Leaflet epidermal studies and taxonomic significance of trichomes in the *Rubus* subgenus *Rubus* (*Rosaceae*) in Iran

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Abstract. This study was to explore the foliar epidermis and trichome diversity of seven *Rubus* species, including *R. caesius*, *R. discolor*, *R. dolichocarpus*, *R. hirtus*, *R. hyrcanus*, *R. persicus,* and *R. sanctus*. The results showed that polygonal cells with straight to curved walls occurred in *R. caesius* of the treated species. Irregular cells with anticlinal straight to curved walls were also observed in *R. sanctus* and *R. discolor*. Sinuous cells were found in the epidermis. Irregular cells with straight to curved anticlinal walls were discovered on the abaxial side of leaves of *R. caesius* and *R. sanctus*. Sinuous cells with undulating walls were observed in the other examined species. No stomata were present on the adaxial leaf sides of the examined species, except in *R. hirtus*. An anemocytic stomata type was observed on the adaxial leaf sides of the studied species. Indumentums were studied and five types of covering trichomes were found: namely, covering trichome, stellate hairs, simple trichome, dentate, trifid branchy, and branchy-stalk trichome. Furthermore, three types of glandular trichomes were present too.

Key words: Anemocytic, irregular cells, leaflets epidermis, *Rubus*

Introduction

Subgenus *Rubus (blackberries, brambles)* belongs to the *Rosaceae* family, subfamily *Rosoideae*, genus *Rubus.* The most thorough taxonomic treatment of *Rubus* (Focke 1910, 1911, 1914) included 429 species from 12 subgenera, the largest being *Rubus* (132 species). In recent classification, various species within subgenus *Rubus* are mainly stated for Europe and adjacent regions, and more than 748 species have been taxonomically characterized (Kurtto & al 2010). The main centers of diversity for these taxa are in Europe, but numerous species are cultivated for their edible fruit and for ornamental purposes (Clarck & al 2013). Taxonomic classification of this subgenus with such large number of described species was a major challenge for the researchers. Generally, the evolution of brambles and blackberries is driven by extensive hybridization, polyploidy and asexual reproduction by seeds (apomixis) (Aalders & Hall 1966; Robertson 1974; Lu 1983; Richards & al. 1996; Sochor & Travnicek, 2016). On the basis of *Flora of Iran*, 10 species were recorded into the *Rubus* subgenus: namely Boissier (1887) *Flora of Orientale*, 12 species; Gilli (1969) *Flora of Iranica*, eight species and five hybrids; and Khatamsaz (1992) *Flora of Iran*, seven species (*R. caesius* L., *R. discolor* Weihe & Nees., *R. dolichocarpus* Juz., *R. hirtus* Waldst. & Kit., *R. hyrcanus* Juz., *R. persicus* Boiss., and *R. sanctus* Schreb.). All these species belong to the Euxino-Hyrcanian

region. However, *R. sanctus* and *R. caesius* have distributed from the Euxino-Hyrcanian to the Irano-Turanian region. Because this plant occurs under diverse environmental conditions, a considerable morphologic variation is found within and among the characterized species. Furthermore, the plant cross-pollinates and, owing to frequent natural hybridization events among the taxa, precise identification and characterization of the species encounters difficulties. In some species, the level of natural variation has not even been documented precisely and it is often difficult to distinguish the different blackberry species. Taxonomic identification of the *Rubus* species was initially based on morphological characteristics (Sochor & al. 2015). Taxonomic value of epidermal morphology and trichome diversity are both well documented in botanical literature (Dilcher 1974; Metcalf 1985).

According to Fell and Rowson (1956, 1960), the results of earlier treatment of three *Rubus* species (*R. idœus* L., *R. fruticosus* L., *R. loganobaccus* L.H. Bailey) showed that the adaxial leaf epidermis has polygonal and curved anticlinal cells, without stomata on the abaxial leaf epidermis, anemocytic stomata type, simple cover trichomes, and few trichomes could be seen on the adaxial leaf surface (Fell & Rowson 1956-1960). Furthermore, simple cover trichomes, curved one-cell trichomes and sunken-cell trichomes??? were visible on the main veins of the abaxial leaf surfaces, petiole and stipule. Researchers regarded stoma as one of the main characters (Miskin et al. 1972), because stomata type and density on the leaves proved valuable characters in this genus. Anatomy of the epidermis in *Rubus* has not been addressed so far in the research literature. Hence, the main aim of this study was to investigate this subgenus on the basis of the anatomy of leaf, stem, petiole, stipule, and pedicle. The genus *Rubus* is distributed in the Hyrcanian regions (Golestan, Gillan and Mazandaran provinces). The abaxial epidermis characters and trichomes were also analyzed comparatively. These characters could contribute to a better taxonomical treatment of this genus.

Material and methods

For the anatomical study, 10 specimens of seven species were collected from different locations in North Iran. Considering the geographical habitats of three northern provinces, treated specimens were collected; the specimens were kept in the Herbarium of the Gonbad-e-Kavous University (GKUH). Identification of specimens was carried out on the basis of *Flora of Iran* (Katamsaz 1992) and *Flora Iranica* (Gilli 1969). In this research, seven collected species and three herbarium specimens were included (Table 1). All materials were boiled for 15 minutes, then fixed in Carnoy solution (alcohols to acetic acid in proportion 3:1). The epidermis was separated with H_2O_2 and acetic acid (1:1), in order to prepare the leaves. Materials were kept warm in a tube with previous lotions (the above-mentioned solutions???) for 4 hours, than the leaves were cleaned. After cleaning, the materials were washed in distilled water. Epidermis separation followed. Epidermal samples were stained with 2 % aceto-carmine and were mounted on microscopic glass slides. In order to cross-section the trichome, the above-mentioned procedures were repeated and cross-section samples were made. Slide sections were studied and photographed by Nickon. Some characters (stomata length/weight, number of stomata, number of epidermal cells) were measured with Image Tools ver. 3.0 and Axio Vision 4.8. (Table 2)

Table 1. Location of the studied specimens.

Taxon	Location	Voucher number
R. caesius L. 1	Iran-Mazandaran: Ramsar, Ghasem Abad Sofla, 50 m a.s.l, Kasalkheh, Habibi & Mahdiyani.	803012-GKUH
R. caesius L. 2	Iran-Mazandaran: Nosaher Botany, in farmland. Amini & Zare.	5642
R. discolor Weihe and Nees.	Iran-Gillan: Astara to Ardabil road, Heiran, 500 m a.s.l, Kasalkheh,Habibi.	803055-GKUH
R. dolichocarpus Jaz.	Iran-Golestan: Golestan Forest, 500 m a.s.l, Kasalkheh & Mahdiyani.	803120-GKUH
R. hirtus Waldst and kit. 1	Iran-Mazandaran: Sanghdeh Forest, 1360 ma.s.l, Kasalkheh & Mahdiyani.	803135-GKUH
R. hirtus Waldst and Kit. 2	Iran-Mazandaran, Kojur, Veser village.	1680
R. hyrcanus Juz.	Iran-Gillan: Asalem to Khalkhal road, 1030 m a.s.l, Kasalkheh & Mahdiyani.	803146-GKUH
R. persicus Boiss. 1	Iran-Golestan: Kordkoy, Emam Reza Forest, 200 m a.s.l, Mahdiyani.	803150-GKUH
R. persicus Boiss. 2	Iran-Mazandaran, Nosahr to Noor, Sisangan Forest, Zare.	1833
R. sanctus Schreber.	Iran-Gillan: Loshan to Jirande, Bivarzin village, 1040 m a.s.l, Kasalkheh.	803235-GKUH

Table 2. Leaf epidermal anatomical features of the *Rubus* subgenera.

Species	Shape of cells	Adaxial epidermis: Pattern of anticlinal walls	Stomatal apparatus cell	Stomata index (mm^2)	Stomata density (mm^2)	Abaxial epidermis: Pattern of anticlinal walls	Stomatal apparatus cell	Stomata index (mm^2)	Stomata density (mm^2)	Stomata size (µm)	Stomata type
R. caesius 1	Pol	Str-Curv	Absent	–	–	Str-Curv	Present	18±0.03	329.2±26.02	18.94×12.52	Anemocytic
R. caesius 2	Pol	Str-Curv	Absent	–	–	Str-Curv	Present	21±0.00	391.7±7.21	16.03×13.61	Anemocytic
R. discolor	Irr	Str-Curv	Absent	–	–	Sin	Present	21±0.02	150±50.00	23.97×17.41	Anemocytic
R. dolichocarpus	Sin	Sin	Absent	–	–	Sin	Present	8±0.01	129.2±19.09	22.01×16.32	Anemocytic
R. hirtus 1	Sin	Sin	Present	0.008	12.5	Sin	Present	10±0.02	195.8±50.52	40.61×30.67	Anemocytic
R. hirtus 2	Sin	Sin	Present	0.02	12.5	Sin	Present	16±0.04	237.5±66.14	36.14×28.68	Anemocytic
R. hyrcanus	Sin	Sin	Absent	–	–	Sin	Present	6±0.05	229.2±7.21	24.55×15.90	Anemocytic
R. persicus 1	Sin	Sin	Absent	–	–	Sin	Present	8±0.01	366.7±57.74	20.74×18.81	Anemocytic
R. persicus 2	Sin	Sin	Absent	–	–	Sin	Present	15±0.03	404.2±80.36	30.26×23.29	Anemocytic
R. sanctus	Irr	Str-Curv	Absent	–	–	Str-Curv	Present	17±0.05	429.2±26.02	18.35×18.14	Anemocytic

Irr: Irregular; **Pol**: Polygonal; **Str-Curv**: Straight to Curved; **Sin**: Sinuous.

Results

Epidermal cell description

Epidermal and stomata characters of the leaves, such as cell shape, anticlinal wall patterns, stomata index, density, size, and types were examined (Table 2). There were different types of epidermal cells: regular polygonal, wavy and sinuous cells could be seen and anticlinal walls differ from the straight wavy and sinuous shapes. There are polygonal cells with straight to wavy anticlinal walls on the adaxial leaf side of *Rubus caesius* (Figs. 1- A1, B1). Curved and wavy cell walls were seen in *R. dolichocarpus*, *R. hirtus* and *R. hyrcanus* (Figs 1- D1, E1, F1, G1, H1, I1). Abaxial leaf epidermal cells were irregular, with straight to sinuous anticlinal walls in *R. sanctus* and *R. caesius* (Figs 1- A2, J2). The cells in the other examined species had curved-wavy walls (Figs 1- C2, D2, E2, F2, G2, H2, I2). No stomata were seen on the adaxial surface of the examined species, except in *R. hirtus* (Figs 1- E1, F1). All studied species had stomata on the abaxial leaf surface. All treated specimens were of the anemocytic stomata type, surrounded by 4-7 auxiliary cells (Figs 1- A2, B2, C2, D2, E1, E2, F1, F2, G2, H1, I2, J2). The largest in size stomata were observed in the *R. hirtus* population and the smallest were observed in the *R. caesius* and *R. sanctus* populations. The maximum stomata index and density were registered in *R. caesius* and *R. sanctus* (Table 2). Trichome structures were examined and recorded as valuable characters (Table 3).

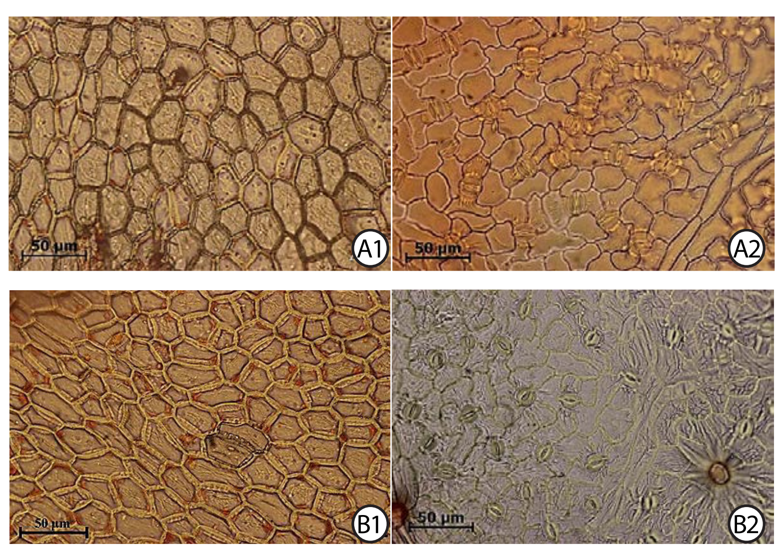

Fig. 1. Epidermal cells on the adaxial and abaxial side of the specimens' leaves: shape, size, anticlinal wall, stomata and trichomes under light microscopy. **A1**, **A2**. *R. caesius* (Ramsar population specimens), **B1**, **B2**. *R. caesius* (Noshar population specimens).

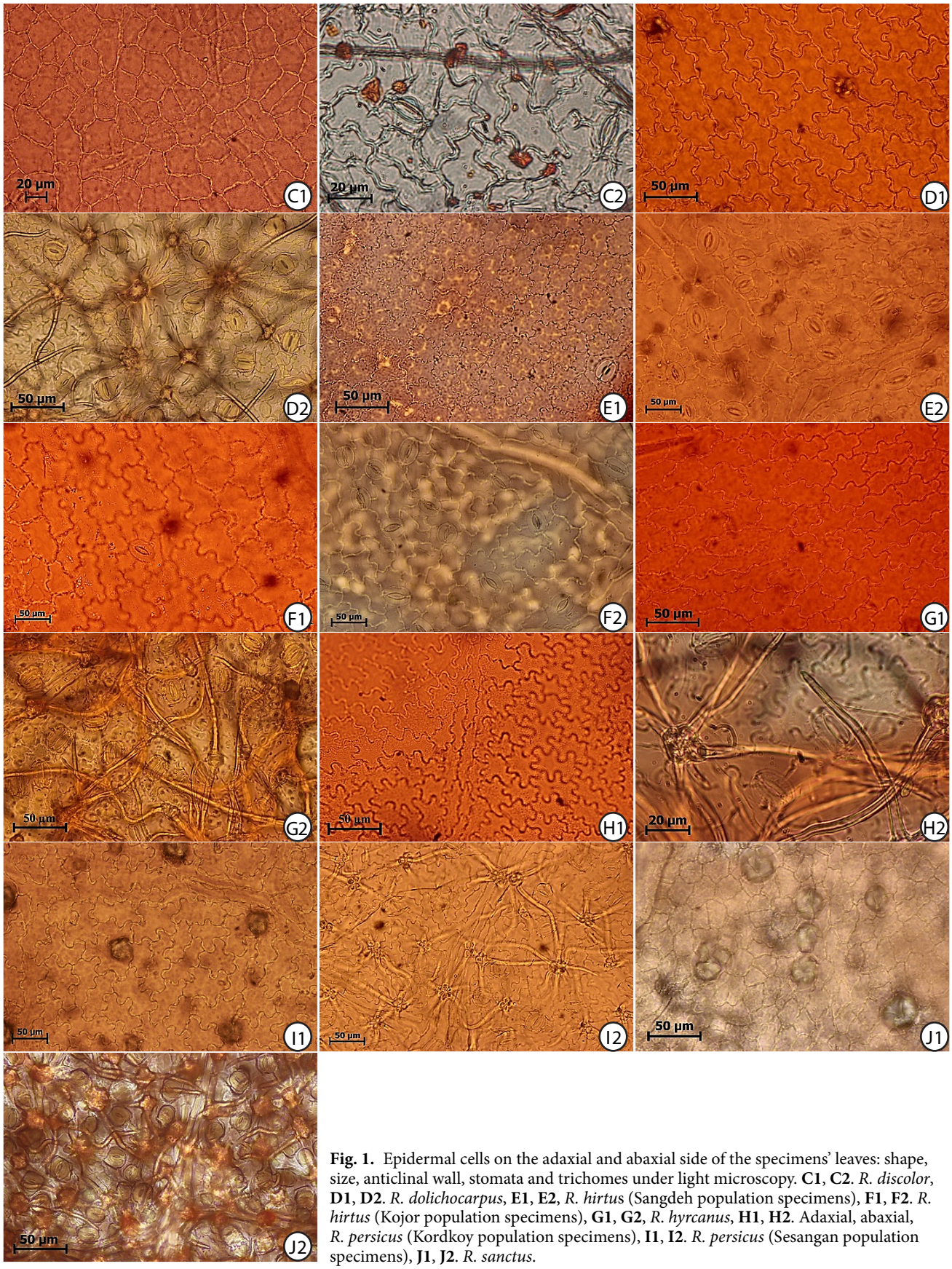

Fig. 1. Epidermal cells on the adaxial and abaxial side of the specimens' leaves: shape, size, anticlinal wall, stomata and trichomes under light microscopy. **C1**, **C2**. *R. discolor*, **D1**, **D2**. *R. dolichocarpus*, **E1**, **E2**, *R. hirtus* (Sangdeh population specimens), **F1**, **F2**. *R. hirtus* (Kojor population specimens), **G1**, **G2**, *R. hyrcanus*, **H1**, **H2**. Adaxial, abaxial, *R. persicus* (Kordkoy population specimens), **I1**, **I2**. *R. persicus* (Sesangan population specimens), **J1**, **J2**. *R. sanctus*.

Table 3. List of studied specimens according to presence (+)/absence (–) of trichomes.

Taxa	Organ	Simple unicellular	Stellate	Trifid branchy	BranchyStalk	Dentate	Bent glandular	Uniseriate multicellular stalk	Multiseriate multicellular stalk
R. caesius 1	Adaxial ep	+	–	–	–	–	–	+	–
	Abaxial ep	+	+	–	–	–	+	+	+
	Petiole	+	+	–	–	–	–	+	–
	Stem	+	+	–	–	–	–	–	–
	Stipule	–	–	–	–	–	–	–	–
	Pedicle	+	+	–	–	–	–	+	+
R. caesius 2	Adaxial ep	+	–	–	–	–	–	+	–
	Abaxial ep	+	+	–	–	–	+	+	+
	Petiole	+	+	–	–	–	–	+	–
	Stem	+	+	–	–	–	–	–	–
	Stipule	–	–	–	–	–	–	–	–
	Pedicle	+	+	–	–	–	–	+	+
R. discolor	Adaxial ep	+	–	–	–	–	–	+	–
	Abaxial ep	+	+	–	–	–	–	+	+
	Petiole	+	+	–	–	–	–	+	+
	Stem	+	+	–	–	–	–	+	+
	Stipule	+	+	–	–	–	–	+	–
	Pedicle	+	+	–	–	+	–	+	+
R. dolichocarpus	Adaxial ep	+	–	–	–	–	–	+	–
	Abaxial ep	+	+	–	–	–	+	+	+
	Petiole	+	+	–	–	–	–	+	–
	Stem	+	+	–	–	–	–	+	–
	Stipule	+	+	–	–	–	–	+	+
	Pedicle	+	+	–	–	–	–	+	+
R. hirtus 1	Adaxial ep	+	–	–	–	–	–	+	–
	Abaxial ep	+	+	–	–	–	+	+	+
	Petiole	+	+	–	–	–	–	+	–
	Stem	+	+	–	–	–	–	+	–
	Stipule	+	+	–	–	–	–	+	+
	Pedicle	+	+	–	–	+	–	+	+
R. hirtus 2	Adaxial ep	+	–	–	–	–	–	+	–
	Abaxial ep	+	+	–	–	–	+	+	+
	Petiole	+	+	–	–	–	–	+	–
	Stem	+	+	–	–	–	–	+	–
	Stipule	+	+	–	–	–	–	+	+
	Pedicle	+	+	–	–	+	–	+	+
R. hyrcanus	Adaxial ep	+	–	–	–	–	–	+	–
	Abaxial ep	+	+	–	–	–	–	+	+
	Petiole	+	+	–	–	–	–	–	–
	Stem	+	+	–	–	–	–	–	–
	Stipule	+	+	–	–	–	–	+	–
	Pedicle	+	+	–	–	–	–	+	+
R. persicus 1	Adaxial ep	+	–	–	–	–	–	+	–
	Abaxial ep	+	+	+	+	–	–	+	+
	Petiole	+	+	–	–	–	–	+	+
	Stem	+	+	–	–	–	–	–	+
	Stipule	+	+	–	–	–	–	+	–
	Pedicle	+	+	–	+	+	–	+	+
R. persicus 2	Adaxial ep	+	–	–	–	–	–	+	–
	Abaxial ep	+	+	+	+	–	–	+	+
	Petiole	+	+	–	–	–	–	+	–
	Stem	+	+	–	–	–	–	–	–
	Stipule	+	+	–	–	–	–	+	+
	Pedicle	+	+	–	+	–	–	+	+
R. sanctus	Adaxial ep	+	+	–	–	–	–	+	–
	Abaxial ep	+	+	–	–	–	–	+	+
	Petiole	+	+	–	–	–	–	–	–
	Stem	+	+	–	–	–	–	+	–
	Stipule	+	+	–	–	–	–	+	+
	Pedicle	+	+	–	–	–	–	+	+

Trichome type description

Trichomes were studied at light-microscopy level. Five types of covering trichomes and three types of glandular hairs were identified:

– simple unicellular

This unicellular type, thicker at the base, maul-shaped and elongated was observed on the leaf, petiole, pedicle and stipule of all examined species, except in *R. caesius*. (Figs 2- A, B, C, D, V).

– stellate

Unicellular, barbate and uncinate, this type was seen on the adaxial leaf side of *R. sanctus* and on the abaxial leaf side, petiole and pedicle of all examined species, as well as on the stipule of all species, except in *R. caesius* (Figs 2- D, E, F, G, U).

– trifid branchy

Unicellular, barbate and unpaired, this trichome type was observed on the abaxial veins of *R. persicus* (Fig. 2- H).

– branchy-stalk

With a multicellular base, this trichome was branchy at the base of stalk. It was observed on the abaxial side of veins of *R. persicus* (Fig. 2- I).

– dentate

This type of trichome was noticed on the pedicle of *R. discolor* and *R. hirtus* (Fig. 2- W).

– curved at a right angle, with a multicellular uniseriate stalk (bent glandular hairs)

This glandular trichome had curved multicellular stalks and two glands at the tip. The type was encountered on the abaxial leaf side and veins of *R. caesius*, *R. dolichocarpus* and *R. hirtus* (Figs 2- K, L, M).

– glandular trichome with uniseriate multicellular stalk

The stalk of this trichome had one row of cells and a rounded tip. This trichome was seen on the adaxial and abaxial leaf sides and pedicle of *R. sanctus*, *R. hirtus* and *R. dolichocarpus*, the petiole of *R. discolor* and *R. dolichocarpus*., and the stipules of *R. caesius* (Figs 2- O, R, X).

– glandular trichome with biseriate-multiseriate and multicellular stalk

The stalk of this trichome had many rows of cells, and a rounded tip. This type was seen on the abaxial side of pedicle of all examined species. It was also seen on the petiole of *R. discolor* and stipule of *R. dolichocarpus*, *R. hirtus*, *R. persicus,* and *R. sanctus* (Figs 2- N, P, Q, T, V, U).

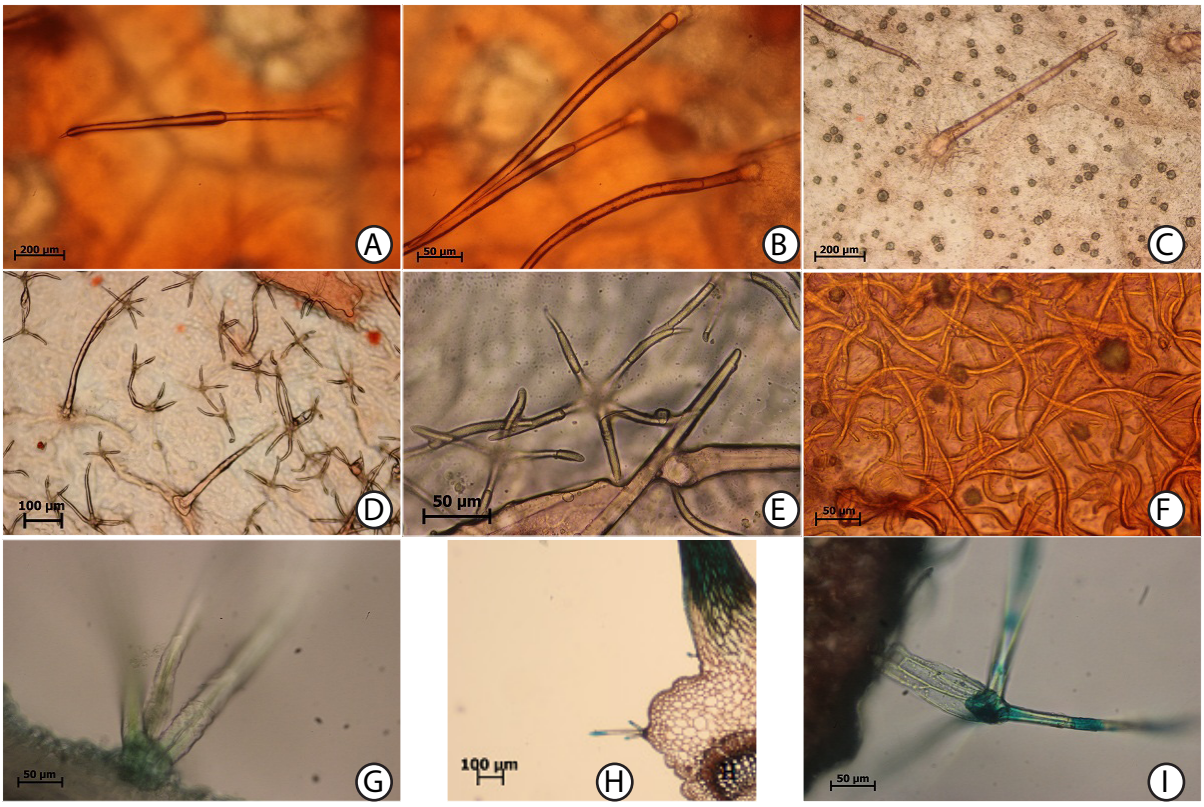

Fig. 2. Trichome types revealed by light microscopy: **A**, **B**. Adaxial epidermis of *R. hirtus*; **C**. Adaxial epidermis of *R. persicus*; **D**, **E**. Adaxial epidermis of *R. sanctus*; **F**. Abaxial epidermis of *R. persicus*; **G**. Stem; **H**, **I**. Abaxial leaf side of *R. dolicocarpus*.

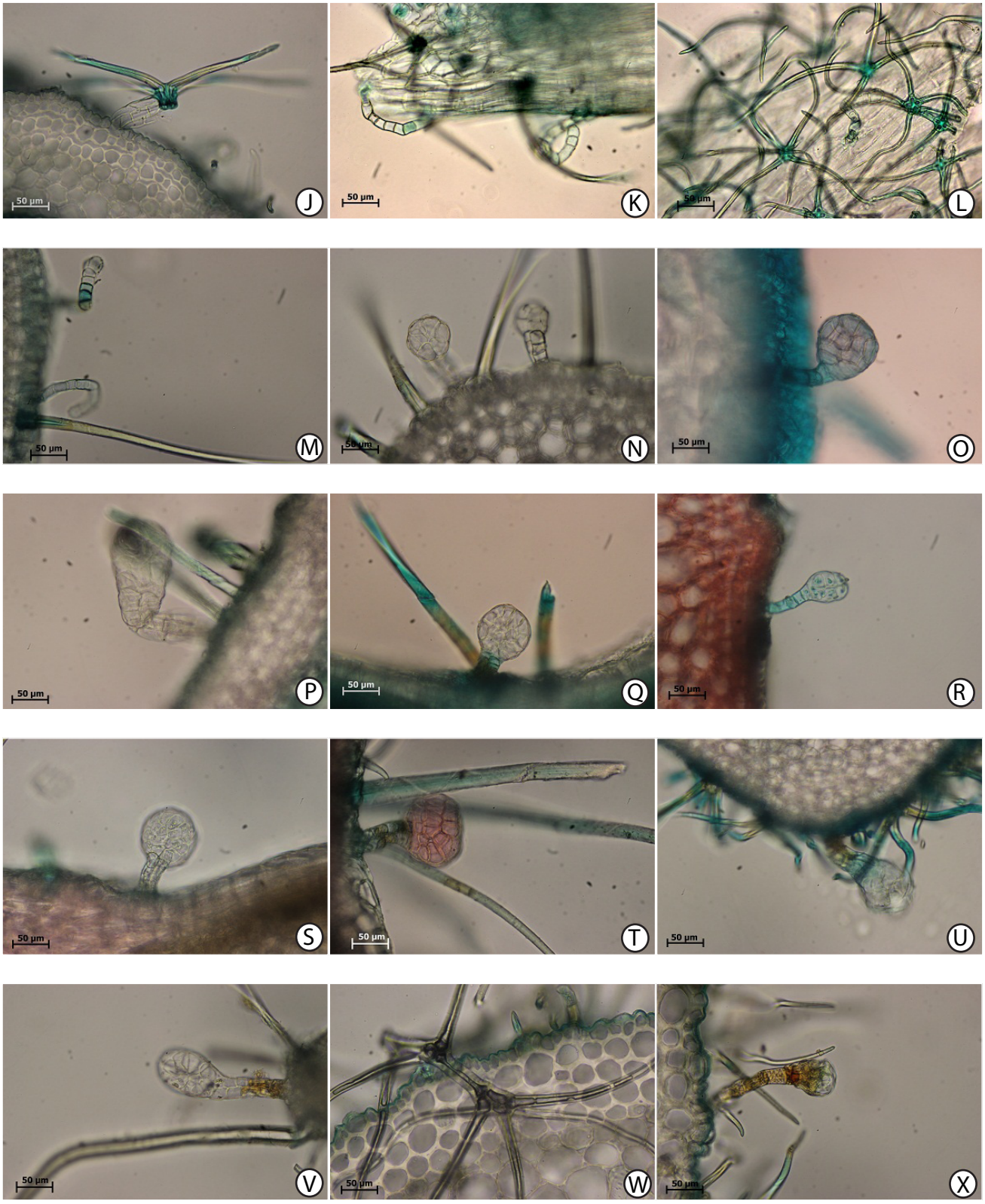

Fig. 2. Trichome types revealed by light microscopy: **J**. Pedicle; **K**, **J**, **L**. Abaxial leaf side of *R. persicus*, *R. caesius* and *R. dolicocarpus*; **M**, **N**. Abaxial leaf side of *R. caesius*; **O**. Adaxial leaf side of *R. discolor*; **P**. Stem, **Q**, **R**. Abaxial leaf side of *R. hirtus* and *R. persicus;* **S**, **T**. Abaxial leaf side of *R. sanctus*; **U**, **V**. Stipule of *R. dolichocarpus* and *R. sanctus*; **W**, **X**. Pedicle of *R. discolor;* **S**, **T**. Abaxial leaf side of *R. sanctus*; **U**, **V**. Stipule of *R. dolichocarpus* and *R. sanctus*; **W**, **X**. Pedicle of *R. discolor*.

Discussion

In this study, 10 specimens of seven *Rubus* species were investigated. The examined characters, such as anticlinal walls, thrichome and stomata type on the leaves and petiole, were regarded as valuable taxonomic characters at the species level. In the population of *R. caesius* in North Iran, polygonal cells were noticed on the adaxial side of leaves, while irregular cells with straight to curved walls occurred on the abaxial side of leaves. On the adaxial leaf side of *R. discolor* and both leaf surfaces of *R. sanctus,* irregular-straight to curved cells were observed. Sinuous cells with wavy walls were observed on both leaf surfaces in the other studied species.

No stomata were seen on the adaxial leaf sides of the studied species, except in *R. hirtus*, where they occurred with low density. All treated species demonstrated an anemocytic type of stomata. The data were in favor of other researchers (Fell & Rowson 1956, 1960) that 3 European *Rubus* were treated . Stomata play a significant role as valuable differentiating characters at different levels of plant ecology, taxonomy and physiology. Furhtermore, the stomata type, density and structure may affect plant physiology, water efficiency and biomass (Luo & Zhou 2001). Genetic factors form the stomata structure (Teare & al. 1971; Miskin & al. 1972). Current research has shown a high mean length-to-width rate in the stomata of *R. hirtus*, *R. hyrcanus* and *R. discolor,* while the lowest rate was traced out in *R. persicus* and *R. sanctus*, *R. caesius*, *R. discolor,* and *R. sanctus*.

Stomata density was also one of the main characters measured in 1 mm^2. Low stomata density was measured in the Irano-Turanian and Hyrcanian regions. Locality and habitats of the species also significantly affect the stomata density.

In terms of forest and unforested areas, stomata density is grouped into two types: forest and unforested. It is clear that humidity in the forest areas can affect the stomata density because the plants do not suffer from drought in such areas. Generally, plants resort to different strategies so as to cope with ecological factors. Similarly, leaf size in this study was small in the unforested areas; therefore, the plants increase the stomata density. The findings in the current study are in accord with those in other studies relating to the stomata and structure (Miller 1983). High stomata density was observed in *R. sanctus* and low density in *R. dolichocarpus* and *R. discolor*. Some scientific reports have shown that genetic and habitat factors can play a significant role for stomata. Some ecological factors such as water availability, leaf size, stomata size, and density also have a say (Blum & al. 1981; Scheettle & Rochelle 2000).

Trichome was another closely studied character. This character could be affected by such factors as drought and UV irradiation (Agrawal & Fishbein 2006). Results of the investigation of trichomes in this project have shown that simple covering and glandular trichomes with one row of cells were present on the adaxial leaf side of all examined species. Furthermore, stellate hairs occurred in *R. sanctus*, which differed from the other species. Stellate and simple covering trichomes of different density were observed on the abaxial leaf side of all examined species. Few glandular trichomes with one or two rows of cells were noticed in all species. Branchy trichomes with stalk were recorded only in *R. persicus.* There were two types of trichomes on the petioles of the species: simple, stellate and glandular trichomes. Fell and Rowson (1960) reported the following trichome types: simple, branchy and glandular trichomes, with one or many rows of cells. In the current research, trichomes of high density were traced out in *R.sanctus* and of low density in *R.caesius* and *R.hirtus.* The overall morphological characteristic of the leaves, such as size, stomata and trichomes, were related to habitat adaptation (Zhang & Marshall 1995). Stomata structure and trichomes in *Rubus* showed that they were valuable taxonomical and ecological characters and could be used as scientific distinguishing characters in botanical and other studies.

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