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ANTIOXIDANT CAPACITY OF PEA (*PISUM SATIVUM* L. CV. RAN) PLANTS DETERMINED BY DIFFERENT ANALYTICAL METHODS

Lyubomira Y. Atanasova, Ivan G. Goshev*, Boryana D. Mihaylova*

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Abstract

The change in the antioxidant capacity (AOC) of pea plants during vegetative growth was followed by means of hydroxyl radical averting capacity (HORAC), oxygen radical absorbance capacity (ORAC) and Folin–Ciocalteau (F–C) assays. During the studied period the pea plants developed all leaf stages before flower formation; root and shoot biomasses enhanced, which was accompanied by an increase of AOC. Highest AOC was localized in the green organs such as leaves, especially those in the growing apical part; stem and root AOC were lower. AOC increased during the organ growth; it was expressed in different magnitude by the three assays. Most likely the assays reflected the antioxidant properties of different compounds. The contribution of potential non-enzymatic antioxidants in the applied assays was discussed.

Key words: *Pisum sativum* L. cv. Ran, antioxidant capacity (AOC) assays, HORAC, ORAC, F–C assay

Introduction. Reactive oxygen species (ROS) are oxygen forms with higher reactivity than molecular oxygen, which are normally produced in virtually all intracellular organelles or compartments [1–5]. Growing evidence suggests that ROS act as signals in the cell to respond to developmental and environmental stimuli [2, 4, 5]. At optimal conditions, ROS are in low concentration, however, the unfavourable environment enhances their production that can lead to a process referred to as oxidative stress [1–5]. Plants possess enzymatic and non-enzymatic antioxidant mechanisms commonly termed antioxidant system, which is underlying the total antioxidant capacity (AOC) [6]. Considerable research work was done to develop methods to characterize the antioxidant properties of foods or biological systems; the chemistry, advantages and limitations of these assays are still discussed [6–8]. Variation between plant species and genotypes to tolerate various environmental stresses was linked to leaf AOC and metabolism; the AOC was recommended as useful screening criterion for the plant tolerance to unfavourable environment [9]. As a valuable food, pea is a species often used for study of its tolerance to stress environment. Cultivar Ran 1 is older Bulgarian early summer-grown pea possessing tolerant photosynthetic apparatus and male gametophyte viability [10] that can be due to features in the antioxidant system. The purpose of this study was to examine the AOC along the pea plant during its vegetative growth. We used three assays which reflect different features of the non-enzymatic antioxidant system in order to obtain more detailed picture of the protective efficacy in the plant.

Materials and methods. Pea plants (*Pisum sativum* L., cv. Ran) were grown on Hoagland–Arnon nutrient solution in climatic chamber at 20 °C, light intensity of 100 $\mu mol.m^{-2}.s^{-1}$ and a day/night photoperiod of 12/12 hours. Leaf, stem and root samples were collected once, after the development of the three true leaf stages (first harvest), and then, after the formation of five true leaf stages (second harvest). The fresh weight (FW) and dry weight (DW) (80 °C until constant dry weight) of the samples were measured. The samples for AOC assays were fixed with liquid nitrogen, lyophilized and kept at deep freezing until use. Each sample (about 50 mg DW) was extracted (4 h) in acetone/water/acetic acid (70/29.5/0.5 v/v) at room temperature under stirring. The extracted mixture was centrifuged at cooling and the clear supernatant was used immediately, or, if necessary, it was kept overnight at deep freezing.

HORAC assay is based on in situ generation of free hydroxyl radicals due to catalytic decomposition of hydrogen peroxide in the presence of divalent cobalt (CoF_2) at 37 °C and pH 7.4 (75 mM sodium phosphate buffer) [11]. Certain parameters and ratios of the basic procedure were changed to improve the experimental design. The reaction was performed in 10 mm light path quartz fluorescence cells on Perkin elmer LS5 fluorimeter equipped with thermostated

cell holder. Fluorescein-disodium salt was used to monitor the generation of free hydroxyl radicals and their scavenging by pea sample substances (excitation wavelength of 493 nm; emission wavelength of 518 nm, observation period of 30 min). Gallic acid (3,4,5-trihydroxy- benzoic acid, GA) was used as a standard. The results were expressed as micromole GA equivalents (GAe) per gram sample DW.

ORAC assay was carried out on the same equipment, in the same buffer and also applied fluorescein disodium salt as a fluorescent probe [12]. The peroxyl radical generation was achieved by decomposition of AAPH (2,2′-azobis-2-methyl-propanimidamide dihydrochloride) at 37 °C and pH 7.4. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as standard. The results were expressed as micromole Trolox equivalents (Te) per gram sample DW.

Folin–Ciocalteau (F–C) assay is based on the reaction of phenolic compounds with the Folin–Ciocalteau reagent [13]. Gallic acid was used for construction of a standard curve. The results were expressed as microgram GAe per gram sample DW.

The sigmoidal fluorescence decay curves were obtained for the standards and tested pea samples (in three replications) and their net areas under curves (AUC) were calculated. The antioxidant potential of each pea sample was evaluated by formulae for comparison of blank, standard and sample AUC [11].

Results and discussion. The AOC of organs from pea plants during their vegetative development was estimated by three assays termed HORAC, ORAC and F–C assays; the values together with the data for dry mass accumulation are shown on Table 1. There are several important trends in the obtained results. Firstly, during the studied period the plant developed all leaf stages before flower formation; root and shoot biomasses (expressed as dry mass content) enhanced, which was accompanied with enhancement of AOC. Secondly, the highest AOC is localized in the green organs such as leaves, especially those in the apical part, while stem and roots AOC are lower. Thirdly, the increase of AOC during the growth progress is expressed in different magnitude by the three assays. Most likely the assays reflect the antioxidant properties of different compounds.

HORAC values reflect the prevention ability of the pea organs against the generation of hydroxyl radical in the non-enzymatic reactions described by Fenton and Haber-Weiss. Hydroxyl radical is generated by the catalytic decomposition of hydrogen peroxide in the presence of transition metal ions (Fe^{+2}, Cu^{+2}, Zn^{+2}, etc.), and its scavenging is not under enzymatic control at physiological conditions. The elimination proceeds not via direct scavenging but by removal of the reactants necessary for the generation (preventive manner), i.e. either decomposition of hydrogen peroxide, or chelation of the metal ion [2, 11]. In

T a b l e 1

Changes in the biomass accumulation and antioxidant capacities of pea plants during the vegetative development

	Content of biomass (mg DW . plant $^{-1}$)		HORAC (μmol GAe . g^{-1} DW)		ORAC (μmol Te . g^{-1} DW)		F-C (μmol GAe . g^{-1} DW)	
	First harvest	Second harvest	First harvest	Second harvest	First harvest	Second harvest	First harvest	Second harvest
Primary root	12 ± 1	14 ± 2	122 ± 10	231 ± 15	37 ± 3	52 ± 5	16 ± 2	16 ± 3
Lateral roots	17 ± 3	37 ± 3	130 ± 7	89 ± 9	33 ± 3	46 ± 4	15 ± 2	14 ± 2
Stem (up to 3rd node)	21 ± 3		196 ± 10		117 ± 6		35 ± 5	
Stem (up to 3rd node)		19 ± 2		251 ± 10		211 ± 10		35 ± 4
Stem (up to 5th node)		21 ± 3		219 ± 10		251 ± 10		25 ± 2
Leaves (three true leaf stages)	56 ± 4		244 ± 10		220 ± 10		58 ± 2	
Leaves (1–2 true leaf stages)		23 ± 2		500 ± 10		552 ± 15		59 ± 3
Leaves (3–5 true leaf stages)		72 ± 5		724 ± 15		590 ± 15		54 ± 3
Apical shoot part	11 ± 1	26 ± 3	241 ± 5	811 ± 15	192 ± 5	544 ± 15	50 ± 3	71 ± 5

this sense, the present results (Table 1) show that pea organs contain compounds expressing considerable metal chelating activity. Another important observation is that HORAC values differ between roots and shoot organs, i.e. this assay is sensitive to the type of organ and its age. Among the natural antioxidants, phenolic compounds that can chelate metals showed HORAC values which were dependent on the chelating ability [11]. Flavonoids with catechol, 4-oxo, and 50–OH arrangements strongly inhibited Fenton-induced oxidation; the same effect was much higher for isoforms without these features [11]. Strong antioxidants such as Trolox, vitamin C, melatonin, did not react significantly [11]. Flavonoids accumulated mostly in surface organs and their epidermal cells at sunlight [3]. Pea leaf guard and epidermal cells were predominant sites for accumulation of flavonol glycosides and anthocyanins; major soluble phenolic compound in mature pea leaf was quercetin glucoside [14]. The glucose and rutinose groups influenced positively the HORAC values as their hydroxyl groups may increase the chelating ability [11]. The preventive AOC of root system can also be attributed to the reactivity of the hydroxyl substituents in the structure of certain phenolic compounds such as catechins, proanthocyanidins and leukoanthocyanidins, which are important for the symbiotic interactions of pea and other Leguminosae plants [15]. Compared to phenolic acids, such compounds demonstrated higher HORAC values [11]. The HORAC values of pea roots obtained in our study (Table 1) could be attributed to the presence of similar compounds.

ORAC assay also demonstrates obvious differences in AOC of pea leaves, stem and roots as well as changes in organ AOC during the growing (biomass accumulation). The values (Table 1) indicate the ability of pea antioxidants to quench peroxyl radical using hydrogen donation [7]. The assay is widely applied in the food industry to measure AOC against peroxyl radical of traditional chain-breaking antioxidants such as flavonoids, vitamin E, ascorbate (vitamin C), β-carotene, glutathione, etc. [8], and rarer – for assessment of plant AOC at changing environment [9]. By means of the used procedure, water-soluble AOC of pea organs was estimated and expressed as equivalents of Trolox. Among flavonoids those with catechol structure in the B-ring strongly inhibited the lipid peroxidation [11]. Tocopherols and tocotrienols (vitamin E) are able to donate their phenolic hydrogen to lipid free radicals, which inhibits the chain propagation during lipid oxidation [3–5, 16]. In cooperation with carotenoids, tocopherols react with other ROS such as singlet molecular oxygen that prevents the lipid peroxidation [16]. Along the plant, tocopherol composition and content differed; pea contained only alpha-tocopherol which prevailed in the apical part and decreased downward the stem [17]. The tocopherol protection against lipid peroxidation is enhanced by ascorbate-glutathione cycle which allows tocopherol recycling [16]. At normal conditions, organs such as leaves

maintain ascorbate and glutathione in a high reduction state [2–5]. The ascorbate pool was large in pea leaves grown at high light intensities, especially embryonic axes synthesized and accumulated it rapidly; almost all root cells contained ascorbate as well [18]. Glutathione function in the antioxidative defence is to regenerate the ascorbate via the ascorbate-glutathione cycle [3–5]. Besides, it can directly scavenge the hydrogen peroxide (thus acting as preventive oxidant) and reacts non-enzymatically with other ROS such as singlet oxygen, superoxide and hydroxyl radicals [3–5]. Briefly, HORAC and ORAC assays assessed different aspects of antioxidant properties in pea organs. The metal-chelating capacity of preventive antioxidants was evaluated by the first assay while the second one measured the function of chain-breaking antioxidants. Therefore, the pea organs do not have the same HORAC and ORAC values.

To characterize another part of pea antioxidant profile, the F–C assay was used. This electron-transfer-based assay, also named total phenolics assay, was standardized to measure the reducing capacity of total phenols and other oxidation substrates [8, 13]. The values (Table 1) show differences between the reducing capacities of leaves, stem and roots. Besides the increase in the shoot apical part from the second harvest, there is no significant change in the reducing capacities of the other plant parts through the studied period. Most likely the compounds with reducing ability did not alter substantially. Generally, the green organs possess higher level of such compounds when compared to roots. These compounds need to be investigated additionally. We assume for now that the soluble phenol fraction brings about the reducing ability of the pea organs. The slower accumulation, observed in our study too, was explained to be due to more cost and complex phenolic manufacture [19]. Actually, the phenolic compounds contribute essentially to the antioxidant defence; their efficiency is considered to be much higher than that of tocopherol or ascorbate [20].

By means of HORAC, ORAC and F–C assays, we measured common and specific features of the AOC of the pea organs. The results indicate that the antioxidant properties of pea non-enzymatic antioxidant system are dynamic and have potential to change in response to growth conditions. By means of three different assays, we demonstrate that the green part, and mostly the youngest (apical) one, is a rich antioxidant source. It should be emphasized that the assays estimate the antioxidant function of different compounds belonging to several molecular families. That is why it is recommendable to use several methods to obtain a more detailed picture of the type and distribution of antioxidant compounds and their protective efficacy in plants. The quantification of AOC by plant organ and its developmental stage is important for the food industry too, especially for the production and preservation of its nutritional value. In the future research we will test the ability of pea

organs and sites rich in antioxidants to reduce the risk of disorders provoked by oxidative stress.

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Institute of Plant Physiology and Genetics
Bulgarian Academy of Sciences
Acad. G. Bonchev Str., Bl. 21
1113 Sofia, Bulgaria
e-mail: ly_atanasova@yahoo.com

**Institute of Organic Chemistry*
with Centre of Phytochemistry
Bulgarian Academy of Sciences
Acad. G. Bonchev Str., Bl. 9
1113 Sofia, Bulgaria