

NON-ENZYMATIC ANTIOXIDANTS CONTENT IN SEVERAL SPECIES COLLECTED FROM SALT MARSHES FROM DOBROGEA

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Abstract: Salt tolerant plants have been the subject of different studies about the mechanisms of salt tolerance at biochemical level. They developed different mechanisms to cope with abiotic stress effects, also by increasing antioxidant activity. Results of this study include polyphenols and flavonoids contents from several species: *Plantago lanceolata* L., *P. coronopus* L., *P. maritima* L. (*Plantaginaceae*), and *Spergularia media* (L.) C. Presl (*Caryophyllaceae*), during vegetative and flowering time. The plant material has been collected from salt marshes located in Dobrogea region (Sulina, Murighiol and Histria). The level of non-enzymatic antioxidant compounds measured in both vegetative and flowering stages in almost all locations generally show higher values for *Plantago* species compared with *Spergularia*. Within these compounds, the polyphenols biosynthesis is more noticeable than that of flavonoids. The level of total polyphenols was higher or unchanged in salt tolerant plants during the vegetative phase compared with flowering stage. Contrarily, the flavonoids content was lower during the vegetative phase in *P. coronopus*, while in *P. maritima* and *Spergularia*, flavonoids content was found to be slightly higher than in plants during flowering.

INTRODUCTION

Salt tolerant plants (many of them being designated as halophytes, *sensu stricto*) are equipped with well-defined adaptive mechanisms that enable them not only to withstand periodical high salinity, but also to complete their entire lifecycles at high salinities (Flowers *et al.*, 1986). These species show immense diversity in terms of occupied habitats, ecological spectra and ability to tolerate the abiotic stress conditions, and have a very large distribution among the taxa of flowering plants (Grigore, 2012; Grigore *et al.*, 2014). All these factors imply a serious issue when trying to find an operational definition of halophytes and trying to discriminate between different species with respect to their salt tolerance (Grigore *et al.*, 2010). It is evident from the existing experimental data that salt stress affects the integrity of cellular membranes, activities of enzymes and the functioning of the plant photosynthetic apparatus (Serrano *et al.* 1999). An important cause of this damage is production of reactive oxygen species (ROS) (Smirnov, 1993). ROS are routinely generated during normal plant metabolic processes. ROS are highly reactive and, in the absence of a protective mechanism in plants, can cause serious damage to different aspects of cell structure and function (Jithesh *et al.*, 2006). Halophytes are known for their ability to withstand and quench these toxic ROS, or decomposing peroxides since they are equipped with a powerful antioxidant system that includes enzymatic and non-enzymatic components (Ksouri *et al.*, 2007). Non-enzymatic components such as polyphenols and flavonoids there are generally stimulated in response to biotic/abiotic stresses (Dixon and Paiva, 1995; Naczki and Shahidi, 2004) such as salinity (Navarro *et al.*, 2014). Their presence in the diet appears to be associated with lower occurrence and lower mortality rates of several human diseases (Andersan *et al.*, 2001). Halophytes are naturally salt-tolerant plants that may be potentially useful for economical (oilseed, forage, production of metabolites) applications (Parvaiz and Satyawati, 2008).

The Earth's total surface area covers about 13.2 billion ha, but no more than 7 billion ha are arable and 1.5 billion are cultivated (Massoud, 1981). Of the cultivated lands, about 340 million ha (23%) are saline (salt-affected) and another 560 million ha (37%) are sodic (sodium-affected) (Tanji, 2002). Salinity is one of the most severe environmental factors limiting the productivity of agricultural crops, because most crops are sensitive to salinity induced by high concentrations of salts in the soil (Pitman and Läuchli, 2002). The cost of salinity to agriculture was estimated to be about 12 billions USD per year (Ghassemi *et al.*, 1995), but perhaps this value will be greater, since it is expected that soil salinity to increase continuously.

The aim of this work is to determine the non-enzymatic antioxidants evidenced by the contents of total polyphenol and flavonoids in four salt tolerant species during vegetative and flowering stages. Plant material has been collected from saline habitats located in South-East of Romanian (Dobrogea region).

MATERIAL AND METHODS

Plant material

Plant species have been collected during vegetative and flowering stages, during April-May and June-July of 2013 (corresponding to vegetative stage and anthesis, respectively). Non-enzymatic antioxidants contents of salt tolerant

plants has been investigated; plants have been collected from several saline habitats located in South-East of Romanian (Dobrogea region): Sulina, Murighiol and Histria. The collected species were: *Plantago coronopus* L., *Spergularia media* (L.) C. Presl (from Sulina), *P. maritima* L., *S. media* (from Histria), *P. lanceolata* L. and *S. media* (from Murighiol, around the "Saraturile" Lake). The species collected from Sulina originate from two habitats: a littoral area (designated as "Z I") and a habitat located at 1000 m away from littoral line (designated as "Z II"). It was hypothesized in this case that the same species growing in different ecological conditions (especially soil humidity that influences soil salinity) is susceptible to have a different response in relation to abiotic factors. All species, apart from *P. lanceolata* are considered true halophytes (Grigore, 2012), while *P. coronopus* is regarded as a psammo-halophyte, due to its adaptations and habitats where it vegetates (Grigore *et al.*, 2012). Moreover, *P. maritima* and *Spergularia* are succulent-leaves plants (Grigore *et al.*, 2014; Grigore, unpublished data). Traditionally, *P. lanceolata* is not regarded as typical halophyte, although there are plenty of data showing that it also vegetates in saline environments (Grigore, 2012 and references therein) and even in high salinity conditions (Piernik, 2012); it is a supporting halophyte (Țopa 1954; Andrei and Serbănescu, 1965) and neohalophyte (Bucur *et al.*, 1961).

Total polyphenols contents assay

The total polyphenols content was determined through a slightly modified Folin-Ciocalteu method (Singleton *et al.*, 1999). Folin-Ciocalteu reagent was added to appropriately diluted samples and mixed thoroughly. After four minutes, 15% Na₂CO₃ was added. After two hours, the absorbance of resulted coloured solution was determined at 765 nm, against the blank (distilled water). The amount of the total polyphenols content was expressed as mg gallic acid equivalents (mg GAE/g DW) (R²=0.99).

Total flavonoids content assay

The flavonoids content was measured following a spectrophotometric method (Dewanto *et al.*, 2002). Briefly, methanol extracts were appropriately diluted with distilled water. Initially, 5% NaNO₂ solution was added to each test tube; after five minutes, 10% AlCl₃ solution was added and then 1.0MNaOH was added after six minutes. Finally water was then added to the test tube and mixed vigorously. Absorbance of resulting pink-coloured solution was read at 510 nm against the blank (distilled water). Flavonoids content was expressed as mg catechin equivalents (CE) per g of dry weight (mg CE/g DW) (R²=0.98). Each sample has been used in three replicates. Three readings were taken for each sample and the result averaged. To calculate and to graphically represent the statistical indices, the Microsoft Office Excel 2003 software of Windows XP operating system was used.

RESULTS AND DISCUSSION

The figures 1 and 2 show the heterogeneous responses of non-enzymatic compounds represented by total polyphenols and flavonoids contents in four salt tolerant species collected from three different saline areas, located in South-East of Romania (Dobrogea).

The total polyphenols content in all species taken into study from these areas registered a high variability. Thereby, the total polyphenols content in species collected from Dobrogea region show the highest and lowest values in *P. coronopus* (ZI) and *S. media* respectively, both in vegetative and flowering phase. Thus, in plants during vegetative phase the total polyphenol content, calculated as gallic acid equivalent (GAE) ranged from 4.33-37.56 mg GAE/g DW while during flowering phase, it varied from 4.11- 28.75 mg GAE/g DW (Fig. 1).

In *P. maritima* collected from Histria, the total polyphenols content show no significant differences during vegetative and flowering phases. Our results are not in accordance with those obtained by Medinim *et al.*, 2011 who found for halophyte *Limonium densiflorum* collected from one of semi-arid region of Tunisia that the polyphenols content was lower during vegetative stage than during flowering.

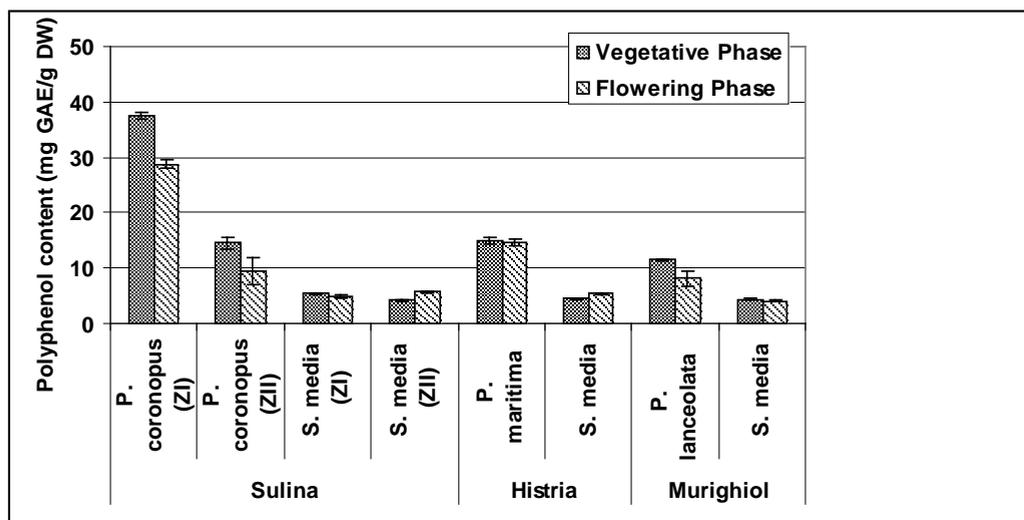


Fig. 1. Total polyphenols content in several species from different salt marshes from Dobrogea

Previous studies (Ksouri *et al.*, 2008, Medini *et al.*, 2011) have shown that in plants phenolic content and antioxidant activities depend on biological factors (species, organ, and physiological development stage) and abiotic factors. On the other hand, Aghae *et al.* (2013) showed that the total phenol content of extract of *Tanacetum balsamita* was not dependent by growing stages (vegetative, flowering and after-flowering stages), but it was rather influenced by the different methods used for drying (microwave, oven, sun and shade) plant material.

In regard to *P. coronopus* collected from Sulina, a difference in polyphenol content values was found in samples from two different habitats: littoral area (Z I) and the habitat located at 1000 m away from littoral (Z II). The results indicated that polyphenol content was 1.6 fold higher in *P. coronopus* leaves collected from littoral area (Z I) as compared to more inland area (ZII) (Fig. 1). Perhaps the salt stress is more intense in this region, close to the littoral, as the salinity values and sun exposure may be higher than towards inland areas, less exposed to harmful abiotic factors. This could explain why species from littoral area (Z I) have a higher concentration of total polyphenols that may be involved in the protection of the cells against the oxidative stress. In addition, attention should be paid on the higher content of polyphenols in leaves from plants sampled in vegetative phase (both littoral and inland areas). Perhaps the early stages of development are more sensitive to abiotic factors than mature individuals; for instance, it is well known that younger tissues of plants (especially in seedlings) are more sensitive to salinity exposure (Waisel, 1972).

The content of polyphenols in *S. media* collected from Sulina (both habitats), Histria and Murghiol remains mainly unchanged, when plants were collected from different habitats in Sulina, and other collecting areas from Histria and Murghiol. Moreover, the polyphenols biosynthesis was found to be similar in plants sampled during vegetative and flowering stages. *Spergularia media* is a C₃ halophyte, with succulent leaves (Grigore *et al.*, 2014) and perhaps the polyphenols synthesis acts as a constitutive trait and not as an inducible one, since the

polyphenols accumulation follows a linear trend. In *P. maritima*, there are not significant differences in polyphenols synthesis in plants collected in vegetative and flowering stages; in the case of *P. lanceolata*, the phenols content is slightly higher in vegetative phase.

There is some evidence of the induction of phenolic metabolism in plants as a response to multiple stresses (Michalak, 2006). For instance, during heavy metal stress, phenolic compounds can act as metal chelators and on the other hand, phenolics can directly scavenge molecular species of active oxygen (Sharma *et al.*, 2011).

Flavonoids content usually show a more uniform biosynthesis within investigated species (Fig. 2). In the vegetative phase, the lowest content of flavonoids, expressed as mg catechin/g DW was observed for *S. media* (collected from Murghiol) (3.62 mg catechin/g DW) whereas the highest amount was recorded for *P. maritima* (8.03 mg catechin/g DW). On the other hand, in the flowering phase, the values of flavonoids ranged between 8.07 and 3.85 mg catechin/g DW in *P. coronopus* (ZI) and *S. media* (sampled from Sulina), respectively. Interestingly, flavonoids content was recorded to be higher in vegetative phase than during flowering in *S. media* (Sulina - both collecting points and Histria), and in *P. maritima*. In *P. lanceolata*, the differences values from the two different stages are insignificant.

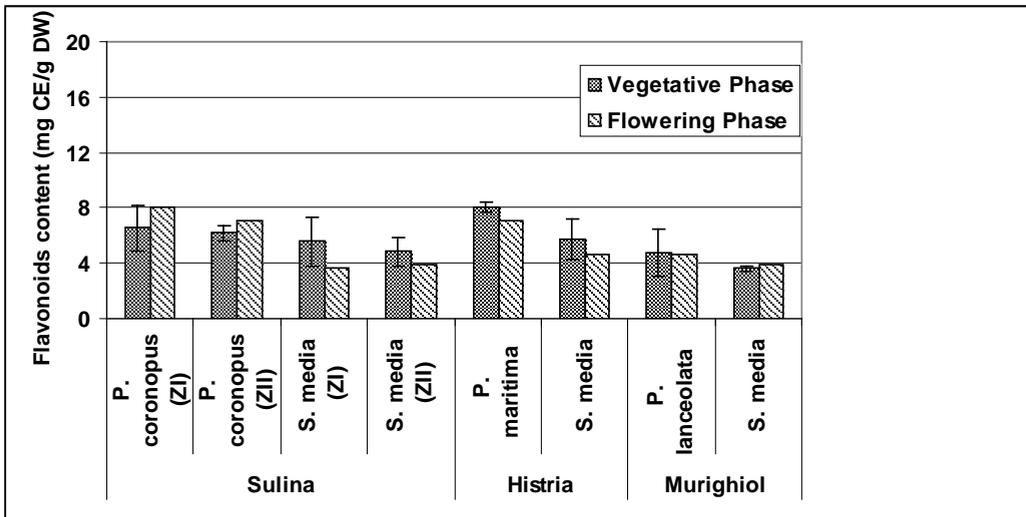


Fig. 2. Flavonoids content in several species from different salt marshes from Dobrogea

It is known that stress condition caused an accumulation of secondary metabolites like flavonoids and thus both polyphenol and flavonoid contents have an important role in plant defense mechanisms (Dixon and Paiva, 1995).

Flavonoids usually accumulate in the plant vacuole as glycosides, but they also occur as exudates on the surface of leaves and other aerial plant parts. One of the most actively studied properties of flavonoids is their protection against oxidative stress (Parvaiz and Satyawati, 2008).

Since different stresses have in common the generation of reactive oxygen species (Mittler, 2006), it has been postulated that flavonoids are synthesized to effectively counter the stress-induced oxidative damage (Di Ferdinando *et al.*, 2012). Flavonoids may act as antioxidant factors by both preventing the generation of ROS (through their ability to chelate transition

metal ions such as Fe and Cu - Brown *et al.* 1998; Agati and Tattini 2010) and scavenging ROS when formed (Ryan *et al.*, 2002; Tattini *et al.*, 2004; Agati *et al.*, 2007; Jaakola and Hohtola, 2010).

Nevertheless, our results could suggest that there is no clear correlation between flavonoids biosynthesis and plant species, in terms of occupied habitat or taxonomical inclusion. Perhaps there is a correlation with polyphenols content and enzymatic activity, considered as a first line of defense against oxidative stress; in addition, there is an inverse tendency in *P. coronopus*, which accumulates flavonoids in higher content during flowering stage, and *vice versa* in the case of polyphenols biosynthesis.

Our previous study (Ivan and Oprica, 2013), reported a close value regarding the contents of total phenolic and flavonoids, correlated with special environmental condition.

Recent studies (Ksouri *et al.*, 2012; Buhmann and Papenbrock, 2013) have shown the potential of halophytes as a source of valuable secondary metabolites with likely economic value.

However, there is no clear and strong correlation between increasing the concentration of valuable secondary metabolites and the antioxidant capacity in plants subjected to changes in salinity concentration (Boestfleisch *et al.*, 2014). In this context, further investigation will bring new insights in respect to capacity of salt-tolerant plants to produce important secondary metabolites with proven economic value.

CONCLUSIONS

The results revealed that in all investigated *Plantago* species, the polyphenol content was higher than in *Spergularia*, both in vegetative and flowering phases; the highest value was recorded for *P. coronopus*, collected from littoral area, a value significantly higher than in the same species, but collected from inland area. This could suggest that here is an inducible response, in contrast with *Spergularia*, where the uniformity of recorded values indicates a possible constitutive way of response to abiotic factors. Flavonoids biosynthesis shows no clear pattern of biosynthesis; in *P. coronopus*, its value is lower during vegetative stage, while in *P. maritima*, *P. lanceolata*, and *Spergularia*, its value is higher during the vegetative phase.

Overall, responses of non-enzymatic compounds (represented by total polyphenols and flavonoids contents) in analyzed salt tolerant plants seems to be dependent on species, developmental stage and assumed ecological conditions of habitats where from plant material has been collected.

REFERENCES

- Aghae M., Alizadeh M., Saadatian M., Sajad R., K. Jangjou, (2013). Effects of growing stages and drying methods on quality of shahsparam (*Tanacetum balsamita* L.). In: Intl J Agri Crop Sci., 5(2): 168-172.
- Agati G., Tattini M., (2010): *Multiple functional roles of flavonoids in photoprotection*. New Phytol., **186**:786–793.
- Agati G., Matteini P., Goti A., Tattini M., (2007): *Chloroplast-located flavonoids can scavenge singlet oxygen*. New Phytol., 174: 77-89.
- Andersan K.J., Teuber S.S., Gobeille A., Cremin P., Waterhouse A.L., Steinberg F.M., (2001): *Walnut polyphenolics inhibit in vitro human plasma and LDL oxidation*. J. Nutr. 131: 2837-2842.
- Andrei M., Serbanescu Gh., (1965): *Contribuții la cunoașterea florei și vegetației de la Lacul Sărat – Brăila*. An. Șt. Univ. București, seria Științele Naturii-Biologie, **14**: 65-79.
- Boestfleisch C., Wagenseil N. B., Buhmann Anne K., Seal C. E., Wade E. M., Muscolo A., Papenbrock J., (2014): *Manipulating the antioxidant capacity of halophytes to increase their cultural and economic value through saline cultivation*, AoB PLANTS, 6 : plu046doi: 10.1093/aobpla/plu046.
- Brown J.E., Khodr H., Hider R.C., Rice-Evans C.A., (1998): *Structural dependence of flavonoid interactions with Cu²⁺ ions: implication for their antioxidant properties*. Biochem J., **330**:1173–1178.

- Bucur N., Dobrescu C., Turcu GH., Lixandru GH., Tesu C.,** (1961): *Contribuții la studiul halofitiei plantelor din pășuni și fânețe de sărătură din Depresiunea Jijia-Bahlui* (partea a III-a). Stud. și Cerc.(Biol. și Șt. Agr.), Acad. R.P.Române, filiala Iași, **12**, 1: 169-190.
- Dewanto V., Wu X., Adom K.K., Liu R.H.,** (2002): *Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity*, J. Agric. Food Chem., **50**: 3010-3014.
- Di Ferdinando M., Brunetti C., Fini C., Tattini M.,** (2012): *Flavonoids as antioxidants in plants under abiotic stresses*. In Abiotic stress responses in plants: metabolism, productivity and sustainability, ed. by Ahmad P., Prasad M. N. V., Springer Science+Business Media.
- Dixon R.A., Paiva N.,** (1995): *Stress-induced phenylpropanoid metabolism*, Plant Cell **7**: 1085-1097.
- Flowers T.J., Hajibagheri M.A., Clipson N.J.W.,** (1986): *Halophytes*. Quart. Rev. Biol., **61**: 313-337.
- Ghasemi F., Jakeman A. J., Nix H. A.,** (1995): *Salinisation of land and water resources: human causes, extent, management and case studies*. UNSW Press, Sydney, Australia, and CAB International, Wallingford, UK: 1-526.
- Grigore M.N.,** (2012): *Romanian Salt Tolerant Plants. Taxonomy and Ecology*. Edit. Tehnopress, Iasi.
- Grigore M.N., C. Toma, M. Boscaiu,** (2010): *Dealing with halophytes: an old problem, the same continuous exciting challenge*. An. Șt. Univ. „Al. I. Cuza”, s. II.a. Biol. Veget., **56** (1): 21-32.
- Grigore M.N., Toma C., Boscaiu M., Zamfirache M.M., Ivănescu L.,** (2012): *Anatomical and ecological observations on psammo-halophytes species (Eastern part of Spain)*. Lucr. Șt. (Horticultură), USAMV „Ion Ionescu de la Brad”, Iași, **55** (2): 19-24.
- Grigore M.N., Ivănescu L., Toma C.,** (2014): *Halophytes. An integrative anatomical study*. Springer, Cham, Heidelberg, New York, Dordrecht, London.
- Ivan M. A., Zamfirache M. M., Grigore M. N., Oprică L.,** (2012): *Determination of antioxidant enzymatic activity in several halophytes from Dobrogea area*, An. Șt. Univ. „Al. I. Cuza”, secț. Genetică și Biol. Mole., **12** (3): 47-52.
- Jitesh M.N., Prashanth S.R., Sivaprakash K.R., Parida A.K.,** (2006): *Antioxidative response mechanisms in halophytes: their role in stress defence*. Journ. of Genetics, **85**(3): 237-254.
- Ivan M., Oprica L.,** (2013): *Study of polyphenols and flavonoids contents of some halophytes species collected from Dobrogea region*, Bulletin of the Transilvania University of Brasov, Series II: Forestry, Wood Industry, Agricultural Food Engineering, **6** (55) No.2, 121-128.
- Jaakola L., Hohtola A.,** (2010): *Effect of latitude on flavonoid biosynthesis in plants*. Plant Cell Environ., **33**:1239–1247.
- Ksouri R., Megdiche W., Debez A., Falleh H., Grignon C., Abdely C.,** (2007): *Salinity effects on polyphenol content and antioxidant activities in leaves of the halophyte Cakile maritima*, Plant Physiology and Biochemistry **45**: 244-249.
- Ksouri R., Megdiche W., Falleh H., Trabelsi N., Boulaaba M., Smaoui A., Abdely C.,** (2008): *Influence of biological, environmental and technical factors on phenolic content and antioxidant activities of Tunisian halophytes*, C. R. Biol., **33**: 865–873.
- Massoud F. I.,** (1981): *Salt affected soils at a global scale and concepts for control*. FAO Land and Water Develop. Div., Tech. Paper, Rome, Italy: 1-2.
- Faten Medini F., Ksouri R., Hanen Falleh, Wided Megdiche, Najla Trabelsi, Chedly Abdely,** **2011**, *Effects of physiological stage and solvent on polyphenol composition, antioxidant and antimicrobial activities of Limonium densiflorum*, Journal of Medicinal Plants Research, **5**(31), 6719-6730.
- Michalak, A.,** (2006): *Phenolic compounds and their antioxidant activity in plants growing under heavy metal stress*. Pol. J. Environ. Stud., **15**:523–530.
- Mittler R.,** (2006): *Abiotic stress, the field environment and stress combination*. Trends Plant Sci., **11**:15–19.
- Naczk M., Shahidi F.,** (2004): *Extraction and analysis of phenolics in food*, J. Chromatogr.A **1054**: 95-111.
- Navarro J.M., Flores P., Garrido C., Martinez V.,** (2006): *Changes in the contents of antioxidant compounds in pepper fruits at ripening stages, as affected by salinity*, Food Chem. **96**: 66-73.
- Parvaiz A, Satyawati S.,** (2008): *Salt stress and phyto-biochemical responses of plants-a review*. Plant Soil Environ **54**:89-99.
- Piernik A.,** (2012): *Ecological pattern of inland salt marsh vegetation in Central Europe*. Wydawnictwo Naukowe, Uniwersytetu Mikolaja Kopernika, Torun.
- Pitman M. G., Läuchli A.,** (2002): *Global impact of salinity and agricultural ecosystems*. In: Läuchli A & Lüttge U (Eds.) Salinity: Environment-Plants-Molecules. Kluwer Academic Publishers, New York, Boston, Dordrecht, London, Moscow: 21-5.
- Ryan K.G., Swinny E.E., Markham K.R., Winefield C.,** (2002): *Flavonoid gene expression and UV photoprotection in transgenic and mutant Petunia leaves*. Phytochemistry, **59**:23–32.
- Serrano R., Mulet J., Rios G., Marquez J., de Larrinoa I., Leube M., Iratxe M., Amparo P. A., M. Proft, Roc Ros, C. Montesinos** (1999): *A glimpse of the mechanisms of ion homeostasis during salt stress*. J. Exp. Bot. **50**, 1023–1036.

- Sharma P., Jha A.B., Dubey R.S.**, (2011): *Oxidative stress and antioxidative defense systems in plants growing under abiotic stresses*. In *Handbook of plant and crop stress*, third edition, ed. by Pessaraki M., CRC Press, Taylor&Francis Group, Boca Raton, London, New York, pp. 89-138.
- Singleton V.L., Orthofer R., Lamuela-Raventos R.M.**, (1999): *Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent*, *Methods Enzymol.*, 299:152-178.
- Smirnoff N.**, (1993): *The role of active oxygen in the response of plants to water deficit and desiccation*. *New Phytol.* **125**, 27–58.
- Tanji K. K.**, (2002): *Salinity in the soil environment*. In: *Salinity: Environment-Plants-Molecules*, ed. by Läuchli A., Lüttge U., Kluwer Academic Publishers, New York, Boston, Dordrecht, London, Moscow: 21-51.
- Tattini M., Galardi C., Pinelli P., Massai R., Remorini D., Agati G.**, (2004): *Differential accumulation of flavonoids and hydroxycinnamates in leaves of Ligustrum vulgare under excess light and drought stress*. *New Phytol.*, **163**:547–561;
- Topa E.**, (1954): *Vegetatia terenurilor sarate din R. P. Romana*, *Natura*, **6** (1) : 57-76.
- Waisel Y.**, (1972): *Biology of halophytes*. Academic Press, New York and London.

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