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A Field Screening of a Pomegranate (*Punica granatum*) Ex-Situ Germplasm Collection for Resistance against the False Spider Mite (*Tenuipalpus punicae*)

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Abstract: Mite management is a major problem in pomegranate (*Punica granatum* L.) cultivation in the arid and semi-arid regions of India and other Asian countries. The aim of this work was to investigate the susceptibility to the false spider mite (*Tenuipalpus punicae*) in a germplasm collection of Indian pomegranates. A field screening of 73 accessions allowed to define different classes of susceptibility (from very low to very high) based on the percentage of infested leaves. Twenty-two accessions, representative of the empirically identified five susceptibility classes, were further tested. The field screening against the mite, extended to another two years, showed that the infestation level did not display a significant interaction with the growing season, and highly correlated between the different growing seasons. The analysis of the tree vegetative growth (height, canopy size, and stem diameter), main phytochemical classes (total phenolics, flavonoids, and tannins) and the antioxidant activity of the leaves indicated strong significant negative correlations between the infestation level and the biochemical traits. Multidimensional reduction of the measured traits revealed that the extreme classes of susceptibility to mites are mainly separated according to the accumulation of phytochemicals in leaves. This work, for the first time, allowed the identification of pomegranate germplasm with low susceptibility to *T. punicae*, with positive and useful implications for the establishment of new orchards, plant breeding, and the identification of allelochemicals of the leaves directly affecting mites.

Keywords: pest; host resistance; breeding; fruit crop; phytochemicals; leaves; selection; Acari

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1. Introduction

Pomegranate (*Punica granatum* L., Lythraceae, formerly Punicaceae) is a vigorous perennial woody plant trained in agriculture as a shrub. This species has the tendency to develop multiple stems from the ground level when left unpruned, and it rapidly develops a bushy appearance. Pomegranate is cultivated for its strongly colored fruit, a berry with a hard pericarp (husk) and a spongy mesocarp containing numerous seeds surrounded by a fleshy and juicy seedcoat (sarcotesta). Usually, the plant is trained to develop three to five main stems, with branches pruned to a vase-shaped system [1], although in recent years different structures, based for instance on a trellised single stem, are also being used [2]. The height of the plant is often contained well below four meters, especially because fruit picking is typically manual.

Pomegranate probably originated in Central Asia and it has been almost invariantly considered a minor fruit crop, despite its adaptability to less-than-optimal conditions [3]. Nonetheless, this species is increasingly grown worldwide. Traditional production regions share long, dry, and hot summers, and comprise countries around the Mediterranean basin (e.g., Turkey, Tunisia, Morocco, Spain, Israel, Greece, and Italy) and in the Middle and Central Asia (e.g., Iran, Iraq, and India, the country with the largest cultivated area in the world) [4]. In the last decades, increasing awareness of the health benefit of the plants in the western world, along with developments in agro-food processing (e.g., industrial and domestic juice extraction) and related new commercial products (jellies, puree, syrups, powder, juice blends and concentrate), has increased the demand for the fruits, ultimately favoring the diffusion of pomegranate cultivation also in countries such as China, USA, South Africa, Chile and Argentina [2,5].

Pomegranate diversity is expected to be high considering its long history of cultivation, geographical diffusion, drought tolerance, ability to survive if abandoned, and ease of propagation (the use of rootstocks is uncommon). Germplasm collections have been established in various countries [6–9]. India is considered an important centre of diversity also because its large geographical extension and related different climatic zones, and various collections were established [6,7]. These resources are important also because pomegranate breeding is active in India, with various cultivars employed for the international market [2]. Currently, cultivation is mainly based on released germplasm although in some areas, like in western Himalaya, highly adapted local varieties are largely employed [10,11].

Biotic stress of pomegranate largely varies between the cultivation regions [12]. Among arthropods, the mite *Tenuipalpus punicae* Pirtchard et Baker 1958 (Acari: Tenuipalpidae), known as pomegranate false spider mite, can easily reach the pest status in arid and semi-arid areas of North-western India (e.g., Rajasthan, Gujarat), around the Mediterranean basin (e.g., Egypt and Israel), and in other Asian countries [13,14]. In Iraq, this species is considered the most serious pest of pomegranate in dry areas [15]. Mites of the genus *Tenuipalpus* preferentially feed on the underside of the leaves [16]. Leaves attacked by *T. punicae* are easily recognized because of their reddish color (rusty leaves) and the presence of individuals at different developmental stages usually close to the midrib [15,17]. Large populations of *T. punicae* can cause premature leaf senescence and abscission, negatively affecting yield and fruit quality because of a higher risk of fruit sunburns [18]. Mites are mostly active in the period from early spring (March) to October, with the highest population density in summer (depending on temperature and drought spells), and with adult females overwintering during the cold season [14,15].

The screening and selection of the existing diversity is an essential step to improve the sustainability of agriculture. Specifically, resistant cultivars are an integral part of Integrated Pest Management (IPM) strategies and are highly desirable to increase economic returns and limit the environmental impact of chemical control strategies. The evaluation of the resistance level in crop germplasm may provide useful information on the main features that govern this trait, fostering more detailed molecular studies on selected plant features and genotypes. Cultivated pomegranate varieties show large variation not only in peel color and juice acidity [2], but also in different phytochemicals, including those with an established role in resistance to biotic stress [19]. For example, it has been proposed that polyphenol level influences the susceptibility of pomegranate fruits to the fungal pathogen *Pilidiella granati* (syn. *Coniella granati*) [20]. Moreover, host plant resistance in agriculture can be elicited by plant bioregulators [21] and consequently, its characterization is important to increase the efficacy of standard cultural control practices.

Pomegranate varieties have been analysed to describe the susceptibility to fungal diseases such as the anthracnose by *Colletotrichum gloesporoides*, using a detached leaves bioassay [22], and the dry fruit rot and leaf spot caused by *P. granati* in field conditions [23]. Cultivated and wild accession have also been screened for susceptibility against bacterial pathogens such as *Xanthomonas axonopodis* pv. *Punicae* Tanuja [24]. Regarding arthropods, a field investigation was carried out on cultivated germplasm for resistance against the carob

moth *Ectomyelois ceratoniae* Zeller (Lepidoptera: Pyralidae), a major pest in the Middle East attacking fruits [25]. All these works suggested that in the cultivated germplasm of pomegranate the variability of the susceptibility to biotic stress is sufficiently ample to define distinct classes, typically ranging from very high to very low percentages of attack [22–25].

The aim of this work was to analyze a collection of pomegranate accessions against the false spider mite *Tenuipalpus granati* Sayed (Acari: Tenuipalpidae) to identify the less susceptible genotypes. Moreover, we also investigated some morphological traits related to plant vigor and the total content in leaves of main classes of phytochemicals known to be involved in plant-pest interaction, to infer some associations with the susceptibility level of the accessions.

2. Materials and Methods

2.1. Experimental Site, Plant Material and Management

The experiment was carried out on pomegranate plants belonging to the ex-situ germplasm collection at the experimental station of the Central Institute for Arid Horticulture (ICAR), in Bikaner, India (28°06′45.0′′ N 73°20′52.4′′ E; 235 m above sea level). The open-field collection includes 73 cutting-propagated accessions obtained from different sources, whose origin is reported in Supplementary Table S1. Plants (six per accession) were spaced 4 m × 6 m (corresponding to a planting density of 417 plants/ha) and trained to a multi-stem system with 3–4 stems per plant. Plants were managed according to practices generally adopted for commercial fruit production and without pesticide treatments. Each season, plants were pruned after fruit harvesting during winter (January–February) and uniform intercultural operations followed (i.e., desuckering, weeding, irrigation, fertilization). Pruned materials were collected and disposed of outside the experimental field. Weeds were removed around the plant basin by spade as per need, while harrowing was done with tractor-driven disc harrow in between the lines of plants in February, July, September, and November. Irrigation was applied through a drip irrigation system uniformly to all plants. Recommended dose of manure and fertilizers (i.e., 45 kg FYM, 625 g N, 250 g P and 250 g K) per plant per year were applied. A micronutrient solution (2% iron, 2% manganese, 5% zinc, 0.5% copper, 0.05% molybdenum and 0.5% boron, on *w/w* basis) was applied twice as foliar spray, one month and two months after fruit set. Weather data were recorded at a meteorological station located in the campus (approximately 500 m away from the experimental field) and are reported in Supplementary Figure S1.

2.2. Field Screening of Mite Infestation on Pomegranate Accessions

The data of mite infestation were recorded in the second half of September, at the peak of rusty leaves appearance that follows the highest population density of the pest. Fifty leaves per accession were randomly selected at shoulder height from three plants per accession, sealed in plastic bags, and immediately transported to a laboratory. Each leaf was visually inspected for the presence of infestation by *T. punicae* to calculate the percentage of infested leaves per accession (PIL). Mites were separated from leaves with the help of a fine paintbrush and examined using a stereo binocular microscope at 20× magnification (Radical Scientific Equipment Ltd., Ambala Cant, India). For taxonomic identification, mite samples were preserved in 70% alcohol and sent to the Insect Biosystematic Section, Division of Entomology, ICAR-Indian Agricultural Research Institute, New Delhi.

In 2018, a screening of mite infestation was carried out on the 73 accessions available in the collection (Supplementary Table S1). Using the infestation data measured in the first season, a subset of 22 accessions was selected as representative of the different infestation classes. The accessions were: Achik Dana, Agah, AHPG-C3, Basin Seedless, Bhagwa, Dholka, Dorsata Malus, Goma Khatta, Gul-e-Shah Rose Pink, HP Collec., IC-318712, Jalore Seedless, Jodhpur Red, Kajaki Anar, P-23, Phule Arakta, Saharanpur), Saih Sirin, Speen Sacarin, Sur Sukker, Tujetis EC 4347, and Uthkal. The PIL of these accessions was measured in the 2019 and 2020 seasons, along with vegetative and phytochemical traits.

2.3. Plant Vegetative Traits

Five trees of each of the 22 pomegranate selected accessions were used to measure plant height, canopy diameter, and stem diameter. Canopy diameter of each plant was measured in two orthogonal directions (North-South and East-West). Canopy perimeter was estimated assuming its shape as an ellipse having as axes the two measured diameters. Plant height and canopy diameters were assessed with a measuring tape. The basal diameter of all the main stems was measured using a Digital Vernier Caliper (model 300 mm, Mitutoyo, Kawasaki, Japan).

2.4. Phytochemical Traits of the Leaves

Ten healthy leaves of each of the selected 22 accessions were collected (as for the infestation analysis), weighed, cut into small pieces, and freeze-dried. Weighed dried tissue was extracted with a 70% methanol solution. The total phenolics in extracts were estimated following the Folin-Ciocalteu method as previously described [26]. The total phenolics content was expressed as mg gallic acid equivalents (GAE)/g DW. All samples were analyzed in triplicates. Total flavonoids content in extracts was determined by the aluminum chloride colorimetric assay as reported [27] with minor modifications. A volume of extract (1 mL) was mixed with 0.3 mL each of 5% $NaNO_2$, 10% $AlCl_3$ and 3.4 mL of 1 M NaOH. The mixture was incubated for 15 min at room temperature and spectrophotometrically read at 510 nm. The total flavonoid content was expressed as mg catechole equivalent (Cat. E)/g DW. The assays were carried out in triplicate. Total antioxidant activity of the extracts was assayed using an already described CUPRAC method with some modifications [28]. In this assay, 1 mL each of cupric chloride (10 mM), ethanolic neocuproine (75 mM) and ammonium acetate (1 M; pH 7.0) were mixed simultaneously in a test tube containing 2 mL of distilled water followed by 100 µL of methanolic extract. These mixtures were incubated in the dark for 30 min at room temperature and then, absorbance was measured at 450 nm. Ascorbic acid was used as calibration standard, and results were expressed as mg ascorbic acid equivalent (AAE)/g DW. The assays were done in triplicate. The content of condensed tannins was determined as reported [29] using catechin as a reference standard. A volume of 1 mL ethanolic extract was added to 3 mL of 4% vanillin in methanol and 1.5 mL concentrated hydrochloric acid. After incubation for 15 min at room temperature in the dark, the absorbance was measured at 500 nm. The condensed tannin content was expressed as mg catechin equivalent per gram DW. All chemicals were purchased from Sigma-Aldrich Chemicals Private Limited, Bangalore (India). Spectrophotometric analyses were performed with a Shimadzu UV-1601 (Shimadzu, Kyoto, Japan) using 1 cm quartz cuvettes.

2.5. Statistical Analysis

The significance of the effect of the growing season (GS), the accession (A), and the GS × A interaction on the PIL and the vegetative traits (measured in 2019 and 2020) were assessed with a two-way ANOVA procedure. The significance of the effect of the accession on the PIL (measured in 2018 during the mass screening) and on leaf composition parameters (measured in 2019) was assessed with one-way ANOVA procedure. The Tukey's honestly significant difference (HSD) test was used for mean separation ($p \leq 0.05$). Before running the ANOVAs, the arcsine square root transformation was applied to the PIL data.

For the data collected in the mass screening, the frequency distribution of the 73 accessions in PIL classes was analyzed studying the probability density function calculated with the Kernel density estimation method. Pearson correlation coefficient (n = 3) between the percent infested leaves, the vegetative traits, and the leaf composition parameters was calculated using the data collected in 2019. The correlation matrix was represented as a correlogram. The Spearman's rank-order correlation coefficient (n = 3) was calculated between the percent infested leaves measured in 2019 and in 2020 (in the 22 selected accessions). In addition, differences between accessions in the vegetative traits and leaf composition were studied with a Principal Component Analysis (PCA) using, as input, seven original

variables: plant height, canopy diameter, stem diameter, and leaf polyphenols, flavonoids, tannins, and antioxidant activity. Statistical analyses and graphical representation were done using R 4.2.

3. Results

3.1. Screening of Pomegranate Accessions

The percentage of infested leaves (PIL) significantly differed among the 73 pomegranate accessions analyzed ranging between 5.8% (HP Collec.) and 63.3% (Bedana Suri) (Table 1).

Table 1. Percent infested leaves (PIL) and assigned infestation severity class (ISC) (very light, VL: PIL $\leq$ 8%; light, L: 8% < PIL $\leq$ 20%; moderate, M: 20% < PIL $\leq$ 32%; severe, S: 32% < PIL $\leq$ 44; very severe, VS: PIL > 44%) measured in 73 accessions of pomegranate. Means followed by a common letter (lower or upper case) are not significantly different according to the Tukey test ($p \leq 0.05$).

Accession	Infestation (%)	ISC	Accession	Infestation (%)	ISC
A K Anar	22.5 ± 1.4 opqrstuv	M	Jalore Red	32.5 ± 1.4 hijklm	S
Achik Dana	17.5 ± 1.4 tuvwxyz	L	Jalore Seedless	23.3 ± 0.8 nopqrstu	M
Agah	16.7 ± 0.8 uvwxyzA	L	Jodhpur coll.	37.5 ± 1.4 efghi	S
AH-PG-H3	32.5 ± 1.4 wxyzABCD	L	Jodhpur Red	27.5 ± 1.4 klmnopqr	M
AHPG-C1	14.2 ± 0.8 BCDE	L	Jyoti	25.8 ± 0.8 lmnopqrs	M
AHPG-C3	8.3 ± 0.8 wxyzABCD	L	Kabul	42.5 ± 1.4 def	S
AHPG-C4	14.2 ± 0.8 mnopqrst	M	Kabul Kohinoor	14.2 ± 0.8 wxyzABCD	L
AHPG-H1	25.0 ± 1.4 ghijkl	S	Kajaki Anar	55.0 ± 1.4 bc	VS
AHPG-H2	33.3 ± 0.8 hijklm	S	Kalisirin	17.5 ± 1.4 tuvwxyz	L
AHPG-H4	14.2 ± 0.8 wxyzABCD	L	Kandhari	15.8 ± 1.7 uvwxyzAB	L
Alah	30.0 ± 2.9 ijklmno	M	Khog	37.5 ± 1.4 efghi	S
Banaras collect.	37.5 ± 1.4 efghi	S	Kurvi	38.3 ± 1.7 efgh	S
Basin Seedling	57.5 ± 1.4 ab	VS	Malta	16.7 ± 0.8 uvwxyzA	L
Basin Seedless	9.2 ± 0.8 ABCDE	L	MR 599	15.8 ± 0.8 uvwxyzAB	L
Bedana Sedana	21.7 ± 0.8 pqrstuvw	M	Mridula	37.5 ± 1.4 efghi	S
Bedana Suri	63.3 ± 2.2 a	VS	Muskat	40.8 ± 0.8 defg	S
Bedana Thin Skin	29.2 ± 0.8 jklmnop	M	P-13	29.2 ± 0.8 jklmnop	M
Bhagwa	29.2 ± 0.8 jklmnop	M	P-21	14.2 ± 0.8 wxyzABCD	L
Boseka Link	15.8 ± 0.8 uvwxyzAB	L	P-23	44.2 ± 0.8 de	VS
Coimb. White	43.3 ± 1.7 def	S	P-26	25.8 ± 0.8 lmnopqrs	M
Crenedo de Elecho	12.5 ± 1.4 xyzABCDE	L	Patna-5	37.5 ± 1.4 efghi	S
Dholka	15.0 ± 1.4 vwxyzABC	L	Phule Arakta	37.5 ± 1.4 efghi	S
Dorsata Malus	47.5 ± 1.4 cd	VS	Ruby	17.5 ± 1.4 tuvwxyz	L
EC-12613	20.8 ± 0.8 qrstuvw	M	Saharanpur	25.0 ± 1.4 mnopqrst	M
EC-62812	30.8 ± 0.8 hijklmn	M	Saih Sirin	42.5 ± 1.4 def	S
G-137	30.8 ± 0.8 hijklmn	M	Sirin	11.7 ± 0.8 yzABCDE	L
Ganesh	28.3 ± 2.2 jklmnopq	M	Speen Danedar	29.2 ± 0.8 jklmnop	M
GKVK-1	11.7 ± 0.8 yzABCDE	L	Speen Sacarin	7.5 ± 1.4 CDE	VL
Goma Khatta	14.2 ± 0.8 wxyzABCD	L	Sur Sukker	20.0 ± 1.4 rstuvwx	L
Gul-e-Shah	14.2 ± 0.8 wxyzABCD	L	Surat Anar	15.8 ± 2.2 uvwxyzAB	L
Gul-e-Shah Red	14.2 ± 0.8 DE	VL	Surkh Anar	35.8 ± 0.8 fghij	S
Gul-e-Shah Rose Pink	6.7 ± 0.8 wxyzABCD	L	Tebest	19.2 ± 2.2 stuvwxy	L
Gulsa Red	21.7 ± 0.8 pqrstuvw	M	Tujetis EC 4347	15.0 ± 1.4 vwxyzABC	L
HP Collec.	5.8 ± 0.8 E	VL	Uthkal	10.8 ± 0.8 zABCDE	L
IC-318712	55.8 ± 0.8 ab	VS	Yercaud	47.5 ± 1.4 cd	VS
IIHR 12/1	34.2 ± 0.8 ghijk	S	Yercaud Local	44.2 ± 0.8 de	VS
IIHR 19/10	42.5 ± 1.4 def	S			

In absence of previous information on the classification of the severity of *T. punicae* infestation, we divided our germplasm in five classes according to the distribution of the PIL (Figure 1).

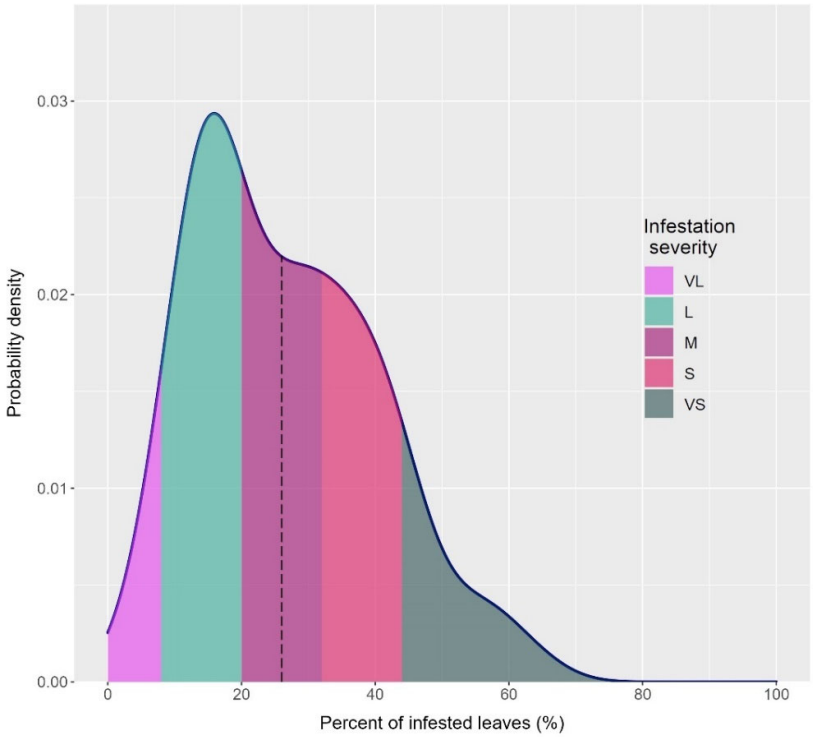

Figure 1. Frequency distribution of the 73 pomegranate accessions into classes of percent of infested leaves (PIL). Different colors indicate the infestation severity class as indicated in the color scale on the right-hand side (very light, VL: PIL $\leq$ 8%; light, L: 8% < PIL $\leq$ 20%; moderate, M: 20% < PIL $\leq$ 32%; severe, S: 32% < PIL $\leq$ 44; very severe, VS: PIL > 44%). The blue continuous line indicates the probability density function calculated with the Kernel density estimation method. The dashed vertical line indicates the PIL value (26%) adopted to center the middle bin (M). Bin size was 12%.

The middle class was centered on the mean PIL value of the population (rounded at 26%). The bin size (12%) was then calculated dividing the observed maximum level of infestation (around 60%) by the number of classes desired. Therefore, the accessions were divided into the following classes of infestation severity: very light (VL: PIL $\leq$ 8%), light (L: 8% < PIL $\leq$ 20%), moderate (M: 20% < PIL $\leq$ 32%), severe (S: 32% < PIL $\leq$ 44) and very severe (VS: PIL > 44%). According to this classification, three accessions (4%) had very light (VL) infestation intensity (Gul-e-Shah Rose Pink, HP Collec., Speen Sacarin), whereas 37% of the accessions (27 accessions) had a low infestation severity (L) (Table 1). The infestation intensity was moderate (M) in 18 accessions (25%), severe (S) in 17 accessions (23%) and very severe (VS) in 8 accessions (11%) (Table 2).

Table 2. Effect of the growing season, accession, and their interaction on vegetative traits of pomegranate plants. Separately for each source of variation and within each column, means followed by a common letter are not significantly different according to the Tukey test ($p \leq 0.05$).

Source of Variation	Plant Height (m)	Canopy Perimeter (m)	Stem Diameter (cm)
Growing season (GS)			
2019	1.30 ± 0.03 b	3.50 ± 0.09 b	1.48 ± 0.04 b
2020	1.59 ± 0.03 a	4.74 ± 0.11 a	1.78 ± 0.05 a
Significance	***	***	***
Accession (A)			
Achik Dana	1.12 ± 0.04 j	3.28 ± 0.13 kl	1.43 ± 0.09 defgh
Agah	1.48 ± 0.02 defgh	3.26 ± 0.12 kl	1.36 ± 0.10 fgh
AHPG-C3	1.59 ± 0.14 bcd	4.63 ± 0.54 d	1.57 ± 0.09 defg
Basin Seedless	1.33 ± 0.02 i	4.07 ± 0.19 fg	1.81 ± 0.09 bcd
Bhagwa	1.90 ± 0.03 a	5.75 ± 0.18 a	1.98 ± 0.10 abc
Dholka	1.52 ± 0.07 cdef	4.49 ± 0.25 de	1.25 ± 0.07 gh
Dorsata Malus	1.40 ± 0.05 fghi	3.90 ± 0.46 ghi	1.68 ± 0.10 cdef
Goma Khatta	1.54 ± 0.03 cde	4.70 ± 0.23 cd	1.67 ± 0.10 cdef
Gul-e-Shah Rose Pink	1.47 ± 0.11 defgh	3.77 ± 0.16 ghij	1.39 ± 0.09 efgh
HP Collec.	1.49 ± 0.05 defg	4.27 ± 0.27 ef	1.81 ± 0.12 bcd
IC-318712	0.99 ± 0.02 k	3.16 ± 0.07 l	1.57 ± 0.12 defg
Jalore Seedless	1.37 ± 0.02 ghi	3.65 ± 0.26 ij	2.28 ± 0.09 a
Jodhpur Red	1.61 ± 0.04 bc	4.68 ± 0.21 d	1.51 ± 0.10 defg
Kajaki Anar	1.12 ± 0.03 j	3.19 ± 0.09 l	1.46 ± 0.07 defgh
P-23	1.32 ± 0.09 i	4.26 ± 0.51 ef	1.44 ± 0.08 defgh
Phule Arakta	1.70 ± 0.03 b	5.36 ± 0.11 b	1.83 ± 0.11 bcd
Saharanpur	1.40 ± 0.06 fghi	4.02 ± 0.40 fgh	2.01 ± 0.10 abc
Saih Sirin	1.37 ± 0.11 ghi	3.70 ± 0.31 hij	1.46 ± 0.10 defgh
Speen Sacarin	1.51 ± 0.07 cdef	4.07 ± 0.14 fg	1.78 ± 0.09 bcde
Sur Sukker	1.42 ± 0.16 efghi	3.83 ± 0.45 ghij	1.27 ± 0.08 gh
Tujetis EC 4347	1.36 ± 0.17 hi	3.55 ± 0.51 jk	1.08 ± 0.08 h
Uthkal	1.83 ± 0.21 a	5.04 ± 0.74 bc	2.11 ± 0.14 ab
Significance	***	***	***
GS × A	***	***	n.s.

*** and n.s. indicate significant differences at $p \leq 0.05$, $p \leq 0.001$ and not significant ($p > 0.05$) according to the two-way ANOVA, respectively.

3.2. Mite Infestation Severity in the Selected 22 Accessions

The severity of the infestation of the representative subset of 22 accessions was measured in the following two consecutive years. The growing season (GS) and the accession (A) significantly affected the PIL, whereas this parameter was not affected by the GS × A interaction (Supplementary Table S2). Mean PIL was only slightly higher in 2020 than in 2019 (25.1% and 24.4%, respectively), but all the 22 accessions under investigation had similar infestation severities in the two growing seasons. The Spearman's rank-order correlation coefficient calculated between the percent infested leaves measured in 2019 and in 2020 was very high ($\rho = 0.997$; $p < 0.01$). Consistently with the selection of a range of susceptibility levels, the PIL largely varied among accessions, ranging between 5.25% (HP Collec.) and 58.0% (IC-318712) (Figure 2).

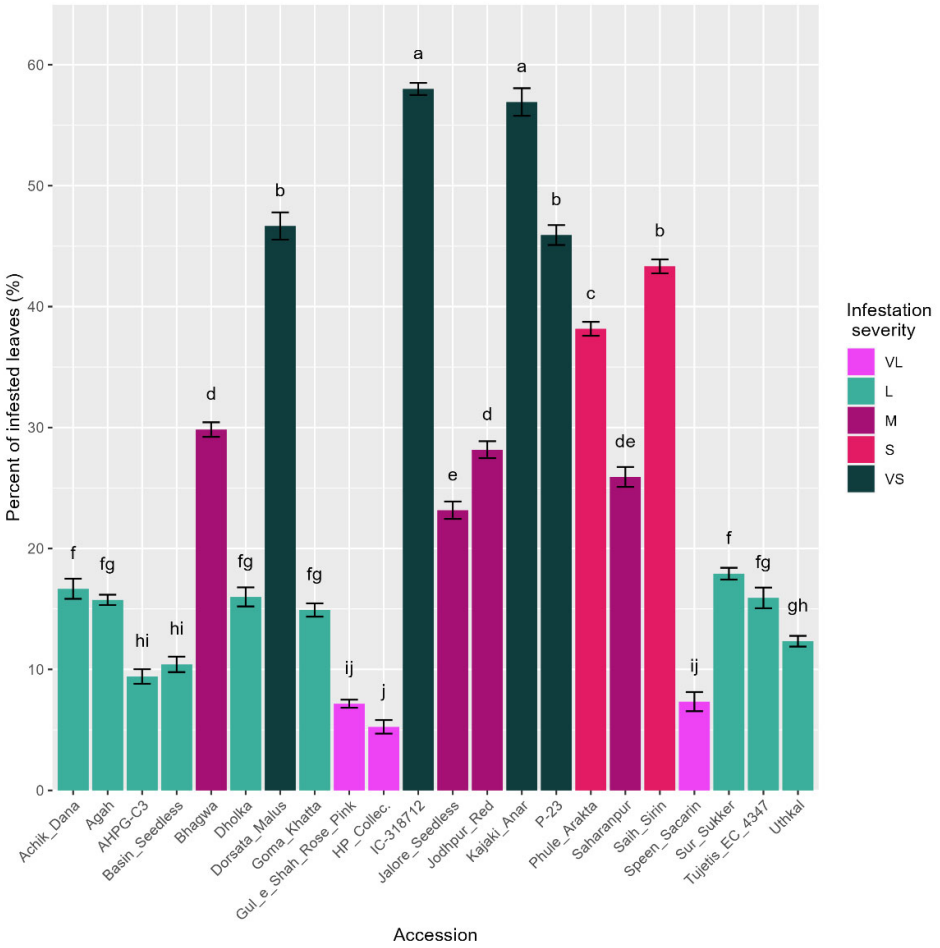

Figure 2. Percent of infested leaves measured in 22 pomegranate accessions (n = 50). Different colors indicate the infestation severity class according to the color scale on the right-hand side. Bars with a common letter indicate that differences between accessions are not significant according to the Tukey test ($p \leq 0.05$).

Three accessions (Gul-e-Shah Rose Pink, HP Collec., and Speen of Sacarin) had the lowest PIL (ranging between 5.3% and 10.4%, measured in HP Collec. and Basin Seedless, respectively) (Figure 2). Infestation severity was low (L) in other nine accessions (Achik Dana, Agah, AHPG-C3, Basin Seedless, Dholka, Goma Khatta, Sur Sukker, Tujetis EC 4347, and Uthkal) ranging between 9.4% (AHPG-C3) and 17.9% (Sur Sukker). Mite infestation was severe (S) and very severe (VS) in two (Phule Arakta, and Saih Sirin, with a mean PIL of 40.8%) and four accessions (Dorsata Malus, IC-318712, Kajaki Anar, and P-23, with a mean PIL of 51.9%), respectively. The other four accessions (Bhagwa, Jalore_Seedless, Jodhpur_Red, and Saharanpur) had moderate (M) infestation severity (mean PIL = 26.8%).

3.3. Vegetative Traits of the 22 Selected Accessions

Plant height, canopy perimeter, and stem diameter were significantly affected by the GS and the A, whereas the GS × A interaction had a significant effect only on plant height and canopy perimeter (Table 2). All the vegetative parameters increased from 2019 and 2020.

Plant height and canopy perimeter ranged, respectively, between 1.0 and 1.9 m and between 3.2 and 5.8 m. For both parameters, the lowest and the highest values were measured in IC-318712 and Bhagwa, respectively. Kajaki Anar plants together with IC-318712 had the most severe mite infestation. They were also relatively short (1.1 m) and had a small canopy perimeter (3.2 m). Trees with the lowest mite infestation (e.g., Gul-e-Shah

Rose Pink, HP Collec., and Speen Sacarin) had intermediate height, canopy perimeter, and stem diameter. The smallest and the largest stem diameters were found in Tujetis EC 4347 and Jalore Seedless, respectively (Table 2). Intermediate stem diameters were also measured in IC-318712 and Kajaki Anar plants.

3.4. Leaf Phytochemical Composition of the 22 Selected Accessions

Leaf phenolics, flavonoids, tannins, and antioxidant activity significantly differed among accessions (Table 3) ranging, respectively, between 34.10 and 49.44 mg GAE/g, between 2.47 and 4.48 mg Cat. E/g, between 3.45 to 9.87 mg Cat. E/g, and between 289.7 and 571.5 mg AAE/g. Leaf phenolics was highest in AHPG-C3, Gul-e-Shah Rose Pink, HP Collec., and Speen of Sacarin (an average 49.1 mg GAE/g), accessions that had a PIL $\leq$ 9.42%. Basin Seedless, Dorsata Malus, and Kajaki Anarhad the lowest leaf phenolic concentration (an average of 35.5 mg GAE/g). The lowest and the highest leaf flavonoid concentrations were measured, respectively, in Basin Seedless and HP Collec., whereas the lowest and the highest leaf tannin concentrations were found in AHPG-C3 and HP Collec., respectively. The leaves of Gul-e-Shah Rose Pink had the highest antioxidant activity, whereas this parameter was lowest in Dorsata Malus, IC-318712, and P-23 (Table 3). The three accessions with VL percent of infested leaves (Gul-e-Shah Rose Pink, HP Collec., and Speen of Sacarin) had leaf phenolics, flavonoids, tannins, antioxidant activity, respectively 26%, 53%, 111%, 68% higher than the four accessions with VS infestation intensity (Dorsata Malus, IC-318712, Kajaki Anar, and P-23) (Table 3 and Figure 2).

Table 3. Leaf total phenolics, flavonoids, tannins, and antioxidant activity in 22 accessions of pomegranate. Within each column, means followed by a common letter are not significantly different according to the Tukey test ($p \leq 0.05$).

Accession	Total Phenolics (mg GAE/g DW)	Flavonoids (mg catechin equi./g DW)	Tannins (mg catechin equi./g DW)	Antioxidant Activity (mg AAE/g DW)
Achik Dana	46.31 ± 0.38 abc	3.04 ± 0.04 defg	8.02 ± 0.05 c	460.6 ± 3.2 d
Agah	47.37 ± 0.47 ab	3.28 ± 0.04 cde	6.76 ± 0.04 fg	445.3 ± 1.7 de
AHPG-C3	49.44 ± 0.42 a	3.66 ± 0.05 bc	3.45 ± 0.02 n	497.1 ± 2.2 bc
Basin Seedless	36.18 ± 0.50 g	2.47 ± 0.07 i	8.62 ± 0.03 b	509.6 ± 2.5 bc
Bhagwa	43.74 ± 0.34 bcd	3.35 ± 0.08 cd	5.67 ± 0.04 i	392.3 ± 3.0 ghi
Dholka	45.50 ± 0.32 abcd	3.19 ± 0.03 def	5.89 ± 0.03 i	410.2 ± 4.2 fgh
Dorsata Malus	36.27 ± 0.63 g	2.66 ± 0.08 ghi	4.19 ± 0.04 l	309.3 ± 2.7 m
Goma Khatta	38.50 ± 0.43 efg	2.84 ± 0.06 efghi	6.56 ± 0.03 gh	458.0 ± 2.7 d
Gul-e-Shah Rose Pink	48.94 ± 0.41 a	3.72 ± 0.05 bc	8.11 ± 0.04 c	571.5 ± 3.2 a
HP Collec.	49.00 ± 0.68 a	4.48 ± 0.05 a	9.87 ± 0.04 a	522.1 ± 3.0 b
IC-318712	43.33 ± 1.93 bcd	2.87 ± 0.03 efghi	3.86 ± 0.02 m	289.7 ± 2.7 m
Jalore Seedless	42.68 ± 0.39 bcde	2.83 ± 0.02 fghi	7.25 ± 0.03 de	387.1 ± 3.5 hij
Jodhpur Red	45.73 ± 0.47 abcd	3.03 ± 0.04 defg	7.39 ± 0.07 d	426.2 ± 2.7 ef
Kajaki Anar	34.10 ± 0.58 g	2.51 ± 0.02 hi	4.16 ± 0.04 lm	337.8 ± 1.9 l
P-23	37.92 ± 0.41 fg	2.61 ± 0.02 ghi	4.78 ± 0.03 k	298.1 ± 1.5 m
Phule Arakta	42.34 ± 0.24 cdef	3.27 ± 0.10 cdef	5.08 ± 0.03 jk	365.6 ± 3.9 jk
Saharanpur	42.33 ± 0.54 cdef	3.15 ± 0.12 def	7.00 ± 0.04 ef	487.8 ± 2.4 c
Saih Sirin	38.59 ± 0.52 efg	2.70 ± 0.05 ghi	4.80 ± 0.04 k	350.0 ± 3.3 kl
Speen Sacarin	45.63 ± 0.45 abcd	4.05 ± 0.05 ab	8.89 ± 0.04 b	460.5 ± 9.2 d
Sur Sukker	41.36 ± 0.53 def	2.93 ± 0.03 defgh	5.32 ± 0.05 j	372.3 ± 2.8 ijk
Tujetis EC 4347	44.25 ± 0.34 bcd	3.35 ± 0.05 cd	6.99 ± 0.04 ef	368.5 ± 2.6 ijk
Uthkal	44.80 ± 0.47 abcd	3.27 ± 0.06 cdef	6.40 ± 0.03 h	415.3 ± 4.2 fg
Significance	***	***	***	***

*** indicates significant differences at $p \leq 0.001$ according to the one-way ANOVA.

3.5. Correlation Analysis

The correlogram of the quantitative traits under investigation illustrated that there are two main groups of interrelated variables (Figure 3). Phenolics, flavonoids and antioxidants

activity displayed strongly positively pairwise correlations, as expected, but also with the content of tannins. Plant height was strongly positively correlated with canopy diameter ($r = 0.84$; $p = 1.5 \times 10^{-18}$), whereas weaker statistically significant, positive correlations were found between plant height and stem diameter ($r = 0.47$; $p = 7.2 \times 10^{-5}$), leaf flavonoids concentration ($r = 0.25$; $p = 4.1 \times 10^{-2}$) and leaf antioxidant activity ($r = 0.27$; $p = 2.9 \times 10^{-2}$), and between stem diameter and canopy perimeter ($r = 0.34$; $p = 4.8 \times 10^{-3}$) (Figure 3). The three vegetative traits measured in this study did not significantly correlate with the PIL. Conversely, PIL was negatively correlated to leaf phenolics ($r = -0.59$; $p = 2.0 \times 10^{-7}$), flavonoids ($r = -0.60$; $p = 1.0 \times 10^{-7}$), tannins ($r = -0.70$; $p = 7.6 \times 10^{-11}$) and antioxidant activity ($r = -0.82$; $p = 5.5 \times 10^{-17}$).

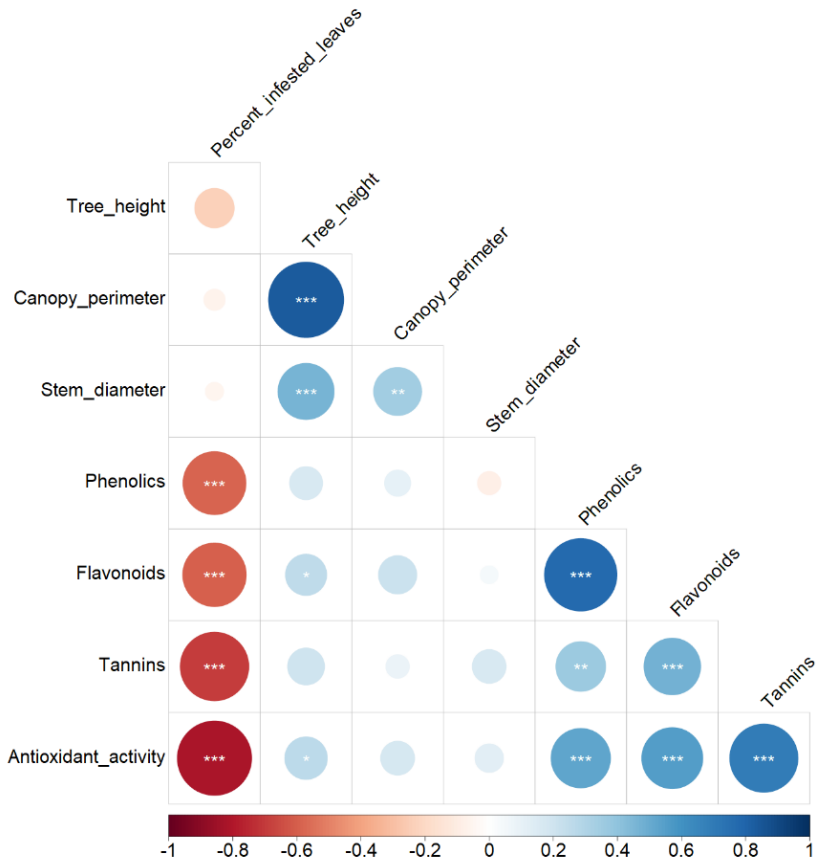

Figure 3. Correlogram representing the Pearson correlation coefficient matrix between percent of infested leaves, vegetative traits, and leaf composition. Asterisks indicate the significance of the Pearson correlation coefficient (*, **, *** correspond to $p \leq 0.05$, 0.01 and 0.001, respectively). Colors indicate different values of the correlation coefficient according to the scale bar reported at the bottom. The size of the circle is proportional to the correlation coefficients.

3.6. Principal Component Analysis

Considering that the morpho-chemical variables under investigation refer to diverse aspects of the tree, we investigated whether their multidimensional reduction could depict a classification of the germplasm resembling the one based on the severity of infestation. The PCA, performed on all the morphological and biochemical traits of the 22 accessions extracted seven principal components (Table 4). The first two principal components explained 61% of the total variance and were the only having an eigenvalue higher than 1. Therefore, they were selected for further analyses and graphical representation of the accession resemblance. The first principal component had a strong positive correlation with leaf phenolics, flavonoids, tannins, and antioxidant activity, whereas the second was strongly and positively correlated to canopy perimeter and stem diameter (Table 4).

Table 4. Eigenvalue, percent of explained variance and correlation with the original variable of the seven principal components (Dim.) extracted.

Principal Component	Eigenvalue	Variance Explained (%)	Correlation						
			Plant Height	Canopy Perimeter	Stem Diameter	Total Phenolics	Flavonoids	Tannins	Antioxidant Activity
Dim. 1	2.89	41.3	0.416 ^{NS}	0.146 ^{NS}	−0.043 ^{NS}	0.805 ***	0.862 ***	0.759 ***	0.854 ***
Dim. 2	1.42	20.3	−0.001 ^{NS}	0.851 ***	0.825 ***	−0.114 ^{NS}	−0.042 ^{NS}	0.066 ^{NS}	−0.012 ^{NS}
Dim. 3	1.00	14.3	0.636 **	0.289 ^{NS}	−0.388 ^{NS}	−0.438 *	−0.312 ^{NS}	0.201 ^{NS}	0.171 ^{NS}
Dim. 4	0.82	11.8	0.637 **	−0.124 ^{NS}	0.200 ^{NS}	0.205 ^{NS}	0.205 ^{NS}	−0.446 *	−0.283 ^{NS}
Dim. 5	0.43	6.2	0.123 ^{NS}	−0.375 ^{NS}	0.330 ^{NS}	−0.175 ^{NS}	−0.067 ^{NS}	0.356 ^{NS}	−0.063 ^{NS}
Dim. 6	0.27	3.8	0.017 ^{NS}	−0.116 ^{NS}	0.135 ^{NS}	0.029 ^{NS}	−0.206 ^{NS}	−0.209 ^{NS}	0.385 ^{NS}
Dim. 7	0.16	2.3	0.032 ^{NS}	0.027 ^{NS}	−0.013 ^{NS}	0.269 ^{NS}	−0.262 ^{NS}	0.097 ^{NS}	−0.097 ^{NS}

*, **, ***, and NS indicate significant differences at $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$ and not significant ($p > 0.05$) correlation.

The distribution of the accessions on the two-dimensional plane identified by the selected principal components was consistent with the variation in the susceptibility to the mite (Figure 4). Interestingly, accessions that had very light (VL) and very severe (VS) infestations were further apart along the first principal component and present in the opposite quadrants. While the group of accessions with very low infestation severity was mainly separated along the first dimension, the group with very severe infestation was separated along the second dimension. Accessions with L or M infestation severity clustered in the middle of the plot, without a clear separation of the two groups. Nonetheless, the accessions of the M group were more spread along the second dimension compared to those of the L group.

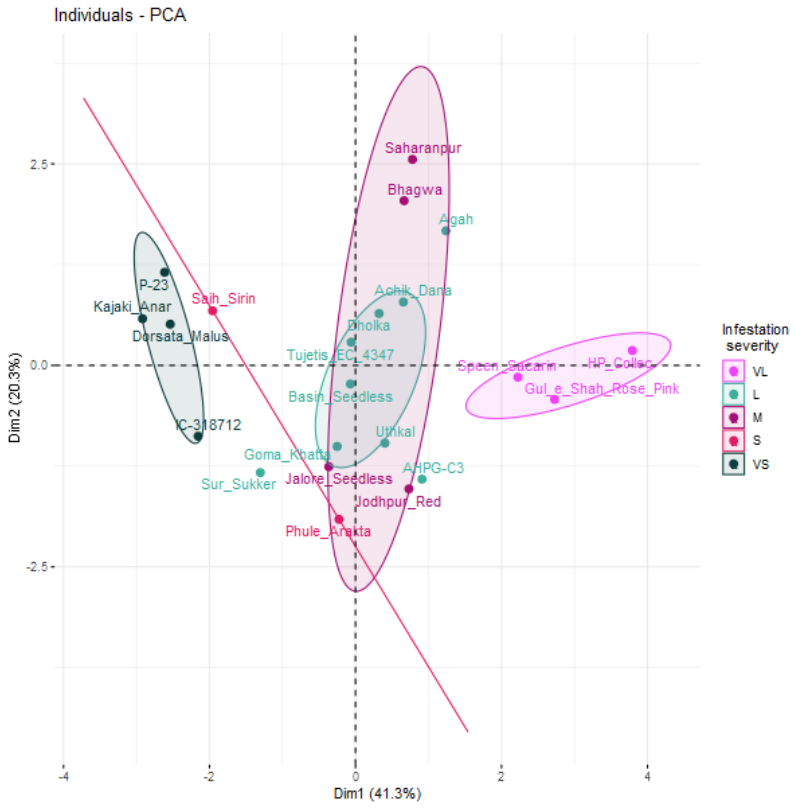

Figure 4. Bi-dimensional PCA plot of the 22 pomegranates accessions according to the morphological and phytochemical analysis. Accessions are colored according to the five classes of infestation severity (VL: very low; L: low; M: medium; S: strong; VS: very strong). For each class the graph displays the confidence interval ellipse (99%) around the mean point (not shown). The plot also reports the percentage of variance explained by each dimension.

4. Discussion

The rising interest of consumers for strongly colored fruits and juices rich in phytonutrients and the adoption of new training systems have boosted the interest of professional growers for pomegranate, especially as a complementary source of income in already running fruit farms. On the other hand, pomegranate-specialized cultivation has increased the occurrence of pest outbreaks. This tree, however, is expected to provide interesting economic results in regions with hot summers and low annual rainfall mainly because of low maintenance costs (e.g., limited cultural practices and off-farm input) [4]. It is therefore important to select suitable germplasm to reduce the need for chemical intervention, particularly for orchards in sub-optimal conditions (e.g., hot semi-arid regions). The present study indicated a broad range of variation for the susceptibility to the false spider mite *T. punicae* infestation. As expected, none of the accessions could be considered fully resistant. This variability was recorded in an ample germplasm collection growing in the same environment and managed with the same standard practices. Moreover, the susceptibility of pomegranate accessions was relatively stable across different years, without significant interaction with the growing season. All this strengthens the expectation that the phenotype has a genetic base, and it will be therefore useful for other agricultural locations and ultimately, for breeding, because classic breeding programs for insect resistance typically start from screening several genotypes in a common environment before the more time-demanding crossing and multi-environment field trials [30]. In relation to the observed infestation level, it is necessary to add that our results refer to a condition in which host preference might also have a role because of the varietal mixture. Nonetheless, *T. punicae* is a polyphagous mite [12] and therefore, host preference should have a relatively limited influence. For phytophagous insects, it has been proposed that oligophagous species have a stronger preference for high-quality plants than species with a broader diet [31]. Still, it is not possible to fully exclude that in commercial farming (i.e., with one or a limited set of cultivars) the percentage of infestation may be different.

Variability in the susceptibility to other pests such as insects, fungal pathogens and bacteria was previously described in pomegranate. However, to our knowledge, this is the first systematic study on the resistance to the false spider mite, and it is not possible to compare the infestation levels with the literature. Probably, the more closely related study is on carob moth susceptibility, which was assessed on 19 pomegranate varieties of Iranian origin [25]. More recently, a demographic laboratory study on the same insect also revealed significant differences among the 11 Iranian varieties under investigation [32].

The host resistance of a plant is mainly influenced by the morphological (e.g., physical) and biochemical (e.g., phytochemicals) traits [33], and different studies highlighted that these features are interconnected [34,35]. Although our screening was not designed to identify the factors that underline the different susceptibility, it was interesting that the infestation level of the leaves negatively correlated with the amount of the measured phytochemical classes. Interspecific variation of secondary metabolites in pomegranate has been largely documented for fruits but correlations with leaf composition are, in comparison, more limited [36,37]. Pomegranate leaves are rich in bioactive compounds, especially polyphenols, and among the various organs and tissues of the plant, they have the highest antioxidant activity [37]. Leaf extracts have also antibacterial activity [38]. With reference to plant-insect interactions, it has been demonstrated that the amount of anthocyanins, phenolics, and tannins in the fruit juice negatively correlated with the demographic indices of reared *E. ceratoniae* [32]. Considering that phenolics, flavonoids, tannins and antioxidant activity have been all linked to the resistance to insects, it will be interesting in the future to test if the accessions accumulating these phytochemicals display a lower susceptibility to other mite species (e.g., *Tenuipalpus granati*, *Aceria granati*) and non-mite arthropod pests of the leaves common in temperate regions (e.g., *Aphis punicae*, *A. gossypii*, and *Siphoninus phillyreae*) [1,39–41].

Principal component analysis of the morpho-chemical variables indicated that accessions were mainly separated by the phytochemical composition of the leaves. Crucially, this

separation was consistent with the susceptibility classification. In particular, the first principal component highly correlated with each of the biochemical classes under investigation and the antioxidant activity. Moreover, the intermediate classes of percentage of infestation were not well separated by the variance reduction procedure, and mostly spread along the second component (correlated with the analyzed morphological traits). In our work, the infestation classes were established according to the distribution of the accessions and the need of having enough classes for further testing. Typically, the number of germplasm classes is set around 4–5, to have sufficient variability for screening for resistance to biotic stress and studying its mechanisms. Our classification was empirically established, but the observed clustering of the accessions of the intermediate infestation levels and the overlap of the confidence intervals imply that different grouping strategies may not lead to different conclusions.

Overall, the correlation and the PCA analyses suggest that the largest differences in the infestation levels are due to the secondary metabolites of the leaves, but also that other characteristics, yet to be identified, should be considered. Our focus was on morphological traits that could relate to the overall status of the tree (e.g., they express its aptitude and strength towards vegetative growth), under the assumption that a higher accumulation of phytochemicals may reflect an overall healthier plant status and responsiveness to suboptimal conditions. Nonetheless, while, as expected, canopy perimeter, plant height and stem diameter were positively correlated, they did not display a significant relation with the infestation, as well as with the phytochemical composition. It should be added that further studies focusing, for instance, on the leaf morphological characteristics are necessary to reveal more specific cues on the mechanical and anatomical features that contribute to the different susceptibility to *T. punicae* [42]. Finally, our PCA results generated the hypothesis that the extreme classes in susceptibility to the false spider mite can be identified by analyzing the phytochemical content of the leaves, information that, after cross-validation of the factor structure in pomegranate breeding lines, could strongly accelerate the selection of segregating populations.

5. Conclusions

Our study illustrated that there is sufficient variability in pomegranate germplasm to breed for mite-resistant cultivars. In addition, our work revealed some biochemical traits of the leaves that associated with the susceptibility to the *T. punicae* infestation, paving the way to a more detailed identification of the allelochemicals that specifically affect pest performance. From an applied perspective, we identified some already cultivated varieties (Achik Dana, Agah, Basin Seedless, Dholka, Goma Khatta, Gul e Shah Rose Pink, Speen Sacarin, Sur Sukker, Uthkal) that could be employed for the establishment of new orchards in areas where the false spider mite can assume the role of pest, and some other non-commercial accessions (AHPG-C3, HP collec., Tujetis EC 4347) that can be used in breeding programs. In the future, it will be also interesting to extend the correlation between the amount of certain phytochemicals with the susceptibility to other polyphagous foliar pests. Lastly, although it is noteworthy that the infestation level can be highly contained by selecting appropriate germplasm, it is also necessary to underline that the adoption of accessions resistant to *T. punicae* should be always supported by adequate IPM and crop management strategies.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture12101686/s1, Figure S1: Climate charts. Average maximum (red line) and minimum (blue line) monthly temperature (dashed lines) in °C and monthly rainfall (continuous grey line) in mm during 2019 and 2020. Table S1: Pomegranate accession collection available at the experimental station of ICAR-Central Institute for Arid Horticulture (Bikaner, India) and their source of collection. Table S2: Two-way analysis of variance for the percentage of infested leaves.

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