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Comparative Study of the Chemical Composition of *Zostera marina* L. and *Zostera nana* Roth from the Black Sea

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Diosmetin, diosmetin-7-0-glucoside, luteolin-7-0-glucoside, four phenolic acids, eight sterols and three groups of glycolipids have been identified in *Zostera marina* and *Zostera nana*. Differences were detected between *Z. marina* in subgenus *Zostera* and *Z. nana* in subgenus *Zosterella*, primarily in the flavonoid and volatile constituents. *Zostera marina* showed only traces of diosmetin-7-0-glucoside; but *Zostera nana* had three flavonoids: diosmetin, diosmetin-7-0-glucoside and luteolin-7-0-glucoside. *Zostera marina* contained the normal parafins but *Z. nana* contained the alkylated benzenes, $Pb(Et)_4$ and $PbMe(Et)_3$.

Introduction

The Black Sea differs significantly from other seas, the main differences being the lower salinity (18‰) and the presence of hydrogen sulphide. Our earlier investigations showed that some Black Sea algae and invertebrates contain terpenoids, characteristic for terrestrial plants, and no halogenated terpenoids (Hadjieva *et al.* 1987, 1989), which we tried to explain as due to the lower salinity. In order to investigate further this phenomenon we undertook a study on the chemical composition of *Zostera marina* L. and *Z. nana* Roth, which are the only seagrasses inhabiting the Black Sea. These higher plants are widespread in other seas and there is a substantial number of publications on their chemical composition. Sulphated flavones have been discovered in many *Zostera* species, including *Z. marina* and *Z. nana* (Harborne 1975, Harborne and Williams 1976, McMillan *et al.* 1980). Some phenolic acids have also been found in seagrasses (Zapata and McMillan 1979, Quackenbush *et al.* 1986, Buchsbaum *et al.* 1990). The total fatty acid composition of *Z. marina* and *Z. nana* was investigated (Dembitsky *et al.* 1991) but there is no data on the composition of the individual lipid classes.

Materials and Methods

Plant material

Zostera marina and *Z. nana* samples were collected in September 1992 from the coastal area near the village of Ravda, at a depth of 3 m. The samples were immediately dipped in ethanol and transported to the laboratory.

Isolation and analysis

After three extractions with ethanol the combined extracts were concentrated, diluted with water and extracted consecutively with hexane, dichloroethane, ethyl acetate and n-butanol. Half of the hexane extract was evaporated and the residue mixed with water and subjected to steam distillation. The volatile compounds were extracted from the distillate with ether. They were analyzed by gas chromatography/mass spectrometry (GC/MS) (30 m fused silica capillary column, coated with SPB-1 and connected with JEOL JGC – 20K gas chromatograph, directly coupled to a JEOL JMS D-300 mass spectrometer, temperature programme 60–280° rising at 6° min^{-1}).

The remaining part of the hexane extract was subjected to column chromatography (CC) on silica gel, eluted with increasing concentrations of acetone in hexane. The sterol fraction obtained was analysed further by GC and MS.

The dichloroethane extract was subjected to CC on silica gel, eluted with increasing concentrations of methanol in dichloroethane. Flavonoid aglycones, steryl glycosides and individual groups of glycolipids were isolated. Steryl glycosides were hydrolyzed by boiling with 2N HCl and the sterols obtained investigated by mass spectrometry. Different glycolipid groups were subjected to transesterification with acetyl chloride in methanol (Christie 1973) and the fatty acid methyl esters obtained analyzed by gas chromatography (Pye Unicam gas chromatograph with 30 m fused silica capillary column, coated with Silar).

The ethyl acetate extract was subjected to CC on silica gel and elution with increasing concentrations of methanol in dichloroethane. The flavonoid glycosides obtained were subjected to acidic hydrolysis with boiling 2N HCl (2 h), the aglycones obtained were

extracted with ethyl acetate and sugars – with n-butanol.

Phenolic acids in the butanol extract were identified by thin layer chromatography (TLC) and comparison with authentic samples.

Results and Discussion

Volatile constituents

Until now there was no information concerning the presence of volatile constituents in aquatic higher plants. Such compounds are usually extracted with unpolar solvents and for this reason we subjected only the hexane extracts from both *Zostera* species to steam distillation. The mixtures of volatile compounds obtained were investigated further by GC/MS and the results obtained are summarized in Table I.

There are significant differences in the composition of volatile compounds in the two plants. In *Zostera marina* they consist mainly of normal parafins. The presence of methyl palmitat in both plants is of special importance, because fatty acid methyl esters are rarely found in plants and algae. Methanol had not been used in this investigation, which excludes the formation of artifacts.

In *Zostera nana* we identified some unusual constituents including the alkylated benzenes, $Pb(Et)_4$ and $PbMe(Et)_3$. All these compounds are gasoline constituents with the exception of $PbMe(Et)_3$, which could be produced by biological oxidation of tetraethyl lead, followed by decarboxylation. These compounds probably originate from the gasoline pollution in the Black Sea. The seagrasses grew together in the collection area and the presence of gasoline constituents only in *Z. nana* showed that these compounds were selectively extracted from the sea water and/or stored in this plant. If this is so, *Z. nana* could be used for the monitoring of gasoline pollution in the sea, as well as for the purification of sea water.

Table I. Composition of volatile constituents from *Zostera marina* L. and *Zostera nana* Roth. The percentage figures refer to the ion current generated by the compound in the mass spectrometer.

Compound	*Zostera marina*	*Zostera nana*
Propyl benzene	–	>1
Methylethyl benzene	–	2
Trimethyl benzene	–	8
o- or m-Xylene	–	17
p-Xylene	–	13
$C_{11}H_{24}$	1	–
$C_{12}H_{26}$	12	–
$C_{13}H_{28}$	1	1
$C_{14}H_{30}$	6	1
$C_{15}H_{32}$	3	–
$C_{16}H_{34}$	>1	–
$C_{17}H_{36}$	4	3
$C_{18}H_{38}$	>1	–
$C_{19}H_{40}$	3	–
$C_{22}H_{46}$	>1	–
$C_9H_{19}Cl$	1	1
$CH_3(C_2H_5)_3Pb$	–	2
$(C_2H_5)_4Pb$	–	6
$C_{17}H_{35}OH$	>1	–
Methyl palmitate	9	>1

Flavonoids

The TLC analysis of dichloroethane and ethyl acetate extracts from both seagrasses showed some differences in their flavonoid composition. Flavonoids predominated in *Z. nana*, where we identified diosmetin, diosmetin-7-0-glucoside and luteolin-7-0-glucoside. They were identified on the basis of their spectral behaviour, acid hydrolyzis and comparison with authentic samples. In *Z. marina* only traces of diosmetin-7-0-glucoside were detected. This difference in the flavonoid concentrations of the two seagrasses is in agreement with the stronger antibacterial activity of *Z. nana* extracts (Stefanov *et al.*, unpublished results).

Diosmetin and luteolin have been found earlier in many seagrasses, including *Zostera marina* and *Z. nana*, but almost exclusively in a conjuagated form as sulphates (Harborne and Williams 1976, McMillan *et al.* 1980). Harborne (1975) suggested that some plants inhabiting the sea or coastal area may adapt to the inorganic salts in the environment and the conjugation of phenolic compounds as sulphates might be one way for transfer, inactivation or storage of inorganic sulphates. According to our knowledge we found for the first time underivatized flavonoid glycosides in seagrasses, while our search for flavonoid sulfates showed their absence in *Z. marina* and *Z. nana*, as indicated by Harborne (1975). This is in agreement with the hypothesis of Harborne (1975), because the salinity of Black Sea is only half that of other seas (about 18‰ against 34‰) and there must be a lower pressure for inactivation of sulphates. This is also in agreement with our earlier hypothesis that the absence of halogenated terpenoids in the Black Sea organisms investigated is related to the low salinity of the Black Sea (Hadjieva *et al.* 1987, 1989).

Phenolic acids

Caffeic, rosmarinic, chlorogenic and ferulic acids were identified in the butanolic extracts of both seagrasses by thin layer chromatography. These acids have been found earlier in the plants investigated (Zapata and McMillan 1974, Quackenbush *et al.* 1986, Buchsbaum *et al.* 1990).

Sterols

There are no data on the sterol composition of the Zosteraceae. We isolated free sterols from *Z. marina*

and *Z. nana* and investigated them by gas chromatography and mass spectrometry. The results obtained are summarized in Table II.

It is evident that independently of their marine habitat both seagrasses investigated have a sterol composition, characteristic for terrestrial plants, sitosterol being the main sterol constituent. In *Z. nana* the C-24 alkylation is weaker than in *Z. marina*, but C-22 dihydrogenation is more intense. The composition of steroidal glycosides appeared to be close to that of the free sterols.

Glycolipids

Glycolipids were isolated from the dichloroethane extract and separated by column chromatography into three main groups – monogalactosyl diglycerol (MGDG), digalactosyl diglycerol (DGDG) and sulphoquinosil diglycerol (SQDG). The fatty acid composition of these glycolipid groups, isolated from *Z. marina*, was investigated after treatment with acetyl chloride in methanol and gas chromatographic investigation of the methyl esters obtained. The results obtained are summarized in Table III.

Table II. Free Sterols from *Zostera marina* and *Zostera nana*.

Sterols	*Zostera marina*	*Zostera nana*
	%	%
Stanols	trace, mainly sitostanol	trace, mainly sitostanol
Δ^{22}-Cholesterol	3.6	7.0
Cholesterol	3.6	7.0
Campesterol	2.1	1.7
Stigmasterol	5.4	11.3
Sitosterol	87.7	75.0
i-Fucosterol	1.0	4.7

Table III. Fatty acid composition of glycolipids in *Zostera marina*.

Fatty acid	MDGD	DGDG	SQDG
	%	%	%
14 : 0	4.8	3.0	–
16 : 0	20.0	27.0	14.1
16 : 1	4.8	trace	trace
16 : 3	15.0	6.0	4.0
18 : 0	3.3	trace	trace
18 : 1	3.3	trace	trace
18 : 2	13.3	24.0	10.1
18 : 3	31.6	39.0	68.4
18 : 4	–	–	trace
20 : 1	–	trace	trace
20 : 4	1.6	trace	trace
20 : 5	1.6	trace	trace

It is evident that the fatty acid composition of the glycolipids from *Z. marina* is closer to that of terrestrial plants than to algae, where polyunsaturated fatty acids predominate.

On the basis of sterol and fatty acid composition we can conclude that in seagrasses the metabolism is closer to that of terrestrial higher plants than to algae – *e. g.*, it is determined by the genotype and not by the ecotype.

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References

Buchsbaum, N., T. F. Short and C. P. Donald. 1990. Phenolic – nitrogen interactions in eelgrass, *Zostera marina* L.: possible implications for disease resistance. *Aquatic Botany 37*: 291–297.

Christie, W. W. 1973. The preparation of derivatives of lipids. *In*: *Lipid Analysis*. Pergamon Press. Oxford. pp. 85–102.

Dembitsky, V. M., E. E. Penchenkina-Shubina and O. A. Rozentsvet. 1991. Glycolipids and fatty acids of some seaweeds and marine grasses from the Black Sea. *Phytochemistry 30*: 2279–2283.

Hadjieva, P., S. Popov, B. Budevska, A. Dyulgerov and St. Andreev. 1987. Terpenoids from a Black Sea Bryozoan *Conopeum seuratum*. *Z. Naturforsch. 42c*: 1019–1022.

Hadjieva, P., M. Konaklieva, S. Andonova, S. Popov, K. Beremlyski and S. Dimitrova-Konaklieva. 1989. *Enteromorpha* species as terpenoid source for some Black Sea invertebrates. *V. Int. Conf. Chem. Biotechn. Biol. Active Nat. Prod.* (Varna, Bulgaria) *2*: 89–93.

Harborne, J. B. 1975. Flavonoid sulphates: A new class of sulphur compounds in higher plants. *Phytochemistry 14*: 1147–1155.

Harborne, J. B. and C. A. Williams. 1976. Occurence of sulphated flavones and caffeic acid esters in members of the fluviales. *Biochem. Syst. Ecol. 4*: 37–41.

McMillan, C., O. Zapata and L. Escobar. 1980. Sulphated phenolic compounds in seagrasses. *Aquatic Botany 8*: 267278.

Quackenbush, R. C., D. Bunn and W. Lingren. 1986. HPLC determination of phenolic acids in the water soluble extract of *Zostera marina* L. *Aquatic Botany 24*: 83–89.

Zapata, O. and C. McMillan. 1979. Phenolic acids in seagrasses. *Aquatic Botany 7*: 307–317.