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Protein mobilisation and proteolytic activities in root tubers of *Asphodelus aestivus*

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Abstract: We studied protein mobilisation in *Asphodelus aestivus* Brot. (Liliaceae) during a 1-year period. The activities of 3 proteolytic enzymes, endopeptidase (EP), leucine aminopeptidase (LAP), and carboxypeptidase (CP), together with soluble protein levels and total nitrogen and water content in root tubers were investigated. We found that protein mobilisation continued throughout the year. The highest levels of the 3 proteolytic enzymes were found in different months: LAP in December (1.19 ± 0.19 µmol pNA/g FW), CP in February (14.19 ± 1.13 µmol Leu/g FW), and EP in May (5.18 ± 0.12 U/h). Activities of LAP and CP generally reached their higher levels in January–April, namely in spring, but EP activity was higher in March–May. Activities of all 3 enzymes remained at lower levels during the asphodel's inactive life phase. The soluble protein contents were associated with the proteolytic activities. The total N contents were generally constant, but decreased in April. Our results show that the proteolytic activities in root tubers of *Asphodelus aestivus* continue to increase up to the start of flowering in early spring, and they can be an important nitrogen resource at the beginning of the generative development phase.

Key words: Endopeptidase, leucine aminopeptidase, carboxypeptidase, Mediterranean

1. Introduction

Knowledge of plant traits is necessary for understanding the ecosystem responses to natural and human-induced environmental changes (Hüseyinova et al., 2013). Geophytes are important life forms among plants with specialised structures in specialised environments. Their regenerating buds are on underground storage organs, and their life cycle includes a dormant period that can extend from a few weeks to most of the year, as defined by Raunkiaer (1934). Geophytes are widespread in the Mediterranean part of the world because they can survive the summer drought (Dafni, 1996; Debussche et al., 2004).

The Mediterranean geophytes are of 2 types: spring-flowering species that display leaves and flowers simultaneously (synanthous geophytes), and autumnal-flowering species that develop flowers before leaves and leaves after the flowering (hysteranthous geophytes) (Dafni et al., 1981a). The synanthous geophytes have periods of growth, storage, flowering, and dormancy, whereas the hysteranthous geophytes go dormant after storing the food reserves such that vegetative and reproductive stages do not occur at the same time (Dafni et al., 1981a, 1981b).

According to Pantis (1993), *Asphodelus aestivus* Brot. could be categorised as intermediate; it is synanthous, but its reproduction depends partly on stored reserves and partly on annual production. *Asphodelus aestivus* is one of the important geophytes growing in degraded areas of the Mediterranean environment (Pantis et al., 1994). When it covers the degraded parts of the eastern Mediterranean basin, these types of ecosystems appear like deserts, and they are called asphodel geophyte-deserts or asphodel semideserts (Naveh, 1973; Ayyad and Hilmy, 1974; Le Houerou, 1981).

Asphodelus aestivus is a competitive ruderal stress-tolerant strategist species, which can be found on agricultural slopes, near roads, and in calcareous soils in pastures (Pantis et al., 1994). *Asphodelus aestivus* is an important plant and it has become the dominant life form in some degraded Mediterranean ecosystems, including those resulting from excessive grazing and fire (Ayyad and Hilmy, 1974; Le Houerou, 1981; Pantis and Margaris, 1988). Pantis and Margaris (1988) reported that the dominance of *Asphodelus aestivus* in degraded areas reflects both its capacity to face the peculiarities of the Mediterranean climate and its resistance to the most common disturbances in habitat.

Geophytes mainly store one or more storage compounds in their underground storage organs. They generally accumulate carbohydrates, starch, soluble sugars, glucomannans, and fructans (De Hertogh and Le Nard,

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1993; Miller et al., 1997). Shewry (2003) suggested 2 roles for tuberous roots: first, to store carbon and nitrogen in forms that can be mobilised when required; and second, to sprout and produce new plants as propagules. Proteins are hydrolysed to their amino acids by the sequential action of 3 types of enzymes: endopeptidases, aminopeptidases, and carboxypeptidases (Callis, 1995). Endopeptidases hydrolyse the internal bonds of polypeptides and produce smaller peptides. Aminopeptidases and carboxypeptidases remove the terminal amino acid from the opposite termini of the peptide chain (Bewley and Black, 1982). Plant species generally suffer from dehydration during the long periods of drought in the Mediterranean region. Geophytes are adapted to these seasonal changes in habitat. They are able to dehydrate and remain dormant during long drought periods in summer and then become active after rehydration in autumn (Dafni et al., 1981a; Kamenetsky et al., 2005). *Asphodelus aestivus* root tubers both store and exploit water and nutritional elements, and they protect the plant from drought stress and environmental hazards (Pantis, 1993; Sawidis et al., 2005).

Bewley (2002) suggested that the storage proteins may serve as a reserve nitrogen source in the roots of several perennial species, especially among the members of Compositae, Euphorbiaceae and Leguminosae. In some species, the amount of root proteins fluctuates seasonally. The storage proteins usually compose a small part of the total nitrogen pool within the root; their accumulation is related to overwintering. Cyr and Bewley (1989, 1990a, 1990b) suggested that, in herbaceous perennial species, some proteins can be used as a temporary nitrogen source to support plant growth when needed.

There is substantial information about the seed proteins and mobilisation of seed proteins by proteinases (Chin et al., 1972; Mitsuhashi et al., 1984; Munoz et al., 1994; Kırmızı and Güleryüz, 2006). However, little information exists about the reserve proteins in the storage organs of geophytes (Guimaráes et al., 2001; Frankova et al., 2006; Kamenetsky et al., 2005). The storage organs of geophytes also act as sources of nitrogen, sulphur, and carbon (Shewry, 2003), and the proteins can be hydrolysed to amino acids by a number of proteinases (Kırmızı and Güleryüz, 2007). Sakar et al. (2010) reported the seasonality of the *Asphodelus aestivus* nitrate reductase activity (NRA) and the high nitrate assimilation capacity in all plant parts, including flowers and tuberous roots. The high nitrate assimilation of *Asphodelus aestivus* could be related to its adaptation to the Mediterranean environment. Information about protein mobilisation in geophytes is very scarce; furthermore, the protein mobilisation in *Asphodelus aestivus* tubers has not previously been studied, whereas some traits such as reproductive development were studied previously (Vardar et al., 2013). We aimed to understand the effect of protein mobilisation in root tubers on the life cycle of this plant. For this reason, we measured 3 proteolytic enzyme activities, endopeptidase (EP), leucine aminopeptidase (LAP), and carboxypeptidase (CP), and the seasonal fluctuations of these 3 proteolytic enzyme activities in the root tubers of *Asphodelus aestivus* during 1 year.

2. Materials and methods

2.1. Plant material and study area

Asphodelus aestivus Brot. is a perennial tuberous root geophyte of the family Liliaceae and grows around the Mediterranean basin (Pantis and Margaris, 1988). Two main phenological phases are distinguished within the life cycle of *Asphodelus aestivus*: a photosynthetically active phenophase, lasting from leaf emergence (early autumn) to the senescence of the above-ground plant parts (late spring), and an inactive phenophase, which lasts until leaf emergence (Pantis et al., 1994). The over-wintering root tubers of *Asphodelus aestivus* develop flat leaves from the shoot apex in February and March and flowering stalks with 60–200 flowers in April and May. Senescence happens in June before the fruit maturation, but the majority of the tuberous roots remain attached to the mother plant (Pantis et al., 1994). In western Anatolia, it is the first species appearing after a fire and is the first stage in secondary succession (Pirdal, 1989).

The study area was located in Bursa, Turkey. It has Mediterranean climate conditions. The annual mean temperature, mean humidity, and total precipitation values are 14.6 °C, 69%, and 696.5 mm, respectively. The precipitation regime is winter-spring-autumn-summer (Rehder et al., 1994).

2.2. Sampling

During a 1-year period (09.05.2007 to 06.05.2008), plant samples were collected from a natural habitat of degraded maquis vegetation near the village of Taşpınar in Bursa Province, Turkey. Sampling was conducted in a 10 × 10 m area from 3 different plants on each sampling date, and all the experiments were repeated at least 3 times. After the tuberous roots were dissected from the plants, they were washed with tap water and then with distilled water.

2.3. Total nitrogen and water contents

Subsamples of the plant material were dried in an oven until the weight became constant and were then used for total nitrogen analysis according to the Kjeldahl method (Steubing, 1965) and for determination of the water content. Tissue water contents were also used for calculation of enzyme activities and protein determination as total volume in plant tissue. The remainder of the fresh plant material was kept at –70 °C in a freezer and used for the extraction of enzyme activity and protein determination.

2.4. Enzyme assays

Homogenisation was carried out at 0 to 4 °C. Tuberous roots were homogenised in a chilled mortar and an Ultra Turrax homogeniser with cold 50 mM Tris-HCl (Tris-hydroxymethylaminomethane-hydrochloride) buffer, pH 7.4, containing 10 mM 2-mercaptoethanol (2-ME). The homogenate was passed through 4 layers of cheesecloth and centrifuged at 4 °C at 20,000 × *g* for 20 min. The supernatant was collected, and a portion of it was dialysed for 24 h against 50 mM sodium acetate buffer, pH 5.4, containing 10 mM 2-ME. All 3 proteolytic activities were assayed according to the method given by Kırmızı and Güleryüz (2006). L-Leucine 4-nitroanilide was used as the substrate for the LAP activity assays. In the reactions, 2 mL of 2 mM substrate was incubated with 0.1 mL of enzyme at 37 °C for 20 min. LAP activity was defined as the micromoles of p-nitroanilide released into the reaction medium per gram of fresh weight (μmol pNA/g FW) under the experimental conditions. EP activity was assayed using 1% azocasein in 75 mM sodium acetate buffer, pH 5.4, containing 5 mM 2-ME as the substrate. EP activity was expressed as the amount of enzyme required to cause an increase of 1.0 in absorbance under the assay conditions, expressed as U/h. The substrate for CP was N-CBZ-Phe-Ala at a concentration of 2 mM in 25 mM citrate phosphate buffer, pH 5.0, containing 0.5 mM EDTA. The reaction mixture was incubated at 37 °C in a water bath for 1 h. Enzyme activity was expressed as micromoles of amino acid released per gram of fresh weight under the experimental conditions. The total soluble protein content of homogenates was determined with Coomassie Brilliant Blue according to Bradford (1976) using bovine serum albumin as the standard. The results were expressed as mg/g FW.

2.5. Statistical analyses

The data presented are the means of 4 replications performed on each of the 3 samples. The differences among the investigated enzyme activities were tested by one-way ANOVA. Tukey's honestly significant difference test was used to determine the differences among the days. All statistical analyses were based on a significance level of 0.05 (SPSS 16.0).

3. Results

Variations in proteolytic activities during a 1-year period of *Asphodelus aestivus* growth are shown in Figures 1–3 and Table 1.

3.1. EP activity

The EP activity patterns during a 1-year period are shown in Figure 1. The differences of the EP activities on different sampling dates were significant ($P < 0.05$). EP activity was high in the spring months from March to May (Table 1). The highest activity was found in May 2007 (5.18 ± 0.12 U/h).The lowest EP activity was found in February (2.32 ± 0.08 U/h).The activity seemed to be generally constant from June to February (Figure 1).

3.2. LAP activity

LAP activity changed significantly during the growth period ($P < 0.05$). LAP activity had 3 peaks, in September, December–January, and March–April (Table 1). The highest activity was found in December (1.19 ± 0.19 μmol pNA/g FW). The activity was lower in May, July, and October, but it started to increase in December (Figure 2).

3.3. CP activity

The highest CP activity was found in February (14.19 ± 1.13 μmol Leu/g FW). CP activity increased in February and March and had a smaller peak in July and September

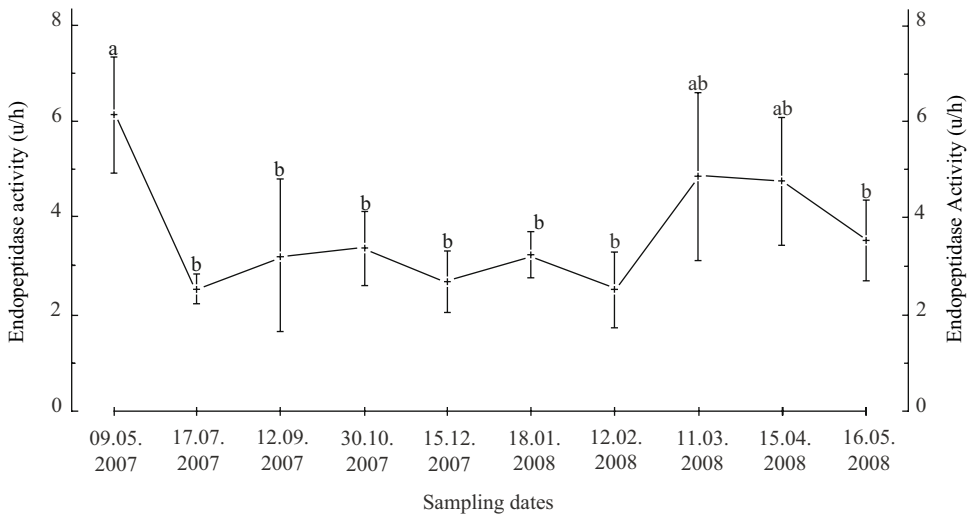

Figure 1. Seasonal fluctuations of endopeptidase (EP) activity in root tubers of *Asphodelus aestivus*. The values with the same letters are not significantly different at α = 0.05. Values are means ± standard errors.

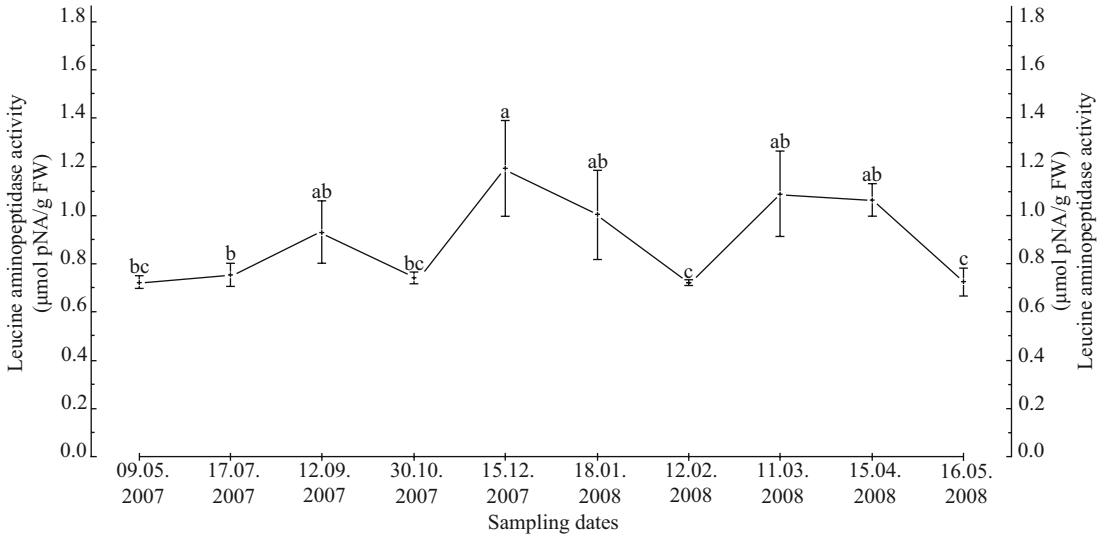

Figure 2. Seasonal fluctuations of leucine aminopeptidase (LAP) activity in root tubers of *Asphodelus aestivus*. The values (means ± standard errors) with the same letters are not significantly different at α = 0.05.

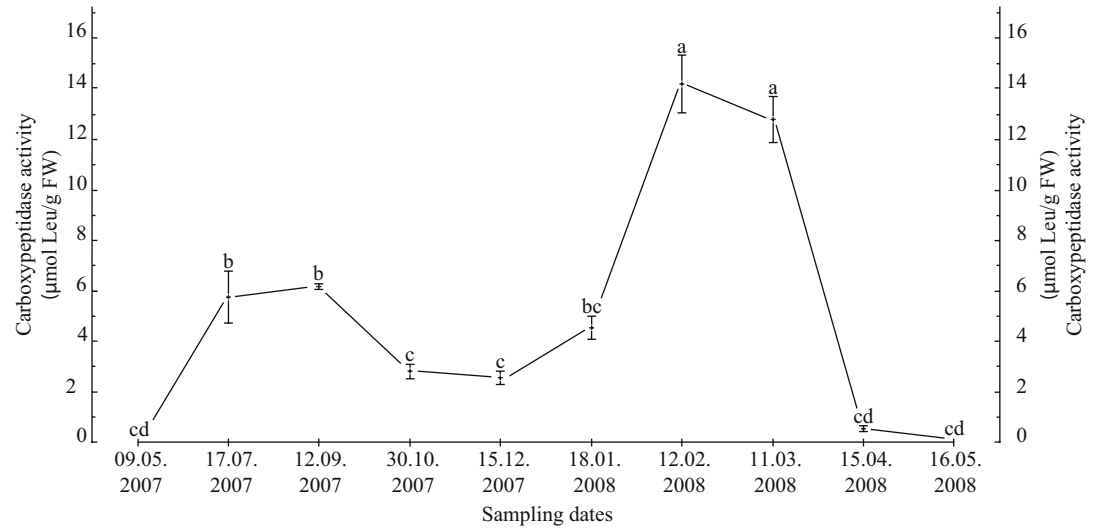

Figure 3. Seasonal fluctuations of carboxypeptidase (CP) activity in root tubers of *Asphodelus aestivus*. The values (means ± standard errors) with the same letters are not significantly different at α= 0.05.

(Table 1). The differences among the mean CP activities were significant ($P < 0.05$). There was no activity in May. The activity was generally constant from June to February (Figure 3). The lowest activity was found in April (0.53 ± 0.04 µmol Leu/g FW).

3.4. Total soluble protein content

The soluble protein content was higher during the period from July to December, and afterwards it decreased to lower values. The highest soluble protein content was found in October (0.25 ± 0.05 mg/g FW) and the lowest soluble protein content was found in March (0.04 ± 0.01 mg/g FW) (Table 2).The differences among the means were significant ($P < 0.05$).

3.5. Total N and water content

The total N contents were generally similar; the lowest total N was found in May 2008. The highest total N content was found in January (3.0 ± 0.2%). The total N contents were significantly different ($P < 0.05$; Table 2). Water contents were expressed on a fresh weight basis. The water contents decreased to the lowest values in July and September and increased thereafter (Table 2).

4. Discussion

In our study, the proteolytic enzyme activities showed seasonal fluctuations. While CP and LAP activities were generally higher in the spring, EP activity was higher in

Table 1. The seasonal means of proteolytic activities and soluble proteins in *Asphodelus aestivus* tubers (means ± standard errors).

Periods	EP (U/h)	LAP (μmol pNA/g FW)	CP (μmol Leu/g FW)	Soluble protein (mg/g FW)
May–July 2007	3.91 ± 1.41	0.74 ± 0.02	2.88 ± 1.19	0.15 ± 0.10
September–December 2007	2.88 ± 0.70	0.96 ± 0.09	3.85 ± 0.51	0.20 ± 0.07
January–March 2008	2.86 ± 0.71	0.94 ± 0.09	10.51 ± 1.37	0.09 ± 0.05
April–May 2008	4.19 ± 0.51	0.89 ± 0.08	0.26 ± 0.11	0.07 ± 0.04

March and May (Figures 1–3). According to Pantis (1993), the spring period up to March supports the flowering phase, whereas the period between spring and summer, up to June, supports seed production. The above-ground parts of the plant become senescent later on. The Mediterranean climate is characterised by its periodicity, and *Asphodelus aestivus* has adapted its phenological phases to this habitat. The root tubers of *Asphodelus aestivus* play an important role in storing and utilising water and nutrients, protecting the plant from drought stress and environmental hazards (Pantis, 1993; Sawidis et al., 2005). Tissue water content was decreased in May–September and increased in October–April (Table 2). These values were in accordance with the climatic conditions of the study area, which is characterised as a Mediterranean climate.

Franková et al. (2006) found that proteolysis continued throughout the year in mother and daughter corms of *Colchicum autumnale* L. Aminopeptidase and endopeptidase were the most active peptidases in *Colchicum autumnale*. They found the highest proteolytic activities during the late autumn and winter period as well as at the end of the plant's life cycle. They concluded that active protein turnover occurs during the autumnal stage.

Table 2. The mean values of some parameters measured in tubers of *Asphodelus aestivus* during a 1-year period. The values (means ± standard errors) with the same superscripted letters are not significantly different at α = 0.05.

Sampling dates	Soluble protein (mg/g FW)	Total N (%)	Water content (%)
09.05.2007	$0.09^{bc} \pm 0.04$	$1.8^{ab} \pm 0.40$	$80^{c} \pm 0.70$
17.07.2007	$0.21^{ab} \pm 0.06$	$1.9^{ab} \pm 0.10$	$74^{d} \pm 1.50$
12.09.2007	$0.15^{abc} \pm 0.01$	$2.7^{a} \pm 0.20$	$66^{d} \pm 1.40$
30.10.2007	$0.25^{a} \pm 0.05$	$2.8^{a} \pm 0.50$	$84^{bc} \pm 0.60$
05.12.2007	$0.20^{ab} \pm 0.03$	$2.5^{a} \pm 0.20$	$86^{ab} \pm 0.30$
08.01.2008	$0.09^{b} \pm 0.01$	$3.0^{a} \pm 0.20$	$91^{a} \pm 0.50$
12.02.2008	$0.14^{abc} \pm 0.02$	$2.5^{a} \pm 0.3$	$93^{a} \pm 0.60$
11.03.2008	$0.04^{bc} \pm 0.01$	$2.5^{a} \pm 0.00$	$89^{a} \pm 0.30$
15.04.2008	$0.08^{bc} \pm 0.01$	$1.3^{b} \pm 0.00$	$91^{a} \pm 0.60$
06.05.2008	$0.06^{bc} \pm 0.03$	$0.7^{b} \pm 0.00$	$87^{ab} \pm 0.60$

Similarly, we found that proteolysis in *Asphodelus aestivus* tuberous roots continued throughout the year (Table 1), but the activities of certain enzymes were expressed during certain periods of time. For example, EP remained high between March and May (Figure 1), LAP was high from December to April (Figure 2), and CP was high in winter and spring (Figure 3). These high levels of exopeptidase (LAP and CP) activity coincide with the flowering and early fruit development as reported by Sawidis et al. (2005). High activity of EP is generally found in the same period as the plant's seed production or fruit development. The mobilisation of tuber proteins in *Asphodelus aestivus* could reflect the seasonality of this plant, and proteolytic enzymes might have a role in the plant's adaptation to the periodicity of the Mediterranean climate. The water contents also reflect the environmental conditions. For example, in July to August, which is the drought season, the water content of the root tubers was at the lowest level (Table 2). At the same time, the above-ground plant parts had died.

Total soluble sugar levels increased from January to June, and the proline content was high in January and June in *Asphodelus aestivus* (Rhizopulou et al., 1997). The dormant tubers of *Asphodelus aestivus* were found to take up N, K, Ca, and Na from the soil (Pantis, 1993). As was suggested by Pantis (1993), the reproduction of the plant might depend on the stored reserves from previous years. In contrast, flowering and seed production depend mainly on annual production and nutrient availability.

Our results indicated that all 3 proteolytic enzyme activities were higher in early spring, and during the summer, their activities were at lower levels. This coincides with the plant's inactive life phase.

Although onion and garlic belongs to the same genus, their protease activity patterns are not the same (Lin and Yao, 1995). The casein hydrolysing activity of garlic was higher after germination than before germination. However, aminopeptidase activity was higher in the dormant garlic bulb. It is probable that these enzymes provide high rates of protein biosynthesis in developing bulbs. We found similar results for *Asphodelus aestivus* tubers. The EP activity (the substrate was also casein in our case) was found to be lower after germination than before,

whereas the exopeptidase activities (LAP and CP) were near their highest values after germination. The soluble proteins in *Asphodelus aestivus* peaked in July–December and then decreased; the soluble proteins might have been used in growth-related processes (Table 1). For example, the products of proteolytic activities might be utilised to support nitrogen requirements for early flowering.

Shewry (2003) suggested that tuber storage proteins may have dual actions. They may serve as storage compounds to be used in sprouting, and they may protect tubers from abiotic stresses as well as biotic stresses like pathogens. The high nitrate assimilation capacity of *Asphodelus aestivus* showed the seasonal pattern of the NRA in above-ground plant parts, but in tuberous roots there was no change in the NRA (Sakar et al., 2010). The high soluble protein content in asphodel tubers could be associated with the continuing proteolytic activities rather than the nitrate assimilation. The amino acids or products of the proteolytic cleavage can be used to meet the metabolic demands of the storage tissue (Kırmızı and Güleryüz, 2006).

Our results indicate that storage proteins of *Asphodelus aestivus* are an important nitrogen source and can be used for the growth processes needed for both the first and second phases of the plant's development. Similar results were found previously by Franková et al. (2006). An understanding of the mobilisation of tuber proteins by the proteolytic enzymes contributes to our knowledge of the basic features of Mediterranean geophytes such as *Asphodelus aestivus*. Seasonal fluctuations of proteolytic activities in the tubers of this species seem to be synchronised with the Mediterranean climate. The results from this study can help our understanding of the developmental stages, their relationship with proteolytic activities, and their influence on the domination of *Asphodelus aestivus* in the degraded areas.

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