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ECOPHYSIOLOGICAL AND ANATOMICAL CHARACTERISTICS OF THE SUBTROPICAL SHRUB *ZANTHOXYLUM ACANTHOPODIUM* (RUTACEAE) IN CONDITIONS OF A TEMPERATE CONTINENTAL CLIMATE (SERBIA)

TAMARA RAKIĆ[1], JASMINA ŠINŽAR-SEKULIĆ[1], BILJANA FILIPOVIĆ[2], VANJA TADIĆ[3],
BRANKA STEVANOVIĆ[1], and KIT TAN[4]

[1]*Institute of Botany and Jevremovac Botanical Garden, Faculty of Biology, University of Belgrade,* 11000 Belgrade, Serbia
[2]*Siniša Stanković Institute for Biological Research,* 11000 Belgrade, Serbia
[3]*Dr. Josif Pančić Institute for Medicinal Plant Research,* 11000 Belgrade, Serbia
[4]*Institute of Biology, University of Copenhagen,* 2200 Copenhagen, Denmark

Abstract — The evergreen shrub *Zanthoxylum acanthopodium* DC. (Rutaceae), originating from warm temperate and subtropical Asia, has existed successfully in the Jevremovac Botanical Garden in Belgrade for more than 80 years. The seasonal pattern of water management in leaves, electrolyte leakage, essential oil composition, and leaf anatomy were examined in order to understand the resistance and viability of this subtropical shrub in the temperate continental climate of Belgrade, Serbia.

Key words: Adaptation, cell membrane damage, oil composition, leaf anatomy, *Zanthoxylum acanthopodium*, (Rutaceae)

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INTRODUCTION

The genus *Zanthoxylum* DC. (Rutaceae) comprises c. 200 species of aromatic deciduous and evergreen trees and shrubs native to warm temperate subtropical regions of the world. The genus as a whole and some species in particular have much ethnobotanical importance and are used as medicinal plants and sources of pharmaceutical and cosmetic raw material. They are especially prized as spices (Samant and Dhar, 1997). “Sichuan pepper” includes a complex of Asiatic taxa with a distribution from the Himalayan region to Southern and Eastern Asia. The fruits, young leaves, and sometimes the bark are widely used as spices, unlike those of the American and African species. The taste is strong and pungent, resembling the flavor of lemon, anise or mint (Chyau et al., 1996).

Zanthoxylum acanthopodium DC. is an evergreen shrub occurring from Northern India and the Tibetan highlands across to Eastern and Southeast Asia (Bangladesh, Bhutan, China, Myanmar, Cambodia, Vietnam, Thailand, and Malaysia). It is found at altitudes of c. 1000 to 2500 m or even higher than 3000 m. In China, it is fairly common in the provinces of Yunnan and Sichuan, while in India it is mentioned as widely distributed but decreasing in numbers (Nayar and Sastry, 1987).

Within its native habitat, *Z. acanthopodium* grows on calcareous limestone in warm and humid subtropical conditions (climate categorized as Type II according to Walter et al., 1975). The regime varies, with heavy precipitation in the humid south to lower rainfall and cooler temperatures further north. In general, the annual rainfall is 1500 mm and several areas in the distribution range usually receive more than 2000 mm, the greatest amounts falling in the summer. The average annual temperature is almost always higher than 25°C. Seasonally humid, evergreen-broadleaved vegetation is characteristic of such climatic conditions.

The first shrub of *Z. acanthopodium* was planted in the open grounds of the University Botanical

Garden in Belgrade more than 80 years ago. The shrubs flower annually from the end of March till the beginning of May, producing short dense clusters of greenish-yellow flowers (Fig. 1). The reddish-purple fruits ripen in autumn (from September to October) and remain on the branches throughout the winter. Since *Z. acanthopodium* is naturally dioecious, the fruiting shrubs in the Botanical Garden in Belgrade must be female. However, the viability of seeds from the shrub has not been well studied and the Botanical Garden plants may in fact be agamospermous (D. X. Zhang, pers. comm., 2007). Monitoring the status during flowering might reveal whether staminate and pistillate flowers can revert to being functionally fertile.

Despite its different natural habitat, the shrub and its vegetatively propagated descendants in the Belgrade Botanical Garden tolerate well the great temperature differences between Belgrade's hot summers with an average maximum of 35°C (or even above 40°C) and the very cold winters, when temperatures drop to below minus 25°C (Bogojević, 1968; Jovanović, 1994). The hot summer months are accompanied by moderate drought, while a real semi-arid period begins from the end of July to September. May and June are the months with the highest rainfall, which, however, rarely exceeds an average annual value of 100 mm (Fig. 2).

The Belgrade region belongs to a transitional arid (continental) temperate climate zone (Type VI/VII according to Walter et al., 1975). The cold winter months are distinguished by shorter or longer periods of frost and snow cover. Annual temperature amplitudes are c. 40°C, but sometimes they exceed 60°C. *Zanthoxylum acanthopodium* retains its leaves throughout these conditions and apparently does not suffer irreversible damage. The leaves fold under stress, during both summer drought and

Fig 1. A) *Zanthoxylum acanthopodium* fruiting in Belgrade Botanical Garden; B) large imparipinnate five-foliolate leaf of *Z. acanthopodium*.

dry winter frost, but they recover rapidly with the smallest favorable improvement in the environment in the same manner as resuscitated leaves of true poikilohydrous plants (Black and Pritchard, 2002). Our interest was thus directed to a study of the plant's leaf anatomy, its changes in water status during summer and winter, and the effects of drought and freezing on plant tissues as indicated by the rate of solute leakage. We also analyzed the composition of the leaf essential oil and its significance for the plant during unfavorable climatic periods in Belgrade.

MATERIALS AND METHODS

Ecophysiological investigations of the shrub from the Belgrade Botanical Garden were carried out in August 2003 (the hottest and driest summer period) and during January to February 2004 (the coldest winter time). Voucher specimens of the plant (Accession No. 16251) are deposited in the Herbarium of the Institute of Botany and Botanical Garden in Belgrade (BEOU) and in Copenhagen (C).

Leaf anatomy

Permanent slides of leaf sections were prepared using a standard histological method (Ruzin, 1999). The leaves were fixed overnight in Navashin's fluid, dehydrated through a series of ethyl alcohols, and then embedded in paraplast. Transverse leaf sections 5 μm thick were cut using a Reichert sliding microtome and (after removal of the paraplast) double stained in safranin and hematoxylin. The preparations were examined with a light microscope (Leica DMLS with attached Canon digital camera). A scanning electron microscope (JEOL JSM-35) was used to examine the leaf surface structure.

Water relations

Water relations were computed from quantitative determinations of total and relative water content

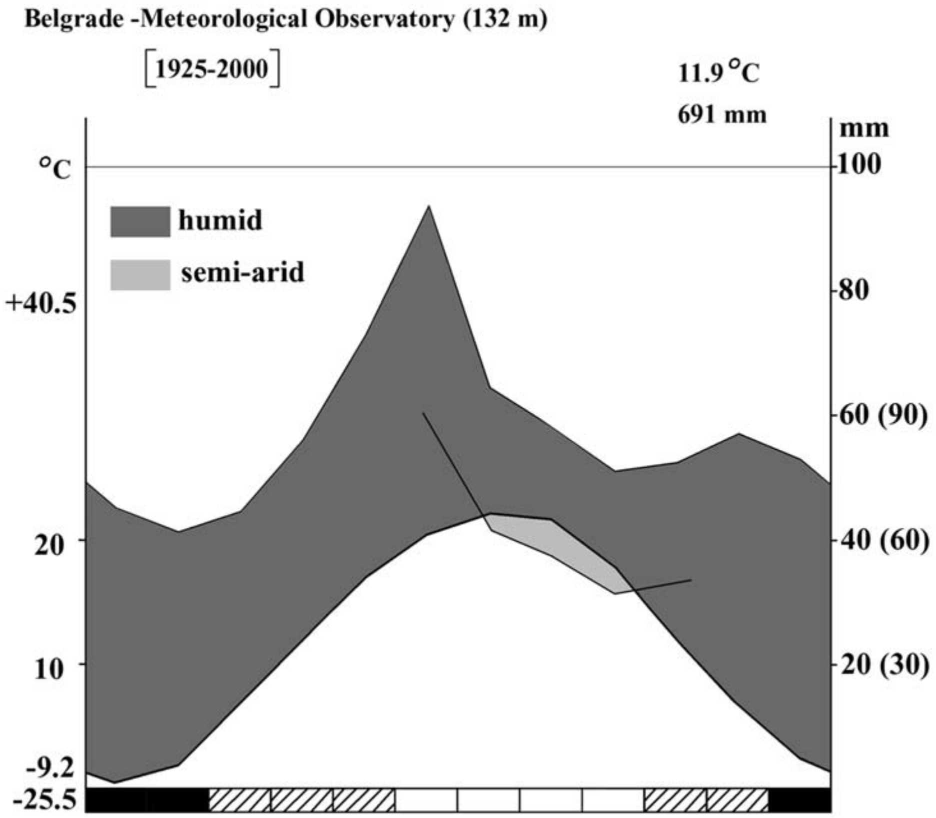

Fig 2. Climatic diagram of Belgrade.

according to Slavik (1974). The water and osmotic potential of leaves was determined following Walter and Kreeb (1970) and Scholander et al. (1965), and expressed as the mean value of three replicates for each treatment.

The total water content (TWC) at a given time was measured according to the following equation and expressed as a percentage:

$$TWC = [(\text{fresh weight} - \text{dry weight})/\text{dry weight}] \cdot 100$$

The relative water content (RWC) of leaves was expressed as a percentage of the water content at saturation according to the equation:

$$RWC = [(\text{fresh weight} - \text{dry weight})/(\text{saturated weight} - \text{dry weight})] \cdot 100$$

The water potential (Ψw) was measured using a pressure chamber (PMS Instrument, Corvallis, USA) following Scholander et al. (1965), while the osmotic potential (Ψs) was obtained from a semi-micro-osmometer (KNAUER).

Electrolyte leakage

Ion conductivity was measured according to Vasquez-Tello et al. (1990) using leaf disks that were submitted for four hours to the drought stress induced by PEG 600 (polyethylene glycol) and mannitol solutions of the same osmotic potential of - 1.68 MPa. After PEG and mannitol osmotic treatment, the disks were rinsed and floated on distilled water, ion conductivity being measured every hour during the next 10 hours. The leaf disks were then warmed at 80°C for four hours and the total amount of electrolytes released was measured. Electrolyte conductivity (mS) was measured with a conductometer (HI 8733, Hanna, Tokyo). The control leaf disks were treated only with distilled water. In order to integrate differences between the control leaves and the total electrolyte content of PEG- and mannitol-treated leaves, leakage was expressed using the index of injury (Id%), calculated according to the equation of Flint et al. (1966):

$$Id = (Rt/Ro) \times 100$$

where $Rt = EC/EC_{total}$ for PEG- and mannitol-treated specimens; $Ro = EC/EC_{total}$ for control specimens; EC = ion conductivity at one-hour intervals; and EC_{total} = ion conductivity after a 10-hour period.

Essential oil analysis

Essential oils were obtained from fresh leaves by steam distillation in a Clevenger type glass apparatus. Qualitative and quantitative analyses of essential oils obtained from leaves taken in the summer (X) and winter (Y) periods were performed by gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS). Gas chromatographic analysis was carried out on a gas chromatograph of the HP 5890 (series II) type, fitted with a FID and HP-5 column measuring 25 m x 0.32 mm under the following conditions: film thickness, 0.52 mm; carrier gas, H2; flow rate, 1 mL/min; injector temperature, 250°C (split ratio = 1: 50); and detector temperature, 280°C. The temperature program was 40-280°C (with a heating rate of 4°C/min).

The GC/MS analysis was performed using a chromatograph of the Hp G1800C (GCD Series II) type, equipped a with split/splitless injector and HP-5MS column (cross linked 5% PH ME Siloxane) measuring 30 m x 0.25 mm under the following conditions: film thickness, 0.25 mm; carrier gas, H2; flow rate, 0.9 mL/min; injector temperature, 250°C (split ratio = 1: 50); and detector temperature, 280°C. The temperature program was 40-280°C (with a heating rate of 4°C/min). Acquisition was carried out in scan mode from 45: 450.

Component analysis: the identification of each compound was carried out by comparison of their retention times with the retention times of standard substances and by matching mass spectral data of oil constituents with those in MS libraries (NBS library/ Wiley), using a computer search and literature. Retention times of determined components match Kovat's index (KI) on a DB-5. The percentage of each oil component was computed from GC (FID) peak areas without using correction factors.

RESULTS

The leaves of *Z. acanthopodium* are large, five-foliolate, imparipinnate, and markedly xeromorphic. The leaf area is reduced with the leaf rachis winged between the leaflets (Fig. 1). Spines are present on the abaxial side of the leaf midvein and on the stem and shoot. During summer drought and winter desiccation, the leaflets as well as the winged leaf rachis fold towards the lower (abaxial) surface.

The leaves are quite thick, varying from 221 to 278 µm in transverse section. They are hypostomatic, with the stomata either on level with the other epidermal cells or slightly raised above the leaf surface (Figs. 3A and 4A). A prominent cuticle is present on both leaf surfaces, covering the enlarged epidermal cells filled with dense mucilaginous substances. Both external and internal secretory structures were

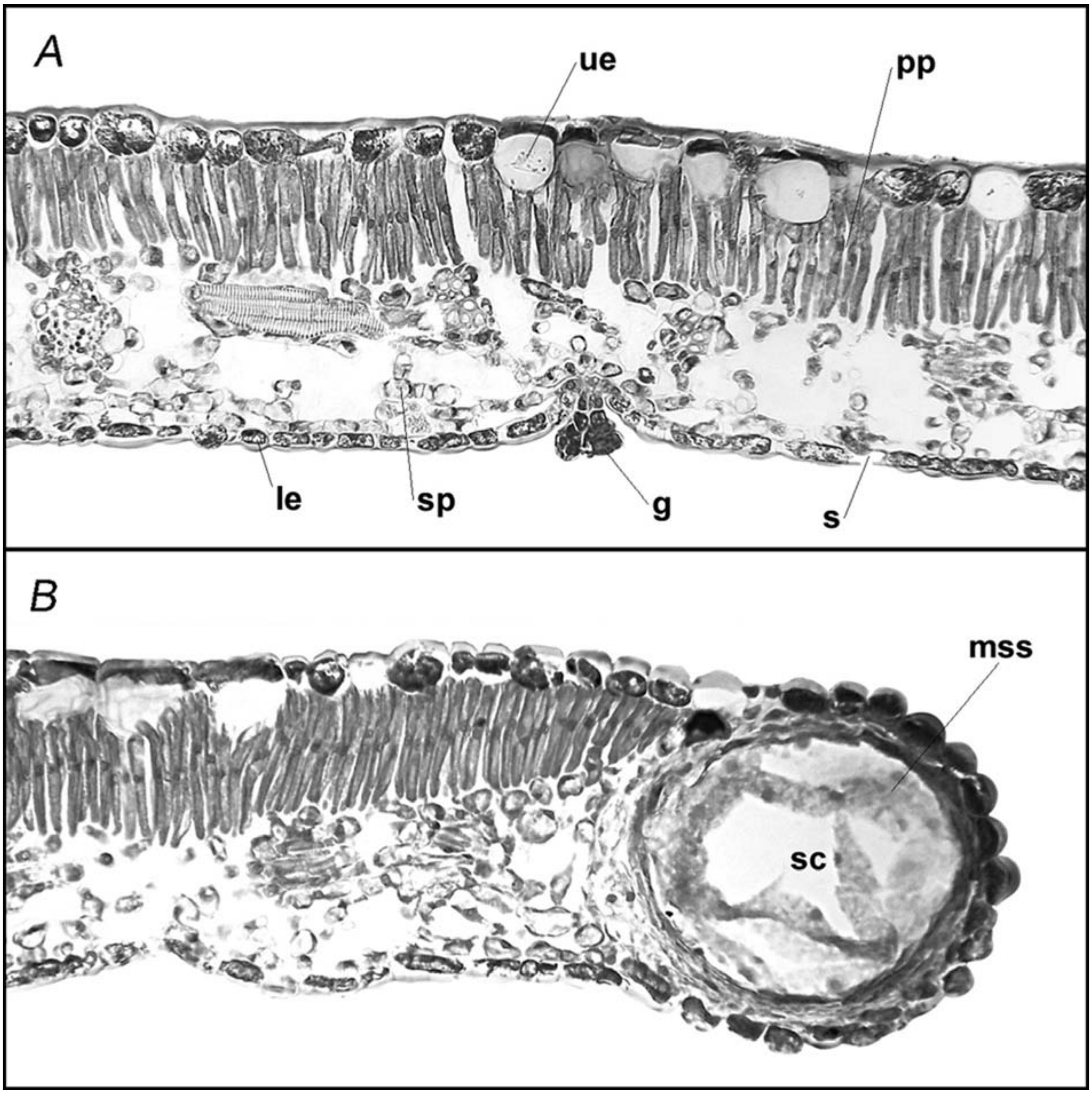

Fig 3. A) Leaf transverse section (ue - upper epidermal cells, le - lower epidermal cells, pp - palisade parenchyma, sp - spongy parenchyma, g - peltate gland, s - stomata); B) leaf transverse section (sc - secretory cavity, mss - marginal secretory structure).

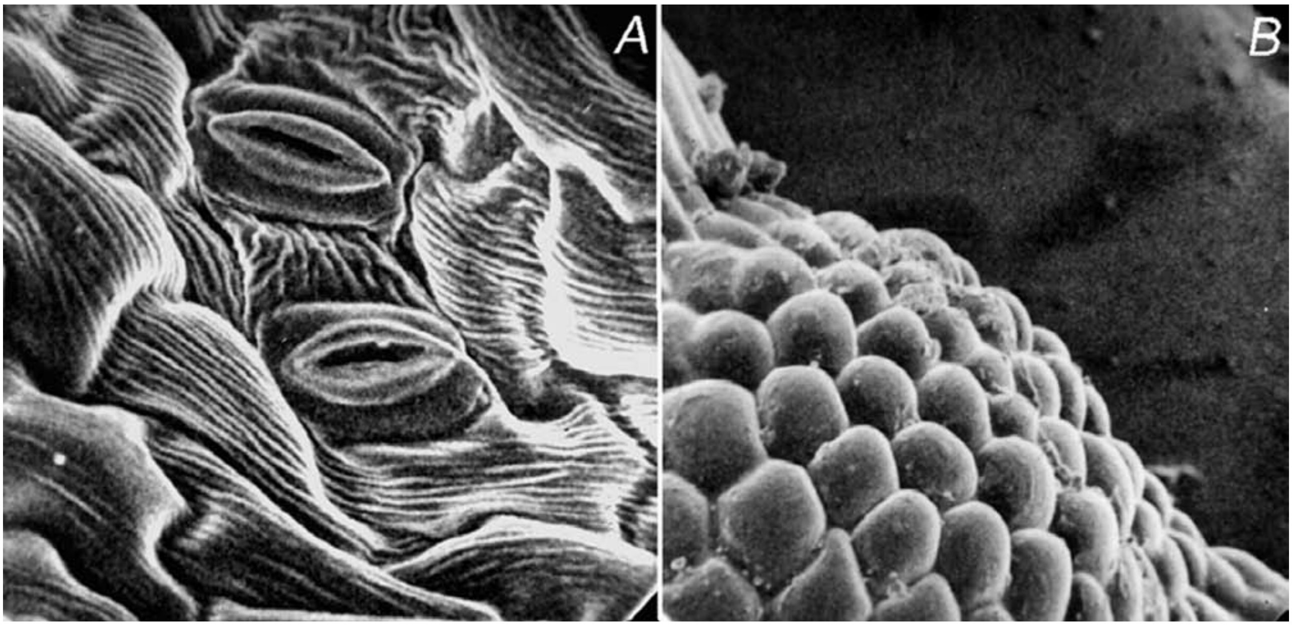

Fig 4. A) Stomata and cuticle surface of lower epidermal cells (SEM x 2000); B) surface of cells creating the marginal secretory structure (SEM x 500).

observed. The external secretory epidermal appendages are numerous multicellular biseriate peltate glands found on the abaxial side (Fig. 3A), while the large internal secretory cavities are situated at the leaf margin (Figs. 3B and 4B). Palisade tissue of these dorsiventral leaves comprises only a single layer of compact elongated cells, whereas the spongy tissue is composed of loosely arranged cells with large intercellular spaces. The ratio of palisade to spongy tissue is 1: 1. Numerous vascular bundles are present in the leaf mesophyll. The largest (midvein) vascular bundle has the additional development of sclerenchyma and secondary conducting tissue.

Zanthoxylum acanthopodium showed obvious seasonal fluctuation of its water relations parameters. In general, summer values of total and relative water content (71 and 87%, respectively), as well as of the water and osmotic potential (- 1.1 and - 1.9 MPa, respectively), were higher than those recorded

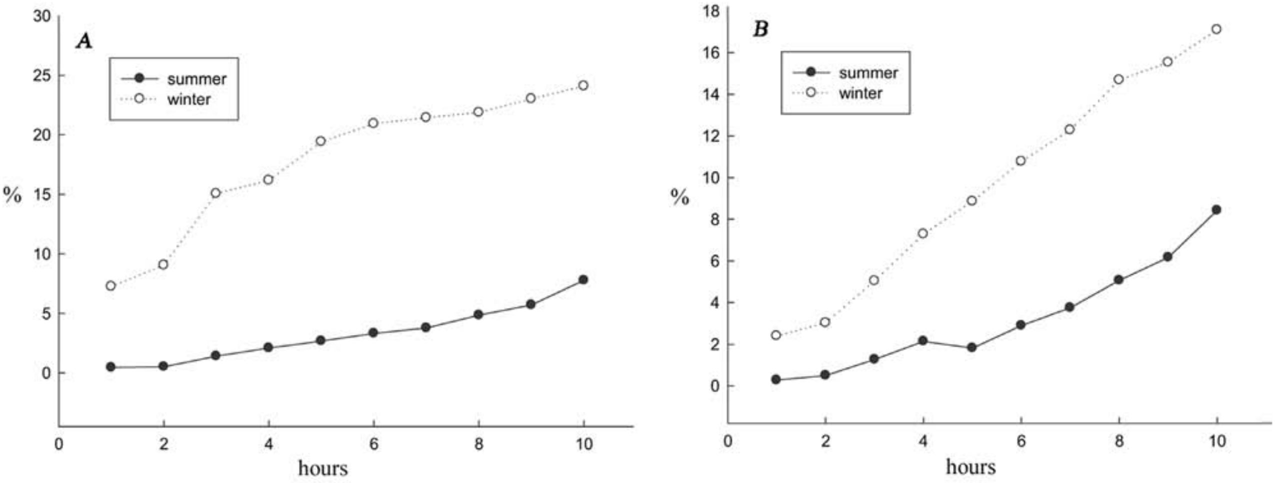

Fig. 5. Electrolyte leakage of leaves stressed in (A) PEG 600 and (B) mannitol during summer and winter (expressed by the index of injury).

Table 1. Values of water relations parameters.

	TWC (%)	RWC (%)	Ψw (MPa)	Ψs (MPa)
summer	71	87	- 1.1	- 1.9
winter	63	56	- 3.1	- 2.0

Table 2. Essential oil compounds from *Z. acanthopodium* leaves. tr < 0.5%, X - summer, Y - winter

No.	COMPONENT	DB 5	KI	X %	Y %
	Monoterpenoid compounds				
	Monoterpene hydrocarbons				
1	Thujene	0307	0931	0.1	0.1
2	α-Pinene	0319	0939	0.7	0.7
3	Sabinene	0379	0976	6.4	5.2
4	β-Pinene	0386	0980	0.4	0.4
5	β-Myrcene	0408	0991	2.3	2.5
6	α-Phellandrene	0435	1005	1.1	1.3
8	α-Terpinene	0457	1018	0.1	0.2
9	p-Cymene	0471	1026	0.1	tr
10	β-Phellandrene	0482	1031	-	0.1
11	Limonene	0481	1031	14.8	18.3
12	*(Z)*-β-Ocimene	0498	1040	tr	tr
13	*(E)*-β-Ocimene	0519	1050	0.1	1.6
14	γ-Terpinene	0545	1062	0.4	0.4
15	*(Z)*-Sabinene hydrate	0560	1068	0.5	0.5
16	Terpinolene	0608	1088	0.3	0.3
17	p-Menth-1,3,8-triene	0661	1111	0.2	-
	Oxidized monoterpenes				
	Aldehydes				
18	Citronellal	0758	1153	-	0.2
19	Dihydromyrtenal	-	-	0.3	0.3
20	Neral	0980	1240	-	0.1
	Alcohols				
21	*(E)*-Sabinene hydrate	0629	1097	tr	0.7
22	Linalool	0632	1098	0.8	tr
23	Thujanol-3	0791	1166	-	0.7
24	Terpin-4-ol	0820	1177	1.1	1.1
25	α-Terpineol	0852	1189	0.9	0.6
26	Myrtenol	0867	1194	2.5	1.9
27	Nerol	0949	1228	0.2	0.2
28	Citronellol	0950	1228	-	tr
29	*(E)*-Myrtenol	1026	1258	0.5	0.8
	Ethers				
30	1,8-Cineole	0485	1033	33.0	37.4
	Esters				
31.	Bornyl acetate	1099	1285	0.1	0.1
32	trans-Pinocarvyl acetate	1135	1297	0.2	-
33	cis-Pinocarvyl acetate	1165	1309	-	0.6
34	trans-Myrtenol acetate	1347	1381	-	0.2

Table 2. Continued.

No.	COMPONENT	DB 5	KI	X %	Y %
	Ketones				
35	Pinocarvone	0781	1162	0.8	-
36	cis-3-Pinocamphone	0.809	1173	tr	tr
37	Piperitone	1011	1252	-	tr
	Sesquiterpenoid compounds				
	Sesquiterpene hydrocarbons				
38	α-Copaene	1334	1376	-	0.1
39	β-Elemene	1375	1391	0.3	0.3
40	β-Caryophyllene	1442	1418	2.6	2.2
41	α-Humulene	1527	1454	tr	0.3
42	Germacrene D	1594	1480	4.3	4.7
43	β-Selinene	1608	1485	-	0.1
44	Byciclogermacrene	1632	1494	1.2	1.3
45	Germacrene A	1653	1503	0.6	-
46	γ-Cadinene	1676	1513	0.2	0.1
47	δ-Cadinene	1700	1524	0.1	0.1
48	Germacrene B	1777	1556	0.1	0.1
49	epi-byciclosesquiphellandrene	-	1565	0.2	-
	Oxidized sesquiterpenes				
50	*(E)*-Nerolidol	1796	1564	0.4	0.3
51	α-Eudesmol	2000	1652	0.2	0.1
52	*(Z, E)*-Farnesol	2106	1697	-	0.4
	Aliphatic volatile organic compounds				
53	Hexyl acetate	0439	1008	tr	tr
54	Nonanal	0643	1102	0.2	0.4
55	n-Decanal	0893	1204	-	1.2
56	*(E)*-n-Decanol	1063	1272	-	tr
57	2-Undecanone	1116	1291	4.8	0.9
58	2-Undecanol	-	-	-	0.3
59	Undecanal	1156	1306	-	0.1
60	n-Pentadecane	1646	1500	-	0.2
61	1-methyl-ethyl-tetradecanale	-	-	-	0.6
	Aromatic volatile compounds				
62	Phenyl ethyl propionate	1265	1336	-	0.1
	TOTAL			83.1	89.7

during winter (Table 1). Hence, winter leaves are distinguished not only by very low RWC (56%), but also by conspicuous reduction of the water potential (- 3.1 MPa).

Exposure of leaf disks to hyper-osmotic solutions, PEG 600 and mannitol, caused a steady increase of electrolyte leakage from leaf disks during a period of 10 hours. Injury indices indicate increased electrolyte leakage in leaves during the winter period and thereby suggest possible occurrence of cell membrane damage (Fig. 5).

The composition of essential oil did not vary greatly from summer to winter, either quantitatively or qualitatively. Its content in leaf samples was 0.019 ml/100 g of dry weight and 0.017 ml/100 g dry weight in summer and winter, respectively. The most abundant constituents were sabinene, limonene, 1,8-cineole, and germacrene D (Table 2). However, some differences were found in the contents of limonene (X = 14.8%, Y = 18.3%), 1,8-cineole (X = 33.0%, Y = 37.4%), and *(E)*-β-ocymene (X = 0.1%, Y = 1.6%), these being rather less in summer than in winter leaves. The amount of 2-undecanone was significantly higher in leaves during August 2003 (X = 4.8%) than in February 2004 (Y = 0.9%).

DISCUSSION

Zanthoxylum acanthopodium undoubtedly is a xerophyte with xeromorphic leaf characteristics. Its structural and functional features are adaptations carried over from its subtropical origin in habitats of mild, moist, and warm climate conditions. It has thick glabrous sclerophyllous leaves that remain green throughout the year in both favorable and adverse seasons. The leaves have a well-developed cuticle, numerous rather raised stomata, and various glandular structures. All these features contribute to reduction of water loss by leaf surface transpiration. During summer drought and winter frost, the leaves of *Z. acanthopodium* fold toward the lower surface due to turgor decrease, primarily in cells of the spongy tissue. Hence, the appearance of the whole leaf changes, as not only individual leaflets fold up, but also the winged rachides, rendering the plant more tolerant to unfavorable environmental conditions. By this means, the lower leaf surface with stomata is protected and stomatal transpiration is reduced. Wrinkling of the leaves is a more pronounced structural response to conditions of water stress, and this is observed in poikilohydrous members of the Gesneriaceae (Stevanović, 1986; Stevanović et al., 1992; Živković et al., 2005). *Zanthoxylum acanthopodium* folds its leaves during desiccation, but quickly recovers even with small ambient improvements, e.g., increased humidity in the summer or a day's winter warming. Such slight changes act immediately to sustain the water status of the plant.

Peltate glands containing essential oils are located on the abaxial leaf surface. These, as well as the contents of secretory cavities at the leaf margin, contribute to general regulation of the transpiration rate and moderate any large water imbalance disturbances. Secretory substances and the type of secretory structure of this species and the genus as a whole are simultaneously of ecological, protective, and defensive significance, as well as being important for taxonomic diagnosis (Metcalfe and Chalk, 1950).

Water relation parameters indicate that *Z. acanthopodium* adapts better to summer drought in the Belgrade area than to freezing temperatures in winter, when lack of ground water is an additional problem. We presume that in its original habitat, even in high mountain regions, it is exposed throughout the year to less pronounced extremes in humidity and temperature than it currently experiences in the Belgrade Botanical Garden.

The water deficit provoked by osmotically active substances such as PEG 600 and mannitol serves as a physiological test for screening the resistance of plants to drought stress. As a rule, the onset of structural and functional changes in cell membranes is followed by increased solute leakage and appears to be well correlated with the drought sensitivity of the species (Vasquez-Tello et al., 1990). The remarkable electrolyte leakage observed during the winter period, i.e., the high injury indices obtained in winter (which are much higher than during the summer) suggests that this plant is primarily susceptible to winter desiccation followed by frost. However, it is also important to know the extent to which leakage represents damage to the xeromorphic leaves. This plant's injury indices are roughly similar to the degree of solute leakage occurring in the desiccation-tolerant *Ramonda serbica* and *R. nathaliae* (Gesneriaceae), as well as in the drought-resistant moss *Thamnobryum alopecurum* (Stevanović et al., 1997-1998; Šinžar-Sekulić et al., 2005). Relatively stable and fairly low values of the injury index indicate remarkable tolerance to water deficit. This correlates well with the functional protective mechanisms of water stress resistance in evergreen leaves of *Z. acanthopodium*. During the winter in Belgrade (corresponding to the dry season in its native origin), this shrub exhibited significantly lower values of the water and osmotic potential as a result of proper osmotic adjustment. Its advanced physiological and structural adaptations reveal a capacity to resist unfavorable conditions.

The qualitative and quantitative composition of essential oils, in which the leaves of this aromatic subtropical plant abound, varies little during seasonal climate changes. The main components of the essential oils in *Z. acanthopodium* are 1,8-cineole, limonene, sabinene, germacrene D, and β-caryophyllene. In other species of the same genus, other compounds such as (Z)-3-hexenol, citronellal, citronellol, bicyclo-germacrene, and germacrene D are dominant (Chyau et al., 1996; de Abreu Gonzaga et al., 2003; Facundo et al., 2003; Setzer et al., 2005; de Moura et al., 2006). A few compounds, ones such as germacrene D, β-myrcene, and β-ocimene, can be mentioned as the main common constituents of essential oils in leaves of the various species of this genus. The leaves and fruits contain mostly terpenoids, limonene, and sabinene, which are the citrus-scented compounds responsible for much of the fragrance. It is supposed that in the original habitats of *Z. acanthopodium*, there are no great changes in the composition of volatile essential oils over the year, owing to the moderate subtropical climate with constant temperature and humidity conditions. A slight increase in the quantity of essential oils during the winter can be regarded as a specific response of the species made possible by its origin, genetics, and capacity for acclimatization to seasonal changes, the cold period of Belgrade's temperate continenal climate corresponding to the drier part of the year in its natural subtropical area. Higher amounts of essential oils during winter in Belgrade could be related to low temperature and local weather peculiarities less favorable for evaporation of volatile compounds and therefore be a factor regulating the heat load and water deficit (Lakušić et al., 2006).

All data obtained so far indicate that *Z. acanthopodium* behaves as an exceptionally healthy and thriving evergreen shrub in Belgrade, growing, expanding, blooming, and fruiting every year. Flourishing under temperate continental climate conditions, it is exposed to great seasonal temperature and humidity fluctuations, as well as to periods of water deficit in both the hot summer and the cold winter (winter desiccation). It is evident that the shrub's xeromorphic anatomical adaptations and physiological adjustments (in the water potential, osmotic potential, and amounts of some essential oils) have enabled this plant to increase its resistance, persist, and recover from any disadvantageous changes that may have arisen during stressful periods in the past at its present locality. The question remains as to whether and to what extent the plant was injured during its initial adaptation for survival in a new environment far from its native land.

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ЕКОФИЗИОЛОШКЕ И АНАТОМСКЕ КАРАКТЕРИСТИКЕ СУПТРОПСКЕ ВРСТЕ *ZANTHOXYLUM ACANTHOPODIUM* (RUTACEAE) У УСЛОВИМА УМЕРЕНО КОНТИНЕНТАЛНЕ КЛИМЕ (СРБИЈА)

ТАМАРА РАКИЋ[1], ЈАСМИНА ШИНЖАР-СЕКУЛИЋ[1], БИЉАНА ФИЛИПОВИЋ[2], ВАЊА ТАДИЋ[3],
БРАНКА СТЕВАНОВИЋ[1] и КИТ ТАН[4]

[1]*Институт за ботанику и Ботаничка башта „Јевремовац“, Биолошки факултет, Универзитет у Београду,* 11000 Београд, Србија
[2]*Институт за биолошка истраживања „Синиша Станковић“,* 11000 Београд, Србија
[3]*Институт за проучавање лековитог биља „Др Јосиф Панчић“,* 11000 Београд, Србија
[4]*Институт за биологију, Универзитет у Копенхагену,* 2200 Копенхаген, Данска

Вечно-зелени жбун врсте *Zanthoxylum acanthopodium* DC. (Rutaceae) пореклом из Азије већ осамдесет година успешно напредује у Ботаничкој башти “Јевремовац”, Београд, Србија У циљу бољег разумевања добре адаптираности ове суптропске врсте на умерено-континенталне климатске услове Београда, праћене су сезонске промене у хидричком стању листова (водни и

осмотски потенцијал, релативни садржај воде), пропуштање електролита кроз плазма мембрану, карактеристике етарског уља као и анатомска грађа листа. Комплекс структурних (ксероморфни листови) и физиолошких одлика (промене водног и осмотског потенцијала као и количина етарског уља) омогућавају овој врсти да побољша резистентност, опстане и опорави се од евентуалних оштећења која се могу догодити током стресног периода.