Seasonal changes in antioxidant activity, flavonoid, anthocyanin and phenolic compounds in *Flavoparmelia caperata* (L.) Hale and *Physcia dubia* (Hoffm.) Lettau from Babol forest sites in north of Iran

Mahlagha Ghorbanli*, Tannaz Amirkian Tehran and Maryam Niyakan

Department of Biology, Faculty of Science, Islamic Azad University, Gorgan Branch, Gorgan, Iran

Abstract

The epiphytic lichen *Flavoparmelia caperata* were collected randomly from mountain forest sites of Firouzja (north of Iran) at 780m altitude, 36° 05' N latitude and 52° 40' E longitude. Also *Physcia dubia* was collected randomly from forest sites of Daronkola (north of Iran) at 220m altitude, 36° 21' N latitude and 52° 43'E longitude. The study was aimed at recording the effects of seasonal changes on antioxidant enzymes activities and flavonoid, anthocyanin and phenolic compounds the two lichen species. The results showed that peroxidase activity in both species increased significantly in winter. The activity of polyphenol oxidase and ascorbate peroxidase in both species and catalase in *P. dubia* were increased in summer. In spring, polyphenol oxidase activity increased significantly more than in winter in *Physcia dubia*. Phenolic compounds in *Flavoparmelia caperata* increased significantly in winter compared to spring and summer, but in *Physcia* dubia they increased significantly in summer compared to winter and spring. Flavonoid and Anthocyanin compounds in *Flavoparmelia caperata* increased significantly in winter compared to spring and summer. In *Physcia dubia,* Flavonoid decreased significantly in spring compared to winter and summer, and anthocyanin increased significantly in winter compared to spring and summer. The results revealed that seasonal acclimation depended on the changes in antioxidants enzymes activity, flavonoid, anthocyanin and phenolic compounds in lichens.

Keywords: antioxidant enzymes, flavonoid, anthocyanin, Phenolic compounds, *Flavoparmelia caperata*, *Physcia dubia*, Seasonal changes.

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Introduction

A lichen is a long-lived symbiotic association between a heterotrophic mycobiont and an autotrophic photobiont resuming active metabolism any time of the year when air humidity, dew, or rain is present in sufficient amount for hydration (Lange et al., 1999; Lange, 2003). Also at boreal and temperate latitudes with strongly seasonal climates, lichen can photosynthesize and grow even during periods of cold season (Lange, 2003), when higher plants survive in a dormant state (Adams et al., 2002; Oquist and Huner, 2003). Lichen abundance in nature, increases substantially with increasing

*Corresponding author
E-mail address: mghorbanli@gorgaiau.ir
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latitude which increases seasonality with respect to temperature and light (Longton, 1988). Such trait suggests that lichens are not only opportunistic organisms utilizing temporal windows of favorable temperatures and humidity at various light intensities, but also flexible organisms in the sense that they may repeatedly acclimate over recurring annual cycles (Mackenzie et al., 2001; Schofield et al., 2003). Various types of seasonal acclimation in lichens have been reported (Kershaw and Macfarlane, 1980). But in general, lichen acclimation is not well studied. Acclimation may take place in the mycobiont and/or the photobiont. Recent studies have shown that the mycobiont acclimates, for example, by a uv-B induced fungal synthesis of sun-screening cortical pigments like melanic compounds (Gauslaa and Solhaug, 2001; Nybakken et al., 2004) and parietin (Solhaug et al., 2003). Compared to higher plants, lichens may experience less oxidative stress during high light periods in winter, simply because of their documented ability to assimilate significant amounts of carbon during autumn and winter (Lange, 2003). Therefore, lichens may need less photosynthetic acclimation than other plants to excessive light in cold season (Vrablikova et al., 2006). Lichen physiology is strongly related to both micro and macro-climatic factors, since it is well known that relative air humidity and rainfall are the main sources of water supply for these organisms (Giordani and Incerti, 2007). In recent years several studies have pointed out the role of climatic factors in the relationship between lichen physiology and distribution (Kappen, 1988; Van Herk et al., 2002; Brunialti and Giordani, 2003). During the normal processes of growth and development, plants are subject to different types of stress such as drought, heat, ultraviolet light, air pollution and pathogen attack (Paliyath et al., 1997) as well as seasonal changes. Plants can develop protective mechanisms under these conditions to tolerate damage (Syvacy and Sokmen, 2004). Antioxidants are compounds that can delay/ inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidative chain reactions (Velioglu et al., 1998). Many changes in physiological and biochemical parameters have been observed during the exposure of plants to low temperatures: modified levels and activities of enzymes from various metabolic pathways, accumulation of carbohydrates, amino acids, soluble proteins as well as appearance of new isoforms of proteins and altered lipid membrane composition (Apostolova and Yaneva, 2006). Low temperature induced overproduction of reactive oxygen species has been shown to bring about serious cellular damage by rapidly reacting with DNA, lipids and proteins (Sattler et al., 2000). The activity of both peroxidase and ployphenol-oxidase showed an increase in the over-wintering organs of each genotype at the beginning of dormancy, while the enzymes activity decreased with the oncoming of spring. This change emphasizes the probability of the protective roles these enzymes play in enhancing the frost tolerance of the over-wintering organs (Citadin et al., 2002). The changes in enzyme activity seem to be an indicator of the end of dormancy and start of growth as described by many authors (EL-Mansy and Walker, 1969; Lasheen and Chaplin 1971; Kenis, 1976; Bassuk and Howard, 1981; Marquat et al., 1999; Citadin et al., 2001). Both enzymatic and non-enzymatic systems are involved in protecting plants against oxygen toxicity (Pastori and Foyer, 2002). The enzymatic system consists of a set of enzymes like superoxide dismutase (SOD), peroxidase (pox) and catalase (CAT), while the non-enzymatic system includes some antioxidants, such as ascorbate, carotenoids and proline (Prasad, 1996). The antioxidant activity of plant parts is mainly contributed to by constituent anthocyanin, phenolic and flavonoid compounds (Syvacy and Sokmen, 2004).

Anthocyanins are the red, purple and blue flavonoid pigments produced mainly in plant epidermal cells, where they accumulate in vacuoles, causing the tissue to become colored (Kim et al., 2006). In a number of warm and cool climate species, a range of maturation following exposure to adverse environmental factors indicated the interaction of irradiance and low temperature (Janda et al., 1996). The biosynthetic pathway of polyphenolic compounds is closely related to that of anthocyanins (Haslam, 1998).Climatic changes like low or high temperature stress promote production of phenolic compounds (Dixon and Paiva, 1995). The

flavonoid content of plants is also known to vary quantitatively and qualitatively depending on growth stage, degree of senescence, season and geographical location (Chaves et al., 1993). Specific flavonoids are also produced in responds to wounding stress, herbivores, strong light, especially UV-B (Cuadra et al., 1997) and temperature stress (Chaves et al., 1997). In this study, the effects of seasonal changes in the activities of antioxidant compounds and their roles in acclimation of lichens were investigated.

Materials and Methods

The epiphytic lichen *F. caperata* was collected randomly from the mountain forest site Firouzja region, in the north of Iran, 780m altitude, 36° 05' N latitude and 52° 40' E longitude, standing on 0.28 slopes. The other species *P.dubia* was collected randomly from a the forest site Daronkola region, in the north of Iran, 220m altitude, 36° 21' N latitude and 52° 43'E longitude, standing on 0.1 slopes.

Lichen extraction

One gram lichen was homogenized with 4 ml extraction solution for 30 minutes. Lichen enzymatic extraction solution was carried out by Korori (1989) method.

Total peroxidase assay

Total peroxidase activity was determined by Korori (1989) method. The absorbance was measured spectrophotometrically (Spectronic 20 Genesys TM) at 530 nm wavelength against blank samples. Enzyme activity was estimated based on OD min^{-1} g^{-1} FW.

Catalase measurement

Catalase enzyme activity was determined using Chance and Maehly (1955) method. The absorbance was measured by a spectrophotometer at 240 nm wavelength. Enzyme activity was estimated based on OD min^{-1} g^{-1} FW.

Assay of polyphenoloxidase

Polyphenoloxidase was estimated using Manoranjan and Bandhumishra (1975) method. Its absorbance was measured spectrophotometrically at 430nm wavelength. Enzyme activity was calculated based on OD min^{-1} g^{-1} FW.

Ascorbate peroxidase assay

Ascorbate peroxidase was determined by the method described by (Arrigoni et al., 1992). Then the absorbance was measured spectrophotometrically at 265nm. Enzyme activity was estimated based on OD min^{-1} g^{-1} FW.

Flavonoid and anthocyanin measurement

Flavonoid and Anthocyanin were determined using Nouges and Baker (2000) method. The absorbance was measured spectrophotometrically at 300 and 530nm wavelengths. Flavonoid and Anthocyanin compounds were calculated based on OD min^{-1} g^{-1} FW.

Phenolic compounds assay

Phenolic compounds were estimated through the method described by Matta and Giai, (1969). The absorbance was measured spectrophotometrically at 640nm. Phenolic compounds were calculated base on mg ^{1} g^{-1} FW.

Statistical analysis

The statistical analysis of the obtained data was performed using Spss software (version 15), with one way analysis of variance (ANOVA) and the statistical significance of results was measured using Duncan test. The graphs were designed using Excel software.

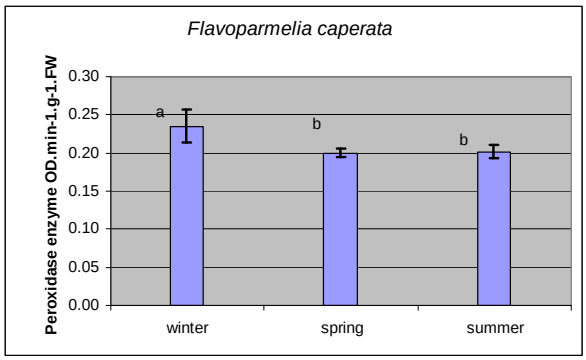

Fig. I. Seasonal Peroxidase activity of *F. caperata* (Mean ± SE)

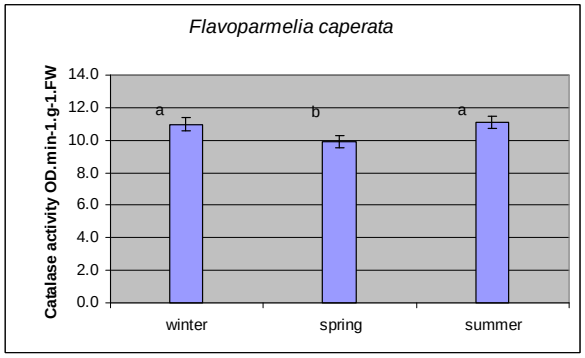

Fig. II. Seasonal Catalase activity of *F. caperata* (Mean ± SE)

Results

Seasonal changes and antioxidant enzymes activity

The total activity of the enzymes showed a typical seasonal pattern in lichens. The activity of peroxidase in *F. caperata* and *P. dubia* showed a significant increase at P≤0.05 levels in winter compared to spring and summer (Figs. I, V). The activity of catalase in both species showed a significant reduction at P≤0.05 in spring compared to winter and summer (Figs. II, VI) but in *P. dubia* catalase it was increased significantly in summer compared to winter and spring at P≤0.05 (Fig. VI). The activity of ascorbate peroxidase in *F. caperata* and *P. dubia* showed significant increase at P≤0.05. This increase in summer was more than other seasons while the enzyme activity decreased significantly in spring (Figs. IV, VIII). The activity of polyphenol oxidase was increased significantly in spring compared to winter in *P. dubia* (Fig. VII), and both species showed significant increase at P≤0.05 in summer compared to winter and spring (Figs. III, VII).

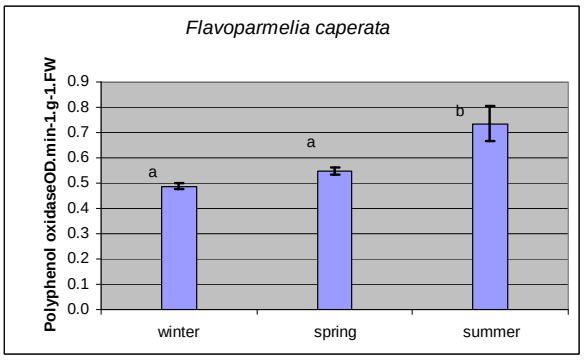

Fig. III. Seasonal Polyphenol oxidase activity of *F. caperata* (Mean ± SE)

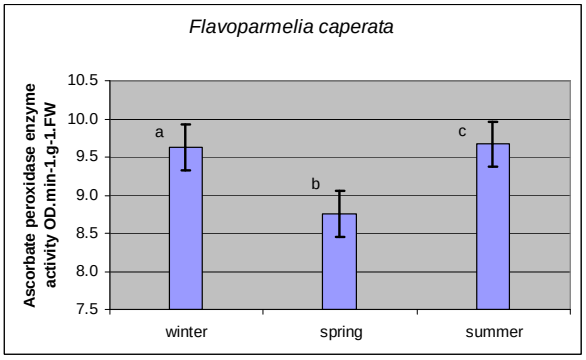

Fig. IV. Seasonal Ascorbate peroxidase activity of *F. caperata* (Mean ± SE)

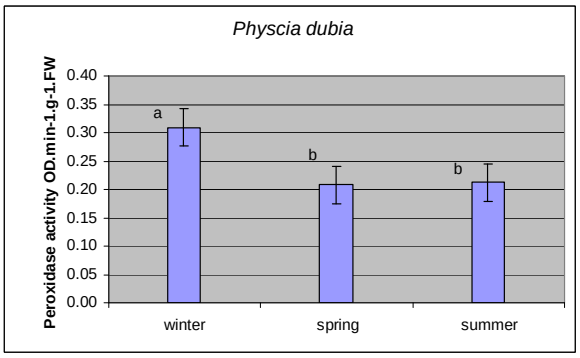

Fig. V. Seasonal Peroxidase activity of *P.dubia* (Mean ± SE)

Seasonal changes and flavonoid, anthocyanin and phenolic compounds in *F. caperata*

Total phenolic constituents of *F. caperata* were lowest in spring and highest in winter. More precisely, total phenolic compounds showed a significant increase at P≤0.05 in winter and summer compared to spring, and a significant increase in winter compared to summer (Fig. IX).

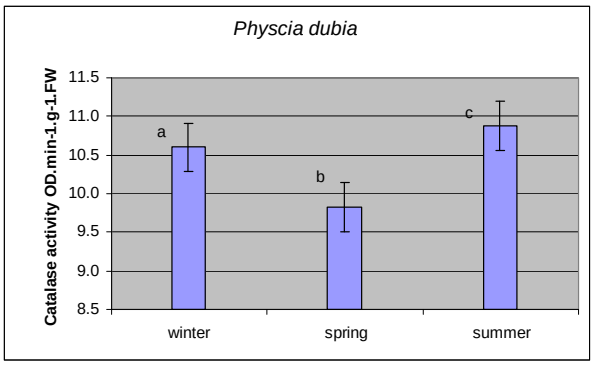

Fig. VI. Seasonal Catalase activity of *P.dubia* (Mean ± SE)

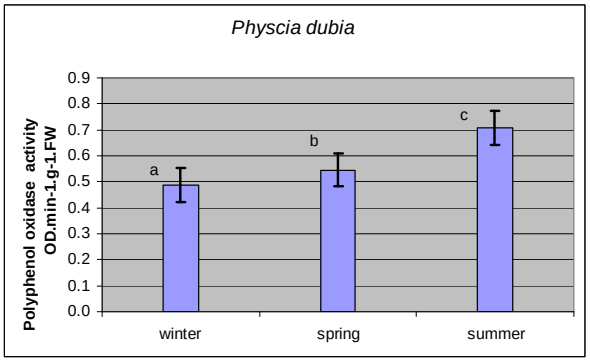

Fig. VII. Seasonal Polyphenol oxidase activity of *P.dubia* (Mean ± SE)

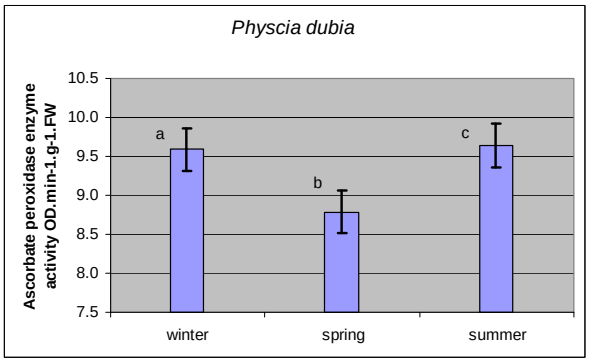

Fig. VIII. Seasonal Ascorbate peroxidase activity of *P.dubia* (Mean ± SE)

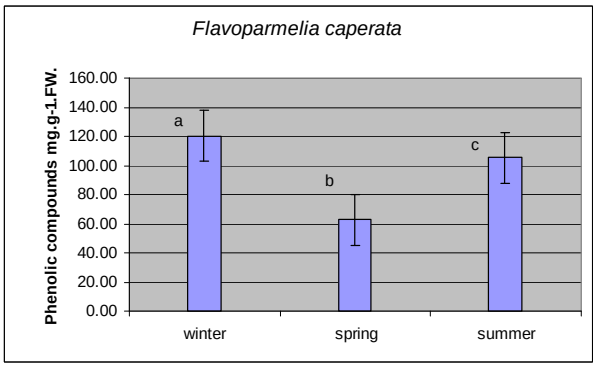

Fig. IX. Seasonal phenolic compounds of *P.dubia* (Mean ± SE)

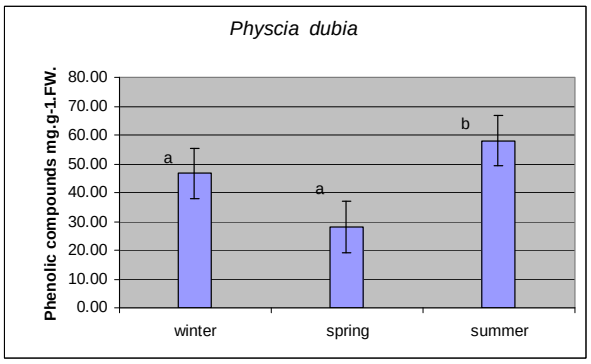

Fig. X. Seasonal phenolic compounds of *P.dubia* (Mean ± SE)

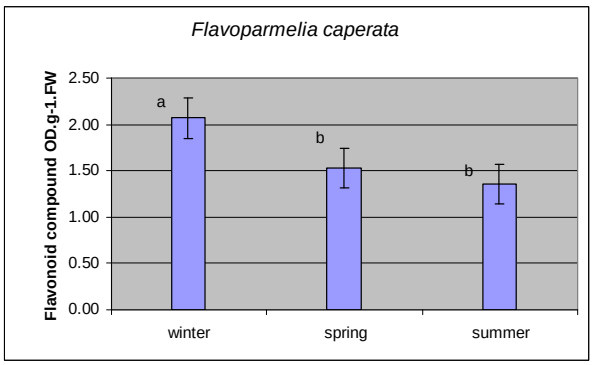

Fig. XI. Seasonal total Flavonoid compounds of *F. caperata* (Mean ± SE)

Total flavonoid content in *F. caperata* increased significantly at P≤0.05 in winter compared to spring and summer, and decreased insignificantly at P≤0.05 in summer compared to spring (Fig. XI). Production of anthocyanin in *F. caperata* significantly rose at P≤0.05 in winter compared to spring and summer, and decreased insignificantly at P≤0.05 in summer compared to spring (Fig. XII).

Seasonal changes and flavonoid, anthocyanin and phenolic compound in *P. dubia*

Production of phenolic compounds in *P. dubia* increased significantly at P≤0.05 in summer compared to winter and spring, but increased insignificantly in winter in comparison with spring (Fig. X).

Flavonoid content decreased significantly at P≤0.05 in spring compared to winter and summer (Fig. XIII). Anthocyanin content on the other hand, increased significantly at P≤0.05 in winter compared to spring and summer (Fig. XIV).

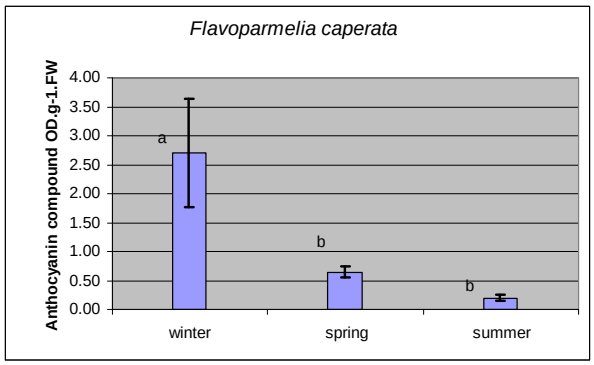

Fig. XII. Seasonal Anthocyanin compound of *F. caperata* (Mean ± SE)

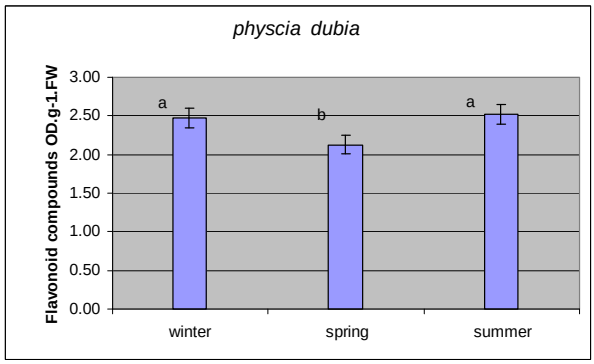

Fig. XIII. Seasonal total Flavonoid compounds of *P. dubia* (Mean ± SE)

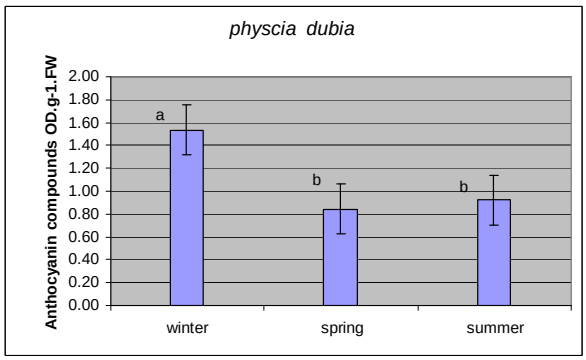

Fig. XIV. Seasonal Anthocyanin compounds of *P. dubia* (Mean ± SE)

Discussion

In winter, low temperatures (cold and frost) have profound negative impacts on plants. Many changes in physiological and biochemical parameters have been observed during the exposure of plant to low temperatures (Apostolova and Yaneva, 2006). Low temperature induced overproduction of reactive oxygen species which have been shown to bring about serious cellular damage by rapidly reacting with DNA, lipids, and proteins (Sattler et al., 2000).

The protective system against oxidative stress in plants involves several enzymes such as: peroxidase, catalase, ascorbate peroxidase and polyphenol oxidase. Peroxidases are enzymes that are widely distributed in the plant kingdom and can be found in vacuoles, tonoplast, plasmalemma and inside and outside the cell wall. These enzymes have a variety of functions as evidenced by the presence of several isoenzymes in plant cell compartments. They are involved in plant hormone regulation (Gaspar, 1985) and control defense mechanisms, cell elongation, polymerization of extension cross-linkage of cell wall polysaccharides, lignin biosynthesis (Vitali et al., 1998) and the suberization processes (Gaspar et al., 1991). Cell wall peroxidase is also induced by pathogen infection and considered to play an important role in cell wall lignification during the hyper sensitive reaction (Mehdy, 1994). Peroxidase participates in a variety of plant defense mechanisms in which H_2O_2 is often supplied by an oxidative burst (Lamb & Dixon, 1997). The activity of peroxidase in *F. caperata* and *P. dubia* showed a more significant increase in winter than spring and summer. The activity of peroxidase showed an increase in over-wintering organs, while the enzyme activity decreased with the oncoming of spring. This change emphasizes the probability of the protective roles these enzymes play in enhancing the frost tolerance of the over-wintering organs (Citadin et al., 2002). Considering the fact that catalase is the major enzyme destroying hydrogen peroxide, the higher specific activity and presence of the cellular isoforms in the intercellular compartment may indicate the possible involvement of catalase in the freezing tolerance by controlling the hydrogen peroxide concentration in the compartment (Baek et al., 2000). The activity of catalase in *F .caperata* and *P. dubia* showed a significant increase in winter compared to spring. The activity of polyphenol oxidase showed an increase in the over wintering organs, while the enzyme activity decreased with the oncoming of spring (Szalay et al., 2005). The maximal activity of polyphenol oxidase measured in buds of plum decreased in the middle of winter, and started to

rise again in spring (Szecsko et al., 2002).Our findings did not support this investigation, The activity of polyphenol oxidase in *P. dubia* showed a significant increase in spring compared to winter. Ascorbate peroxidase is involved in the detoxification of H_2O_2 which may also contribute to enhanced freeze tolerance in the Zea mays leaves (Asada, 1997). This finding corresponded with our investigation, the activity of ascorbate peroxidase showed a significant increase in winter compared to spring in both species. Desiccation stress increases the degree of reactive oxygen species (ROS) in plant cells (Elstner and Osswald, 1994). So this information corresponded with our experiment because total activity of polyphenol oxidase, ascorbate peroxidase in both species and catalase in *P. dubia* were highest in summer for the protection of plants. Evidences showed a positive correlation between increasing anthocyanin content and the increase of tolerance to photoinhibition in low temperature stress in the needle pine (Krol et al., 1995). Recent investigations have shown that anthocyanin synthesis increased and cell growth was suppressed by decreasing temperature in strawberry (Wei et al., 1998). These findings correspond with our experiment. The total anthocyanins content of *F. caperata* and *P. dubia* were highest in winter or in low temperature stress for the protection of the plant. Pigment contents decreased progressively during summer in leaves at sunshine and remained stable in leaves at shadow (Syvacy and Sokmen, 2004). This finding corresponded with our experiment. Total anthocyanin content in *F. caperata* was lowest in summer.

Total phenolic and flavonoid compounds in *F. caperata* were increased in winter more than other seasons. But in *P. dubia* this compounds experienced an increase summer more than other seasons. In general, phenolic compounds are well- known, as high level antioxidant constituents because of their high ability to scavenge toxic free radicals and reactive oxygen species. Interestingly, the phenolic contents in five Antarctic lichens species were found to be much higher than in black and white pepper. (Bhattarai et al., 2008). Total phenolic compounds of *Morus nigra* and *Morus alba* were lowest in summer (Syvacy and Sokmen, 2004). This finding corresponds with our experiment where phenolic compounds in two different lichens decreased significantly in spring.

Finally total flavonoid content was reported to be highest in spring, and it depended on light contents or day length (Haribal et al., 2001). However our results did not support this idea.

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