Enzymatic Activity and Non-enzymatic Antioxidants Content in Several *Plantago* Species (from Valea Ilenei Nature Reserve), During Different Phenophases

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Traditional use of Plantago species as herbal remedies is based on their anti-toxic, antimicrobial, anti-inflammatory, anthelmintic, diuretic properties. Plantain species are widely used as alternative therapeutic tools for the prevention or treatment of many diseases. The aim of this study is to evaluate the enzymatic activity (superoxide dismutase and peroxidase) and non-enzymatic antioxidants (total polyphenols and flavonoids) of three Plantago species (P. lanceolata L., *P. media* L. *and P. schwarzenbergiana* Schur*) collected in different phenophases from Valea Ilenei (Iasi, Romania) nature reserve.*

Key words: Plantago, total polyphenols, flavonoids, enzymes

Plantaginaceae s. l. is a widespread family that comprises about 90 genera and 1900 species mainly distributed in temperate regions [1]. *Plumbaginaceae* constitute a well-represented cosmopolitan family in the temperate zones of the Northern Hemisphere and showing preferences for arid or saline, often coastal, environments [2]. Some *Plantago* species, such as *P. lanceolata* L. and *P. major* L. are almost ubiquitous weeds, and, in some cases, agricultural pests [3]. Many *Plantago* species are halophytic [4-6]. In Romania, there are 16 confirmed species of *Plantago* [7]; an extended characterization of *Plantago* species from Romania has been conducted by Ianovici et al. (2010) [8]. Plantain (*Plantago* sp.) plants include diverse group of species differing in salt tolerance [5], some of which are adapted to live in saline wetlands, which makes the genus *Plantago* a good model for comparative studies on the responses salinity stresses [9].

Leaves of *Plantago* species have astringent, anti-inflammatory, antioxidant, anti-fungal, anti-cancer, antibacterial, spasmolytic, antiviral, antihyperlipidemic, antitoxic, immunostimulant, epithelising, pro-coagulant, anthelmintic, hepatoprotective, antidepressants properties [8, 10, 11].

As regards the phytochemicals, *Plantago* genus contains many biologically active compounds, making of them major medicinal herbs. For example, several *Plantago* species contain mucilage polysaccharides (plantaglucide, glucomannon PMII, PMIa), lipids, caffeic acid derivatives (caffeic acid, chlorogenic acid, plantamajoside R, aceteoside R, ferulic acid, p-cumaric acid and vanillic acid), monoterpenoids (linalool), flavonoids and flavone glucosides (aspigenin, scutallarin, plantagonin, baicalein, luteolin, luteolin 7 glucoside, hispidulin 7 glucuronide), iridoid glycosides (aucubin, catapol, gardoside, geniposidic acid, mayoroside, melittoside), as well as terpenoids (loliolid, oleanolic acid and ursolic acid) and tannins [12,-16]. Alkaloids (indicain, plantagonin) and some organic acids have also been detected [17-19].

Natural products such as herbs or fruits become popular in recent years because generally these contain not only mineral and primary metabolites but a diverse array of secondary metabolites with antioxidant properties such as carotenoids, terpenoids, phenolics include flavonoids and phenolic acids [20, 21].

Non-enzymatic antioxidants as well as antioxidant enzymes are known to counteract the effect of ROS and RNS. These antioxidants are known to diffuse free radicals leading to limited risk of oxidative stress. At cellular and molecular level they inactivate ROS and under specific low concentration inhibit or delay oxidative processes by interrupting the radical chain reaction [22].

The common structural feature flavonoids is the diphenylpropane moiety, which consists of two aromatic rings linked through three carbon atoms that together usually form an oxygenated heterocycle. Phenolic compounds acting as antioxidants may function as terminators of free radical chains and as chelators of redox-active metal ions that are capable of catalyzing lipid peroxidation [23].

One of the most actively studied properties of flavonoids is their protection against oxidative stress [24, 25]. For example, flavonoids are ideal scavengers of peroxyl radicals due to their favorable reduction potentials relative to alkyl peroxyl radicals and thus, in principle, they are effective inhibitors of lipid peroxidation [25]. Of particular importance is the hydrogen (electron) donating ability of a flavonoid molecule which acts to scavenge a reactive radical species, and is primarily associated with the presence of a B-ring catechol group (dihydroxylated B-ring). One important structural feature which is partly responsible for the antioxidant properties of flavonoids involves the presence of 2,3 unsaturation in conjugation with a 4-oxo group in the C-ring. In addition, the presence of functional groups involving both hydroxyl groups of ring-B and the 5-hydroxy group of ring-A are all important contributors in the ability of flavonoids to chelate redox-active metals and thus prevent catalytic breakdown of hydrogen peroxide (Fenton chemistry) [22].

The aim of this study is to examine the enzymatic (superoxide dismutase and peroxidase) activity and non-enzymatic (total polyphenol and flavonoids) antioxidants content of three *Plantago* species (*P. lanceolata* L., *P. media* L. and *P. schwarzenbergiana* Schur), which have been

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collected in different (vegetative, flowering and fruiting) phenophases.

Experimental part

Plant material

Leaves of *Plantago* species (*P. lanceolata* L., *P. media* L., *P. schwarenbergiana* Schur) were seasonally sampled during May 2012 (vegetative stage), June-August 2012 (flowering stage) and October 2012 (fruiting stage), in a randomized way from saline areas of Valea Ilenei (Iasi, Romania) nature reserve. The plant material for each species has been collected from five different individuals.

Salt areas from Valea Ilenei (Leþcani) nature reserve occupy a surface of about 10 ha; this nature reserve is located 4 km from Leþcani railway station, next to Iaºi-Dorohoi railways at the confluence of Valea Ilenei and Bahlui rivers (fig. 1). Yet it is a small nature reserve, several species are included in the *Red Book* of Iasi district, such as *Plantago schwarzenbergiana* [26], a typical halophyte restricted only to Eastern part of the Europe [27, 4], as a pannonical species [7]. Salt areas from Valea Ilenei nature reserve do not have a uniform distribution; their zonality is strongly influenced by local environmental factors: soil humidity and salinity, rainfall and elevation of salt-affected surfaces [28]. This fact can explain the presence of non-halophytic *P. lanceolata* and *P. media* in this area, even they have a certain degree of salt tolerance [5].

Fig. 1. Localization of Valea Ilenei (Iasi) nature reserve (adapted from Grigore and Toma, 2014)

Chemical and reagents

Folin–Ciocalteu reagent, sodium carbonate anhydrous (Na_2CO_3), gallic acid, sodium nitrite solution ($NaNO_2$), aluminum chloride hexahydrate solution ($AlCl_3.6H_2O$), were purchased from Fluka (Switzerland). Nitroblue tetrazolium, Folin-Ciocalteu phenol reagent, riboflavine, o-dianisidine, hydrogen peroxide and catechin were purchased from Sigma-Aldrich (Germany). Sulfuric acid (H_2SO_4), Coomasie Blue G250, albumin fraction V (from bovine) and were obtained from Merck (Germany). The used reagents were of analytical grade and the aqueous solutions were prepared with double distilled high purity water.

Enzymes and non-enzymatic antioxidants extraction

Homogenized plantlets with disodium phosphate buffer (pH-7) were used for determination of superoxide dismutase (SOD) and peroxidase (POD) activities. For total polyphenol and flavonoids the dried samples were extracted with methanol.

Superoxide dismutase (SOD) activity assay

Superoxide dismutase activity was measured by its ability to inhibit the photoreduction of nitroblue tetrazolium (NBT) as described by Winterbourn et al, (1975) [29]. Absorbance was recorded at 560 nm using UV-Vis 1700 Shimadtzu Pharma Spectrophotometer. One unit of SOD activity is defined as that amount of enzyme required to inhibit the reduction of NBT by 50% under the specified conditions.

Peroxidase (POD) activity assay

Determination of POD activity is based on the measurement of color intensity of o-dianisidine oxidation product with hydrogen peroxide in presence of peroxidase. Colour intensity was measurement at 540 nm. One POD unit corresponds to the enzyme amount catalyzing decomposition of 1µmolH_2O_2/min, in optimal conditions [30].

Protein content assay

The determination of soluble protein content was done according to Bradford method [31]. All the enzymes activities were reported as U/mg protein.

Total polyphenol content assay

The total polyphenols content was determined by using a modified Folin-Ciocalteau method SINGLETON et al., (1999) [32]. The absorbance of resulting bleu-colored solution was read at 765 nm after two hours, against the blank (distilled water). The amount of the total polyphenolic content was expressed as mg gallic acid equivalent (mg GAE/g DW) (R^2=0.99). Three readings were taken for each sample and the result averaged.

Flavonoids content assay

The flavonoids content was measured following a spectrophotometric method DEWANTO et al., (2002) [33]. Absorbance of resulting pink-colored solution was read at 510 nm against the blank. Flavonoids content was expressed as mg catechin equivalent (mg CE/g DW) (R^2 =0.98). Three readings were taken for each sample and the result averaged.

Statistical analysis

Data were presented as means and standard errors (SE). All the samples were analyzed in triplicate; the average and the standard errors SE were calculated using the Excel software package.

Results and discussions

Our results show that enzymatic (superoxide dismutase, peroxidase) activity and non-enzymatic (total polyphenols, flavonoids) antioxidants content have different patterns in investigated species (figs. 2-5).

Enzymatic activity

One of the most effective intracellular enzymatic antioxidants is superoxide dismutase (SOD), which constitutes the first line of defence against ROS by playing a critical role in cellular defence against oxidative stress. This enzyme catalyzes the dismutation of $O_2^{\bullet-}$ to O_2 with remarkably high reaction rates by successive oxidation and reduction of the transition metal ion and to the less-reactive species H_2O_2. The excess O_2^- generated under stress condition could disproportionate into H_2O_2 by the action of SOD, which is then metabolised by the CAT, POX and the components of the ascorbate-glutathione cycle [34].

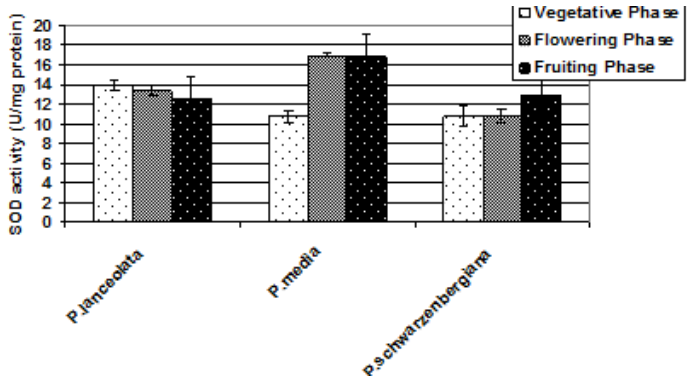

Fig. 2. SOD activity in three *Plantago* species measured in different growth stage

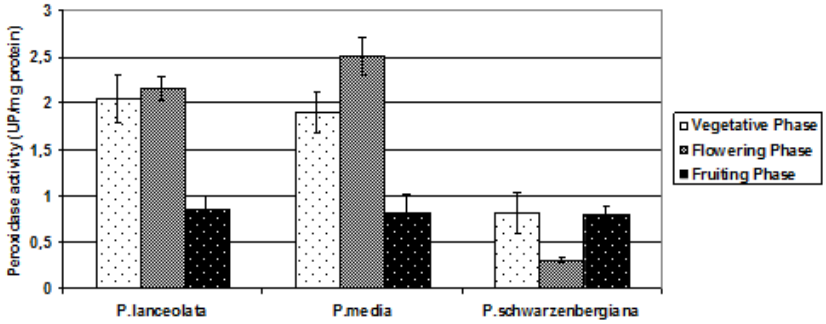

Fig. 3. POD activity in three *Plantago* species measured in different growth stage

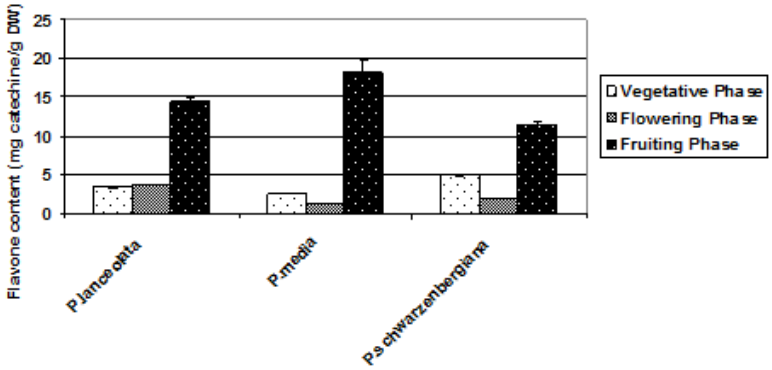

Fig. 4. Flavonoids content in three *Plantago* species measured in different growth stage

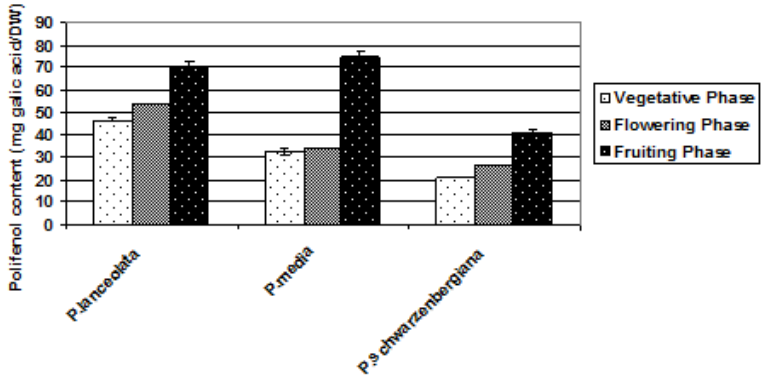

Fig. 5. Total polyphenols content in three *Plantago* species measured in different growth stage

In our study, SOD activity varied both according to considered phenophases, as well to different species. However, there is no uniform pattern regarding SOD activity in investigated species. From the three species, SOD activity was lower at vegetative stage only for *P. media* and *P. schwarenbergiana*, as compared with *P. lanceolata.* In *P. lanceolata,* the SOD activity was slightly diminished from vegetative to fruiting stage (from 13.96 U/mg protein to 12.54 U/mg protein), in a gradual manner (fig. 2).

In the other species (*P. media* and *P. schwarzenbergiana*), SOD activity has been found to be lower in vegetative phase than in flowering and fruiting phenophases; this trend is more noticeable in *P. media*, wnere SOD activity shows similar values in flowering and fruiting phase, rather than in *P. schwarzenbergiana*, where differences from vegetative and flowering phases are quite negligible. Overall, the halophytic *P. schwarzenbergiana* displayed the lowest values of SOD activity in all phenophases, within all the species.

In *P. lanceolata* and *P. media*, there is a similar pattern in respect with POD activity; both species registered the highest values for flowering phenophases, followed by vegetative and fruiting, respectively. Overall, within all three species, POD activity was lower in *P. schwarzenbergiana*, followed by *P. media* and *P. lanceolata* in vegetative phase; from all investigated species, this value was the smallest in fruiting species, with slightly similar values for all species.

Non-enzymatic antioxidants

Regarding the flavonoids content (fig. 4), there is a clear pattern for their biosynthesis in each species, individually considered. In all cases, the content is greatly higher in fruiting phenophase, followed by vegetative phase (especially in the case of *P. schwarzenbergiana* and *P. media*) and finally, with smallest value recorded in flowering phase - except for *P. lanceolata*, where this value is only slightly increased as compared with that measured for vegetative phase.

An even clearer pattern was noticed in respect with total polyphenols content (fig. 5), where it has been found to be greater in fruiting phase, followed by flowering and vegetative, respectively, considered for each individual species. In addition, total phenolics have the greatest value in fruiting phase in *P. media*, followed by *P. lanceolata* and *P. schwarzenbergiana*.

In our previous researches we found that in all investigated *Plantago* species (*P. coronopus, P. maritima* and *P. lanceolata*), the total polyphenol content was higher than in other species, both in vegetative and flowering phases [35].

Regarding the total polyphenols and flavonoids, Rugna et al. (2013) [36] also found that phenol production in *Smilax campestris* is related to the time of the year and to the phenological condition in the plant; they found that the leaf gradually increases phenol production throughout the year. However, despite the similar pattern, attention should be paid on the fact that there are different species and different climatic and phenological conditions. In different species of *Artemisia* and *Hypericum*, flavonols showed the same qualitative composition in all phenophases, with a peak value during flowering stage [37-39]. Phenological variation in the antioxidant phytochemicals (total phenol and total flavonoids) has been also recorded for the mature green and ripe brown pods of *Tetrapleura tetraptera*, with higher values for ripe brown pods [40].

Lowman and Box (1983) [41] found that phenol contents were significantly different among the five age classes of leaves of five species of Australian rain forest trees; four species showed an accumulation of total phenolics, despite some fluctuations; an interesting observation is that sun leaves had up to twice the levels of total phenolics than shade leaves. This is also consistent with Irondi et al. (2013) [40] hypothesis, which attributed the elevated total phenolic content to longer exposure of the ripe brown pods to sunlight.

Duenas et al. (2006) [42] stated that phenolic compounds are synthesized in response to light, while Jaakola et al. (2004) [43] reported that the expression of the genes involved in flavonoids biosynthesis is activated by increased light. In our case, the higher content of polyphenols and total phenolics in investigated *Plantago* species might be also attributed to exposure of sun light, due to specific ecological conditions from Valea Ilenei (Iasi) nature reserve [28].

Conclusions

Antioxidant enzymatic activity in the three *Plantago* species shows a uniform behaviour in respect with different phenophases; only the peroxidise activity was significantly lower in *Plantago schwarzenbergiana*, a fact perhaps correlated with its halophytic character.

Results obtained from the present study clearly indicate that there is a variation of total polyphenols and flavonoids in all investigated *Plantago* species during different phenophases. Overall, the highest values were registered in fruiting stage, suggesting that this may be correlated with prolonged exposure to sun light.

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