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COMPARATIVE MORPHOLOGICAL AND BIOCHEMICAL STUDIES OF *SALVADORA* SPECIES FOUND IN SINDH, PAKISTAN

FARZANA KOREJO[1,2], SYED ABID ALI[2,*], SYEDA SALEHA TAHIR[1], MUHAMMAD TAHIR RAJPUT[1] AND MUHAMMAD TUAHA AKHTER[2]

[1]*Institute of Botany, University of Sindh, Jamshoro, Sindh, Pakistan*
[2]*HEJ Research Institute of Chemistry, International Centre for Chemical and Biological Sciences (ICCBS), University of Karachi, Karachi-75270, Pakistan*
[*]*Corresponding author: E-mail: abid.ali@iccs.edu*

Abstract

Salvadoraceae is a small family comprising of three genera viz., *Azima*, *Dobera* & *Salvadora*. *Salvadora* 10 species are distributed mainly in the tropical and subtropical regions of Africa and Asia. In Pakistan it is represented by a single genus *Salvadora* with so far, two morphologically distinct species i.e., *S. persica* L. and *S. oleoides* Decne. In the present investigation, a comparative and comprehensive leaf, branch, fruit, seed, and pollen grain macro and micro morphological characters have been analyzed and complemented with chemotaxonomy of the seed proteins as biochemical markers for identifications. As expected taxonomical characters within the *Salvadora* species revealed great vegetative morphological differences, especially plant length and width. Floral morphological characters appear to be more stable, except the fruit colours which are different. Furthermore, sizes and the anatomical characters of the leaf, branch, seed and pollen grain studied by scanning electron microscopy revealed that in contrast to *S. oleoides* Decne much intra-species variation exist in *S. persica* L. and at least two types and/or varieties are available in Sindh, Pakistan.

Introduction

The genus *Salvadora* belongs to the family *Salvadoraceae*, comprising of three genera (i.e. *Azima*, *Dobera* & *Salvadora*) and 10 species distributed mainly in the tropical and subtropical region of Africa and Asia (Mabberely, 2008). The natural habitats are near mangroves, in saline lands, swamps, thorn shrubs, desert flood plains and grassy savannah, in seasonally wet sites and along drainage lines in arid zones. *Salvadora* species are also found near riverbanks where ground water level is high indicating its tolerance to a wide range of water, soil and soil pH and perhaps the main reason for its widespread nativity (Zodape & Indusekhar, 1997; Ahmad, 2007). In Pakistan this family is represented by a single genus *Salvadora*, with two species i.e., *S. persica* L., and *S. oleoides* Decne (Qureshi 1972; Khan & Qaiser, 2006). *Salvadora* species are perennial shrub or trees with simple, opposite, petiolate leaves, inflorescence densely fascicled or laxly panicled, axillary terminal. Flowers are minute, pale green sessile or sub-sessile, bisexual tetramerous. Fruits have single seed, are globose drupe with persistent calyx (Watson & Dallwitz, 1992; Panday, 2004). Furthermore, fruits of *S. persica* L., are red or white on maturation whereas, *S. oleoides* Decne have reddish brown fruits. Leaves are smaller and more in number in *S. persica* L., compared to *S. oleoides* Decne. Both *Salvadora* species are deep rooted mesomorphic xerophytes as well as facultative halophytes with high salt tolerance (Hooker, 1887; Jafri, 1966; Qureshi, 1972; Khan & Qaiser, 2006). Around the world *S. persica* L., is more famous by the brand name of

Miswak and the tree is referred as the toothbrush tree and extensively used as toothbrush (Almas, 2002; Al-Otaibi *et al.,* 2003; 2004). *S. persica* L., is also culturally more important both in local knowledge systems and major religions. It is one of the identified plants from among the seventeen plants families that are cited in the Holy Quran (Khafagi *et al.,* 2006).

The *Salvadora* species have a number of proven medicinal applications and almost all parts have been found to be pharmaceutically important (Almas, 2002; Almas *et al.,* 2005; Darmani *et al.,* 2006). The leaves, root bark, fruits and seeds are used for the treatment of cough, fever, asthma and as purgative. Roots are also used for chest diseases while, latex used for treating sores (Mahar & Malik, 2001; Savithramma *et al.,* 2007). The young roots, stems and branches are used as toothbrush (Darmani *et al.,* 2006). The plant holds strong antiulcer (Sanogo *et al*., 1999) antifungal (Al-Mohaya *et al.,* 2002; Hamza *et al.,* 2006), anti-parasitic, antiviral (Ali *et al.,* 2002), and/or antibacterial (Sofrata *et al.,* 2008) properties. It also includes other workings like mordant, cleanser and coarseness which encourage its utilization in most of dental treatments and cleansers (Almas, 2002; Al-Otaibi *et al.,* 2003; 2004; Almas & Al-Zeid, 2004; Almas *et al.,* 2005; Darmani *et al.,* 2006). The young branches and leaves are also favorite fodder for camels because of the high water content (15-36%). Oil from seed is used in rheumatic pain, diabetes and/or spleen and stomach disorders. The fruits are sweet and peppery in taste with pungent smell and eaten when ripe for medicinal purposes. The oil extracted from the seeds is pale green in color and not meant for edible purposes. The most vital aspect of oil is its constituency of low percentage of C8 and C10 fatty acids that holds a great economic significance (Duhan *et al*., 1992; Panday, 2004). For example *S. persica* L., appears to be a potentially valuable oilseed crop for saline and alkali soils, since the seeds contain 30-40% of oil rich in lauric (C12) and myrestic (C14) acids used in soap, detergent, candles and cosmetic industry (Reddy *et al.,* 2008). Likewise, seeds of *S. oleoides* Decne contain 40-45% oil, and fruits are also found to be rich sources of calcium (Duhan *et al.,* 1992; Zodape & Indusekhar, 1997). In view of multifaceted utilization, both species of *Salvadora* are included in restoration programs of many developing countries in Africa and Asia (Khan & Qaiser, 2006; Khan, 2009).

The wide ranging medicinal, ecological, social and economic importance on the one hand and declining population in Pakistan on the other necessitates systematic studies including intra-species variations within the genus *Salvadora* found in Sindh, Pakistan. As mentioned earlier, family *Salvadoraceae* in Pakistan represented by a single genus i.e., *Salvadora* with two major species *S. persica* L., and *S. oleoides* Dence identified on the basis of simple macromorphology (Qureshi, 1972), and supported by the pollen micromorphology by the studies of Perveen & Qaiser (1996). In the present communication, a comparative and comprehensive leaf, branch, fruit, seed and pollen grain macro and micro morphological characters have been studied and complemented with chemotaxonomy of the seed proteins as biochemical markers for identifications. In contrast to *S. oleoides* Decne much intra-species variation have been observed in *S. persica* L. and at least two types and/or varieties are available in Sindh, Pakistan.

Materials and Methods

For morphological and biochemical investigation both fresh and herbarium materials were used. Leaves, flowers, fruits, seeds and pollen material were collected from the plants growing in the field at the different localities of Sindh, Pakistan. The flowering and

fruiting times were also observed besides size and weight of fruit and seed measurements. List of voucher specimen of *Salvadora* species deposited at the University of Sindh Herbarium, USH) are as follows:

***Salvadora oleoides* Decne:** Botanic Garden University of Sindh Jamshoro, F. Korejo, 25-04-2007(SUH701) Hussainabad, Hyderabad F. Korejo, 30-04-2006(SUH702) Village Sehra Taluka Moro, District Noshahro Feroze, F. Korejo, 10-05-2007(SUH703), Right Bank of K.B. Feeder, Near Kotri, F. Korejo, 10-06-2007(SUH704).

***Salvadora persica* L. (with red fruit):** Botanic Garden University of Sindh Jamshoro, F. Korejo, 4-11-2006(SUH705) Hussainabad, Hyderabad F. Korejo, 20-11-2007(SUH706), Village Sehra Taluka Moro, District Noshahro Feroze, F. Korejo, 01-01-2007(SUH707), Right Bank of K.B. Feeder, Near Kotri, F. Korejo, 10-01-2007(SUH708).

***Salvadora persica* L. (with white fruit):** Botanic Garden University of Sindh Jamshoro, F. Korejo, 30-02-2007(SUH709), Hussainabad Hyderabad, F. Korejo 30-02-2007(SUH710), Village Sehra Taluka Moro, District Noshahro Feroze, F. Korejo, 01-03-2007(SUH711), Right Bank of K.B. Feeder, Near Kotri, F. Korejo, 05-03-2007(SUH712).

For macromorphological studies leaves, branch, flowers and seeds of the *Salvadora* species were examined by simple microscope (Kyowa SDZ-P StereoZoom, Japan). For the study of pollen grains, slides were prepared following classical acetolysis method as described by Erdtman (1960). For micromorphological studies by SEM, sample of leaf, branch, seed, pollen were mounted on to the stub with double sided cellotape and coated with gold in a sputtering chamber (Auto-Coater JFC-1500 Joel, Japan) and were examined under scanning electron microscope (JSM 6380A Joel, Japan) at the Centralized Science Laboratories (CSL) University of Karachi. In case when species have distribution in different regions, 5-10 specimens from each region were examined in order to account the whole range of variation in micromorphological characteristics. The terminology used for pollen description is in accordance with Erdtman (1952, 1960), Kremp (1965), Faegri & Iversen, (1989), and Perveen & Qaiser, (1996).

For the chemotaxonomy of the *Salvadora* species, seeds - the natural reservoirs of storage proteins were selected for finger printing and identification of protein markers (Moreira *et al*., 1993; Sharifnia & Assadi, 2003; Kharazian, 2008). The seeds were collected after carefully removing pulp from the fruits. The seeds were dried in shady places at room temperature. Seeds were ground with the help of mortar pestle and the resulting powder air dried over filter paper for 24 hours. Major seed storage proteins (i.e. albumin, globulin, prolamin and glutelin) from the seed flour was ideally extracted and estimated as described by Sammour (1999). For protein finger printing under physiological conditions, seed flour was extracted (1:10; w/v) in buffers of three different pH (i.e. 50 mM; Sodium acetate pH4.5, Sodium phosphate pH6.8 and Tris-HCl pH8.5), extraction was performed at 4ºC for over night. Extracts were filtered (Whatmann No. 1 filter paper) and the filtrates were further centrifuged at 14000 rpm (Biofuge Primo R, Heraeus, Japan) for one hour (4ºC). The supernatants obtained after centrifugation were stored at -20ºC until further use. Protein concentration was determined by the Lowry (1951) method.

The crude extracts (*ca*. 15µg) were subjected to SDS-PAGE analysis (Mini-PROTEAN® 3 Cell, Bio-Rad Lab, UK) under non-dissociating and dissociating/denaturing conditions (pH 8.8 in presence of SDS and ß-mercaptoethanol) as

described by Laemmli (1970). The crude seed extracts were subjected to 10% resolving gel and 5% stacking gels (140V) and at the end of electrophoresis the gel were stained with 0.2% Coomassie Brilliant Blue R-250. The chromatographic behavior of the crude protein extracts (*ca*. 1mg/ml) were also analyzed by fast protein liquid chromatography (ÄKTA-design, Amersham Biosciences, UK) using a size exclusion column (TSK2000 SW, 7.5 x 300mm, Tosoh Bioscience, Japan). The column was equilibrated and eluted in 50 mM Tris-HCl buffer, pH 8.5, containing 0.01% NaN_3. The flow rate was maintained at 1ml/min and the eluate was monitored at 280 nm.

Result and Discussion

Salvadora persica L. (with red fruit)

Macromorphological properties: A tall shrub attaining the size of small or medium size tree (2-6 m high). Leaves are simple, opposite, petiolate, ovate. Inflorescence axillary panicled. Flowers pale-greenish in color, complete actinomorphic tetramerous. Sepal lobes fused half of the length, rounded. Petals 4 in number, connate half way, lobes ovate or oblong. Stamens 4 in number, inserted at the base of corolla. Filaments alternating; Petals basifixed; anther ovate. Ovary, 1-locular glabrous, obvate, erect; Style absent; stigma more or less peltate. Fruit pulpy, drupe globose, pink to red with shinny surface. Seeds dark brown (Table 1, Fig. 1).

Micromorphological properties

Leaves: Both surfaces smooth, without any definite pattern, traces of non-oriented wax present. Stomata anomocytic and mostly sunken, more on upper surface (Fig. 1, 2A).

Branches: branches drooping; pale green, glabrous, and with ridges through out the length (Fig. 3A).

Seeds: Globular, 4. 05 µm long and 4.05 µm wide, dark brown, variously pattern, having dull or shinny surface. The seed surface shows scabrate pattern. The cells are arranged in irregular and regular circular rows, and mostly pitted with defined lines (Fig. 4A).

Pollen grains: Prolate tricolpate, 10.8 µm long and 4.2 µm wide. Colpus, tending deeply sunken forming elongated fold or sutures. Surface pattern is foveate, shallow and deep pits with wavy ridges. Exines constitute thick sexine (Fig. 5A).

Salvadora persica L. (with white fruit)

Macromorphological properties: A tall, shrub attaining the size of small or medium sized tree (2-6 m high). Leaves simple, opposite lanceolate, petiolate, glaucous. Inflorescence densely panicled. Flowers greenish-yellow in color, pedicellate complete actinomorphic, tetramerous hypogynous. Sepal lobes fused half of the length with rounded apex. Petals 4, connate half way, lobes ovate or oblong. Stamens 4 inserted at the base of corolla; filaments alternating petals lobes; anther basifixed, ovate; Ovary 1-locular, glabrous, obovate, erect, style absent, stigma more or less peltate. Fruit a drupe, globose, green in young white after maturation. Seed light brown and roughly globular (Table 1, Fig. 1).

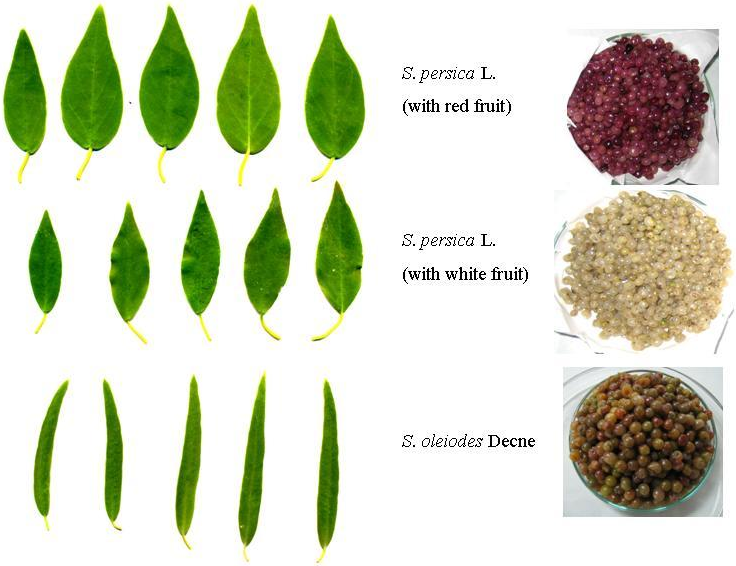

Fig. 1. Comparison of the leaves and fruits of the *Salvadora* species. See also table 1 and text for details.

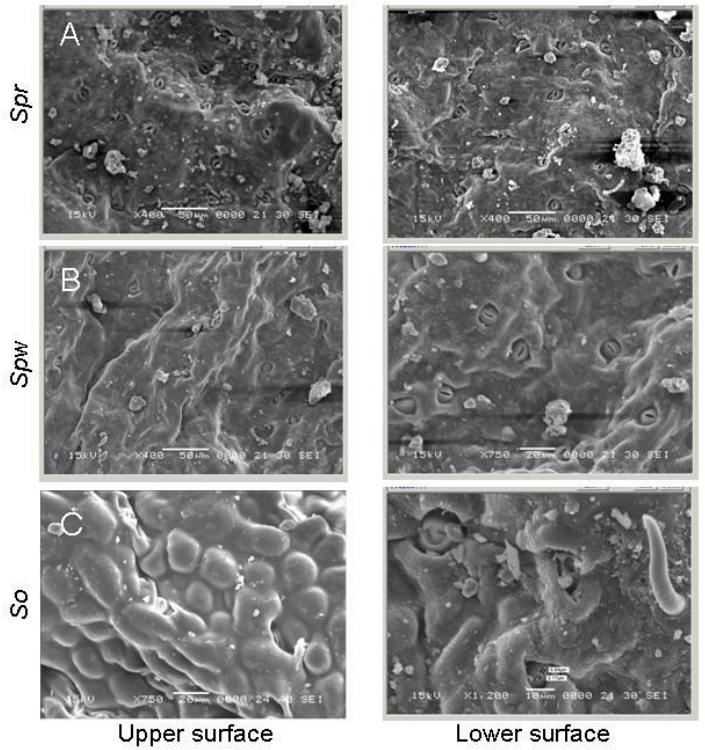

Fig. 2. Scanning electron micrographs of the leaves of *Salvadora* species. *A* - *S. persica* L. (with red fruit), *B* - *S. persica* L. (with white fruit), *C* - *S. oleoides* Decne. Upper and lower surfaces of the leaves are shown in left and right panels, respectively.

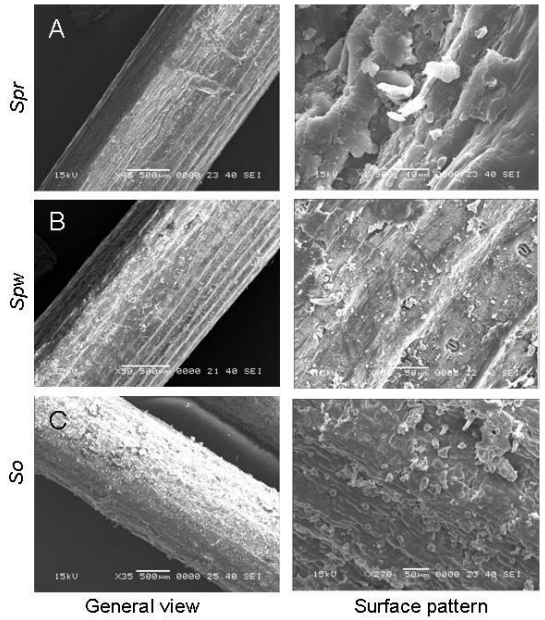

Fig. 3. Comparison of the SEM micrographs of the branch patterns of *Salvadora* species. A - *S. persica* L. (with red fruit), *B* - *S. persica* L. (with white fruit), *C* - *S. oleoides* Decne. A general view and details of the branch surface patterns are shown in left and right panels, respectively.

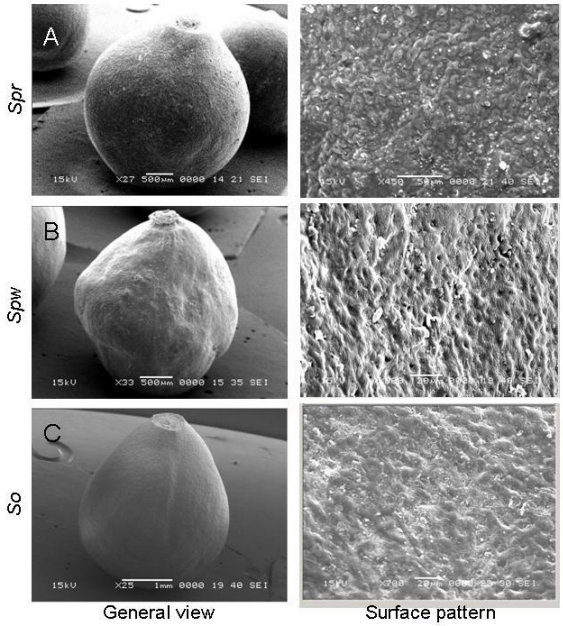

Fig. 4. Scanning electron micrographs of the seeds of *Salvadora* species. *A* - *S. persica* L. (with red fruit), *B* - *S. persica* L. (with white fruit), *C* - *S. oleoides* Decne. A general view and details of the seed surface patterns are shown in left and right panels, respectively.

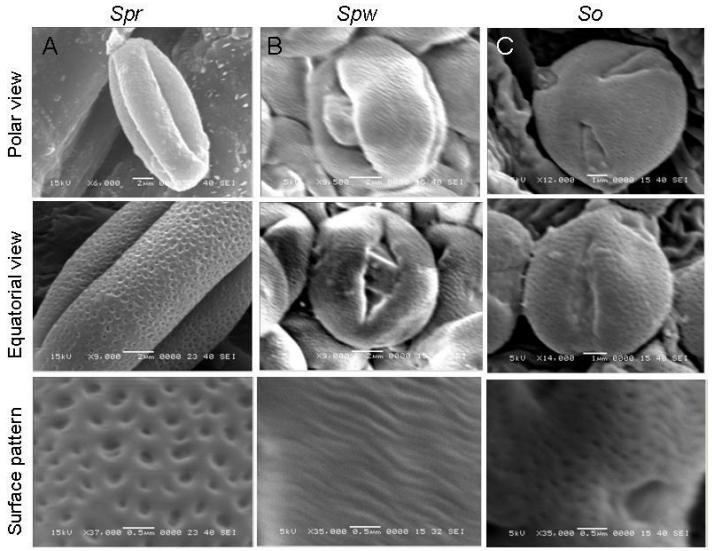

Fig. 5. Morphological characteristics of the pollen grains of *Salvadora* species by SEM. *A* - *S. persica* L. (with red fruit), *B* - *S. persica* L. (with white fruit), *C* - *S. oleoides* Decne. A polar view (top), equatorial view (middle) and surface pattern (bottom) are shown.

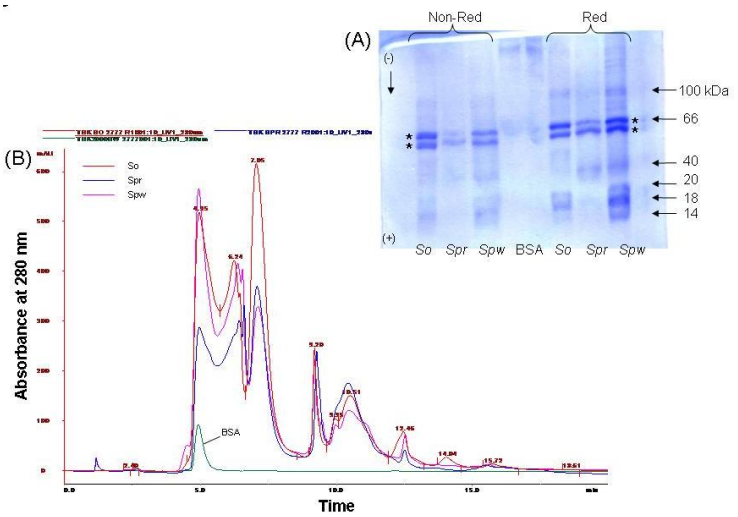

Fig. 6. Biochemical characterization of the seed proteins as biochemical markers of *Salvadora* species analyzed by SDS PAGE and SEC FPLC. *A* - electrophorogram of the crude seed protein extracts (*ca.* 15µg) subjected to 10% SDS-PAGE under non-reducing and reducing conditions. The arrow indicates the direction of electrophoretic mobility and asterisks marked the common protein markers. *B* - Separation profiles of the same samples (*ca.* 1mg/ml) subjected to SEC FPLC column equilibrated and eluted with 50 mM Tris-HCl buffer, pH8.5. Flow rate was maintained at 1ml/min and the eluate was monitored at 280nm. Abbreviations used; So**,** *S. oleoides* Dence (red trace); Spr, *S. persica* L. (with red fruit; blue trace) and Spw, *S. persica* L. (with white fruit; magenta trace). Bovine serum albumen (BSA) 1mg/ml was also used as standard (green trace).

Micromorphological properties:

Leaves: Both surfaces of the leaf marked with wrinkles show some stratification, leaf surface (both lower and upper) forming somewhat prominent ridges. Stomata are sunken and almost of the same size and uniformly distributed on both surfaces (Fig. 1, 2B).

Branches: The branches are spreading; light green in color, surface shows some stratification, stomata present, same sized and sunken (Fig. 3B).

Seeds: Small, globular, 4.5 µm long and 4.05 µm wide, light brown in color with distinct pattern scabrate having irregular cells arrangement, few with defined lines, cells are sunken and forming pit-like structure, inter spaces are filled with striate pattern forming ridges through the length (Fig. 4B).

Pollen grains: pollen spheroidal tricolporate 6.5 µm long and 5.85 µm wide colpi elongated broad elliptic mostly with converging acute ends having a distinct circular protruding ora in the center and pollen surface shows light wavy pattern. Sexine layer thicker than nexine (Fig. 5B).

***Salvadora oleoides* Decne**

Macromorphological properties: A shrub attaining the size of a small tree, 2-6m high, much branched, leaves simple opposite petiolate, linear. Inflorescences axillary, branched panicled. Flowers greenish-white in colour, complete, actinomorphic pedicellate, bisexual. Sepal lobes fused half of the length, with rounded apex, Petal 4, connate halfway, obovate or oblong. Stamens 4, attached at the base of petal lobes, filaments alternating, anther dorsifixed. Style absent; stigma peltate short; ovary obovate, erect. Fruits globose, brownish – pink or dark brown when mature. Seeds brown obovate rough (Table 1, Fig. 1).

Micromorphological properties:

Leaves: Both surfaces with stomata, sunken and almost the same size and uniformly distributed on both surfaces. This specie has very distinct pattern on both the surface of leaf as compared to other species of *Salvadora*. Epidermal cells are of two different shapes, few are circular and bulging, and others are forming rows of elongated suppressed cells. Both surfaces of leaf show trichomes which are very sparsely distributed on the surface but found more near to the margin. Trichomes are simple non glandular and unicellular (approximate size 35.1-36.2µm). The presence of trichome on leaf surface is a distinguishing character of this species (Fig. 1, 2C).

Branches: branches spreading; stiff, yellow green, surface glaucous and with trichome. The presence of trichomes on branch surface is a distinguishing character of this species (Fig. 3C).

Seeds: Globular, 6. 54 µm long and 5.24 µm wide, brown in color. Seed surface show reticulate pattern with small pites. The cells are regularly or irregularly arranged in circular rows (Fig. 4C).

Pollen grains: Pollens are spheroidal tricolpate, 6.02 µm long and 6.54 µm wide. Aperture broad, lanceolate to elliptical with concave surface and some-what bulging center. Exine is thicker then sexine (Fig. 5C).

Biochemical properties of *Salvadora* sp (a chemotaxonomic approach): Seeds are the natural reservoirs of proteins including; storage (e.g. albumin, globulin, prolamin & glutelin), functional enzymes (e.g. phosphatases, proteases & lipases) and their inhibitors (e.g. STI & LBTI) etc. thus can serve as biochemical or chemotaxonomic markers for the differentiation of plant species and/or varieties (Sharifnia and Assadi, 2003; Kharazian 2008). Quantification of the concentrations of major seed storage proteins revealed subtle variation both in term of total proteins (mg/g) as well as the percent yield of individual protein in seed flour (Table 2). On the other hand, crude proteins extracted in 50 mM Tris-HCl buffer, pH8.5 revealed a maximum total protein concentration of *ca* 21-37.5 mg/g. Samples were subjected to comparative SDS-PAGE analysis under non-dissociating and dissociating/denaturing conditions. The electrophoretic pattern revealed two protein bands of approximate molecular masses of 60 and 66 kDa in all the tested samples indicating that these conserved proteins can serve as markers for the genus *Salvadora* (Fig. 6A, Table 1). Some minor high molecular mass bands (>100 kDa) appeared in *S. persica* L. (with white fruit) which were not observed in other two species. Likewise, some low molecular mass protein bands were differentially present and absent in different *Salvadora* species. Notably, a minor band of 40 kDa could be detected in both S. *persica* L. (with red fruit) and *S. persica* L. (with white fruit) but not in *S. oleoides* Decne and can serve as specie specific marker in this case. Similarly, low molecular mass protein bands (between 14-20 kDa) were totally absent in *S. persica* L. (with red fruit) but present in low quantities in *S. oleoides* Decne, while in very high quantities in *S. persica* L. (with white fruit).

The chromatographic behavior of these protein samples with identical extraction conditions (i.e. 50 mM Tris-HCl buffer, pH8.5) were also analyzed by size exclusion fast protein liquid chromatography. Separation profiles of the SEC FPLC results of the *Salvadora* species reveal identical separation pattern i.e. same number of peaks but with different protein concentrations as reflected by UV absorbance (Fig. 6B). Cumulatively, biochemical techniques independently complement each other and suggest that the three samples having common proteins marker belongs to the same genus *Salvadora* while, other minor proteins and their variable concentrations nicely support the differences within *Salvadora* species.

Conclusions

Most of the earlier taxonomic work on the genus *Salvadora* recognized two species i.e., *S. oleoides* Decne and *S. persica* L., from Pakistan (Qureshi, 1972). Later Perveen & Qasier, (1996) confirm the two *Salvadora* species based on pollen morphology. In the present studies some intra-species variations within *S. persica* L., have been observed in leaf, branch, fruit, seed, and pollen characteristics. Description of the genus *Salvadora* with special reference to seed proteins as biochemical markers (chemotaxonomy) besides fruit colour, seed, leaves, branch and pollen macro and micromorphology (via light and scanning electron microscopy) were established which reveal subtle variations in characteristics (Table 1). *S. oleoides* Decne showed very distinct linear leaf shape and could be clearly distinguished from *S. persica* L., which had ovate leaves or lanceolate.

Likewise, the leaf surface pattern of the *Salvadora* species also confirms the subtle differences (Fig. 1, 2). The fruit colour of fully mature *S. oleoides* Decne is brown while the fruit color of *S. persica* L., is red and/or white as reported earlier (Qureshi, 1972). The pollen grains of *S. oleoides* Decne are spheroidal tricolpate, whereas pollens of *S. persica* L., (with red fruit) was prolate with tricolpate aperture and that of *S. persica* L., (with white fruit) was spheroidal tricolporate (Fig. 5). These results are also in accordance with the previous studies of Perveen & Qasier, (1996). Furthermore, micromorphology and pollen attributes of *S. persica* L., (with white fruit) does not match with *S. persica* L., (with red fruit) as well as *S. oleoides* Decne. Currently, seed macro and micromorphological studies appear to be an excellent added tool for systematic studies of plants (Dadandi *et al.,* 2009; Özcan & Zorlu, 2009). In the present studies, both branch and seed features revealed remarkable differences within the *Salvadora* species (Fig. 3, 4). Likewise, the biochemical analysis utilizing seed proteins as biochemical markers for identification also reveals differences in total protein concentrations (Table 2) and band patterns (Fig. 6A-B) and clearly indicates the possibility of two subspecies and/or varieties of *S. persica* L., in Sindh, Pakistan. Our data are also well supported by the earlier reports of Verdcourt (1964) suggesting much variation within *S. persica* L. compared to *S. oleoides* Decne. The presence of at least two varieties (i.e. *S. persica* L., var *indica* Wight & var *tuticornica*) are found in Pakistan and also reported from India (Rao & Chakraborti, 1996).

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