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Stress Sensitivity of Correlation between POD and PPO Activities in Plants

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Abstract – In the leaf extracts of two plant species (*Quercus robur* L. and *Phaseolus vulgaris* L.), peroxidase (POD) and polyphenol oxidase (PPO) activities have been measured by the methods of Shannon et al (1966) and Flurkey and Jen (1978). The oxidative activities regarded as semi-empirical biochemical variables have distributions in the plant foliage and between them a linear correlation has been observed. In this work the resultant oxidative activities of POD and PPO isoenzymes of plant foliages, the measuring uncertainties of their values and their correlation are interpreted. For the effects of cold shock and lack of illumination, significant alterations in the correlation have been revealed that are also reflected by the parameters of the regression. The correlation of POD and PPO has been established to be stress sensitive by the application of covariance analysis (ANCOVA).

plant stress / enzyme correlation / *Quercus robur* L./ *Phaseolus vulgaris* L. / peroxidase / polyphenol oxidase

Kivonat – Növényi oxidatív aktivítás-korreláció stressz-érzékenysége. Két növényfaj (*Quercus robur* L. és *Phaseolus vulgaris* L.) lombozatából peroxidáz (POD) és polifenol oxidáz (PPO) aktivitásokat határoztunk meg Shannon et al (1966), ill. Flurkey-Jen (1978) módszerével. A félempirikus biokémiai változóknak tekintett oxidatív aktivitások a növényi lombozatban eloszlással rendelkeznek. Értékeik között lineáris korrelációt tapasztaltunk. Dolgozatunkban a POD és PPO izoenzimek eredő oxidatív aktivitásait, értékeik bizonytalanságát és korrelációjukat egyaránt értelmezzük. Hideg sokk és fényhiány hatására a regressziós paraméterek által visszajelzett szignifikáns változásokat észleltünk a POD versus PPO korrelációban. A kovariancia analízis (ANCOVA) alkalmazásával ez a korreláció statisztikai értelemben stressz-érzékenynek bizonyult.

növényi stressz / enzimkorreláció / *Quercus robur* L. / *Phaseolus vulgaris* L. / peroxidáz / polifenol oxidáz

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1 INTRODUCTION

The plant cells have a complex antioxidant system. Its primary role is to minimize the amount of hydrogen-peroxide produced in the SOD-ASC-GSH cycle during the photosynthesis (Polle 2001) and to oxidize some metabolites, respectively. Various peroxidases (POD isoenzymes) and polyphenol oxidases (PPOs) take part in the oxidation of the metabolites. The higher plants have several classes and types of PODs, reaching up to 160 isoenzymes in some plants such as cereals (Dunford 1999). The same is true for PPO (Mayer 2006). In their activity controls, there were found both up and down regulated isoenzymes (Boss et al. 1994, Kim et al. 2001, Li – Steffens 2002, Thipayong et al. 2004, Wang – Constabel, 2004). In cases of these two enzyme classes it is necessary to emphasize the activity overlapping between them, too. For example, 10 isoenzymes of PODs in tobacco leaves can also produce PPO activity (Sheen–Calvert 1969).

The plant antioxidant enzyme system has been proved to be stress sensitive. The stress sensitivity of these two enzyme groups, the POD and the PPO enzymes have in turn been experienced in a numerous cases of environmental and stress effects. Their susceptibility can be related to their gene transcriptions influenced by abiotic and biotic stress factors. Their investigations are very important part in the researches of plant physiological states and these state alterations (Sárdi–Stefanovits-Bányai 2006, Jaleel et al. 2009). The knowledge about the stress dependence of POD and PPO activities has continuously been broadening. Recently, in the investigations of *Catharanthus roseus* (Jaleel et al. 2008), cowpea (Chandra et al. 2007), spinach (Ozturk et al. 2008), *Polygonum vivparum* (Wang et al. 2009) and bean (Sárdi – Stefanovits – Bányai 2006), the POD and/or PPO activities are significantly altered by sodium chlorite, triadimefon, *Rhizoctonia solani* inoculation, ethephon, different altitudes and *Pseudomonas savastanoi* infection. However, the relative large standard deviations of the results greatly limit the characterization of stress with enzyme activities. In many cases, the establishment of significant difference between the two means requires many simultaneous experiments, that can improve the statistical comparability. Based on determination of enzyme activities, a substantial condition of stress qualifying conception is to decrease the standard deviation of the measurements. The reduction of standard deviations demands that we know the reasons and the origin of the activity differences. The standard deviation of the enzyme activity results from the stochastic error of analytical methods, uncertainty of fixation of the developmental stage and different intensities of metabolisms. The difference in metabolic intensities among the cells has the dominant role. In consequence, efficiency of the stress investigations carried out by the measurement of enzyme activities can be augmented if there is a possibility to decrease or avoid metabolic intensity deviations of biological samples.

Observations of the existence or non-existence of the correlations between biochemical variables are important establishments that suggest a synchronized potential regulation of the variable values in the metabolism. Seeking the origin of the correlations, the conditions of the existence of state-dependent correlation have been declared in one of the recent articles and a theoretical equation was successfully deduced to the linear regression symbolizing state-dependent correlation of two biochemical variables (Németh et al. 2009a):

$$x_1 = \frac{\sigma_1}{\sigma_2} x_2 + \frac{\sigma_2 \mu_1 - \sigma_1 \mu_2}{\sigma_2} \qquad (1)$$

where $\mu_i = \lim_{n\to\infty}\left(\overline{x_i}\right) = \lim_{n\to\infty}\left(\frac{1}{n}\sum_{j=1}^{n} x_{i_j}\right)$, $\overline{x_i}$ is the mean of variable x_i,

$\sigma_i = \lim_{n\to\infty}\left(s_i\right) = \lim_{n\to\infty}\left(\sqrt{\frac{\sum_{j=1}^{n}\left(x_{i_j} - \overline{x_i}\right)^2}{n-1}}\right)$ and s_i is the ith empirical standard deviation,

where, x_1 and x_2 are the biochemical variables in the correlation (e.g. metabolite concentrations, enzyme activities, etc). μ_1 and μ_2 are the expected values of the variables x_1 and x_2. σ_1 and σ_2 are the theoretical standard deviations of distributions of variables x_1 and x_2. Applying equation 1 to enzyme concentrations, the relationship of enzyme correlation is obtained:

$$[E_1] = \frac{\sigma_{E_1}}{\sigma_{E_2}}[E_2] + \frac{\sigma_{E_2}\mu_{E_1} - \sigma_{E_1}\mu_{E_2}}{\sigma_{E_2}} \qquad (2)$$

where, μ_{E_1} and μ_{E_2} are the means of concentration distributions of E_1 and E_2 enzymes. σ_{E_1} and σ_{E_2} are the theoretical standard deviations of the distributions of enzyme concentrations.

The correlations belonging to the same sampling time are considered as state-dependent correlations. State-dependent correlation is a concept that is based on the physiological state of organism. The slope and the intercept of state-dependent regression can alter in time. It makes an opportunity to characterize the physiological states or state alterations with the correlation of biochemical variables. The state-dependent correlation is regarded as sensitive to state alteration if the slope and/or the intercept of regression straight line, ergo if the theoretical standard deviations and/or the expected values of distributions of biochemical variables can significantly change during the state alteration (Németh et al. 2009a). By a correlative investigation in this paper, we show that the physiological stages of biological systems can be distinguished from each other in a more sensitive way in relation to only the comparisons of the means of correspondent variables. By using the ANCOVA method statistical identity or deviation has effectively been established among the state-dependent regressions of some plant stresses.

Resultant activities of both POD and PPO isoenzymes are often tracked by spectrophotometrical methods (Chisari et al. 2008, Nunes et al. 2007, Toralles et al. 2005; Rajeswari – Paliwal 2008). In these protocols, the *in vitro* abilities of the crude leaf extract, which degrade *o*-dianizidine and catechol substrates, have primary been monitored. The correlative approximation has been extended to the investigation of the resultant activities of peroxidase (POD) and polyphenol oxidase (PPO) isoenzymes. Via a model investigation, the existence of the correlation of resultant oxidative activities was discussed. Moreover, the correlations of some biochemical variables (carbohydtrate contents, levels of quaterneric ammonium compounds, etc) have been proven to be susceptible to some plant stresses. As a result of this phenomenon, the stress syndromes of some plants have been characterized with the state-dependent regressions having various slopes and intercepts (Németh et al. 2009b). Thus, for the investigation of stress sensitivity we have tried to choose these two universal activities whose roles are related to high-flux backbone of the metabolism and which can be stress sensitive. The alterations of POD and PPO activities have been investigated in the leaves of a herbaceous plant and a forest tree species exposed to cold shock and lack of illumination.

2 MATERIALS AND METHODS

2.1 Plants

Individual characteristics of the investigated pedunculate oak trees (*Quercus robur* L.) located in the Botanical Garden at the University are the next: (a) first tree. No 1.; GPS:47.67780 ^{o}N. 016.54928 ^{o}E; age: 27 years; (b) second tree. No 2; GPS:47.67960 ^{o}N. 016.57364 ^{o}E; age: 32 years; third tree. No 3; GPS: 47.67963 ^{o}N. 016.57360 ^{o}E; age: 32 years.

Peroxidase (POD) and polyphenol oxidase (PPO) activities were determined in pedunculate oak seedlings with six leaves, in the leaves of pedunculate oak trees and also in leaves of large white bean plants (*Phaseolus vulgaris* L.). All experiments were performed at the Department of Chemistry of the University of West Hungary. Fresh acorns originated from West Hungary (Vitnyéd) were stored in refrigerator at 2°C and 80% relative humidity until used.

The germination of oak acorns and bean seeds for growing seedlings and plants were carried out in a wet bed of Florasca B soil (Florasca Ltd. Sopron. Hungary) under greenhouse condition.

2.2 Extraction of enzymes

All reagents came from Merck Chemical Co. (Germany) and Reanal Chemical Co. (Hungary). After sampling the sample clean-up was carried out immediately and all measurements were made in triplicate. The fresh leaves were homogenized with equal quantity of quartz sand. For enzyme extraction, the samples of 1.20 g were extracted with 15.00 cm^3 amounts of K-Na-phosphate buffer (pH = 6.0) solutions at 4°C for 20 minutes and then centrifuged at 6000 rpm for 10 minutes.

2.3 Assay for oxidative activity of POD isoenzymes

Resultant activity of POD isoenzymes (EC. 1.11.1.7) with *o*-dianizidine as chromogen reagent was determined by spectrophotometry at 480 nm. The reaction mixture contained 1.7 mL of buffer (pH=6.0), 30 μL of 0.3 % H_2O_2 and 20 μL of *o*-dianizidine as substrates. The reactions were started by the addition of 10 or 20 μL of extracts (Shannon et al. 1966). The increase was followed for 4 minutes. 1 POD Unit = 0.01 absorbance unit·min^{-1}.

2.4 Assay for oxidative activity of PPO isoenzymes

By monitoring of the increase in absorbance at every 10th second with a Hitachi U-1500 spectrophotometer at 420 nm, resultant activity of PPO isoenzymes (EC 1.10.3.1) with catechol substrate was measured. The increase was tracked for 3 minutes. The reaction mixture contained 1mL of buffer (pH=6.0), 1 mL of 0.2 M catechol as a substrate and 0.5 mL of extract (Flurkey–Jen 1978). All measurements were performed at room temperature. Each sample was assay in triplicate. 1 PPO Unit = 0.001 absorbance unit·min^{-1}.

2.5 Protein determination

All enzyme activities were expressed as units per microgram of protein weight (Uμg protein^{-1}). The determination of protein amounts in the extracts was carried out with Bradford's method (Bradford 1976).

3 RESULTANT EFFECT OF ISOENZYMES

Biochemical researches with reductionist approximation had a penchant for the application of spectrophotometrical technique in order to detect the enzymes and to confirm their ubiety within a given tissue, respectively. During enzyme catalysis, chemical conversion of the substrates added to the extract of biological sample to the products could be tracked according to Beer's law of spectrophotometry. For detecting the activities of various enzymes a lot of photometrical protocols have been developed. Until the middle of the seventies, the spectrophotometrical protocols had been dominant and quickly feasible indirect methods of enzyme kinetical investigations. Determination of the values of enzyme activities on the basis of biochemical reaction models (e.g. Michaelis-Menten kinetic, Ping-pong mechanism, etc) was taken back to quantitative alterations of substrate or product in time. Since finding of the existence of isoenzymes, these bioanalytical protocols have become semi-empiricals because they are directly not able to distinguish the different isoenzymes or their catalitical properties. With spectrophotometrical investigations of the activities, POD and PPO isoenzymes degrading the same substrate can not be distinguished from each other. The investigation of enzyme activities has been relocated to the analytical techniques including the separation of isoenzymes (e.g. protein electrophoresis). In spite of development of isoenzyme investigations, the applications of spectrophotometrical activity measurements has not sunk into oblivion since the semi-empirical information provided by them are/were characteristic of the biological systems. Physiological states can be characterized and their significant differences can be revealed by the measurements of semi-empirical activities, respectively. Spectrophotometrical activity protocols integrate the activities of the isoenzymes. They reflect the resultant effect of the isoenzymes as the activity of a hypotethical enzyme, wich substitute the roles of each isoenzymes in the investigated biochemical reaction. The resultant effect of the isoenzymes can be interpreted as a specific capability or activity of biological extract. The effects of POD and PPO isoenzymes in the sample extract on given substrates (e.g. catechol and o-dianizidine) are named as substrate specific oxidative activities of *in vitro* biological system.

The reaction rate (v) depends on the converting capability of biological system (Ψ) and the biochemical driving force (φ). The driving force of biochemical reactions, $\varphi([S])$ is influenced by substrate concentration and the value of Ψ is determined by product of rate constant (k) and enzyme concentration ($[E_0]$). The rate of ith reaction catalyzed by i^{th} isoenzymes:

$$v_i = \frac{d[P]_i}{dt} = \Psi(k_i, [E_{i0}]) \cdot \varphi([S]) = k_i [E_{i0}] \cdot \varphi([S]), \quad i = 1, 2, ..., n \tag{3}$$

where $\varphi([S]) \in [0, 1]$. n is the number of isoenzymes. $[P]$ is the biochemical product. $[S]$ is the substrate concentration, $[E_{i0}]$ is the total amount of ith isoenzyme and k_i is the rate constant of product formation in the ith reaction.

If the substrate concentration is much larger than the values of Michaelis constants of their isoenzymes ($[S] >> K_{M1}, K_{M2}, ..., K_{Mi}, ..., K_{Mn},$) the biochemical reaction rates are particularly propotion to the concentrations of their isoenzymes ($\varphi([S]) \approx 1$). Anyway, this limitation is the necessary condition of the application of spectrophotometrical activity measurements. The overall reaction rate is determined by the amount of the rates of independent reaction steps.

$$\begin{aligned} v &= v_1 + v_2 + ... + v_i + ... + v_n = (k_1[E_{10}] + k_2[E_{20}] + ... + k_i[E_{i0}] + ... + k_n[E_{n0}]) \cdot \varphi([S]) \\ &= k^*[E_0]^* \cdot \varphi([S]) \approx k^*[E_0]^* \end{aligned} \tag{4}$$

$$v = v\big|_{\varphi([S])\approx 1} \approx k_1[E_{10}] + k_2[E_{20}] + ... + k_n[E_{n\S}] \tag{4.1}$$

v is the resultant reaction rate measured in *in vitro* manner. Adapted the nomenclature of specific enzyme reactions to this reaction rate, it can be regarded as the resultant activity of the isoenzymes E_1, E_2, ..., E_n, or as the converting activity of the extract.

$$[E_0]^* = [E_{10}] + [E_{20}] + ... + [E_{i0}] + ... + [E_{n0}] \tag{4.2}$$

$$k^* = \frac{k_1[E_{10}] + k_2[E_{20}] + ... + k_i[E_{i0}] + ... + k_n[E_{n0}]}{[E_{10}] + [E_{20}] + ... + [E_{i0}] + ... + [E_{n0}]} \tag{4.3}$$

3.1 Uncertainty of resultant activities

Stochastic error and/or measuring precision ($\pm \Delta v$) of the reaction rate v is collectively influenced by the values of rate constants k_1, k_2, ..., k_n , which are equivalent to turnover numbers, and stochastic deviations ($\pm \Delta[E_i]$) of isoenzyme activities E_1, E_2, ..., E_n. Applied the law of error propagation to equation (4.1) we obtained the equation (5).

$$\Delta v \approx k_1\Delta[E_1] + k_2\Delta[E_2] + ... + k_n\Delta[E_n] \tag{5}$$

On the basis of equation (5), model values of Δv, which have been determined at the combinations of identical and different hypothetical rate constants (cases A, B and C in the *Table 1*) and the same values of $\Delta[E_i]$ deviations (hypothetical absolute error: ± 0.1 or ± 1), respectively, are shown in *Table 1*.

Table 1. Effect of the turnover numbers (ki) and stochastic error of isoenzyme concentrations (Δ[Ei]) on the resultant reaction rate in a hypothetical case of three isoenzymes.

Combinations of reaction rates		**A**			**B**			**C**		
$v = \Sigma v_i = constant$		i								
		1	2	3	1	2	3	1	2	3
$\Delta[E_i]$	$[E_i]$ k_i	$[E_1]=1$ $k_1=1$	$[E_2]=1$ $k_2=1$	$[E_3]=1$ $k_3=1$	$[E_1]=5$ $k_1=0.1$	$[E_2]=2$ $k_2=1$	$[E_3]=5$ $k_3=0.1$	$[E_1]=1$ $k_1=0$	$[E_2]=3$ $k_2=1$	$[E_3]=1$ $k_3=0$
± 0.1	$v_i=k_i[E_i]$	1	1	1	0.5	2	0.5	0	3	0
	$\Delta v_i=k_i\Delta[E_i]$	±0.1	±0.1	±0.1	±0.01	±0.1	±0.01	0	±0.1	0
	$\Delta v=\Sigma\Delta v_i$		**± 0.3**			**± 0.12**			**± 0.1**	
± 1	**$\Delta v=\Sigma\Delta v_i$**		**± 3**			**± 1.2**			**± 1**	

With the hypothetical value ± 0.1 of Δ[Ei] such a biological system (e.g. blood) is represented, in which the concentrations of the isoenzymes can be considered nearly constant. The absolute error is about ± 10% of the means or smaller than this limit. In these systems the stochastic deviations of isoenzyme quantities can primary be originated from the noises of gene operations (McAdams – Arkin 1999, Thattai – van Qudenaarden 2001, Arkin et al. 1998). In a hypothetical case of Δ[Ei] ≤ ± 1, in turn, such biological tissues (e.g. plant foliage) are modelled where the isoenzyme concentrations E1, E2, ..., En have some distributions. The absolute error is about ± 100% of the means or smaller than this limit. In the cases of the errors of both 10% and 100% , the value of Δv is minimal if

the conversion of the substrate S to product P is carried out by only one isoenzyme. In case of collective catalysis of more isoenzymes (n), if the rate constants were equivalent (k1 = k2 = ... = ki = ... = kn) then Δv would be equivalent to nkiΔ[Ei]. The value of Δv will decrease if one of the rate constants becomes dominant with respect to the rest (kj >> k1, k2, ..., ki ,...., kn; j ≠ i). Besides the constancy of resultant reaction rate, the larger is the dominancy of kj, the smaller will be Δv.

The occurrence of this latter case within the metabolism is supported by reference data (Masanori et al. 1984, Chemnitius et al. 1982, Wu – Marletta 1988, Thongsook – Barrett 2005, Bogdanovic et al. 2005). Moreover, it has been established in our own experiments that the distributions of resultant oxidative activities cover the domains of about one magnitude order surrounding their expected values similarly to the distributions of other biochemical variables. These phenomena may also reflect the fact that the metabolic regulation mechanisms make every effort to minimize the uncertainty of biochemical reactions. During the improvement of control of metabolic fluxes the deacrease of flux noises must have ensured evolutional advantage to the organisms (Savageau 1976, Heinrich – Schuster 1996).

3.2 Correlation and state dependence of resultant activities

In a former paper it has already been proven that the values of two biochemical variables can produce state dependent linear correlation if their distributions are identical and if the regulation of their values in the metabolism is synchronized (Németh et al. 2009a). There is a linear correlation between the resultant activities of isoenzymes if the mentioned conditions are valid for the dominant isoenzymes or for all isoenzymes. In that case if some environmental state alteration is able to induce significant alteration in the physiological state of the organism then the isoenzyme activities can also change in significant manner. If, during the alteration of physiological state, the identity of the distributions and the synchronization of regulation of the activities to each other are maintained then the new environmental state will be characterized by a new correlation differing from the previous one. Various environmental states can be distinguished from each other by the correlation of resultant isoenzyme activities being susceptible for state alteration, for example, by the correlation of different oxidative activities of leaf extracts. According to the isoenzymes determining them, these oxidative activities are specific and significantly independent from each other. The oxidative activities measured by us were specific according to their POD and PPO isoenzymes. In such situations the correlations of oxidative activities are represented by regression straight lines. To distinguish them from each other is possible by using the covariance analysis (ANCOVA) (Sváb 1967). In the next, representing and modelling various environmental effects, oxidative activities, their linear correlations and covariance analysis of regression straight lines of the correlations are reported.

4 RESULTS AND DISCUSSION

To study the correlation between the oxidative activities produced by peroxidase and polyphenol oxidase isoenzymes we chose three pedunculate oak trees (*Quercus robur* L.) at the Botanical Garden of the University of West Hungary (Sopron, Hungary). The sampling of the leaves of the three trees was variously designed. Different leaf samplings were applied to get information about their effect on the investigated enzyme correlation. Six leaves from each tree were cut off and then they were analysed at once.

In case of the first tree (No 1) a random sampling of leaves was taken from the same branch. The leaves of the second tree (No 2) originated from six different branches. The leaf samples from the third tree (No 3) were gathered without assigning branches. The leaf samplings were taken for three days when the values of climatic parameters (e.g. temperature: 20,6 ± 0,9 °C; moisture: 68 ± 1 %, atmospherical pressure: 1012 ± 4 kPa; sunshine duration: 1.5 ± 0.2 h;) could be regarded as nearly the same. The protein contents of leaf samples provided the confidence interval of 7.24 ± 0.95 mg/g dry weight independent of the individual character of oak trees. Thus, the protein content in the leaves has been considered as constant in the statistical sense. The three trees were growing on brown earth soil.

The linear regression of enzyme activities provided the straight lines with about 0.93 values of the coefficients of determination, r^2. The results of enzyme activities are shown in *Table 2*. The influence of the trees (and different leaf samplings) on the correlations were investigated by covariance analysis (ANCOVA) and we found some answers to the next four questions.

I. May a total equation of linear regression be applied to incorporated results of the leaf samplings?
II. May the regression coefficients of different samplings be considered as the same? (Is there a significant deviation between the slopes of the straight lines?)
III. Are the regression of the mean enzyme activities and the regressions of the trees identical with each other?
IV. May the corrected PPO activities, treating them as independent variables, be regarded as almost the same?

With respect to the assumed normal distribution of enzyme activities, the ANCOVA analysis was carried out (Sváb 1967) by StatsDirect v2.6.5 software (StatsDirect Ltd, Altrincham, UK). These two covariance methods can give the answers to the question mentioned above. The statistical equations of applied covariance method can be found in the Supplemental Data.

After fitting straight lines in terms of the formulation of $[PPO] = b[POD] + a$ to the values of POD and PPO specific activities, to respond to our questions the investigations of following hypotheses are necessary:

I. H_0: $b_T \neq 0$ and H_a: $b_T = 0$, respectively. If the condition of $b_T \neq 0$ is met then there is a total linear relationship in the incorporated set of enzyme activities. (b_T – he slope of the total linear regression)
II. For the second question, the H_0: $b_W = b_1 = b_2 = b_3$ and respectively, H_a: $b_W \neq b_1 \neq b_2 \neq b_3$ hypotheses have to be checked, where b_W is the slope of the regression within the classes (or of the trees) and b_i is the slope of ith class.
III. For the third case the investigation of H_0: $b_B = b_W$ resp. H_a: $b_B \neq b_W$ hypothesis give some answers. (b_B – the slope between the classes)
IV. H_0: $Y'_1 = Y'_2 = Y'_3$ is the hypothesis of the equivalence of corrected PPO specific oxidative activities.

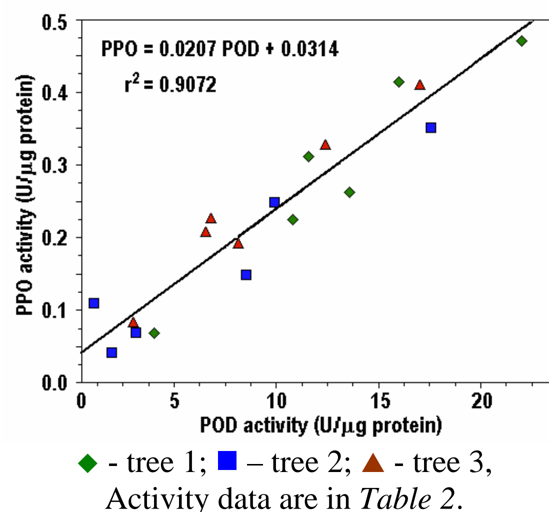
The parameters of ANCOVA are shown in *Table 2* and their results and statements are recorded in *Table 3*.

On the basis of ANCOVA, the leaf sampling did not show any influence on the investigated enzyme correlations. There was no significant deviation in the slopes and interceptions of the linear relationships. Thus, the incorporated set of activity data pairs can be characterized by a common regression straight line. The existence of the total regression is independent of the sampling and of the entity of the oak trees. The total correlation relationship between POD and PPO specific activities independent of the leaf sampling and the individual character of the tree is depicted in *Figure 1*.

Table 2. Activities (U µg protein-1) of POD and PPO isoenzymes in the leaf samples of Quercus robur L. and their statistical data. (Statistical equations can be found in Supplemental Data)

$j = 1...k$	*j* – the number of investigated oak trees								
$i = 1...n$	1.			2.			3.		
$k = 3$	Leaves from the same branch of the oak tree 1.			Leaves from six various branches of the oak tree 2.			Leaves from the tree 3 by chance.		
$n = 6$	POD	PPO		POD	PPO		POD	PPO	
i	X_{i1}	Y_{i1}	$X_{i1}Y_{i1}$	X_{i2}	Y_{i2}	$X_{i2}Y_{i2}$	X_{i3}	Y_{i3}	$X_{i3}Y_{i3}$
1	4.014	0.068	0.2729	8.496	0.150	1.2744	6.532	0.206	1.3456
2	22.018	0.470	10.349	1.074	0.110	0.1181	6.790	0.224	1.5210
3	13.564	0.262	3.5538	17.520	0.352	6.1670	3.018	0.082	0.2475
4	10.836	0.224	2.4273	3.120	0.070	0.2184	8.130	0.190	1.5447
5	11.594	0.312	3.6173	1.950	0.042	0.0819	17.034	0.408	6.9499
6	16.024	0.414	6.6339	9.882	0.250	2.4705	12.414	0.326	4.0467
Mean:	13.008	0.292	4.4756	7.007	0.162	1.7217	8.986	0.239	2.6093
□(…) :	78.050	1.750	26.853	42.042	0.974	10.330	53.918	1.436	15.656
□(…^{2}) :	1193.5	0.61308	182.78	491.48	0.22767	45.83	608.24	0.4082	71.25
[□(…)]^2 :	6091.8	3.063	721.12	1767.5	0.9487	106.72	2907.2	2.0621	245.10

Calculated statistical data			
Grand means	SST(X) = 611.02	SST(Y) = 0.2875	SPT(XY) = 12.6240
	SSB(X) = 112.22	SSB(Y) = 0.0508	SPB(XY) = 2.2781
G(X) = 10.236	SSW(X) = 498.80	SSW(Y) = 0.2367	SPW(XY) = 10.3459
G(Y) = 0.2447	$SW(X)_1$ = 178.20	$SW(Y)_1$ = 0.1027	PW(XY)1 = 4.0891
G(XY) = 3.1082	$SW(X)_2$ = 196.89	$SW(Y)_2$ = 0.0696	PW(XY)2 = 3.5056
	$SW(X)_3$ = 123.72	$SW(Y)_3$ = 0.0645	PW(XY)3 = 2.7512



◆ - tree 1; ■ – tree 2; ▲ - tree 3,
Activity data are in *Table 2*.

Figure 1. Linear correlation between POD and PPO specific activities from different pedunculate oak trees and various leaf sampling designs. Total correlation equation: PPO = 0.0207 POD + 0.0314, r^2 = 0.9072; (Global solar radiation (GSR) = 4.82 MJ·m^{-2}·day^{-1})

Table 3. Variance table for the investigation of homogenity of the linear regressions. (F - variable of F test; Fcv - criterion value of F test at α = 0.05 significance level)

	α= 0.05	Deviation from linear regression				
i	Source of variation	D_i	DFD_i	$MD_i=D_i/DFD_i$	*F*	F_{cv}
1	summarized for the samples *($D_1 = D_C$)*	0.01928	12	0.00160		
2	within samples collectively *($D_2 = D_W$)*	0.02213	14	0.00158		
3	between samples *($D_3 = D_B$)*	0.00454	1	0.00454	$\frac{MD_3}{MD_1} = 2.84$	4.60
4	for total regression *($D_4 = D_T$)*	0.02669	16	0.00167		
	Null Hyphothesis					
5	H_0: $b_T \neq 0$; There is a common regression equation *($D_5 = D_T - D_C$)*	0.00741	4	0.00185	$\frac{MD_5}{MD_1} = 1.16$	3.26
6	H_0: $b_1 = b_2 = b_3 = b_W$; The slopes are almost the same *($D_6 = D_W - D_C$)*	0.00285	2	0.00143	$\frac{MD_6}{MD_1} = 0.90$	3.89
7	H_0: $Y'_1 = Y'_2 = Y'_3$; Corrected *Y* means are almost the same *($D_7=D_T - D_W$)*	0.00456	2	0.00228	$\frac{MD_7}{MD_2} = 1.44$	3.74
8	H_0: $b_W = b_B$; *($D_8 = D_T - D_B - D_W$)*	0.00002	1	0.00002	$\frac{MD_8}{MD_2} = 0.01$	4.60

The distributions of activities of POD and PPO isoenzymes were checked by Shapiro-Wilk test and the characterization of its distribution is depicted in the *Figure 2* by van der Waerden's methods (Buchan 2000). Note that in the plain of normal scores versus enzyme activities, scattering patterns of the points along their fitted straight lines are very similar. This similarity results from necessary condition of type identity of the distributions (Németh et al. 2009a). In the case of meeting the perfect type identity of the distributions, the normal scores of POD and PPO would provide the linear regression with r^2=1 (see *Figure 2c*).

$$NS = \Phi(p),\ p = \frac{R}{n+1} \tag{7}$$

where *NS* is the normal score for an observation, *R* is the rank for that observation, *n* is the sample size and $\Phi(p)$ is the pth quantile from the standard normal distribution (Buchan 2000).

The results of Shapiro-Wilk test applied to the significance level of α = 0.05 did not contradict our hypothesis for the normal distributions of enzymes activities. As can be seen in the *Figure 2* the normal scores of activities determined by van der Waerden test versus the values of the activities represents linear relationships with a very good approximation.

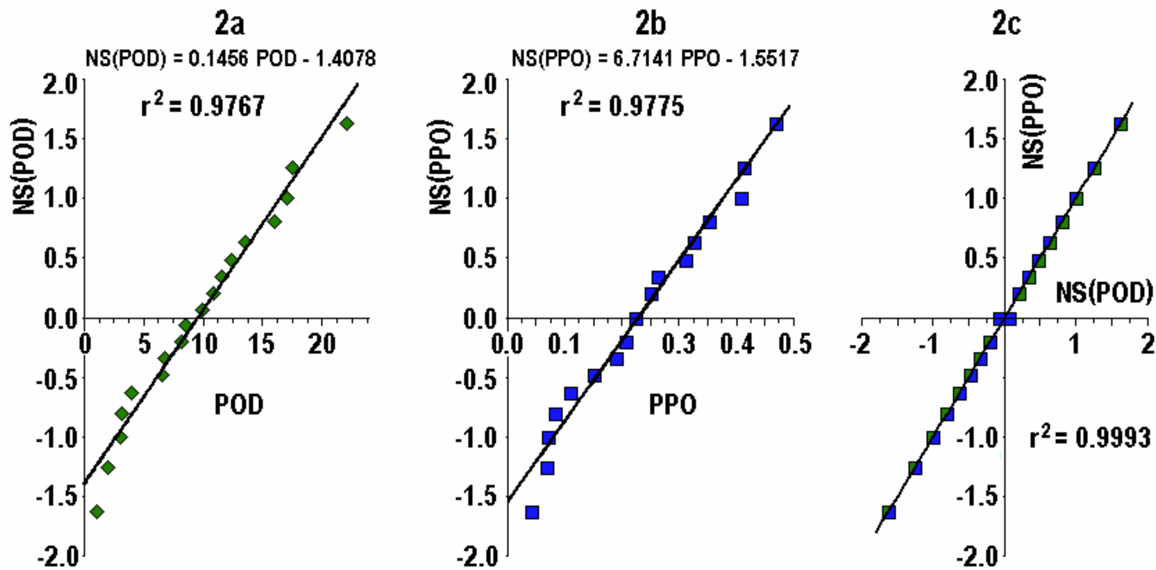
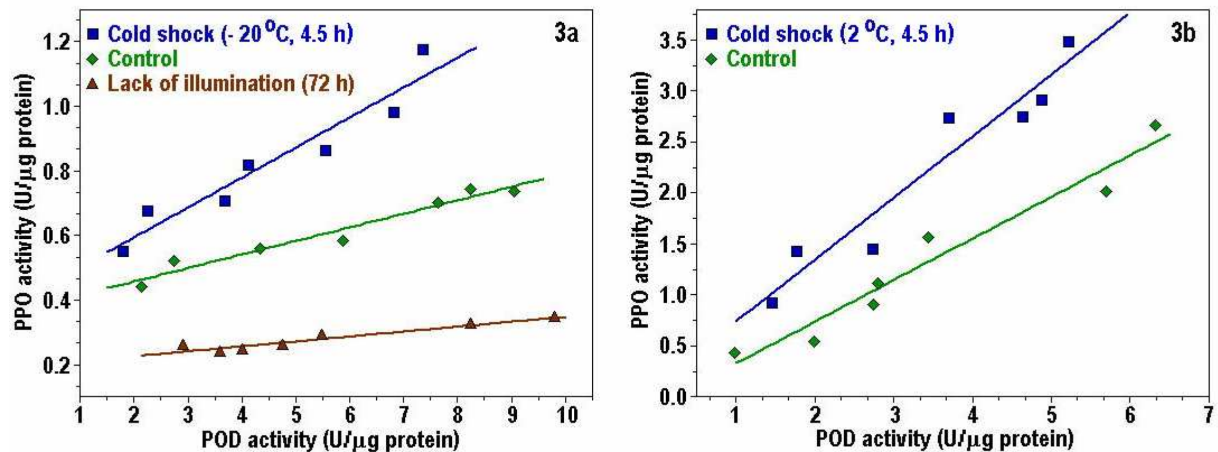


Figure 2. Investigation of distributions of POD and PPO activities in Table 2 by van der Waerden's test. (NS(POD), NS(PPO) – normal scores of enzyme activities POD and PPO)

The correlation between the resultant oxidative activities of POD and PPO isoenzymes, which showed an independence of tree entity, and which was found in pedunculate oak trees, has also been mapped for a herbaceous plant species and for the leaves of large white bean plant (*Phaseolus vulgaris* L.). Since the cold shock and absence of illumination are able to change some enzyme activities (Li et al. 2006, Prestano et al. 2004, Cohen et al. 1984, Fair et al. 1974) the role of these environmental effects on POD-PPO oxidative activity correlation has been investigated. For control and stress investigations oak acorns and bean seeds were germinated and full-grown bean plants and oak seedlings were exposed to the similar effects. Linear correlations between POD and PPO specific activities of both the oak seedlings and bean plants were found (*Figure 3*).

The ***measured*** protein contents in the control state, cold shock and lack of illumination of bean plants and oak trees were in the confidence intervals of 75.65 ± 3.97 and 6.32 ± 0.30 mg/g dry weight (□ = 0.05).

As an alternative statistical evaluation, the regression straight lines, whose data are depicted in the *Tables 4 and 5,* were compared to each other by the ANCOVA of StatsDirect v2.6.5 software (StatsDirect Ltd., UK). The results of this evaluation are enclosed in the sections II. and III. of Supplemental Data. From them it can be established that the investigated environmental effects induced significant alterations in the regression parameters of oxidative activities POD and PPO. These environmental conditions are characterized by significantly different POD versus PPO correlations. This correlation has been proven to be stress sensitive. With respect to the classical comparison of activity means, the stress sensitivity of the correlation can reveal extra information about the measured data. Note that, for example in the case of *Figure 3b*, statistical *t*-test is not able to detect any differences between the POD or PPO values because there are almost total overlaps in their confidence intervals.



3a: Pedunculate oak seedling.

■ - cold shock{-20 °C, 4.5 h}, PPO = 0.0929 POD + 0.4081, r^2 = 0.9206;
◆ - control, PPO = 0.0419 POD + 0.3737, r^2 = 0.9529;
▲ - lack of illumination{72 h}, PPO = 0.0153 POD + 0.1948, r^2 = 0.9195

3b: large white bean plant.

■ - cold shock{2 °C, 4.5 h}, PPO = 0.6043 POD + 0.1415, r^2 = 0.9258;
◆ - control, PPO = 0.4074 POD - 0.0766, r^2 = 0.9477

Figure 3. Effects of the cold shock and lack of illumination on the PPO versus POD correlations

5 CONCLUSION

Linear correlations between the activities of POD and PPO isoenzymes in leaf samples from oak trees as well as bean plants have been established. The linear relationship independently of the individual character of the species is exactly able to reflect the synchronic regulation of these isoenzymes in leaf metabolism. If there is small POD specific activity in the leaves, then small value of PPO specific activity will be related to it. This correlation has been assumed to reveal some important information characterizing the plant species and to eliminate the individual characteristics of the investigated plant or tree.

Comparing the confidence intervals of the activities the effect of environmental conditions on the metabolism in the leaves could not always be detected. On the contrary, the slope of a linear relationship between the resultant oxidative activities is stress sensitive. The cold shock and the lack of illumination were able to alter the enzyme correlation characterizing a steady state of the metabolism in significant way. The linear character of the regressions obtained after the stress effect can be related to the property of the plant leaf cells that were able to find a new steady state for their metabolism.

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SUPPLEMENTAL DATA

I. Equations of covariance analysis (ANCOVA)

Equations (1-12) are necessary to calculate the sums of squares for statistical evaluations. X_{ij} is the POD activity of ith leaf of jth tree shown in the *Table 1*. Y_{ij} is the PPO activity belonging to X_{ij}.

(1) $$SST(X) = SS_{Total(X)} = \sum_{j=1}^{k}\sum_{i=1}^{n}\left(X_{ij} - \overline{\overline{X}}\right)^2$$

(4) $$SST(Y) = SS_{Total(Y)} = \sum_{j=1}^{k}\sum_{i=1}^{n}\left(Y_{ij} - \overline{\overline{Y}}\right)^2$$

(2) $$SSB(X) = SS_{Between(X)} = n\sum_{j=1}^{k}\left(\overline{X}_j - \overline{\overline{X}}\right)^2$$

(5) $$SSB(Y) = SS_{Between(Y)} = n\sum_{j=1}^{k}\left(\overline{Y}_j - \overline{\overline{Y}}\right)^2$$

(3) $$SSW(X) = SS_{Within(X)} = \sum_{j=1}^{k}\sum_{i=1}^{n}\left(X_{ij} - \overline{X}_j\right)^2$$

(6) $$SSW(Y) = SS_{Within(Y)} = \sum_{j=1}^{k}\sum_{i=1}^{n}\left(Y_{ij} - \overline{Y}_j\right)^2$$

(7) $$SW(X)_j = \sum_{i=1}^{n} X_{ij}^2 - \frac{\left(\sum_{i=1}^{n} X_{ij}\right)^2}{n}$$

(8) $$SPT(XY) = SP_{Total(XY)} = \sum_{j=1}^{k}\sum_{i=1}^{n}\left(Y_{ij} - \overline{\overline{Y}}\right)\left(X_{ij} - \overline{\overline{X}}\right)$$

(9) $$SW(Y)_j = \sum_{i=1}^{n} Y_{ij}^2 - \frac{\left(\sum_{i=1}^{n} Y_{ij}\right)^2}{n}$$

(10) $$SPB(XY) = SP_{Between(XY)} = n\sum_{j=1}^{k}\left(\overline{Y}_j - \overline{\overline{Y}}\right)\left(\overline{X}_j - \overline{\overline{X}}\right)$$

(11) $$SPW(XY) = SP_{Within(XY)} = \sum_{j=1}^{k}\sum_{i=1}^{n}\left(Y_{ij} - \overline{Y}_j\right)\left(X_{ij} - \overline{X}_j\right)$$

(12) $$PW(XY)_j = \sum_{i=1}^{n} X_{ij} Y_{ij} - \frac{\sum_{i=1}^{n} X_{ij} \sum_{i=1}^{n} Y_{ij}}{n}$$

Deviations (13-16) for ANCOVA analysis in Table 2.

(13) $$D_W = SSW(Y) - \frac{SPW(XY)^2}{SSW(X)}$$

(14) $$D_B = SSB(Y) - \frac{SPB(XY)^2}{SSB(X)}$$

(15) $$D_T = SST(Y) - \frac{SPT(XY)^2}{SST(X)}$$

(16) $$D_C = \sum_{j=1}^{k}\left[SW(Y)_j - \frac{PW(XY)_j^2}{SW(X)_j}\right]$$

II. Results of ANCOVA of *Table 4* by Stats Direct v2.6.5 software

Table 4. Resultant oxidative activities (U μg protein-1) of perosidase (POD) and polyphenol oxidase (PPO) in the leaves of Quercus robur L. seedlings under different enviromental conditions (Figure 3a)

j	1.		2.		3.	
j = 1…*k*	Control		Cold shock (-20 °C)		Lack of illumination	
i = 1…*n*	POD	PPO	POD	PPO	POD	PPO
i	X_{i1}	Y_{i1}	X_{i2}	Y_{i2}	X_{i3}	Y_{i3}
1	4.345	0.559	4.118	0.821	3.587	0.238
2	2.736	0.522	5.543	0.866	2.895	0.259
3	7.631	0.703	2.243	0.676	9.789	0.345
4	5.872	0.585	6.803	0.982	5.487	0.287
5	2.134	**0.441**	7.357	1.178	3.996	0.245
6	9.056	0.737	1.783	0.553	8.235	0.322
7	8.235	0.745	3.667	0.707	4.763	0.259

Grouped linear regression

Source of variation	SSq	DF	MSq	VR	
Common slope	0.229150	1	0.229150	135.240204	P < 0.0001
Between slopes	0.098859	2	0.049430	29.172567	P < 0.0001
Separate residuals	0.025416	15	0.001694		
Within groups	0.353425	18			

Common slope is significant

Difference between slopes is significant

Slope comparisons

Slope 1 (Xi1) versus *Slope* 2 (Xi2) = 0.04189 *versus* 0.092859
Difference (95% CI) = 0.050969 (0.029801 to 0.072137)
t = 2.956198 P = 0.0098

Slope 1 (Xi1) versus *Slope* 3 (Xi3) = 0.04189 *versus* 0.015259
Difference (95% CI) = 0.026631 (0.00743 to 0.045831)
t = -5.132126 P = 0.0001

Slope 2 (Xi2) versus *Slope* 3 (Xi3) = 0.092859 *versus* 0.015259
Difference (95% CI) = 0.077599 (0.055851 to 0.099348)
t = 7.605173 P < 0.0001

Covariance analysis

Source of variation	YY	xY	xx	DF
Uncorrected				
Between groups	1.063725	-1.782643	6.006368	2
Within	0.353425	5.060858	111.770938	18
Total	1.417151	3.278216	117.777305	20

Source of variation	SSq	DF	MSq	VR
Corrected				
Between groups	1.201629	2	0.600815	82.187254
Within	0.124275	17	0.007310	
Total	1.325905	19		

P < 0.0001

Corrected Y means ± SE for baseline mean predictor of 5.25119

Y' = 0.592116	± 0.032534
Y' = 0.860065	± 0.032879
Y' = 0.26639	± 0.032398

Line separations (common slope = 0.045279)

Lines not parallel

Line 1 (Xi1) versus *Line* 2 (Xi2) vertical separation = -0.267949
95% CI = -0.36657 to -0.169328
t = -5.732288 (17 df) P < 0.0001

Line 1 (Xi1) versus *Line* 3 (Xi3) vertical separation = 0.325726
95% CI = 0.229255 to 0.422198
t = 7.123604 (17 df) P < 0.0001

Line 2 (Xi2) versus *Line* 3 (Xi3) vertical separation = 0.593675
95% CI = 0.495652 to 0.691699
t = 12.778037 (17 df) P < 0.0001

III. Results of ANCOVA of Table 5 by Stats Direct v2.6.5 software

Table 5. Resultant oxidative activities (U μg protein-1) of peroxidases (POD) and polyphenol oxidases (PPO) in the leaves of Phaseolus vulgaris L. plants under control and cold shock conditions (Figure 3b)

j	1.		2.	
$j = 1 \ldots k$	Control		Cold shock (2 °C)	
$i = 1 \ldots n$	POD	PPO	POD	PPO
i	X_{i1}	Y_{i1}	X_{i2}	Y_{i2}
1	2.745	0.906	1.454	0.932
2	2.803	1.110	3.694	2.739
3	0.978	0.435	5.213	3.483
4	5.693	2.012	4.624	2.747
5	6.328	2.660	1.765	1.435
6	3.431	1.562	2.727	1.453
7	1.986	0.542	4.872	2.916

Grouped linear regression

Source of variation	**SSq**	**DF**	**MSq**	**VR**	
Common slope	8.477383	1	8.477383	138.358472	P < 0.0001
Between slopes	0.333158	1	0.333158	5.43743	P = 0.0419
Separate residuals	0.612712	10	0.061271		
Within groups	9.423253	12			

Common slope is significant
Difference between slopes is significant

Slope comparisons

Slope 1 (Xi1) versus *Slope* 2 (Xi2) = 0.407409 *versus* 0.604322
Difference (95% CI) = 0.196912 (0.008756 to 0.385069)
t = -2.33183 P = 0.0419

Covariance analysis

Source of variation	YY	xY	xx	DF
Uncorrected				
Between groups	2.997463	0.178145	0.010587	1
Within	9.423253	17.563323	36.387443	12
Total	12.420716	17.741468	36.398031	13

Source of variation	SSq	DF	MSq	VR
Corrected				
Between groups	2.827135	1	2.827135	32.878208
Within	0.945869	11	0.085988	
Total	3.773004	12		

P = 0.0001

Corrected Y means ± SE for baseline mean predictor of 3.450929	
Y' = 1.331416	± 0.110841
Y' = 2.230298	± 0.110841

Line separations (common slope = 0.482675)	
Lines not parallel	
Line 1 (Xi1) versus *Line* 2 (Xi2) vertical separation = -0.898881	
95% CI = -1.243918 to -0.553845	
t = -5.733952 (11 df)	P = 0.0001