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THERMOSTABILITY OF PEROXIDASE IN LEAVES OF *BUXUS SEMPERVIRENS* L. UNDER THE INFLUENCE OF HYPERTHERMIC SHOCK

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The aim of this study was to investigate the thermal stability of total activity and peroxidase (PO) isoenzymes isolated from boxwood *Buxus sempervirens* (L.) leaves subjected to hyperthermic shock (HTS) at temperatures in the range of 53-90°C for 5 min. It was demonstrated that PO in leaves exposed to HTS of 53°C, 55°C and 57°C exhibits high resistance to these temperatures, the activity being at the level of the control. Although, the total activity and intensities of PO isoenzymes distributed in 4 zones in a vertical gradient (4-7%) of polyacrylamide gel (PAAG) gradually decreased with increasing HTS of temperatures above 58°C. However, treatment of leaves with HTS at 90°C for 5 min resulted in complete suppression of PO, the activity of which was not detected.

Keywords: *boxwood leaves, hyperthermic shock, activity, peroxidase, isozymes.*

TERMOSTABILITATEA PEROXIDAZEI DIN FRUNZELE DE *BUXUS SEMPERVIRENS* L. SUB INFLUENȚA ȘOCULUI HIPERTERMIC

Scopul acestui studiu a fost de a investiga stabilitatea termică a activității totale și a izoenzimelor peroxidazei (*PO*) izolate din frunzele de cimișir *Buxus sempervirens* (L.) supuse șocului hipertermic (ȘHT) la temperaturi în intervalul 53-90°C cu durata de 5 min. S-a demonstrat că PO în frunzele expuse la ȘHT de 53°C, 55°C și 57°C manifestă rezistență înaltă la temperaturile respective, activitatea fiind la nivelul martorului. Deși activitatea totală și intensitățile izoenzimelor PO distribuite în 4 zone în gradient vertical (4-7%) de gel de poliacril amidă (GPAA) au diminuat gradual odată cu majorarea ȘHT la temperaturi peste 58°C. Însă, tratarea frunzelor cu ȘHT de 90°C cu durata de 5 min. a condus la suprimarea completa a PO, activitatea căreia n-a fost detectată.

Cuvinte-cheie: *frunze de cimișir, șoc hipertermic, activitatea, peroxidaza, izoenzime.*

Introduction

The growth and development of plants in natural conditions are very often exposed to unfavorable environmental factors, including the action of extreme temperatures. Global warming, accompanied by the development of intolerable meteorological conditions, is becoming an increasingly frequent phenomenon in Moldova.

Temperature stress has a detrimental effect on plant growth and metabolism, since these processes have optimal temperature limits for each plant species. Heat stress is defined as an increase in temperature above a critical threshold for a period of time sufficient to cause irreversible damage to plant growth and development [1, 2]. The response of plants to high temperature shock (HTS) varies depending on dose and duration of heat stress, as well as the type of plant species [3].

Boxwood *Buxus sempervirens* (L.) is an evergreen shrub or small tree, widely distributed in southern Europe, North Africa and Western Asia [4]. Many studies on *Buxus* have demonstrated the ethnomedicine, phytochemistry and pharmacology of the genus *Buxus* [5-7]. Boxwood shrubs are also widely used for ornamental purposes in gardens, parks, or as hedges. In addition, Boxwood leaves are widely used as a model plant in various research experiments [8].

Boxwood, like most plants, is susceptible to environmental stress factors that cause the formation and accumulation of reactive oxygen species (ROS) in cells, including hydrogen peroxide (H_2O_2), which leads to changes in cellular homeostasis and the development of oxidative stress [9].

To resist the destructive effects of oxidative stress, to adapt to them and, at the same time, to maintain their vital potential, plants must develop the ability to activate protective and adaptive mechanisms to stressful conditions. Protection of plants against the destructive effects of oxidative stress, caused by excess of ROS, including H_2O_2, is achieved by an efficient antioxidant system [10].

As numerous studies show, boxwood, like most plants, has a wide range of defense mechanisms, including non-enzymatic and enzymatic antioxidants, which help it adapt to extreme temperatures caused by the effects of global warming [9, 11-14].

Among the antioxidant enzymes, peroxidases (PO), which are the most important of them, play an important role in regulating the intracellular level of H_2O_2 [7,10]. Though PO is a multifunctional enzyme presents in most plants [7,15-16], under stress conditions its main function is antioxidant – reducing the level of H_2O_2 in tissues. The thermosensitivity of this enzyme is species-specific, which has been demonstrated for tissues of different plant taxonomic groups [17-22].

Studying of the boxwood leaves PO activity in different seasons of the year [12] demonstrated that PO activity tends to correspond to the resistance of boxwood leaves to the action of seasonal temperatures, reaching the highest level of enzymatic activity in summer at an ambient temperature of +37°C and in winter at an ambient temperature of -25°C. Regardless of the season, one-year-old leaves manifested more resistance to the environmental temperature factor, determined by the season, compared to two-year-old leaves. The results of these studies indicate the importance of determining in laboratory conditions the maximum thermal tolerance of PO in boxwood leaves at high temperatures, in order to establish supraoptimal temperatures caused by current climate warming, to which boxwood could adapt.

Since boxwood has leaves of different ages (1, 2 and 3 years) it has been proposed as a model plant for studying the specificity of adaptation to different seasonal conditions, depending on the age of the leaves [9]. Such studies are of great importance for identifying early antioxidant reactions involved in reducing oxidative stress and annihilation of hydrogen peroxide, which largely determine the adaptive response of boxwood to the action of extreme environmental temperatures, which are constantly changing.

The aim of this study was to investigate changes in the total activity of PO and the composition of its isoenzymes in boxwood leaves subjected to short-term hyperthermic shock HTS) of varying intensity.

Materials and methods

One-year-old leaves of the evergreen boxwood *Buxus sempervirens* L. (Fig. 1) were used in the study. Leaves collected during the winter months (January, February) from plants grown in native conditions in the immediate vicinity of the Institute of Plant Genetics and Physiology, MSU were used in a fresh state immediately, by immersing them in water with a temperature in the range of 53-90°C for 5 min. The control leaves were immersed in water with a temperature of 25°C.

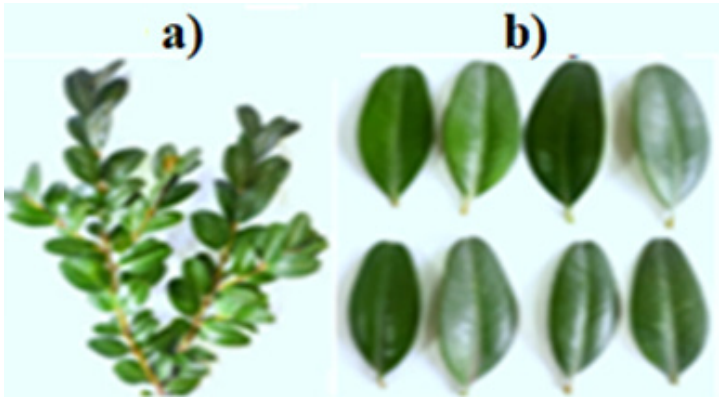

Figure 1. A twig of Buxus sempervirens L. with leaves of diffeent ages (a) and one-year-old leaves (b), used in experiments with short-term exposure to hyperthermic shock

The selected boxwood leaves were placed in test tubes containing 10 ml of deionised water, which were subsequently placed in the water thermostat (Universal ultrathermostat "UTU-4"), where they were subjected to thermal shock at temperatures of 53-90°C for 5 min. After completion of each hyperthermic shock tested, leaf samples were placed in cold water and stirred for 2 hours in a mixer (Wstrzasarka universal type WU-4, Poland) at room temperature (25°C). To establish the response of PO to the application of hyperthermic shock, a temperature higher than 50°C was chosen, because such temperatures were used in previous studies conducted with oak leaves with

different doses of thermal shock [9]. Leaves that were obtained at each hyperthermic shock temperature tested were analysed for total PO activity and its isoenzymes composition.

Enzyme extraction. Enzyme extracts from control and experimental boxwood leaves (exposed to hyperthermic shock (HTS) were obtained by homogenizing them at low temperature (4°C) in 0,05 M sodium phosphate buffer with 1 mM EDTA and 1% (w/v) polyvinylpolypyrrolidone, pH 6.8 and then centrifuged at 15,000 g for 15 min at 4°C. The resulting supernatants represented crude enzyme extracts used to measure PO activity and its isoenzyme composition.

Enzyme activity assays. Peroxidase activity was determined according to the method [23] by monitoring the increase in absorbance at 334 nm as benzidine was oxidized in the presence of H_2O_2.

Electrophoresis for isozymes assays. Samples were subjected to discontinuous polyacrylamide gel (PAAG) electrophoresis under nondenaturing, nonreducing conditions as described by Laemmli [24] with some modifications. Electrophoretic separation of PO isoenzymes was performed at 4°C using vertical gradient (4-7%) PAAG for 4-5 h in 0,04 M Tris (hydroxymethyl) aminomethane-glycine buffer (pH 8.3). Protein bands displaying peroxidase activity were revealed utilizing 1,3 Mm benzidine (Sigma, SUA) and 0,05% H_2O_2 in 20 Mm acetate buffer, pH 4.2.

Results and discussion

In this work to establish the response of PO to the application of hyperthermic shock (HTS), a temperature higher than 50°C was chosen, because such temperatures were used in previous research conducted with oak leaves with different doses of thermal shock [9].

From the data presented in Fig. 2 it is obvious that the activity of PO in the leaves of boxwood exposed to HTS at temperatures of 53ºC, 55ºC and 57ºC for 5 min does not change significantly 25 min after temperature shock. A day after recovery from the expose to HTS, a decrease in PO activity is observed in all variants. Heat treatment resulted in significant loss of PO activity. But subsequent incubation of treated leaves at room temperature (25°C) allowed for reactivation of PO activity.

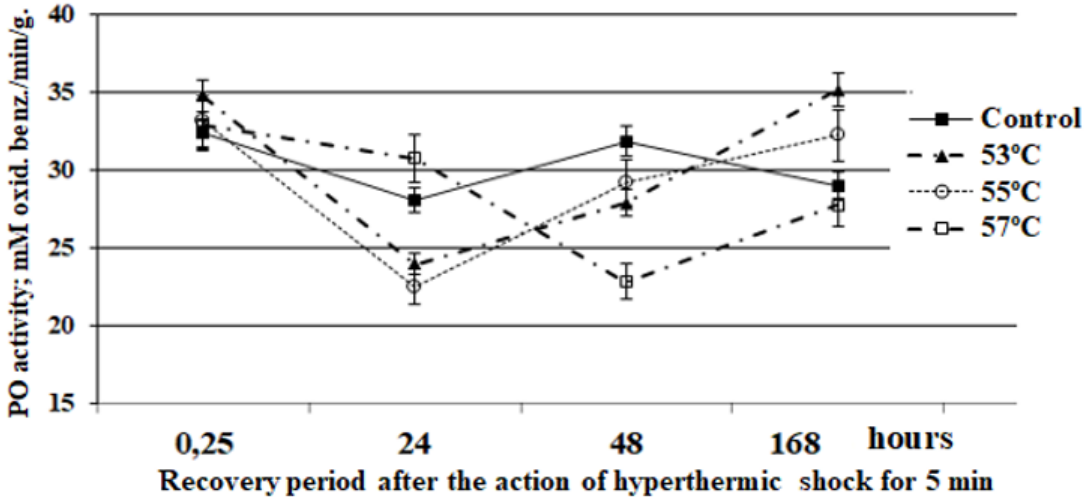

Figure 2. Changes in PO activity during the recovery period after treating bowood leaves with HTS of 53ºC, 55ºC and 57ºC for 5 min

During the subsequent recovery period after 24 hours, the PO activity in the leaves treated with HTS of tested temperatures increases, reaching a level lower than the initial one (Fig. 2). A study [17] of some aspects of the kinetics of partial inactivation of horseradish PO at 70°C, 90°C or 110 °C showed recuperation of PO activity. However, holding for 24 hours at 50°C almost completely prevented the recovery of enzyme activity upon subsequent cooling, and the degree of regeneration depended on the duration of time at the inactivation temperature [17]. Using spectral methods to study thermal inactivation of PO, it was suggested that reversible inactivation is due to the reassociation of the thermally displaced prosthetic heme group with the protein portion of the enzyme [17].

Testing the thermal stability of PO from boxwood leaves after expose to temperatures higher than 57°, up to 90ºC for 5 minutes, showed a gradual loss of enzymatic activity (Fig. 3). It can be observed that starting with the use of temperatures of 58ºC and above, a gradual decrease in PO activity occurs, with complete inactivation of its activity at a temperature of 90ºC (Fig. 3).

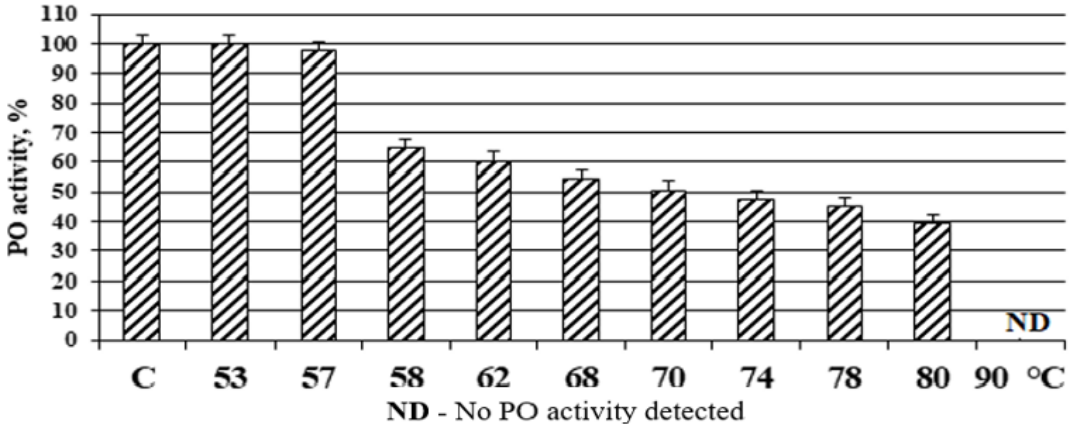

Figure 3. Thermostability of PO from boxwood leaves exposed to hypethermic shock of different intensity (53°-90ºC) with a duration a 5 min

Results on the characterization of the soluble (SPO), ionically (IBP) and covalently(CBP) bound peroxidase from leaves and hearts of artichoke showed that the three PO forms have different heat sensitivity; the bound forms (IBP and CBP) are characterized by a greater heat stability than the soluble one, and leaf PO forms showed greater heat, then head ones [19].

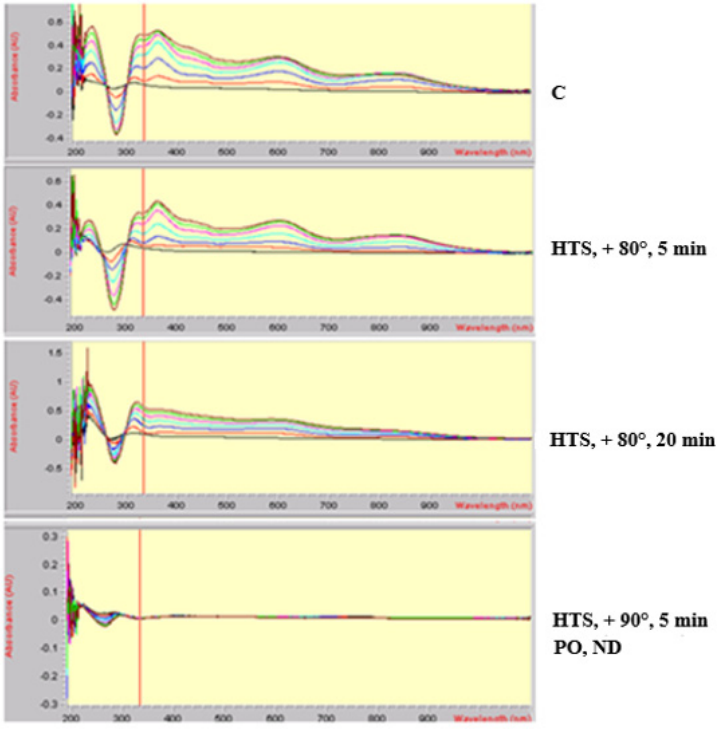

Figure 4. PO spectra in enzymatic extracts from boxwood leaves that were treated with HTS at 80ºC for 5 and 20 min, respectively, and 90°C for 5 min, obtained from apsoption at 340 mm of benzidine oxidation in the presence of H_2O_2 recorded by UV-Vis spectrophotometer. C - control, ND - no PO activity

Since the treatment of boxwood leaves with a temperature of 80ºC results in 60% enzymatic inactivation, we proposed to increase the duration of exposure to HTS at 80ºC, taking the time of 5 and 20 min, and testing the expose of leaves to HTS at 90°C for 5 min. Monitoring of PO reaction spectra in boxwood leaves after exposure to HTS at 80ºC for 5 and 20 min, respectively, and at 90ºC for 5 min is shown in Fig. 4. From the data presented in this figure, it is evident that PO exhibits enzymatic activity even when boxwood leaves are exposed at HTS of 80ºC for 20 min. This indicates that boxwood leaf PO is resistant to high temperatures.

Spectral analyses of the course of the PO-catalysed reaction with benzidine as a substrate in the presence of H_2O_2 (Fig. 4) demonstrated that the PO spectra from control leaves and from leaves exposed to the HTS action of 80ºC for 5 min are analogous. While the PO spectrum in boxwood leaves exposed to 80ºC for 20 min is different from that of leaves treated with HTS of 80ºC for 5 min. This may indicate that the application of HTS for more than 5 min leads to changes in the formation of the enzyme-substrate complex and the develop-

ment of the enzymatic reaction. And when boxwood leaves are exposed to a temperature of 90ºC for 5 min, as can be seen from the spectrum in Fig. 4, peroxidase activity is irreversibly suppressed, the formation of the enzyme-substrate complex does not happen and the development of the enzymatic reaction does not occur. So, boxwood leaf peroxidase activity is irreversibly inhibited by high temperature of 90ºC**.** The two-phase processes of inactivation and regeneration of PO in horseradish may be due to the presence of PO isoenzymes with different sensitivities to high temperatures [18]. Soluble and membrane-bound PO extracted from red cabbage, demonstrated that membrane-bound PO is more thermostable than soluble one, losing >90% of relative activity after 5 min of incubation at 76,6°C and 30,2°C, respectively [25]. Thermal inactivation of PO in tomatoes was also performed at temperatures of 63, 64, 65, 66 and 67 °C [21].

It is supposed, that data on the role of PO as a marker enzyme in stress conditions are contradictory and unclear, due to the existence of a large number of isoenzymes in cellular compartments [26].

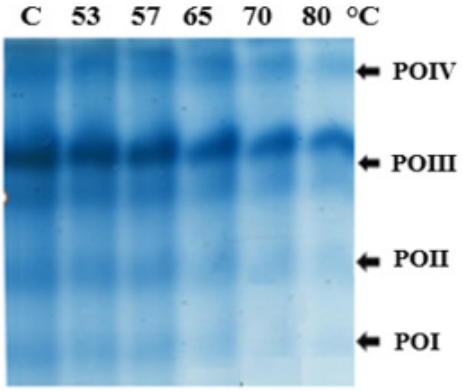

Figure 5. Separation of PO isoenzymes rom boxwood leaves after their exposure to the action of HTS of different intensity. HTS was performed with temperatures in the range 53-80°C with a duration of 5 min. HTS - hyperthermic shock, C - control

Our determination on the changes in the isoenzimatic spectrum of PO under the influence of HTS of different intensity (53-80°C) are presented in Fig. 5. Peroxidase isozymes in soluble protein fractions undergo visible quantitative and qualitative changes depending on the temperature value of HTS used for leaf treatment (Fig. 5). The isoenzyme patterns (Fig. 5) indicated the clear distinction of the influence of different HTS temperatures on the pattern of PO isoforms in soluble proteins extracted from boxwood leaves exposed to HTS action for 5 min. The pattern differs in the electrophoretic mobility and intensity of the bands demonstrating PO activity. As can be seen from the electrophoresis of extracts from boxwood leaves, there are several areas of localization of PO isoenzymes. Under the influence of HTS, the intensity of PO isoforms decreases in all I-IV zones. The most pronounced decrease in the intensity of the bands is observed in the POI and POII zones, especially at HTS 65°C, 70°C and 80°C, respectively. The isoforms from zones III and IV also decrease, but they are more abundant, compared to the isoforms from zones I and II. Thus, by the method of gradient polyacrylamide gel electrophoresis, it was established that the composition and intensity of the bands corresponding to PO activity in one-year-old leaves exposed to HTS of different intensities (53-80°C) differ. This is probably due to the physiological state of the leaves. The maximum intensity of PO isoforms was recorded in boxwood leaves exposed to HTS at temperatures of 53°C and 57°C.

The changes recorded in the total activity of PO and its isoforms caused by the action of HTS determine their participation in the oxidation-reduction processes in cells and their contribution to establishing the level of resistance to extreme temperature shock. The obtained results expand the understanding of the response of PO activity and its isoenzymes to heat stress.

The high resistance of PO to hyperthermic stress indicates the possibility of using boxwood leaves, similar to PO from palm, soybean and others [17, 7], as a source of PO for various industrial branches.

Conclusions

Treatment of boxwood leaves with hyperthermic shock (HTS) at a temperature of 53°C, 55°C and 57°C for 5 min caused practically no significant changes in the activity and composition of the PO isoenzymes in the fraction of soluble proteins isolated from leaves exposed to HTS.

Application of HTS at 57-80°C for 5 min caused a gradual decrease in the total activity and intensity of PO isoenzymes in boxwood leaves. However, in leaves treated at 90°C for 5 min, PO activity was not detected.

The obtained results showed that PO from boxwood leaves exhibits relatively high PO stability at high temperatures shock, which indicates the possibility of using PO from boxwood leaves, similar to PO from palm, soybean and others, as a source of PO for various industries.

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