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Full Length Research Paper

Different interactions catalase and peroxidase enzymes with minerals to response seasonal variation in leaves and twigs of oak trees

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The ecological system of forests is diverse and complex. To obtain information in this field, admissible parameters for characterisation and evaluation is required for research strategies. To gain more knowledge about this topic, useful biostatistics and multivariate statistical analyses were to be developed and applied. In current research, detail of minerals and enzyme activities were investigated plus correlation between them. Adult trees of *Quercus brantii* were selected in oak forests at 2300 m above sea level. In months of March, May, July, September, November and January, sampling was done from twigs and leaves. The content of Ca, Cu, Fe, K, Mg, Mn, Na and activity of CAT and POD were estimated in samples. Factor analysis showed enzyme activities as basal principle in adaptation of twigs and leaves, also some relation were observed between macro nutrients as other affective principles in photosynthesis. Between investigated micro nutrients, Fe had important role in twigs. Cluster analysis using standardized k-means algorithm indicated seasonal clusters in separate positions by two markers. Clusters were similar in separate seasons.

Key words: Clustering, enzyme, nutrients, multivariate statistical analyses, oak, season.

INTRODUCTION

Oaks are known by high drought resistance and durability against cold and hot temperatures. By this aspect, this tree delivers a very interesting study object for physiological developments enzymes and mineral balance in the seasonal cycle over a year. Scientists have been suggesting that some enzymes play an important role in several physiological processes including pathogen resistance, genetic type, response to stress (Yazici et al., 2007; Chandra and Dubey, 2010), and lignin biosynthesis (Onnerud et al., 2002).

Although numerous researches have been conducted on PODs, the precise function of the isoenzymes is undetermined (Gulen and Eris, 2004). The PODs are merely classified as acidic, neutral and alkaline, according to their isoelectric points (Tang and Newton, 2005). Each group corresponds to a special function in the cell. Furthermore, Polle and Chakrabarti (1994), Chaitanya et al. (2002) and Mazorra et al. (2002) reported that POD patterns and activity are related to the appearance of trees and their physiological status caused by stress situation as extreme temperature and drought. Under water deficiency, an increase in activities of

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Abbreviations: Ca, Calcium; **Cu,** copper; **Fe,** iron; **K,** potassium; **Mg,** magnesium; **Mn,** manganese; **Na,** sodium; **CAT,** catalase; **POD,** peroxidase.

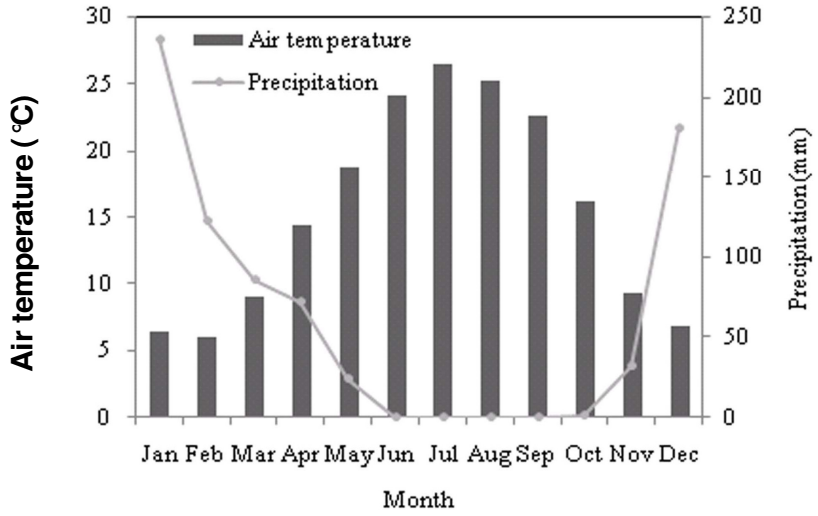

Figure 1. Monthly precipitation and mean air temperature at the research site.

superoxide dismutase, CAT and glutathione reductase has been reported (Iturbe-Ormaetxe et al., 1998). Significant change of POD and CAT activity as a marker in pine direct shoot has been observed (Tang and Newton, 2005). Also, enzymes are affected by environment, genotype and their interaction (Law et al., 2001; Lucash et al., 2007; Shabannejad et al., 2009).

Cycling of nutrients is a fundamental component in the functioning of forest ecosystems and in fact, the key to the survival of any forest ecosystem lies in its efficient nutrient cycling (Palviainen et al., 2004). The nutrients accumulated in a forest ecosystem depend on the type of forest species present, density, age, basal area, altitude, climatic conditions as well as on soil and moisture conditions (Gessler et al., 1998, 2002). Mineral nutrients have many functions in plants; they are for example, constituents of plant tissue, co-factors in enzymes, osmotic regulators, disease resistance, constituents of buffer systems, and regulators of membrane permeability (Holzmueller et al., 2007).

However, most studies on enzymes and minerals productivity have been focused on young, fully expanded leaves, ignoring the effect of twigs (Han et al., 2008).

In many species, the season-related with enzymes and minerals reserves are mobilized in trees to fuel maintenance respiration during winter, build twigs, leaves and support regeneration in spring and to provide the energy expended in making adaptive responses to other seasonal and environmental factors (Barbaroux and Breda, 2002; Barbaroux et al., 2003; Grassi and Magnani, 2005; Han et al., 2008).

To use statistical inference of agricultural problems in landscape analysis, stratification of land into relatively homogenous regions using an objective aggregation approach is necessary (Metzger et al., 2005; Williams et al., 2008). Multivariate statistical analyses and crop growth simulation models have been used to predict crop yields (Leilah and Al-Khateeb, 2005; Hargrove and Hoffman, 2005; Williams et al., 2008). Here, we defined factors as areas of similarity combined in twigs and leaves. Principle component analysis (PCA), factor analysis and multivariate clustering were used in combination to delineate these topics (Leilah and Al-Khateeb, 2005; Golicher et al., 2008).

The purpose of this study was to delineate seasonal clustering factors using a quantitative analytical approach. The aim was to identify different combinations of enzymes, minerals and environmental factors as distributed spatial units of similar potentials and limitations, and that would have broad application to numerous plants.

MATERIALS AND METHODS

Site description

The study was carried out in a site in high forest with *Q. brantii* at an altitude of 2300 m above sea level (31° 30′ 13″ N, 50° 35′ 25″ E). Soil texture was 58% sand, 17% silt, 24% clay, 26.4 ppm P, 1.673% OC and 91 mg C/100 g soil microbial biomass, with pH 7.6. The climate is semi arid with unpredictable temperature fluctuation in both summer and winter. Rainfall and snowfall begin from mid-autumn and continue until spring March/April and the climate is dry from May until October. Long-term averages precipitation and temperature were according to Figure 1.

Plant materials

Twenty four elite trees were selected; trees had normal phenotypic and physiology situation. The selection of trees was done in March. Selected individuals were seeding crop and had 25 to 35 cm diameter at breast height. Height, crown diameter and diameter at breast height of them were measured in July (Appendix, Table 1). The samplings were performed before, during and after a vegetative period in March, May, July, September, November and

January. At each sampling time, the current year twigs and leaves were cut from all 24 selected trees. The samples were held under cold conditions and transferred immediately to the laboratory to carry out the following biochemical and chemical analyses.

Enzymes assay

Crude extraction was conducted using Ebermann and Stich (1982) method. Briefly, 0.1 g of sample was homogenized in 0.3 ml extraction buffer (1.2 g l^{-1} Tris, 3.8 g l^{-1} Borax, 3.6 g l^{-1} sodium chloride, 2 g l^{-1} ascorbic acid, 50 g l^{-1} polyethylene glycol 2000, 2 g l^{-1} Na_2EDTA, pH = 7.5) and was kept for 48 h at 4°C. The samples were centrifuged at 3,000 rpm, for 15 min in 4°C. The supernatant was used for POD and CAT activity assay. POD activity was determined according to the procedure of Chance and Maehly (1955) at 25°C with spectrophotometer (at the wave length of 420 nm) following the formation in a 3 ml reaction mixture containing 0.32 ml pyrogallol solution (5%), 0.32 ml 0.1 M phosphate buffer (pH = 6.0), 2.1 ml distil water, 0.16 ml H_2O_2 0.5% (w/w), and 0.1 ml enzyme extract.

The total POD activity values reported are the average of three measurements. Specific activity of POD is expressed in terms of pyrogallol units. One pyrogallol unit will form 1.0 mg purpurogallin from pyrogallol in 20 s at pH 6.0, at 20°C. CAT activity was measured spectrophotometrically based on the reduction of H_2O_2 by Eising and Gerhardt (1989). CAT activity was detected (at the wave length of 240 nm) in a 100 ml 0.1 M phosphate buffer (pH = 7.0) containing 200 µl hydrogen peroxide 3% (w/w) plus 50 µl sample solution. One unit was defined as the decomposition of 1 mmol H_2O_2 per min per gram DW (Dry Weight). The total CAT activity values reported are the average of three measurements. POD and CAT activity were reported and expressed in arbitrary units (unit min^{-1} mg^{-1} DW).

Elements assay

Elements composition of leaves and twigs were determined on the basis of procedure suggested by Palma et al. (2000). Oven-dried plant samples were ground in a Wiley Mill and digested in perchloric and nitric acid (1:5, v/v). The digest was analyzed for total Ca, K, Mg, Na, Fe, Mn and Cu, following the standard procedure described in Handbook of reference methods for plant analysis edited by Kalra (1998).

Statistical analyses

All statistical analyses were done using SAS 9.0 (SAS Institute Inc., Cary, NC, USA. 2001), SPSS 13 for Windows (SPSS Inc., Chicago, IL, USA. 2002), MINITAB 14 (Minitab Inc., PA, USA. 2003) and JMP 7.0 (SAS Institute Inc. 2007) softwares for more certitude. Experimental data obtained from different months were analyzed by Analysis of Variance (ANOVA) using the GLM procedure, employing Statistical Analysis System (SAS). Significant differences between mean values obtained from at least three independent experiments were made with the Duncan test at 5% level of probability. Each value was presented as means standard errors (SE) of the mean, with a minimum of three independent replicates.

To identify the potential factors affecting enzymes and nutrients, principle-component factor analysis with Varimax rotation was used (Leilah and Al-Khateeb, 2005). The factor analysis was computed based on a correlation matrix to determine the effect of different months on concentration ranges of enzymes, macro and micro nutrients. The number of factors was determined using a screen plot and then identifying where the elbow in the curve occurs (Liu et al., 2007). Differences in the remaining proportion of the nine variables at the end of seasons were determined by Duncan comparisons ($P < 0.05$). In this study, clustering was created using non-hierarchical standardized k-means algorithm following a procedure described by Iniguez et al. (2005). Because of the Euclidean assignment method, the k-means algorithm tends to fit globular clusters of equal size in data space. Thus, all large ecoregions share a similar upper limit within group variance and have a similar maximum radius around each centroid (Hargrove and Hoffman, 2005).

Although other clustering methods (that is, Ward's, average, single, or complete linkage) are available, the uniform heterogeneity across ecoregions provided by k-means prevents the creation of side-by-side ecoregions that have vastly different within-region variance (Hargrove and Hoffman, 2005). Four months data were used for clustering only (May, July, September and November) because twig and leaf were observed together, and 3D clustering used by Hoffman et al. (2005).

RESULTS AND DISCUSSION

Enzymes activities

Peroxidase (POD)

During the investigated period, twig samples POD amount was around 1.7 unit min^{-1} mg^{-1} DW in spring, but in leaf fall months (Figure 2a) decreased extremely and were significant (Table 2). The amount of POD was high in May because fresh twigs and leaves have been produced (Figure 2a). In the following period, during drought and high temperature, the activity of POD was reduced dramatically (Figure 2a). After this period and at the fall of leaves, an increase to an acceptable level of POD activity in samples during winter time was observed (Figure 2a). This situation confirms lignification reduction during drought and high temperature; probably for better water mobility followed by a period of more lignification with reduction of water mobility and stabilization of oak tree system (Chaitanya et al., 2002; Onnerud et al., 2002; Mazorra et al., 2002; Gulen and Eris, 2004). Subsuming these results, we can assume that the POD system of oak trees have been adapted to long drought and high temperature in the research site. In this regard, the results confirm those ones of authors who declared that POD will result in more stability and adaptability against stress situation as drought, temperature or climate condition on high sea level (Yazici et al., 2007; Chandra and Dubey, 2010).

Catalase (CAT)

Significant CAT activity occurred only in May (new twigs and development of leaves) and in November (before leaf fall) (Table 1). Tang and Newton (2005) and Shabannejad et al. (2009) have reported CAT and POD as markers for *in vitro* shooting stage in pine and eucalypt. Figure 2b shows CAT activity alterations in oak twigs and leaves during different vegetative phases.

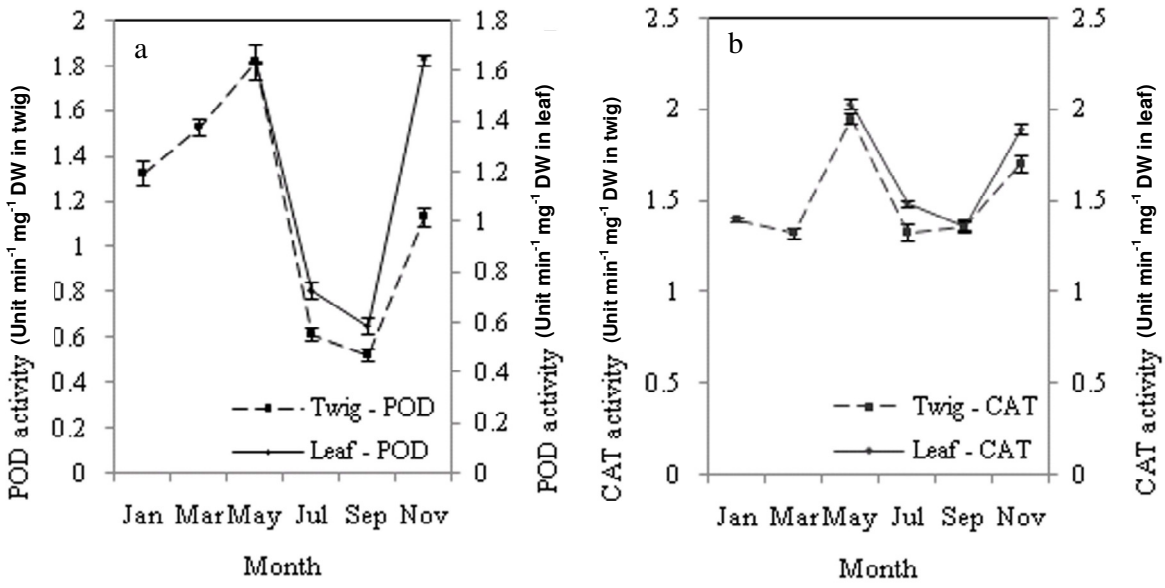

Figure 2. *Quercus brantii* twigs and leaves POD (a) and CAT (b) activity in seasonal variations. Vertical bars indicate means ± SE (Standard Error); n = 24.

Table 1. Enzyme activity in twigs and leaves of *Quercus brantii*.

Enzyme activity		Twig		Leaf	
		POD	CAT	POD	CAT
Month	Jan	1.327±0.054^{c}	1.400±0.013^{c}	-	-
	Mar	1.532±0.039^{b}	1.329±0.027^{c}	-	-
	May	1.820±0.003^{a}	1.949±0.028^{a}	1.638±0.068^{a}	2.034±0.027^{a}
	Jul	0.619±0.025^{e}	1.332±0.043^{c}	0.727±0.035^{b}	1.484±0.014^{c}
	Sep	0.524±0.026^{e}	1.368±0.037^{c}	0.588±0.031^{c}	1.364±0.024^{d}
	Nov	1.136±0.039^{d}	1.708±0.044^{b}	1.646±0.020^{a}	1.897±0.030^{b}

Data are presented as the means ± SE (Standard Error); n = 24. Different superscripts indicate significant differences ($\alpha < 0.05$) by ANOVA between months in enzyme activity. Units for each enzyme are according to Figure 2.

Differences in CAT activities between oak twigs and leaves could hardly be observed (Figure 2b) but in leaves, the CAT activity had significant differences in all seasons (Table 1).

Some studies reported the effect of environmental conditions (*in vivo* and *vitro*) on CAT and POD (Law et al., 2001; Tian et al., 2003; Lucash et al., 2007; Shabannejad et al., 2009). CAT, as well as POD expressed the highest activity in samples during leaf expansion and formation of new twigs in May (Figure 2b). During the following dry period and winter time, the level of activity differed hardly, but delivered a good support for the lignification by POD. Yet it turned out that this system is not useable as a parameter for stress situation. CAT can disproportionate H_2O_2 to H_2O and O_2 and can be assessed as a further indicator for stress situation (Hegedus et al., 2001). The reduction of CAT activities during drought period was reported by Iturbe-Ormaetxe et al. (1998).

Macro nutrients

Calcium (Ca)

For both samples, Ca concentration was high (Table 2). The levels were definitely higher in twigs than that of in leaves (Table 2). Ca content declined extremely in new twigs in May (Figure 3a). With the beginning and continuation of the drought period (May to November), the Ca content increased continuously until September; and then remained almost constant until March (Figure 3a). Ca is a structural component in plant cell walls (Sun et al., 2009; Lautner and Fromm, 2009). Studies have shown that Ca is involved in the regulation of plant responses to various environmental stresses, including heat (Palma et al., 2000; Jiang and Huang, 2001; Hong-Bo et al., 2008; Sun et al., 2009). For leaves, a similar cycle could be observed (Figure 3a). Minimum and maximum levels of Ca were 8 to 27 mg g^{-1} DW in twig

Table 2. Nutrient concentrations in twigs and leaves of *Quercus brantii*.

Nutrients cycling		Ca	K	Mg	Na	Fe	Mn	Cu
					Twig			
Month	Jan	28.563±1.549^{a}	1.138±0.106^{d}	1.580±0.070^{b}	210.404±13.224^{c}	134.150±11.322^{ab}	62.984±6.057^{a}	15.014±0.533^{b}
	Mar	28.792±1.636^{a}	6.201±0.329^{c}	1.884±0.090^{b}	354.491±13.421^{a}	148.720±7.021^{a}	46.790±4.214^{bc}	6.835±0.525^{d}
	May	8.538±0.888^{c}	14.175±0.256^{a}	2.134±0.080^{b}	340.336±11.733^{a}	150.794±6.784^{a}	29.147±3.135^{d}	10.998±0.597^{c}
	Jul	17.909±1.194^{b}	8.719±0.264^{b}	1.713±0.060^{b}	210.241±14.325^{c}	104.546±6.109^{cd}	37.282±3.701^{cd}	5.385±0.412^{e}
	Sep	26.202±1.323^{a}	8.460±0.210^{b}	9.514±0.570^{a}	295.262±13.396^{b}	92.337±5.649^{d}	52.631±4.534^{ab}	6.208±0.349^{de}
	Nov	26.100±1.815^{a}	1.420±0.065^{d}	1.847±0.040^{b}	258.772±13.744^{b}	125.023±6.214^{bc}	62.346±4.114^{a}	16.403±0.430^{a}
					Leaf			
Month	May	4.101±0.290^{d}	8.572±0.278^{a}	1.165±0.034^{c}	216.997±8.47^{d}	266.318±13.934^{b}	38.052±4.687^{c}	8.523±0.669^{b}
	Jul	6.997±0.469^{c}	5.768±0.339^{b}	1.419±0.081^{bc}	281.587±20.15^{c}	307.120±24.618^{b}	37.523±3.743^{c}	3.735±0.487^{c}
	Sep	16.320±0.625^{a}	7.933±0.345^{a}	8.737±0.209^{a}	1193.208±7.93^{a}	341.625±38.257^{b}	58.050±6.215^{b}	3.350±0.303^{c}
	Nov	13.150±0.997^{b}	2.267±0.273^{c}	1.707±0.087^{b}	451.605±28.097^{b}	663.528±45.965^{a}	83.306±8.881^{a}	17.064±1.364^{a}

Data are presented as the means ± SE (Standard Error); n = 24. Different superscripts indicate significant differences ($\alpha < 0.05$) by ANOVA between months in nutrient concentrations. Units for each nutrient are according to Figure 3

and 4 to 16 mg g^{-1} DW in leaf (Figure 3a). Also in leaves, Ca was in different Duncan groups during seasons (Table 2). Some suggest that Ca may be involved in signal transduction (Nirmal et al., 2010) and gene expression (Jiang and Huang, 2001; Hong-Bo et al., 2008) under oxidative and heat stress. In this connection, Palma et al. (2000) reported that a higher lignification process decreases the cell-wall plasticity and in consequence reduces the cell developing process particularly the elongation of woody cells.

Potassium (K)

Potassium (K) is the major cell electrolyte and is important for the balance of charge in the cells; it is involved in plant growth, helps to regulate stomates, special gate cells in leaves that open and close in order to allow air and water vapour to pass into and out of the plant; it is also required for every major step of protein synthesis, is responsible for synthesis of starch by activating starch synthesize, maintains electrical charge balance at the site of ATP production and may influence the transport of sugars (Palma et al., 2000; Egilla et al., 2001; Brossa et al., 2009; Ache et al., 2009; Escalante-Pérez et al., 2009).

In the building phase of new twigs (May), the level of K (14 mg g^{-1} DW) was twice as high as in the following period of dryness in summer (Figure 3b). Similarly to this results, the highest level of K was found in fresh leaves (8 to 10 mg g^{-1} DW) in March, dropping down to a low level in the next months during the dry period (Figure 3b). As well in twigs as in leaves, K reached its lowest level at the time of leaf fall in November (1 mg g^{-1} DW) and in the following period in wintertime (Figure 3b). After this time, K was mobilized anew and the content in twigs increased before buds burst in May and March (Figure 3b). Similar changes of K in leaves during seasonal period were obtained by Palma et al. (2000), Sardans and Penuelas (2007), Sardans et al. (2008) and Ache et al. (2009). As to Palma et al. (2000), Egilla et al. (2001), Escalante-Pérez et al. (2009) and Nirmal et al. (2010), a sufficient K level delivers more resistance against environmental stress, particularly against chill, heat, drought and pests. This could be one of the reasons for the high level of K in twigs and leaves during the long period of drought with high temperatures between May and September, Duncan group of an included twig in May but in leaf, May and September months, respectively (Table 2).

Magnessium (Mg)

This mineral did not show considerable seasonal variability, the Mg content was always lower, higher volume is in September for both samples only (Figure 3c). This difference was significant before shrivel of twigs and leaves in September (Table 2). In both samples, the concentration of Mg increased continuously from the fresh

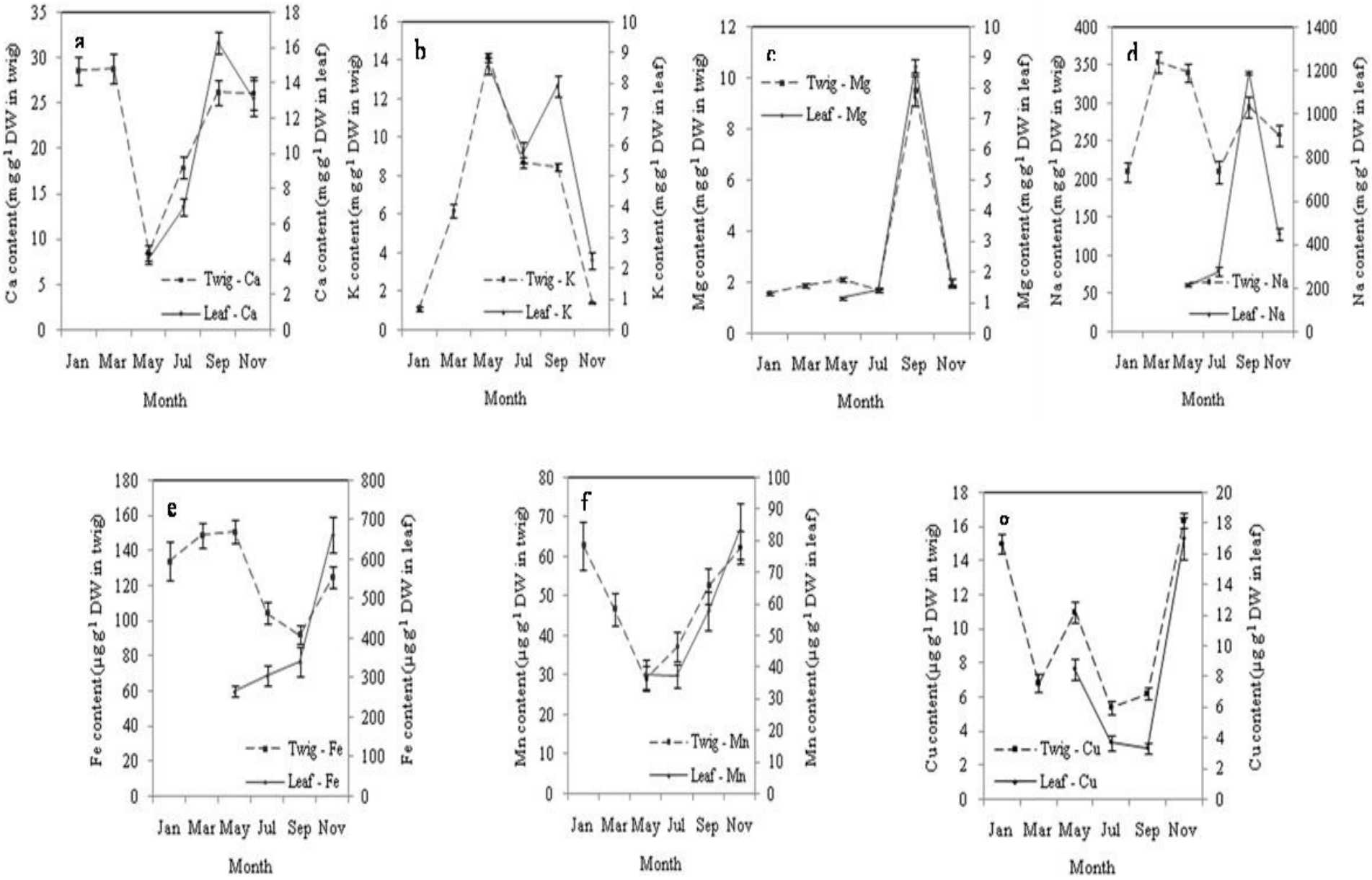

Figure 3. *Quercus brantii* twigs and leaves Ca (a), K (b), Mg (c), Na (d), Fe (e), Mn (f) and Cu (g) concentrations in seasonal variations. Vertical bars indicate means ± SE (Standard Error); n = 24

building process of leaves in May, during the hot period in summer, till the leaf fall in November (Figure 3c) which is the fact that higher Ca concentrations reduce the availability of Mg support (Palma et al., 2000; Thomas and Sprenger, 2008; Nirmal et al., 2010). Mg level in twigs and leaves had decreased extremely between September and November (Figure 3c). Mostly, the found concentrations were in the range 9 mg g^{-1} DW in maximum and 2 mg g^{-1} DW in minimum volumes and were clearly higher than the required limit concentrations of Mg for optimal production of chlorophyll in leaves (Nirmal et al., 2010). The macronutrient Mg is the central element of chlorophyll and a cofactor for enzymes in the carbon fixation process of photosynthesis (Black et al., 2008; Thomas and Sprenger, 2008). Moreover, it is included in carbohydrate metabolism, protein, fat and nucleic acid synthesis and what is more, is involved in active membrane transport in cells (Palma et al., 2000; Clair et al., 2005; Black et al., 2008; Thomas and Sprenger, 2008).

Sodium (Na)

Figure 3d shows Na amount was high and significant in new twigs (Table 2). Both samples had similar procedure during leaf expansion (Figure 3d). An important stage is the increase of Na level (six times the amount: 1200 mg g^{-1} DW) before leaf fall in September suddenly (Figure 3d). Significant difference in the Na amount in leaves of September was found (Table 2). Some research reported different behaviours in various species (Palma et al., 2000; Escalante-Pérez et al., 2009; Nirmal et al., 2010). Na is an activator and co-factor for stress adaptation system, controls the water movement in cells and is necessary for cell growth and division (Escalante-Pérez et al., 2009; Sun et al., 2009; Ding et al., 2010).

Micro nutrients

Iron (Fe)

The studied oak population in both samples did not show Fe deficit at all, however the soil pH was above 7 (site description). The concentration of Fe in twigs is reduced during drought period (Figure 3e); then reached its original content before dry season (150 µg g^{-1} DW) (Figure 3e). Fe content decrease is parallel to leaf vegetation (Figure 3e). This difference was significant before formation of new twigs in March and in the beginning, new vegetation period in May (Table 2). In leaves, Fe amount was around 300 µg g^{-1} DW in spring and during the dry months, but increased extremely (twice the amount: 680 µg g^{-1} DW) before leaf fall in November (Figure 3e), this was significant (Table 2). These levels were extremely high in comparison to literature values of *Quercus* species by Thomas and Sprenger (2008) and with other species dy Jiménez et al. (2007, 2009). Fe is the key element in ferredoxin, POD, CAT, and cytochrome oxidase. Fe deficiency in leaves produced yellowing between the veins (chlorosis), and bad soil conditions (too high pH in the forest area) are often the reason (Grotz and Guerinot, 2006; Jiménez et al., 2007). The reason is that the soil texture is not too heavy and the oak tree roots are adapted to this soil conditions (site description).

Manganese (Mn)

The concentrations of Mn in leaves and twigs were between 30 to 80 µg g^{-1} DW with an increasing level from the building process of leaves and twigs in May to the fall of leaves in November (Figure 3f). Significant difference was found in November (Table 2). The Mn level of leaves, May till July stayed constant, had an increased trend during vegetation period (Figure 3f). Generally, the contents of Mn in leaves and twigs were lower than in other tree species (Jiménez et al., 2007; Fernando et al., 2006a, b). Mn is important in photosynthesis and is involved in oxidation-reduction processes, decarboxylation and hydrolysis reactions in cells (Clair and Lynch, 2005; Fernando et al., 2006a, b; Thomas and Sprenger, 2008). Significant differences of twig Mn content were observed in November and January (Table 2). But the studied oak trees were remarkably adapted to the low level of Mn in the soil of these extreme regions according to Fernando et al. (2006a, b). On the other hand, Clair et al. (2005) and Thomas and Sprenger (2008) showed that an excess of Mn could impose negative influence of tree growth and photosynthetic activity.

Copper (Cu)

During one seasonal period, Cu content in leaves and twigs showed similar results. The concentration of Cu in the leaves varied from 3 µg g^{-1} DW (throughout dry season) to 19 µg g^{-1} DW (before leaf fall), which for leaves were 4 to 17 µg g^{-1} DW (Figure 3g). Both leaves and twigs possessed the lowest level during the dry period and showed the highest concentration before leaf fall and during winter time. Silk et al. (2006) found similar results in *Bromus carinatus*. Important stage is reduced Cu before leaf growth in twig (Figure 3g). Cu had decreased trend during leafs development (Figure 3g), but the 3-fold increasing of Cu in the twigs and leaves in autumn and winter time (sampling of November and January) can be explained by the fact that Cu is a very important factor in the lignification process (Figure 3g). Significant differences were only observed in November

Table 3. First three principal components and factors.

Components		Sample					
		Twig			Leaf		
Eigen value		3.503	2.0821	1.452	3.838	2.545	0.876
Proportion		0.389	0.231	0.161	0.427	0.283	0.097
Cumulative		0.389	0.621	0.782	0.427	0.709	0.807
PCA eigen vector		**PC 1**	**PC 2**	**PC 3**	**PC 1**	**PC 2**	**PC 3**
	Ca	0.369	0.311	-0.237	0.269	-0.455	-0.141
	K	-0.258	-0.521	-0.133	0.199	0.402	-0.546
	Mg	0.257	-0.195	-0.604	0.456	-0.153	-0.312
	Na	-0.253	-0.028	-0.659	0.430	-0.242	-0.297
Variable	Fe	-0.386	0.129	-0.223	-0.136	-0.478	0.244
	Mn	0.198	0.439	-0.267	-0.019	-0.436	-0.062
	Cu	-0.208	0.586	0.037	-0.318	-0.343	-0.288
	POD	-0.502	0.098	0.015	-0.432	-0.096	-0.362
	CAT	-0.426	0.170	-0.055	-0.431	0.019	-0.466
Factor eigen vector		**Factor 1**	**Factor 2**	**Factor 3**	**Factor 1**	**Factor 2**	**Factor 3**
	Ca	-0.169	0.193	-0.135	**0.692**	0.146	-0.220
	K	0.110	0.046	0.095	0.116	0.066	**0.921**
	Mg	-0.155	**0.922**	-0.131	**0.920**	0.286	0.169
	Na	0.179	0.193	0.242	**0.946**	0.265	0.070
Variable	Fe	0.230	-0.137	**0.911**	0.038	-0.108	-0.278
	Mn	-0.080	0.081	0.002	0.122	-0.048	-0.118
	Cu	0.312	-0.226	0.148	-0.094	-0.390	-0.242
	POD	**0.573**	-0.291	0.303	-0.319	**-0.880**	-0.119
	CAT	**0.929**	-0.118	0.192	-0.378	**-0.815**	0.031

Factors are rotated through varimax procedure. Bold characters were estimated significant. Others principal components and factors do not show.

for two samples (Table 2). This fact was confirmed by Lombardi and Sebastiani (2004), Silk et al. (2006) and Grotz and Guerinot (2006) who could show that the lignification of cell walls was defected by Cu deficiency and in root by Shi et al. (2009). Cu is localized to 50% as plastocyanin in chloroplasts and involved in POD catalysed reactions, particularly in laccases (oxidases) and oxidoreductase processes (Herbik et al., 2002; Lombardi and Sebastiani, 2004; Chen et al., 2004; Shi et al., 2009).

Multivariate statistical analyses and clustering

Principal component analysis (PCA) identified 9 components for each sample (Data not shown). Eigenvalues for first three components were upper one in twigs but first two components were upper one in leaves (Table 3). The first three components covered 78% of variation in twigs and 80% in leaves (Table 3). Factor analysis in format rotated components (Varimax) with three components showed the role of important variables in each factors. In twigs, POD and CAT had most roles in factor 1, Mg and Fe were effective in factors 2 and 3, respectively (Table 3). Law et al. (2001), Onnerud et al. (2002), Tian et al. (2003), Tang and Newton (2005), Lucash et al. (2007), Yazici et al. (2007), Shabannejad et al. (2009) and Chandra and Dubey (2010) reported enzymes activities as major principles in adaptation to environmental stresses and lignification. Solidarity actions between Mg and Fe are important factors in photosynthesis (Palma et al., 2000; Clair et al., 2005; Black et al., 2008; Thomas and Sprenger, 2008) also, Mg have promoter role for enzyme activity (Palma et al., 2000; Thomas and Sprenger, 2008; Nirmal et al., 2010) and Fe is base of heme in enzymes (Chaitanya et al., 2002; Mazorra et al., 2002).

Figures 2a, b, 3c and e revealed that the variables have increased in May and decreased in July. In leaves, efficacious components were Ca, Mg, and Na and are in factor 1, enzymes in factor 2 and K in factor 3 (Table 3). Most of the role of factor 1 is in cell wall, for the increase in tolerance to environmental stresses (Jiménez et al., 2007, 2009). Also, enzymes have more activity in interaction of environmental conditions and stresses (Yazici et al., 2007; Shabannejad et al., 2009; Chandra and Dubey, 2010) but K is a bridge between factor 1 and 2 because it is recognized as a cell electrolyte controller

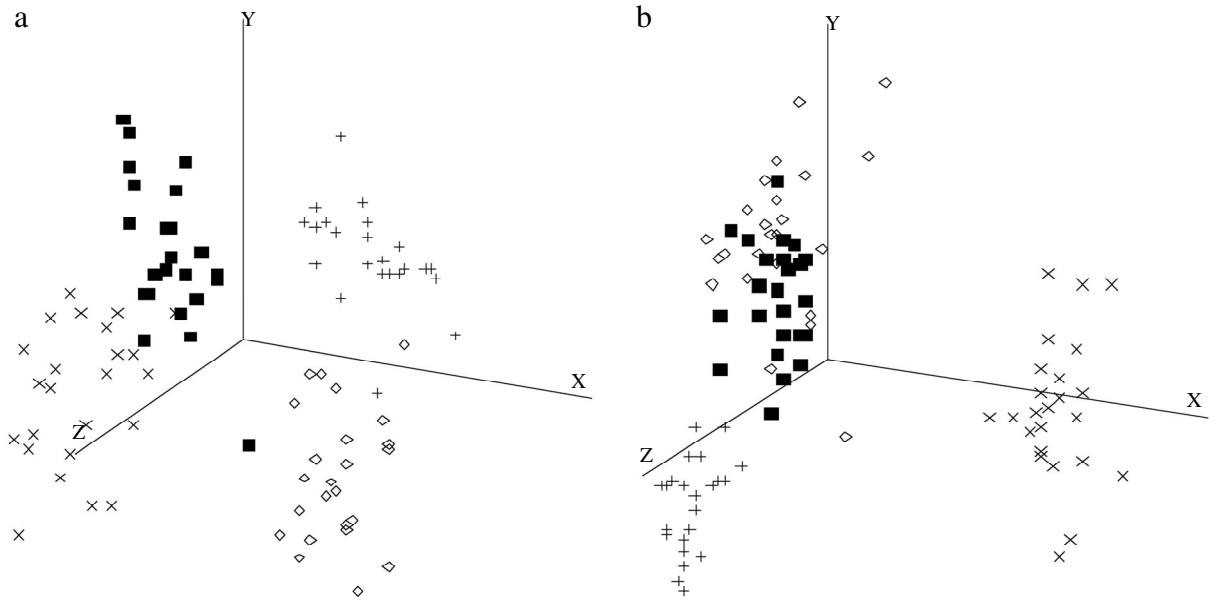

Figure 4. Cluster analysis marked by months in twigs (a) and leaves (b). Characters +, ■, × and ◇ show May, July, September and November months, respectively. X, Y and Z are rotated factors 1, 2 and 3, respectively. Each month included 24 samples

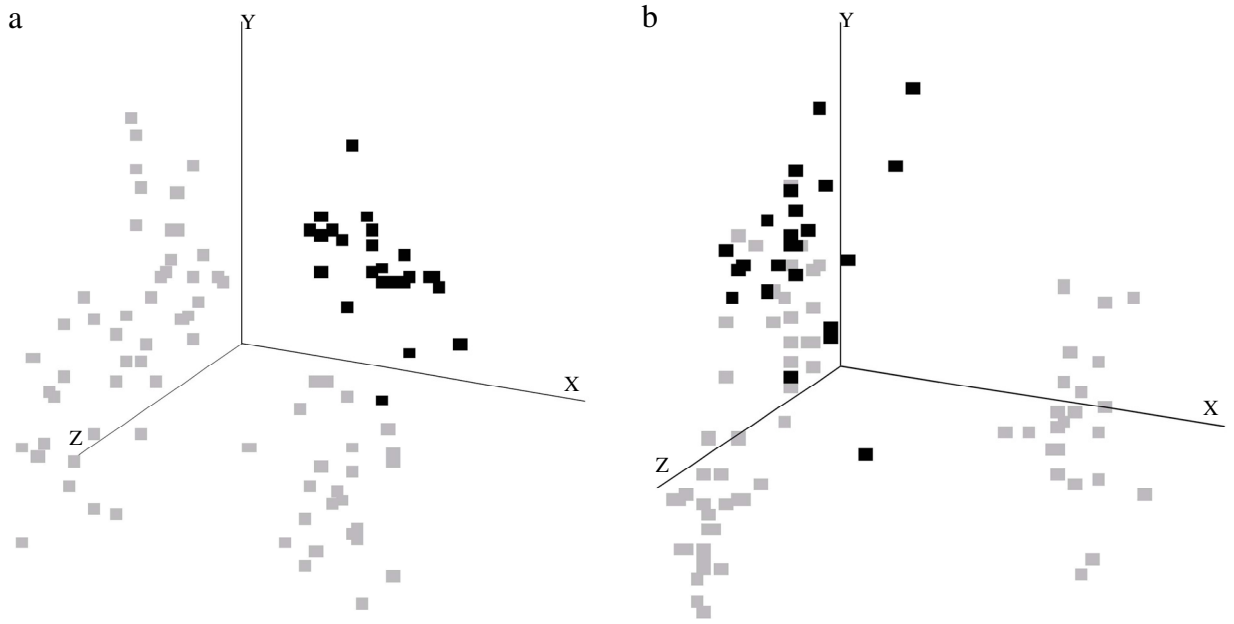

Figure 5. Cluster analysis marked by non-hierarchical standardized k-means algorithm in twigs (a) and leaves (b). Characters ■ and ■ show cluster 1 and 2, respectively. X, Y and Z are rotated factors 1, 2 and 3, respectively. In twigs; cluster 1 included 25 sample and 71 samples in cluster 2 also in leaves; cluster 1 included 24 sample and 72 samples in cluster 2.

element (Palma et al., 2000; Egilla et al., 2001; Ache et al., 2009).

Clustering marked by months showed clear separation to any season in each sample (Figure 4). In twigs and leaves; three months had rotation (May, September and November) and July had almost fixed situation in factor plans (Figure 4a and b). July had the maximum air temperature and was the driest season of year in the research site (Figure 1). The key position stability in this month can be the environmental factors (Palma et al.,

2000; Silk et al., 2006; Liu et al., 2007; Sardans et al., 2008; Nirmal et al., 2010). On the other hand, clustering marked by standardization data put variables in two clusters. Cluster 1 included data from May; also July, September and November were in cluster 2 in twigs (Figure 5a). Starting the leaf generation would be in May, hence trees will transport important variables from twigs to buds and new leaves (Palma et al., 2000; Sardans et al., 2008; Nirmal Kumar et al., 2010) thus 2 clusters will be here.

In leaves, November data separated in cluster 1 while other months were in cluster 2 (Figure 5b). In November, leaves will be falling; all variables are shared into fruits, autumn's leaves or returned to twigs (Palma et al., 2000; Barbaroux and Bréda, 2002; Barbaroux et al., 2003; Jiménez et al., 2007, 2009; Nirmal et al., 2010).

Conclusion

The present study and the discussed results can provide more understanding of the physiological status of oak trees (*Q. brantii*) in forests at high sea level (2,300 m) under extreme climatic conditions. The accumulation of enzymes, macro, and micro nutrients in a forest ecosystem mainly depends on the type of forest, in particular the tree species, density, age, base area, altitude, and the climate, soil and humidity. The use of multivariate statistical analyses using crop-relevant environmental parameters is data-driven and arguably as valid as the input data.

By using best available and most recent data, the resulting agro-ecosystems provide an updated view of the agricultural environment with specific relevance to crop growth and production. Because quantitative validation procedures are currently unavailable, future research has to concentrate on the influence of environmental variation captured by our delineation method in relation to a range of crop options. Future research efforts could also examine expected crop performance under altered climate regimes.

Our method provides both a visual and analytical foundation for exploring physiological scale of oaks. The major findings of this study are:

1. Multivariate statistical analyses showed strong effect of enzymes, macro nutrients and Fe in separate seasonal clusters;
2. POD and CAT have role in adaptation of samples to climate changes so are basal principles at clustering;
3. Clearly, macro nutrients are effective in separate seasons in leaves. Since photosynthesis is in leaves, we can tell that photosynthesis and/or content of photosynthesis is a principal factor in clustering;
4. Fe as micro nutrient is a significant variable in twigs formation. Perhaps lignification is a function in this sample;
5. Some correlations between the variables are mentioned in results and discussion but other relations are unknown;
6. Twigs and leaves are reasonable sample materials; in summary, twigs reflect the metabolic situation of the whole tree system whereas leaves are responsible for the photosynthetic activity;
7. Although seasonal variations had effect on variables in each month but July had fixed position for twig and leaf on cluster plot. Authors were compatible that July is passing-time during plant yearly life for variables stability and completion growth period. On the other hand, all of the investigated variables have changed as at the month of May. Hence finding two separated clusters could be right based on plants yearly life.

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APPENDIX

Table 1. Some trees characteristics in research site.

Trees number	Height (m)	Crown diameter (m)	Diameter at breast height (m)
1	12	8.2	39
2	7.5	5.85	22.5
3	11.4	9.65	36
4	10.8	8.6	30.5
5	10.6	7.8	29
6	10.1	6.7	31.5
7	9.8	7.3	33.5
8	11	7.15	35
9	10.6	6.8	33.5
10	8.4	5.85	28
11	9.5	6.75	33.5
12	8.6	4.5	25
13	6.1	7.25	30
14	6.8	5.35	15
15	7.7	9.1	29
16	8.9	8.1	28.5
17	9	7.7	27
18	7.7	8.6	25
19	10.5	5.65	37
20	7.55	7	30
21	4.5	6.2	15.5
22	5.9	5.4	26
23	6.5	5.35	24
24	5.7	4.85	27
Average	8.63	6.9	28.79