experiments.

Determination of growth traits

To determinate fresh weight (FW), root, leaf and stem samples were washed off with water to remove soil and blotted gently with soft paper towel to remove any free surface moisture. Fresh weights were determined immediately and dry weights (DW) were measured after drying in an oven at 60 °C for 48 h. Saturated masses of fresh tissues were determined by keeping them in water for 24 h, followed by drying in an oven at 60 °C for 48 h until constant weight was achieved.

To measuring the relative water content (RWC) of leaves, the FW of the leaves was first measured, then the leaves were immersed in distilled water for 48 h in the dark at 4 °C and their saturation weight (SW) was measured. The leaves were then placed in an oven at 60 °C for

48 h and their DW was measured [20]; RWC is calculated from the following equation:

$$RWC\ (\%) = [(FW-DW)/(SW-DW)]100$$

Determination of morphoanatomical parameters in roots

Root samples were fixed in formalin 37%-acetic acid 100%-ethanol 95% (FAA and stored at 4 °C until sectioning. Transverse sections were obtained using a rotating microtome. The sections were stained with methyl blue and methyl green 1% for cellulose and lignin, respectively. Samples were mounted in glycerol and examined with an Olympus microscope (BH 2). Morphoanatomical parameters including, the diameter of root, root epidermis, root cortex and root central cylinder were measured. The measurements were carried out using Image J Software.

Total alkaloid assessment

The content of total alkaloid was determined depending on the method which was described as follows. 1 g dry weight was soaked with 80 mL acetic acid (0.05%), and they were sealed for 18 h. The resulting solution was passed through Whatman No. 3 filter paper. They were washed with chloroform for several times. In the next step, the soluble pH was reached about 7 using ammonia. 10 mL sample solution was put in the 50-mL centrifuge tube and 5 mL potassium buffer was added. Then 5 mL bromocresol green solution. The resulting solution was extracted with 5 mL, 8 mL, and 10 mL of chloroform. The chloroform phase solution was transferred to a 25 mL balloon and then reached volume by chloroform. Finally, absorbance of solution was read at 415 nm. The total alkaloid content was calculated by using atropine standard.

Trigoneline measurement

Dry tissues (0.5 g) of plants were ground with methanol in a mortar and pestle. After incubation at 25 °C for 22 h, the homogenates were centrifuged at 3000 rpm and the supernatant was collected. After complete evaporation of the methanol by rotary evaporator, the methanol-soluble extracts were dissolved in 1 mL methanol. The samples after filtering were used for determination of trigonelline by HPLC [21]. The HPLC system equipped with a reverse-phase C18 column (OSD-UG-5, 4.6 mm × 250 mm × 10 µm) and UV detector. Samples were eluted using the mobile phase of methanol: distilled water (50:50, v/v), adjusted to pH 5 with hydrochloric acid and delivered at a flow rate of 1.0 mL/min. Detection was carried out at 267 nm at room temperature (25 ± 1 °C). The injection volume was 40 µL for all runs. The trigonelline accumulation was determined using a calibration curve compared with standards and a co-chromatogram of the standards and samples. Standard cure and chromatogram of trigonelline were showed in Figure 1.

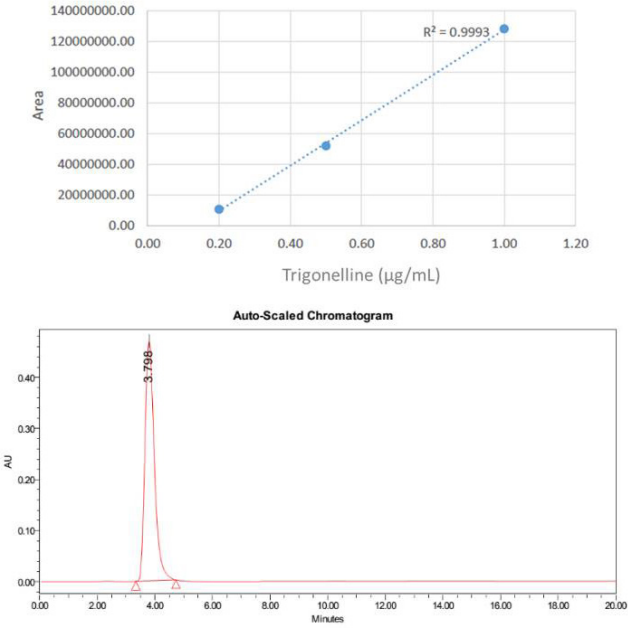

Figure 1. Standard cure and chromatogram of trigonelline.

Statistical methods

All data were analyzed with statistical software SPSS version 19. The means were compared by Duncan's test at the 0.05 level of confidence.

3. Results and Discussion

Drought stress is one of the main abiotic stresses that influences plant growth and biomass production. Roots and shoots were followed by measuring length, FW and DW in *T. persica* plants under different drought treatments. The results shown in Table 2 indicated that drought stress deteriorated all growth parameters include shoot length, shoot FW, shoot DW, root FW, root DW, leaf area and RWC, except root length as compared to control. The largest decrease in growth parameters occurred at 25% FC. These consequences might be owing to the declination of photosynthesis, reduced cell turgidity, enhanced evapotranspiration, declined CO_2 assimilation due to stomatal closure, and finally, decreased cell division under drought stress [22,23]. Enhancement of root length was as a defense strategy to deal with drought conditions. A similar decrease in growth has been previously witnessed under drought stress in *Fagopyrum tataricum* [24]. Rezayian et al. [25] showed that drought stress reduced growth in *Brassica napus*.

Table 2. Effect of different irrigation levels on growth parameters in *T. persica* plant

Field capacity (%)	Shoot length (cm)	Shoot FW (mg)	Shoot DW (mg)	Root length (cm)	Root FW (mg)	Root DW (mg)	Leaf area (cm^2)	RWC (%)
100	20.26±1.50 a	82±6.24 a	4.03±2.95 a	4.8±0.70 b	21±2.22 ab	8±1.30 ab	4.32±0.99 a	67.89±2.11 a
75	13.06±2.01 ab	52±5 b	1.60±1.21 a	5.06±0.70 b	25±1.11 a	9±0.99 a	1.49±0.12 b	49.57± 1.2 ab
50	11.66±1.2 b	64±6 b	6.33±5.50 a	5.56±0.81 ab	18±1.01 c	6±0.87 c	1.61±0.32 b	38.67±2.45 bc
25	11.93±1.65 b	60.33±7.7 b	1.60±1.31 a	6.83±0.76 a	10± 1.06 c	5±0.43 c	0.43±0.08 c	21.47±1.09 c

Mean ± SE based on three replicates for growth parameters are presented

Root traits influence the amount of nutrient and water absorption, and are essential for preserving crop yield under drought conditions [26]. Cross section of *T. persica* root was studied to assess the anatomical adaptations of this plant to be acclimatized under drought stress. There were significant changes in anatomical features of root of *T. persica* plants imposed to various levels of drought (Figure 2 and Table 3). By examining the cross sections of root from control and treated plants, it was observed that thickness of epidermal and endodermal layer was increased as compared to control. On the other hand, thickness of central cylinder and parenchyma enhanced in drought-treated plants. The vascular bundle of drought-exposed plants was significantly greater in diameter and number than the controls. According to obtained data, drought caused a significant increment in the root diameter in plants exposed to drought stress compared with the controls.

Table 3. The results of anatomical measurements of *T. persica* root under different irrigation levels

Field capacity (%)	Epidermal thickness (µm)	Endodermal thickness (µm)	Central cylinder thickness (µm)	Parenchyma thickness (µm)	Vascular bundle number	Vascular bundle diameter (µm)	Root diameter (µm)
100	9.53±1.50 b	1.81±0.24 c	138.23±2.15 b	303.33±6.70 c	3.33±0.57 c	138.33±18.9 b	783±8 c
75	15.53±2.01 a	3±0.90 b	182±3.21 a	338.66±2.70 b	4.33±1.52 c	182±12.12 a	829±19 b
50	15±1.52 a	2.5±0.96 b	189±2.50 a	383.66±2.61 a	6.66±1.15 b	189±14.93 a	66.3 ±15 a
25	14.66±2.65 a	4.23±0.87 a	146±3.31 b	351±3.56 a	8.66±1.15 a	146±23.71 b	824±18 b

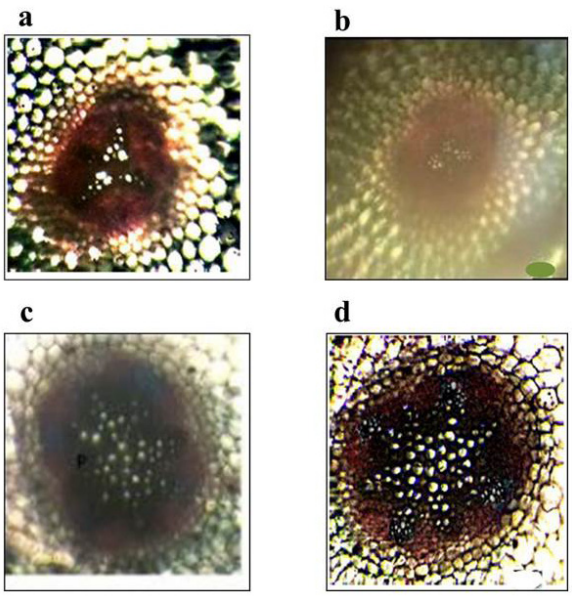

Figure 2. Cross sections of the roots in the drought-treated *T. persica* plants.
a (100% FC), b (75% FC), c (25% FC), d (50% FC).

Under drought conditions, roots expand to aid extract soil moisture which being held at larger surface tension [27]. Also, deep root growth and xylem diameter in roots may enhance the capacity of roots to mine more water in deep soil when water in deep soil is abundant. Roots with greater length support plants to enhance water absorption and conserve plant biomass under drought conditions by enhancing surface area and root length in contact with soil water [28,29].

Our findings propose a complex network of anatomical adaptations such as with increased epidermal and endodermal thickness, enhanced vascular bundle, augmented root diameter end etc. These proprieties are required for the conservation of water potential and energy storage under drought stress which can develop the resistance of *T. persica* to survive in drought conditions. The improvement of endodermal layers around the stele are considered mean to avoid the dryness of meristematic tissues [30]. In this study, root diameter enhanced in *T. persica under* drought condition, it has direct relation with water uptake and has more capacity to explore soil [31,32]. Numerous studies have reported the significance of root system for absorption of water from soil layers under drought condition in different plants such as rice (*Oryza sativa* L.) [33] and wheat [34].

In this experiment, an increase in the production of total alkaloid was observed in aerial parts and roots when *T. persica* plants were kept under severe drought conditions (Figure 3a,b). Our data suggest that the cultivation of *T. persica* in severe drought stress would serve as suitable treatment for accumulating alkaloid. The fact that drought stress causes increases in particular metabolism constituents such as alkaloids has been shown by some authors, such as Ghorbanpour et al. [35] in work with *Hyoscyamus niger*; and Kleinwächter et al. [36] with spices. The up-regulation of the biosynthesis of alkaloids may contribute to decline in the reducing status of the electron transport chain under stress conditions. These compounds have a further role in the dissipation of excess energy and thus inhibit the generation of toxic oxygen radicals [10,37]. Liu et al. [38] stated that drought stress enhanced alkaloid content by increasing the expression of genes involved in their biosynthesis.

Trigonelline content was quantified in aerial parts and roots of *T. persica* plants under drought conditions (Figure 4a,b). The findings demonstrated that trigonelline content increased in the plants exposed to moderate (5% FC) and severe (25% FC) drought stress. An increase in trigonel-

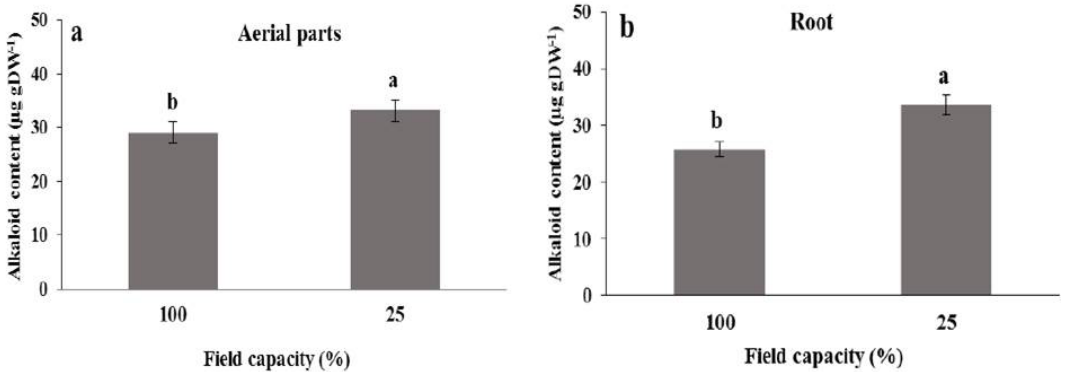

Figure 3. Effect of drought stress on alkaloid content in aerial parts (a) and root (b) of *T. persica*. Values are means ± SE of three replicates. Different letters indicated significant ($p<0.05$) differences.

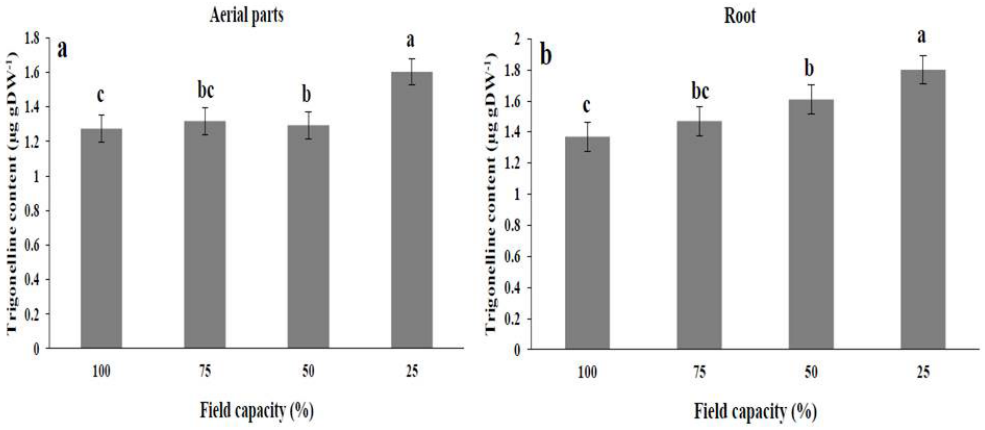

Figure 4. Change in trigonelline content in aerial parts (a) and root (b) of *T. persica* by different drought stress. Values are means ± SE of three replicates. Different letters indicated significant ($p<0.05$) differences.

line content in drought conditions compared to control supports the protecting role of this biomolecule under unsuitable environmental conditions. This increase in severe drought level was higher compared to moderate drought level. Possibly, the more accumulation of secondary metabolites in plants in a stressed environment occurred to inhibit too enormous production of reactive oxygen species (ROS) and corresponding injuries by photoinhibition [4,39]. Some studies have presented the role of trigonelline in the mechanism of plant defense [40,41]. Dadrasan et al. [42] and Zamani et al. [43] reported that drought stress enhanced trigonelline accumulation in *T. foenum-graecum*. The demand for secondary metabolites from plants for the medicinal industry coupled with the low yields necessitate abiotic or biotic factors augmenting the potential to produce beneficial phytochemicals. Therefore, due to the importance of trigonelline, drought stress can be used for the accumulation of this valuable substance in *T. persica plant.*

In conclusion, this study showed that drought stress decreased *T. persica* growth and induced changes in anatomical characteristics such as thickness of epidermal, endodermal, vascular bundle, central cylinder and parenchyma in *T. persica* roots. Drought stress enhanced alkaloid content and trigonelline content in *T. persica* plants. Therefore, it is possible to use this stress to increase the valuable metabolites in this plant.

Conflicts of Interest

The authors declare no potential conflicts of interest regarding the publication of this paper.

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EDITORIAL

Research Advances of Anthocyanin Accumulation in Plants Tissues

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The color of plants is mainly due to the rich content of chlorophylls, carotenoids, betalains, flavonoids, and other substances. Anthocyanins as an important group of flavonoids, have an important effect on the color of plant tissues, especially fruit color [1,2]. In the early 1900s, a summary of Mendelian studies on pea coloration was given by Weldon [3]. Since then, the anthocyanin biosynthetic pathway has been continuously refined, until it was first summarized in plants in detail [4]. Anthocyanins are important secondary metabolites in plants, and the metabolic starting material is phenylalanine produced from glucose breakdown. Finally, various anthocyanins resulting from glycosylation modifications, are furtherly modified by methylation accomplished by O-methyltransferases (OMTs) to produce final products such as malvidin, peonidin and petunidin [5-7].

Generally, the anthocyanins formed in the cytoplasm will be transferred to the cell vacuole for storage to avoid degradation and display multiple colors. Recent years, researchers also pay attention to the decisive effect of anthocyanin vacuolar transport on fruit anthocyanin accumulation. At present, the research on anthocyanin transport is mainly focused on the study of multidrug and toxic compound extrusion (MATE) and glutathione-S-transferase (GST) transporters. Previous report revealed the key role of MATE in the process of transferring anthocyanins from small vesicles to vacuoles in transgenic hairy roots of grapes [8]. The important effect of GST on anthocyanin transport was successively demonstrated on fruits such as apple, peach and so on [9,10].

The expression of plant anthocyanin biosynthetic genes is regulated by MYB-bHLH-WD40, called MBW complex, with a relatively conserved mechanism. The MBW complex is formed by specific interactions between

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MYB transcription factors, bHLH transcription factors and WD40 proteins, and its interaction was validated in apple fruit [11]. Studies on the regulatory mechanism of anthocyanin formation in *Arabidopsis*, have shown that MYB transcription factors such as AtMYB11/12/111 have an activating effect on the expression of the early genes of anthocyanin biosynthesis, AtCHS, AtCHI, AtF3H and AtF3'H, and the expression of the late synthesis genes, AtDFR, AtLDOX, and AtUFGT, is regulated by the MBW complex formed by AtMYB75/90/113/114 and so on [12,13]. It follows that MYB transcription factors play a crucial role in the regulation of anthocyanin biosynthetic genes. In addition, the stimulation of external environment can cause dramatic changes in anthocyanin accumulation in plant tissues. The effect of light exposure on anthocyanin accumulation is mainly determined by 2 factors, light intensity and wavelength [14]. Temperature can significantly affect the accumulation of anthocyanins and has an effect on its stability [15,16]. Drought stress, exogenous nitrogen and other treatments can also affect fruit coloration [17,18]. Furthermore, anthocyanin accumulation is also influenced by phytohormone signaling pathways such as ethylene (ETH), jasmonic acid (JA), abscisic acid (ABA), auxin and so on [19-22].

Conflict of Interest

There is no conflict of interest.

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ARTICLE

Enumeration of Family Fabaceae from Sechu Tuan Nalla Wildlife Sanctuary, Chamba District, Himachal Pradesh (India)

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ABSTRACT

An account of 20 species under 11 genera of the family Fabaceae is presented based upon a thorough study of the collected specimens and field surveys in this paper from Sechu Tuan Nalla Wildlife Sanctuary, Chamba district, Himachal Pradesh. Of these, five taxa are reported first time from the Chamba district of the state. The updated nomenclature of the species, local name if any, a brief description of the plant, flowering and fruiting period, distribution in the study area, habitat and ecology and specimen examined have been provided.

1. Introduction

The Sechu Tuan Nalla Wildlife Sanctuary, a high-altitude sanctuary that lies in Sechu valley, a minor sub-valley of the major Pangi valley is located at the extreme northwest end of Chamba district of Himachal Pradesh. The wildlife sanctuary is located in the inner Trans Himalayan region between two great mountain ranges i.e. The Great Himalayan Range and Pir Panjal Range in the Chamba district of Himachal Pradesh. The sanctuary is situated within the geo-coordinates of North Lat. 33°10′55” N & Long. 76°43′24” E East Lat. 32°57′31” & Long. 76°46′38” E, South Lat. 32°49′49” N & Long. 76°45′00” E West Lat. 32°54′18” & Long. 76°31′22” E (Figure 1A-C). It is one of the innermost valleys of the Great Himalayas. It is bounded by the interstate boundary of Jammu and Kashmir in Northern and Lahaul-Spiti district (Himachal Pradesh) on the North-eastern and South-eastern sides.

Due to inaccessibility and difficult geographic conditions, this area had not been included in the earlier floristic surveys of the Chamba district [1-6]. Therefore, as such, no literature or any kind of comprehensive published document about the floristic diversity of the sanctuary is available for reference and use by forest officials and various other government agencies. Hence, an attempt has been made to document the members of the family Fabaceae from the study area.

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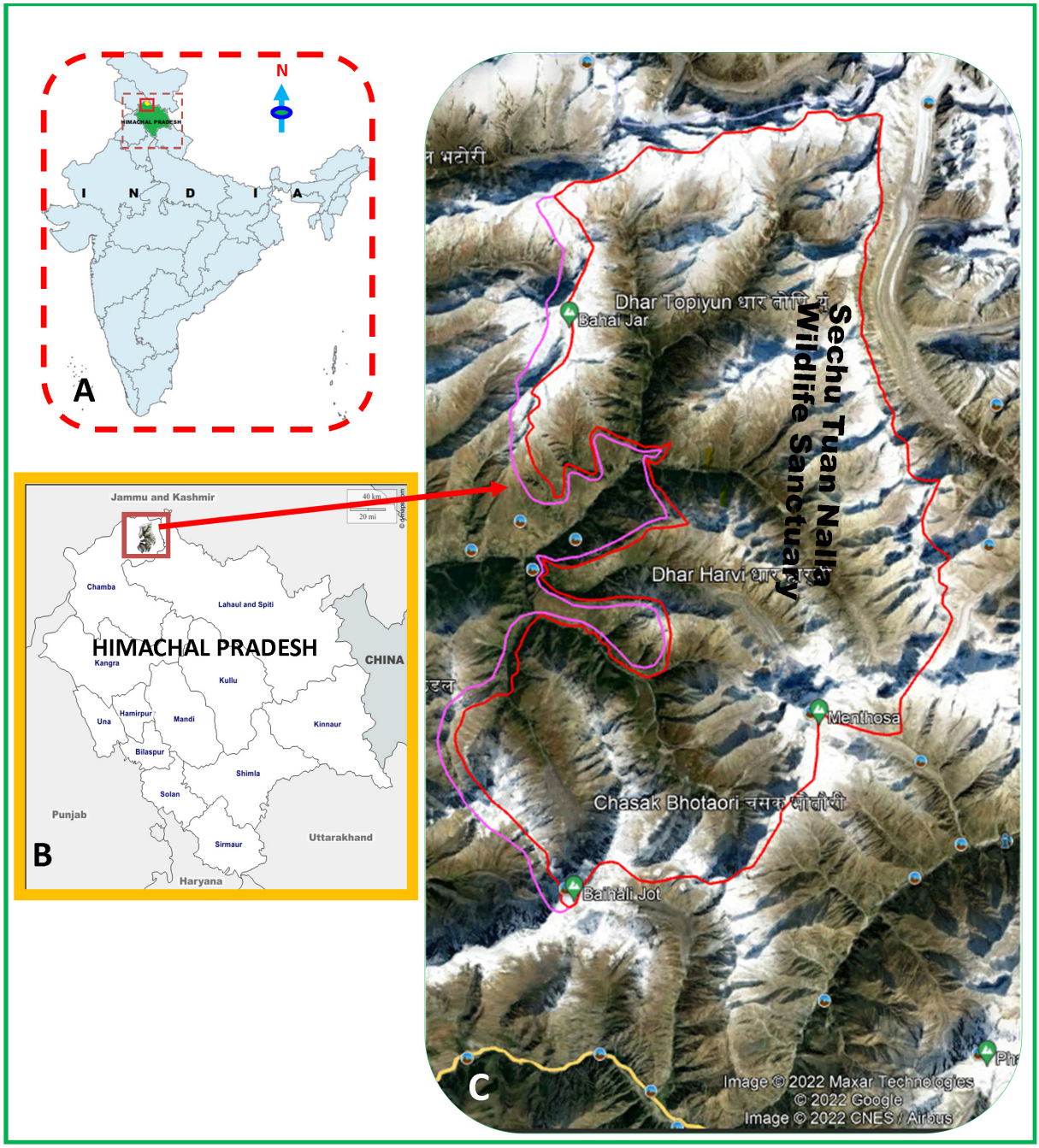

Figure 1. Map for the localization of the area in India and in the district. A) Himachal Pradesh in India; B) Chamba district and Sechu Tuan Nalla Wildlife Sanctuary in Himachal Pradesh India; C) Sechu Tuan Nalla Wildlife Sanctuary (Red colour indicates the boundery of the sanctuary, and the area between purple and red outline is eco-sensetive zone of the sanctuary)

2. Materials and Methods

During the field surveys of Sechu Tuan Nalla Wildlife Sanctuary, Chamba district, Himachal Pradesh the author collected members of the family Fabaceae for assessment of the floristic diversity of the sanctuary. The collected specimens were processed and preserved as suggested by Jain & Rao [7]. Later on, the specimens were identified and an attempt has been made to bring out a systematic account of the family Fabaceae from the study area. Updated nomenclature of the species, local name if any, a brief description of the plant, flowering and fruiting period, distribution in the study area, habitat and ecology and specimen examined have been provided. The specimens are deposited in the herbarium of Botanical Survey of India, Northern Regional Centre, Dehradun (BSD).

3. Results and Discussions

This study revealed that the sanctuary inhabits 20 species under 11 genera of the family Fabaceae. All the reported genera fall under the subfamily Papilionoideae DC. as proposed by The Legume Phylogeny Working Group [8]. Genera and species are arranged alphabetically.

Enumeration

(1) ASTRAGALUS L., Sp. Pl. 2: 755. 1753.

1) Astragalus chlorostachys Lindl. in Trans. Hist. Soc. 7: 249. 1828; Sanjappa, Legum. India 85. 1992; Sud. Kumar & P.V. Sane, Legum. South Asia 225. 2003; H. Singh & M. Sharma, Fl. Chamba 258. 2006; Deroliya & al., Ann. For. 69. 2019.

Perennial herbs, to 1.2 m tall; stems much branched, stipules free, lanceolate. Leaves imparipinnately compound. Flowers pale yellow or pinkish-white, dense, in axillary, pedunculate racemes. Pods turgid, glabrous.

Flowering & Fruiting: July-September.

Distribution in Sanctuary, habitat and ecology: Common, on moist rocky slopes between 2800 m- 3100 m.

Specimen examined: Harbi Dhar, *P. Kumar* 127310; Sechu Dhar, *P. Kumar* 127744; Along Triund Nalha towards Chogalu Dhar, *P. Kumar* 128192.

2) Astragalus coluteocarpus subsp. **chitralensis** Wenn., Mitt. Bot. Staatssamml. München 30: 52.1992; Kumar & Sane, Legum. South Asia: Checkl. 225. 2003; Podlech & Zarre, Tax. Rev. gen. Astragalus (Legum.) Old World 1: 247. 2013; L.B. Chaudhary, Rev. gen. Astragalus L. (Leguminosae-Papilionoideae) India 52. 2018. *A. coluteocarpus* var. *glaber* Ali, Kew Bull. 1958: 304. 1958; Sanjappa, Legum. India 86. 1992.

Perennial herbs or small shrubs; nearly 2 m high, stems erect, leaves 8 cm-18 cm long, imparipinnately compound. Inflorescence 5 cm-20 cm long, axillary, long peduncled raceme, many-flowered. Flowers c. 15 mm long, pale yellow and maroon-purple in some parts of corolla and calyx. Pods young 10-15 × 1.5-2 mm, stipitate, oblong, pointed at both ends, glabrous.

Flowering & Fruiting: July-September.

Distribution in Sanctuary, habitat and ecology: Not common, only few individuals found in Betula forest, under partly shady moist slopes at elevation between 3230 m-3701 m. (Figure 2A)

Specimen examined: Sidhani Dhar, *P. Kumar* 132531; On way to Sechu from Murch, near bridge, *P. Kumar* 127575; Topiyun Dhar, *P. Kumar* 127656; Along Triund Nalha towards Chogalu Dhar, *P. Kumar* 128161.

3) Astragalus himalayanus Klotz., B. Reise Pr. Waddem. 160, t. 4. 1862; Sanjappa, Legum. India 89. 1992; Sud. Kumar & P.V. Sane, Legum. South Asia 230. 2003; Deroliya & al., Ann. For. 70. 2019.

Annual herbs, ascending or suberect, 25 cm-55 cm high; stems slender, well branched, pubescent. Leaves imparipinnately compound. Flowers purplish, in axillary, pedunculate racemes. Pods c 1 cm long, unilocular, pubescent.

Flowering & Fruiting: June-September.

Distribution in Sanctuary, habitat and ecology: Common, on open grassy slopes, open forest area along river, sandy places, open dry slopes in Pine forest, alpine scrub, moist open slopes and dry sandy river beds between 2800 m-4000 m.

Specimen examined: Eco-sensitive Zone near Tuan, *P. Kumar* 127140; Along Triund Nalla towards Chogalu Dhar,127200; Harbi Dhar, *P. Kumar* 127314; Pepe Nalla, Chasakh Bhatori, *P. Kumar* 127474; Along Sindhmarh Nalla upwards, *P. Kumar* 127619; Topiyun Dhar, 127668; Along Triund Nalha, *P. Kumar* 127841; Sidhani Dhar, *P. Kumar* 127970; Eco-sensitive zone, around Hillu and Tuan villages, *P. Kumar* 128056.

4) Astragalus malacophyllus Benth. ex Bunge, Astrag. 1:36. 1868. 2:61. 1969; Sanjappa, Legum. India 91. 1992.

Perennial herbs; stems branched from base. Leaves imparipinnately compound. Flowers in an axillary peduncled raceme, yellow. Pods c. 11 mm-13 mm long, silky, sessile.

Flowering & Fruiting: May-August.

Distribution in Sanctuary, habitat and ecology: Not common on open grassy slopes up to 3800 m.

Specimen examined: Towards North of Bhatori Seri along Sindhmarh Nalla, *P. Kumar* 127554.

Note: Podlech & Zarre [9], Kumar & Sane [10], and Deroliya *et al*. [11], have treated this species as synonym under the *A. rhizanthus* Royle ex Benth, however Chaudhary [12], considered it as two separate species.

5) Astragalus melanostachys Benth. ex Bunge, Astrag. 1:21. 1868. 2:22. 1869; Sanjappa, Legum. India 91. 1992; Sud. Kumar & P.V. Sane, Legum. South Asia 234. 2003; Deroliya & al., Ann. For. 70. 2019.

Perennial herbs, to 15 cm-55 cm tall; stems branched, branches many from the base. Leaf imparipinnately compound. Flowers in an axillary pedunculate raceme. Pods sessile, globular or ovoid.

Flowering & Fruiting: June-August.

Distribution in Sanctuary, habitat and ecology: Not common, in moist gassy places near stream up to 3408 m.

Specimen examined: Along Triund Nalha towards Chogalu Dhar, *P. Kumar* 128119.

6) Astragalus rhizanthus Royle ex Benth. in Royle, Ill. Bot. Himal. Mount. 200. 1835; Sanjappa, Legum.

India 93. 1992; Sud. Kumar & P.V. Sane, Legum. South Asia 239. 2003; Deroliya & al., Ann. For. 71. 2019.

Perennial herbs; rootstock woody; aerial stem almost absent or reduced with crowded internodes. Leave; imparipinnately compound. Flowers yellow in clustered, sessile or shortly peduncled racemes; Pods 1.2 cm-2.0 cm long, sessile, densely silky.

Flowering & Fruiting: June-July.

Distribution in Sanctuary, habitat and ecology: Common, on dry river beds and grassy slopes in alpine areas between 2900 m-4000 m. (Figure 2B)

Specimen examined: Pepe Nalla, Chasakh Bhatori, *P. Kumar* 127437; Pepe Nalla, Chasakh Bhatori, *P. Kumar* 127473; Pepe Nalla, Chasakh Bhatori, *P. Kumar* 127498; Sidhani Dhar, *P. Kumar* 128006; Eco-sensitive zone, around Hillu and Tuan villages, *P. Kumar* 128046.

7) Astragalus tecti-mundi subsp. **orientalis** Podlech in Sendtnera 7: 178. 2001; Kumar & Sane, South Asia Legum.: Checkl. 242. 2003. *Astragalus frigidus* (L.) A. Gray in Proc. Amer. Acad. Arts 6: 219. 1864; Sanjappa, Legum. India 87. 1992; Sud. Kumar & P.V. Sane, Legum. South Asia 242. 2003; Deroliya & al., Ann. For. 70. 2019.

Perennial herbs, to 30 cm-55 cm high; stems erect, simple, glabrous. Leaf imparipinnately compound. Inflorescence an axillary pedunculate raceme. Pods c. 2.2 cm-2.7 cm long, pubescent, unilocular, stiptate.

Flowering & Fruiting: July-September

Distribution in Sanctuary, habitat and ecology: Occasional, in alpine meadows and moist places along streams between 3500 m-3900 m. (Figure 2C)

Specimen examined: Along Sindhmarh Nalla upwards, *P. Kumar* 127617; Along Triund Nalha towards Chogalu Dhar, *P. Kumar* 132513.

(2) CICER L., Sp. Pl. 1: 738. 1753.

Cicer microphyllum Royle, Ill. Bot. Himal. Mount. 200. 1835; Sanjappa, Legum. India 113. 1992; Sud. Kumar & P.V. Sane, Legum. South Asia 140. 2003; Deroliya & al., Ann. For. 73. 2019.

Jangli Mattar

Perennial herbs; stems ribbed, zig-zag, branched. Leaves pinnate. Inflorescence 1-2 flowered; flowers purple or white. Pod 22 mm-29 mm long, beaked.

Flowering & Fruiting: June-August.

Distribution in Sanctuary, habitat and ecology: occasional, in sandy places 2900 m-3600 m.

Specimen examined: Topiyun Dhar, *P. Kumar* 127658; Sechu Dhar, *P. Kumar* 127717; Harbi Dhar, *P. Kumar* 127839.

Uses: Fruits are eaten raw.

(3) HEDYSARUM L., Sp. Pl. 2: 745. 1753.

1) Hedysarum astragaloides Benth. in Hook.f., Fl. Brit. Ind. 2: 146. 1876; Sanjappa, Legum. India 183. 1992; Sud. Kumar & P.V. Sane, Legum. South Asia 266. 2003; Lal & al in J. Jpn. Bot. 89: 233. 2014; Deroliya & al., Ann. For. 76. 2019.

Perennial herbs, to 60 cm tall; stems erect. Leaves compound; leaflets 22-30, 1.7-2.3 cm long, narrowly ovate to elliptic, obtuse, mucronate, glabrescent above, pubescent below. Inflorescence a dense raceme; flowers yellowish. Pod stipitate, 1-2 jointed, joints oblong, membranous, wing crisped on the lower side and obscure above.

Flowering & Fruiting: June-August.

Distribution in Sanctuary, habitat and ecology: occasional, in moist alpine grassy slopes 3400 m-3826 m. (Figure 2D) Endemic to India [Western Himalayas (Himachal Pradesh, Jammu & Kashmir, Uttarakhand)].

Specimen examined: Pepe Nalla, Chasakh Bhatori, *P. Kumar* 127422; Eco-sensitive zone, around Hillu and Tuan villages, *P. Kumar* 128057; Along Triund Nalha towards Chogalu Dhar, *P. Kumar* 128128.

2) Hedysarum microcalyx Baker in Hook.f., Fl. Brit. India 2: 147. 1876; Sanjappa, Legum. India 184. 1992; Sud. Kumar & P.V. Sane, Legum. South Asia 267. 2003;Deroliya & al., Ann. For. 76. 2019.

Perennial herbs, tufted, spreading, to 55 cm high; stems branched, grooved, Leaves imparipinnately compound. Flowers purple, in racemes. Pods 2-3-jointed, long-stipitate, membranous, entire, both sutures distinctly margined.

Flowering & Fruiting: July-October.

Distribution in Sanctuary, habitat and ecology: occasional, along streams and in moist places in thickets between 2700 m-3700 m. (Figure 2E) Endemic to India [Western Himalayas (Himachal Pradesh, Jammu & Kashmir, Uttarakhand)].

Specimen examined: Towards North of Bhatori Seri along Sindhmarh Nalla, 127557; Way to Sidhani Dhar, *P. Kumar* 127775.

Note: Inspite of the fact that it is a purely high-altitude species, in literature it is also shown to distributed in Punjab (altitude range 150 m-550 m).

(4) LATHYRUS L., Sp. Pl. 2: 729. 1753.

1) Lathyrus emodii (Wall. ex Fritsch) Ali in Biologia. 11(2): 4. 1965; Sud. Kumar & P.V. Sane, Legum. South Asia 415. 2003; Deroliya & al., Ann. For. 77. 2019. *Orobus emodii* Wall. ex Fritsch in Sitzungsber. Akad. Wissensch. Wien. Math.-Naturw. Classe. 104: 489. 1896. *Lathyrus laevigatus* subsp. *emodii* (Wall. ex Fritsch) Ohashi in H. Hara & al., Enum. Fl. Pl. Nepal 2: 123.

1979; Sanjappa, Legum. India 201. 1992.

Perennial herbs, to 90 cm high; stems suberect, branched. Leaves petioled, pinnately compound; Flowers cream or pink, turning yellow, in axillary racemes. Pods c 6 cm × 0.8 cm, cyclindrical, glabrous.

Flowering & Fruiting: July-September

Distribution in Sanctuary, habitat and ecology: not common in moist shady places in forest up to 2918 m.

Specimen examined: Sidhani Dhar, *P. Kumar* 128001.

2) Lathyrus humilis (Ser.) Fisch. ex Spreng., Syst. Veg. 3: 263. 1826; Sanjappa, Legum. India 201. 1992; Sud. Kumar & P.V. Sane, Legum. South Asia 416. 2003; Deroliya & al., Ann. For. 77. 2019. *Orobus humilis* Ser. in DC., Prodr. 2: 378. 1825. *Lathyrus ovatus* Royle ex Benth. in Royle, Ill. Bot. Himal. Mts. 1: 200. 1835.

Perennial herbs; stems suberect, branched. Leaves paripinnately compound. Flowers purple to slightly blue, 2-6-flowered, in racemes. Pods c 5 cm × 0.5 cm, cylindrical, glabrous.

Flowering & Fruiting: June-July.

Distribution in Sanctuary, habitat and ecology: Occasional, in partly shady places in forest up to 2881 m. (Figure 2F)

Specimen examined: Sechu Dhar & Eco-sensitive Zone, *P. Kumar* 127939; Sidhani Dhar, *P. Kumar* 127991.

(5) LESPEDEZA Michx., Fl. Bor.-Amer. 2: 70, t. 39. 1803.

Lespedeza juncea (L.f.) Pers., Syn. Pl. 2:318. 1807; Sanjappa, Legum. India 203. 1992; Sud. Kumar & P.V. Sane, Legum. South Asia 211. 2003; H. Singh & M. Sharma, Fl. Chamba 280. 2006; Deroliya & al., Ann. For. 78. 2019. *Hedysarum junceum* L.f., Decas prima Pl. t.4. 1762. *Lespedeza aitchisonii* Ricker in Lingnan Sci. J. 20:199. 1942.

Undershrub, up to 1 m tall; stems branched. Leaf trifoliolate. Inflorescence 2-4-flowered pedunculate umbel; flowers pale yellow or pink. Pod 2.5 mm-3.0 mm long, silky.

Flowering & Fruiting: July-September.

Distribution in Sanctuary, habitat and ecology: occasional, on dry open slopes between 2900 m-3400 m.

Specimen examined: Harbi Dhar, *P. Kumar* 127328; Along Triund Nalha towards Chogalu Dhar, *P. Kumar* 128169.

(6) LOTUS L., Sp. Pl. 2: 773. 1753.

Lotus corniculatus L., Sp. Pl. 775. 1753; Sanjappa, Legum. India 205. 1992; Sud. Kumar & P.V. Sane, Legum. South Asia 291. 2003; H. Singh & M. Sharma, Fl. Chamba 281. 2006; Deroliya & al., Ann. For. 78. 2019 (publ. 2022).

Perennial herbs, prostrate, ascending or decumbent, variable; stems branched. Leaves petioled, 5-foliolate. Flowers pale-yellow or orange, 3-6-flowered, in peduncled umbels. Pods c 3 cm × 0.4 cm, cylindrical, straight, glabrous.

Flowering & Fruiting: July-September.

Distribution in Sanctuary, habitat and ecology: common, on open dry and sandy slopes between 3000 m-3900 m. (Figure 2G)

Specimen examined: Eco-sensitive Zone near Tuan, *P. Kumar* 127133; Pepe Nalla, Chasakh Bhatori, *P. Kumar* 127493.

(7) MEDICAGO L., Sp. Pl. 2: 778. 1753.

1) Medicago falcata L., Sp. Pl. 779. 1753; Sanjappa, Legum. India 209. 1992; Sud. Kumar & P.V. Sane, Legum. South Asia 403. 2003; Deroliya & al., Ann. For. 78. 2019.

Perennial herbs up to 80 cm long; stem erect or procumbent. Leaves compound. Inflorescence a peduncled raceme. Pods 8 mm-11 mm long, c. 2.5 mm broad, nearly straight to crescentic.

Flowering & Fruiting: March-August.

Distribution in Sanctuary, habitat and ecology: occasional, on open slopes up to 3006 m. (Figure 2H)

Specimen examined: Eco-sensitive Zone near Tuan, *P. Kumar* 127106.

2) Medicago lupulina L. Sp. Pl. 779. 1753; Sanjappa, Legum. India 209. 1992; Sud. Kumar & P.V. Sane, Legum. South Asia 404. 2003; H. Singh & M. Sharma, Fl. Chamba 282. 2006; Deroliya & al., Ann. For. 78. 2019.

Annual or perennial herbs, up to 60 cm long; stems prostrate or ascending. Leaves compound. Inflorescence an axillary, pedunculate raceme; flowers yellow. Pods 2 mm-3 mm, curved.

Flowering & Fruiting: July-September.

Distribution in Sanctuary, habitat and ecology: occasional on moist slopes up to 3606 m.

Specimen examined: Along Jamboo Nalha, *P. Kumar* 127936.

(8) OXYTROPIS DC., Astragalogia 24, 66; 19, 53.1802.

Oxytropis lapponica (Wahl.) Gay, Flora 10: 30. 1827; Sanjappa, Legum. India 205. 1992; Sud. Kumar & P.V. Sane, Legum. South Asia 256. 2003; Deroliya & al., Ann. For. 79. 2019. *Phaca lapponica* Wahl. Veg. Helv. 131. 1813. *Oxytropis lapponica* var. *xanthantha* Baker in Hook.f. Fl. Brit. India 2: 137. 1876.

Perennial herbs, to 8 cm-30 cm tall; rootstock woody; aerial stem with 2 or more apparent internodes. Leaves compound. Flowers in many flowered racemes; flowers pale purple. Pod cylindric, stipitate inflated, unilocular.

Flowering & Fruiting: June-September.

Distribution in Sanctuary, habitat and ecology: common, in moist open slopes near glacier, alpine grassy, open dry and sandy slopes between 2700 m-4000 m.

Specimen examined: Sechu Dhar, *P. Kumar* 127282; Pepe Nalla, Chasakh Bhatori, *P. Kumar* 127405; Along Sindhmarh Nalla upwards, *P. Kumar* 127578; Along Sindhmarh Nalla upwards, *P. Kumar* 127612; Topiyun Dhar, 127694; Harbi Dhar, *P. Kumar* 127833; Along Triund Nalha, 127880, 128198; Sidhani Dhar, *P. Kumar* 127978,132545; Eco-sensitive zone, towards Sidhani bia Mujh village, *P. Kumar* 128024; Eco-sensitive zone, around Hillu and Tuan villages, *P. Kumar* 128042.

(9) THERMOPSIS R. Br., Hort. Kew., ed. 2, 3: 3.1811.

Thermopsis barbata Royle, Ill. Bot. Himal. Mts. 1: t. 32. 1834; Sanjappa, Legum. India 261. 1992; Sud. Kumar & P.V. Sane, Legum. South Asia 403. 2003; Deroliya & al., Ann. For. 83. 2019.

Annual or perennial herbs, tufted, to 20 cm high; stems stout, branched from base. Leaves shortly petioled, trifoliolate. Flowers deep purple, showy, crowded, in axillary and terminal racemes. Pods linear-oblong, hairy.

Flowering & Fruiting: July-September.

Distribution in Sanctuary, habitat and ecology: occasional, on moist open slopes up to 3500 m. (Figure 2I)

Specimen examined: Along Triund Nalha towards Chogalu Dhar, *P. Kumar* 128178.

(10) TRIFOLIUM L., Sp. Pl. 1: 764. 1753.

Trifolium repens L., Sp. Pl. 767. 1753; Sanjappa, Legum. India 263. 1992; Sud. Kumar & P.V. Sane, Legum. South Asia 412. 2003; H. Singh & M. Sharma, Fl. Chamba 291. 2006; Deroliya & al., Ann. For. 83. 2019.

Perennial herbs, trailing, glabrous; stems slender, prostrate, branched. Leaves petioled, trifoliolate. Flowers white, fragrant, many-flowered, clustered, in pedunculate, globose racemes forming heads. Pods minute, linear, included, 3-4-seeded.

Flowering & Fruiting: July-September.

Distribution in Sanctuary, habitat and ecology: common, in moist places, 3000 m-3200 m. (Figure 2J)

Specimen examined: Sechu Dhar, *P. Kumar* 127291; Harbi Dhar, *P. Kumar* 127835.

(11) TRIGONELLA L., Sp. Pl. 1: 776. 1753.

Trigonella emodi Benth. in Royle, Ill. Bot. Himal. Mts. 1: 197. 1835; Sanjappa, Legum. India 264. 1992; Sud. Kumar & P.V. Sane, Legum. South Asia 413. 2003; Deroliya & al., Ann. For. 84. 2019.

Kuchona

Perennial herbs, erect or ascending; stems much branched. Leaves pinnately trifoliolate. Flowers yellow, in axillary pedunculate condensed racemes. Pods c 1.4 cm × 0.2 cm, linear-oblong, straight, glabrous, veins prominent.

Flowering & Fruiting: July-September.

Distribution in Sanctuary, habitat and ecology: common, in moist places sandy river beds 2900 m-3600 m.

Specimen examined: Sidhani Dhar, *P. Kumar* 127996; Eco-sensitive zone, around Hillu and Tuan villages, *P. Kumar* 128058; Along Triund Nalha towards Chogalu Dhar, *P. Kumar* 128148.

Uses: Young shoots are used as vegetable.

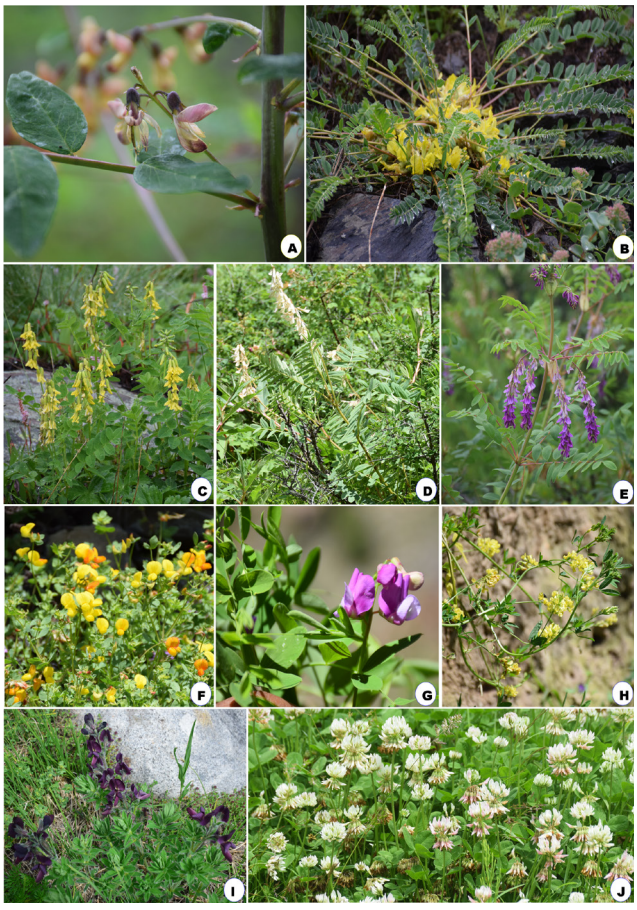

Figure 2. Field photographs: A) *Astragalus coluteocarpus* subsp. *chitralensis* Wenn.; B) *Astragalus rhizanthus* Royle ex Benth.; C) *Astragalus tecti-mundi* subsp. *orientalis* Podlech; D) *Hedysarum astragaloides* Benth. in Hook.f.; E) *Hedysarum microcalyx* Baker; F) *Lathyrus humilis* (Ser.) Fisch. ex Spreng.; G) *Lotus corniculatus* L.; H) *Medicago falcata* L.; I) *Thermopsis barbata* Royle; J) *Trifolium repens* L.

4. Conclusions

This study reveals that Sechu Tuan Nalla Wildlife Sanctuary harbours a considerable number of taxa of the family Fabaceae, which is 2.98 percent of the total taxa of Fabaceae of the state [6], whereas it is 24.39 percent of the total taxa of Fabaceae of the district Chamba [1,9]. Two species namely *Hedysarum astragaloides* Benth. and *H. microcalyx* Baker are endemic to North-West Himalayas. Five taxa *viz*., *Astragalus coluteocarpus* subsp. *chitralensis* Wenn., *A. melanostachys* Benth. ex Bunge, *A. tecti-mundi* subsp. *orientalis* Podlech, *Hedysarum microcalyx* Baker, and *Oxytropis lapponica* (Wahl.) Gay are reported first time from Chamba district, Himachal Pradesh.

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Conflict of Interest

Authors declare no conflict of interests.

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