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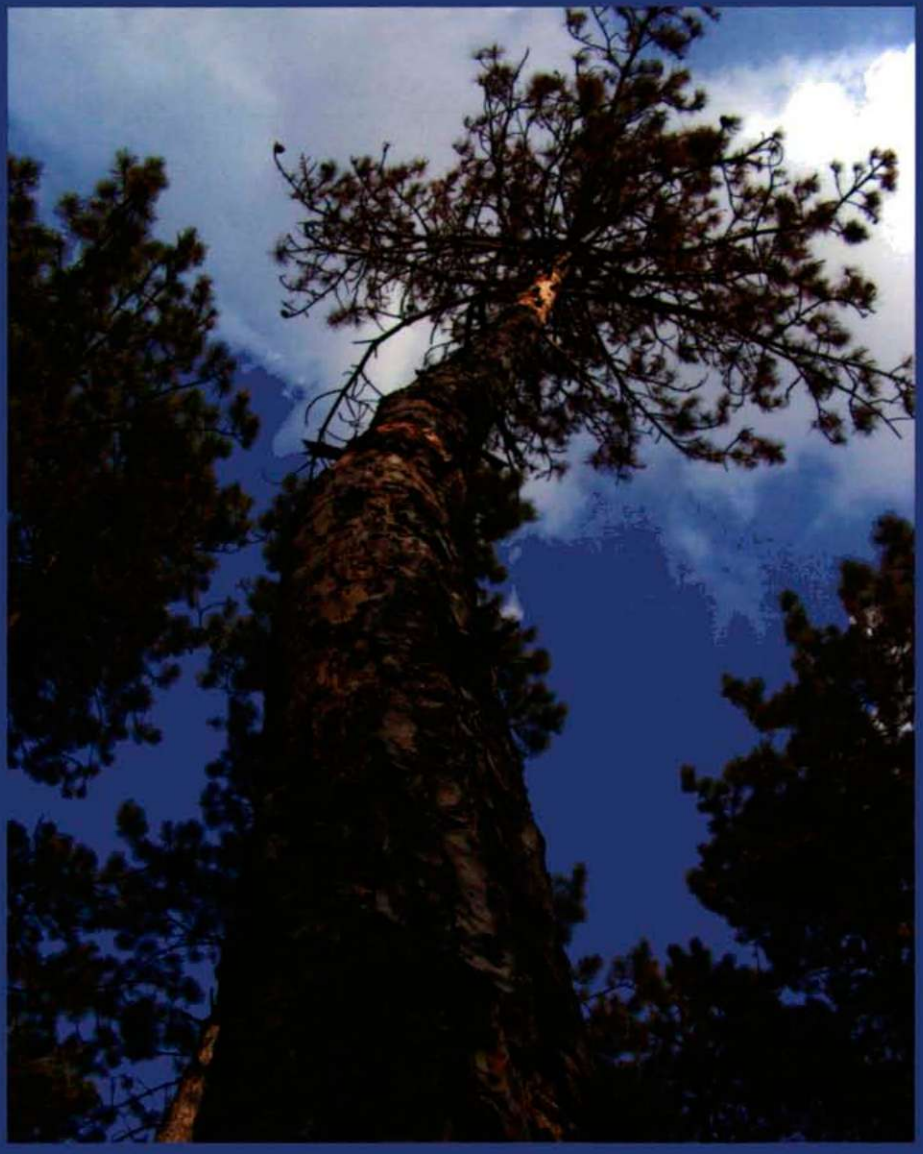

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REVIEW ARTICLE

Bursaphelenchus xylophilus, the pinewood nematode: its significance and a historical review

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ABSTRACT This paper reports on the biology, introduction, spread, damage and the control of *Bursaphelenchus xylophilus* (STEINER and BUHRER, 1934) NICKLE, 1970. Presenting a significant economic threat, the pinewood nematode is a unique quarantine status species of the genus *Bursaphelenchus*. The risk of its spread in European coniferous forests is especially high where the insect vectors are present. In Hungary, the pests *B. mucronatus* and *B. vallesianus* are present as well as its vector species *Monochamus*. Considering the health status of domestic planted pines, the severity of storm-damages, global warming, the increased volume of imported wooden packages and insect migration, the likelihood of *B. xylophilus* invading Hungary and finding favourable conditions keeps increasing. **Acta Biol Szeged 55(2):213-217 (2011)**

KEY WORDS

Bursaphelenchus xylophilus
Hungary
Monochamus sp.
pathway
spread
survey

The North American pinewood nematode, *Bursaphelenchus xylophilus* was identified as the main causal agent of the Pine Wilt Disease (Kiyohara and Tokushige 1971). A serious invasive and destructive species, it is listed as a quarantine pest in the legislation of more than 40 countries; is on the EPPO (European and Mediterranean Plant Protection Organization) A2 list (no.158.) and on the EU II/A2 list meaning that it is a regulated pest of quarantine significance present in at least one EPPO Memer State (PPO 211).

Taxonomic position, Bursaphelenchus species, hosts and symptoms

Its taxonomic position is the following: Nemathelminthes, Nematoda, Secernentia, Tylenchida, Aphelenchina, Aphelenchoidoidea, Parasitaphelenchidae, Bursaphelenchinae. The *Bursaphelenchus* genus contains about one hundred species (Hunt 2008), which are split into six groups, namely *xylophilus, hunti, aberrans, eidmanni, borealis* and *piniperdae* (Ryss et al. 2005). Braasch (2008) expanded the *xylophilus* group with species described after 2000 so this group currently contains *B. xylophilus, B. fraudulentus, B. mucronatus, B. kolymensis, B. conicaudatus, B. baujardi, B. luxuriosae, B. doui* and *B. singaporensis*. Presenting a significant economic threat to conifers, *B. xylophilus* is a unique quarantine species of *Bursaphelenchus* genus. With the help of its vector, *Monochamus* spp., this nematode is responsible for the Pine Wilt Disease, yet it does not cause significant mortality to native conifers in North America (Linit 1988). Pine Wilt Disease first appeared on the EPPO quarantine list in 1986. Effective early detection is a prerequisite for surveillance and eradication programs, as well as for the establishment of standards for pest risk analysis and the determination of pest-free areas (Schrader and Unger 2003). Most *Bursaphelenchus* species live on woody plants – mainly Coniferales: *Pinus, Abies, Chamaecyparis, Cedrus, Larix, Picea, Pseudotsuga* – and are mycophagus, while some species are phytophagus. A known exception among coniferous plants is *Thuja plicata*, which is considered to be immune to *Monochamus* spp. and thus, to the nematode. The most important feature of the damage is a sudden wilting. On infection, trees display the following symptoms: at first the production of oleoresin in the wounds significantly drops, secondly the transpiration of leaves decreases and finally stops, and later yet, the needles show yellow and red discoloration. All infection leads to the death of infected trees. Mortality rate is expected to peak from late August, not earlier than 30-50 days after the first symptoms. A tree may contain 10 million nematodes within its trunk, branches and roots (Braasch 1983). When scarred, healthy trees cover the surface of the scar with resin within a short time while infected trees produce less, if any, resin at all. However, these symptoms atypical and do not necessarily indicate the presence of the nematode: they might be caused by physical factors or by other pathogens. At the moment, there are no known symptoms to aid visual distinction between trees that are dying from Pine Wilt Disease and those dying for other reasons. It is important to note that the infection may or may not result in the quick appearance of symptoms: coniferous trees may remain symptomless for a considerable period of time (in experimental studies, up to 14 years). Latent infection is a key feature of the biology of nematode infestations. The pine nematode has gained importance throughout the European Union, especially in

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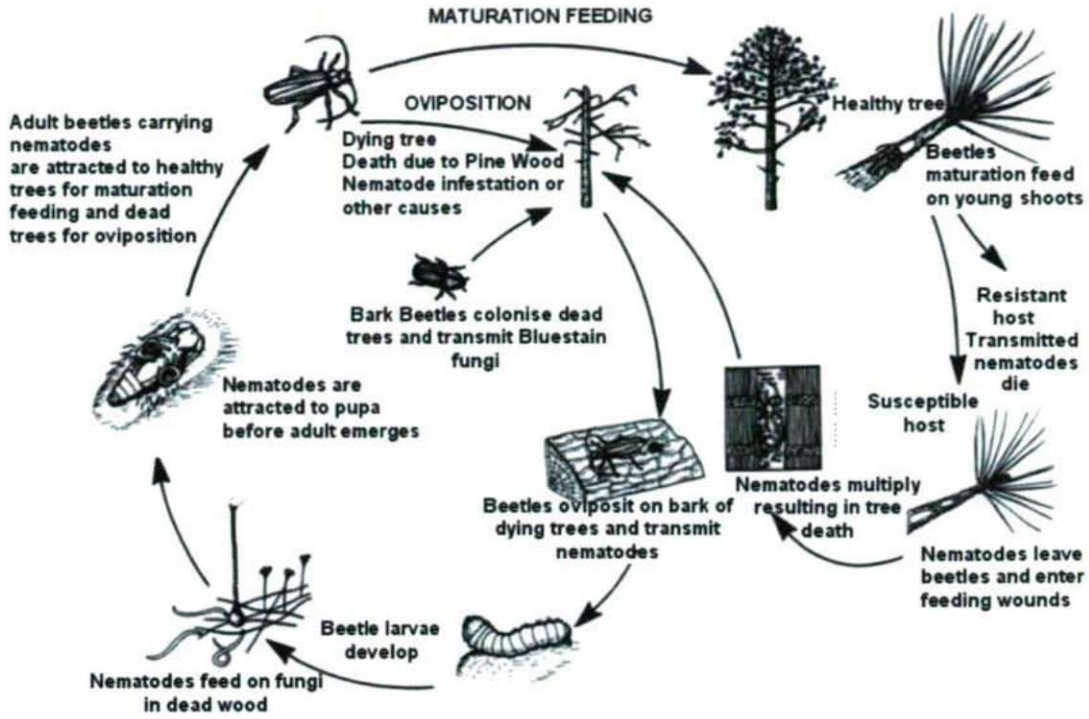

Figure 1. Transmission cycle of *B. xylophilus* by its *Monochamus* sp. insect vector (Wingfield 1987).

Northern countries (European Commission 2009).The only known solution to stop spreading the infection is to burn the infected plants after cutting. The infected plant dies within 1 to 3 months. Live *B. xylophilus* can also be found in roots, even when the upper parts of the tree are already dead, dried out or cut. Depending on climatic conditions and on the speed of desiccation of the wood, nematodes can be detected in trees up to 3 years after the trees died from Pine Wilt Disease (Malek and Appleby 1984). *B. xylophilus* can be found in coniferous plants for planting, cut branches, wood, isolated bark and wood shavings, but never in needles, cones or seeds. Under favourable conditions (25°C) this nematode can complete its life cycle from egg to adult in just four to five days (Ishibashi and Kondo 1977; Mamiya 1984).

Phytosanitary risk, pathway: vector and human

Pinewood nematode is a major threat to European pine forests today with an estimated mortality risk of >50% of pine trees in southern Europe. Their introduction and spreading may have a highly destructive effect on European forests (~82 million ha in the EU). The risk of its spreading in European coniferous forests is especially high where the insect vectors are present. *B. xylophilus* is transmitted from infected trees into healthy ones mainly by *Monochamus* beetles (Coleoptera: Cerambycidae). Alive individuals of the nematode species can be identified to species level directly from the bodies of their vectors. In healthy trees, nematodes favour the place of maturation feeding of *Monochamus* beetles, whereas on infected, or on dead trees they assemble where *Monochamus* lay their eggs (Fig .1). The pinewood nematode is transported in its fourth stage of development to new host trees by longhorn beetles (Cerambicydae). *Monochamus* vectors damage the trees during maturation feeding by chewing on young shoots and during finishing development by boring at least 3-mm wide holes under the bark. One usually finds the typical flat-headed *Monochamus* larvae /apodous/ under the bark or within oval larval galleries (grub holes). While the natural spread of nematode by insect vectors is limited (Braasch 2000), coniferous wood consignments and wood packaging material may both contain the nematode and the vector(s), making these the primary methods of spreading the nematode. To obtain adults and larvae for identification, one has to search for them in wood and in the insect vector. The number of nematodes per each vector may total from 15,000 to 230,000. Once the host tree is diseased, the food source for the pinewood nematode weakens and the pest starts feeding on various wood-inhabiting fungi such as blue-stain fungi, although these fungi, including *Trichoderma* spp. living on dead pines are unsuitable for *B. xylophilus* for propagation (Kobayashi et al. 1975). For a short period after having been introduced by vectors during oviposition or maturation feeding, nematodes are found in the vicinity of these locations.

They spread and multiply rapidly in all parts of the infected tree except in needles, cones and seeds. Nematodes have been observed to invade the root system as well, where they are able to survive for a certain period once the tree has died, dried or cut out. Under unfavourable climatic or host conditions however, the nematode infection does not achieve its usual systemic form (that is, the infection established in the crown does not spread) (European Commission 2009). *Monochamus* species prefer physiologically weakened trees. *B. xylophilus* were found on or within the bodies of cerambycid beetles (of the following genera: *Acalolepta*, *Acanthocinus*, *Amniscus*, *Arhopalus*, *Asemum*, *Corymbia*, *Neacanthocinus*, *Rhagium*, *Spondylis*, *Uraecha* and *Xylotrechus)* and on and within other coleopterans (eg *Chrysobothris*, *Hylobius*, *Pissodes*) (EPPO/CABI 1996). According to a 2001 publication on the European appearance and spreading of *Bursaphelenchus* species by Braasch, not all insect vectors are completely known, but the most important transmitters belong to families *Scolitydae, Cerambycidae* and *Curculionidae.*

Dispersal and economic impact

B. xylophilus was introduced to Eastern Asia and Western Europe by human activities and international trade of untreated and contaminated wood. Outside its native range, the nematode is one of the most important pests of pine trees and other conifers worldwide. It is the casual agent of Pine Wilt Disease, which, in certain conditions leads to the death of infected trees (Kobayashi et al. 1984; Kishi 1995; Evans et al. 1996). With timber consignments imported from the American continent, *B. xylophilus* was introduced to Asia in the late 1970's. The pest rapidly spread and has became one of the most important forest pests in Japan, China, Taiwan, Korea, and also in European countries, namely in Portugal and Spain (OEPP/EPPO, 1986; EPPO/CABI, 1996; Evans et al. 1996; Anonymous 2008). *B. xylophilus* is widespread in Canada and USA (Ryss et al. 2005; Sutherland 2008) and it appeared in Mexico as well (Dwinell 1993). In Japan tens of millions of dollars are spent annually to control *B. xylophilus* (Kulinich and Kolossova 1993). In 2000, approximately 580,000 hectares of pine forest that is 28% of the total pine forest area in Japan were estimated to have been infected by the species (Mamiya 2004). The nematode spreads easily by wood moving in trade, either as a commodity (live plants, log, sawn timber etc.), or as wood packaging material (being transported with other commodities), not meeting the requirements of ISPM No. 15 'Regulation of Wood Packaging Material in International Trade' (FAO 2009). *B. mucronatus* which is morphologically and biologically very similar to *B. xylophilus* does not infect trees of European native pine forests but its presence indicates the potential penetration of the pine wood nematode. According to speculations, *B. mucronatus* may threat Northern American coniferous stands when introduced with imported European (or Russian) pinewood into the USA. *B. mucronatus* is wide-spread in European pine forests and in Japan (Mamiya and Enda 1972). Damage done by *B. xylophilus* is the most significant where the average temperature exceeds 20°C in July and August (Braasch and Enzian 2004). Both *Bursaphelenchus* species contain a number of different pathogenic strains with various effect on coniferous species. It is very difficult to tell a harmful strain from a non-harmful one. North Europe has had import restrictions on coniferous timber and wood chips since the 1980's, when *B. xylophilus* was discovered in pine chips imported from Canada and the USA. Later, those restrictions were adopted by the EU and applied to most countries of Europe (Dwinell 1997). To prevent the introduction of *B. xylophilus* and its vectors from infected countries into the EU, a number of phytosanitary regulations (Directive 77/93 updated as 2000/29/EC) were implemented. Nevertheless in 1999, the first establishment of European pinewood nematode was found in Portugal (Økland et al. 2010). Findings of *B. xylophilus* in wood pallets exported from Portugal to other European countries triggered measures, and the European Commission banned Portuguese imports of all coniferous wood that were not proved of having undergone the required heat treatment (European Union 2008). *B. xylophilus* has been present in Portugal since 1999 and the infection was not stopped ever since despite of the important amounts of European Union subvention of nearly 24 Million Euros between 2001-2009 (EPPO 2009). The most important insect vector in continental Portugal is *Monochamus galloprovincialis*. The main transmitters of pinewood nematodes are *Monochamus* beetles and each geographical region has its own *Monochamus* species: for example *M. alternatus* (Japan and China), *M. saltuarius* (Japan and Korea), *M. carolinensis* and *M. titillator* in North America and *M. galloprovincialis* in Portugal (Schröder et al. 2009). In Portugal, *Pinus pinaster* is the coniferous species most affected by Pine Wilt, corresponding to an area of about 1 million ha (34% of the total forest) (Mota et al. 1999; Sousa et al. 2001). In Spain 344,000 Euros were spent in 2009 and 3 Million Euros in 2010 for control measures (EPPO 2009). In 2009 several countries identified live *B. xylophilus* individuals and their vectors in consignments of timber and in wood packing material arriving from Portugal.

Phytosanitary measures and survey

In compliance with EU regulations, measures against the pest include monitoring coniferous forests and coniferous wood, wood packing, nursery plants and coniferous plant material in international trade (OEPP/EPPO 2009a). Import restrictions vary among Member States. One of the recommended treatments is the correct use of heat treatment (HT) that kills both the vector and the nematodes as the wood reaches a core temperature of 56°C for a minimum of 30 minutes (Dwinell 1990 and 1997). EU Member States shall annually conduct official national surveys for pinewood nematode on

susceptible plants and for vector species occurring in their territory and on susceptible wood and bark originating in their territory, to determine whether there is any evidence of the presence of *B. xylophilus* in their territory or parts of their territory where pinewood nematodes were not yet detected. In Hungary the Central Laboratory for Pest Diagnosis, Central Agriculture Office, Directorate of Plant Protection and Soil Conservation has been conducting nation-wide surveys since 2003 for B. *xylophilus* in coniferous forests, within 25 km radius around pine forests and at all potential sources of danger (wood depot, airport, border post, international transport lines). Morphological and molecular diagnostic tests were also conducted according to international protocols (OEPP/EPPO 2009b, c) (Tóth 2010). As the chance to detect *B. xylophilus* on trees that seem healthy is very low, survey for the pest should be linked to trapping and testing for *Monochamus* beetles (European Commission 2009). Surveillance may include trapping and testing of *Monochamus* beetles. Official survey detected no individuals of *B. xylophilus* and other European *Bursaphelenchus* species between 2003 and 2010. While Hungary is free from the quarantine pest pinewood nematode (Tóth and Elekes 2011), *B. mucronatus* and *B. vallesianus* as well as the vector species *Monochamus* are present.

Conclusions

Despite the worldwide importance of *B. xylophilus* as a quarantine pest and its economic importance in forestry and associated industries, including wood packaging industry, the biology, ecology, long-term survival and development of *B. xylophilus* in wood packaging materials is still largely unknown (Sousa et al. 2011). In Hungary, the pests *B. mucronatus* and *B. vallesianus* are present as well as its vector species *Monochamus*. Considering the health status of domestic planted pines, the severity of storm-damages, global warming, the increased volume of imported wooden packages and insect migration, the likelihood of *B. xylophilus* invading Hungary and finding favourable conditions keeps increasing. The only protection against *B. xylophilus* is prevention, trapping and early detection. Monitoring may include trapping and testing of *Monochamus* beetles. Where the presence of *B. xylophilus* is confirmed, the strictest quarantine measures are to be taken: all infected trees should be totally destructed in a several km radius.

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ARTICLE

ISSR, RAPD and agronomic study in some F1 and F2 cotton genotypes

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ABSTRACT Cotton is an important economic crop plant with diploid and tetraploid cultivars. Hybridization is one of the main breeding strategies in cotton breeding producing new genotypes and also increasing the genetic diversity in cotton germplasm available. The present study considers agronomic and molecular study of genetic variations in thirteen F1 and F2 cotton genotypes (*Gossypium hirsutum*) obtained by crossing the cotton cultivars Bellizovar, No. 200, Siokra, Sindose and Tabladilla. Out of 30 RAPD primers used 19 primers produced 191 reproducible bands/loci out of which 63 bands were polymorph. Eight ISSR primers used produced 86 reproducible bands, out of which 27 bands were polymorph and 59 bands were monomorph. Some bands were present in the F1 progenies but absent in the F2 progenies of the same genotype. The mean values of gene diversity (H) and Shanon's Information Indices (I) for ISSR markers in the F1 progenies were 0.27 and 0.40 respectively, while the same values in F2 progenies were 0.18 and 0.26 respectively. Similarly the mean values of H and I of the F1 progenies for RAPD markers were 0.07 and 0.11 respectively, while the same values in F2 progenies were 0.09 and 0.13 respectively. UPGMA and NJ dendrograms grouped the F1 and F2 progenies of Siokra X Belilzovar together, standing from the other genotypes due to their genetic differences. The use of present finding in planning future hybridization is discussed.

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KEY WORDS

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Hybridization is one of the main breeding strategies in cotton breeding producing new genotypes and also increasing the genetic diversity in cotton germplasm available. The genetic variations induced by hybridization may lead to the formation of unique gene combinations necessary for new superior cultivars. This is important when we consider the fact that the extensive cultivation of closely-related cultivars by producers could result in vulnerability to pests and diseases due to loss of genetic diversity which brings about the genetic erosion (Sheidai et al. 2008; Van Esbroeck and Bowman 1998).

Cotton is an important economic and fiber crop, grown in 70 countries in the world including Iran. Both diploid (*Gossypium herbaceum*) and tetraploid (*G. hirsutum*) cotton cultivars are cultivated in the country. Tetraploid cotton (*Gossypium hirsutum*) has genome constitution of AADD (2n = 52) (Menzel and Brown 1978) and is one of the world dominating cotton cultivars.

Different molecular markers including RAPD (Random Amplified polymorphic DNA) as well as ISSR (Intersequence Simple Repeats) have been used for studying genetic diversity, hybridization and the occurrence of somaclonal variation in cotton (Wajahatollah and Stewart 1997; Kumar et al. 2003; Vafaie-Tabar et al. 2003; Mehetre et al. 2004; Dongre 2007; Rana et al. 2007; Preetha and Raveendren 2008; Sheidai et al. 2008; Wei et al. 2008; Tafvizei et al. 2010). The present report considers molecular diversity of F1 and F2 progenies of five cotton cultivars not studied before by us.

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Materials and Methods

Plant materials

For homogeneity of parent genotypes' seeds, selfing crosses have been carried out for two continues generations/years before future studies. Thirteen F1 and F2 cotton genotypes (*Gossypium hirsutum*) obtained by crossing the cotton parental genotypes Bellizovar, No. 200, Siokra, Sindose and Tabladilla, were cultivated in three rows of 10 m length with 20 cm interplant distance, in the experimental field of Gorgan Cotton Research Center of Iran, according to a completely randomized design (CRD) with 3 replications.

Total genomic DNA was extracted from fresh leaves using the CTAB method by Murry and Tompson (1980) with the modification described by De la Rosa et al. (2002).

RAPD analysis

Thirty decamer RAPD primers of Operon technology (Alameda, Canada) belonging to OPA, OPB, OPC, OPH, OPI and

OPM sets were used in this study. The PCR reaction mixture consisted of 20 ng template DNA, 1 x PCR buffer (10 mM Tris-HCL pH 8.8, 250 mM KCL), 200 µM dNTPs, 0.80 µM 10- base random primers and 1 unit of *Taq* polymerase, in a total volume of 25 µl. DNA amplification was performed on a palm cycler GP-001 (Corbet, Australia). Template DNA was initially denatured at 92°C for 3 min, followed by 35 cycles of PCR amplification under the following parameters: denaturation for 1 min at 92°C, primer annealing for 1 min at 36°C and primer extension for 2 min at 72°C. A final incubation for 10 min at 72°C was performed to ensure that the primer extension reaction proceeded to completion.

The PCR amplified products were separated by electrophoresis on a 2% agarose gels using 0.5 X TBE buffer (44.5 Mm Tris/Borate, 0.5 Mm EDTA, pH 8.0) or 6% polyacrylamide gels. The gels were stained with ethidium bromide and visualized under UV light (Sambrook et al. 2001). A 100 bp DNA ladder (GeneRuler, Fermentas) was used as the molecular standard in order to confirm the appropriate RAPD markers. These markers were named by primer origin, followed with the primer number and the size of amplified products in base pairs.

ISSR assay

Eight ISSR primers used are UBC807, UBC810, UBC811, UBC823, UBC832, UBC834, UBC849 and (CA)9GT commercialized by UBC (the University of British Columbia). PCR reactions were performed in a 25 µL volume containing 10 mM Tris–HCl buffer at pH 8; 50 mM KCl; 1.5 mM MgCl2; 0.2 mM of each dNTP; 0.2 µM of a single primer; 20 ng genomic DNA and 3 unit of *Taq* DNA polymerase (Bioron, Germany). Amplifications reactions were performed in Techne thermocycler (Germany) with following program: 5 min initial denaturation step 94°C, 30 s at 94°C; 1 min at 50°C, 1 min at 72° C. The reaction was completed by final extension step of 7 min at 72°C. Amplification products were visualized by running on 2% agarose gel, following ethidium bromide staining. Fragment size was estimated by using a 100 base pairs (bp) molecular size ladder (Fermentas, Germany).

Data analyses

RAPD and ISSR bands obtained were treated as binary characters and coded accordingly (presence =1, absence = 0). Jaccard similarity as well as Nei,s genetic distance (Nei 1973) was determined among the cultivars studied and used for grouping of the genotypes by UPGMA (Unweighted Paired Group with Arithmetic Average) and NJ (Neighbor Joining) clustering methods and ordination based on principal coordinate analysis (PCO) (Podani 2000; Weising et al. 2005). The fit of dendrograms obtained were checked by bootstrapping using 100 replications. Bayesian clustering was also performed on RAPD and ISSR data by using Markov chain Monte Carlo (MCMC) method (Hall 2001; Weising et al. 2005). NTSYS Ver. 2.02 (1998) and DARwin ver. 5 (2008) were used for clustering and PCO analyses. Bayesian clustering was performed by MrBayes ver. 3.1 (2005).

In total 8 agronomic characters (Table 1) were studied which were subjected to the analysis of variance (ANOVA) followed by the Least Significant Test (LSD) to show significant difference among the cotton genotypes.

Results

RAPD analysis

Out of 30 RAPD primers used, 19 primers produced reproducible bands. In total 191 RAPD bands (loci) were obtained out of which 63 bands were polymorph while 128 bands were common in the cotton genotypes studied. Among the primers used OPI-12 and OPA-05 produced the highest number of bands (17 & 16 respectively), while primers OPA-11 produced the lowest number of bands (2). The Primers OPM-19 and OPH-07 produced the highest number of polymorphic bands (8 and 7 respectively). The primers OPI-18, OPA-09, OPH-07, OPC-12, OPA-05, OPA-13, OPM-19 and OPC-08 produced 1 unique band while, the other primers produced no unique band at all.

Some of the cultivars showed the presence of specific bands, for example band No. 5 (1250 bp) of the primer OPA-09 was specific for the F1 progeny of No. 200 X Belilzovar, band No. 1 (3000 bp) of the primer OPM-19 was specific for the F2 progeny of Sindose X Belilzovar, band No. 5 of the primer OPI-18 (900 bp) was specific for the F1 progeny of Siokra X Tabladilla, while band No. 1 of the primer OPA-05 (2300 bp) occurred only in the F1 progeny of No. 200 X Belilzovar.

Some bands were present only in two genotypes, for example band No. (1600 bp) of the primer OPM-10 occurred in the F1 and F2 progenies of No. 200 X Belilzovar, band No. 2 (750 bp) of the primer OPI-16 occurred in F2 progeny of Sindosa X Belilzovar and F1 progeny Tabladilla X Belilzovar, while bands No. 1 and 2 (750 and 1000 bp) of the primer OPC-09 occurred in the F1 progeny Tabladilla X Belilzovar and F2 progeny of No. 200 X Tabladilla.

Some bands were present in all the genotypes except one, for example bands N0. 6 (1200 bp) of the primer OPA-09 was only absent in the F1 progenies of Siokra X Tabladilla and bands No. 2, 3 and 4 (2500, 2100 and 2000 bp respectively) of the primer OPM19 were absent only in the F2 progenies of No. 200 X Tabladilla.

Some RAPD bands were present in the F1 progenies but absent in the F2 progenies of the same genotype. For example bands No. 1 and 2 (1800 and 1400 bp respectively) of the primer OPI-16 and bands No. 7 and 13 (1000 and 400 bp respectively) of the primer OPA-11 occurred in the F1 progenies of Siokra X Belilzovar but were absent in the F2 progenies. On the other hand band No. 2 (1700 bp) of the primer OPC-O4 was absent in the F1 progenies of Siokra X

Table 1. Agronomic characters in cotton genotypes studied.

		Pod weight gram	Pod No.	Yield Kg/hectare	Kill%	Kill length mm	Microner mg/inch	Strength g/tex	Elonga-tion%
N x Bel F2	Mean	138.10	14.93	3016.66	39.34	26.60	5.03	30.53	6.30
	N	3	3	3	3	3	3	3	3
	Std. Deviation	22.70	4.98	500.53	1.23	1.20	0.28	1.06	0.10
N x SK F2	Mean	136.46	12.20	2886.66	41.37	30.10	4.83	30.33	6.50
	N	3	3	3	3	3	3	3	3
	Std. Deviation	5.02	1.56	735.27	2.97	1.58	0.30	0.90	0.20
N x Tab F2	Mean	150.10	14.10	3103.33	39.26	29.36	4.56	31.60	6.60
	N	3	3	3	3	3	3	3	3
	Std. Deviation	10.82	5.40	746.48	3.04	1.33	0.25	1.22	0.10
Sin x Bel F2	Mean	128.96	14.46	3220.00	42.82	27.90	4.96	29.33	6.40
	N	3	3	3	3	3	3	3	3
	Std. Deviation	9.83	1.13	888.43	3.62	0.45	0.25	2.60	0.00
SK x Bel F2	Mean	131.26	13.80	2631.66	41.79	27.60	5.03	28.76	6.20
	N	3	3	3	3	3	3	3	3
	Std. Deviation	5.78	2.27	210.73	2.89	2.26	0.23	0.72	0.10
SK x Tab F2	Mean	146.96	14.00	3106.66	40.46	30.30	4.96	29.80	6.50
	N	3	3	3	3	3	3	3	3
	Std. Deviation	9.30	3.90	1188.87	3.38	2.71	0.11	0.98	0.01
Tab x Bel F2	Mean	150.26	13.06	3520.00	39.60	29.36	4.86	29.60	6.66
	N	3	3	3	3	3	3	3	3
	Std. Deviation	71.13	0.61	633.79	2.97	2..34	0.11	2.19	0.56
N x Bel F1	Mean	144.53	13.40	2780.00	38.69	25.53	5.00	27.43	6.13
	N	3	3	3	3	3	3	3	3
	Std. Deviation	10.55	2.47	985.95	1.32	1.13	0.20	1.96	0.25
N x SK F1	Mean	148.10	11.96	2900.00	39.82	27.53	5.030	30.83	6.43
	N	3	3	3	3	3	3	3	3
	Std. Deviation	6.33	3.39	807.27	2.13	5.77	0.15	0.30	0.23
N x Tab F1	Mean	141.43	13.20	2523.33	39.95	28.53	5.02	29.83	6.36
	N	3	3	3	3	3	3	3	3
	Std. Deviation	14.3	2.30	614.35	1.82	1.06	0.17	0.83	0.15
Sin x Bel F1	Mean	125.26	18.10	2793.33	42.40	28.30	4.93	29.96	6.33
	N	3	3	3	3	3	3	3	3
	Std. Deviation	13.78	2.15	780.14	2.69	2.26	0.005	1.50	0.20
SK x Bel F1	Mean	134.33	13.63	2483.33	39.41	26.10	4.86	25.83	5.93
	N	3	3	3	3	3	3	3	3
	Std. Deviation	13.87	2.08	431.08	0.53	1.05	0.25	1.90	0.28
SK x Tab F1	Mean	146.23	12.63	2970.0	40.200	26.86	4.96	28.13	6.13
	N	3	3	3	3	3	3	3	3
	Std. Deviation	10.11	3.35	431.08	2.77	1.56	0.15	2.30	0.25
Tab x Bel F1	Mean	142.10	14.30	2970.00	40.22	26.23	5.00	28.83	6.10
	N	3	3	3	3	3	3	3	3
	Std. Deviation	4.03	3.70	648.61	1.30	2.19	0.17	3.11	0.55
Total	Mean	140.29	13.84	2911.30	40.38	27.88	4.94	29.34	6.32
	N	42	42	42	42	42	42	42	42
	Std. Deviation	12.21	2.95	690.36	2.40	2.03	0.22	2.03	0.30

Abbreviations: N =No. 200 Bel =Belilzovar, SK = Siokra, Tab = Tabladila and Sin = Sindose.

Belilzovar but was present in the F2 progenies.

In Sindose X Belilzovar, band No. 1 (3000 bp) of the RAPD primer OPM-19 and band No. 4 and 17 (1500 and 300 bp respectively) of the primer OPA-05 occurred in the F1 progenies but was absent in the F2 progenies while, bands No. 3 (750 bp) of the RAPD primer OPI-16, band No. 8 (250 bp) of the primer OPB-12 and bands No. 8 and 9 (1400 and 750 bp respectively) of the primer OPC-12 were absent in the F1 progenies but occurred in the F2 progenies.

The mean values of gene diversity (H) and Shanon's Information Indices (I) of the F1 progenies for RAPD markers were 0.07 and 0.11 respectively, while the same values in F2 progenies were 0.09 and 0.13 respectively. These indices determined in the crosses in which Siokra genotype was the pollen parent were 0.08 and 0.11, in the crosses in which No. 200 genotype was the pollen parent were 0.08 and 0.12, in the

Figure 1. NJ tree of RAPD data. (numbers on the tree branches are bootstrap values).

crosses in which Sindose genotype was the pollen parent were 0.02 and 0.04, and in the crosses in which Tabladilla genotype was the pollen parent were 0.02 and 0.03 respectively.

The genetic diversity indices determined for RAPD markers in the crosses in which Belilzovar was the female parent showed almost similar values of H and I in all crosses, *i.e.* Siokra X Belilzovar (H=0.08, I=0.12), Sindose X Belilzovar (H=0.07, I=0.09), Tabladilla X Belilzovar (H=0.07, I=0.11) and No. 200 X Belilzovar (H=0.11, I=0.15) compared to that of other crosses.

Similarly in the crosses in which Tabladilla was the female *i.e.* Siokra X Tabladilla, H value was 0.12 and I=0.16 while, in No. 200 X Tabladilla H value was 0.12 and I=0.17.

UPGMA and NJ dendrograms as well as Bayesian clustering of RAPD data produced similar results supported by PCO ordination plot (Fig. 1). The Cophenetic correlation of NJ tree was higher (r=0.98) and showed almost good bootstrap values, therefore it is discussed bellow.

In general 3 major clusters were obtained. The F1 and F2 progenies of Siokra X Belilzovar formed the first major cluster standing far from the other genotypes. The second major cluster is comprised of two subclusters in which, F1 and F2 progenies of Sindose X Belilzovar formed the first subcluster, while F1 and F2 progenies of Tabladilla X Belilzovar formed the second subcluster.

The second major cluster is comprised of 3 subclusters, F1 and F2 progenies of Siokra X Tabladilla formed the first subcluster, while F1 and F2 progenies of No. 200 X Tabladilla formed the second subcluster and F1 and F2 progenies of No. 200 X Belilzovar formed the third subcluster.

The branch lengths of NJ tree based on RAPD markers were longer for the F1 progenies compared to that of F2 progenies in Tabladilla X Belilzovar, Siokra X Tabladilla and No. 200 X Belilzovar.

ISSR analysis

The eight ISSR primers used in this experiment produced 86 reproducible bands, out of which 27 bands were polymorph and 59 bands were monomorph. The highest number of polymorphic bands was obtained for the primer UBC849 while ISSR primers UBC807 and UBC823 produced only 1 polymorphic band. The ISSR primer UBC832 was the only one primer producing a single specific band.

The ISSR band No. 4 (1900 bp) of the primer (CA)7GT occurred only in two genotypes of F2 progeny of Siokra X Belilzovar and F1 progeny of Siokra X Tabladilla, while the ISSR bands No. 6 and 8 (800 and 600 bp respectively) of the primer UBC832 occurred only in the F1 progeny of No. 200 X Siokra.

The ISSR band No. 7 (1500 bp) of the primer (CA)7GT and band No. 5 (800 bp) of the primer UBC811 occurred in all the genotypes except in F2 progeny Siokra X Tabladilla and No. 200 X Tabladilla.

Some ISSR bands were present in the F1 progenies but absent in the F2 progenies of the same genotype, for example bands No. 3 and 5 (1000 and 800 bp respectively) of the ISSR primer UBC811 and bands No. 1 and 2 (200 and 900 bp respectively) of the primer UBC834 occurred in the F1 progenies of Siokra X Belilzovar but were absent in the F2 progenies of this cross. On the other hand, band No. 3 (1500 bp) of the ISSR primer UBC807, band No. 1 (1500 bp) of the primer UBC832 and bands No. 4 and 13 (1900 and 1000 bp respectively) of the primer (CA)7GT were absent in the F1 progenies of Siokra X Belilzovar but were present in the F2 progenies of this cross.

Similarly in Sindose X Belilzovar band No. 3 (1500 bp) of the ISSR UBC832 and band No. 3 (1500 bp) of the primer UBC807 occurred in the F1 progenies but were absent in the F2 progenies. Band No. 1 (1600 bp) of the primer UBC810 occurred in the F2 progenies but was absent in the F1 progenies. In Tabladilla X Belilzovar also bands No. 6 (1200 bp) of the ISSR primer UBC842, band No. 3 (1300 bp) of the primer UBC832 occurred in the F1 progenies but were absent in the F2 progenies, while band No. 3 (1500 bp) of the ISSR UBC807 was only present in F2 progenies of the same genotype.

The mean values of gene diversity (H) and Shanon's Information Indices (I) for ISSR markers in the F1 progenies

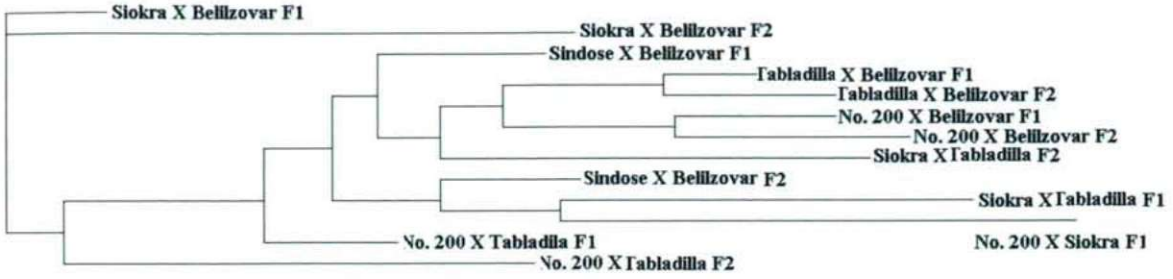

Figure 2. NJ tree of ISSR data.

were 0.27 and 0.40 respectively, while the same values in F2 progenies were 0.18 and 0.26 respectively.

These genetic diversity indices determined in the crosses in which Siokra genotype was the pollen parent were 0.22 and 0.32 respectively, in the crosses in which No. 200 genotype was the pollen parent were 0.23 and 0.34, in the crosses in which Sindose genotype was the pollen parent were 0.06 and 0.09, and in the crosses in which Tabladilla genotype was the pollen parent were 0.07 and 0.09 respectively.

These genetic diversity indices determined for ISSR markers in the crosses in which Belilzovar was the female parent showed very low and almost similar values of H and I in all crosses i.e. Siokra X Belilzovar (H=0.04, I=0.05), Sindose X Belilzovar (H=0.03, I=0.04), Tabladilla X Belilzovar (H=0.02, I=0.03) and No. 200 X Belilzovar (H=0.02, I=0.03).

Similarly in the crosses in which Tabladilla was the female parent, *i.e.* Siokra X Tabladilla the H value was 0.04 and I = 0.06 while, in No. 200 X Tabladilla, H value was 0.03 and I = 0.04.

UPGMA and NJ dendrograms as well as Bayesian clustering of ISSR data produced similar results supported by PCO ordination plot. The Cophenetic correlation of NJ tree was higher (r=0.96) and showed about 65% bootstrap values, therefore it is discussed bellow (Fig. 2).

In general 3 major clusters were obtained. The F1 and F2 progenies of Siokra X Belilzovar formed the first major cluster standing far from the other genotypes, supporting RAPD tree result. The second major cluster is comprised of F1 and F2 progenies of Tabladilla X Belilzovar, F1 and F2 progenies of No. 200 X Belilzovar and F1 and F2 progenies of Sindose X Belilzovar as well as Siokra X Belilzovar. The third cluster is formed by F1 and F2 progenies of No. 200 X Tabladilla, supporting RAPD tree result.

The branch lengths of NJ tree based on ISSR markers were longer for the F2 progenies compared to that of F1 progenies in Siokra X Belilzovar, Tabladilla X Belilzovar, No. 200 X Belilzovar and No. 200 X Tabladilla.

Agronomic data

The ANOVA followed by LSD test showed significant difference for most of the agronomic characters studied, for example, pod weight differed significantly (P<0.05, Table 1) between F2 progenies of No. 200 X Tabladilla and F1 and F2 progenies of Sindose X Belilzovar, it also differed between F2 progenies of Tabladilla X Belilzovar and F1 and F2 progenies of Sindose X Belilzovar. Similarly the mean No. of pods differed significantly (P<0.05) between the F1 progenies of Sindose X Belilzovar and F1 and F2 progenies of No. 200 X Siokra, and also with F1 plants of Siokra X Tabladilla. The mean kill length differed significantly (P<0.05) between the F2 progenies of No. 200 X Belilzovar and F2 progenies of No. 200 X Siokra and also with the F2 progenies of Siokra X Tabladilla.

UPGMA dendrogram of agronomic data showed the highest value of Cophenetic correlation (r=0.98) and is discussed here. In general 4 major clusters are formed. In the first cluster the F2 progeny of the Sindose X Belilzovar stands alone far from the other genotypes. This genotype showed the highest values of pod weight and number, yield, percentage of kill and strength compared to those of other genotypes (Table 1).

In the second major cluster, the F1 plants of Siokra X Belilzovar, Sindose X Belilzovar and No. 200 X Belilzovar as well as F2 progeny of Siokra X Belilzovar join each other due to similarity in micronaire and elongation percentage. The first two genotypes show more similarity and join each other with a lesser distance compared to the other two genotypes of this cluster.

The F2 progenies of No. 200 X Tabladilla and Siokra X Tabladilla also show similarity in the pod No., yield, micronaire and elongation percentage forming the third major cluster. In the fourth major cluster, three genotypes *i.e.* F1 and F2 plants of Tabladilla X Belilzovar and F1 plants of Siokra X Tabladilla show more similarity and join each other, the F1 plants of No. 200 X Siokra and No. 200 X Tabladilla show join each other, while the F2 progeny of No. 200 X Belilzovar joins the other genotypes in this cluster with some distance. The members of this cluster show similarity in kill %, micronaire and elongation percentage.

The grouping obtained by agronomic tree partly agrees with RAPD and ISSR trees. For example, F1 and F2 progenies of Siokra X Belilzovar show close affinity in RAPD and

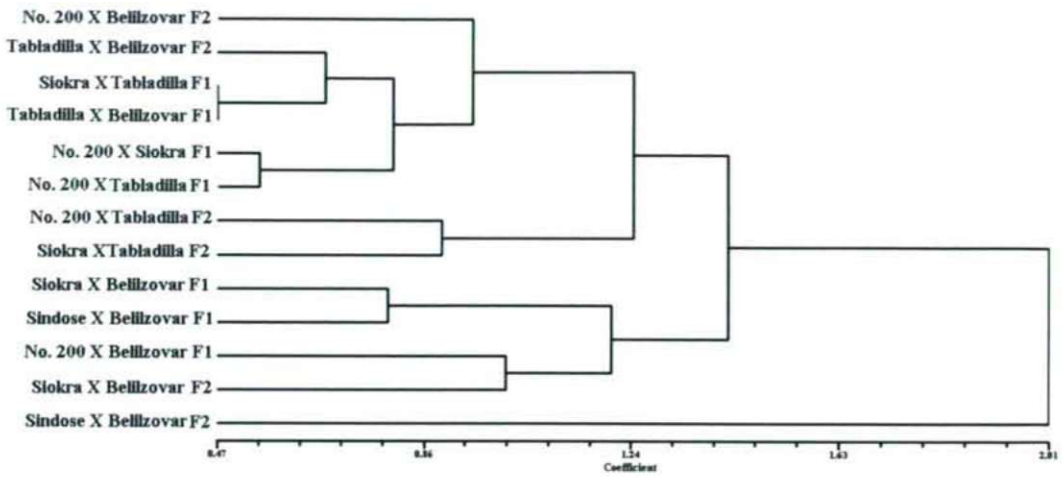

Figure. 3. UPGMA tree of agronomy data.

ISSR tree and also join each other with some distance in the agronomic tree. The same is true for F1 and F2 progenies of Tabladilla X Belilzovar and N0. 200 X Tabladilla as well as Siokra X Tabladilla genotypes.

Discussion

The presence of RAPD and ISSR polymorphic bands in F1 and F2 progenies of the cotton cultivars studied indicates the presence of genetic polymorphism in these genotypes which may be used in planning hybridization in cotton. Moreover, the occurrence of specific bands/loci only in some of the cultivars illustrates the occurrence of unique insertion/deletion in DNA material of these genotypes.

The number of RAPD and ISSR bands and degree of polymorphism obtained in F1 and F2 progenies of cotton cultivars studied is almost in agreement with the other studies performed in cotton. For example, Wei et al. (2008) used ISSR markers to study genetic diversity in 48 cotton accessions including *G. barbadense* and *G. hirsutum*, obtaining 92 ISSR bands out of which 72 bands were polymorph. UPGMA tree of ISSR data separated different genotypes of cotton collected from different provinces in China. Similarly Rana and Bhat (2005) studied genetic diversity among different Indian diploid and tetraploid cotton cultivars by RAPD markers and showed that diploid cultivars show greater genetic diversity than tetraploid cultivars. Vafaie-Tabar et al. (2003) reported 79% average genetic similarity among Indian tetraploid cotton cultivars while, Rana and Bhat (2005) reported 74% average genetic similarity. Other studies on tetraploid cotton cultivars outside India reported similar ranges of average genetic similarity (Rana and Bhat 2005). The average genetic similarity obtained in the present study for ISSR markers is 80% while the same value for RAPD markers is 85% indicating the presence of greater genetic similarities among the cotton genotypes studied, indicating the presence of a narrower genetic diversity in these cultivars which should be considered for future hybridization program. We may use more distant genotypes for future hybridization to improve the degree of genetic polymorphism available in Iran. The relatively low values of gene diversity (H) and Shannon's Information Indices (I) in RAPD and ISSR markers in both F1 and F2 progenies also support the above said conclusion. However, the genetic diversity values obtained are much higher in the crosses with Siokra and No. 200 genotypes as the pollen parents and are very low in the crosses in which the Belilzovar and Tabladilla genotypes are the female parents. Therefore these points should also be considered in the future hybridization program. Chen et al. (2004) also reported the effects of reciprocal differences in morphological and RAPD characters in interspecific hybridization in Cucumis.

A longer branch length of NJ tree based on molecular data indicates the occurrence of a higher degree of molecular changes in these genotypes, which seems in some cases it occurs in F1 plants but surprisingly in some cases it occurs in the F2 plants. We usually expect a higher degree of genetic variation in F1 progenies compared to that of F2 plants, but in case of RAPD markers a higher degree of molecular changes occur in F2 plants of Tabladilla X Belilzovar, Siokra X Tabladilla and No. 200 X Belilzovar and in the case of ISSR markers, it happened for F2 plants of Siokra X Belilzovar, Tabladilla X Belilzovar, No. 200 X Belilzovar and No. 200 X Tabladilla. This may happen due to frequent genetic recombination, molecular insertion/deletion, etc. in the F2 progenies. As we can see the crosses showing this phenomenon in both RAPD and ISSR sequences are mainly Tabladilla X Belilzovar and No. 200 X Belilzovar, therefore we may consider these crosses as more vulnerable to molecular changes.

Dendrograms obtained in both RAPD and ISSR markers almost are in agreement, indicating the genetic distinctness

of the F1 and F2 progenies of each hybrid genotype as they each form a separate cluster and also show that the F1 and F2 progenies of Siokra X Belilzovar differ greatly from the other genotypes in both RAPD and ISSR loci. These are also partly supported by the agronomic tree obtained. It is expected to see some difference between agronomic/ morphological dendrogram and that of molecular data as agronomic/ morphological characteristics are mainly of polygenetic nature affected greatly by environmental factors (Preetha and Raveendren 2008).

As stated before there were bands which occurred in the F2 progenies but were absent in the F1 progenies of the genotypes studied. Since, even single base change at the primer annealing site is manifested as appearance or disappearance of RAPD and ISSR bands, these bands may indicate the occurrence of genetic changes in the genome of the progenies either through the loss or rearrangement of some of their nucleotides. Chromosomal crossing over during meiosis may result in loss of primer attachment pair sites in the offspring leading to novel RAPD pattern in the offspring (Smith et al. 1996). Sushir et al. (2008) carried out cytogenetic and RAPD analysis of F1 and F2 progenies of the interspecific cross between *Gossypium arboretum* X *G. anomalum* and reported that among nine F2 segregates, F2-1 progeny plants showed one additional band than F1 and F2-5 progeny plant showed the recombination event. On the contrary in plants F2-6 and F2-8 loss of priming sites happened showing that recombination between A and B genomes of *G. arboreum* and *G. anomalum* respectively is possible (Sushir et al. 2008). Similarly Tafvizei et al. (2010) reported the occurrence of RAPD band in the hybrids not observed in their parents and also absence of RAPD bands in the hybrids which were present in their parents in cotton hybrids. Wang et al. (2004) also reported the occurrence of RAPD bands in the parental genotypes of *Fagopyrum* which were not observed in the hybrid obtained. They also observed the appearance of some RAPD bands absent in the hybrids which were not present in the parental genotypes due to genetic rearrangements. Therefore the present study reveals genetic differences of the cotton genotypes obtained by hybridization and also identify some of the parental genotypes which can be used as pollen parents in the further hybridization program and cotton breeding.

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ARTICLE

Molecular characterization of Iranian *Dracocephalum* (Lamiaceae) species based on RAPD data

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ABSTRACT Taxonomic relationship and genetic diversity among 17 accessions belonging to six Iranian *Dracocephalum* L. species and one accession of *Lallemantia* as closely related genus was analyzed using RAPD markers. Forty RAPD markers were used and only twelve of them gave reproducible polymorphic bands among the accessions studied. In total 262 bands were produced out of which 10 bands were monomorphic and 252 bands were polymorphic. Among the taxa investigated *Dracocephalum kotschyi* (Damavand population) showed the highest number of RAPD bands (144), while *D. multicaule* (Zanjan population) showed the lowest number (95). UPGMA cluster analysis showed efficacy of RAPD data to differentiate the species at molecular level. *Dracocephalum polychaetum* and *D. surmandinum* as two different species in Flora of Iranica revealed a close relationship with *Dracocephalum kotschyi* and formed a mixed subcluster. The RAPD analysis offered rapid and reliable tools for the estimation of inter- and intra specific variability in *Dracocephalum*.
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KEY WORDS

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genetic diversity
RAPD
labiatae
Iran

The genus *Dracocephalum* L. (Lamiaceae) consists of around 60 species distributed in the temperate regions of the Northern Hemisphere. In the flora of Iran, the genus is represented by eight species, which are mainly distributed in the northern and central parts of the country, belonging to the Irano- Turanian phytogeographical region (Rechinger 1982). With the exception of the widespread endemic species *D. kotschyi* L. and the cultivated one *D. moldavica*, the rest of the species (namely *D. polychaetum*, *D. surmandinum*, *D. multicaule*, *D. subcapitatum* and *D. aucheri*) exhibit more or less highly restricted distributional patterns in Iran. The first four medicinal and most scented perennial herbs have some morphological characters in common and in some treatments they were considered to be subspecies from *D. multicaule*.

Recently, much attention has been paid to the *Dracocephalum* genus and its chemical constituents because of their diverse activities, such as anticancer, antioxidant, antihypoxic, and immunomodulatory activities (Zeng et al. 2010). Random Amplified Polymorphic DNA (RAPD) is one of the molecular markers widely used to study genetic diversity in plants and to study the species relationships (Bogani et al. 1994; Oxelman 1996; Sanz-Cortés 2001; Çelebi et al. 2008; Mirjalil et al. 2009; Sheidai et al. 2010; İkinci and Oberprieler 2010). As far as our literature could ascertain, little research has been performed within *Dracocephalum* using molecular markers. Therefore, a project was initiated to evaluate the genetic diversity within *Dracocephalum* and ascertain the discriminating potency of RAPDs to distinguish inter- and intra-species relationship.

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Material and Methods

Plant material

The aerial parts of plants were collected during the flowering stage (2004–2010) as shown in Table 1. Leaf samples dried in silica gel and were stored at -20°C until required.

DNA extraction

Total genomic DNA was extracted from leaves dried in silica gel or taken from herbarium specimens with the DNeasy plant mini kit DNA extraction (QIAGEN) following the manufacturer's protocol.

Screening and PCR amplification with RAPD primers

Random primers (decamers) from the Operon series were tested for the amplification of DNA. In total twelve primers were used in this study viz., OPA04, OPA15, OPB03, OPC04, OPC06, OPH07, OPI18, OPM10, OPM11, OPM19, OPR06 and OPR12. The PCR reactions were carried out in 20µl reaction volume containing 20-25ng/µl DNA, 25pM primer, 10µl PCR master mix (Ampliqon) and 8.0 µl of nuclease free

Table 1. Locality and voucher information of the taxa studied.

No.	Taxa	Code	Locality	Voucher
1	*Dracocephalum polychaetum*	POL	Kerman, Babini village, Hazar mountain, 3400m., 7 May 2008, *Gholipour, Kanani & Mirtajedini*	1276 (MPH)
2	*D. polychaetum*	POH	Kerman, Chatroud, Horjond-Sodkoh, 2300m., 27 June 2007, *Gholipour*	1229 (MPH)
3	*D. kotschyi*	KLA	Mazandarn, Haraz road, Polur towarsd Lasem, *Sonboli*	1217 (MPH)
4	*D. kotschyi*	KDA	Tehran, Damvand mountain, 3200m., 6 July 2007, *Gholipour*	1218 (MPH)
5	*D. kotschyi*	KDE	Yasuj, Sisakht, Dena,3200 m.,16 June 2007, *Sonboli, Kanani & Gholipour*	1163 (MPH)
6	*D. kotschyi*	KDI	Alborz, Dizin, *Sonboli & Gholipour*	1299 (MPH)
7	*D. kotschyi*	KBO	Khorasan, Bodjnurd, *Joharchi*	37610 (FUMH)
8	*D. surmandinum*	SUR	Esfahan, Semirom, Surmand mountain,2900 m., 18 June 2007, *Sonboli, Kanani & Gholipour.*	1220 (MPH)
9	*D. multicaule*	MUN	Ardabil, Neor, *Gholipour*	1349 (MPH)
10	*D. multicaule*	MUG	West Azarbaiejan, Khoy, Qotour, Mirzagol Valley, 2300, 3 June 2008, *Sonboli, Gholipour & Kazempour*	1307 (MPH)
11	*D. multicaule*	MUK	West Azarbaiejan, Khoy, Firuragh road, Passak, *Moussavi & Tehrani*	22807 (IRAN)
12	*D. multicaule*	MUZ	Zanjan, Soltaniyeh, *Moussavi & Termeh*	22809 (IRAN)
13	*D. subcapitatum*	SUS	Khorasan, Shirvan, Reshvanloo, *Joharchi*	22812 (FUMH)
14	*D. subcapitatum*	SUN	Semnan, Chashm, Nizva mountain, 3200m., 12 July 2007, *Gholipour & Sonboli*	1219 (MPH)
15	*D. subcapitatum*	SUE	Khorasan, Shirvan, Nemanloo, *Joharchi*	22813 (FUMH)
16	*D. subcapitatum*	SUK	Khorasan, Kalat, *Joharchi*	20864 (FUMH)
17	*D. moldavica*	MOL	West Azarbayejen, Urmia,Ashena abad village,1700m., 12 July 2007 , *Sonboli & Mojarrad*	1221 (MPH)
18	*Lallemantia peltata*	LAL	West Azarbayejen, Takab, Maeen Bolagh pass, 1700m., *Sonboli*	273 (MPH)

distilled water. The amplification was conducted in a thermocycler (BioRad Mycycler) and programmed for an initial denaturation step of 1 minute at 94°C, followed by 44 cycles of denaturation at 94°C for 30 seconds, primer annealing at 40°C for 1 minute and extension at 72°C for 2 minutes. Final extension was carried out at 72°C for 7 minutes and a hold at 4°C temperature. PCR products were resolved on 1.0% agarose gel, in 1.0X TAE buffer at 70 V for 3 hours and then stained with ethidium bromide (0.5µg/ml). Gel with amplification fragments was visualized and photographed by using gel documentation system (Vilber Lurmat, Infinity).

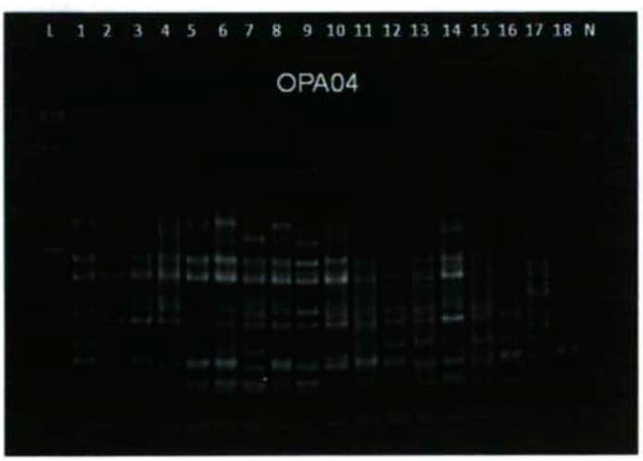

Figure 1. RAPD profiles of 18 accessions of *Dracocephalum* using the primer OPA04. L: represent molecular weight size marker (1 kb ladder). N: negative control. The numbers represent different accessions according to Table 1.

Statistical analysis of RAPD data

For cluster analysis, the correlation coefficient was selected as a measure of similarity among all accessions, and the Unweighted Pair Group Method with Arithmetical Averages (UPGMA) was used for cluster definition. Only distinct, reproducible, well-resolved fragments, in the size range from 250 bp to 3.0 kb, were considered and scored as present (1) or absent (0) for each RAPD reaction. Genetic similarity between pairs was estimated by the Dice coefficient (Sneath and Sokal, 1973). This approach seems to be more appropriate for a genetic character, since the lack of a common band should not imply a similarity in genetic terms. Dendrograms were constructed by cluster analysis based upon the UPGMA of the SPSS ver. 9.0 software.

Results and Discussion

Out of a total of 40 different primers screened, only twelve primers produced reproducible and polymorphic bands for all populations representing the taxa studied (Fig. 1). In total 262 bands were produced out of which 10 bands were monomorphic and 252 bands were polymorphic. Among the taxa investigated *Dracocephalum kotschyi* (Damavand population) showed the highest number of RAPD bands (144), while *D. multicaule* (Zanjan population) showed the lowest number (95). The number of informative polymorphic bands per

Table 2. RAPD polymorphic reproducible bands among the taxa studied.

Primer name	Primer sequence	TB	PB	MB	SB	PP (%)
OPA04	5′ AATCGGGCTG 3′	25	25	0	9	100
OPA15	5′ TTCCGAACCC 3′	14	14	0	2	100
OPB03	5′ CATCCCCCTG 3′	25	25	0	6	100
OPC04	5′ CCGCATCTAC 3′	19	17	2	2	89.5
OPC06	5′ GAACGGACTC 3′	14	12	2	1	85.7
OPH07	5′ CTGCATCGTG 3′	24	23	1	5	95.8
OPI18	5′ AATGCGGGAG 3′	27	27	0	2	100
OPM10	5′ TCTGGCGCAC 3′	28	28	0	3	100
OPM11	5′ GTCCACTGTG 3′	29	26	3	5	89.6
OPM19	5′ CCTTCAGGCA 3′	13	12	1	6	92.3
OPR06	5′ GTCTACGGCA 3′	21	20	1	4	95.2
OPR12	5′ ACAGGTGCGT 3′	23	23	0	3	100
Total		262	252	10	49	

TB, Total bands; PB, Polymorphic bands; MB, Monomorphic bands; SB, specific bands; PP, Polymorphism percentage.

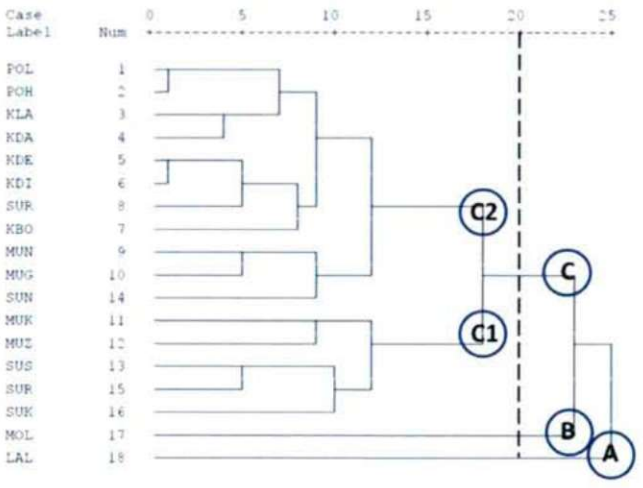

Figure 2. UPGMA dendrogram of 18 accessions of *Dracocephalum* based on 12 RAPD primers. The bar on the bottom represents similarity index based on Jaccard's coefficients. The codes represent different accessions according to Table 1.

primer ranged from 12 for primers OPC06 and OPM19 to 28 for primer OPM10 (Table 2). An average of 21 bands was amplified per sample and primer. The UPGMA dendrogram obtained from cumulative cluster analysis of twelve primers matrix using Jaccard's similarity coefficient clearly delineated all 18 accessions of taxa studied. The combined dendrogram delineated 18 accessions into three main clusters (Clusters A, B and C in Fig. 2).

The first cluster (A) formed by *Lallemantia peltata*, which is considered here as outgroup taxon. The second cluster (B) contained a morphologically distinct species of the genus *Dracocephalum*, i. e. *D. moldavica*. Whereas, the third cluster (C) had two subclusters (C1 and C2), the first subcluster (C1) contained three populations of *D. subcapitatum* (SUS, SUE and SUK) and two populations of *D. multicaule* (MUK and MUZ) while the second subcluster (C2) composed of mixed grouping of accessions belonging to *D. kotschyi*, *D. polychaetum*, *D. surmandinum*, *D. multicaule* (MUN and MUG) and *D. subcapitatum* (SUN).

From the chemotaxonomic point of view, limonene and perilla aldehyde were the main components of *D. polychaetum*, *D. surmandinum*, *D. multicaule*, *D. kotschyi* and *D. subcapitatum* (Sonboli et al. 2010), while citral and geranyl acetate was found to be the principal essential oil constituent of *D. moldavica* (Sonboli et al. 2008). This phytochemical differentiation is in accordance with the results of molecular RAPD data, in which *D. moldavica* is clearly separated from other *Dracocephalum* species (Fig. 2).

DNA-based markers provide precise information on genetic diversity because of the independence of the confounding effects of environmental factors (Powell et al. 1995). RAPD markers are based on random priming, which randomly screen various regions of the genomic DNA. In this study, RAPD marker system revealed high levels of polymorphism among the *Dracocephalum* species indicating its effectiveness for evaluating intra- and inter-specific genetic diversity in the genus *Dracocephalum*. The significance of wild genetic diversity using DNA based markers like RAPD and its efficacy have also been reported for *Satureja hortensis* (Hadian et al. 2008), *Silene* sect. *Auriculatae* (Sheidai et al. 2010) and *Whitania* species (Mirjalili et al. 2009) from Iran.

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ARTICLE

Morphological investigations on anthers and pollen grains of some quince cultivars

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ABSTRACT In the present study we investigated some pollen morphological characters and anther size of *Cydonia oblonga*. Anthers of cultivars with apple- vs. pear-shaped fruits were also compared. In 2005 the anthers of cultivars with oblate vs. suboblate pollen showed significant differences. Anthers of the oblate group were notably shorter and smaller in size, than those of the suboblate group. Equatorial area of pollen in the former group was also smaller. In 2006 P/E ratio of pollen also correlated with length, width and size of anthers. However, shape and size of pollen grains did not show any relationship in the latter year. Equatorial area of pollen grains was not connected with anther size in 2005-2006. Fruit shape correlated with pollen shape only in 2005. P/E ratio of pollen in cultivars with apple-shaped fruits was lower than in cultivars with pear-shaped fruits. We could not demonstrate any relationships between anther morphology and fruit shape of the investigated quince cultivars.
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KEY WORDS

anther
Cydonia oblonga
fruit shape
pollen

The 3-whorled androecium of *Cydonia oblonga* L. consists of 15-20 stamens with purplish filaments and light yellow anthers (Halmágyi and Keresztesi 1975). Size of anthers is influenced by flower size (Delph et al. 1996). The level of pollen production is directly related to anther size and inversely related to pollen grain size (Lopez et al. 1999). In apple (*Malus domestica* Borkh.), which belongs to the same subfamily as quince, Kocsis-Molnár et al. (1994) reported that the mean width of anthers was 1.71 mm and their length was 2.46 mm in 1989, while in 1990 they measured 1.98 mm and 2.66 mm, respectively. Benedek et al. (1989) found that the size of apple anthers was 2.20x1.53 mm and their relative surface was 3.37 mm^2.

Quince has yellow pollen grains (Mohácsy and Porpáczy 1958). Shape and size of pollen often change with variable air temperature and humidity and other environmental factors (Thakur and Thakur 1970). Pollen size of apple increases with higher levels of ploidy (Fogle 1977; Orosz-Kovács 2001). Pollen size also depends on pollination strategies of plants (Harder 1998). According to Baker and Baker (1979) pollen grains collected by honeybees are smaller than those which are transferred by butterflies and birds.

Structure of stigma, length and thickness of style can also be related to pollen size (Cruden and Lyon 1985; Kirk 1993).

In subfamily Maloideae (Rosaceae) pollen shape is highly diverse. Typical pollen grains are oblate or spheroidal, with a triangular or circular equatorial outline (Halbritter and Schneider 2000). Quince possesses spheroidal pollen grains (P E, P/E=0.88-1.14), which are round in equatorial view (Erdtman 1966, Halbritter and Schneider 2000). According to the classification of Erdtman (1966), the trizonocolporate pollen grains of quince were considered as large (50-100 µm) by Erdtman (1966) and Sótonyi et al. (2000), contrasting the view of Halbritter and Schneider (2000), who classified quince pollen in the medium (26-50 µm) category. According to Sótonyi et al. (2000), length of *C. oblonga* pollen changed between 50.4-54.4 µm, its width was in the range 23.6-24.1 µm, and the average length and width ratio was 2.19. In comparison, the mean length of pollen grains in apple was 44 µm, mean width 23.1 µm and mean shape index was 1.9 (Sótonyi et al. 2000).

Quince cultivars are divided into two groups on the basis of their fruit shape. They have apple-shaped [*C. oblonga* var. *maliformis* (Mill.) Schneid.] or pear-shaped [*C. oblonga* var. *pyriformis* (Dierb.) F. Zinn.] fruits (Nyéki 1990). Besides the above two varieties, cultivars were also bred from *C. oblonga* var. *lusitanica* (Mill.) Schneid. for fruit growing. This variety possesses pear-shaped fruits (Mohácsy and Porpáczy 1958). However, intermediate fruit types also occur which have fruit shape between apple and pear (Nyéki 1990). The same trees can bear apple-shaped or pear-shaped fruits year by year in turns. It is connected partially with the number of fully developed seeds. Fruits, which contain less seed, are more elongated. Shape and size of fruits can also be influenced by fruit density and flowering time (Soltész 1998, Nyéki 2004).

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Table 1. Length (L), width (W) and size (L*W) of anthers in the investigated quince cultivars in Újfehértó in 2005-2006. Data are shown as mean±SE.

Cultivars	Anther length (µm)		Anther width (µm)		Anther size (L*W) (mm²)	
	2005	2006	2005	2006	2005	2006
'Angers'	3298.46±74.53	3446.62±22.98	1312.37±26.77	1364.61±22.98	4.34 ±0.17	4.70±0.14
'Apple-shaped Dunabogdány'	3259.86±24.78	3392.03±29.50	1368.51±27.68	1337.32±29.50	4.46 ±0.08	4.53±0.09
'Apple-shaped wild quince'	-	3712.52±34.38	-	1424.65±34.38	-	5.31±0.22
'Aromate'	3779.19±30.07	3694.02±28.57	1375.53±38.37	1400.41±28.57	5.20±0.15	5.18±0.12
'Bereczki'	3484.44±62.56	3413.30±13.61	1368.51±15.69	1381.27±13.61	4.76±0.07	4.71±0.07
'Champion'	3768.67±62.38	3670.41±19.61	1477.29±17.70	1449.22±19.61	5.57±1.48	5.32±0.13
'Constantinople'	3445.84±80.49	3454.42±25.79	1350.97±31.06	1337.35±25.79	4.66±1.80	4.63±0.14
'Mezőtúri'	-	3729.11±19.72	-	1419.58±19.72	-	5.30±0.10
'Pear-shaped Bólyi'	3670.41±52.36	-	1410.62±21.44	-	5.18±1.29	-
'Pear-shaped Dunabogdány II'	3670.41±30.14	-	1438.69±10.46	-	5.28±0.04	-
'Pear-shaped Noszvaji'	3576.26±38.74	3639.79±16.64	1441.61±17.52	1419.55±16.64	5.16 ±0.09	5.17±0.08

Table 2. Results of one-way ANOVA test. Differences between quince cultivars possessing oblate vs. suboblate pollen grains in some morphological features of androecium (length (L), width (W) and size (L*W) of anthers, equatorial area of pollen) in 2005. SS – sum of squares, MS – mean squares, F – value of Fisher test, p – significance level. Df=16, critical value of F test=4,5431; * indicates significant difference at $p < 0.05$.

Factors	SS	MS	F	p
Anther length (µm)	1472313.08	376923.71	5.1615*	0.0382
Anther width (µm)	102916.67	20280.95	3.6814	0.0743
Anther size (L*W) (mm2)	7.86E+12	1.99E+12	5.0926*	0.0394
Equatorial area of pollen (µm2)	128987.45	53563.42	11.3626*	0.0039

The present study was based on the hypothesis that a relationship exists between certain pollen morphological characters (shape and size) and anther size. Our additional aim was to investigate and compare the anther morphology of cultivars with apple- vs. pear-shaped fruits.

Materials and Methods

The studied material was taken from the quince genebank of the Research and Extension Centre for Fruitgrowing, Újfehértó, Hungary during 2005-2006. Anther size, pollen shape and size was studied in 11 quince cultivars. 5 of the investigated cultivars had apple-shaped fruits (Ang - 'Angers', A Dun - 'Apple-shaped Dunabogdány', A wildq - 'Apple-shaped wild quince', Const - 'Constantinople', Mezt - 'Mezőtúri'), while 6 cultivars (Aro - 'Aromate', Ber - 'Bereczki', Cham - 'Champion', P Bóly - 'Pear-shaped Bólyi', P Dun - 'Pear-shaped Dunabogdány II', P Nosz - 'Pear-shaped Noszvaji') possessed pear-shaped fruits. Samples were collected from 30-70 pollen shedding flowers per tree, from 2–3 trees per cultivar. 100-500 data were measured per cultivar.

Freshly gathered anthers were investigated with stereo microscope (Carl Zeiss, Jena) immediately. Their size was measured with an ocular micrometer. Pollen samples were prepared with the fixation and isatine staining method elaborated by Gulyás and Pálfi (1986). The slides were examined with light microscope (NIKON H600L Eclipse 80i), and micrographs were taken with the software Spot Basic 4.0. For analyzing shape and size of pollen grains software 'UTHSCSA Image Tool' was used.

All statistical analyses of variance (ANOVA) were performed using Statistica 5.1 software. Differences between cultivars that had different pollen shape or fruit shape was analyzed by using a one-way ANOVA (p 0.05). Pearson correlation analysis was also employed to compare pollen size, pollen shape and anther size.

Results and Discussion

Mean length of anthers (L) in various cultivars changed between 3259.86±24.78 µm and 3779.19±30.07 µm in 2005. The smallest values were measured in the flowers of cv. 'Apple-shaped Dunabogdány'. The longest anthers could be found in the flowers of cv. 'Aromate'. In 2006 again, cv. 'Apple-shaped Dunabogdány' possessed the shortest anthers (3392.03±29.50 µm). The longest anthers (3729.11±19.72 µm) could be observed in the case of cv. 'Mezőtúri' (Table 1). Anther length fluctuated 6-30 % per cultivar during the two years.

Mean width of anthers (W) was also very diverse. Its fluctuation rate was 5-35 % in 2005-2006. In 2005 the narrowest anthers (1312.37±26.77 µm) occurred in the flowers of cv.

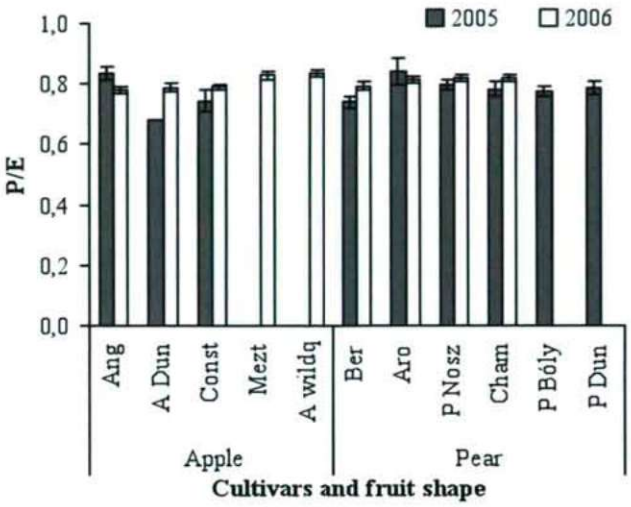

Figure 1. P/E ratio of pollen grains in the investigated quince cultivars in Újfehértó in 2005-2006.

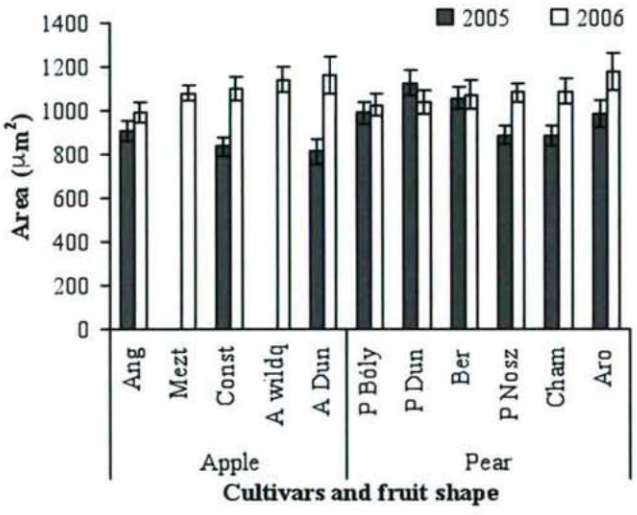

Figure 2. Equatorial area of pollen grains in the investigated quince cultivars in Újfehértó in 2005-2006.

'Angers' and the widest in cv. 'Champion' (1477.29±17.70 µm). In 2006 their width varied between 1337.32±29.50-1449.22±19.61 µm. The smallest values were measured in the flowers of cv. 'Apple-shaped Dunabogdány' and the largest again in cv. 'Champion' (Table 1).

Size of anthers (length * width, L*W) changed between 4.34-5.57 mm² in 2005, and 4.53-5.32 mm² in 2006. Smallest anthers were measured in cv. 'Angers' and 'Apple-shaped Dunabogdány' in the two years, respectively. Largest anthers could be observed in the flowers of cv. 'Champion' in both years (Table 1).

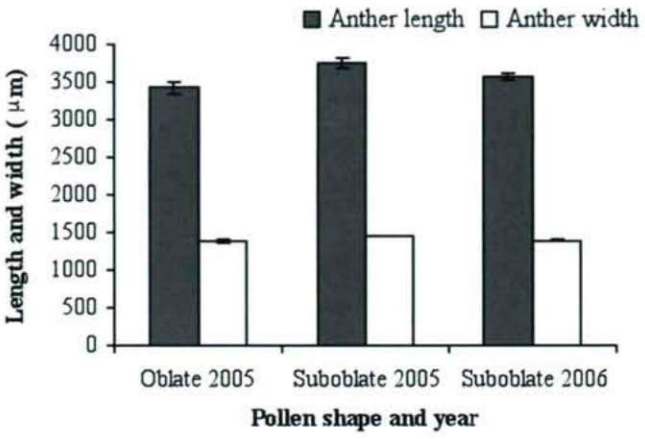

Figure 3. Length and width of anthers in quince cultivars possessing oblate vs. suboblate pollen grains in Újfehértó in 2005-2006.

Shape of the pollen grains was typified with the ratio of the length of their polar (P) and equatorial (E) axis (P/E) and it was ranked by the evaluation system elaborated by Erdtman (1952). According to this ratio, the investigated quince cultivars could be divided into two groups. They possessed oblate (P/E=0.5-0.75) or suboblate (P/E=0.75-0.88) pollen grains. Oblate group could be distinguished only in 2005. In this year P/E ratio of pollen varied between 0.68 and 0.84. The smallest values were reached by cv. 'Apple-shaped Dunabogdány' and the largest ones could be counted in cv. 'Aromate'. Three cultivars, namely 'Apple-shaped Dunabogdány', 'Bereczki' and 'Constantinople', belonged to the oblate category. The other investigated cultivars were characterized by suboblate pollen grains. In 2006 all quince cultivars had suboblate pollen. Their P/E ratio was 0.78-0.83. Pollen grains of cvs. 'Apple-shaped Dunabogdány', 'Bereczki' and 'Constantinople' were typified by the smallest values also in the latter year. The highest P/E ratios were reached by cvs. 'Apple-shaped wild quince' and 'Mezőtúri' (Fig. 1).

In 2005 equatorial area of pollen changed between 813.48±56.94 µm² and 1126.46±56.32 µm². The smallest pollen grains could be found in the anthers of cv. 'Apple-shaped Dunabogdány', while the largest ones could be detected in cv. 'Pear-shaped Dunabogdány II'. In 2006 area of pollen was larger, than in the previous year. The smallest pollen grains (992.36±49.62 µm²) were possessed by cv. 'Angers' and the largest ones (1175.73±82.30 µm²) by cv. 'Aromate'(Fig. 2).

Comparing 2005 and 2006, it can be established that size of anthers was very similar in the two years. Nevertheless, shape and equatorial area of pollen grains was usually smaller in 2005 than in 2006 (Table 1, Figs. 1-2).

In 2005 we compared the anthers of the cultivars which had oblate and suboblate pollen and significant differences could be noticed between these groups. Anthers of the oblate

Table 3. Results of one-way ANOVA test. Differences between quince cultivars with apple-shaped vs. pear-shaped fruits in some morphological features of androecium (length (L), width (W) and size (L*W) of anthers, pollen shape (P/E), equatorial area of pollen) in 2005-2006. SS – sum of squares, df – degree of freedom, MS – mean squares, F – value of Fisher test, p – significance level, F crit. – critical value of F test. * indicates significant difference at $p < 0.05$.

Factors and year	SS	df	MS	F	p	F crit.
Anther length (µm) 2005	412530.94	11	58203.28	1.6426	0.2289	4.9646
Anther length (µm) 2006	160718.18	8	7332.22	0.3346	0.5811	5.5914
Anther width (µm) 2005	33753.20	11	2864.19	0.9273	0.3583	4.9646
Anther width (µm) 2006	12770.27	8	2867.40	2.0269	0.1975	5.5914
Anther size (mm2) (L*W) 2005	2.10E+12	11	2.55E+11	1.3803	0.2673	4.9646
Anther size (mm2) (L*W) 2006	8.69E+11	8	9.05E+10	0.8140	0.3969	5.5914
Pollen P/E 2005	1.27E-02	8	9.92E-03	25.0555*	0.0016	5.5914
Pollen P/E 2006	4.66E-03	11	5.74E-05	0.1249	0.7311	4.9646
Equatorial area of pollen (µm2) 2005	86830.28	8	37338.28	5.2810	0.0551	5.5914
Equatorial area of pollen (µm2) 2006	42471.35	10	2420.05	0.5438	0.4796	5.1174

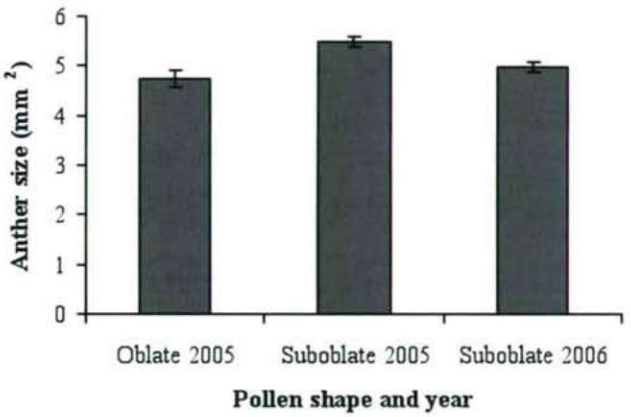

Figure 4. Size of anthers (L*W) in quince cultivars possessing oblate vs. suboblate pollen grains in Újfehértó in 2005-2006.

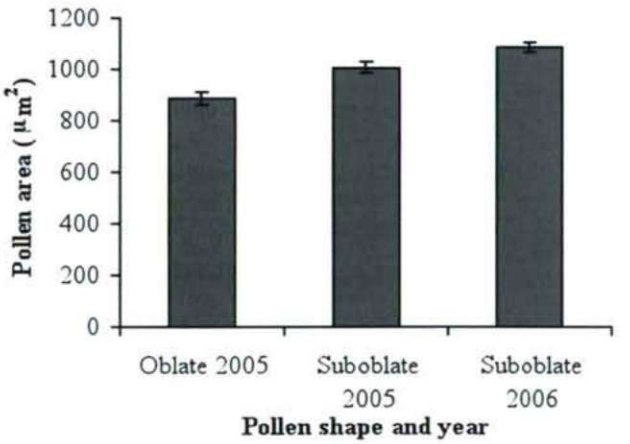

Figure 5. Equatorial area of pollen in quince cultivars possessing oblate vs. suboblate pollen grains in Újfehértó in 2005-2006.

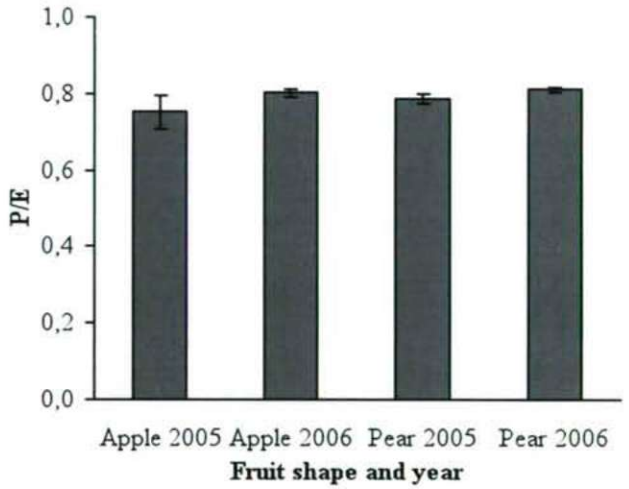

Figure 6. P/E ratio of pollen in quince cultivars having apple-shaped vs. pear-shaped fruits in Újfehértó in 2005-2006.

group were notably shorter and smaller in size, than those of suboblate group (Figs. 3-4). Equatorial area of pollen in the former group was also smaller (Fig. 5, Table 2). None the less all investigated quince cultivars had suboblate pollen in 2006, in which year P/E ratio of pollen also correlated with length (r^2=0.8925), width (r^2=0.7633) and size (r^2=0.9058) of anthers. Cvs. 'Angers', 'Apple-shaped Dunabogdány', 'Bereczki' and 'Constantinople', whose pollen grains could be characterized by lower P/E ratios, had smaller anthers than other investigated cultivars (Fig. 1, Table 1). However, shape and size of pollen grains did not show any relationship (r^2=0.3239) with each other in 2006. Similarly, equatorial area of pollen grains was not connected with anther size either in 2005 (r^2=0.2782) or in 2006 (r^2=0.0005).

Cultivars with apple-shaped vs. pear-shaped fruits did not differ from each other in respect of length, width and size of

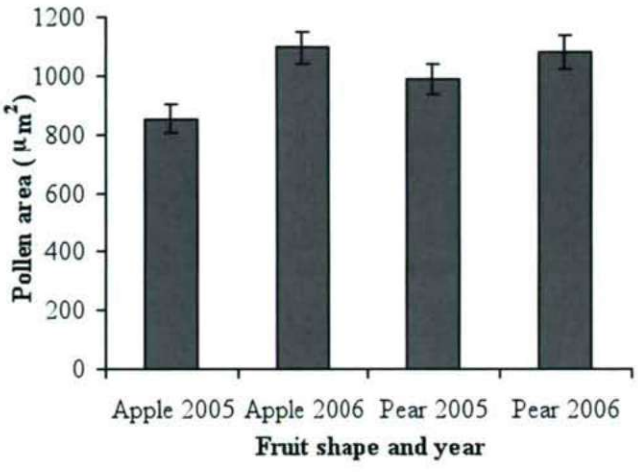

Figure 7. Equatorial area of pollen in quince cultivars having apple-shaped vs. pear-shaped fruits in Újfehértó in 2005-2006.

anthers (Table 3). In contrast, correlation was found between pollen shape (P/E) and fruit shape in 2005 (Fig. 6, Table 3), when P/E ratio of pollen in cultivars having apple-shaped fruits was lower than in cultivars having pear-shaped fruits. Cultivars with apple-shaped fruits usually had oblate pollen grains, whereas cultivars with pear-shaped fruits developed suboblate pollen (Fig. 1). However, in 2006 this relationship could not be observed. Although in 2005 pollen area of apple-shaped cultivars seemed to be smaller than that of pear-shaped ones (Fig. 7), no statistically significant relationship could be demonstrated between pollen size and fruit shape of cultivars in any of the years (Table 3).

In summary, positive correlation was found between anther size and pollen shape, as well as between anther size and equatorial area of pollen. Anthers of apple-shaped vs. pear-shaped quince cultivars also differed from each other. P/E ratio of pollen grains was lower in cultivars with apple-shaped fruits than in the case of pear-shaped cultivars.

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ARTICLE

Leaf epidermal investigations on *Festuca pratensis* Huds. subspecies

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ABSTRACT This work deals with the examination of the leaf epidermal anatomy of two subspecies of Meadow fescue: *Festuca pratensis* Huds.: subsp. *pratensis* and subsp. *apennina*. As a result of our studies carried out on Central European natural populations of different origin, it can be concluded that in the case of both microtaxa, in all the examined populations the upper and lower epidermis exhibits structural differences. The variation between the populations manifest in the differences in dimensions of certain cells of the epidermis, in the size and amount of silica cells, hairiness, and the size of the bristles. The difference of the two subspecies on the basis of epidermal structure is the length of stoma complexes, the number and dimension of bristles as well as the length of the epidermis cells. **Acta Biol Szeged 55(2):237-241 (2011)**

KEY WORDS

leaf epidermis
Festuca pratensis subsp. *pratensis*
F. pratensis subsp. *apennina*

The broad-leaved forage grass taxa from the temperate zones belong to the section *Bovinae* Fries. ap. Anders of the genus *Festuca* L. The populations and subspecies of *Festuca pratensis* Huds. (Meadow fescue) deserve special attention as genetic reserve materials (Borill et al.1977; Tyler 1988, Chapmann 1996). The diversity of populations of Meadow fescue is of scientific and economic importance, not only because they are constituents of the natural flora, but as germplasm sources or breeding materials for useful cultivars and biotypes as well (Kovács 1994).

In the line of natural biological materials, the genetically and ecologically stable subspecies (microtaxa) of Meadow fescue are the most important: *Festuca pratensis* Huds. subsp. *pratensis* and *F. pratensis* Huds. subsp. *apennina* (De Not) Hegi [Syn.: *Festuca apennina* Notaris, *F. pratensis* subsp. *apennina* (Notaris) Hackel ex Hegi]. The presence and separation of the two subspecies in Central Europe is taxonomically accepted (Markgraf-Dannenberg 1980). The Meadow fescue subspecies (microtaxa) differ morphologically mainly in the openness of sheaths, the width of the leaf lamina, the structure of the lemma, the presence or absence of teeth and arista (Aiken et al. 1991; Connert 1994; Ciocârlan 2009). The populations of the two subspecies show significant variations in chromosome number. Populations belonging to the basic type (*F. pratensis* subsp. *pratensis*) are usually diploids (2n = 14). They prefer the lower hilly collin-submontane landscapes. The other subspecies (*F. pratensis* subsp. *apennina*) has mostly tetraploid (2n = 28), rarely aneuploid (2n = 21, 25) populations, and usually exist in mountain and sub-alpine habitats (the Carpathians, the Alps, Apennines) (Borill et al. 1977; Kovács 1982; Tyler 1988). At the same time the populations' gene source research and plant anatomy studies concerns mostly the basic type (Toma et al. 1982; Kovács and Dani 1999). Major leaf anatomical and biological studies were published also regarding the related species of *F. arundinacea* populations (Cenci et al. 1990; Ueyama 1992; Gibson and Newman 1997).

As a continuation of our previous research (Kovács and Dani 1999; Dani and Kovács 2007), the aim of the present work is to study further populations, with particular regard to the finer details of the leaf epidermal structures (costal and intercostal cell rows, stoma complexes, trichomas, etc.), constant and changing properties and characteristics.

Materials and Methods

The individuals of the populations of the two subspecies (microtaxa) were collected in the years 2008 and 2010 during flowering period. The sample specimens of the populations were collected within the natural vegetation units of Central Europe (Eastern Alpes-Dolomites) and in the area of the Carpathian Mountains, usually 325-1600 m asl. (*F. pratensis* subsp. *apennina:* populations No.: 32, 33, 35, 36, 37, 39, 40, 41, 48 and *F. pratensis* subsp. *pratensis* No.: 42, 44, 46, 47, 49, 50, 56 (Table 1). The sampling was made from of the middle part of the flag leaf. The collected material was fixed in 70% alcohol and stored in a Flemming and Strasburger's (alcohol/glycerol/water, ratio = 1:1:1) preservative mixture. The epidermic peels for light microscope examinations of the epidermal tissue were prepared by manual technique, dyed with Erlich's acid haematoxylin stain solution and, after dehydration, were covered with Canada balsam (Metcalfe 1960; Mihalik et al. 1999; Rudall 2004). The epidermis peels were

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Table 1. The geographical origin of the samples and the habitat characteristics.

Taxon name	Population name and number of sample	Altitude above sea level (m)	Habitat-types
Festuca pratensis subsp. *apennina*	Sesto-32	950-1000	Tall herb communities along the streams
	Sesto-33	950-1000	Tall herb communities along the streams
	Beluno-35	1350	Mesophilic mountain meadow
	Croce-36	1600	Marshes/wet meadows
	Cortina d'Ampezzo-37	1300	Marshes/wet meadows
	Falzarego-39	1700	Wet mountain meadow
	Cernadoi-40	1350	Wet mountain meadow
	Arabba-41	1600	Wet mountain meadow
	Borsa-48	1420	Upland marshes
Festuca pratensis subsp. *pratensis*	Zalaszántó-42	340	Wet meadow
	Verecke-44	820	Mountain meadow with red fescue
	Gyergyószentmiklós-46	730	Upland marshes
	Kalibáskő-47	910	Mountain meadow with red fescue
	Veresvíz-49	950	Mountain meadow with red fescue
	Lemhény-50	560	Mesophilic meadow
	Koloska-56	325	Wet meadow

Table 2. The size and the number of trichomes.

Taxon name	Number of sample	The size of trichomes (µm)				The number of trichomes (pcs/mm²)	
		Adaxial epidermis		Abaxial epidermis			
		length	width	length	width	Adaxial epidermis	Abaxial epidermis
F. *pratensis* subsp. *pratensis*	42	27,21	10,17	33,50	15,35	29	6
	44	32,77	10,86	34,91	16,40	13	5
	46	27,91	11,65	25,49	10,97	8	5
	47	37,26	12,62	0,00	0,00	52	0
	49	34,41	12,98	29,41	13,02	15	8
	50	34,22	14,09	27,66	13,86	10	3
	56	27,77	10,18	27,71	11,69	9	13
F. *pratensis* subsp. *apennina*	32	38,19	13,81	44,99	19,72	51	8
	33	61,73	15,58	0,00	0,00	100	0
	35	38,98	12,50	0,00	0,00	24	0
	36	42,34	13,28	0,00	0,00	60	0
	37	41,47	12,80	49,02	22,88	63	22
	39	42,50	12,68	64,30	21,79	61	39
	40	35,00	10,67	0,00	0,00	29	0
	41	61,91	11,80	0,00	0,00	71	0
	48	61,91	25,52	78,61	22,51	16	22

Table 3. The size and the number of stoma complexes.

Taxon name	Epidermis side	Stomatal complexes length	Stomatal complexes width	The number of stoma complexes per 1mm²
F. pratensis subsp. *pratensis*	Adaxial epidermis (µm)	24,21 – 37,17	13,14 – 18,20	32-94
	Abaxial epidermis (µm)	31,31 - 35,51	20,77 – 29,53	15-45
F. pratensis subsp. *apennina*	Adaxial epidermis (µm)	25,84 - 47,55	12,99 – 24,22	21-86
	Abaxial epidermis (µm)	32,19 – 49,05	22,01 – 33,28	4-36

examined and photographed with Labophot Nikon 2A optical microscope (Ellis 1976). The histological measurements (costal and intercostal cells' length, width, stoma complexes' length and width, stoma density/1mm^2, density of the hair cells/1mm^2) were carried out using an Olympus DP-Soft 3.1 type image processing system. Averages were calculated from

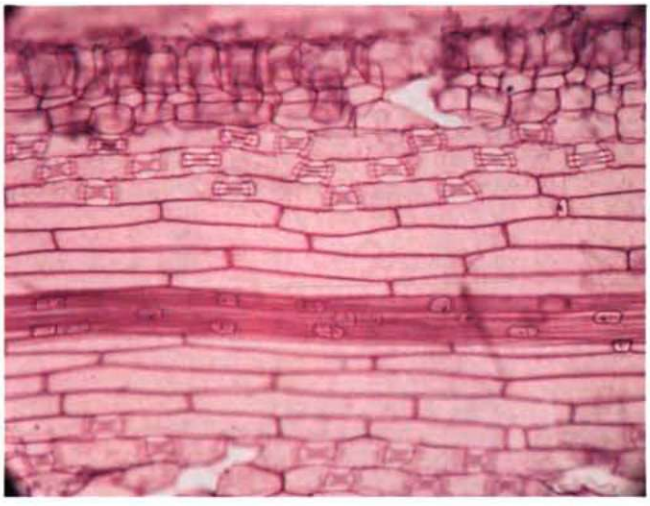

Figure 1. Microscopic photograph of the *F. pratensis* subsp. *pratensis*-46 adaxial epidermis (100x).

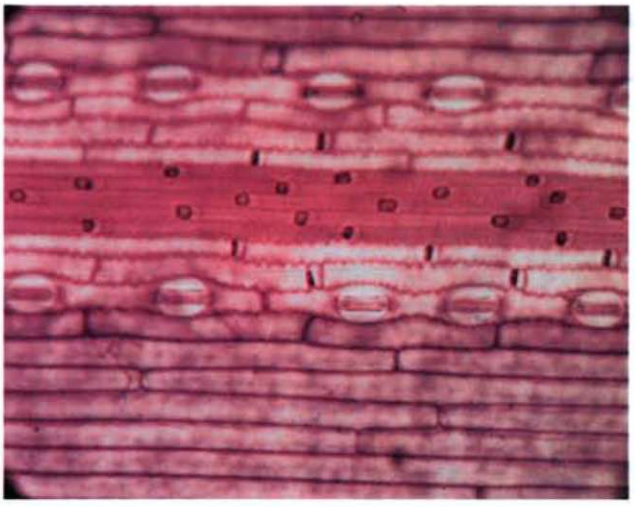

Figure 3. Microscopic photograph of the *F. pratensis* subsp. *pratensis*-48 abaxial epidermis (100x).

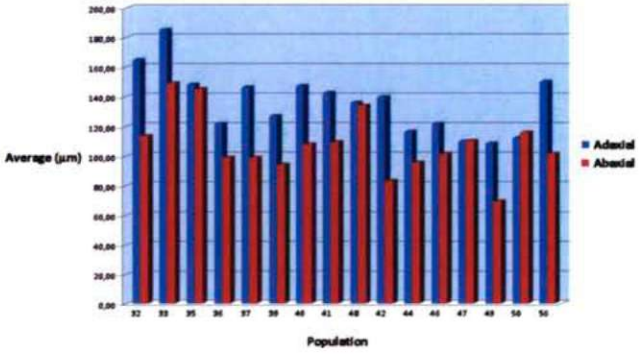

Figure 2. The length of the cells in the costal zone (µm).

50 measurements per population. The dataset made from the numerous photos were analysed with Microsoft Excel. The leaf epidermis' surface structures were examined in images prepared on a Tesla BS 300 scanning electron microscope form fresh, green leaf material.

Results and Discussion

In the examination of the Meadow fescue leaf epidermis, the histological features of the surface of the lamina, the constant and variable features and the characteristics of the epidermal structures were well defined. In each population, in the costal zone of the adaxial (upper) leaf epidermis, there are an average of 2-6 rows of elongated cells (the length being multiple of the width), their radial wall being straight. The cells are on average 108 to 185 µm long and 4.5 to 5, 9 µm wide. Between the long cells there are single silica cells, of mostly rectangular, elongated rectangular shape, rarely square (Fig. 1). The silica cell characteristics (length, width and distribution) will be studied later on.

The costal zone of the abaxial (lower) zone of the epidermis is formed by 3-10 rows of elongated rectangular-shaped cells that in contrast to the adaxial side's costal zone, are wavy (radial walled). Regarding their size, in the case of most populations, they are shorter (Fig. 2) and wider than the costal cells of the adaxial side. The cells are on average 70 to 185 µm long and 5 to -7, 9 µm wide. Compared to the adaxial epidermis, the number of silica cells is greater and they are typically occurring in pairs with phellem cells (Fig. 3).

The intercostal zones of the adaxial epidermis have 15 to 19 cell rows. Directly next to the vein zone, there are 3 to 5 cell rows, of rectangular and even more often having an elongated hexagonal shape, running straight, containing no stoma complexes. The cell lengths are 144-354 µm, the width 10 to 19 µm. Silica cells are rarely found between these cells (The population 36 is an exception to that). After these rows, there are 2 to 4 (rarely 5, e.g. population 50) cell rows of rectangular shape and straight-running, however shorter than in the foregoing (41 to 84 µm long and 9.5 to 16, 5 µm wide) accompany the articular (bulliform) cells on both sides. There are stoma complexes between the cells (Fig. 4.).

The abaxial epidermis, due to the lack of articular cells is more even than the upper epidermis, but the fields above the vein (costal) and between the veins (intercostal) are also well distinguishable. The intercostal zones are composed of 14 to 27 cell rows. Next to the costal zone, an average of 2 or 2 to 3 (rarely 1) cell rows, without stoma complexes, and, unlike the upper side, these cells are strongly wavy and of rectangular shape. The cells are all the same length or longer and in all populations they are unambiguously wider than the cells of the vein zone. Compared to the cells next to the upper side's

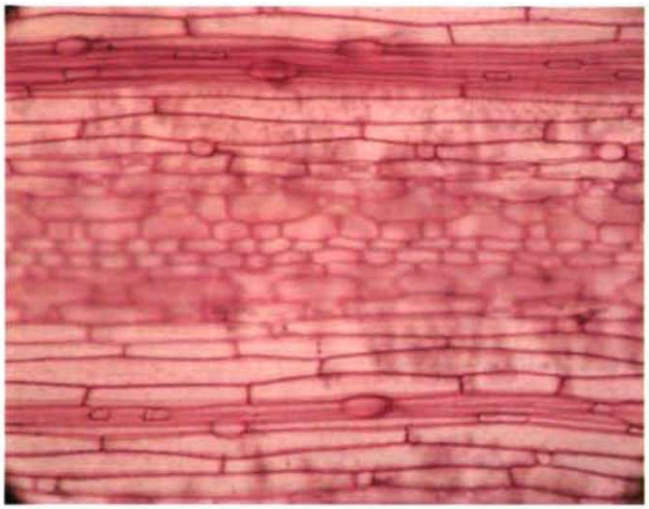

Figure 4. Microscopic photograph of the *F. pratensis* subsp. *apennina*-39 adaxial epidermis (100x).

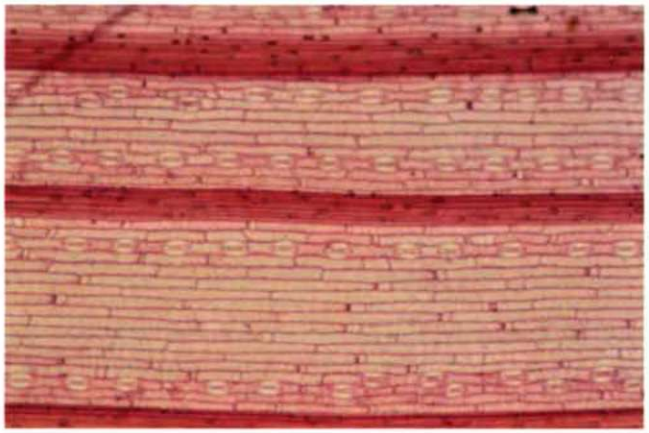

Figure 5. Microscopic photograph of the *F. pratensis* subsp. *apennina*-39 abaxial epidermis (100x).

costal zone, these are shorter (90 to 250 μm) and in most of the populations are narrower (7 to 15 μm) as well.

The number of the rows containing stomata in the lower epidermis intervein zone is mostly 1-1, more rarely 1-2 or 2-2. The cells between the stoma complexes are rectangular, their radial wall is also wavy. The length of the cells is 46 to 96 μm, and compared to the upper side's cells between the stomata, they are varied among the populations (longer or shorter), but their width in most populations is greater (12 to 17 μm). In most populations, in the abaxial epidermis we could observe 1 or 2 wavy cell rows containing stoma complexes in the intervein zone. In all cases the silica cells occur in pairs with the phellem cells, in the rows free of stomata cells and in those contaning stomata as well (Fig. 5.). In the middle of the intercostal zone, between the rows containing stomas, in

Figure 6. Scanning EM photograph of the *F. pratensis* subsp. *apennina*-33 adaxial epidermis (100x).

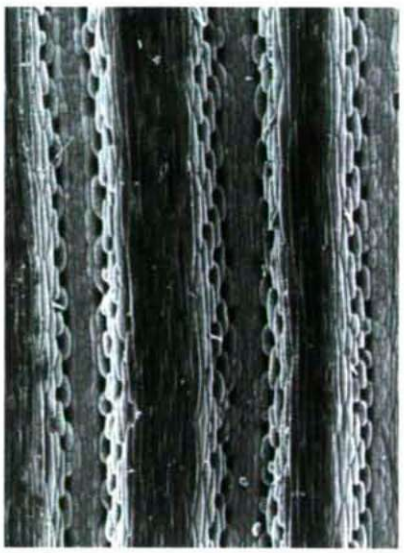

Figure 7. Scanning EM photograph of the *F. pratensis* subsp. *pratensis*-44 adaxial epidermis (100x).

each population, there are straight-running, rectangular cells – these are the longest cells in the zone.

The dumbbell-shaped guard cells of the 'Graminea' type stoma complexes are surrounded by slightly arched side cells, therefore the stoma complex has a flat bun shape. However, on the abaxial epidermis the stomatal cells are domed, more arched than on the adaxial side and the stoma complexes are more round. The length of the stoma complexes is similar on the lower and upper sides, their width is in all cases larger on

the lower side than those on the upper side. The *F. pratensis* subsp. *apennina* populations' stoma comlexes are in average longer both on upper and lower side (except for populations 37 and 39), than those of *F. pratensis* subsp. *pratensis*. The number of stoma complexes per 1mm^2 in all populations is greater in the upper epidermis than the number on the lower epidermis, the number varying from 21 to 94 pcs/mm^2.

Related to the epidermal micromorphology, the scanning electron microscopy studies carried out on fresh epidermal segments reveal finer details in the structure of the epidermis. These are less detectable in the traditional epidermis peels. Bristles occur on the upper epidermis in both the costal and intercostal zones. The upper side of all populations is hairy. On the lower side we did not find any trichomas in six populations (population 33, 35, 36, 40, 41, 47). The populations of subspecies *F.. pratensis* subsp. *apennina* from the Dolomites region (Alpes) are on average more hairy. The bristles of *F. pratensis* subsp. *apennina* are longer than in the populations of *F. pratensis* subsp. *pratensis* that is anyway less hairy.

In conclusion we can remark that in all the examined populations the upper and lower side epidermis exhibits structural differences. The variation between the populations manifest in the differences in dimensions of certain cells of the epidermis, in the size and amount of silica cells, hairiness, and the size of the bristles. The differentiation of the two subspecies (*F. pratensis* subsp. *pratensis* and subsp. *apennina*) on the basis of epidermal structure is done according to the length of stoma complexes, the number and dimension of bristles as well as the length of the epidermis cells.

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ARTICLE

Nectary structure of *Cotoneaster roseus*

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ABSTRACT Cotoneasters are widely planted as ornamentals, which at the same time can serve as a sources of nectar for honey bees and bumble bees. The present study gives a detailed description of the nectary anatomy of *Cotoneaster roseus*. The floral nectary is located within the receptacle, with well distinguishable regions of the epidermis, glandular tissue and nectary parenchyma. Modified nectary stomata are at level with or below the epidermis; the glandular cells are arranged in 3 to 4 subepidermal layers; and calcium oxalate crystals are typical in the nectary parenchyma. Data are provided on the size and thickness of the nectar gland, which can be significant factors determining the nectar producing ability of the flowers.
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KEY WORDS

cotoneaster
fire blight
honey bee
glandular tissue
nectary, stoma

Cotoneasters (*Cotoneaster* spp.) belong to the *Rosaceae* family, comprising both deciduous and evergreen shrubs that are widespread in the temperate regions of Europe and Asia, as well as in North-Africa (Fryer and Hylmö 2009). In Hungary various cotoneasters are frequently planted in gardens, parks and in the vicinity of various institutions or along roads, due to the ornamental value of their leaves, flowers and fruits alike.

Despite their small size, the flowers are able to secrete substantial amounts of nectar, with a fairly high sugar content of 15-40%, which makes them attractive both for honey bees (*Apis mellifera*) and bumble bees (*Bombus* sp.) (Corbet and Westgarth-Smith 1992; Weryszko-Chmielewska et al. 2003, 2004). Being a valuable bee pasture, planting cotoneaster shrubs would be desirable and profitable for apicultural purposes. On the other hand, cotoneasters belong to the genera being the most susceptible to fire blight (van der Zwet and Keil 1979; Roberts et al. 1998), and bees play an important role in transmitting the causing agent *Erwinia amylovora*. Therefore, cotoneasters should be avoided in the vicinity of orchards (Corbet and Westgarth-Smith 1992).

Different species produce highly varying amounts of nectar, which can be explained partly by the structural differences of their nectar glands, and partly by the actual environmental circumstances that will determine the volume and sugar concentration of the nectar produced by the flowers in the given year. The nectary of only a few cotoneaster species has been described so far: Weryszko-Chmielewska et al. (2003, 2004) reported on the anatomy and nectar production of the flowers in *C. hjelmquistii*, *C. lucidus* and *C. nanshan*. The present study gives the detailed anatomical description of the floral nectar glands in *C. roseus*, a species that has previously not been characterised from this aspect.

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Materials and Methods

The flowers of *C. roseus* Edgew. were sampled on two occasions, in May 2007 and June 2010, in the Botanical Garden in Vácrátót. Flower samples were dehydrated in ascending ethanol series, then embedded in paraplast (2007) or Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany), a hydroxyethylmetachrylate based resin (2010). From the blocks 10 µm thick medial longitudinal sections were cut with a rotary microtome (Anglia Scientific 325). Sections were stained with toluidine blue, and mounted in Canada balsam. Slides were investigated with a NIKON ECLIPSE 80i microscope, and micrographs were taken with SPOT BASIC 4.0. Nectary area and thickness (at the thickest part of the gland) were measured with Image Tool 3.0 in 10 and 20 flowers in 2007 and 2010, respectively. Data were analyzed with Microsoft Excel.

Results

The floral nectary of *C. roseus* is lining the adaxial surface of the receptacle, between the ovary and the base of the stamens (Fig. 1). The protruding, automorphic gland can be easily distinguished from the surrounding tissues. In the longitudinal section of the flower the nectary epidermis cells are square or rectangular (Fig. 2). The guard cells of nectar secreting stomata are located at the same level as the epidermal cells (mesomorphic type, Fig. 3) or slightly below the level of the epidermis (xeromorphic type, Fig. 4). Subepidermally 3 to 4 layers of small, isodiametric cells can be observed, comprising the glandular tissue of the nectary (Fig. 2). Below the

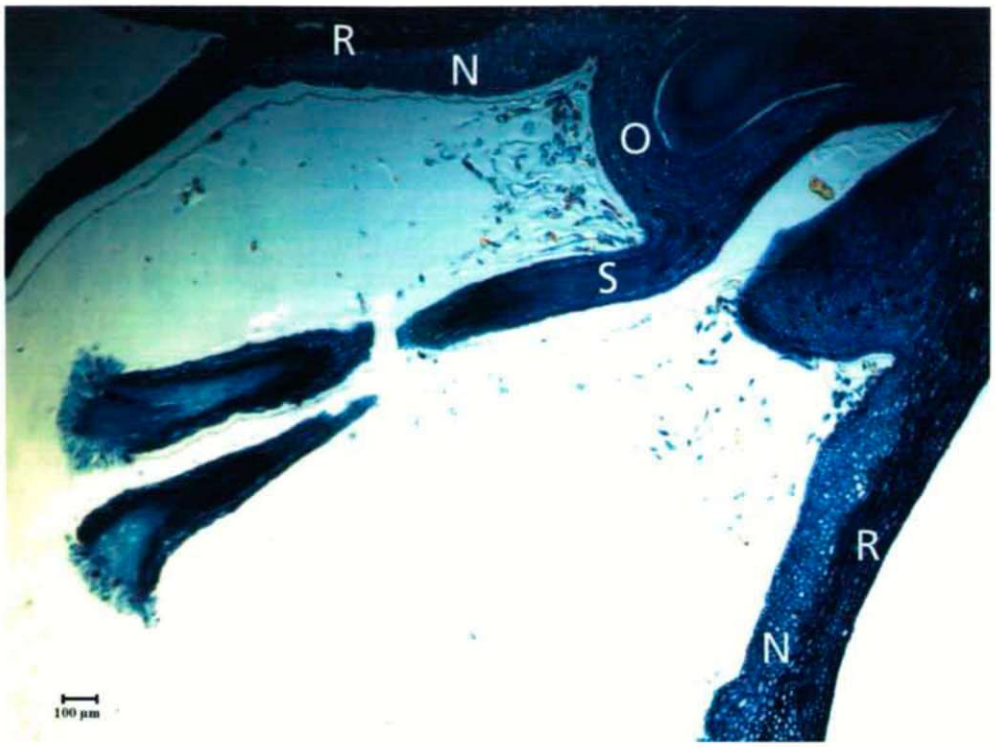

Figure 1. Automorphic nectary of *Cotoneaster roseus* in the longitudinal section of the flower. R: receptacle, N: nectary, S: style, O: ovary.

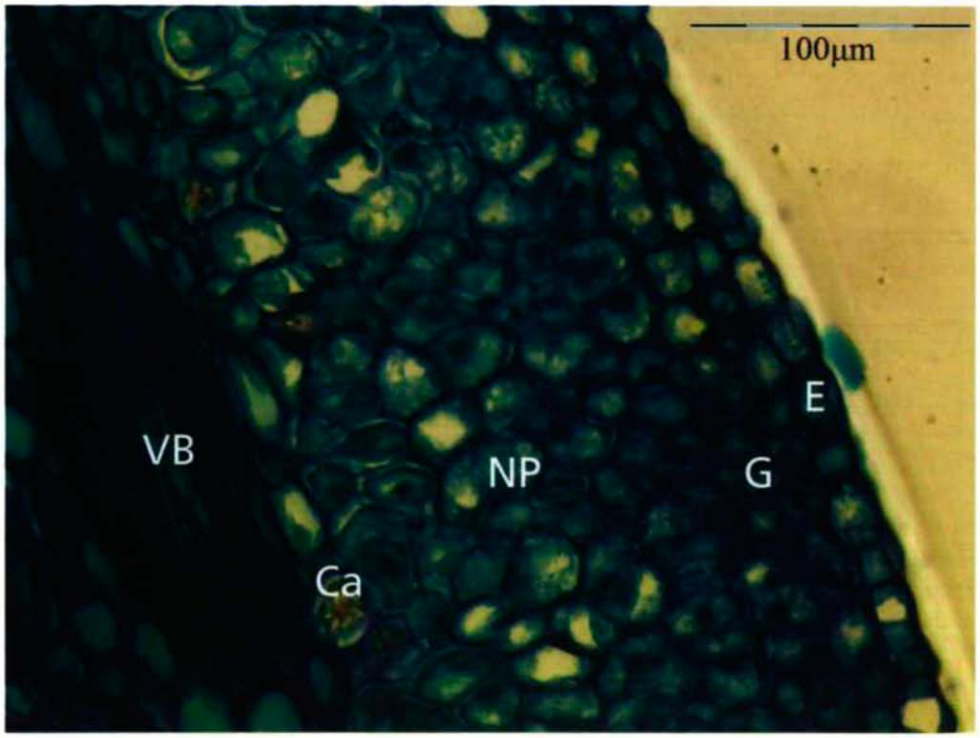

Figure 2. Structure of the floral nectary in *Cotoneaster roseus*. E: nectary epidermis, G: glandular tissue, NP: nectary parenchyma, Ca: calcium oxalate druse, VB: vascular bundle.

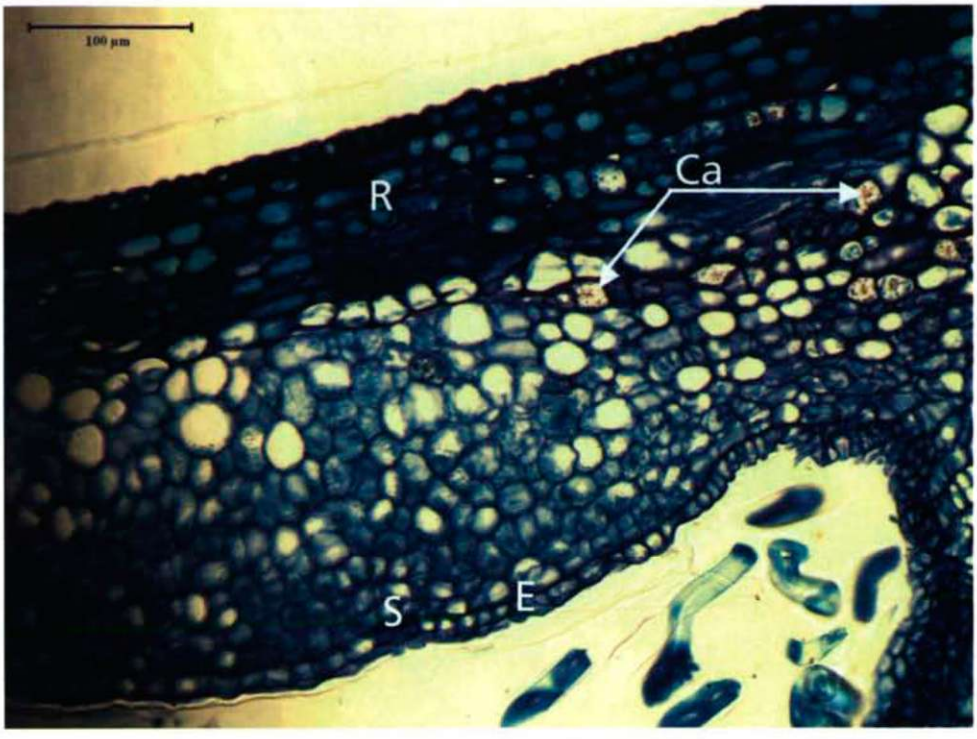

Figure 3. Nectary of *Cotoneaster roseus* with mesomorphic stoma. E: nectary epidermis, S: nectary stoma, R: receptacle.

glandular tissue the larger cells of the nectary parenchyma can be seen. The parenchymatous tissue of both the gland and the receptacle often contains idioblasts with calcium oxalate druses (Figs 2 and 3). Directly beneath the nectary parenchyma vascular bundles can be observed, where the annular cell wall thickening of xylem elements is characteristic (Figs 2 and 4).

The average size (area) of the nectary was below 200,000 µm² in both years of study (Table 1). From the 13 Cotoneaster species studied in 2007, *C. roseus* possessed the second smallest nectar gland (Farkas et al. 2010). The mean thickness values of the nectary – measured at the thickest part of the gland – were around 200 µm in both years (Table 1), and fell between 190 and 225 µm, measured at *C. lucidus* and *C. nanshan*, respectively (Weryszko-Chmielewska et al. 2004). From the cotoneasters studied by us in 2007, *C. roseus* was classified among taxa with thin nectar gland, whereas thickness values reached 240 to 250 µm in the group with thick nectary (Farkas et al. 2010).

Table 1. Area and thickness of the nectary in *Cotoneaster roseus* in 2007 and 2010.

	Mean ± SD (2007)	Mean ± SD (2010)
Area of nectary (µm²)	186284.26 ± 25186.11	185077.30 ± 23860.28
Thickness of nectary (µm)	207.27 ± 16.05	192.34 ± 17.72

Discussion

Weryszko-Chmielewska et al. (2004) found a positive correlation between nectary size and nectar weight, as well as between the number of stomata in nectary epidermis and the weight of nectar. Similarly, our earlier experience and further authors (Gulyás and Kincsek 1982; Orosz-Kovács and Gulyás 1989; Orosz-Kovács et al. 1990; Petanidou et al. 2000; Chwil and Weryszko-Chmielewska 2009) also suggest that taxa with larger nectary and/or thicker glandular tissue produce more nectar than those with smaller glands and/or thinner glandular tissue. On the basis of the above relationship *C. roseus* is supposed to secrete low volumes of nectar. In the view of other, contradictory studies (Weryszko-Chmielewska et al. 1996), however, further investigations are needed to confirm if such a correlation exists between the size of glandular tissue and nectar production in *C. roseus*, as well. Although less nectar production and the expected smaller degree of bee attraction is disadvantageous from the aspect of apiculture, at the same time it can reduce the chances of bees transmitting fire blight causing bacteria in the vicinity of orchards. Additionally, due to attracting smaller number of bees, planting of C. roseus

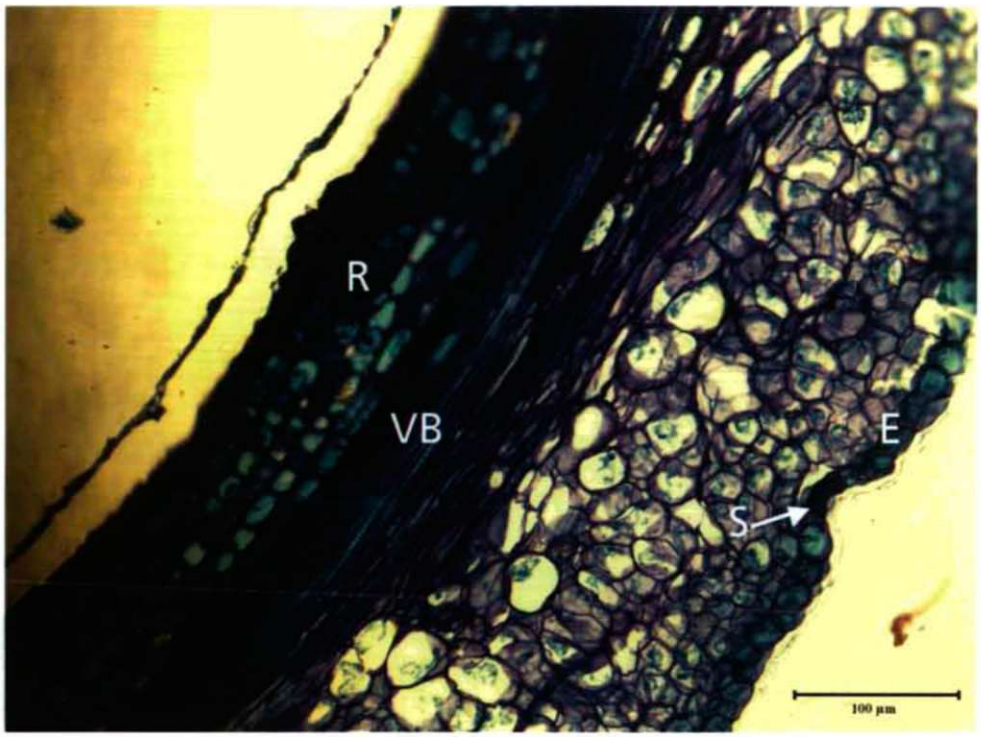

Figure 4. Nectary of *Cotoneaster roseus* with xeromorphic stoma. E: nectary epidermis, S: nectary stoma, VB: vascular bundle, R: receptacle, Ca: calcium oxalate druse.

seems to be safer along roads, in parks and around childcare institutions.

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ARTICLE

Histological study of quercus galls of *Neuroterus quercusbaccarum* (Linnaeus, 1758) (Hymenoptera: Cynipidae)

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ABSTRACT The aim of this study is to reveal the histological structure of galls induced by *Neuroterus quercusbaccarum* and to identify accumulated secondary metabolism products and storage materials in gall tissue by histochemical methods. The galls induced by *N. quercusbaccarum* showed a concentrical layer tissue structure under light microsope. Directly next to the chamber, a protein and lipid containing nutritive tissue can be found, which is covered by sclerenchyma layer consisted of two large cell plates in the lenticular galls of the unisexual generation, while the bisexual generation induced grape shaped galls lack lignified sheaths. Our results confirm previous findings. The outer layer of the gall is the voluminous parenchymatic cortex with a supplying vascular network and covered epidermis. We proved with histological methods, that the galls really contain accumulated nutritives -proteins, lipids and starch- in large quantities. The concentration of these nutrients from the chamber toward the border of inner-gall, show a decreasing gradient in the case of proteins and lipids, and an increasing gradient for starch. We present the differences and similarities in histological structure among galls induced by two generations of *N. quercusbaccarum* and the well studied *Biorrhiza pallida*.

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KEY WORDS

gall
Neuroterus quercusbaccarum
Biorrhiza pallida
histology

The finest way of manipulation of nutritive plants is done by gall-generating herbivorous insects. These force their nutritive plants for abnormal growing and on this, the plant forms a special formation, so-called gall. Galls have been defined in many ways, but most commonly a gall is: an abnormal cell proliferation or cell size growing in the plant tissue. It is caused by the activity of some sort of living organism to use it as food supply or hiding place. When we define it, it is important that these organisms induce the growth of the gall but they don't create it (Csóka 1997). Formation of cynipid galls is a complex interaction between cynipid gall wasps and host plants (most frequently *Quercus* and *Rosa* species). To understand this interaction we need to study the ecology of gall-inducing insects (Szabó 1992; LeBlanc and Lacroix 2001), the structure and development of the galls and examine the cytological, biochemical and physiological properties of gall tissues (Shorthouse and Rohfritsch 1992; LeBlanc and Lacroix 2001; Harper et al. 2004).

In the case of closed galls of the *Cynipidae* species we discriminate one- and many-chambered galls depending on the number of larvae living in them. Regarding the two species we studied, *Neuroterus quercusbaccarum* prefers the one-chambered galls, while the common *Quercus* species herbivore *Biorrhiza pallida* generates the typical many-chambered galls (Shorthouse and Rohfritsch 1992; Harper et al. 2004). The formation and structure of leaf galls of unisexual generation of *N. quercusbaccarum* is well studied (Rohfritsch 1992). The bisexual generation induces nutrient plants to form grape-shaped leaf or inflorescence galls. We have less information about the formation and structure of these galls (Harper et al. 2004).

It is known that in gall development there are four basic stages: initiation, growth and differentiation, maturation and at last dehiscence (Rohfritsch 1992). It was revealed by the example of *Diplolepis rosae* that in the vicinity of the eggs it is needed to have parenchyma cells which are able to divide, and these generate a callus (called plastem) which will form the chamber by the destruction of cells. The other condition is the presence of a vein from the cells of which a „vascular cambium" develops into the tissue of the plastem and supplies nutrition to the gall by differentiating to vascular tissue elements (Rohfritsch 1992).

The most conspicuous structural characteristic of the differentiated galls of *Cynipidae* is the presence of concentric layers made of different cells around the larval chamber. The nutrient tissue and the lignified sheath constitute the "inner gall" and the "outer gall" is formed by the parenchymatic

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cortical tissue and the epidermis. The size of "outer gall" can vary between taxons (Bronner 1985).

It is known that in the cells surrounding the larval chamber, active protein synthesis takes place and they become nutritive cells which are situated either in patches around the larval chamber or in one region. In the early stage of development, the nutrient tissue is not well structured enough. As the appetite of the larva increases, the nutrient tissue is getting more homogeneous and at the end it surrounds the whole larval chamber. The multiplying cells situated under the nutritive cells (cambial zone) generate radial parenchyma cell layers to grow the gall and renew nutritive layers. By the end of the larval state, the cambial zone and nutritive tissue disappear, only a few residuals of cells border the sclerenchyma sheath (Bronner 1985; Rey 1992; Rohfritsch 1992).

In the outer gall radial vascular tissue grows from the peripheral vascular tissue to the cambial area and proceeds toward the chamber. The cambium laces parenchyma cells, which in the beginning, contain starch too. Later mitosis ends and due to cell differentiating, peripheral cells lignify. Sclerenchyma cell layers often differentiate next to the vascular tissue, causing concentric layers in the gall. The mature outer part of the gall (cortex) is made of well-differentiated peripheral vascular tissue, parenchyma and epidermis. The peripheral vascular tissue of the cortex establishes the connection between the host plant and the gall. The mature cortex often stores water or air and also may contain tannin and lignin. Accessories formed on the outer side of the gall (spines, glandular hairs, etc.) probably play a role in defense against parasites (Askew 1984).

The primary goal of this study is tissue examination of galls induced by *Neuroterus quercusbaccarum* and to examine how the original structure changes during gall generation (structural changes of tissues), furthermore the detection of accumulating secondary metabolism products and stored nutrients within gall tissue with histochemical methods. We show the histological structure of galls generated by *N. quercusbaccarum* with the help of models found in literature (*Diplolepis rosae,* Rohfritsch 1992), confirmed by our own experimental results. We will discuss about the bud gall of *Biorrhiza pallida*, another well studied cynipid model (Rey 1992), especially to emphasize the differences.

Materials and Methods

Collecting site and time of plant samples

The galls developed on leaves and catkins of *Quercus robur* induced by the bisexual generation of *Neuroterus quercusbaccarum* were collected in the Botanical Garden of University of Debrecen several times between 04-25.05.2006., 10-25.05.2007. and 10-15.04.2011. We examined the galls induced by the unisexual generation of *Neuroterus quercusbaccarum* on *Quercus robur* leaves collected in the Botanical Garden of the University of Debrecen. Date of collection: 07.09.2007.

The bud galls of the unisexual generation of *Biorrhiza pallida* were collected from nutritive plant *Quercus pubescens* from the "Kőtenger" area of Balaton-highland on 06.05.2006. and in the Botanical Garden of the University of Debrecen on 03.05.2007.

Preparing plant materials

We collected healthy leaves and galls for examination. For the histochemical study, we created preparations from fresh plant matter manually, and with the help of a freezing accessory and microtome (Leica Jung Histoslide 2000 microtome).

We examined the preparations with Olympus BX50 and Olympus Provis AX70/A light microscopes, the microscopic photos were taken by OLYMPUS Camedia 4040 and DIGITAL SP350 cameras.

With microchemical or histochemical methods, we could create chemical reactions which cause differences in some details of the preparation (e. g. in colour). From the quality of the difference, we could draw conclusions from the chemical nature of the cell or tissue structures and detect special products of cells (Sárkány and Szalai 1964).

We used the following histochemical methods in order to

identify proteins: after treatment with potassium-iodide solution, the protein containing cell organelles (aleurone grains) got different shadings of yellow and brown (Sárkány and Szalai 1964, Wanner 2004),

identify starch: potassium-iodide solution caused a violet-blue or bluish-black coloration of starch grains (Sárkány and Szalai 1964, Wanner 2004),

identify tanninic acid: the tanninic acid containing cytoplasm became greyish-blue to 3% ferrichloride solution (Sárkány and Szalai 1964),

identify fatty oil and volatile oil drops: these drops became brick-red to Sudan III treatment, furthermore it coloured the suberinic cell walls too (Mihalik et al. 1999).

We used the method of colouring with congo red to stain the cytoplasm and the cellulose cell wall (Mihalik et al. 1999).

Results

The histological properties of the grape-shaped leaf galls generated by the bisexual generation of *Neuroterus quercusbaccarum*

The unisexual generation of *Neuroterus quercusbaccarum* lays its eggs into the flower-buds of the male catkin or near the still differentiating vein of the developing leaf, ensuring the fast cell multiplying generating a one chambered gall. The galls we studied were generated in leaves (Figs. 1.B-D) and catkin (Figs. 1.F-H) of *Quercus robur.*

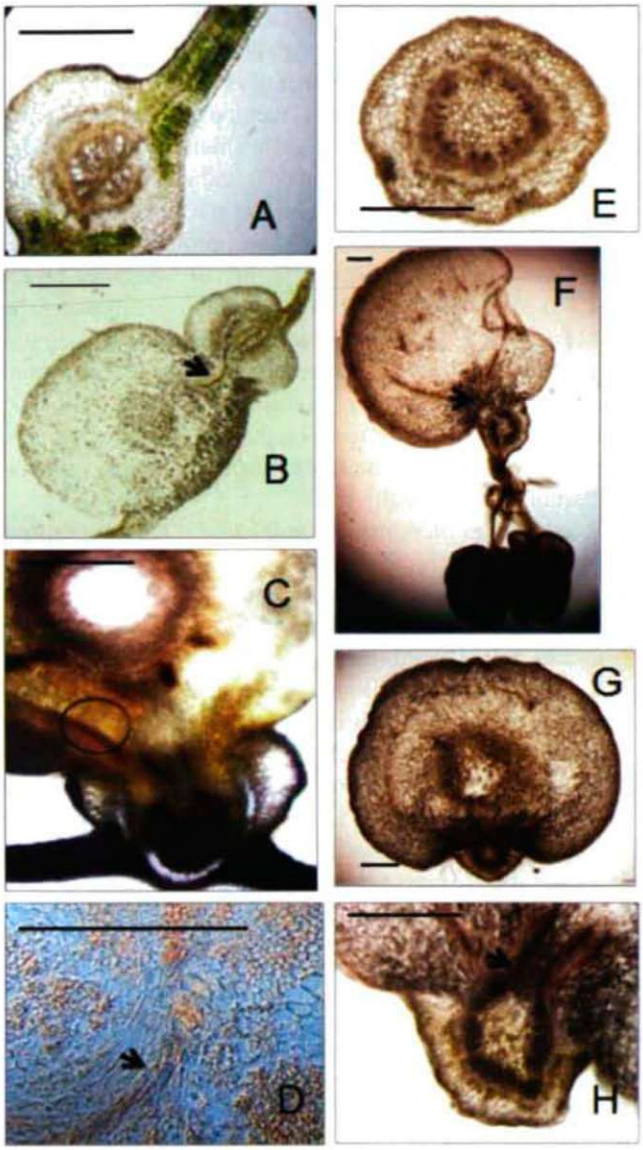

Figure 1. Formation of leaf and flower galls generated by the bisexual generation of *Neuroterus quercusbaccarum* on *Quercus robur*. Cross section of untouched leaf (A) and inflorescence axis (E) of *Quercus robur*. Gall generation next to (B) and above (C) the vein of leaf. Gall generating on the inflorescence axis opposite to flower (F) or replacing flower (G). Differentiation of vascular elements to ensure the transport between the vascular system of leaf/ inflorescence axis and the gall (D and H, arrows). Bars: 400 µm.

The untouched leaves of *Quercus robur* show a typical dorsiventral structure, the chloroplast (chlorophyll) content of the chlorenchyma is very high. The epidermis cells are large, covered by a thick cuticle, but no epidermal accessories can be seen (Fig. 1A). The extended vascular tissue (xylem and phloem) of the main vein is braced by sclerenchyma, and under the epidermis, collenchyma too. The vascular tissue of xylem is made of two direction-differentiated tracheal rows, which refers to secondary thickening and presumes the presence of meristema (cambium). However the vein grid of the leaves is usually built of collateral-closed bundles.

Our leaf samples showed two types of gall-generation (Figs. 1B and C). In the first type, the gall generated directly next to the main vein (Fig. 1B), while in the second type, above the vein (Fig. 1C). Both types have the two conditions of gall-generation: first, the presence of multiplying parenchyma cells near the eggs, in this case the clorenchyma of the leaf, second, the presence of a vein, from which a "vascular cambium" develops into the tissue of plastem (Rohfritsch 1992), which ensures the nutrition of the gall by differentiating into vascular bundles (Figs. 1.B and D, black arrows).

In the parenchymatous tissue of the axis of male catkin there is a good development of vascular system with many rows of tracheal elements, which seemed to grow by new elements produced by the ring-formed cambium (Fig. 1E). The vascular system of catkin axis is in connection with the bundles of flower peduncles (Fig. 1F). This connection can form as well, when a gall replaces completely a flower (Fig. 1G and H, black arrow) or when it develops opposite to a flower (Fig. 1F, black arrow).

In most cynipid galls it is possible to find three kinds of tissues surrounding the larval chamber; a nutritive tissue lining the larval chamber, a sheath of lignified cells, and a parenchymatous zone (or cortex). This structure could be seen at the bud galls induced by the bisexual generation of *Biorrhiza pallida* (Rey 1992). In the case of this type of gall, the parenchyma grows significantly and makes the gall soft and spongy. The gall becomes spherical, will turn from red to white. The nutritive tissue and the starch containing cell layers (nutritive parenchyma) around the larval chamber are closed by sclerenchyma cells (Fig. 2E), and this inner-gall is surrounded by the above mentioned spongy parenchyma. However, we did not find the layer of sclerenchyma cells in the leaf galls and flower galls generated by the bisexual generation of *Neuroterus quercusbaccarum* (Figures 2A-D). The layer of lignified cells (sclerenchyma) is missing from both the still multiplying (Figs. 2A and C) and the differentiated galls (Figs. 2B and D). The chamber is surrounded only by a parenchymatic nutritive tissue. The outer- and inner-galls can be still distinguished, because differentiation of a cambial zone is characteristic in the growing gall to ensure hyperplasia (Figs 2A and B., marked by red arrows).

According to cytological examinations, in the grape shaped gall of the bisexual generation of *N. quercusbaccarum*, the cells between the larva chamber and the gall cortex showed similar cytological features (Rohfritsch 1992). This seems to be true to the young leaf galls (Fig. 2A.) and the ones developed in the catkin inflorescence (Fig. 2C). However, in galls from the end of May, the inner gall is divided into at least 2 different concentric layers. It is easy to distinguish the lighter part of nutritive cells around the chamber which

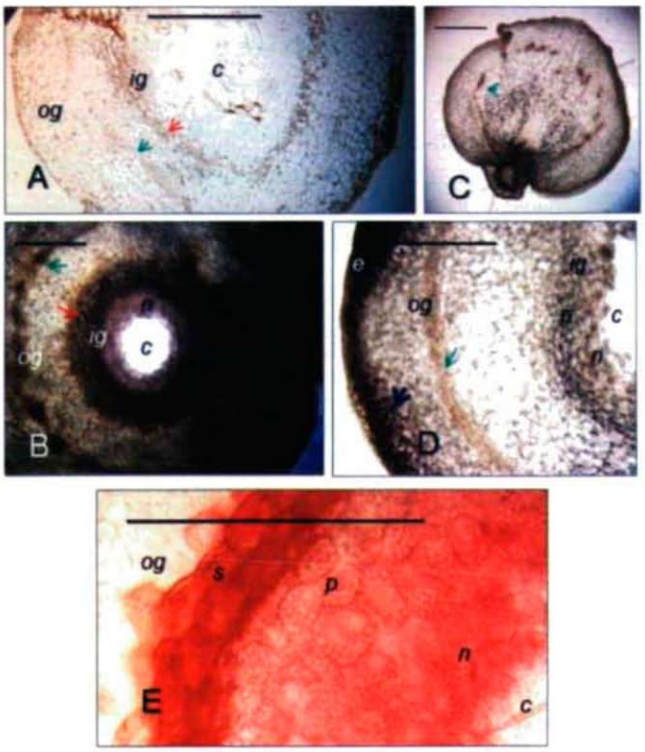

Figure 2. The growth and maturation stages of cynipid gall formation induced by the bisexual generations of *Neuroterus quercusbaccarum* (A-D) and *Biorrhiza pallida* (E). Formation of leaf gall induced by *N. quercusbaccarum* at the beginning of May (A) shows that the chamber (*c*), inner gall (*ig*) and outer gall (*og*) start separating. Later, in the middle of May (B), within the epidermis and the wide parenchymatic cortex (*og*) in the inner gall (*ig*) around the chamber (*c*) the layers of nutritive parenchyma cells (*p*) and of inner nutritive cells (*n*) are differentiated. In differentiated galls the inner and outer galls are separated (A and B, red arrow) by brownish cells with thin cell-walls instead of sclerenchymatised cells. In the cortex, the differentiating tracheal elements form a new transport system (A and B, green arrow). Galls on the catkin-axes induced by the bisexual generation of *N. quercusbaccarum* show the same stages of formation and layers of differentiated cells. They were collected at the end of April (C) and at the beginning of May (D) and have the concentric layers of epidermis (*e*), cortical parenchyma (*og*) with starch grains in the outer part (blue arrow) and with transport network (green arrow), the layers of nutritive parenchyma cells (*p*) and the inner nutritive cells (*n*) of the inner gall (*ig*) around the chamber (*c*). The gall with multilocular larval chambers induced by bisexual generation of *Biorrhiza pallida* in the flower-bud of *Quercus pubescens* (E) has good detectable layers of different cells around the chambers: outer gall (*og*), sclerenchyma (*s*), parenchyma cells with starch (*p*), nutritive tissue (*n*), chamber (*c*). The roughly thickened and sclerenchymatised cell walls became dark-red stained by congo red solution. Bars: 400 μm.

is covered by a zone of darker cells - this can be the layer of starch containing cells – followed by radially elongated parenchyma cell layers belonging to the outer-gall/cortex. The parenchymatic wide cortex is divided into two equal parts by the layer of brownish cells (Fig. 2A-D, green arrows). This could be the peripheral differentiating vascular network, because they are in connection with the vascular bundles of the leaf and catkin axis (Figs. 2B and C). In the spherical gall of another cynipid, *Liposthenes glechomae* similar vascular bundles were detected by Bronner (1992).

With the aid of histochemical methods, we found that the cells next to the chamber store lipids in higher quantities (Fig. 3A), but large drops of lipids can be found in the parenchyma cells of the inflorescence galls creating plastem as well (Fig. 3B). Potassium-iodide staining shows proteins in the cells around the chamber and starch in the cells of outer layers (Fig. 3C). We experienced that during the formation of galls (leaf- and inflorescence-galls as well) starch grains firstly appear in the cells under the epidermis, where the normal subepidermal cells are rich in chloroplasts (Fig. 3D). The histochemical staining of fresh preparations showed that the galls really do accumulate spare nutritives: lipids, proteins and carbohydrates equally.

According to literature, the galls have a high concentration of starch, which depletes toward the larval chamber, the typical nutritive tissue does not contain starch. Lipid gradient shows the opposite tendency. The lipid content depletes in all *Cynipidae* galls toward the periphery of the nutritive tissue, while the nutritive cells around the larva contain many lipid drops (Bronner 1977). This observation was affirmed by the preparations of galls induced by the bisexual generations of *Neuroterus quercusbaccarum* (Figs. 2B, D and Figs. 3A-D) and *Biorrhiza pallida* (Fig. 2E, further data not shown). The lipid drops are the mixtures of unsaturated triglycerides and oil bodies and are not spherosomes. While the larva is alive, there is no starch in the inner layers of the nutritive tissue, but after the larva dies, starch soon appears and the lipids form large drops (Bronner 1980).

It can be traced in older, mature galls, that the nutritive tissue of the chamber is getting used up during the larva development (Figs. 3E and G) and the wall of parenchymatic cells thicken unevenly around the chamber (Figs. 3E-H). But from the chamber towards the epidermis the walls of parenchymatic cells stay thinner. The wall of the epidermic cells are also thin, there can be groups of cells accumulating anthocyanins among them, which make the surface of the gall spotted (Fig. 3F).

Properties of the disk shaped gall induced by the unisexual generation of *Neuroterus quercusbaccarum*

The stages of the development and the histological structure of lenticular galls caused by the unisexual generation of *N. quercusbaccarum* is well studied (Rohfritsch 1992). The larval chamber develops near a vein between the palisade and the spongy clorenchyma of the dorsiventral leaf. In the differentiated lenticular gall around the chamber, narrow nutritive tissue and parenchyma covered by sclerenchyma layers are characteristic (Rohfritsch 1992).

We compared our preparations to the study about the for-

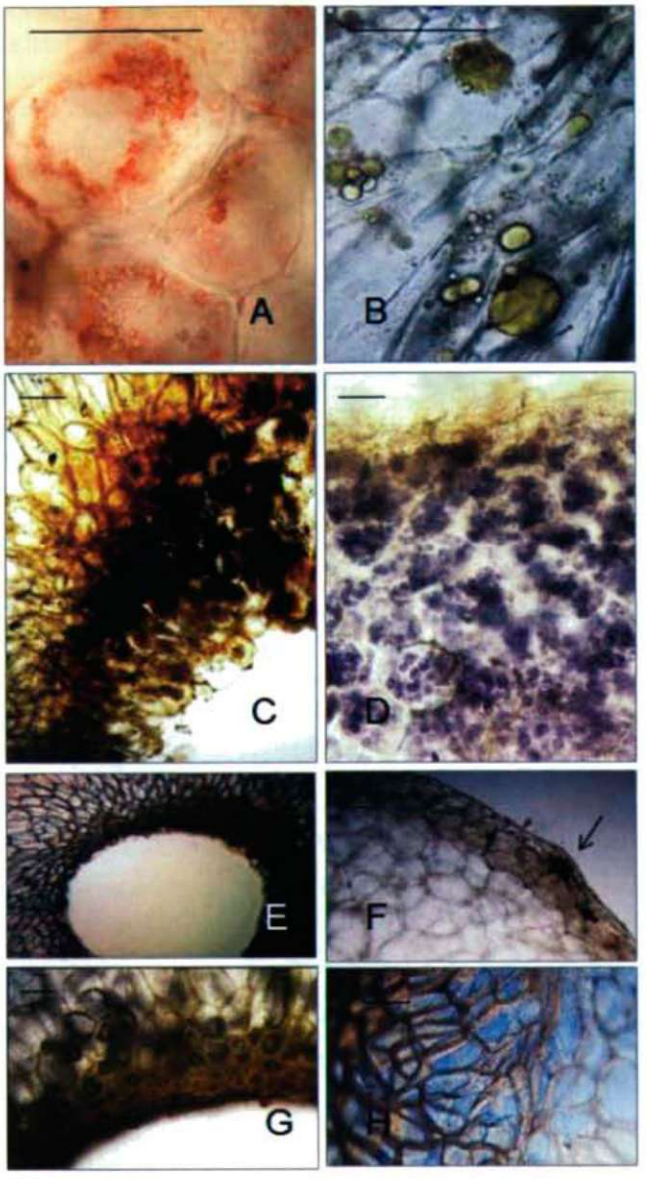

Figure 3. Differentiation- and accumulation processes in the galls on leaves (A, C, E, G) and on catkin-axes (B, D, F, H) induced by the bisexual generations of *N. quercusbaccarum*. Cross sections coloured by potassium iodide (C, D, G) and Sudan III. solutions (A), the other ones are native preparations. Lipid drops in the cells around the chamber coloured red by Sudan III (A) in a leaf-gall, and the large drops of lipids in parenchyma cells of the inflorescence-galls creating a plastem (B, uncoloured preparation). Cells around the larval chamber in reaction with potassium-iodide (C): the protein bodies are stained yellow, the starch grains dark-purple. We experienced that starch firstly appears in the cells under the epidermis in inflorescence galls (D). In the matured galls the cells around the larval chamber are empty (E and G) and the galls consist mainly of parenchyma cells with reticulate secondary cell walls. The walls are less thickened from inner gall (E and G) toward the periphery of the gall (H and F). The epidermis cells on the gall surface contain anthocyanins (F, arrow). Bars: 20 μm.

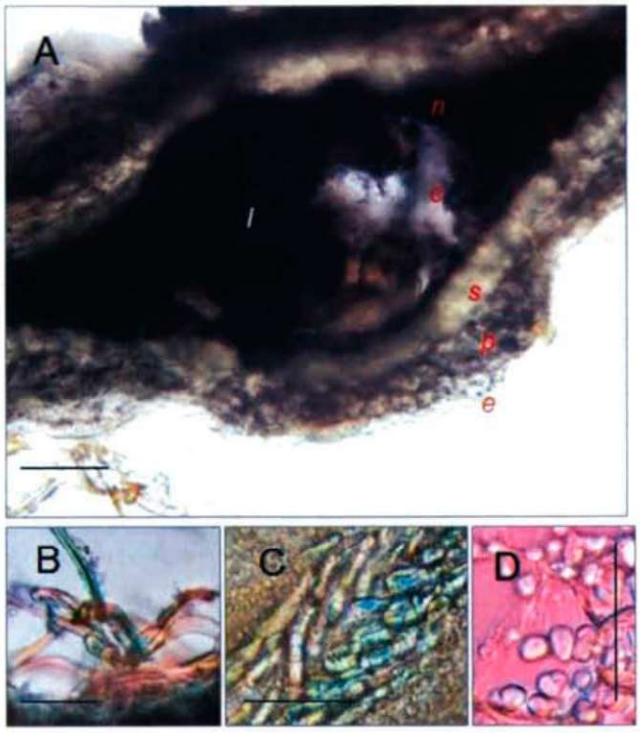

Figure 4. Disk shaped/lenticular leaf gall induced by the unisexual generation of *Neuroterus quercusbaccarum*. A: Our preparation shows a mature gall on the leaf: larva (*l*), chamber (*c*), nutritive tissue (*n*), sclerenchyma (*s*), parenchyma (*p*), epidermis (*e*). It may be more than 7 weeks after oviposition. Cross section made in the middle of the gall and coloured by $FeCl_3$-solution, the dark colour shows the high tanninic acid content. Bar: 300 μm. B: Typical stress-response of the host-plant is the abnormal hair accessory on the gall surface. C: Differentiation of sclerenchyma cells around the chamber in polarized light. D: Chloroplasts turned into starch grains in the chlorenchyma of the host-leaf, examined in polarized light. Bars: 30 μm.

mation of this gall by Rohfritsch (1992).We concluded that the development of galls we collected show the mature state, before falling (Figs. 4A-D). *Quercus* species are very rich in tannins and they can be detected with histochemical methods around the chamber in galls induced by *N. quercusbaccarum* (Fig. 4A) and *B. pallida* (data not shown) as well. On the surface of lenticular gall, abnormal hairs appeared (Fig. 4B), in the middle of leaf mesophyll cells showing sclerification could be seen (Fig. 4C) and starch grains instead of chloroplasts were detected (Fig. 4D).

Discussion

From the four basic stages of gall development we focused on the growth with differentiation and maturation of cynipid galls. The best examples of prosoplasmic galls are those induced by cecidomyiids and cynipids, because these well organized and differentiated galls are the results of defensive reactions of host plants and specific adaptations to the requirements of the insects (Rohfritsch 1992). The "oak apple" a multilocular, spongy, spring gall formed on buds of *Quercus pubescens* by the bisexual generation of *Biorrhiza pallida*

was used as reference, being perhaps the best known cynipid model (Rey 1992; Harper et al. 2004) to compare the galls of the two generations of *Neuroterus quercusbaccarum*. The most prominent structural characteristic of cynipid galls is the presence of concentric layers of differentiated cells around the larval chamber (nutrient cells, nutrient parenchyma and sclerenchyma), that can be shown on our preparation from gall of bisexual generation of *B. pallida* (Fig. 2E).

The bisexual generation of *N. quercusbaccarum* develops in the spherical, monolocular, spring gall, called "currant gall" induced on *Quercus* leaf and in male catkin (Figs.1A-H. and Figs. 2A-D). The lenticular autumn leaf galls of the unisexual generation of *N. quercusbaccarum* are also developed near veins of *Quercus* leaves. Both have nutritive tissue and nutritive parenchyma around the larval chamber, but the parenchyma of the lenticular gall is much thinner (Figs. 2B, D and Fig. 4A). The spherical galls of *N. quercusbaccarum*, similarly to the oak apple of *B. pallida*, develop an extended callus-like parenchymatic tissue via realignment of the cells of clorenchyma tissue. In the growing plastem, chloroplasts transform into amyloplasts, and a vascular cambium produces tracheal elements for a vascular network (Figs. 1, 2A-D, Fig. 3D).

The differentiated currant galls show the concentric layered histological structure of inner-gall, which are mostly layers of lipid-, protein- and starch accumulating cells (Figs. 2.B-E, Figs. 3A-C, Figs.4A and D). Harper et al. (2004) investigated the cytological and biochemical background of formation of cynipid galls. Among the inner-gall proteins the putative carboxyl carrier protein, which is involved in triacylglycerol lipid synthesis gave a rich source of energy in the inner-gall cells, revealed differential expression throughout development (Harper et al. 2004). They claimed that the spherical galls of *B. pallida, N. quercusbaccarum* and *Cynips quercusfoliis* have the same patterns of development, including sclerenchyma layers. However we found that in the spherical galls induced in *Quercus* leaf and in male catkin of *N. quercusbaccarum* these sclerenchyma layers do not differentiate. In the young gall only a row of small parenchymatic cells marks the border between the differentiating inner- and outer-gall (Fig. 2A, red row), maybe it is a cambial zone characteristic on several cynipid galls (Rey 1992). We can read that in case of the galls with no sclerenchyma, a layer of tannin containing cells can appear (Rohfritsch 1992). Synthesis of tannins is general in *Quercus* species and we can detect them in a wide stripe round the chamber in galls induced by both generations of *N. quercusbaccarum* (Fig. 4A) and *B. pallida* (data not shown) as well. We believe that our results are in good agreement with the notice of Rohfritsch (1992) about the missing of a lignified sheath in the grape shaped gall of the bisexual generation of *N. quercusbaccarum*. The cambial zone appearing near larval chamber organizes the compensation of the nutritive parenchyma and nutritive cells.

We proved by different histochemical methods that the galls really contain nutriments: proteins, lipids and starch in large quantities. The concentration of lipids and proteins shows a decreasing gradient toward the periphery of the gall, while the concentration of starch shows an increasing gradient from the chamber to the periphery (Figs. 3A-D). The results of histological studies of these galls and the histological identifying of accumulated nutrition and secondary tannins altogether were compatible with the common statements about *Cynipidae* galls (Bronner 1977). In differentiated galls around the inner-gall an extended, spongy tissue of large parenchyma cells with vascular network is the characteristic outer-gall structure (Figs. 2B-D). The vascular elements that can be found in the gall are either the remains of the original vascular bundles or products of the cambium generated during gall formation and are essential for gall development. We detected typical plant stress responses during the gall formation induced by wasps (Figs. 3F and 4.A-C). We confirm that during the stages of gall development, active cell multiplying (hyperplasia), cell growing (hypertrophy), differentiating and cell destruction jointly shape the gall structure.

Little data can be found in literature about the grape shaped galls of *Neuroterus quercusbaccarum* compared to other types. Therefore our results might contribute to a better understanding of the structure of this gall type.

Acknowledgments

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ARTICLE

Studies on elemental composition and antioxidant capacity in callus cultures and native plants of *Vaccinium myrtillus* L. local populations

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ABSTRACT The biological and medical effects of bilberry fruit (*Vaccinium myrtillus* L.) are mainly due to high anthocyanin content of tissues. Calli containing anthocyanins, derived from bilberry plants, may represent a potential source of natural colouring matter, pharmaceutical and natural antioxidants. In the present study we investigated the occurrence of differences in elemental composition and antioxidant capacity of the three local populations of mountain bilberry collected in the western region of Romania (Arieseni, Retezat and Sebes Valley) in order to compare the anthocyanin production of plant and callus tissues originated from various plant populations. It was found that K, Fe and Zn content was higher in calli than in intact plant leaves. The excess of latter two microelements, Fe and Zn can induce oxidative stress, and, as a result of the accumulation of reactive oxygen species, various antioxidant mechanisms. The total antioxidant capacity of callus cultures determined by FRAP method (ferric reducing antioxidant power) could be enhanced as a function of increasing adenine sulphate (AS) concentration in the culture medium and it depended on the origin of mother plants. The leaves of intact plants contained higher amount of total non-protein sulfhydryl groups than calli, and the decrease was especially significant in tissue cultures originated from the Retezat region. In contrast, depending on the AS concentration, the anthocyanin content could increase in callus cultures. The tissues originated from various populations exhibited different AS concentration optimum. This suggests that bilberry callus cultures can be a suitable source of the anthocyanins.
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KEY WORDS

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anthocyanins
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intact plants
Vaccinium myrtillus L

Lowbush bilberry belongs to the genus *Vaccinium* (Ericaceae family), which contains about 400 species. *Vaccinium myrtillus* L. is native to Europe and North America. This plant is a component of ground layer vegetation in forests of cold and temperate climate zones and forests at higher elevations in Southern Europe, and it has an important role in the nutrient fluxes of natural ecosystems. It was also found that the elemental composition and the accumulation of phenolic compounds in bilberry leaves were suitable indicators of heavy metal stress and it could effectively indicate the elemental content of soil (Mróz and Demczuk 2010).

Bilberry is considered to be an important nutritional resource for humans. The fruits and leaves are rich in phenolic compounds, especially in anthocyanins and other antioxidants (Martz et al. 2010). It was found that the antioxidant capacity of blueberry (*Vaccinium corymbosum* L.) cultivars could be influenced by the genotype, but other factors (e.g. growing season, location, the age of plants, storage condition of samples) can also affect these parameters (Piljac-Žegarac et al. 2009). Over the years, a series of chemical analyses have revealed these health-beneficial compounds in bilberry fruits, however, the underlying genetic diversity and the variation in biochemical composition between populations and *in vitro* callus cultures, remain to be thoroughly investigated. The aim of the present study was to investigate the differences in the elemental composition, total antioxidant capacity, anthocyanin and total non-protein thiol content of intact leaves and calluses derived from various populations of bilberry collected in the western part of Romanian mountains.

In vitro propagated callus cultures can become an alternative to plants grown in their native environment due to the fact that under controlled conditions, plant tissues can produce significant amounts of metabolites of interest. Moreover, the antioxidant activity and the content of macro- and microelements may represent parameters that indicate the occurrence of somatic variability in the callus, this fact being important in selecting the cell lines of interest.

The hormone balance and chemicals applied in the culture medium are important for the selection of callus lines with high antioxidant capacity. Therefore, we also would like to

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determine whether the application of adenine sulphate (AS) in the culture medium favors growth and antioxidant capacity of callus cultures established from various native populations of bilberry.

Materials and Methods

Plant material representing native populations of bilberry was collected from three sites of Carpathian mountains in western Romania, in Arieseni, Retezat and Sebes Valley districts. Because of the difficulties of the representative sampling for elemental analysis the fully expanded leaves of naturaly growing plants were collected two times in June 2011. Five plants were selected each time and were transported to laboratory in an icebox. Then the leaves were detached and after washing with double distilled water, they were stored at -80°C until analysis (Kovacheva et al. 2000). To obtain calluses, different types of explants were taken from various tissues of bilberry plants, originated from the three different mountain locations. After surface sterilization the explants were inoculated on the Woody Plant Medium (WPM) (Lloyd & McCown's Woody Plant Medium, PhytoTechnology Laboratories, Lenexa, Kansas, USA), supplemented with 5,24 µM 1-naphthylacetic acid (NAA) and 5 µM benzylaminopurine (BAP) and with different concentrations of AS (Sigma, Chemical Co., St. Louis, Missouri). Concentrations of AS were 99 µM (40 mg/l), 148,51 µM (60 mg/l) and 198,01 µM (80 mg/l). The calluses used in this study were analyzed after three subcultures on the same type of culture medium and under the same hormonal influence.

Determination of macro- and microelement content

The content of macro- (K, Ca, Mg) and microelements (Cu, Zn, Fe, Ni) as well as some heavy metals (Cr, Cd, Pb) in *Vaccinium* calluses and mother plant leaves was determined by atomic absorption spectrometry (AAS) with a Hitachi Z-8200 spectrophotometer (Tokyo, Japan). For each sample, 100 milligrams of dried plant or dried callus tissues were used. Plant material was homogenized and placed in test tubes containing 5 ml of concentrated HNO_3 and 4 ml of 30% H_2O_2 at 200°C for 2 hours. For the determination of metal content, 3 replicas were taken in each experiment for each sample. Metal content in the samples are given in µmol g^{-1} dry mass (DM).

Preparation of samples for biochemical assays

Fresh plant material (0.3 g) was homogenized with 1.2 ml of cool 0.1 M phosphate buffer (K_2HPO_4, pH 7.6) containing 0.1 mM EDTA, and centrifuged for 10 min at 12,000g. Then the supernatant was used for the detection of total antioxidant capacity and total non-protein thiol assays. Non-protein sulfhydryl groups were expressed in glutathione (GSH) equivalents.

Determination of total antioxidant capacity (FRAP)

The total antioxidant activity was determined by FRAP method (Ferric Reducing Activity of Plasma or Ferric Reducing Antioxidant Power) which measure the ferric ion reducing capacity of the cytoplasm (Benzie and Strain, 1996). The plant extract was prepared and the measurements were done according to the modification of Varga et al. (2000) and Szőllősi and Varga (2002). The reaction mixture contained 50 µl plant extract and 1.5 ml FRAP reagent (300 mM acetate buffer, pH 3.6, 10 mM tripyridyltriazine (TPTZ) in 40 mM HCl and 20 mM $FeCl_3$ in ratio 10: 1: 1). Ferric (Fe^{3+}) to ferrous (Fe^{2+}) ion reduction at low pH causes a coloured ferrous-tripyridyltriazine (Fe^{2+}-TPTZ) complex to form. The absorbance was determined with spectrophotometer at 593 nm. The total antioxidant capacity was expressed in units of µmol g^{-1} fresh weight (FW).

Determination of total non-protein thiol content

Total non-protein thiol content was measured using the method of Sedlak and Lindsay (1968). 125 µl of plant extract and 0.5 ml of 5% (w/v) trichloroacetic acid (TCA) were mixed and centrifuged for 10 min at 10,000g. Then the supernatant was used for the measurement by adding 0.4 M Tris buffer (pH 8.9) and 5,5'-dithio*bis* (2- nitrobenzoic acid) (DTNB) and the absorbance was detected by the spectrophotometer at 412 nm. Data are expressed in µmol GSH g^{-1} fresh weight (FW).

Analysis of anthocyanin compounds

Anthocyanin compounds were extracted from 250 mg of fresh callus at 4°C, using 2 ml concentrated methanol (MetOH, 99% v/v) acidified with 1N hydrochloric acid (HCl) in 1:1 ratio according to the assay of Lange et al. (1971). After extraction, the samples were centrifuged for 20 minutes at 12,000g and the supernatant was analyzed with spectrophotometer at 479 nm.

To estimate the anthocyanin-concentration of plant tissues by measuring the absorbance, the anthocyanin contents are expressed in cyanidin-3-glucoside equivalents (mg) and calculated for 1 g fresh weight. The calculation of the concentration was based on Lambert-Beer's law using a molar extinction coefficient of 2.95×10^4.

Statistics

The statistical analysis of results was carried out using STATISTICA 9.0 software. First we executed two-way ANOVA to detect the effect of population and/or treatment on several parameters. Then non-parametric test (Kruskal-Wallis ANOVA) was used to test the differences of means. In order to deter-

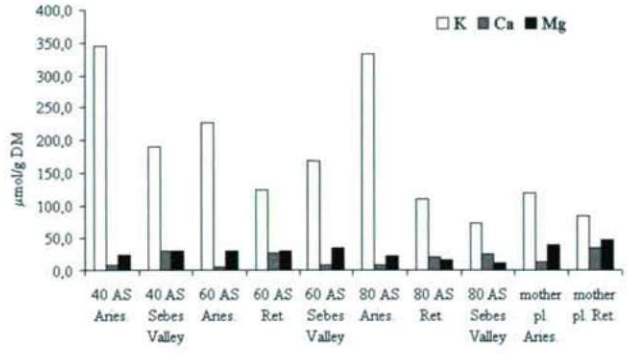

Figure 1. The macroelement (K, Ca, Mg) content in the calluses and the leaves of mother plants of *Vaccinium myrtillus* L. originated from Arieseni, Retezat and Sebes Valley districts. The culture medium was supplemented with 40, 60 and 80 mg l^{-1} adenine sulphate (AS). Data are given in µmol g^{-1} DM (dry mass).

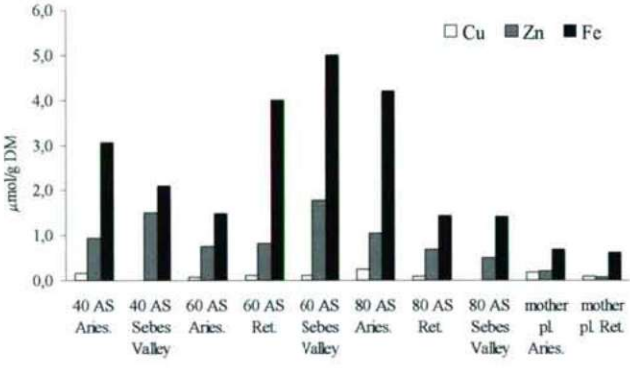

Figure 2. The microelement (Cu, Zn, Fe) content in the calluses and the leaves of mother plants of *Vaccinium myrtillus* L. originated from Arieseni, Retezat and Sebes Valley districts. The culture medium was supplemented with 40, 60 and 80 mg l^{-1} adenine sulphate (AS). Data are given in µmol g^{-1} DM (dry mass).

mine the relationship between the biochemical parameters, a non-parametric analysis of correlation (Spearman's Rank Order Correlation) was used. Data are given in mean values ± standard deviation (SD) and calculated for fresh weight (FW). Level of significance was generally $p<0.05$.

Results

The content of macro- and microelements

The amount of macroelements of the *Vaccinium* calluses derived from several populations had a high diversity and ranged from 73.6 to 346 µmol g^{-1} DM for potassium, 6.8-35 µmol g^{-1} DM for calcium and 11.6-47 µmol g^{-1} DM for magnesium (Fig. 1). The potassium level was usually higher in the calluses than in the mother plants that were regarded as

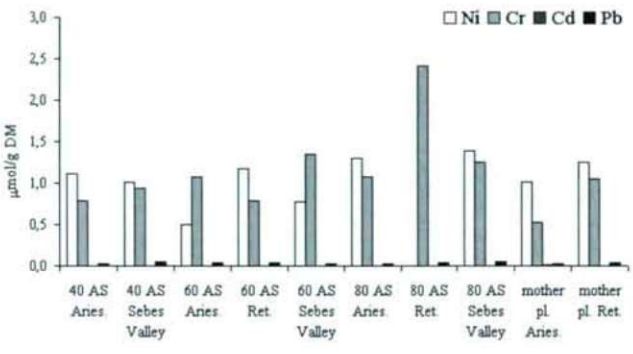

Figure 3. The heavy metal (Ni, Cr, Cd, Pb) content in the calluses and the leaves of mother plants of *Vaccinium myrtillus* L. originated from Arieseni, Retezat and Sebes Valley districts. The culture medium was supplemented with 40, 60 and 80 mg l^{-1} adenine sulphate (AS). Data are given in µmol g^{-1} DM (dry mass).

control (119.5 and 84.1 µmol g^{-1} DM in the samples derived from Arieseni and Retezat, respectively), while the calcium and magnesium content of the mother plants was generally higher than those of the calluses.

Increasing concentrations of AS in the culture medium did not enhance the macroelement content of callus tissues, in contrast, the highest AS concentration (80mg l^{-1}) may result in a decline in K content in certain cell lines.

Copper level of the calluses were similar to those of the mother plants and were very low (0-0.25 µmol g^{-1} DM), but zinc concentrations of the calluses were 5-9-times higher than those of the control (0.06-1.8 µmol g^{-1} DM). At the same time, calluses showed very high iron levels compared to control (0.6-5 µmol g^{-1} DM, Fig. 2).

The amounts of Ni (essential element) and Cr were very low and the concentrations of two other non-essential heavy metals, Cd and Pb were close to zero in all samples (Fig. 3).

The total antioxidant capacity (FRAP)

Since analysis of variance (ANOVA) revealed that the population and the treatment had an effect on the FRAP values, the effect of population being stronger, ($F_{2, 26}$= 20.62, $p<0.001$; Table 1), we compared the data of the different populations (V1= Arieseni, V2= Retezat, V3= Sebes Valley) grown on increasing concentration of AS.

We found significant differences between the populations only at 60 AS treatment (Fig. 4). Surprisingly, FRAP did not show significant correlation neither with free, non-protein thiol level nor the macro- or microelement content.

Total non-protein thiol content of tissues

We found that both the population type and the treatment have an effect on the accumulation on non-protein thiols

Table 1. Results of two-way ANOVA testing the effect of population type and treatment on ferric reducing capacity (FRAP) and total non-protein thiol (GSH) content in *Vaccinium myrtillus* L.

Effect	FRAP	GSH
Population	$F_{2,26} = 20.62^{***}$	$F_{2,26} = 21.25^{***}$
Treatment	$F_{3,26} = 3.84^{*}$	$F_{3,26} = 31.62^{***}$
Popul. x treatm.	$F_{6,26} = 2.83^{*}$	$F_{6,26} = 2.63^{*}$

Significance levels are indicated by * and *** representing $p< 0.05$ and $p< 0.001$.

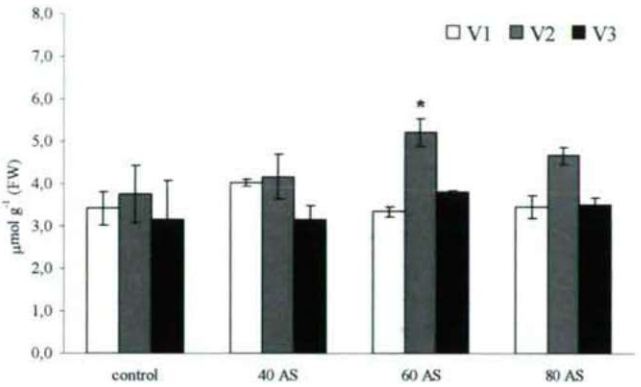

Figure 4. The total antioxidant capacity (FRAP) of the calluses and mother plants (control) of *Vaccinium myrtillus* L. originated from Arieseni (white column, V1), Retezat (grey column, V2) and Sebes Valley (black column, V3) districts. The culture medium was supplemented with 40, 60 and 80 mg l^{-1} adenine sulphate (AS). Data are given in µmol g^{-1} FW (fresh weight). Mean ±SD (n=3). Asterisk (*) refer to significant difference between control and treated plants within the same population, at $p<0.05$.

(Table 1). After further analysis, significant differences were found among the populations in the control samples. Moreover, callus cultures exhibited a decrease in total non-protein thiol content in all populations (Fig. 5) and non-protein thiol concentrations in tissues growing on 40-80 mg l^{-1} AS were rather similar to each other. At the same time, within V2 population, all types of calluses had much lower free SH levels than the intact plant samples. We found significant correlations between total non-protein thiol content of tissues, and elemental composition, a positive correlation coefficient for Mg ($r= 0.59$, $p< 0.001$) and negative values for Zn ($r= -0.70$, $p< 0.001$) and Fe ($r= -0.60$, $p< 0.001$).

Total anthocyanin content

In all calluses and the control plants remarkable differences were found between V2 and V3 populations (Fig. 6). We also found relatively strong positive correlation between total anthocyanin content and FRAP values ($r= 0.60$, $p< 0.001$; Fig. 7). It was also found that depending on the genotype, the anthocyanin contents could increase transiently (V1- white column and V3- black column) as a function AS concentration.

Discussion

In our experiments it was revealed that elemental composition of callus cultures can show significant differences from the tissues of mother plants and in some cases there were significant differences between the tissues originated from different populations.

Increasing the amount of AS (60-80 mg l^{-1}) in the culture medium causes the accumulation of iron in bilberry callus. The tissues also accumulate zinc in smaller amounts, and the

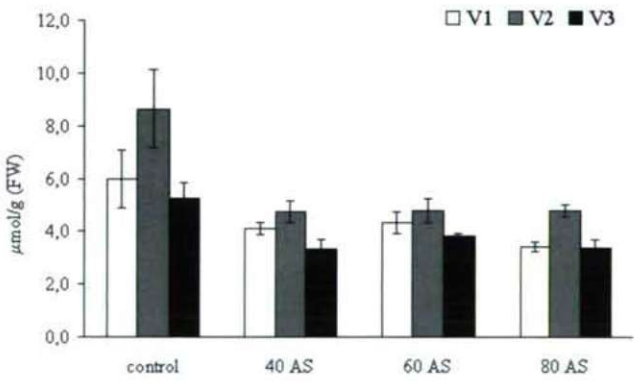

Figure 5. The total non-protein thiol content of the calluses (expressed in glutathione equivalents) and mother plants (control) of *Vaccinium myrtillus* L. originated from Arieseni (white column, V1), Retezat (grey column, V2) and Sebes Valley (black column, V3) districts. The culture medium was supplemented with 40, 60 and 80 mg l^{-1} adenine sulphate (AS). Data are given in µmol g^{-1} FW (fresh weight). Mean±SD (n=3).

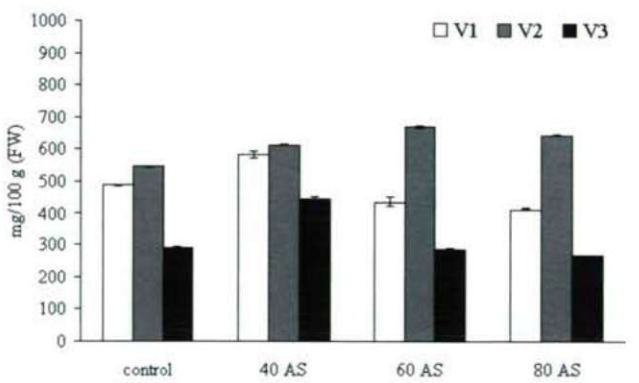

Figure 6. The total anthocyanin content of the calluses and mother plants (control) of *Vaccinium myrtillus* L. originated from Arieseni (white column, V1), Retezat (grey column, V2) and Sebes Valley (black column, V3) districts. The culture medium was supplemented with 40, 60 and 80 mg l^{-1} adenine sulphate (AS). Data are given in µmol g^{-1} FW (fresh weight). Mean±SD (n=3).

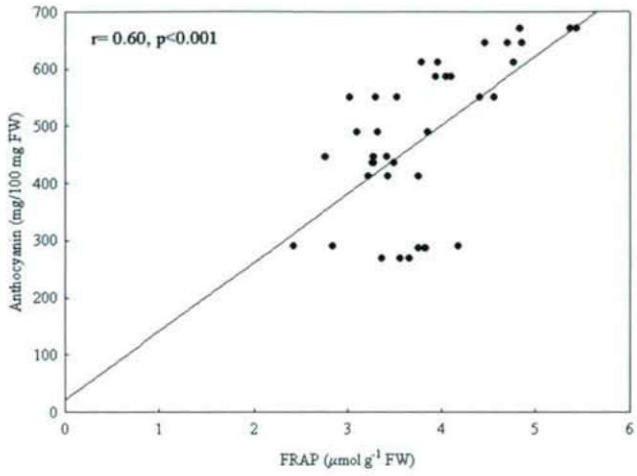

Figure 7. Spearman's Rank Correlation between the total anthocyanin content and FRAP in *Vaccinium myrtillus* L. tissues.

zinc content in callus cultures is higher than in the mother plant. This phenomenon makes the callus tissues usable in treatments to combat the deficit in iron and zinc of food, in form of food or feed dietary supplements.

Regarding the heavy metals, chromium content reaches slightly higher values in the callus tissues than in the mother plants. Growing callus on culture medium which is supplemented with 40 mg l^{-1} and 60 mg l^{-1} AS does not change the content of these non-essential heavy metals compared to the mother plants.

The addition of AS to the culture medium may increase the total antioxidant capacity of bilberry calluses, the effect was most significant in tissues of V2 and V3 populations treated with 60 mg l^{-1} AS.

The pool of non-enzymatic antioxidants and antioxidant enzymes is induced very frequently by oxidative stress itself (Csiszár et al. 2004). However, the non-protein thiol content of calli are lower than those of intact plant tissues suggesting that the cells in tissue culture are probably exposed to a slight oxidative stress due to the relatively high concentrations of plant hormones in the culture media or due to the excess of Fe and Zn in callus tissues. Since glutathione constitutes the highest portion of free, non-protein thiols in plant tissues, we can suppose that GSH content may be exhausted in these tissues as it was found in wheat roots exposed to heavy metal stress generating reactive oxygen species (Tari et al. 2002). The synthesis of phenolic compounds such as anthocyanins is also induced by oxidative stress caused by heavy metals (Mróz and Demczuk, 2010) or H_2O_2-generating chemicals such as salicylic acid (Szepesi et al. 2008).

The anthocyanin content of bilberry calluses is greater than that of the mother plants leaves, under *in vitro* culture conditions used. We suggest that both heavy metal stress and application of AS in the culture medium favors the biosynthesis of these important components with therapeutic value and the accumulation of anthocyanins is a consequence of the generation of reactive oxygen species in callus tissues growing on culture medium supplemented with AS.

Abbreviations

NAA: 1-naphthylacetic acid; BAP: benzylaminopurine; AS: adenine sulphate.

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ARTICLE

Exogenous ascorbate improves antioxidant defense system and induces salinity tolerance in soybean seedlings

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ABSTRACT Germination, growth and antioxidant defense system were investigated under salinity stress and pre-treatment with ascorbate (ASC) in two soybean cultivars SAHAR and DPX. Sterilized seeds were soaked in distilled water or ASC solution (0, 400 mg L^{-1}) for 4 hrs before they were sown in distilled water or NaCl solution (0, 12.5 and 50 mM). Salt stress reduced the growth of both cultivars through reduction in percentage of germination, shoot and root length and dry weight of seedling. ASC induced enhancement in growth of salt-stressed plants coupled with an increase in catalase and peroxidase activity in seedlings only in SAHAR cultivar, and an increase in superoxide dismutase activity in both cultivars. These findings led us to conclude that applied ASC counteracts the adverse effects of salt stress on growth of soybean; however, these effects were cultivar specific.
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KEY WORDS

antioxidant enzymes
ascorbate
salt stress
soybean seedling

Salinity is an increasing problem affecting 20% of the world's cultivated lands and nearly half of the area under irrigation, for that reason, genetic improvement of salt tolerance has become a critical need for the future of agriculture in arid and semi arid regions (Kaviani and Kharabian 2008). Excessive amounts of salt in the soil, most commonly NaCl, have detrimental effects on plant growth and productivity (Reynolds et al. 2005; Zilli et al. 2008; Sobhanian et al. 2010). Salt stress changes the morphological, physiological and biochemical responses of plants. Salinity causes both ionic and osmotic stresses and affects plant growth and development (Munns 2002; Benlloch-Gonzalez et al. 2005). One of the biochemical changes occurring when plants are subjected to salt stress is the production of reactive oxygen species (ROS). ROS causes cellular damage via membrane peroxidation, protein oxidation and DNA damage. To prevent such damages, plants have evolved an effective scavenging system involving antioxidant molecules like carotenoids, ascorbate, glutathione and tocopherols as well as antioxidant enzymes such as peroxidase (POD), superoxide dismutase (SOD) and catalase (CAT) (Bandeoglu et al. 2004). It has also been reported that plants with high levels of antioxidants, whether constitutive or induced, have a greater resistance to oxidative damages induced by ROS (Alqurainy 2007).

In many crop plants natural accumulation of osmoprotectants and other antioxidant compounds is very low and this deficiency can be overcome by their exogenous application (Makela et al. 1998). Exogenous application of plant growth regulators, fertilizers, osmoprotectants and antioxidants have been reported to successfully alleviate the adverse effects of salt stress on plants (Shalata and Neumann 2001; Khadri et al. 2007). ASC is a major water-soluble antioxidant, protecting biologically important macromolecules from oxidative damage caused by hydroxyl radicals, superoxide, and singlet oxygen. In addition to its importance in photoprotection and the regulation of photosynthesis (Smirnoff 2000; Foyer and Noctor 2000), ASC plays an important role in the regulation of cell cycle and several fundamental processes of plant growth and development.

Soybean (*Glycine max* L. Merr.), one of the most popular legumes, is one of the oldest cultivated crops. It is also considered as a good source of vegetable protein and oil since it has the highest level of protein in comparison with the other leguminous plants. It is necessary to investigate certain abiotic factors such as drought and salinity that may limit the soybean yield (Shilpi and Narendra 2005).

The purpose of this study was to determine the influence of ASC pretreatment on soybean seeds subjected to saline stress during germination and growth criteria through change in antioxidant capacity such as superoxide dismutase, catalase and peroxidase activity.

Materials and methods

Plant materials and treatments

Glycine max L. cv. SAHAR and cv. DPX seeds were obtained from Azarbaijan Agricultural Research Center and Natural Resources of Tabriz. Selected seeds were sterilized with 70% ethanol for 2 min and 0.5% sodium hypochlorite

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Table 1. Germination (%), shoot and root length (cm) in two soybean cultivars grown for 10 days under 0, 12.5 and 50 mM NaCl salinity and ASC at 0 or 400 mg l^{-1}. Values are the mean ± SD (n=4). Data of each column within each cultivar indicated by the same letters are not significantly different (P<0.05).

Parameters			Treatments		Cultivar
Root lenght	Shoot lenght	Germination %	NaCl	ASC	
12.9 ± 4.7 ^{a}	10.2 ± 1.1 ^{a}	95 ± 3.8 ^{a}	0	0	
12.7 ± 2.9 ^{ab}	9.4 ± 0.7 ^{ab}	83 ± 3.8 ^{bc}	12.5	0	
4.4 ± 1.6 ^{c}	6.0 ± 1.8 ^{c}	75 ± 6.0 ^{c}	50	0	SAHAR
13.0 ± 5.4 ^{a}	10.1 ±1.2 ^{ab}	93 ± 3.8 ^{ab}	0	400	
15.0 ± 3.9 ^{a}	10.2 ± 1.7 ^{a}	90 ± 2.3 ^{ab}	12.5	400	
7.50 ± 2.0 ^{ab}	7.3 ± 0.6 ^{bc}	85 ± 6.8 ^{bc}	50	400	
14.4 ± 1.2 ^{a}	8.9 ± 0.6 ^{a}	81 ± 8.2 ^{a}	0	0	
12.3 ± 4.2 ^{a}	4.4 ± 0.9 ^{b}	75 ± 8.8 ^{a}	12.5	0	
12.9 ± 4.3 ^{a}	4.0 ± 1.0 ^{b}	72 ± 12.6 ^{a}	50	0	DPX
17.3 ± 5.6 ^{a}	8.0 ± 1.5 ^{a}	86 ± 9.5 ^{a}	0	400	
13.6 ± 2.3 ^{a}	7.8 ± 1.5 ^{a}	80 ± 11.7 ^{a}	12.5	400	
15.0 ± 1.9 ^{a}	8.1 ± 0.8 ^{a}	78 ± 10.9 ^{a}	50	400	

solution for 15 min. Seeds were soaked in distilled water or ASC solution (0, 400 mg l^{-1}) for 4 hrs before they were sown in distilled water or NaCl solutions (0, 12.5, 50 mM). The seeds were allowed to germinate at 25°C in darkness. Seeds were considered to germinate after a radicle emerged from the testa. After 10 days, growth parameters were recorded in controlled growth chamber.

Preparation of extracts for enzyme assays

For shoot crude extract preparation, 1 g tissue was homogenized in 3 ml of 0.1 M phosphate buffer (pH 7.0) at 4°C. The homogenate were then transferred into eppendorf tubes and centrifuged at 14000 g for 20 minutes at 4°C. Supernatant was used for antioxidant enzyme and protein content assays.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed as described by Winterbourn et al. (1976). The reaction mixture contained 0.05 ml of enzyme extract, 2.650 ml of 67 mM potassium phosphate buffer (pH 7.8), 0.2 ml of 100 mM EDTA/ 0.3 mM sodium cyanide (NaCN) and 0.1 ml of 1.5 mM nitroblue tetrazolium (NBT). One unit of enzyme activity was defined as the amount of SOD required to produce a 50% inhibition of reduction of NBT and the specific enzyme activity was expressed as units per milligram of total protein.

Peroxidase (POD, EC 1.11.1.7) was assayed as described by Ghamsari et al. (2007). Assays were carried out at room temperature (~ 20-25°C) using T-60 spectrophotometer (PG Instrument, UK). Reaction mixture contained 3 ml of 0.1 M citrate-phosphate-borate buffer system (pH 7.0), 25 µl of 480 mM guaiacol, 25 µl of 96 mM H_2O_2 and 30 µl of extract. The reaction was started by the addition of extract. Activity of POD was calculated as enzyme protein required for the formation of 1 µmol tetraguaiacol per min.

Catalase (CAT, EC 1.11.1.6) activity was measured according to the method given by Obinger et al. (1997). The assay mixture contained 3 ml of 50 mM citrate-phosphate-borate buffer (pH 7.0), 26 µl of 11.8 mM H_2O_2 and 50 µl of extract. The decomposition of H_2O_2 was followed by the decline in absorbance at 240 nm. One unit of CAT activity represents one µmol of H_2O_2 decomposed per min.

Soluble protein was estimated spectrophotometrically by the dye-binding method (Bradford 1976).

Statistical Analysis

All the values in the figures and tables are the mean of four independent determinations. Differences between control and treated seeds were analyzed by one-way ANOVA followed by Tukey's multiple range test ($P \leq 0.05$).

Results

Effect of salinity and ACS on growth parameters

50 mM NaCl delayed germination and decreased final germination percentage in SAHAR cultivar, but in DPX cultivar there were no significant differences as compared with the control (Table 1). Pretreatment with ASC led to a significant increase in the percentage of germination in DPX cultivar. Increasing NaCl levels decreased shoot length of both cultivar's seedlings. In SAHAR cultivar 50 mM NaCl decreased shoot length by 41% and for DPX by 54.6%. Root length was decreased significantly (66%) in SAHAR cultivar under 50 mM NaCl treatment whereas this parameter remained unchanged in DPX. Salt stress significantly decreased shoot dry weight in both cultivars ($P<0.05$). Root dry weigth increased only in ASC treated DPX cultivar (Table 2).

Effect of salinity and ASC on protein content and antioxidant enzymes

50 mM NaCl decreased protein content of both cultivars seedlings (SAHAR, 41% and DPX, 49%) (Table 2). Activity of

Table 2. Protein content (mg g^{-1} FW), shoot and root dry weight (mg $plant^{-1}$) in two soybean cultivars grown for 10 days under 0, 12.5 and 50 mM NaCl salinity and ASC at 0 or 400 mg l^{-1}. Values are the mean ± SD (n=4). Data of each column within each cultivar indicated by the same letters are not significantly different (P<0.05).

Parameters			Treatments		Cultivar
Root dry weight	Shoot dry weight	Protein content	NaCl	ASC	
18.7 ± 1.50 ^{a}	87.7 ± 6.23 ^{a}	23.52 ± 0.61 ^{b}	0	0	
22.7 ± 1.25 ^{a}	89.2 ± 6.65 ^{a}	24.82 ± 0.22 ^{a}	12.5	0	
15.5 ± 4.04 ^{a}	15.7 ± 5.37 ^{b}	12.62 ± 0.30 ^{d}	50	0	SAHAR
19.5 ± 3.69 ^{a}	119 ± 5.47 ^{a}	21.22 ± 0.34 ^{c}	0	400	
28.5 ± 9.60 ^{a}	107 ± 34.7 ^{a}	24.12 ± 0.71 ^{ab}	12.5	400	
20.2 ± 6.55 ^{a}	104 ± 18.9 ^{a}	23.12 ± 0.30 ^{b}	50	400	
23.5 ± 1.29 ^{abc}	142 ± 7.20 ^{a}	21.82 ± 0.26 ^{d}	0	0	
21.7 ± 2.62 ^{bc}	115 ± 20.4 ^{a}	23.82 ± 0.34 ^{b}	12.5	0	
17.5 ± 5.19 ^{c}	16.7 ± 3.09 ^{b}	6.92 ± 0.35 ^{e}	50	0	DPX
30.5 ± 7.50 ^{ab}	139 ± 24.1 ^{a}	27.82 ± 0.26 ^{a}	0	400	
33.2 ± 5.85 ^{a}	123 ± 32.2 ^{a}	22.82 ± 0.18 ^{c}	12.5	400	
33.5 ± 3.10 ^{a}	127 ± 38.3 ^{a}	21.92 ± 0.42 ^{d}	50	400	

SOD in both cultivars increased in salt stress. In SAHAR and DPX cultivars, 50 mM of NaCl caused significant increase in activity of SOD (P<0.05) compared to control (Fig. 1). ASC increased POD activity rather than NaCl treatment in SAHAR cultivar. However, increasing NaCl concentration with or without ASC caused a significant decrease in POD activity (P<0.05) in comparison with control group of DPX (Fig. 1). CAT activity was decreased in both cultivars under the salt stress. However, applied ASC caused an increase in CAT activity of salt stressed seedlings of SAHAR in comparison with salt stressed group, whereas it decreased in all different treatments of DPX.

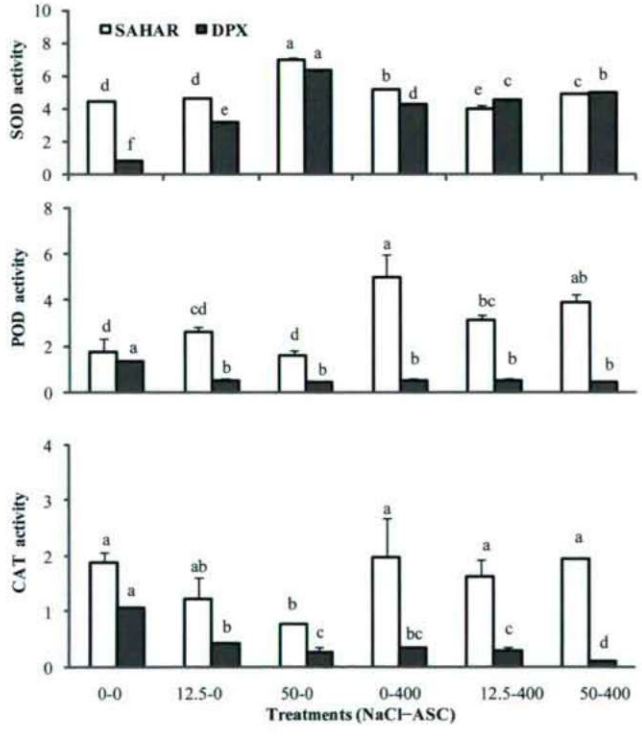

Figure 1. Specific activity of superoxide dismutase (SOD, U mg^{-1} protein min^{-1}), peroxidase (POD, nmol tetraguaiacol mg^{-1} protein min^{-1}) and catalase (CAT, µmol H_2O_2 mg^{-1} protein min^{-1}) in seedlings of two soybean cultivars grown for 10 days under 0, 12.5 and 50 mM NaCl salinity and ASC (ascorbate) at 0 or 400 mg l^{-1}. Data were compared within each cultivar. Values are the mean ± SD (n=4). Bars indicated by the same letters are not significantly different (P<0.05).

Discussion

Growth parameters and protein content

Plants under salt stress use a variety of strategies to neutralize the adverse effects of salt stress on their growth. In the present study growth was significantly decreased in salt-treated seedlings. Similar results on the reduction of soybean growth due to salt stress were reported by Amirjani (2010). Non-enzymatic antioxidants are more important due to their dominant role in plant growth and development in addition to their antioxidant capacity (Khan et al. 2006). Comparison of germination and root length under salinity showed that DPX is a rather more salt tolerant cv than SAHAR cv. There is no published work concerning the expression of salt tolerance in SAHAR and DPX cultivars. In this work, exogenously applied ASC, through pretreatment, caused enhancement in germination percentage and growth of salt stressed seedlings of soybean cultivars. There are several reports, which provide evidence that ASC accelerates cell division and cell enlargement as observed in different plants such as *Pisum* (De Cabo et al. 1996), and *Lupinus albus* (Citterio et al. 1994). These findings and the results of the present study suggest that the growth-promoting effect of ASC may have been due to enhanced antioxidant capacity under salt stress (Athar et al. 2008).

ROS are produced under salt stress that may cause protein denaturation and oxidation (Sajid and Aftab 2009). Soybean plants exposed to 100 and 200 mM NaCl show significant decrease in their protein content (Muthukumarasamy et al. 2000). A remarkable decrease in the protein content of salt stressed radish plants have been reported (Moussa 2004). Also, in this study soybean seedlings showed a significant decrease in protein content when stressed with 50 mM NaCl.

Antioxidant enzymes

It is now well-known that salt tolerance in most crop plants is associated with a more efficient antioxidant system including enzymes (SOD, APX, and CAT) and non-enzyme antioxidants (ASC, tocopherols, salicylic acid and carotenoids) (Athar et al. 2008). The results of this work suggest a protective role of ASC against salt-induced oxidative damage in soybean cultivars. Salinity caused an increase in SOD activity in both cultivars. Similar results of SOD activity reported in salt-tolerant cultivars of pea (Hernandez and Almansa 2002), sugar beet (Bor et al. 2003) and tomato (Koca et al. 2006) under salt stress. In this research the enzyme activity indicated that under saline condition, in both cultivars, only SOD activity increased, therefore we can say that in the seedling stage SOD play an important role in the free radical scavenging system.

The present study demonstrated that ASC may play an important role in salt stress by protecting soybean seedlings from salt-induced oxidative damage through the maintenance and/or increase of the activity of antioxidant enzymes. These findings led us to conclude that applied ASC counteracts the adverse effects of salt stress on growth of soybean; however, these effects were cultivar specific.

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ARTICLE

Effect of light intensity on photosynthesis and antioxidant defense in boron deficient tea plants

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ABSTRACT *Tea* (*Camellia sinensis* (L.) O. Kuntze) plants were grown at adequate (46 μM) or low (<2.5μM) boron (B) supply in the nutrient solution under low (LL, 50 μmol $m^{-2}s^{-1}$), intermediate (IL, 250 μmol $m^{-2}s^{-1}$) and high (HL, 500 μmol $m^{-2}s^{-1}$) light intensities. Boron deficiency hardly affected photochemical events under LL conditions, but alleviated reduction of efficiency of photosynthetic energy conversion in IL and HL plants. The optimum light intensity for CO_2 assimilation was IL for the young and HL for the old leaves. Activity of ascorbate peroxidase and superoxide dismutase and concentration of proline was lower under IL compared to LL and HL conditions. Compared to the old leaves, in the young leaves photochemical events were more protected under excess light and low B supply. Antioxidant defense system involved in the protection of leaves against excess light under IL conditions while thermal dissipation performed this role under HL conditions. Alleviation of high light stress effect on the photochemical events could be attributed to the B deficiency-induced activation of antioxidant defense system.
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KEY WORDS

Camellia sinensis
high light stress
leaf photochemistry
micronutrient deficiency
proline

Boron (B) is an essential micronutrient required for normal growth of higher plants. However, its function in plants has not yet been fully understood (Mengel and Kirkby 2002). Boron is involved in different processes such as vegetative growth, tissue differentiation, metabolic control through regulation of enzymatic reactions, membrane integrity and function, phenolic metabolism, sugar translocation, and nucleic acid synthesis.

It has been shown that B deficiency decreases plant photosynthetic capacity (Zhao and Oosterhuis 2002). Decreased photosynthetic capacity is the result of decreased Hill reaction activity and low intercellular CO_2 concentration (Sharma and Ramchandra 1990), reduced chlorophyll (Chl) content, photosynthetic electron transport rate, photophosphorylation (Plesničar et al. 1997) as well as structural damage (El-Shintinawy 1999). In turnip plants, the photosynthesis apparatus conserved its normal activities and thylakoid constituents were not damaged seriously in B-deprived leaves (Hajiboland and Farhanghi 2010). However, strongly reduced stomatal conductance accompanied by reduced leaf chlorophyll content caused a significant photoinhibition in the leaves of B-deficient turnip plants (Hajiboland and Farhanghi 2010).

Oxidative stress is a central factor in abiotic and biotic stress phenomena that occurs when there is a serious imbalance between the production of reactive oxygen species (ROS) and antioxidant defense capacity (Apel and Hirt 2004). Although B is not a constituent of plants antioxidative defense system, B deficiency like imbalance of other nutrients leads to oxidative stress in plants.

High light intensity is one of the important environmental stresses particularly for shade plants. Various mechanisms operate for protection of the photosynthetic apparatus against damage from the accumulation of excessive energy. Shade plants have low capacities not only for photosynthetic electron transport but also for photoprotective responses such as thermal energy dissipation. In some cases, light stress does not result from high light per se, but rather from an excess of absorbed light beyond that utilized in photosynthesis e.g. in response to factors cause stomatal closure (Demmig-Adams and Adams 1992). Apart from the effects on photosynthesis, phenolics metabolism (Mole and Waterman 1988) and the balance between production and scavenging of ROS (Demmig-Adams and Adams 1992) could be greatly influenced in plants exposed to high light intensity.

Nutritional status of plants has great influence on the tolerance of plants to environmental stresses (Marschner 1995). Because of a wide spectrum of the effect of B deficiency on physiological processes of plants, it is expected that responses of plants to stress factors such as high light intensity would be influenced greatly by their B nutritional status.

Tea (*Camellia sinensis* (L.) O. Kuntze) is cultivated in humid and subhumid of tropical, subtropical and temperate regions of the world. Tea is a shade plant and a canopy of moderate shade provided by shade trees is necessary for an optimum growth and productivity in tea gardens (Janendra

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et al. 2008). Boron deficiency is generally related to high rainfall areas and acid soil conditions common in soils of tea plantations (Shorrocks 1997). To our knowledge, there is hardly any information on the responses of tea plants neither to B deficiency nor to high light stress combined with B starvation.

The objective of this work was study of the effect of low B supply on growth and photosynthesis of tea plants grown under three different lighting conditions. Possible involvement of antioxidant defense system in the response of plants to interaction of low B supply with high light stress was also evaluated.

Materials and Methods

Plants culture and treatments

Seeds of tea (*Camellia sinensis (L.)* O. Kuntze) plants were collected from the garden of Tea Research Station in Fuman (Guilan Province, Iran). Hulled seeds were surface-sterilized with 1% active hypochlorite and germinated on perlite in dark and moistened by distilled water and saturated $CaSO_4$ every day. After the emergence of primary leaves, seedlings were transferred to the light. One month-old seedlings were transferred to the nutrient solution (Ghanati et al. 2005) pH 4.2 containing either low (–B) or adequate (46 µM) B supply (+B) and were grown under three photosynthetic photon flux density (PPFD) conditions including low light (LL, 50 µmol $m^{-2}s^{-1}$ PPFD), intermediate light (IL, 250 µmol $m^{-2}s^{-1}$ PPFD) and high light (HL, 500 µmol $m^{-2}s^{-1}$ PPFD) intensities. The incident PPFD was measured by a quantum sensor attached to the leaf chamber of the gas exchange unit. In order to minimize the B contamination of nutrient solution in –B treatment, 1g L^{-1} of washed B specific resin (Amberlite IRA 743, Fluka; Asad et al. 1997) packed in small textile bags were kept immersed in the nutrient solution throughout the plants cultivation. Nutrient solution and resin bags were replaced every one week. Plants were grown under controlled environmental conditions with a temperature regime of 25°/18°C day/night, 14/10 h light/dark period and a relative humidity of 70/80%.

Plant harvest and analysis

Six weeks after treatment (10 weeks after sowing), plants were harvested. After drying at 70°C for 2 days to determine dry weight, oven-dried samples were transferred to porcelain crucibles and dry-ashed with 10 mg $Ca(OH)_2$ at 550°C for 5h, resolved in 0.5 M HCl and made up to volume by double-distilled water. Boron was determined following the azomethine-H method as described by Lohse (1982). The second youngest, fully expanded leaf together with the third old leaf defined as young and old leaves respectively, were used for all analyses. Before harvest, Chl fluorescence and gas exchange parameters were determined in the attached leaves.

Determination of chlorophyll fluorescence and gas exchange parameters

Chlorophyll fluorescence parameters were recorded using a portable fluorometer (OSF1, ADC Bioscientific Ltd., UK) for both dark adapted and light adapted leaves. An average of 4 records from different parts of each individual leaf was considered for each replicates. Definitions and calculations were described elsewhere (Hajiboland and Amirazad 2010). CO_2 assimilation and transpiration rates were measured in parallel with Chl fluorescence measurements in the same leaf with a calibrated portable gas exchange system (LCA-4, ADC Bioscientific Ltd., UK) between 10:00 and 13:00 under the same PPFD as that of the treatments.

Determination of chlorophyll, carotenoids, anthocyanins and flavonoids

Leaf concentration of Chl a, b and carotenoids were determined according to Lichtenthaler and Wellburn (1985) after extraction of pigments in cold acetone and allowing the samples to stand for 24 h in the dark at 4°C. Anthocyanins and total flavonoid content of leaves were determined according to the methods described before (Hajiboland and Amirazad 2010).

Assay of antioxidant enzymes and related metabolites

Determination of peroxidase (POD, EC 1.11.1.7), ascorbate peroxidase (APX, EC 1.11.1.11) and superoxide dismutase (SOD, EC 1.15.1.1) activity, the amount of malondialdehyde (MDA), H_2O_2 and proline were undertaken according to optimized protocols described elsewhere (Hajiboland and Hasani 2007). Fresh samples were ground in the presence of liquid nitrogen and measurements were undertaken using spectrophotometer (Specord 200, Analytical Jena, Germany). Soluble proteins were determined as described by Bradford (1976) using a commercial reagent (Sigma) and BSA (Merck) as standard.

Experiments were undertaken in complete randomized block design with 4 replications. Statistical analyses were carried out using Sigma Stat (3.02) with Tukey test ($p<0.05$).

Results

Plants dry matter production was increased with increasing light intensity from LL to IL irrespective to the B supply level. Further increase of light intensity from IL to HL did not result in further growth improvement with the exception of root growth of low B plants. Under HL conditions, low B supply did not influence root DW negatively (Fig. 1).

Boron concentration increased with increasing light intensity from LL to IL in leaves but not in roots. In plants grown under HL conditions, B concentration was slightly or significantly lower than IL plants. The same trend was

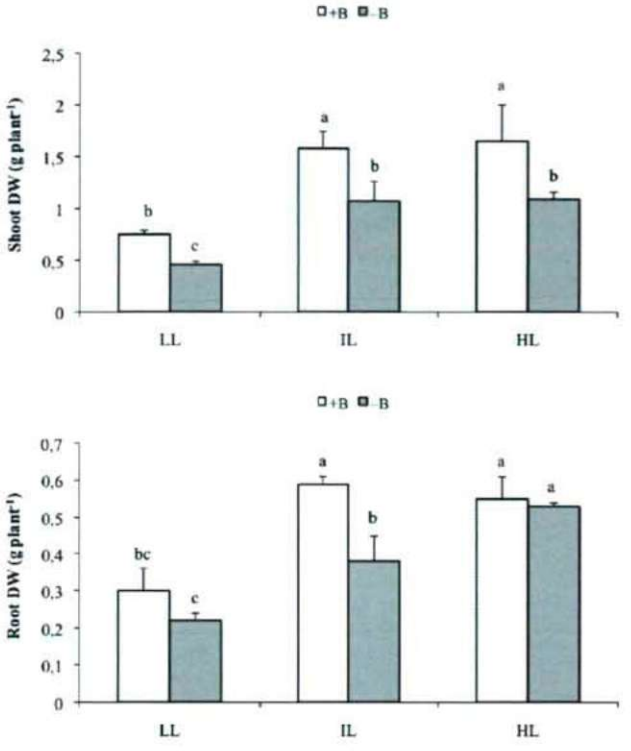

Figure 1. Dry weight (mg plant^{-1}) of shoot and root in tea (*Camellia sinensis* L.) plants grown for six weeks at adequate (+B) or low (–B) boron supply under low (LL, 50 µmol m^{-2}s^{-1}), intermediate (IL, 250 µmol m^{-2}s^{-1}) or high (HL, 500 µmol m^{-2}s^{-1}) light intensity. Bars indicated by the same letter are not significantly different (P<0.05).

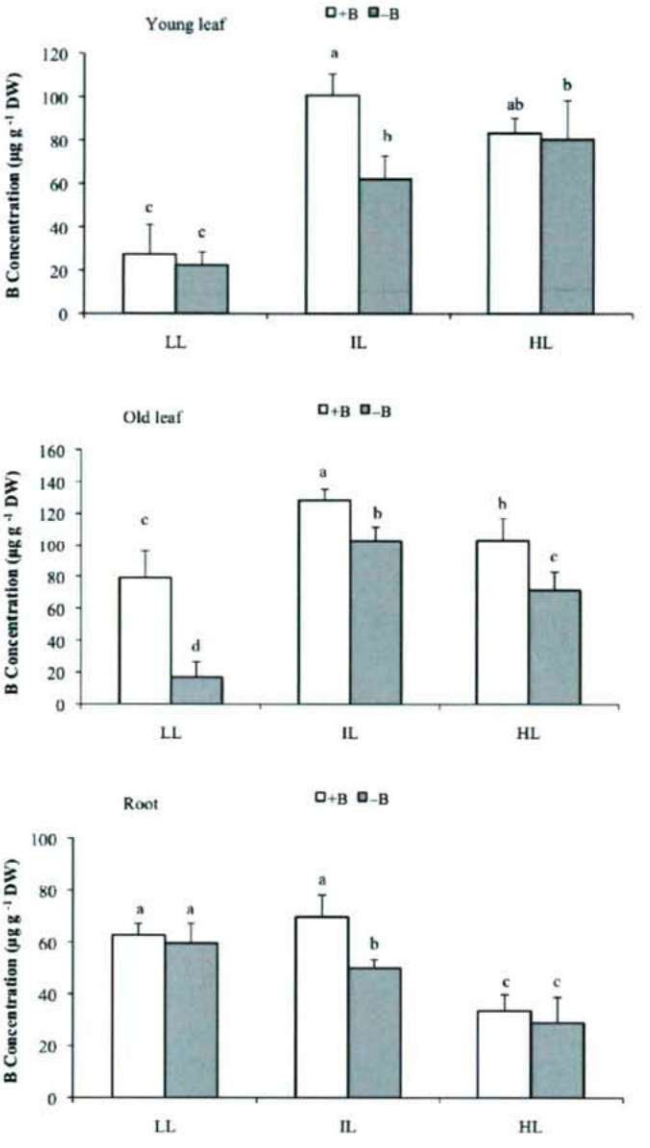

Figure 2. Boron concentration (µg g^{-1} DW) of three different fractions in tea (*Camellia sinensis* L.) plants grown for six weeks at adequate (+B) or low (–B) boron supply under low (LL, 50 µmol m^{-2}s^{-1}), intermediate (IL, 250 µmol m^{-2}s^{-1}) or high (HL, 500 µmol m^{-2}s^{-1}) light intensity. Bars indicated by the same letter are not significantly different (P<0.05).

observed for B content (µg fraction^{-1}) because of parallel changes in the dry weight and B content of fractions under all applied treatments (data not shown).

Under IL and HL compared with LL conditions, concentration of both Chl a and b decreased irrespective to the B nutritional status in both young and old leaves. In LL plants, Chl a and b concentration increased due to low B supply in the young leaf. In the old leaf, however, significant effect of B supply level was observed under IL conditions. In IL and to a lesser extent in HL plants, Chl a content of the old leaf decreased while that of Chl b increased resulting in a reduction of Chla/b ratio from 2.69 to 0.94 in IL and from 3.0 to 2.1 in HL plants due to B deficiency. Leaf carotenoids decreased with increasing light intensity from LL to IL slightly or significant irrespective to the B nutrition of plants. Rising light intensity to HL caused a slight increase of leaf carotenoids in the young and a significant reduction in the old leaf. Boron deficiency caused a significant increase of carotenoids in the young leaf under LL and in the old leaf under IL conditions. Increasing light intensity from LL to IL caused increase in anthocyanins in B-sufficient but not in B-deficient leaves. Under HL conditions, anthocyanins content decreased again. Anthocyanins content of both young and old leaves increased under LL conditions due to low B supply. In general, light intensity and B supply level did not affect flavonoids content of leaves. The exception was significantly lower flavonoids content in the young leaf of B-sufficient HL plants compared with B-deficient ones (Table 1).

In the young leaf, the maximal photochemical efficiency of PSII (F_v/F_m) decreased with increasing light intensity in B-sufficient but not in B-deficient plants. In the old leaf, reduction of F_v/F_m was observed following increased light intensity in both B-sufficient and B-deficient plants. Excita-

Table 1. Leaf concentration of chlorophyll (Chl) a, b and carotenoids (mg g^{-1} FW), anthocyanins (mg cyanidin-3-glucosid g^{-1} FW) and flavonoids (mg g^{-1} FW) in young and old leaves of tea (*Camellia sinensis* L.) plants grown for six weeks at adequate (+B) or low (–B) boron supply under low (LL, 50 µmol $m^{-2}s^{-1}$), intermediate (IL, 250 µmol $m^{-2}s^{-1}$) or high (HL, 500 µmol $m^{-2}s^{-1}$) light intensity. Data in each column within each organ followed by the same letter are not significantly different (P<0.05).

Young leaf	Treatments	Chl a	Chl b	Carotenoids	Anthocyanins	Flavonoids
	+B	2.1±0.4^{b}	0.9±0.0^{b}	191±39^{b}	7.4±1.7^{b}	0.66±0.07^{a}
LL	–B	3.0±0.5^{a}	1.4±0.2^{a}	267±44^{a}	23.1±4.0^{a}	0.65±0.09^{a}
	+B	1.1±0.1^{c}	0.4±0.1^{d}	86±10^{c}	26.0±2.3^{a}	0.55±0.07^{a}
IL	–B	1.2±0.3^{c}	0.5±0.1^{cd}	100±18^{c}	21.6±3.2^{a}	0.65±0.07^{a}
HL	+B	1.8±0.3^{bc}	0.7±0.1^{bc}	134±24^{bc}	4.8±2.3^{b}	0.35±0.05^{b}
	–B	1.1±0.5^{c}	0.6±0.1^{c}	130±26^{bc}	2.8±1.3^{b}	0.52±0.04^{a}
Old leaf	Treatments	Chl a	Chl b	Carotenoids	Anthocyanins	Flavonoids
	+B	4.5±0.4^{a}	1.5±0.2^{ab}	320±44^{ab}	5.2±1.6^{b}	0.89±0.19^{a}
LL	–B	5.1±0.5^{a}	1.8±0.1^{a}	371±32^{a}	27.2±5.1^{a}	1.07±0.23^{a}
	+B	3.5±0.3^{b}	1.3±0.1^{b}	254±19^{b}	29.6±6.2^{a}	0.83±0.13^{a}
IL	–B	1.6±0.5^{c}	1.7±0.3^{a}	331±48^{a}	20.9±6.3^{a}	0.89±0.10^{a}
HL	+B	2.1±0.2^{c}	0.7±0.1^{c}	149±16^{c}	10.9±3.6^{b}	0.76±0.04^{a}
	–B	1.7±0.3^{c}	0.8±0.1^{c}	162±8^{c}	22.3±2.3^{a}	0.78±0.05^{a}

Table 2. Chlorophyll fluorescence parameters including F_v/F_m (maximal photochemical efficiency of PSII), F'_v/F'_m (excitation capture efficiency of open PSII), q_P (photochemical quenching), q_N (non-photochemical quenching) and Φ_{PSII} (quantum yield of PSII) in young and old leaves of tea (*Camellia sinensis* L.) plants grown for six weeks at adequate (+B) or low (–B) boron supply under low (LL, 50 µmol $m^{-2}s^{-1}$), intermediate (IL, 250 µmol $m^{-2}s^{-1}$) or high (HL, 500 µmol $m^{-2}s^{-1}$) light intensity. Data in each column within each organ followed by the same letter are not significantly different (P<0.05).

Young leaf	Treatments	F_v/F_m	F'_v/F'_m	q_P	q_N	Φ_{PSII}
	+B	0.79±0.01^{a}	0.72±0.03^{b}	0.97±0.03^{a}	0.35±0.02^{ab}	0.73±0.01^{a}
LL	–B	0.79±0.01^{a}	0.70±0.01^{bc}	1.00±0.04^{a}	0.45±0.04^{a}	0.73±0.01^{a}
	+B	0.74±0.04^{b}	0.73±0.03^{b}	0.92±0.03^{b}	0.14±0.03^{c}	0.70±0.03^{a}
IL	–B	0.79±0.01^{a}	0.77±0.02^{a}	0.89±0.02^{b}	0.13±0.04^{c}	0.71±0.01^{a}
HL	+B	0.74±0.02^{b}	0.67±0.03^{c}	0.89±0.04^{b}	0.38±0.09^{ab}	0.62±0.02^{c}
	–B	0.77±0.03^{a}	0.72±0.02^{b}	0.88±0.01^{b}	0.29±0.07^{b}	0.66±0.02^{b}
Old leaf	Treatments	F_v/F_m	F'_v/F'_m	q_P	q_N	Φ_{PSII}
	+B	0.80±0.00^{a}	0.80±0.01^{a}	0.97±0.04^{ab}	0.09±0.01^{d}	0.79±0.06^{a}
LL	–B	0.78±0.04^{a}	0.78±0.02^{ab}	0.96±0.02^{ab}	0.08±0.02^{d}	0.76±0.01^{a}
	+B	0.76±0.04^{ab}	0.72±0.04^{b}	0.98±0.03^{ab}	0.09±0.01^{d}	0.79±0.06^{a}
IL	–B	0.75±0.02^{ab}	0.73±0.02^{b}	1.00±0.03^{a}	0.19±0.05^{c}	0.73±0.01^{a}
HL	+B	0.61±0.03^{c}	0.39±0.04^{c}	0.85±0.02^{c}	0.73±0.08^{a}	0.34±0.05^{c}
	–B	0.72±0.03^{b}	0.58±0.02^{d}	0.93±0.03^{b}	0.59±0.07^{b}	0.55±0.02^{b}

tion capture efficiency of open PSII (F'_v/F'_m) decreased by higher light intensity slightly or significant in both young and old leaves irrespective to the B nutritional status. Photochemical quenching (q_P) in the young leaf decreased by higher light intensity. Boron supply level did not affect q_P with the exception of the old leaf in HL plants. The lowest amount of non-photochemical quenching (q_N) was detected under IL conditions. Low B supply reduced this parameter only under HL conditions which was slightly in the young and significant in the old leaf. Quantum yield of PSII (Φ_{PSII}) did not respond to either low B supply or light intensity from LL to IL. However, following exposure of plants to HL conditions, reduction of Φ_{PSII} in both young and old leaves was observed. Φ_{PSII} was higher in B-deficient than B-sufficient HL plants. Reduction of F_v/F_m, F'_v/F'_m, q_P and Φ_{PSII} in HL compared to LL plants were 24%, 51%, 12% and 57% in the old leaf respectively. The corresponding values for the young leaf were considerably lower, 6%, 7%, 8% and 15% respectively (Table 2).

In the young leaf, increasing light intensity from LL to IL increased CO_2 net assimilation rate (A), however, A was not further changed significantly with further increase of light to HL intensity. In the old leaf, in contrast, HL plants have significantly higher A than the IL ones. Boron-deficient leaves had significantly or slightly lower A irrespective to the lighting conditions. In the young leaf, a continues reduction of transpiration rate (E) was observed with increasing light intensity and under B deficiency conditions. In contrast,

Table 3. Gas exchange parameters including net photosynthetic rate (*A*, μmol m^{-2} s^{-1}), transpiration rate (*E*, mmol m^{-2} s^{-1}) and stomatal conductance to water vapor (g_s, (mol m^{-2} s^{-1}) in young and old leaves of tea (*Camellia sinensis* L.) plants grown for six weeks at adequate (+B) or low (–B) boron supply under low (LL, 50 μmol $m^{-2}s^{-1}$), intermediate (IL, 250 μmol $m^{-2}s^{-1}$) or high (HL, 500 μmol $m^{-2}s^{-1}$) light intensity. Data in each column within each organ followed by the same letter are not significantly different (P<0.05).

Young leaf	Treatments	*A*	E	g_s
LL	+B	1.88±0.24^{bc}	2.96±0.27^{a}	0.01±0.00^{b}
	–B	1.73±0.11^{c}	0.70±0.14^{d}	0.01±0.00^{b}
IL	+B	3.31±0.27^{a}	1.94±0.19^{b}	0.54±0.20^{a}
	–B	2.36±0.20^{b}	0.53±0.24^{d}	0.04±0.02^{b}
HL	+B	3.77±0.16^{a}	1.42±0.31^{c}	0.37±0.14^{a}
	–B	1.92±0.26^{bc}	0.38±0.19^{d}	0.06±0.01^{b}
Old leaf	Treatments	*A*	E	g_s
LL	+B	1.63±0.21^{b}	0.53±0.15^{bc}	0.01±0.00^{c}
	–B	0.67±0.08^{d}	0.30±0.09^{c}	0.01±0.00^{c}
IL	+B	1.32±0.06^{bc}	0.53±0.06^{bc}	0.01±0.00^{c}
	–B	1.10±0.05^{c}	0.36±0.11^{bc}	0.02±0.00^{c}
HL	+B	2.48±0.28^{a}	1.39±0.11^{a}	0.28±0.02^{b}
	–B	1.48±0.09^{b}	0.57±0.13^{b}	0.32±0.02^{a}

Table 4. Specific activity of peroxidase (POD, μmol mg^{-1} protein min^{-1}), ascorbate peroxidase (APX, μmol mg^{-1} protein min^{-1}) and superoxide dismutase (SOD, U mg^{-1} protein) in young and old leaves and roots of tea (*Camellia sinensis* L.) plants grown for six weeks at adequate (+B) or low (–B) boron supply under low (LL, 50 μmol $m^{-2}s^{-1}$), intermediate (IL, 250 μmol $m^{-2}s^{-1}$) or high (HL, 500 μmol $m^{-2}s^{-1}$) light intensity. Data in each column within each organ followed by the same letter are not significantly different (P<0.05).

Young leaf	Treatments	POD	APX	SOD
LL	+B	32±9^{a}	124±7^{bc}	3.4±0.6^{b}
	–B	35±3^{a}	171±37^{a}	6.4±0.4^{a}
IL	+B	8±2^{c}	83±19^{cd}	1.5±0.4^{c}
	–B	17±3^{b}	44±12^{d}	3.2±0.4^{b}
HL	+B	18±6^{bc}	105±2^{c}	2.8±0.3^{b}
	–B	5±3^{d}	164±9^{ab}	3.4±0.7^{b}
Old leaf	Treatments	POD	APX	SOD
LL	+B	18±3^{a}	128±19^{b}	6.5±0.2^{a}
	–B	23±2^{a}	169±10^{a}	1.3±0.7^{e}
IL	+B	17±9^{a}	88±21^{cd}	1.4±0.0^{de}
	–B	23±9^{a}	127±9^{b}	5.3±0.8^{b}
HL	+B	19±2^{a}	116±20^{bc}	3.4±0.3^{c}
	–B	19±4^{a}	78±16^{d}	2.5±0.6^{cd}
Root	Treatments	POD	APX	SOD
LL	+B	186±34^{ab}	123±11^{cd}	5.0±1.2^{b}
	–B	214±18^{a}	197±15^{b}	9.0±1.9^{a}
IL	+B	62±21^{d}	70±4^{d}	0.8±0.4^{d}
	–B	175±12^{ab}	87±11^{d}	2.7±0.4^{bc}
HL	+B	151±44^{bc}	299±82^{a}	3.7±0.7^{bc}
	–B	109±23^{cd}	250±28^{ab}	1.4±0.3^{cd}

higher light intensity (500 μmol $m^{-2}s^{-1}$) resulted in greater E in the old leaf and B deficiency effect was significant only in HL plants. Somatal conductance (g_s) in the young leaf increased with increasing light intensity from LL to IL, but was not influenced further at HL conditions. In the old leaf, however, a continuous increase in g_s was observed with increasing light intensity up to HL intensity. Boron deficiency resulted in stomatal closure that was significant in the young leaf under IL and HL conditions and in the old leaf only under HL conditions (Table 3).

Activity of all three studied antioxidant enzymes decreased with increasing light intensity from LL to IL slightly or significant irrespective to the B supply level. Only activity of POD in the old leaf was not affected either by light conditions or B nutritional status. With further rise of light intensity (at HL) a slight or significant increase in the activity of en-

Table 5. Concentration of malondialdehyde (MDA, nmol g^{-1} FW), hydrogen peroxide (H_2O_2, nmol g^{-1} FW), proline (nmol g^{-1} FW) and protein (mg g^{-1} FW) in young and old leaves and roots of tea (*Camellia sinensis* L.) plants grown for six weeks under adequate (+B) or low (–B) boron supply under low (LL, 100 µmol $m^{-2}s^{-1}$), intermediate (IL, µmol $m^{-2}s^{-1}$) or high (HL, µmol $m^{-2}s^{-1}$) light intensity. Data in each column within each organ followed by the same letter are not significantly different ($P<0.05$).

Young leaf	Treatments	MDA	H_2O_2	Proline	Protein
	+B	43±5 ^{cd}	3.4±0.4 ^{b}	213±73 ^{a}	1.4±0.1 ^{ab}
LL	–B	46±6 ^{bc}	4.6±0.4 ^{ab}	73±18 ^{b}	1.4±0.1 ^{ab}
	+B	29±2 ^{d}	6.0±1.2 ^{a}	54±7 ^{b}	1.6±0.2 ^{a}
IL	–B	55±1 ^{b}	3.3±0.5 ^{b}	87±9 ^{b}	1.6±0.2 ^{a}
HL	+B	56±9 ^{b}	5.1±1.4 ^{ab}	78±17 ^{b}	1.2±0.1 ^{ab}
	–B	76±7 ^{a}	5.0±0.4 ^{ab}	183±18 ^{a}	1.0±0.3 ^{b}
Old leaf	Treatments	MDA	H_2O_2	Proline	Protein
	+B	19±4 ^{c}	6.8±0.4 ^{a}	374±59 ^{a}	1.4±0.2 ^{a}
LL	–B	48±4 ^{b}	5.5±0.7 ^{b}	206±47 ^{b}	1.2±0.1 ^{a}
	+B	46±5 ^{b}	3.8±0.6 ^{cd}	65±8 ^{d}	1.4±0.2 ^{a}
IL	–B	60±4 ^{b}	3.5±0.2 ^{cd}	114±9 ^{cd}	0.7±0.1 ^{b}
HL	+B	99±5 ^{a}	4.6±0.9 ^{bc}	108±36 ^{cd}	1.4±0.1 ^{a}
	–B	111±18 ^{a}	2.6±0.2 ^{d}	166±18 ^{bc}	1.5±0.2 ^{a}
Root	Treatments	MDA	H_2O_2	Proline	Protein
	+B	19±5 ^{d}	0.4±0.1 ^{c}	151±32 ^{a}	1.0±0.1 ^{a}
LL	–B	322±6 ^{a}	0.5±0.1 ^{c}	88±14 ^{bc}	0.7±0.2 ^{ab}
	+B	56±3 ^{b}	1.2±0.2 ^{b}	60±8 ^{bc}	0.8±0.0 ^{a}
IL	–B	34±1 ^{c}	0.6±0.2 ^{c}	95±19 ^{b}	0.7±0.2 ^{ab}
HL	+B	40±3 ^{c}	1.6±0.0 ^{a}	51±6 ^{c}	0.4±0.1 ^{b}
	–B	35±7 ^{c}	1.1±0.1 ^{b}	72±4 ^{bc}	0.8±0.2 ^{a}

zymes was observed. Boron deficiency, in general, increased activity of antioxidant enzymes with the exception of POD activity in the young leaf of HL plants (Table 4).

Malondialdehyde (MDA) content in the young leaf decreased with increasing light intensity from LL to IL and increased again with exposure of plants to HL conditions. In the old leaf, however, a continues increase was observed with increasing light intensity. Boron deficiency caused mainly significant or slight increase of MDA content. Concentration of H_2O_2 increased continuously with increasing light intensity in the young leaf and roots, while decreased in the old leaf. Boron deficiency either caused no significant effect on H_2O_2 content or reduced it in all three examined fractions. Exposure of plants to higher light intensity declined proline content of leaves and roots. Boron deficiency caused a significant reduction of proline content in LL plants. In IL and HL plants, in contrast, B deficiency resulted in significant or slight increase of proline in all three tested fractions (Table 5).

Discussion

Considering growth responses of plants to increasing light intensity, it could be suggested that 250 µmol m^{-2} s^{-1} is an optimum light intensity for growth of tea plants. Light intensity of 500 µmol m^{-2} s^{-1} did not lead to greater dry matter production even though was not high enough to act as a stress factor if judged on the basis of dry weight data.

Boron concentration of plants increased with increasing light intensity from LL to IL conditions that could be attributed to increase in transpiration following rise of stomatal conductance. Leaf B concentration seems to be highly related to the overall rate of transpiratory water loss (Marschner 1995). However, slight or significant reduction of leaf B concentration with further increase of light intensity e.g. in HL plants, could be explained by parallel reduction of stomatal conductance only in the young leaf. The cause for reduction of B concentration in the old leaf under HL conditions though increase of stomatal opening is not known and may be the result of re-translocation of B to the young leaves. We observed that mature leaves of tea plants are capable to re-translocate B into young leaves particularly under B deficiency conditions (Hajiboland, et al. unpublished data).

A continues reduction of Chl content with increasing light intensity being more pronounced in the old compared with the young leaf, could be attributed to Chl destruction and degeneration of plastid membranes. On the other hand, under HL conditions concentration of anthocyanins in the young leaf was considerably lower than IL or LL conditions irrespective to the B nutritional status. Young leaves of tea plants similar with many other species are transiently red because of the accumulation of anthocyanins. Two potential functions have been proposed for the foliar anthocyanins including sunscreen photoprotective function against excess visible light and making them less discernible to insect herbivores (Karageorgou and Manetas 2006). The anthocyanin accumulation is thought to mask Chl and/or act as a filter for preventing high light absorption by leaves and thus minimize

photoinhibition (Farrant 2000). Destruction of Chl and anthocyanins under increasing light intensity is obviously one of important reasons for and/or results of susceptibility of tea plants to higher light intensity.

Boron deficiency did not reduce Chl content of the young leaf, conversely, a significant increase was observed under LL conditions. It could be attributed to reduced growth and expansion of the young leaf, therefore, concentration effect. Similarly, increased carotenoids and anthocyanins concentration of both young and old leaves observed particularly in IL plants was likely a concentration effect due to growth impairment of low B leaves. However, for anthocyanins higher synthesis is also an explanation because of the well known accumulation of phenolics in B-deficient plants (Marschner 1995). In contrast, reduction of Chl a and Chl a/b ratio of the old leaf due to B deficiency observed particularly under IL conditions was likely the result of decreased synthesis and/or damage to the plastid membranes.

Significant reduction of maximal photochemical efficiency of PSII (F_v/F_m), excitation capture efficiency of open PSII (F'_v/F'_m), photochemical quenching (q_p) and quantum yield of PSII (Φ_{PSII}) with increasing light intensity indicated the occurrence of photoinhibition and/or a serious damage to PSII. It was suggested that, prior to the occurrence of any damaging processes, photoinhibition takes place which can result from an increase in thermal energy dissipation as a photoprotective process and associated with increase in the pool size of xanthophyll cycle pigments (Demmig-Adams and Adams 1992). Non-photochemical fluorescence quenching (q_N) is a mechanism to prevent or alleviate damage to the photosynthetic apparatus (Müller et al. 2001). In IL plants of our experiment, reduction in the efficiency of photosynthetic energy conversion compared to LL plants was associated with reduction of leaf carotenoids and q_N particularly in the young leaves leading likely to PSII damage. With increasing light intensity, leaf carotenoids content and q_N values increased again caused likely protection of leaves from serious damages. In this case, decrease in PSII efficiency resulted mainly from photoprotective increase in thermal energy dissipation induced by excess of adsorbed light (Demmig-Adams and Adams 1992). It implied that, photosynthetic apparatus in both young and old leaves are more protected against excess energy under high compared with intermediate light intensities.

With a lowered capacity for photosynthetic CO_2 assimilation in B-deficient leaves as the consequence of stomatal closure, the requirement for reducing power and photophosphorylation will be lowered. Accordingly, it was expected the actual PSII efficiency to be down-regulated and surplus excitation energy to be dissipated. However, B-deficient plants had lower qN compared to B-sufficient plants apparently because of improvement in electron transport events without need to thermal energy dissipation. Accordingly, B deficiency alleviated inhibitory effect of excess light on the efficiency of photosynthetic energy conversion in both young and old leaves. One possible mechanism is likely an increased activity of antioxidant enzymes and accumulation of antioxidants such as proline (see below). There are two ways for photoprotection of leaves against excess light, namely removal of excess excitation energy directly within the light-capturing system *i.e.* thermal dissipation, and removal of active oxygen formed in the photochemical apparatus by various components of antioxidant defense system (Demmig-Adams and Adams 1992). It seems likely that, protection of photosynthetic apparatus in tea plants under intermediate light intensity is mainly achieved by antioxidant defense system while thermal dissipation mechanisms act profoundly under higher light intensity.

Our results demonstrated that, the inhibitory effect of increasing light intensity on the leaf photosynthetic pigments and photochemical events was much more expressed in the old leaves. Old leaves were also more susceptible against B deficiency-induced damages to photochemistry and Chl a/b ratio compared to the young leaves.

Increasing light intensity led to increased stomatal conductance, transpiration and CO_2 assimilation rate. For the young leaves, IL conditions seemed to be optimum for stomatal conductance, while in the old leaves HL conditions resulted in more opened stomata compared with IL conditions and greater transpiration and assimilation rate. Net assimilation rate per leaf surface area was depressed by low B supply mainly due to stomatal limitation. Impaired stomatal conductance under B deficiency conditions was reported for other plant species (Han et al. 2008; Hajiboland and Farhanghi 2010). Role of B in stomatal opening has not been investigated. Boron is required for membrane integrity (Cakmak and Römheld 1997), function, activity and expression of H^+-ATPase gene (Camacho-Cristóbal and González-Fontes 2007). Therefore, it is plausible that B deficiency causes reduction of K^+ uptake into guard cells and following loss of membrane integrity, stimulates passive leakage of K^+ from guard cells.

In general, activity of antioxidant enzymes was greater in LL than IL conditions. It was likely the cause for reduction of MDA and H_2O_2 in IL compared with LL plants and resulted in the protection of leaf photochemistry though lower efficiency of xanthophylls cycle-related thermal dissipation. With increasing light from 250 to 500 μmol m^{-2} s^{-1}, activity of enzymes increased again, obviously because of production of more active oxygen radicals. Nevertheless, this activation did not lead to reduction of antioxidants and could not provide enough protection against free radicals and cellular damage was occurred apparently. This could be explained well with reduction of photochemical parameters and damage to reaction centers as judged particularly by reduction of F_v/F_m.

Boron deficiency caused mainly activation of antioxi-

dant enzymes that in some cases were effective in reduction of lipid peroxidation and H_2O_2 content. Proline was one of important components in response of tea plants to both light intensity and B deficiency. It was decreased under IL conditions compared with LL, and increased again with increasing light intensity. Indeed, it reflected the optimum light conditions for plants and was correlated well with plants growth response to light intensity. Boron deficiency increased proline concentration only under IL and HL conditions. Proline accumulation often occurs in plants under variety of stress conditions, but a consensus has not emerged on its role in tolerance to stresses. Some researchers assume that proline accumulation is a symptom of injury which does not confer protection against stresses (Lutts et al. 1996). On the contrary, a relationship between lipid peroxidation and proline accumulation was reported in plants subjected to diverse kinds of stress (Molinari et al. 2007). Proline acts as a free radical scavenger to protect plants away from damage by oxidative stress (Alia and Matysik 2001; Wang et al. 2009). However, excess light responsiveness of proline biosynthesis had not been studied so far. Proline accumulation in plants during stresses such as salinity is controlled by coordinate induction of biosynthesis and inhibition of degradation pathways (Ábrahám et al. 2003). Light dependence of proline synthesis was reported by some authors (Hanson and Tully 1979; Ábrahám et al. 2003). However, effect of high light stress as a factor for increasing proline synthesis has not been studied. Excess light likely exert its effect mainly via blue light absorbing pigments (Briggs and Christie 2002). Effect of excess light on the proline biosynthesis and its role in protection of chloroplast should be investigated further.

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ARTICLE

Generation of reactive oxygen and nitrogen species in pea cultivars under copper exposure

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ABSTRACT Copper is an essential microelement in plants, but its exposure can induce toxicity symptoms such as growth inhibition, chlorosis or necrosis. The aim of this study was to investigate the physiological responses of two pea cultivars (*Pisum sativum* L. cv. Rajnai törpe and cv. Lincoln) to long term copper exposure. Seven-day-old pea plants were treated with 25 or 50 μM $CuSO_4$, in nutrient solution for 14 days. We studied the growth parameters, the metal uptake, the levels of different reactive oxygen species (hydrogen peroxide, H_2O_2 and superoxide radical, $O_2^{\cdot-}$) and reactive nitrogen species (nitric oxide, $NO^{\cdot}$ and peroxynitrite, $ONOO^-$) together with lipid peroxidation and cell death in the meristem cells of pea roots using *in vivo* and *in situ* microscopic methods. Long-term copper exposure resulted in a serious decrease in shoot and root growth of both pea cultivars and the root system proved to be more sensitive to the stressful condition. The reason of higher sensitivity of the root system is that the largest proportion of copper accumulated in it, namely, pea plants exclude the toxic metals from their shoot. Copper treatment induced the elimination of $O_2^{\cdot-}$ and the concurrent H_2O_2 generation in root tips of both cultivars. The level of NO significantly decreased as the effect of Cu^{2+} exposure, while the level of $ONOO^-$ ($+OH^{\cdot}$) enhanced, suggesting the occurrence of the reaction between $O_2^{\cdot-}$ and NO yielding peroxynitrite. As the effect of copper, lipid peroxidation and cell death were detected in the root tips which led to growth inhibition and biomass decrease of pea plants.
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KEY WORDS

long-term copper exposure
reactive nitrogen species
reactive oxygen species
pea cultivar

Copper (Cu^{2+}), an essential microelement is considered to be a major heavy metal for plants which is toxic at high concentrations. It can accumulate in various plant organs, directly causing a decrease in photosynthetic activity, carbohydrate content enhancement, damages of lipids, proteins, DNA or cell death (Shao et al. 2010). In the presence of toxic copper concentrations (3-100 μM) plants show reduced biomass (decrease of the root and shoot volume, stem and leaf size), chlorotic and/or necrotic symptoms and inhibition of shoot and root growth. Chlorophyll content decrease and alterations of chloroplast structure have been found in leaves of spinach, rice, wheat, bean and oregano under copper exposure (refs. in Yruela 2009). In general, legume crops (including pea) are less tolerant to copper compared to cereals and grasses. According to the results published by Palma et al. (1987) antioxidant enzyme activities in cv. Lincoln were higher in response to serious copper exposure than in cv. Granada, which suggests the more intense resistance of cv. Lincoln to copper stress.

Moreover, Cu^{2+} and other transition metals induce the formation of reactive oxygen species (ROS) leading to oxidative stress condition. Hydroxyl radical ($OH^{\cdot}$), having the highest reactivity is able to react directly with biological membranes causing lipid peroxidation. Superoxide radical ($O_2^{\cdot-}$) has a short half-life, it does not penetrate membranes, but it reduces transition metal complexes of Fe^{3+} and Cu^{2+}, thus affecting the activity of metal containing enzymes. Hydrogen peroxide acts as a signal molecule and it may inactivate enzymes by oxidizing their thiol groups (Vranová et al. 2002).

Reactive nitrogen species (RNS) such as nitric oxide radical ($NO^{\cdot}$) and peroxynitrite ($ONOO^-$), can be also produced under different stress conditions. Excessive amount of RNS triggers nitrosative stress, which can damage DNA, lipids, proteins and carbohydrates leading to impaired cellular functions (Corpas et al. 2011). Similarly to ROS, reactive nitrogen species can act as signal molecules inducing defence gene expression. Nitric oxide production was detected in *Brassica juncea* L. Czern. and *Pisum sativum* L. roots under Cu or Cd stress or in Cu-treated *Panax ginseng* roots (Bartha et al. 2005; Tewari et al. 2008; Lehotai et al. 2011). However, NO levels were significantly reduced by Cd in pea leaves and roots after long periods of metal treatments (Barroso et al. 2006; Rodríguez-Serrano et al. 2006, 2009). Exogenous application of NO donor induces enzymatic and non-enzymatic antioxidants, regulates root cell wall decomposition, reduces heavy metal uptake and regulates tolerance-related gene expression under heavy metal stress (Xiong et al. 2010). Nitric

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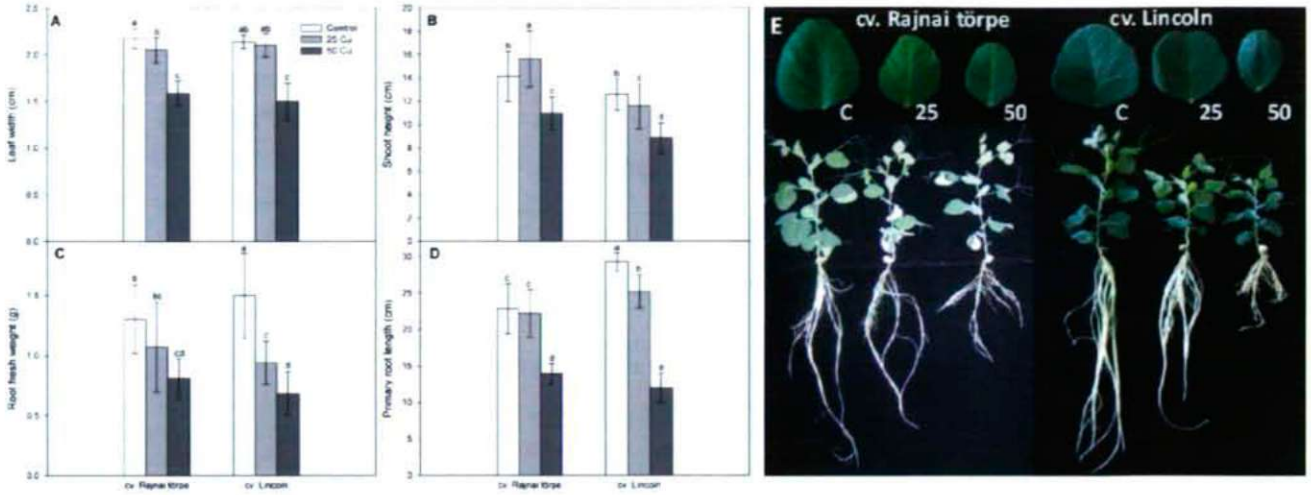

Figure 1. Leaf width (A), shoot height (B), root fresh weight (C) and primary root length (D) of *Pisum sativum* L. cv. Rajnai törpe and *Pisum sativum* L. cv. Lincoln treated with 0, 25 or 50 μM $CuSO_4$ for 14 days. Values are means of 10 plants ±SE. Different letters indicate significant differences ($P<0.05$) according to Duncan's test. Representative images illustating detached pea leaves and intact plants (E).

oxide may act as an antioxidant during heavy metal exposure through reacting with $O_2^{\cdot-}$ and producing peroxynitrite, a less toxic reactive nitrogen species (Hasanuzzaman et al. 2010).

The effect of long term copper exposure on ROS and RNS metabolism of plants is less known, therefore the aim of this study was to investigate the effects of 14-day-long copper treatment on growth, metal accumulation, ROS (H_2O_2, $O_2^{\cdot-}$) and RNS (NO, $ONOO^-$) levels and cell damages in root apices of two pea cultivars (cv. Rajnai törpe and cv. Lincoln).

Materials and Methods

Plant material and growth conditions

Two pea cultivars (*Pisum sativum* L. cv. Rajnai törpe = Petit Provençal and *Pisum sativum* L. cv. Lincoln) were used for our experiments. The seeds were surface sterilized with 5 % (v/v) sodium hypochlorite for 10 min, rinsed and imbibed for 2 h in running water. Seeds were germinated between moisture filter papers at 26°C for 2-3 days. Germs with radicles (about 2 cm) were placed into Hoagland solution (30 germinated seeds per 10 L growth basin). Plants were grown under controlled conditions in greenhouse at photo flux density of 150 μmol m^{-2} s^{-1} (12/12 day/night period) at a relative humidity of 55-60%, and 25 ± 2°C temperature for 7 days. The Hoagland solution contained the following chemicals: 5 mM $Ca(NO_3)_2$, 5 mM KNO_3, 2 mM $MgSO_4$, 1 mM KH_2PO_4. The micronutrient concentrations were: 10 μM H_3BO_3, 1 μM $MnSO_4$, 5 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 0,1 μM $(NH_4)_6Mo_7O_{24}$, 10 μM $AlCl_3$, 10 μM Fe-EDTA. Seven-day-old pea plants were treated with 25 or 50 μM $CuSO_4$ for 14 days. As control, untreated plants were used. All the measurements were done 14 days after treatments. All chemicals were purchased from Sigma-Aldrich (St. Louis MO), unless specified otherwise.

Determination of growth parameters

Shoot height (cm), leaf width (cm) and primary root (PR) length (cm) were determined manually using a scale. Root fresh weight (g) was measured with the help of a compact scale.

Measurement of element contents by atomic absorption spectrophotometry (AAS)

The Cu content in root, stem and leaf samples of pea plants were determined using atomic absorption spectrophotometer (Hitachi Z-8200, Tokyo, Japan). After drying the plant material (90°C, 24 h) 100 milligrams of the samples were measured into destruction tubes. Nitric acid (HNO_3 65% (w/v), Carlo Erba Reagents, Italy) and hydrogen peroxide (H_2O_2 30% (w/v, Reanal, Hungary) were added to the dry material and the samples were destructed in microwave destructor (MarsXpress CEM, Matthews, USA) at 200°C on 1600 W for 20 min. Cooled samples were diluted with distilled water and were transferred to 20 ml Packard glasses. After further sufficient dilution of the samples the element contents were determined by AAS. Values of copper concentrations are given as μmol g^{-1} dry mass (DM).

In vivo and *in situ* light microscopy

For light microscopic investigations Zeiss Axioskope 2000-C (Carl Zeiss, Jena, Germany) stereomicroscope was used. Hydrogen peroxide was detected by 3,3'-diaminobenzidine (DAB) staining method (Guan et al. 2009). Pea root tip segments were incubated for 1.5 h in DAB solution (2 mg L^{-1}) then samples were washed once with 2-N-morpholine-ethansulphonic acid/potassium chloride (MES/KCl) buffer (10^{-3} M, pH 6.15) and were prepared on microscopic slides.

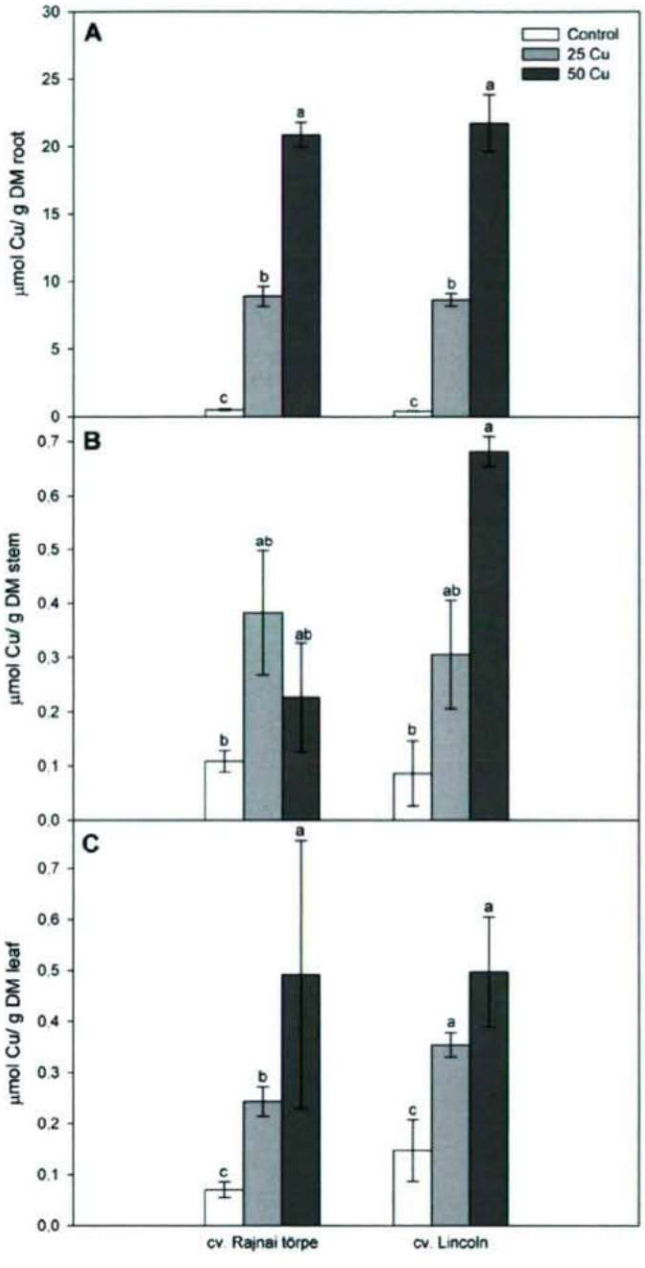

Figure 2. Copper concentration (µmol/g DM) in the root (A), stem (B) and leaf (C) of 0, 25 or 50 µM copper-treated cv. Rajnai törpe and cv. Lincoln. Values are means of 10 plants ±SE. Different letters indicate significant differences (P<0.05) according to Duncan's test.

Detection of superoxide anion was carried out by nitroblue tetrazolium (NBT) staining. Root samples were dyed for 2 h with 0.1 mg/mL NBT (in 0.2 M phosphate buffer, pH 7.6) in the dark. Finally, they were washed once with phosphate buffer. Schiff reagent was applied for the detection of lipid peroxidation and Evans blue was used for the determination of cell death according to Lehotai et al. (2011).

Fluorescent microscopy

For the fluorescent detection of NO, $ONOO^-$ and $O_2^{\cdot-}$ Zeiss Axiowert 200M microscope (Carl Zeiss, Jena, Germany) equipped with a high resolution digital camera (Axiocam HR, HQ CCD, Carl Zeiss, Jena, Germany) and filter set 10 (exc.: 450-490 nm, em.: 515-565 nm) or filter set 9 (exc.: 450-490 nm, em.: 515-∞ nm) was used. FLUAR 5x/0.12 NA objective lens was applied for the investigations. Nitric oxide levels in root tips of pea were visualized by a NO-specific fluorescent dye, 4,5-diaminofluorescein diacetate (DAF-FM DA) according to Pető et al. (2011). Root segments of 1.5- 2 cm length were dyed with 10 µM DAF-FM DA (in 10 mM Tris-HCl buffer, pH 7.4) for 20 min in the dark at 25±2°C and were washed 4 times within 20 min with MES/KCl buffer. Peroxynitrite generation was monitored using aminophenyl fluorescein (APF) according to Lehotai et al. (2011). This fluorophore is suitable for the detection of highly reactive oxygen species (e.g. $ONOO^-$, $OH^{\cdot}$ or OCl^-), but it does not react with NO, $O_2^{\cdot-}$ or H_2O_2. Segments of pea root tips were incubated in 10 µM APF (in 10 mM Tris-HCl, pH 7.4) for 60 min in darkness at room temperature. Samples were washed twice within 30 min with Tris-HCl and were prepared on microscopic slides. Superoxide radicals were detected in segments of pea root, which were incubated at 37°C in darkness for 30 min with 10 µM dihydroethidium (DHE in 10 mM Tris–HCl, pH 7.4) as described by Corpas et al. (2009). Then the root segments were washed twice in the same buffer for 15 min. The intensity of NO-, $ONOO^-$- and $O_2^{\cdot-}$-dependent fluorescence was measured within area of circles with 0.5 mm radii 0.5 mm from the root tip with the help of Axiovision Rel. 4.8 software. The radii of circles were not modified during the experiments.

Statistical analysis

Results are expressed as mean ± SE. Statistical analysis was performed applying SigmaStat 11. software using analysis of variance (ANOVA, P<0.05) and Duncan's test for multiple comparison analyses. All experiments were carried out at least two times. In each treatment at least 10 samples were measured.

Results

The effect of copper on growth and development of pea cultivars

Under control conditions, significant differences were observed in shoot height, root fresh weight and primary root length of the pea cultivars. In the case of control *Pisum sativum* L. cv. Lincoln, longer PR and larger fresh weight was measured compared to cv. Rajnai törpe. In both cultivars 50 µM copper resulted in a serious inhibition of the stem and leaf, while 25 µM Cu^{2+} had no effect on the growth of them. In cv. Lincoln, both copper concentrations significantly de-

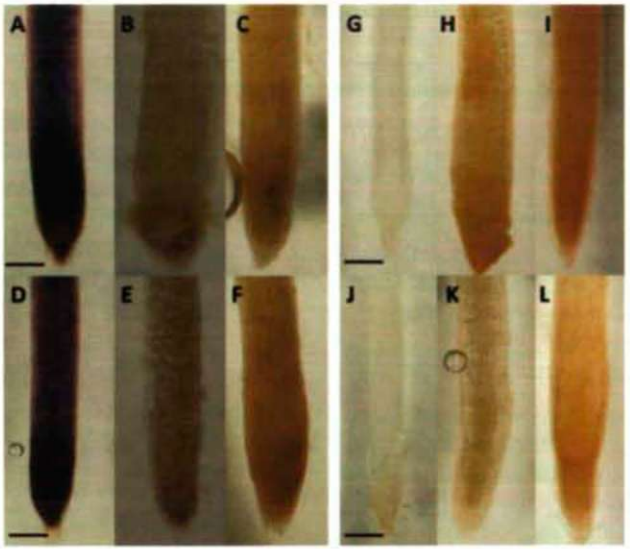

Figure 3. Superoxide (A-F) level in root tips of 0, 25 or 50 µM copper-treated cv. Rajnai törpe (A-C) and cv. Lincoln (D-F). Hydrogen peroxide (G-L) level in root tips of 0, 25 or 50 µM copper-treated cv. Rajnai törpe (G-I) and cv. Lincoln (J-L). Bars = 1 mm.

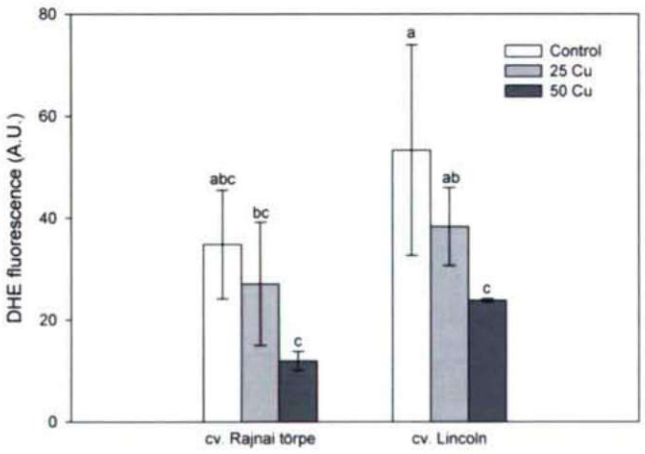

Figure 4. Intensity of superoxide-specific fluorescence (DHE) in root tips of 0, 25 or 50 µM copper-treated cv. Rajnai törpe and cv. Lincoln. Values are means of 10 plants ±SE. Different letters indicate significant differences (P<0.05) according to Duncan's test.

creased PR length, but only 50 µM Cu^{2+} caused PR shortening of cv. Rajnai törpe (Fig. 1).

Copper accumulation in the root and stem system of pea plants

In the case of both cultivars copper concentration within the plants enhanced as the effect of treatments. The largest proportion of the copper uptaken accumulated in the root system, hence 10- and 20-fold increase in Cu^{2+} concentration was

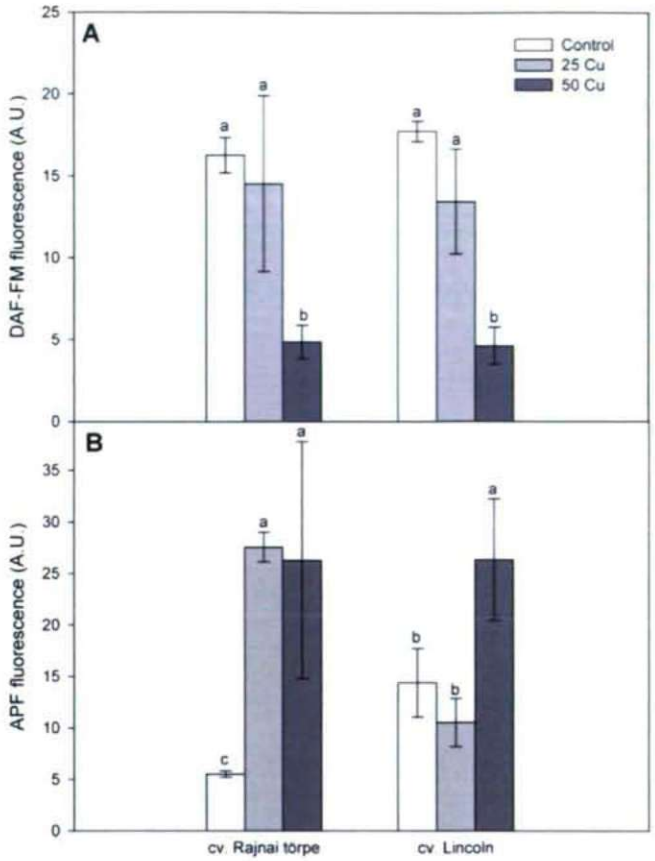

Figure 5. Intensity of nitric oxide- (DAF-FM, A) and peroxynitrite- (APF, B) specific fluorescence in root tips of 0, 25 or 50 µM copper-treated cv. Rajnai törpe and cv. Lincoln. Values are means of 10 plants ±SE. Different letters indicate significant differences (P<0.05) according to Duncan's test.

measured in the roots of 25 and 50 µM copper-treated plants, respectively (Fig. 2A). In the stem of cv. Rajnai törpe Cu^{2+} concentration enhanced moderately compared to cv. Lincoln, where notable (~7-fold) Cu^{2+} accumulation was observed (Fig. 2B). In the leaves of the cultivars, copper accumulated to a similar extent (Fig. 2C).

The effect of copper on ROS and RNS levels of pea root tips

In both cultivars, 25 and 50 µM copper treatment caused the complete elimination of superoxide from root apices, which was indicated by the lack of NBT staining (Fig. 3A-F). Similar results were obtained by dihydroethidium, where the fluorescence intensities significantly decreased as the effect of 50 µM Cu^{2+} in both cultivars (Fig. 4). Concurrently with superoxide elimination, an extensive H_2O_2 accumulation was observed by DAB staining in root tips of cv. Rajnai törpe (Fig 3 G-I), while in cv. Lincoln the degree of copper-induced H_2O_2 formation seemed to be lower (Fig. 3J-L).

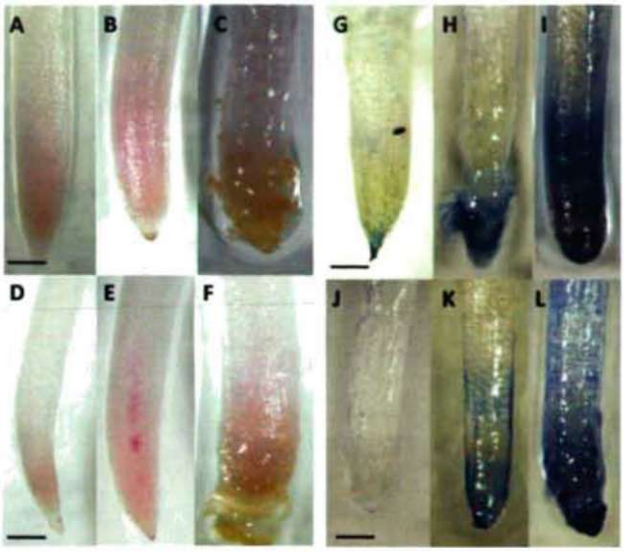

Figure 6. Lipid peroxidation (Schiff reagent staining, A-F) in root tips of 0, 25 or 50 µM copper-treated cv. Rajnai törpe (A-C) and cv. Lincoln (D-F). Cell death (Evans blue staining, G-L) in root tips of 0, 25 or 50 µM copper-treated cv. Rajnai törpe (G-I) and cv. Lincoln (J-L). Bars= 1 mm.

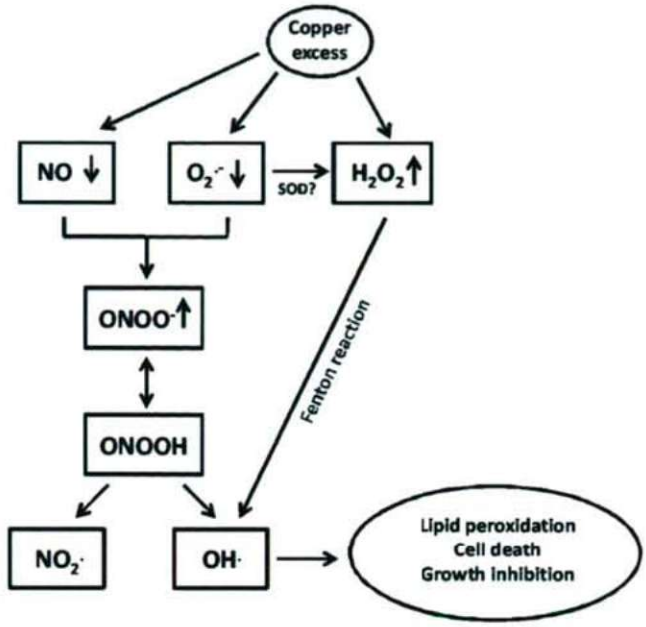

Figure 7. Schematic representation of possible pathways leading to gowth inhibition under copper exposure.

Nitric oxide levels of root apexes were also modified by long-term copper exposure. Interestingly, 25 µM Cu^{2+} had no effect on NO generation, but higher copper concentration (50 µM) resulted in a significant decrease of NO level in cv. Rajnai törpe and cv. Lincoln root tips (Fig. 5A). Furthermore, $ONOO^-$ (+OH^-) –dependent fluorescence significantly increased in cv. Rajnai törpe treated with 25 or 50 µM CuSO4, although in cv. Lincoln roots ONOO- (+OH.) level did not enhance as the effect of 25 µM copper (Fig. 5B). In 50 µM copper-treated root samples of cv. Lincoln 1.6-fold increase of APF-fluorescence was detected compared to control.

Copper exposure induces lipid peroxidation and death of root tip cells

As the effect of 25 and 50 µM Cu^{2+}, lipid peroxidation (detected by Schiff reagent) and cell death (detected by Evans blue) intensified in the root tips, and the damage of 50 µM-copper treated roots were visible. In root tips of cv. Rajnai törpe the Evans blue staining was more pronounced, indicating the strong cell death (Fig. 6).

Discussion

The first physiological process during copper exposure is the uptake of the metal from the environment. Our results showed that copper is accumulated in the root system of both pea cultivars and only a slight increase of Cu^{2+} concentration was observed in the aerial parts of the plant bodies. The inhibition of copper translocation into the leaves (metal exclusion from the shoot) provides an important defence mechanism for the plant against toxicity (Baker 1981). Among the symptoms of copper toxicity the growth inhibition is one of the most characteristic. Long-term copper exposure resulted in a significant decrease in growth parameters of pea plants, and the root system proved to be more sensitive to copper compared to the shoot, which can be explained by the significant copper accumulation within the root system (Lequeux et al. 2010). In the stem of cv. Rajnai törpe less copper accumulated compared to cv. Lincoln, which correlates the slighter decrease of elongation. The root system of cv. Lincoln suffered more serious growth inhibition than cv. Rajnai törpe, which implies that cv. Lincoln is more sensitive to copper exposure. This finding seems to be contrary to that of Palma et al. (1987), where cv. Lincoln was found to be more sensitive compared to cv. Granada. The alterations of ROS and RNS levels were established by *in vivo* and *in situ* staining methods. The complete elimination of superoxide anion from the root tip tissues was detected by NBT staining and was verified by the significant decrease of DHE fluorescence in both cultivars. The concurrent accumulation of H_2O_2 in the root apex suggests the spontaneous or enzymatic dismutation of $O_2^{\cdot-}$ to H_2O_2 under copper exposure. Whereas, in root tips of Cu-treated soybean the significant activation of superoxide dismutase (SOD) was observed (Chongpraditnun et al. 1992), this enzyme may have a role in superoxide detoxification under copper stress. Nitric oxide levels in root tips were significantly decreased in both cultivars as the effect of long-term copper exposure. Heavy metal-induced decrease of NO levels was observed, inter

alia, in pea leaves and roots (Rodriguez-Serrano et al. 2009); however, it must be noted, that the concentration of the applied metal, the treatment condition, the age of the plant and the variety of tissue examined all determine the effects on NO production (Xiong et al. 2010). Related to the decrease of the NO content, it was attractive to hypothesize that superoxide radicals eliminate NO by the reaction yielding peroxynitrite. Aminophenyl fluorescein was applied for the detection of $ONOO^-$ (+OH^-) and significant enhance of the fluorescent intensities was found in root tips of copper-treated cv. Rajnai törpe and cv. Lincoln, which suggests the occurrence of the reaction between $O_2^{\cdot-}$ and NO producing $ONOO^-$. The peroxynitrite anion is in pH-dependent protonation equilibrium with peroxynitrous acid (ONOOH), which decompose resulting nitrogen dioxide ($NO_2^{\cdot}$) and hydroxyl radical ($HO^{\cdot}$) (Virag et al. 2003). Hydroxyl radical can originate also from H_2O_2, because copper as a transition metal is able to catalyze the Fenton-reaction (Rowley and Halliwell 1983). The elevation of OH^- level within the root tip cells (which was partly demonstrated by APF) is responsible for lipid peroxidation process as it was shown by Schiff reagent staining in both cultivars. The damage of membrane lipids leads to cell death in root apex, which results in growth inhibition of the whole organ. The hypotetical pathways leading to growth inhibition under copper exposure are shown in Fig. 7.

Based on our results it can be concluded, that the general responses of the examined pea cultivars to copper stress are similar. However, cv. Lincoln proved to be more sensitive to copper than cv. Rajnai törpe, which can be explained by e.g. potential hormonal disturbances. An important defence mechanism of pea plants is the exclusion of the toxic copper from the aerial organs and the accumulation of it in the root system. At the back of superoxide elimination can be the spontaneous or SOD-catalyzed dismutation of it or the reaction of it with NO to produce peroxynitrite. Nitric oxide is considered to be an antioxidant, because it is able to convert the highly reactive $O_2^{\cdot-}$ to the less toxic $ONOO^-$. However, from the protonated form of $ONOO^-$ (ONOOH) the reactive hydroxyl radical can generate, which contributes to cell death induction and growth inhibition of pea plants.

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ARTICLE

On the terms related to spatial ecological gradients and boundaries

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ABSTRACT Ecological gradients and boundaries are currently in the focus of research interest. A widely accepted terminology, however, is still lacking, thus the use of the terms related to gradients and boundaries continues to be confusing. In this paper, we provide new more elaborated definition of the spatial boundary. We distinguish between the gradient (transition) and the space-segment (transitional zone). Our paper identifies the main difference between the two types of gradients: cline and tone. We discuss the meanings of the synonyms boundary line, boundary zone, edge, margin and border. We review the importance of scale and organizational levels in the field of gradients and boundaries. The article also enlightens the difficulties of vegetation mapping associated with boundaries. At last, we identify some important research topics for the future, where intensive studies are needed **Acta Biol Szeged 55(2):279-287 (2011)**

KEY WORDS

ecotone
ecocline
edge
transitional zone, margin
border

Ecological boundaries and gradients belong to the most current research topics in ecology. Although the area occupied by the boundaries may be small compared to the total landscape or habitat (Cadenasso et al. 2003a), their role is extremely important, because they control the flow of organisms, materials, energy, and information (Wiens et al. 1985; Wiens 1992; Cadenasso and Pickett 2001; Cadenasso et al. 2003b; Strayer et al. 2003). Boundaries can have significant effects on the adjacent patches they separate. According to Fagan et al. (1999), boundaries can influence the within-patch interactions between populations, for example competition or consumer-resource dynamics.

Knowledge about ecological gradients and boundaries plays a significant role in the fields of community ecology and landscape ecology, as well as in nature conservation (Yarrow and Marín 2007). Increased fragmentation due to human activity results in more boundaries (Merriam and Wegner 1992; Boren et al. 1997; Standovár and Primack 2001; Pullin 2002). Responses of boundaries to global changes, especially to global climate change will probably be one of the most important research questions in the upcoming decades (Holland 1988; Weinstein 1992; Neilson 1993; Allen and Breshears 1998; Weltzin and McPherson 2000).

Increasingly confusing is the inconsistent use of the terms linked with boundaries and gradients. A widely accepted terminology is lacking (Jagomägi et al. 1988; van der Maarel 1990; Kolasa and Zalewski 1995; Kent et al. 1997; Baker et al. 2002; Kark and van Rensburg 2006), so it is often difficult to compare the studies carried out by different researchers (Hufkens et al. 2009).

In this paper, we summarize the opinions of several authors, and make an attempt to define the following terms: boundary, boundary line, boundary zone, ecotone, ecocline, edge, margin and border. We also discuss some respects of scales and organizational levels. The article illustrates the difficulties of depicting boundaries on vegetation maps and briefly discusses some possible solutions. Furthermore, we identify some possible research directions where active investigations are most urgent.

Mismatch between nature and our words

A basic property of our thinking is the categorization of things (Proctor 1974; Standovár 1995; Podani 1997). Grouping into categories involves a simplification of the immeasurable variety of nature. This simplification is a consequence of the mismatch between reality and our words (Sainsbury 1995): our words denote discrete categories, while reality does not necessarily come in discrete entities. However, we have to use words that denote discrete categories, because this is the cost we have to pay for finding our way around the world.

Delineation of a boundary between two communities (for example when we draw conventional vegetation maps) is a categorization with spatial constraint. Delineating the boundary involves a simplification of reality. Boundaries are regarded as having no thickness (Zonneveld 1974). The contrast between the two patches (the dissimilarity between the two units) is increased by the map and the patches are

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considered homogenous (Küchler 1974). Thus, classification maps produce oversimplification, because neither gradual changes in the proximity of the boundary nor within-patch inhomogeneities are taken into account.

To reduce such oversimplification, a transitional zone can be drawn on the map between the two neighbouring units (e.g. an edge between a forest and a grassland). In this case, the problem of where to delineate the boundaries of this zone emerges (Csorba 2008).

The cause of the difficulty is that our words referring to vegetation units denote discrete categories, whereas vegetation does not form discrete entities or at least is discrete and continuous at the same time.

Boundary, boundary line, boundary zone

There are three meanings of the term boundary: a temporal, a spatial (topographical), and an abstract (topological) one.

Temporal boundaries can denote rapid changes in time (Westhoff 1974). During succession, communities of a given locality change, and the question can arise, from what point of time changes are sufficient to speak about another community (Gleason 1926; Jax et al. 1998).

Abstract boundaries exist not in a topographical, but in a topological space. Objects are grouped not necessarily with reference to their spatial relationships (that is, two objects that are near to each other in the abstract space may be far in the real space). In vegetation science, such abstract categories are associations. When delineating the abstract boundary of an association, we must determine which association a plot or stand belongs to (Gleason 1926; Proctor 1974; Westhoff 1974; Zonneveld 1974; Ramenskii in Rabotnov 1978).

Boundaries are most frequently defined in the spatial sense. Henceforth we will consider spatial boundaries exclusively. A spatial boundary always separates two neighbouring space-segments (van der Maarel 1976; Cadenasso et al. 2003b; Martín et al. 2006; Peters et al. 2006). The two neighbouring space-segments have to be different from each other from the point of view of the research question (Canny 1981; Cadenasso et al. 2003b; Fagan et al. 2003; Martín et al. 2006; Peters et al. 2006). Difference is offset within the boundary zone (that is, transition occurs here), therefore, gradients within the boundary are always steeper than in either of the neighbouring space-segments (Cadenasso et al. 2003b). The boundary not only separates but also connects, because a boundary through which no fluxes occur probably does not exist in nature (Wiens et al. 1985). The thickness of a boundary is necessarily smaller than the width of the neighbouring patches (Kolasa and Zalewski 1995; Körmöczi and Jusztin 2003; Csereklye et al. 2008).

The definition of the spatial boundary can be given as follows: the spatial boundary is a segment of space separating and at the same time connecting two neighbouring segments of space. The two entities on both sides of the boundary must differ from each other from the point of view of the research question, and their extent has to be much wider than that of the boundary. The boundary is the locality in which the transition occurs from one side to the other.

In accordance with the previous definition, if the two sides of the boundary do not differ, there is no boundary at all. In contrast to Hansen et al. (1988) and Cadenasso et al. (2003a), a hedgerow, a windbreak, a fence or a road is not necessarily a boundary. If these objects are situated in a homogenous matrix (that is, the patches separated by these objects do not differ), or if no transition occurs within them, they are simply landscape elements (van der Maarel 1990) or corridors (Forman 1995). Although corridors and boundaries are to some extent similar in their functions, they differ fundamentally in their structure (Forman 1995).

Boundaries between communities can be relatively sharp or blurred (Gleason 1926; Paczoski in Dąbrowska-Prot et al. 1973; Kent et al. 1997). Boundaries that are sharp at every spatial resolution do not exist. When the resolution gets finer, every boundary becomes blurred (Strayer et al. 2003). A boundary that is like a line at a given resolution will be a zone at a finer resolution (Cadenasso et al 2003b). Of course, at this finer resolution, boundary zones themselves have two boundaries (Kolasa and Zalewski 1995). According to Armand (1992): "Any natural boundary is in reality a transition zone, which has its own two boundaries. They are, in turn, also transition zones with their own boundaries, and so on, endlessly." In short, delineating lines does not correspond to reality, because transitions are gradual (Csorba 2008).

The very same boundary can appear as a line or as a zone, depending on the resolution. The phrases boundary line (Grenzlinie) and boundary zone (Grenzzone, Grenzbereich) (van der Maarel 1976; Forman and Moore 1992) refer to this strange duality.

If the resolution is coarse enough, a community boundary may appear on a map as a line (a one-dimensional object). But every boundary continues below and above the surface, and – as mentioned above – every boundary has width. Therefore, boundaries are three-dimensional structures, with lenght, height, and thickness (van der Maarel 1976; Kolasa and Zalewski 1995; Cadenasso et al. 2003a).

Community gradients and environmental gradients

If we study ecological gradients, it is necessary to distinguish between community gradients (where a kind of change in species composition occurs in space) and environmental gradients (where a kind of change in environmental factors occurs in space). (Of course, population gradients also belong to ecological gradients, but in this paper, we consider only community gradients.) However, it is confusing, that the words ecotone and ecocline are used for both community gradients and environmental gradients. It is clear, that we

Table 1. Terms in the literature denoting environmental gradients and community gradients.

	environmental gradient	community gradient
Küchler (1974), Juhász-Nagy (1986)	ecocline	coenocline
Zólyomi (1987)	ecotone	coenotone
Whittaker (1967, 1975)	complex-gradient, factor-gradient	coenocline
Jagomägi et al. (1988)	causal ecotone	resultative ecotone
Fortin et al. (2000)	environmental ecotone	biotic ecotone

need a term to denote community gradients, and another term to denote environmental gradients. For this purpose, several expressions have been coined (Table 1).

The twin-terms coenocline and ecocline (Küchler 1974; Juhász-Nagy 1986), as well as coenotone and ecotone (Zólyomi 1987) were suggested in Central Europe. Coenotone and coenocline denote the community gradients, while ecotone and ecocline refer to the gradients of the background factors that cause the community gradients.

The terms coenocline and ecocline were already used by Whittaker (1967, 1975), but he used the following terminology: an ecocline is a gradient of ecosystems, that is, a community gradient together with the environmental gradients. In Whittaker's view, a complex-gradient is a gradient of environmental complexes, *i.e.* a gradient of several environmental factors, whereas a factor-gradient is a gradient of a simple environmental factor. Whittaker's fourth term, coenocline denotes a community gradient.

Albeit the words coenotone and coenocline seem to be appropriate to denote community gradients, they were used mainly in the 1970's (Gauch and Whittaker 1972; Westhoff 1974, Noy-Meir 1978; Phillips 1978), whereas nowadays these terms are rarely used (Kleinebecker et al. 2007). It is important to note that the words coenotone and coenocline are often used to denote abstract (that is, topological), and not topographical gradients (for an obvious example, see Zólyomi 1987).

Instead of the above mentioned terms, we may use the phrases of Jagomägi et al. (1988): causal ecotone and resultative ecotone (the first term meaning an environmental gradient and the second a community gradient).

Another possiblity is to use the terms of Fortin et al. (2000): biotic and environmental ecotones. Biotic ecotones or ecoclines refer to the community gradients, whilst environmental ecotones and ecoclines mean the gradients of the background factors.

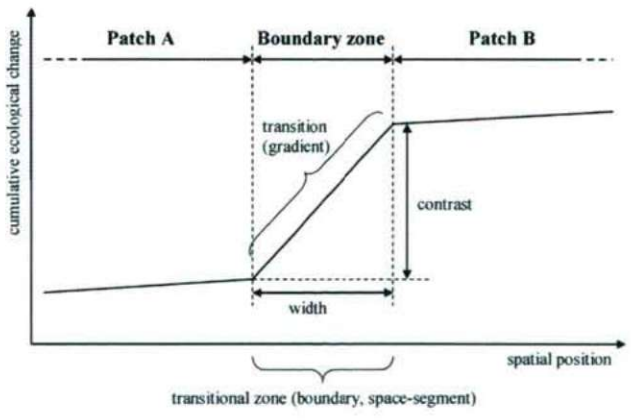

Figure 1. Terminological distinction between transition and transitional zone, and constituents of sharpness (abruptness): contrast and width.

Gradient and zone

A prerequisite of the elimination of the terminological confusion is the distinction between the space-segment and the transition within this space-segment (Erdős et al. 2010). The transition (the gradient) cannot be identical with the transitional zone (a space-segment) (Fig. 1). It is confusing in the literature that the terms ecotone, ecocline, coenotone, and coenocline can denote transitions (*i.e.*, gradients), or transitional zones (*i.e.*, boundaries, space-segments), or both.

Ecotone and coenotone most often denote zones (Clements 1907; van der Maarel 1976; Mészáros et al. 1981; Holland 1988; Jagomägi et al. 1988; Mirzadinov 1988; Swanson et al. 1992; Gosz 1993; Baker et al. 2002; Lévêque 2003), but occasionally zones and gradients at the same time (Odum 1971). The opposite can be seen in the case of the terms ecocline and coenocline, which usually denote gradients (Whittaker 1967, 1975; Phillips 1978; Ricklefs 1980; Kleinebecker et al. 2007) and not very often space-segments (van Leeuwen 1966; van der Maarel 1976) or both (Jeník 1992; Kent et al. 1997).

This difficulty can be solved by using the original meanings of the words tone and cline. The terms ecotone, ecocline, coenotone, and coenocline should be used to denote gradients, and not space-segments! The Greek root "tonus" in the words ecotone and coenotone means tension (Harris 1988; Mirzadinov 1988; Kark and van Rensburg 2006), that is, a gradient between two neighbouring units. The word cline, introduced by Huxley (1938), originally means a gradual transition, a gradient. Westhoff (in van der Maarel 1976) writes: "A vegetational cline is a gradual transition in space of one vegetation type to another."

Different terms should be used for the space-segment and the transitions within this space-segment (Fig. 1). While the boundary is a space-segment, ecotone and ecocline (as well as coenotone and coenocline) are gradients within a space-segment. The gradient is steep in the case of the tone, and it is gradual in the case of the cline. However, as we will see in the next chapter, there are gradients of intermediate steepness and no sharp line exists between tone and cline.

Since these terms denote gradients, they should not be regarded as types of boundaries. For example, if we want to speak about a relatively sharp boundary we should not use the word ecotone. Instead, application of one of the following phrases is suggested: ecotone zone (Churkina and Svirezhev 1995), ecotonal boundary or simply sharp boundary. Similarly (if the gradient within the boundary is more gradual), ecocline zone, ecoclinal boundary or blurred boundary should be used.

Ecological (both community and environmental) gradients can occur within or outside boundaries. A cline, for example, can refer to a whole series of communities along a gradient (Fig. 2a.), but also to a gradual and blurred transition between two contacting communities (Fig. 2b.) (Whittaker 1975). Of course, in both senses there is a gradient, but at different scales. Figure 2a. shows a cline from community A to community E. This gradient of communities is independent of a boundary situation. In Figure 2b., the cline can be found in the space-segment denoted with B. Here, the community gradient is a transition from community A to community C. In this case, the community gradient occurs within the boundary. (Whether B can be recognized as a separate community, is disputable, see below.)

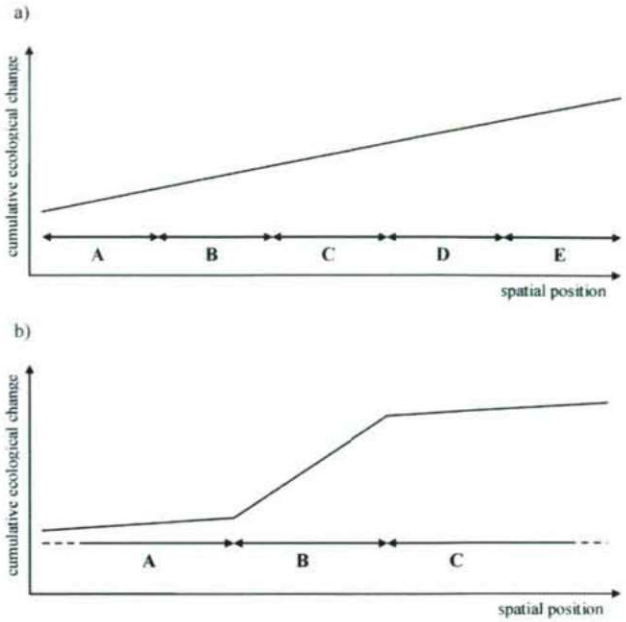

Figure 2. Cline, as community gradient. A cline may be a continuous change of a series of communities (a) or a gradient between two communities (b). In the first case A-E are the communities, and in the second case A and C: communities, B: space-segment of the cline.

Differences between limes convergens and limes divergens

According to van Leeuwen (1966) and van der Maarel (1976, 1990), two main types of boundaries exist: limes convergens (ecotonal boundary) and limes divergens (ecoclinal boundary). Limes convergens is a boundary where several species reach their distributional limits within a narrow zone, forming an abrupt boundary. In contrast, where distributional limits of the species are not so close, a limes divergens develops. Limes convergens and limes divergens can be distinguished based on three attributes: sharpness (abruptness) of the transition, stability of the environmental factors within the boundary, and species diversity: limes divergens is less sharp, more stable and more diverse (van der Maarel 1976, 1990; van Leeuwen 1966).

In the followings, we shall discuss the applicability of the above attributes in distinguishing between limes convergens and limes divergens. According to van Leeuwen (1966) and van der Maarel (1976, 1990), diversity is high only in ecoclinal boundaries, while the diversity of ecotonal boundaries is low. However, there are few studies which support this view. In fact, results are often contradictory (Kark and van Rensburg 2006; Erdős et al. 2011). Moreover, evaluation of the studies is complicated, because authors often do not differentiate between ecotonal and ecoclinal boundaries, so we can not know which boundary type the measured diversity was observed in. Therefore, species diversity within boundaries can not be used in differentiating between limes convergens and limes divergens.

Two attributes remain: stability and sharpness. However, by drawing the types and sub-types of the boundary zones established by van Leeuwen (1966) in a coordinate system, it is obvious that the main difference is in sharpness (Fig. 3). Indeed, most researchers consider ecotonal boundaries to be sharp and ecoclinal boundaries to be blurred (Zonneveld 1974; di Castri and Hansen 1992; Jeník 1992; Kent et al. 1997; Hennenberg 2005).

Sharpness has two constituents: contrast and width (Fig. 1). Contrast means the difference of the communities or environmental factors between the neighbouring patches (Cadenasso et al 2003b; Strayer et al. 2003). The width of the zone is the size of the space-segment in which the difference is offset. The greater the contrast of the neighbouring patches and the smaller the width of the boundary, the greater the sharpness.

Both van Leeuwen (1966) and van der Maarel (1976) emphasize that ecotonal boundary and ecoclinal boundary are extreme types of boundaries between which intermedi-

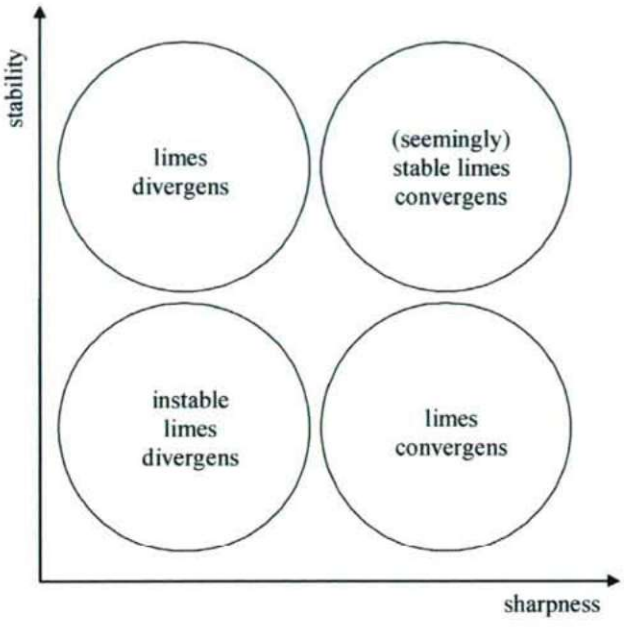

Figure 3. Types of boundaries according to van Leeuwen (1966) in a coordinate system.

ate kinds are possible. Real boundaries identified in nature can be placed on a continuum, the endpoints of which are limes convergens (ecotonal boundary) and limes divergens (ecoclinal boundary).

In sum, tones and clines are gradients; a tone means a steep gradient, while a cline is gradual. There are environmental gradients (*i.e.*, the gradients of the background factors) and community gradients. If we want to speak about boundaries, we should use the phrases ecotone zone, ecotonal boundary or simply sharp boundary (and ecocline zone, ecoclinal boundary or blurred boundary).

Edge, margin, border

The terms edge and boundary are often used as synonyms (Brunt and Conley 1990; Gosz 1991; Laurance et al. 2001; Cadenasso et al. 2003b; Csereklye et al. 2008; Erdős et al. 2011). Margin is also used in the same meaning (e.g. Risser 1995; Kivistö and Kuusinen 2000). It is important to note that edge and edge effect are not the same, as discussed more detailedly in Erdős et al. (2010).

The term border is relatively rarely used in the literature. It usually suggests a thin boundary (Jagomägi et al. 1988; Łuczaj and Sadowska 1997). In our opinion, the word border should be regarded as synonymous with the terms boundary and edge. Moreover, the terms borderline (e.g. Dutoit et al. 2007) or border area (e.g. van Leeuwen 1966) can be used depending on whether the border appears as a line or as a zone at the given resolution.

The importance of scale and organizational levels

The importance of hierarchy and the related topics of scale and organizational levels is widely recognized in ecology (cf. Allen and Starr 1982). Consequently, spatial scales and organizational levels must not be neglected in the case of ecological gradients and boundaries.

Ecological gradients and boundaries occur at severel spatial scales and organizational levels (Holland 1988; Hansen et al. 1988; Jagomägi et al. 1988; Gosz and Sharpe 1989; Gosz 1991, 1993; diCastri and Hansen 1992; Johnston et al. 1992; Risser 1995; Strayer et al. 2003; Peters et al. 2006). For example, according to Peters et al. (2006), ecological boundaries exist between individual plants, between patches of plant populations, and between plant associations. Rusek (1992) distinguishes microecotone, mesoecotone and macroecotone. At the edge of a moss cushion a microecotone can be found, a forest edge is a mesoecotone, whereas biom boundaries form macroecotones. (Ecotone is used in this cited article, as well as in several other articles, as synonymous with boundary.) Szwed and Ratyńska (1991) regard boundaries between plant associations as microecotones and boundaries between vegetation formations (~biomes) as macroecotones. Gosz (1993) identifies five ecotone levels ranging from the individual plants to the biomes.

Gosz and Sharpe (1989) and Gosz (1991, 1993) suggested that different constraints may be responsible for the control of boundaries at different scales. Broad-scale boundaries (e.g. biom boundaries) are formed by climatic parameters (temperature and moisture), whereas the characteristics of fine-scale ecotones are probably determined by site-specific parameters such as soil discontinuities (Gosz and Sharpe 1989; Gosz 1991). Moreover, the number of constraints is increasing towards finer scales, which contributes to the difficulties in the study of fine-scale boundaries (Gosz 1993).

Both the boundary width's order of magnitude and the organizational rank of a given boundary must be lower than those of the two neighbouring units (Mirzadinov 1988). As we have noted earlier in the present article, a boundary must be considerably narrower than the neighbouring patches (Kolasa and Zalewski 1995; Körmöczi and Jusztin 2003; Csereklye et al. 2008). To put it another way, its order of magnitude has to be lower. If boundaries were allowed to possess a width of the same order of magnitude as the neighbouring units, huge amounts of the Earth's surface could be categorized as boundaries (cf. Csorba 2008). This not only would contradict the intuitive meaning of the term boundary, but it would also be undesirable since situation would be hard to manage. The organizational rank of a boundary should also be lower than that of the two contacting units. For example, the boundary between two formation types (e.g. forest and grassland) may be at the association level, whereas the boundary between two associations is at a lower level (Szwed and Ratyńska 1991).

As noted by Jeník (1992), ecotonal structures are only rarely treated as separate plant associations due to their spatial restriction, although considerable debates exist in this respect, mainly in the case of forest edges (cf. Papp 2007).

It depends on the scale whether an entity is recognized as a boundary or not (Küchler 1974; Kolasa and Zalewski 1995). In fact, what is a boundary at a given resolution may be studied as consisting of patches with their own boundaries at a finer resolution (Hansen et al. 1988; Peters et al. 2006). Thus a boundary often has a mosaic structure. Patches dominated by one plant species are quite large in the interior area of a biom, because that plant species can occupy several microhabitats. Approaching the boundary of the biom, more and more species reach their limits of ecological tolerance. Therefore, suitable microhabitats begin to diminish and, as a consequence, patches decrease in diameter. As a result, boundaries often show increased numbers of small patches of plant species (Gosz 1991, 1993). The same phenomenon was observed at much finer scales (Bagi 1997).

Boundaries and vegetation mapping

It is a commonplace that in nature, one can find mosaics consisting of patches. Patches are delimited by boundaries (Laurance et al. 2001; Cadenasso et al. 2003b). Vegetation mapping involves delineating those boundaries (Küchler 1974; Bagi 1997, 1998).

The Zürich-Montpellier phytosociology school (the most wide-spread phytosociology school in Central Europe) implies that vegetation units are discontinuous, that is, their boundaries are relatively sharp (Bagi 1998; Fekete 1998). In fact, as noted earlier, if the resolution of the map is coarse enough, a simple line is appropriate to denote a boundary (Bagi 1997; Csorba 2008). However, if the boundary is blurred and wide at the given resolution, considerable problems arise in vegetation mapping (Lájer 2000). The wider a transition zone, the more complicated to delineate a boundary (Küchler 1974).

If boundary zones are treated as units of their own, vegetation mappers face serious problems. First of all, boundaries with a transitional species composition have no coenological standards, that is, they can not be classified into existing syntaxonomical categories (Bagi 1991, 1997; Seregélyes and Csomós 1995). Second, boundary delineation is vague due to the bistability of perception (Bagi 1997, 1998). Boundaries of species-rich communities are likely to result in greater vagueness (Bagi 1998). Third, floristic composition of boundary zones is highly variable, depending on which of the neighbouring units has a greater influence on it (Bagi 1998).

One possible solution is to give the boundary zone a name as a combination from the names of the two adjoining units (Bagi 1998), which also emphasizes the transitory character of the boundary zone. A second option is to divide the boundary zone into narrow stripes (Bagi 1997). A drawback of this latter method is the very big number of newly created categories (Bagi 1998). According to Küchler (1974), in some instances it is reasonable to omit boundaries on vegetation maps. An excellent example was given by Schmidtlein et al. (2007). In their vegetation map of a mosaic habitat complex in Germany, each pixel had the chance to represent a particular species composition with a unique colour. Thus the method reconciles vegetation mapping and complex reality. However, although this new approach may be promising, conventional vegetation maps depicting boundary lines will be indispensable in several cases (Schmidtlein et al. 2007).

The concept of gradients

There are many concepts related to ecological gradients and boundaries, such as ecotone, ecocline, edge etc. Yarrow and Marín (2007) suggested that the concept of transitional zone is the most general. In our opinion, the concept of gradients deserves consideration, since it is even more general than the concept of transition zones. According to this point of view, not only boundary areas but also other regions can be conceived as consisting of horizontal gradients. There are within-patch and between-patch gradients everywhere. In this sense, the main difference between boundaries and other areas is that the gradient in the boundary is much steeper than elsewhere (Goodall 1963; Whittaker 1967; Cadenasso et al. 2003b). Moreover, the difference between sharp and blurred boundaries lies in the steepness of the gradient in the boundary. This thinking is not confined to one dimension: a gradient in one direction becomes a pattern in more than one direction (Whittaker 1967), that is, every pattern consists of gradients.

Future directions

We identified a number of gaps in the ecology of gradients and boundaries both in theory and practice.

First of all, more long-term studies would be needed in order to gather information on the dynamics of vegetation, including the dynamics of boundaries (cf. Bartha 2008), which is especially important if we want to predict the responses of boundaries to global changes, especially to global climate change.

One of the most important research topics for the future is to link boundary structure with boundary function (Cadenasso et al. 2003a). By this time, it is poorly understood, how structural characteristics of the boundaries determine functions (e.g. diversity, permeability).

There are models predicting responses of variables (e.g. abundance of a species) to edges. For example, Ries et al. (2004) suggested that edge responses depend on the resource distribution between adjoining communities. Although their model proved to perform well, it is clear that much more case studies would be necessary.

Edges may play an important role in nature conservation,

e.g. in the survival of protected and rare plants (cf. Erdős et al. 2011). However, our knowledge is far from being sufficient. For example, the role of transitional areas in the preservation of relict species is judged contradictorily (cf. Zólyomi 1987; van der Maarel 1990).

The definition of edge species is lacking (Lloyd et al. 2000), and there are too few data on the exinstence of edge-related species (cf. Erdős et al. 2011).

Although there are some theories concerning the similarity of temporal and spatial gradients (Whittaker 1967, 1975; Margalef 1979; Neilson 1993; Huntley and Baxter 2006), the connections between the dimensions of space and the dimension of time on the field of ecological gradients have not been studied intensively so far.

Vertical gradients (i.e. gradients between vegetation layers) form another neglected research area, since most studies focus on horizontal gradients.

It was realized early (Ramenskii in Rabotnov 1978) that sometimes abrupt changes in vegetation occur in spite of continuous changes in the environment. Further studies are needed to reveal the possible explanations for these phenomena (for some possible reasons, see Weltzin and McPherson 1999; Fagan et al. 2003; Holt et al. 2005).

One of the most controversial issues in ecology is diversity within boundary habitats. The most wide-spread hypothesis states that diversity is greater in boundary zones than in either of the two contacting communities (e.g. Odum 1971; Pianka 1983; Chiras 1991). Other theories claim that only blurred boundaries support higher diversities, while sharp ones are less diverse compared to the two adjoining communities (e.g. van Leeuwen 1966; van der Maarel 1976, 1990; Brown and Gibson 1983). Unfortunately, field studies concerning plant species diversity within boundary zones are relatively scarce.

Generalizations in the issues mentioned above will be possible only if the different studies are comparable. In our opinion, it would be necessary to describe the key boundary features (the most imortant of them being contrast and width) as precisely as possible in every case study and to use a terminology free from inconsistencies. We hope that the present article has contributed to the elaboration of this new terminology.

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ARTICLE

Hypolipidemic effect of *Terminalia arjuna* (L.) in experimentally induced hypercholesteremic rats

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ABSTRACT The hypolipidemic activity of the 50% ethanol extract of bark of *T. arjuna* were evaluated in rats. The 50% v/v ethanol bark extract at the dose of 40mg/kg body weight, substantially reduced the plasma total cholesterol, triglycerides and LDL cholesterol while HDL cholesterol increased in experimental group in comparison with hypercholesterolemic animal group. Atherogenic index and liver weight of treated animals also showed significant decrease. A significant increase in the activities of lipoprotein lipase and plasma LCAT enhanced hepatic bile acid synthesis and thereby increased degradation of cholesterol to neutral sterols. Furthermore, the activities of lipogenic enzymes like HMG-CoA reductase, glucose-6-phosphate dehydrogenase and malate dehydrogenase were significantly reduced. The bark extract of *Terminalia arjuna* has excellent hypolipidemic activity. The effect of the extract seems to be mediated through increased hepatic clearance of cholesterol, down regulation of lipogenic enzymes and inhibition of HMG- CoA reductase.

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KEY WORDS

atherogenic index
lipid lowering
Terminalia arjuna
neutral sterols
plasma LCAT

Coronary heart disease (CHD) is the main cause of death in western countries and Asia. Among CHDs, ischemic heart disease (IHD) leads to the highest mortality rate. The number of heart patients suffering from IHD worldwide is gradually increasing. About 41% deaths in United States are due to the heart diseases (Dallas 2001). It is well known that the three major risk factors for IHD are hypercholesterolemia; hypertension and smoking. Extensive epidemiological studies have shown that increased blood cholesterol level is a major cause of coronary heart diseases (Gambhir et al. 2001). Studies have also demonstrated the relationship between plasma cholesterol levels and the development of IHD. Hypercholesterolemia is generally, associated with an increase in plasma concentration of LDL and VLDL. Lowering of elevated levels of LDL cholesterol can slow the progression of atherosclerotic lesions (Altschul, 1964). To reduce the risk associated with high serum cholesterol levels, the development of several hypolipidemic drugs and therapies have been adopted intensively in India and other countries. About 70% of total cholesterol in human is synthesized *de novo* and the remaining is supplied by absorption from diet (0.3- 0.5 g/day in human). Several methods are presently practised to control blood cholesterol levels. These include balance of dietary fats; bile acids sequester and use of HMG-CoA reductase inhibitors (statins). HMG-CoA reductase is the key enzyme in the cholesterol biosynthesis pathway and its inhibition has proven to be the most efficient therapy for managing hypercholesterolemia (Alberts et al. 1999).

Many plants in the Indian system of medicine have been reported to be beneficial in hypercholesterolemia. Extracts of various parts of plants like *Allium sativum, Allium cepa, Cucurma longa, Emblica officinalis, Picrorrhiza kurroa* etc. are known to have antihyperlipidemic activities (Anonymus, 1999). *Terminalia arjuna* commonly known as *Arjuna* is a large deciduous tree found all over in India and Himalaya (Dwivedi and Udapa 1989). The bark of *T. arjuna* has been recommended and used as cardiac tonic and bark powder/ decoction is used to treat heart diseases, bone fractures, skin diseases, polyuria, white discharge, giddiness, fever and worms (Anonymus, 1999). In the present study, the ethanol extract of *T. arjuna* bark was investigated for hypolipidemic activity in experimental animals and some possible mechanisms of its lipid lowering properties are discussed.

Materials and Methods

Plant material

Fresh *Terminalia arjuna* bark was obtained from the Toranmal forest of Nandurbar district, Maharashtra, India, in the month of January 2007. The plant was authenticated by Dr. D. A. Patil, Botanist, S.S.V.P.S. College, Dhule, India and a voucher specimen (No. 094) has been deposited in the herbarium of the Department of Pharmacognosy, R.C. Patel College of Pharmacy, Shirpur

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Table1. Effect of 50% ethanol extract of *T. arjuna* on lipid profile of hypercholesterolemic rats.

Animal Groups	Parameters				
	TC (mg/dl)	TG(mg/dl)	HDL(mg/dl)	LDL(mg/dl)	VLDL(mg/dl)
Group A (Control)	68.20±6.22	89.32±3.14	34.82±5.47	16.34±2.56	15.40±1.23
Group B (Hypercholesterolemic)	149.20±9.36*	209.10±16.23*	37.12±7.58*	70.26±8.16	40.00±7.63*
Group C (Hypercholesterolemic plus 50% extract)	70.12±5.95#	90.02±2.56#	35.40±4.25#	16.68±2.56	15.22±3.10#
Group D standard (Atrovastatin 5mg/kg BW)	68.90±5.21	88.32±3.14	34.00±3.89	17.24±3.26	13.19±3.44

Values are expressed as Mean ± SD of each group ($n=6$). The symbols * and # represents statistical significance vs. hypercholesterolemia (Group B) and standard group (Group D) respectively $p< 0.05$.

Preparation of plant material

The shade dried *T. arjuna* bark was coarsely powdered and 200g of coarse powder of 20-40 mesh size was refluxed with 50% v/v ethanol for three hours using Soxhlett's apparatus. The extract was filtered and evaporated in a vaccum evaporator (Buchi, Switzerland) until a gel like residue remained. The amount of residue remained was measured and stored in glass bottle at 4°C and was redissolved in physiological saline at the time of use.

Animal studies

Twelve week old 24 healthy male albino Wistar rats weighing 150 – 160 g each were used. The animal experiments were conducted at the animal house of R. C. Patel College of Pharmacy, Shirpur, India as per internationally accepted principles for laboratory animal use and approved by the Institutional Animal Ethics Committee. All animals were maintained at 27±1°C with 12 h light and dark cycle. The animals were fed with standard diet and water *ad libitum*. Rats were divided in to four groups ($n=6$). Their body weight was monitored on 3 day interval. Group A rats were served as non- treated control and received standard diet plus 1ml saline for six weeks, group B received high fat diet (athero diet) for six weeks whereas group C rats received high fat diet plus 10, 20, 30, 40 and 50mg/kg body weight extract of *Terminalia arjuna* for six weeks. Group D rats received athero diet plus standard atrovastatin tablet at 5 mg each (Alberts et al. 1990). The atrovastatin was used as a reference compound to compare the hypolipidemic effect of the extract. The extract was administered orally through gastric intubation. High fat diet composed of (g/100 g of normal diet) hydrogenated sunflower oil: 20, egg yolk: 35 and cholesterol: 0.5 (Gandhi et al. 1992). After 6 weeks treatment, blood samples were collected by retro orbital sinus and serum was separated.

The acute oral toxicity study

The assay was performed as per the OECD guidelines (OECD-TG, 425, 2002) with albino rats. The age, body weight, acclimatization, randomization, accommodation, environmental condition for the animal during the period of study was as per the standard OECD guidelines. The limit dose of 2000mg/kg body weight of the animal was administered to the test animals ($n=6$) orally through gastric intubation.

Biochemical analysis

The serum and liver tissues were extracted as per the procedure described by Menon et al. (1976). Analysis of serum for total cholesterol (Zlatkis et al. 1953), triglycerides (Gottfried and Rosenberg 1973), and HDL cholesterol (Burnstein et al. 1970) was performed by micro titer plate reader using standard kits (Span Diagnostics, Surat, India). Serum LDL cholesterol concentration was determined using Friedwald formula (Friedwald et al. 1970).

Bile acids and fecal sterols were extracted as per the previously known procedures (Grundy et al. 1965). The bile acids were extracted from liver according to the procedure of Okishio et al. (Okishio et al. 1967). Total bile acids (Palmer 1969), neutral sterols (Ghanbari and Leelamma 1996) and serum lipoproteins (Warnick and Albers 1978) were also estimated. The activity of plasma lecithin cholesterol acyl transferase (LCAT) (EC 2.3.1.43) and lipoprotein lipase was estimated as described earlier (Annie and Kurup 1986). The extent of increase in the cholesterol ester/unesterified cholesterol ratio was taken as a measure of LCAT activity. Activity of glucose -6-phosphate dehydrogenase and malic enzyme was determined as per previously used methods (Annie and Kurup 1986). β hydroxy β methyl glutaryl CoA reductase (HMG CoA reductase EC 1.1.1.34) activity was assayed using the method described earlier (Rao and Ramkrishnan, 1975). The ratio of HMG CoA to mevalonate was taken as an index of enzyme activity which catalyzes the conversion of HMG to mevalonate. The lower the ratio, the higher the enzyme activity (Chitraand Leelamma 1997).

Atrovastatin

The standard atrovastatin tablets were procured from Lupin Pharmaceuticals Limited, Tarapur, India.

Table 2. Effect of 50% ethanol extract of *T. arjuna* on cholesterol deposition, body weight and atherogenic index of hypercholesterolemic rats.

Animal Groups	Parameters		
	Relative liver weight (gm)	Atherogenic index	Body weight (gm)
Group A Control	5.12±1.85	0.91±0.09	287
Group B Hypercholesterolemic	6.90±1.10*	2.97±0.12*	365
Group C Hypercholesterolemic plus 50% extract	5.07±1..93#	0.90±0.08#	290
Group D standard Atrovastatin (5mg/kg BW)	4.90±1.23	0.89±0.02	304

Values are expressed as Mean ± SD of each group (n=6). The symbols * and # represents statistical significance vs. hypercholesterolemia (Group B) and standard group (Group D) respectively $p< 0.05$

Statistical analyses

Statistical significance of data was analyzed using one-way analysis of variance (ANOVA) in Microsoft Excel. Each data value is expressed as the mean ±S.D.of six animals. Statistical differences were considered significant at $p< 0.05$.

Results

The choice of the solvent (50% ethanol) for extraction was based on literature reports on immunomodulatory and antioxidative ((Dwivedi 2007) properties of ethanol extracts of *T. arjuna* bark. The yield of the ethanol extract was 8.5%. Primary phytochemical screening of the ethanol extract of *T. arjuna* bark revealed the presence of triterpenoids, phenols, flavonoids, tannins and saponins in agreement with a previous report (Dwivedi 2007).

Preliminary studies with different doses of the ethanolic extract (10, 20, 30, 40 and 50 mg/kg body weight of rats) indicated a dose dependent decrease in total cholesterol in group C (induced hypercholesterolemic) rats till 40mg/kg body weight dose. The decline in the total cholesterol beyond this does became independent of the dose and was not significant and hence all other parameters were evaluated for animals treated with this dose.

It was observed that keeping the animals on high fat diet significantly increased the total cholesterol (TC), triglycerides (TG), LDL and VLDL cholesterol as compared to the rats on normal diet. Co-administration of bark extract of *T. arjuna* at 40mg/kg body weight resulted in considerable decline in the levels of these parameters. The lipid profile of different groups of animal after the experiment period of 6 weeks is presented in Table 1. The levels of serum TC, TG, VLDL and LDL- cholesterol in the group C animals were significantly lower than group B (animals receiving high fat diet only) and comparable to group D (animals receiving high fat diet plus Atrovastatin at 5mg/kg body weight). On the other hand, serum HDL-cholesterol concentration in group C animals was marginally higher than that of other groups (Table1). The extract of *T. arjuna* at the dose of 40mg/kg body weight lowered the plasma total cholesterol, LDL and TG by 53%, 76.25% and 57% ($p< 0.05$), respectively in group C *vis- a- vis* group B animals and the values were comparable with that of group A and group D.

The liver weight, atherogenic index and body weight in different groups is presented in Table 2. The liver weight in treated group (group C) was significantly lower than that in hypercholesterolemic group (group B) indicating decrease in cholesterol and fat deposition in liver (Table 2). Atherogenic index in group C was three times less than group B and was comparable to group A and D. The body weight of the group B animals showed significant weight gain when fed with high fat diet over the group A animals (normal controls). Treatment with bark extract and standard drug (Atrovastatin) (Alberts et al. 1989) in group C and group D, respectively reduced body weight by approximately 20% compared to group B animals.

The activity of glucose -6-phosphate dehydrogenase, malate dehydrogenase and, HMG-CoA reductase was significantly reduced while activities of plasma LCAT and lipoprotein lipase were enhanced in the animal group fed with the extract (Table 3). The high concentrations of fecal neutral sterols and bile acids in the liver in the treatment group shows the enhanced rate of degradative processes and reduction in intestinal absorption of free cholesterol and other lipids. The significantly higher levels of bile acids in the liver and feces and increased concentrations of fecal neutral sterols provide the evidence for higher rate of degradation.

The acute and oral toxicity studies provide information on health hazards likely to arise from the short term exposure to the test substance by oral route. The results of the toxicological studies showed that the administration of ethanolic extract of *T arjuna* bark by oral route at the limit dose (2000mg/kg

Table 3. Concentration of hepatic and fecal bile acids and sterols, activities of lipogenic and lipolytic enzymes in rats fed with 50% ethanol extract of *T. arjuna*.

Parameters	Animal groups			
	Group A (Control)	Group B (Hypercholesterolemic)	Group C (Hypercholesterolemic plus 50% extract)	Group D (Standard Atrovastatin)
Hepatic bile acids (mg/100 g)	36±2.3	40.6±3.3	70.6±1.5*	39.2±2.2
Hepatic neutral sterols (mg/100 g)	100.3±3.5	105.6±4.5	135.2±3.2*	105.5±5.2
Fecal bile acids (mg/rat/day)	35.9±1.3	38.3±2.5	51.3±2.1*	40.3±3.2
Fecal neutral sterols (mg/rat/day)	90.5±2.6	98.8±3.1	123.5±3.2*	100.2±3.1
Plasma LCAT[A]	28.5±0.6	27.9±0.3	39.4±0.8*	30.2±0.5
Lipoprotein lipase[B]	40.6±1.2	35.3±1.2	56.6±1.6*	45.3±1.2
Glucose-6-phosphate dehydrogenase[C]	64.2±1.3	100.3±3.2	62.2±1.2*	60.1±2.0
Malate dehydrogenase[D]	501.3±8.6	990.3±9.6	453.2±9.6*	503.2±8.3
HMG CoA reductase (ratio of HMG CoA to mevalonate)[E]	4.1±0.2	6.8±03	3.9±0.3*	2.1±0.2

Control group (A) and group B compared with 50% ethanol extract fed group. * $p < 0.05$ [A] % increase in the ratio of ester cholesterol to free cholesterol during incubation [B] μ moles of glycerol liberated /h/g protein [C] One unit is defined as that amount of the enzyme that causes an increase of 0.01in optical density/mg protein. [D] One unit is defined as that amount of the enzyme that causes an increase of 1.0 in optical density/mg protein (Sudheesh et al.1997). [E] Decreased ratio indicates increased activity (Chitra and Leelamma 1997).

body weight) did not produce any signs of toxicity or deaths in experimental animals indicating minimal or no chance of toxicity of the extract at the likely therapeutic dose in human which are lower by several orders of magnitude then no observed adverse effects level (NOAEL).

Discussion

The male albino wistar rats used in the present study were reported as ideal hypercholesterolemic models in previous studies (Mary et al. 2003). The decrease in the cholesterol levels of animals fed with extract may be attributed to increase in the level of serum HDL, increase in the activity of lipoprotein lipase and plasma LCAT, which are known to involve in transport of tissue cholesterol to liver for its excretion. Hence the hypocholesterolemic effect of the extract seems to be mediated through increased hepatic clearance of cholesterol, down regulation of lipogenic enzymes like glucose-6-phosphate dehydrogenase and malate dehydrogenase and cholesterol biosynthetic enzyme HMG-CoA reductase.

It is known that for being effective antihyperlipidemic agent the compound should reduce the plasma levels of LDL cholesterol as it transports 70% of plasma cholesterol in humans. Epidemiological and clinical studies have demonstrated positive correlation in LDL cholesterol level in serum and risk of coronary heart diseases (Kannel et al. 1971). The present report demonstrates significant decrease in plasma LDL-cholesterol level (quantitatively the most important lipoprotein class in control of serum cholesterol level) as a function of treatment by 50% alcoholic bark extract of *T. arjuna* in experimental animals. The reduced triglyceride level in treated animals could be co-related to elevated lipoprotein lipase activity in agreement with the previous report on a mushroom extracted exo-biopolymer (Sugiyama et al. 1995). The treatment may have an inhibitory effect on cholesterol deposition in liver tissues apparently by inhibition or down regulation of HMG-CoA reductase activity resulting in observed lesser relative liver weight in group C animals. The mechanism of hypolipidemic effect of *T. arjuna* is yet to be resolved. However, the previous report of Sinha et al. (2008) suggests that, the hypolipidemic activity may be attributed to inhibition of oxidative stress. Recent finding on Arjunic acid revealed its free radical scavenging potential (Sun et al. 2008). It appears that combination of more than one factor i.e. inhibition of HMG- CoA reductase activity, accelerated fractional turnover of LDL etc. may be responsible for the observed effect (Gandhi et al. 1992). Further the bark of *T. arjuna* is rich in glycosides, alkaloids and saponins and the glycosides are known for their cardioprotective activity (Dwivedi and Udupa 1989). The decrease in the serum total cholesterol concentration may be due to increase in HDL cholesterol which normally facilitates catabolism of excess cholesterol. Comprehensive chemical and pharmacological studies would help discover the exact mechanism of hypolipidemic effect of this indigenous tree and pave the way for its large scale commercial use as a cardio protective/curative drug.

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ARTICLE

Differential inhibition by trifluoperazine of responses of hippocampal CA1 pyramidal cells to NMDA and AMPA *in vivo*

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ABSTRACT The effects of trifuoperazine (TFP), a phenothiazine neuroleptic drug having potent anticalmodulin activity, were studied on the responses of hippocampal CA1 pyramidal cells to N-methyl-D-aspartic acid (NMDA) and (*RS*)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) *in vivo*. Single-unit activity was recorded using multibarrel carbon fiber containing microelectrodes whilst all drugs were delivered by microiontophoresis. NMDA and AMPA were iontophoresed alternately so that they evoked comparable responses in terms of peak heights as peristimulus time histograms were recorded. We observed that changing the stimulation intensity of one classes of receptors (*e.g.* NMDA) greatly influenced the responsiveness of the other (*e.g.* AMPA) and *vice versa*. In the presence of iontophoretically applied TFP responses to both NMDA and AMPA were significantly decreased. More interestingly, NMDA-evoked responses were significantly more inhibited by TFP than responses to AMPA under the same experimental conditions. In our conclusions, these results are due to the inhibition by TFP of the second messenger cascade events leading from NMDA receptors *via* Ca^{2+}/calmodulin to AMPA receptors and, in consequence, for the blocking of phosphorylation of AMPA receptors and their sensitization. It is also likely that the function of NMDA receptors by itself is, at least in part, dependent on the Ca^{2+}/calmodulin-mediated events.
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KEY WORDS

hippocampal CA1 pyramidal cells
single-unit recording
NMDA
AMPA
trifluoperazine
microiontophoresis
calmodulin

Trifluoperazine (TFP), a phenothiazine antipsychotic drug, is primarily used in the treatment of schizophrenia. This compound has been shown to exert a variety of effects in the hippocampus. It antagonizes dopamine D_1 and D_2 receptors (Seeman et al. 1976; Creese et al. 1996) or α_1 adrenergic effects (Huerta-Bahena et al., 1983). Binding studies showed that neuroleptics such as TFP can also compete for serotonin (5-HT), α-adrenergic, and histamine receptors (Peroutka and Snyder 1980). In low concentration (0.1-1 μM), TFP antagonizes nicotinic actions of acetylcholine (ACh) by increasing the rate of desensitization (Clapham and Neher 1984). At concentrations of 1-100 μM, TFP blocks noncompetitively γ-aminobutyric acid (GABA)-gated chloride currents in hippocampal and spinal cord neurons (Zorumski and Yang 1988)

At micromolar concentrations, TFP is also a potent calmodulin inhibitor (Levin and Weiss 1977). Interference with calmodulin-dependent processes may in turn give rise to a wide spectrum of effects. For instance, the release of 5-HT or noredrenaline from hippocampal slices was completely abolished or significantly decreased by TFP (Satoh et al. 1996; Jaffe 1998). The evoked release of acetylcholine (ACh) from slices of hippocampus was decreased in a concentration-dependent manner by preincubation with TFP (Budai and Kasa 1987). In pyramidal neurons freshly dissociated from the rat hippocampal CA1 region, ACh-induced two types of muscarinic current was reversibly and concentration dependently inhibited by TFP (Wakamori et al. 1993). Neuroleptics decrease calcium-activated potassium conductance in hippocampal pyramidal cells. Bath application of a wide variety of neuroleptics was found to depress the slow afterhyperpolarization, which is mediated in these neurons by a calcium-dependent potassium conductance occurring following a burst of spikes (Dinan et al. 1987). Tetanic stimulation or brief exposure to Ca^{2+} produced a long-lasting augmentation of the extracellular excitatory postsynaptic potentials (EPSP) and of the responses of the population spikes in the CA1 region of hippocampal slices. Both forms of potentiation were inhibited by perfusion of TFP, an effect which is unlikely to involve interactions with dopamine or norepinephrine receptors, but rather a potent blockade of calmodulin-mediated events (Mody et al. 1984). Similarly, both somatic population spikes and dendritic EPSP fields were depressed reversibly by TFP when applied by microiontophoresis in the CA1 region of hippocampal slices (Agopyan and Krnjevic 1993).

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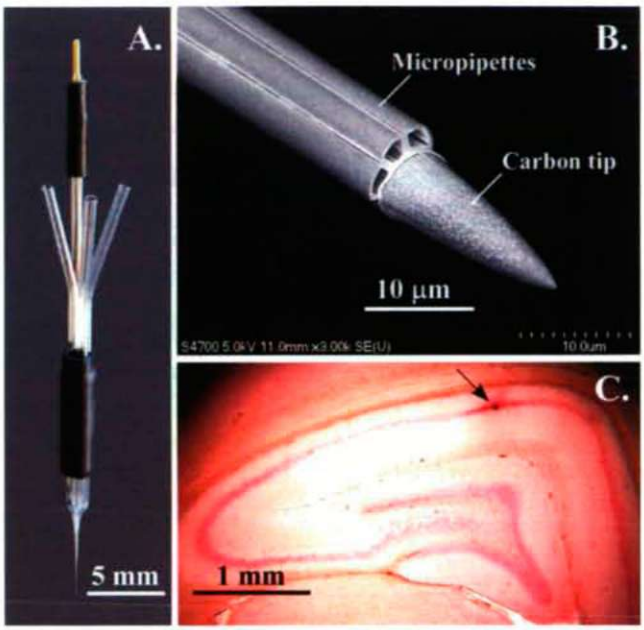

Figure 1. Macroscopic view of a multibarrel carbon fiber microelectrode used in this study (panel A) and the scanning electron micrograph of its tip (panel B). Carbon fiber was used as lead element for recording electrical signals whereas the attached micropipettes allowed iontophoretic application of electrically charged compounds in the near vicinity of the recorded neuron. The site of extracellular single-unit recording (arrow in panel C) was marked by ejection of pontamine sky blue after completing the experiment. Spikes recorded from this site are shown in Figure 2.

Induction of long-term potentiation (LTP) in the CA1 region of hippocampal slices is associated with increased activity of Ca^{2+}/calmodulin-dependent protein kinase II (CaM K II) (Fukunaga et al. 1995, 1996). Neuroleptic drugs such as TFP were able to block LTP almost completely. The ability of neuroleptics to antagonize LTP was more closely related to their ability to block calmodulin than to their relative potencies as dopamine antagonists. It would appear that neither norepinephrine nor adrenergic antagonists influence the amount of LTP elicited by repetitive stimulation; however, drugs which have been shown to interfere with calmodulin-mediated cellular processes do antagonize this phenomenon (Dunwiddie et al. 1982).

The ionotropic glutamate receptors include the N-methyl-D-aspartate (NMDA) receptors, 2-amino-3-hydroxy-5-methyl-oxazole-4-propionic acid (AMPA) receptors and kainic acid (KA) receptors (for recent review, see (Peng et al. 2011). The NMDA receptor is dual voltage and ligand-gated channel which combines with the Mg^{2+} channels to maintain a resting potential state, with this activity being voltage-dependent. Channel activation depends on the depolarization of the postsynaptic membrane and the neurotransmitter release from the presynaptic membrane. When NMDA receptors are open Ca^{2+}streams into the intracellular space, serving as a second messenger to activate a series of biochemical reactions, which may result in the manifestation of LTP. NMDA receptor activation requires the participation of the non-NMDA glutamate receptors, including the AMPA receptors and KA receptors. The non-NMDA glutamate receptors mediate low-frequency synaptic transmission in the resting state, and serve as the main receptor of Na^+, K^+ permeability. The inflow of Ca^{2+} into the postsynaptic membrane can activate a wide range of calcium-dependent enzymes including the CaM K II. After CaM K II activation, the AMPA receptor subtypes of GluR1 are phosphorylated, the AMPA receptors from non-synaptic sites are redistributed to the synaptic site. At the same time, the function of the AMPA receptor also significantly increased, as shown by the increase of the single-channel AMPA receptor synaptic transmission as well as the development of a phosphorylation site.

In the present study, our aim was to test the effects of the potent calmodulin inhibitor TFP on the activity of hippocampal CA1 pyramidal cells evoked by alternate NMDA and AMPA iontophoretic applications *in vivo*. This approach allowed us, at least in principle, to activate the two classes of ionotropic glutamate receptors independently from one another. TFP was also delivered by microiontophoresis having the advantage of affecting only a small sphere around the recorded site instead of affecting all regions of the hippocampus.

Materials and Methods

Animals and surgery

Twelve male Wistar rats (*Rattus norvegicus*) weighing 350–450 g were used in this study. Animals were purchased from the local University owned breeder and were allowed to acclimatize for at least one day before use. There was automatic control of light cycle and temperature. Light hours were from 06 to 18 h and temperature was kept within the target range of 22°C ± 3°C.

After an initial dose (400 mg/kg) of intraperitoneally applied chloral hydrate solution (40 mg/ml in physiological saline) (Sigma, St. Louis, MO), the jugular vein on one side was cannulated with a 60 cm-long PE-50 plastic tubing. A continuous anesthetic inflow was provided through this cannula at a rate of 1-2 ml/hour using diluted (8 mg/ml) chloral hydrate solution as needed during the whole experiment.

The head of the animal was mounted in a stereotaxic frame, the skull was opened above the hippocampus by drilling the bone (antero-posterior: 2.8 to 3.8 mm from bregma; lateral: 2 mm on either side from the midline) (Paxinos and Watson 1998), and the dura mater was carefully removed. The brain surface was always kept moist using physiological saline. All efforts were made to minimize animal suffering. The principles of laboratory animal care (NIH publication No. 85-23) and the protocol for animal care approved by the Hungarian Health Committee (1998) and the European Com-

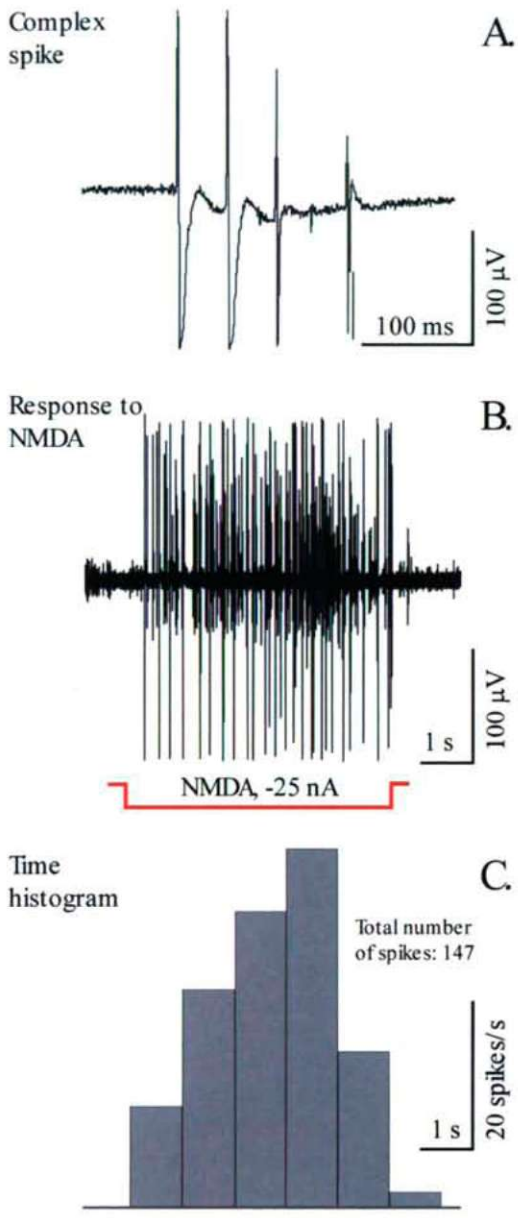

Figure 2. Representative single-unit recording from a hippocampal pyramidal cell located in the CA1 region as shown in Fig. 1, panel C. A complex spike typical to pyramidal cells in this region is shown in panel A. Neuronal spiking in response to iontophoresed NMDA and its computed time histogram, using 1s bin width, of this activity are shown in panels B and C, respectively.

munities Council Directive of 24 November 1986 (86/609/EEC) were followed. The animals were killed by exposure to intravenously applied lethal amount of chloral hydrate.

The level of anesthesia was monitored by tail-flick test (applying noxious pressure to the tail by thumb) and by pupilla reflex (using a drop of physiological saline) throughout the experiment. The venous inflow of anesthetic (2-3 ml/h using 8 mg/ml chloral hydrate in physiological saline) was adjusted correspondingly so that the tail-flick test was just observable.

Extracellular recording and microiontophoresis

Single-unit activity was recorded extracellularly from the hippocampal CA1 neurons by means of a low-impedance (<1 MΩ) 7 µm carbon fiber containing combined recording and iontophoresis microelectrodes (Figs. 1 and 2) (Budai et al. 2007; Budai 2010). Recordings were commenced at least 1 h after surgery from a depth of about 2.1 mm from brain surface. The action potentials (spikes) were amplified using an ExAmp-20K amplifier (Kation Scientific, Minneapolis, MN), filtered, (Budai, 2004) and then monitored using an oscilloscope. A window discriminator (WD-2, Dagan, Minneapolis, MN) was used for spike discrimination. The amplified signals were sampled and digitalized at 80 kHz. The number of action potentials per second was counted by the computer and peristimulus time histograms were calculated using 1 s bin width (for example, see Fig. 2, panel C), displayed in line and digitally stored for off-line analysis.

Iontophoretic drug delivery and experimental data collection were controlled by a PCI-6221 multifunction instrument control and data acquisition board (National Instruments, Austin, TX) placed in a desktop computer and programmed in a LabView environment. Retention and ejection currents were generated using Union-40 iontophoresis pumps (Kation Scientific, Minneapolis, MN).

N-Methyl-D-aspartic acid (NMDA) and (*RS*)-α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid hydrobromide (AMPA) solutions was made at respective concentrations of 25 mM and 10 mM in 100 mM NaCl solution. Their pHs were adjusted to 8.5 using NaOH. Both compounds were purchased from Tocris (Bristol, UK). Trifuoperazine (TFP) was purchased from Sigma (St. Louis, MO) and was dissolved in 100 mM NaCl in a final concentration of 50 mM. Pontamine sky blue dye was from Sigma and was used at 2% in 100 mM NaCl. Microampere iontophoresis was performed using a BAB-501 iontophoresis pump (Kation Scientific, Minneapolis, MN).

NMDA and AMPA were ejected alternately from the multi-barrel electrodes for 5 s in every 2 min using negative currents ranging from 10 to 40 nA. TFP was ejected using positive current of 100 nA. Currents of about 20 nA of opposite polarity were used for all drugs to prevent leakage in between ejection periods. Only cells showing no or a very low basal activity (a few spikes/s at the most) were selected for recording. The control NMDA or AMPA receptor responses were set between 30 and 60 spikes/s.

Recording sites were marked by ejection of pontamine sky blue using 3 µA negative current for 20 min. At the end of the experiment, animals were euthanized with an overdose of chloral hydrate; the brain was removed and immersed in

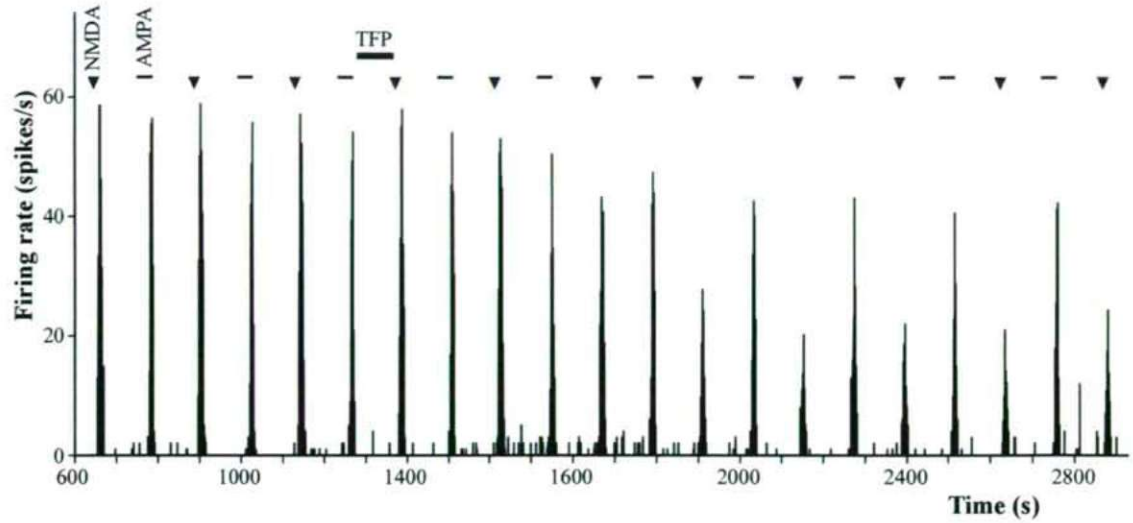

Figure 3. Trifluoperazine (TFP) differentially inhibits NMDA-, and AMPA-induced responses of hippocampal CA1 pyramidal cells. NMDA and AMPA were iontophoresed, alternately, 2 min apart using -45 nA and -20 nA, respectively. TFP was ejected as shown using 120 nA which markedly inhibited responses to either excitatory amino acid analogues. Note the differential effects of TFP on NMDA- versus AMPA-evoked responses. A representative time histogram recording is shown.

10% formalin. Recording locations were histologically verified in 60-µm thin sections counterstained with neutral red. Positions of the Pontamine sky blue marks were established with the stereotaxic atlas of Paxinos and Watson (1998). For example, see Figure 1.

Statistical analysis

Statistical evaluations were performed by using the total number of spikes evoked during each excitation epoch by iontophoretic application of NMDA or AMPA. The background neuronal discharge (or spontaneous activity) was calculated by averaging a 15 s period of ongoing activity preceding and following each excitation epoch, and this value was subtracted from all evoked responses. The total spike number during each excitation epoch was calculated and (Fig. 2, panel C) expressed as a percentage of mean (± S.D.) of the control and compared statistically with the data obtained after iontophoretic TFP application using one-way analysis of variance (ANOVA, with the Bonferroni test for *post hoc* analysis). A P value of <0.05 was considered as significant change. Statistical calculations were performed using a SigmaStat software program.

Results

Pyramidal cells in the hippocampal CA1 region were identified on basis of stereotactic coordinates and typical complex spiking patterns (Fig. 2, panel A) (Kandel and Spencer 1961; Mancillas et al. 1986; Henze et al. 2000; Huang et al. 2010). After finding an adequate neuron, NMDA or AMPA were alternately ejected 2 min apart from one another to evoke cellular firing using ejections currents sufficient to produce a peak firing rate of 30 to 60 spikes/s. Following the establishment of 3 or 4 pairs stable control responses, TFP was iontophoretically delivered using 100 nA for 30 s in between two excitation periods. Responses to NMDA or AMPA were expressed in peristimulus time histograms (Fig. 3) and were quantified by counting the total number of spikes per stimulation period after subtracting the background averaged activity (if any) preceding and following each excitation epoch. Changes in the total number of spikes in NMDA or AMPA responses after TFP application were expressed as percent of their respective controls and pooled over all experiments. At the end of the experiment, recording sites were marked by pontamine sky blue ejection and were verified by histological means (Fig. 1, panel C).

A total of 12 successful experiments were performed in the same number of animals. Adjusting ejection currents for NMDA or AMPA excitation to reach comparable peak heights for both (as seen in Fig. 3) proved to be rather difficult as changing the stimulation intensity of one classes of receptors greatly influenced the responsiveness of the other (data not shown). Iontophoretic application of TFP significantly decreased responses to both NMDA and AMPA (Fig. 3) in 8 of the total 12 pyramidal cells. Maximal inhibition of NMDA- or AMPA-evoked responses were reached 10–16 min after TFP application and remained so during the rest of the approximately 1-h long experiment. Responses to NMDA were decreased to 32 ± 15% of control whereas AMPA responses were decreased to 74 ± 19% (mean ± SD, n=8 for both) of control. Both decreases were significant at $p<0.05$ and $p<0.01$ level, respectively. The ANOVA procedure with the Bonfer-

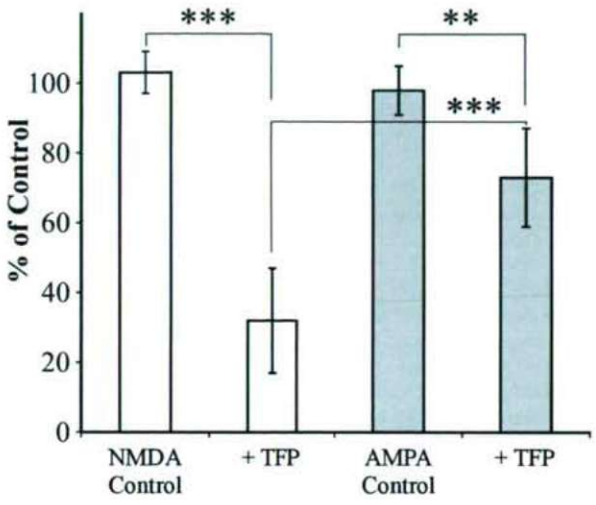

Figure 4. Summary of the effects of trifluoperazine (TFP) on the responses to alternately iontophoresed NMDA and AMPA of hippocampal CA1 pyramidal cells. Note the decrease of the responses in the presence of TFP. Data represent the mean ± SD of 8 experiments. Asterisks denote significant differences by ANOVA (**p< 0.05, ***p< 0.01) as compared to their respective controls or the significant difference between the decrease of NMDA- and AMPA- evoked responses in the presence of TFP. See details in the text.

roni test for *post hoc* analysis revealed a significant difference between the inhibition by TFP of NMDA and AMPA responses. The levels of significance was $p<0.01$ (Fig. 4). The present findings indicate that these two classes of ionotropic glutamate receptors may function in some form of cooperation through a Ca^{2+}/calmodulin-dependent way.

Discussion

We have shown that TFP could significantly decrease responses of hippocampal CA1 pyramidal cells *in vivo* to alternately iontophoresed NMDA and AMPA as compared to their respective controls. Also, inhibition of NMDA responses was significantly greater in the presence of TFP than that of the AMPA responses. Possible explanations for these effects include: (1) TFP prevents the action of tonically released dopamine, noradrenaline, serotonin or ACh (for reviews see (Vizi and Kiss 1998; Lendvai and Vizi 2008)) and/or (2) by binding to calmodulin and protein kinase C (PKC), TFP interferes with the phosphorylation or dephosphorylation of the corresponding ion channels. The former case of a major TFP action mediated through cell surface receptors specialized for a variety of endogenous neurotransmitters is unlikely in the views of most investigators, including ours.

Our observation that selective stimulation of the NMDA-class of the ionotropic glutamate receptors can in fact influence the responsiveness of the AMPA-class of receptors (and *vice versa*) strongly suggests *per se* that there must be an interaction between the function of the two classes of receptors in the hippocampal CA1 pyramidal cells which express calmodulin in large quantities (Palfi et al. 1999, 2002). Most feasibly, this interaction is mediated through second messengers such as CaM K II and PKC. As for the effects of TFP, experimental data show that arginine vasopressin potentiation was blocked by 50 μM TFP, which is consistent with a Ca^{2+}/calmodulin involvement but which might also implicate PKC (Brinton and McEwen 1989). It has also been hypothesized that NMDA receptor activation is positively coupled to adenylyl cyclase *via* Ca^{2+}/calmodulin and are consistent with a role for cyclic AMP metabolism in the induction of NMDA receptor-dependent LTP in area CA1 of the hippocampus. In intact hippocampal slices, TFP blocked the increase in cyclic AMP levels caused by both NMDA application and stimulation of Schaffer collateral fibers. Exposure of hippocampal slices to elevated extracellular potassium to induce calcium influx also caused increased cyclic AMP levels; the increase in cyclic AMP caused by high potassium was also blocked by TFP. (Chetkovich and Sweatt 1993). Inhibition of calmodulin by calmidazolium also produced a differential inhibition of NMDA and KA stimulation of dorsal horn neurons of the spinal cord where NMDA-evoked responses were significantly more inhibited than KA ones (Olah et al. 2007).

Our present experiments may provide evidence *in vivo* for the inhibition by TFP of the second messenger cascade events leading from NMDA receptors *via* Ca^{2+}/calmodulin to AMPA receptors and, in consequence, for the blocking of phosphorylation of AMPA receptors and their sensitization. This blocking, in turn, prevents he AMPA receptors to enable NMDA receptors to become more sensitive to presynaptic glutamate release (or to iontophoretic NMDA ejection in our case) through phosphorylation enzyme activity. In fact, NMDA receptors became less sensitive to ejected NMDA in our experiments after application of TFP and they became more inhibited than AMPA receptors under the same conditions. Based on our experimental data, it is also likely that the function of NMDA receptors itself is, at least in part, dependent on the Ca^{2+}/calmodulin-mediated events.

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There are no explicit length limitations: a normal research article will occupy 4-6 printed pages; reviews might be considerably longer. Authors should submit three sets of the complete manuscript and illustrations, together with a computer disk containing an electronic version of their manuscript. The elctronic file is considered the final material. Both Macintosh and PC versions will be accepted. The disk should be labeled with the date, the first author's name, the file name of the manuscript and the software, disk format and hardware used. Acta Biologica Szegediensis will not return copies of submitted manuscripts and figures. Requests to return original figures will be honored as a courtesy, but cannot be guaranteed. If instructions are not followed, authors will be asked to retype their manuscripts.

Manuscript format

Only good-quality laser printouts will be accepted. All pages should be printed with full double spacing, 2.5 cm margins, and a nonjustified right margin. A standard 12 point typeface (e.g. Times, Helvetica or Courier) should be used throughout the manuscript, with symbol font for Greek letters. Boldface, italics or underlined text should not be used anywhere in the manuscript. Footnotes are not permitted. Each page should be numbered at the bottom as follows:

Page 1. Title page: Complete title, first name, middle initial, last name of each author; where the work was done (authors' initials in parentheses if necessary); mailing address, phone, fax, and e-mail of the corresponding author; a running title of no more than 48 characters and spaces.

Page 2. Abstract: no more than 200 words, followed by 4-6 key words.

Beginning on page 3: Introduction, Materials and Methods, Results, Discussion, Acknowledgments, References, Figure Legends, Tables. Each section should be begun on a new page.

The manufacturer's name and location should be given in parentheses for reagents and instruments. Sources for all antibodies and nucleotide sequences should be indicated. Customary abbreviations in common use need not be defined in the text (e.g. DNA or ATP). Other abbreviations should be defined the first time that they are used. Quantitative results must be presented as graphs or tables and supported by appropriate experimental design and statistical tests. Only SI units may be used. For studies that involve animals or human subjects, the institutional, national or international guidelines that were followed should be indicated.

References

Only work that has been published or is in the press may be referred to. Personal communications should be acknowledged in the text and accompanied by written permission. In the text, references should be cited by name and year, e.g. Bloom (1983) or (Schwarz-Sommer et al. 1990) or (Maxam and Gilbert 1977). In the References, references should be listed alphabetically by first authors (including all co-authors) and chronologically for a given author (beginning with the most recent date of publication). Where the same author has more than one publication in a year, lower case letters should be used (e.g. 1999a, 1999b, etc.). Periods should not be used after authors' initials or abbreviated journal titles (e.g. Acta Biologica Szegediensis should be cited as Acta Biol Szeged). Inclusive page numbers should be used. Examples:

Bloom FE (1983) The endorphins: a growing family of pharmacologically pertinent peptides. Annu Rev Pharmacol Toxicol 23:151-170.

Coons AH (1978) Fluorescent antibody methods. In Danielli JF, ed., General Cytochemical Methods. Academic Press, New York, 399-422.

Maxam AM, Gilbert WA (1977) A new method for sequencing DNA. Proc Natl Acad Sci USA 74:560-564.

Monod J, Changeux J-P, Jacob F (1963) Allosteric proteins and cellular control systems. J Mol Biol 6:306-329.

Schwarz-Sommer Z, Huijser P, Nacken W, Saedler H, Sommer H (1990) Genetic control of flower development by homeotic genes in Antirrhinum majus. Science 250:931-936.

Illustrations

Three complete sets, including a high-quality "original" for publication, must be submitted with the manuscript. The back of each figure or composite plate should be labeled in soft lead pencil, indicating the orientation, the figure number, and the first author's name. The back of the best set should be marked "use for reproduction" or "original". Authors are encouraged to submit digital images of photographs, line drawings or graphs for printing. Most major image editing and drawing/illustrator computer software files (both Macintosh and PC) in TIFF or EPS formats are acceptable. It is particularly important that adequate resolution (at least 300 dpi, preferably 600 dpi) is used in making the original image.

Figure legends

Figures should be numbered consecutively with Arabic numerals. Material in the text should not be duplicated and methods should not be described. The size of scale bars should be indicated when appropriate. The first figure in the text should be referred to as Fig. 1, and so on.

Tables

Tables should be numbered consecutively with Arabic numerals. A brief title should be included above the table. Each table should be printed double spaced, without vertical or horizontal lines, and on a separate sheet. Material in text should not be duplicated and methods should not be described. The first table in the text should be referred to as Table 1, and so on.