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Research Article

Nitrate Reductase Activity in *Verbascum* L. (*Scrophulariaceae*) Species from the Eastern Mediterranean in Dependence on Altitude

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Abstract: Nitrate reductase activity (NRA) was investigated in diferent parts of some *Verbascum* L. species (*Scrophulariaceae*) collected from different altitudes of Uludağ Mountain, Bursa Turkey. NR activities were compared with respect to (1) different plant organs, (2) different *Verbascum* species and (3) samples collected from different altitudes (120–1850 m). The highest levels of NRA were found for all species in the leaves. Significant differences were determined between the samples collected from different altitudes. Despite the NRA is the highest in *V. olympicum* Boiss., it is the lowest in *V. lagurus* Fisch. & Mey. Furthermore the correlation between NRA values in aboveground organs of species and altitude is significant.

Key Words: Nitrate Reductase Activity, *Verbascum* L. species.

Yüksekliğe Bağlı Olarak Doğu Akdeniz Bölgesi *Verbascum* L. *(Scrophulariaceae)* Türlerinde Nitrat Redüktaz Aktivitesi

Özet: Uludağ'ın farklı yüksekliklerinden toplanan bazı *Verbascum* L. türlerinin farklı kısımlarındaki nitrat redüktaz aktivitesi (NRA) araştırıldı. NR–aktiviteleri farklı bitki organlarına (1), farklı *Verbascum* türlerine (2), farklı yüksekliklerden (120–1850 m) toplanan örneklere (3) göre karşılaştırıldı. NRA'nın en yüksek düzeyleri tüm türler için yapraklarda saptandı. Farklı yüksekliklerden toplanan örnekler arasında anlamlı fark tespit edildi. Nitrat redüktaz aktivitesi *V. olympicum* Boiss. türünde en yüksek olmasına karşın, *V. lagurus* Fisch. & Mey. türünde en düşüktür. Ayrıca, araştırılan türlerin toprak üstü organlarındaki NRA değerleri ile yükseklik arasındaki ilişki anlamlıdır.

Anahtar Sözcükler: Nitrat redüktaz aktivitesi, *Verbascum* L. türleri.

Introduction

Nitrogen is quantitatively the most important mineral nutrient for higher plants and it is assumed to be taking up mainly in inorganic forms (NH_4^+, NO_3^-) by the roots. Ammonium together with nitrate ions comprise about 80% of total cations and anions taken up by plant roots (1). The amount of inorganic nitrogen available to plant roots under natural conditions depends on several biotic and abiotic factors, eg. type of soil, climate, season and microbial activity. Therefore, the mineralization of nitrogen in the soil and the absorption by plants are important indicators for the explanation of productivity of ecosystems. It has been explained by using nitrogen–flow modelling that the nitrogen form in grassland ecosystems is a factor to be checked (2).

Most of the ammonium is already incorporated into organic compounds in the roots whereas nitrate is mobile in the xylem and can also be stored in the vacuoles of roots, shoots and storages organs. Ammonium is also mobile in the xylem, but it is more risky to transport ammonium due to the pH dependent balance $NH_4^+ \Leftrightarrow NH_3 + H^+$ and the toxicity of NH_3. However, nitrate, as a plant nutrient has to be reduced to ammonia, in order to incorporate into organic structure. The currently accepted pathway reduction in higher plants is as follows:

$$NO_3^- + 8H^+ + 8e^- \rightarrow NH_3 + 2H_2O + OH^-$$

Two separate enzymes mediate the reduction of nitrate to ammonia: nitrate reductase (NR), which reduces nitrate to nitrite; and nitrite reductase (NIR), reducing nitrite to ammonia (1). The reduction of nitrat catalysed by the NR is the first and rate–limiting step of nitrate assimilation by plants (3). Nitrate reductase is inactivated after only a few hours and it can be induced by addition of nitrate and suppressed by certain amino

acids (4). The nitrate reductase activity is very low under conditions of molybdenum deficiency, because molybdenum is a cofactor of the enzyme. It also contains several prostetic groups including flavinadenindinucleotid (FAD) and cytochrome b_{557} (cyt) and requires either NADH or NADPH as electron donor. In general, when the external NO_3^- supply is low, a high proportion of nitrate is reduced in the roots. With increasing supply of nitrate, the capacity for nitrate reduction in the roots becomes a limiting factor and an increasing proportion of the nitrate is transported to the shoot (1).

In principle, nitrate reduction is possible in roots as well as aboveground organs. Nitrate is reduced preferentially in the leaves of the most herbaceous plants (5, 6). In contrast, woody plants have since recently been considered to reduce nitrate nearly exclusively in the roots (7, 8, 9). Smirnoff et al (10) and Al Gharbi and Hipkin (11) were among the first to show that the leaves of trees from various taxonomic groups (gymnosperms and angiosperms) which grow under natural conditions have also NRA. For more recent data on this question see Gebauer and Schulze (12).

The NRA of a plant is assumed to reflect the nitrate supplying power of its habitat (3). Stewart et al (13) have successfully established the activity of this key enzyme in nitrate assimilation as an indicator of nitrate supply in ecological studies. Lee et al (14) give a summary of means NRA values of plants from different habitats and climatic regions. These values range from 0.2 µmol NO_2/g fresh wt.h in bog plants up to 4.58 µmol NO_2/g fresh wt. h in plants from wasteland sites and agree with results of mineral nitrogen analyses from similar sites.

In this study, we investigated the nitrate reductase activity of some *Verbascum* L. species spread on the different altitudes of northern of Uludağ Mountain Bursa Turkey. Uludağ Mountain is a western extension of Pontic mountain ranges and lying at the intersection of 40° North latitude with 29° East longitude. It is one of the highest peaks at the far Northwest point of Anatolian peninsula. The climate of the mountain changes from base to top, being of Mediterranean type in lower parts which are near to the city of Bursa and rainy, partially mild micro–thermic, with icy winter at higher altitudes (15, 16). The change of Mediterranean, Euro–Siberia and alpine vegetation can be seen very clearly step by step from bottom to the top of mountain.

Our basic aim was to obtain pioneer information about the nitrogen metabolism of some *Verbascum* L. species from different altitudes. We compared the following differences according to NRA values; (1) among species, (2) among organs of same plant, (3) among plant samples collected from different altitudes.

Material and Methods

Material

Fresh plant samples were collected from eight investigation sites in six different altitudes of Uludağ Mountain. The species collected from different altitudes are usually spread on wasteland places of anthropogenic origin, such as around building, roadsides, picnic areas, plantation areas or archaeological sites etc. General characteristics of the sites are shown in Table 1.

Methods

NRA of the plant material was determined according to an *in vivo* test described by Hageman and Hucklesby (17) and Jaworski (18) and modified by Gebauer et al. (19). The method is based on the determination the

Table 1. Habitats of *Verbascum* L. species.

SITE NO		SPECIES	ALTITUDE (m)	SITE NAME	HABITATS
	A_1	*V. bombyciferum* Boiss.			Around the roads and buildings
A	A_2	*V. sinuatum* L.	120	Campus site of Uludağ	Around the roads and buildings
	A_3	*V. lagurus* Fisch. & Mey.		University	Plantation areas
B		*V. bombyciferum* Boiss.	500–550	Bursa–Uludağ road	Shrub lands (Mediterranean formation) and roadsides
C		*V. splendidum* Boiss.	750	Yiğitali Village	Destroyed forest and shrub lands
D		*V. cheiranthifolium* Boiss. var. *cheiranthifolium*	1100	Bursa-Uludağ road (~20 km)	Opened areas around the roads in the forest (Pine and beech)
E		*V. olympicum* Boiss.	1650	Kirazlıyayla	Meadows at picnic areas
F		*V. olympicum* Boiss.	1850	Center of Winter Sports	Opened areas around the hotels

absorbance of nitrite spectrophotometrically which is formed as product of the reduction of nitrate in the incubation medium. Because of the NO_3^- – reductase having a half–life of only a few hours, plants were brought to the laboratory and tested within a few hours. All of the plant samples were in the flowering phase. Plant samples were divided into organs (roots, stems, basal leaves and upper leaves), before the analysis procedure.

NRA test was carried out in two steps: in the first step, discs with 1 cm radius were punched out of the leaves and pieces were cut from the roots and stems of plants previously cleaned with distilled water. These were put in brown flasks containing 5 ml buffer solution [0.08M KNO_3, 0.25M KH_2PO_4 and 1.5 %n–propanol; pH: 7.5; Gebauer et al. (19)]. After a vacuum infiltration (100 bar) and addition of nitrogen gas, plant samples were incubated in a water bath with horizontal shaking at 30 °C in the dark for two hours.

In the second step of the NRA test, nitrite released from plant material into the incubation medium was determined colorimetrically at 540 nm by adding 0.3 ml of 0.1% N–naphthylethylenediamine solution, 0.3 ml of 5% sulphanilamide solution, 0.4 ml distilled water and 1 ml of incubation buffer.

After incubation, plant material was removed from the incubation medium, rinsed with distilled water and dried at 80 °C until weight became constant and then weighed. NRA (µmol NO_2^-/g dry wt h) was calculated by using absorption value and dry weight. The differences between NRA values of the plant samples were tested by Analysis of Variance. Difference groups were distinguished using the Tukey test. Furthermore, NRA values and growth altitude was tested for potential correlation. Analysis of Variance and Regressions were calculated using the MICROSTA statistical package (Ecosoft, 1984). All statistical analyses were based on a significance level of 0.05 (20). "Flora of Turkey and the East Aegean Islands" is referred to for the names of taxa cited in the text (21).

Results

We examined the NRA values in different organs of *Verbascum* L. species. The difference among organs of the same plant, species and plants collected from different altitudes for NRA values were tested by Analysis of Variance and difference groups were formed by Tukey test. The results of statistical analyses are shown in Table 2 and 3. The difference groups among the organs of plants collected from the same habitat, and the plants collected from different habitats were marked by major (A, B, C) and by minor letters (a, b, c, d) respectively (Table 2 and 3).

Table 2. Difference groups among the samples collected from different altitudes (referred by uppercase letters), and the organs of same species (refferred by lowercase latters) regarding to their NRA values (µmol NO_2^-/g dry wt h).

Site No	Species	ROOTS	STEMS	BASAL LEAVES	UPPER LEAVES
		B^{cb}	B^{c}	A^{b}	A^{bcd}
A_1	*V. bombyciferum*	0.057±0.036	0.029±0.018	1.006±0.323	0.892±0.257
		B^{ab}	B^{c}	A^{a}	A^{b}
A_2	*V. sinuatum*	0.510±0.325	0.031±0.044	1.741±0.361	1.767±0.835
		B^{cb}	B^{bc}	A^{bc}	A^{d}
A_3	*V. lagurus*	0.084±0.024	0.052±0.023	0.168±0.012	0.206±0.096
		B^{b}	B^{bc}	A^{a}	A^{bc}
B	*V. bombyciferum*	0.280±0.296	0.092±0.085	1.818±0.258	1.547±0.314
		B^{abc}	B^{b}	A^{b}	A^{bcd}
C	*V. splendidum*	0.345±0.345	0.325±0.298	0.942±0.180	1.81±0.358
		A^{b}	A^{bc}	A^{ab}	A^{d}
D	*V. cheiranthifolium*	0.243±0.183	0.248±0.218	0.368±0.065	0.395±0.156
		BC^{a}	B^{a}	AB^{a}	A^{a}
E	*V. olympicum*	0.670±0.434	0.752±0.458	2.381±1.266	3.538±1.483
		B^{cb}	B^{bc}	A^{a}	A^{ab}
F	*V. olympicum*	0.157±0.095	0.233±0.090	2.083±0.589	2.581±1.076

Table 3. Difference groups among Vesbascum species regarding to NRA values (µmol NO_2^-/g dry wt h) in their different organs.

Species	n	ROOTS	STEMS	BASAL LEAVES	UPPER LEAVES
		a	ab	ab	b
V. bombyciferum	8	0.168±0.228	0.060±0.066	1.412±0.511	0.219±0.440
		a	b	ab	ab
V. sinuatum	4	0.510±0.325	0.031±0.044	1.741±0.361	1.767±0.835
		a	ab	cb	b
V. lagurus	4	0.084±0.024	0.052±0.023	0.168±0.012	0.206±0.096
		a	ab	b	b
V. splendidum	4	0.345±0.345	0.325±0.298	0.942±0.180	1.181±0.358
		a	ab	cb	b
V. cheiranthifolium	4	0.243±0.183	0.248±0.218	0.368±0.065	0.395±0.156
		a	a	a	a
V. olympicum	8	0.414±0.400	0.492±0.413	2.232±0.928	3.059±1.304

Except of *V. cheiranthifolium* Boiss. var. *cheiranthifolium* (site D), significant difference among organs of all examining species were found. Irrespective of the plant species, the lowest NRA values were found for roots. In contrast, the NRA values of leaves (basal and upper leaves) were highest (Table 2). No significant difference was found for the NRA in the roots and in the stems and no significant difference was found for the NRA in the basal leaves and upper leaves.

In general, significant differences among plant samples collected from different altitudes (Table 1) were found. We observed that nitrate reductase activity in all organs of *V. olympicum* Boiss. from site E had the highest NRA values whereas *V. largurus* Fisch. & Mey. from site A_3 had the lowest. Stems of plant samples of *V. bombyciferum* Boiss. (site A_1), *V. sinuatum* L. (site A_2) and *V. lagurus* (site A_3) had lower NRA values than the others (Figure 1 Table 2).

When we compare the NRA values of *Verbascum* species, in general, *V. olympicum* have the highest and *V. lagurus* and *V. cheiranthifolium* var. *cheiranthifolium* have the lowest values (Table 3 Figure 2).

The correlation between NRA values and altitude were tested (Table 4). A significant positive correlation was found between altitude and NRA values of basal leaves (r=0.441), and upper leaves (r=0.562), and stems (r=0.573). No sifnificant correlation was found between NRA values of roots and altitude of plant growth (r=0.202).

Discussion

NRA was the highest in the leaves of all *Verbascum* species investigated. This fit to the general assumption that the nitrate is usually reduced in the leaves of herbaceous plants (5, 6). Nitrate is also reduced in the roots and stems, but the reduction rates were different among *Verbascum* species and also among the samples of the same species harvested from different altitudes. *V. lagurus* (site A_3) NRA in the roots is a little big higher than

Table 4. Simple correlation coefficients between altitude and NRA values in organs [n=32; P<0.001 significant, P>0.001 not significant; Sr: Standart error, (20)] (µmol NO_2^-/g dry wt h).

PARAMETER	r	r^2	$Sr = \sqrt{\frac{1-r^2}{n-2}}$	$t_{0.05}$ (2) 30	$t = \frac{r}{Sr}$	H_0: P=0	Y= a+bx
Altitude and Root (NRA)	0.202	0.041	0.179	2.042	1.128	P>0.001	Y = 0.220+0.0001x
Altitude and Stem (NRA)	0.573	0.329	0.150	2.042	3.820	P<0.001	Y = 0.017+0.003x
Altitude and Basal Leaves (NRA)	0.441	0.194	0.164	2.042	2.689	P<0.001	Y = 0.810+0.001x
Altitude and Upper Leaves (NRA)	0.562	0.316	0.151	2.042	3.722	P<0.001	Y = 0.687+0.001x

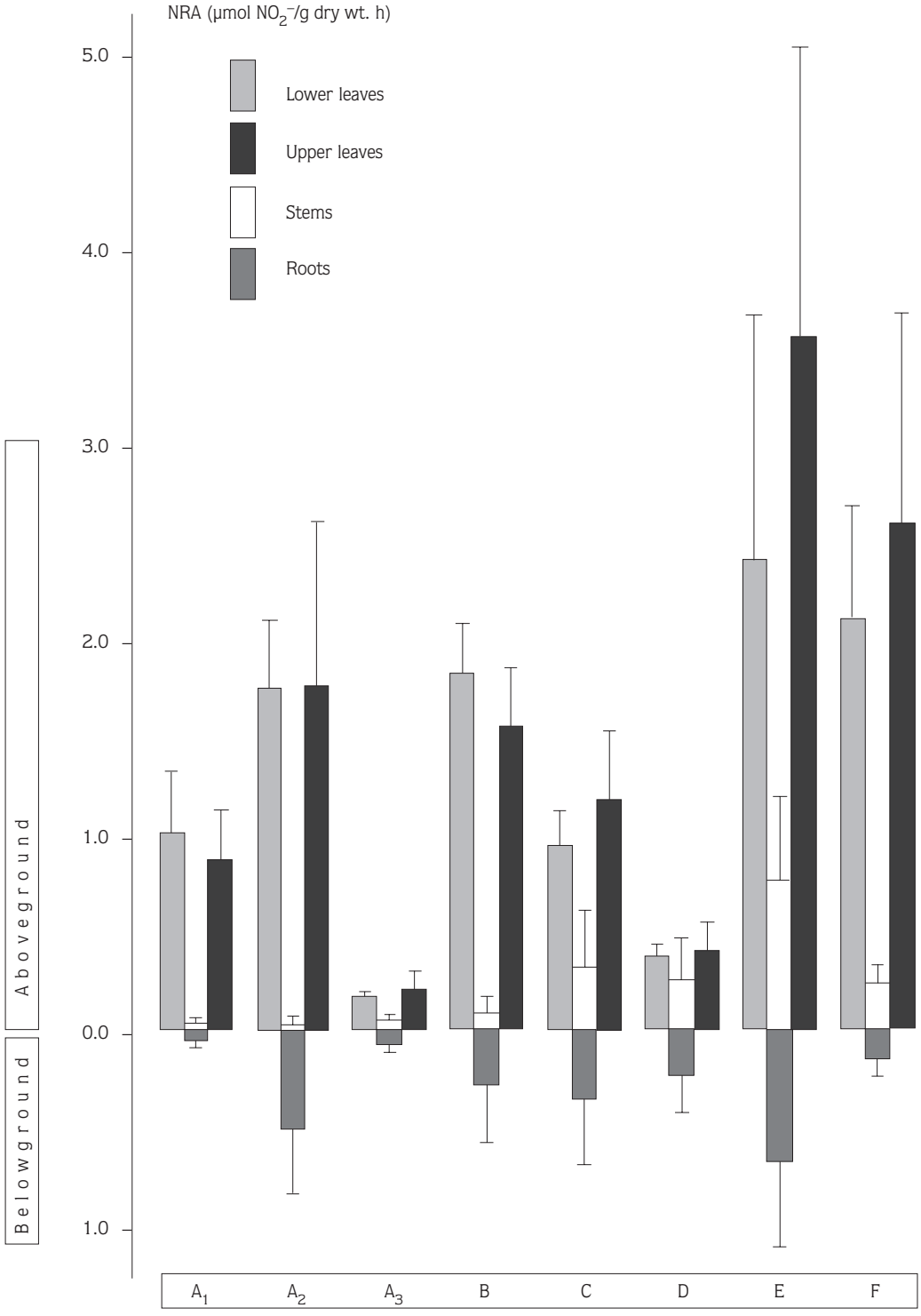

Figure 1. The nitrate reductase activity in organs of the same plants collected from different altitudes. (For explanation on the site numbers see Table 1 and 2).

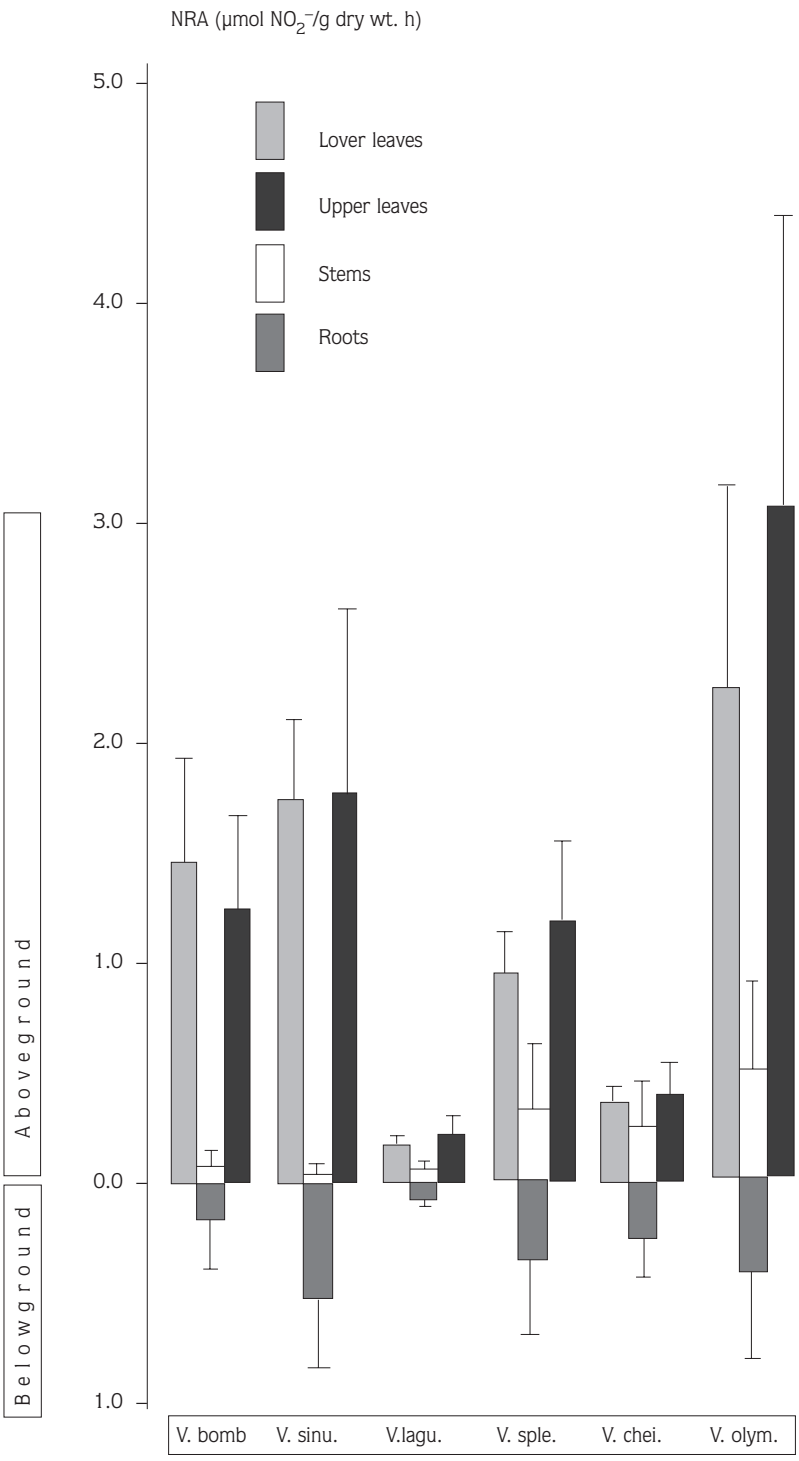

Figure 2. Distribution of nitrate reductase activity among the organs of *Verbascum* L. species.

of the stems, but it was similar to NRA values in the basal leaves (Table 2). Also, there was no significant difference among the NRA values in all organs of *V. cheiranthifolium* var. *cheiranthifolium* (Site D). In general, the NRA in the stems was low in the lower altitudes; but depending on the increasing altitude, it could became almost equal the NRA values in the roots (Figure 1). The reason of this correlation can be a variation in the amount of the nitrate

available in the respective habitats and of the environmental factors changing with altitude. According to Marschner (1) nitrate is reduced in the roots when the external nitrogen supply is low, however, with an increasing supply of nitrate, the capacity of nitrate reduction in the roots becomes a limiting factor and increasing proportion of the total nitrogen is translocated to the shoots in the forms of nitrate.

When the samples *V. olympicum* and *V. bombyciferum* collected from different altitudes were compared, the difference among their NRA values was found to be significant. The NRA values in the samples of *V. bombyciferum* from site B were higher than that from site A_1 and, similar results were observed for *V. olympicum* (Table 2, Figure 1). This difference is probably related to the nitrate supply power of the habitat. According to Lee and Stewart (3), the extreme difference in the induced enzyme activities of ruderal and bog species may indicate that the potential to synthesis nitrate reductase is related to the nitrate supply of the habitat.

We determined that NRA was highest for *V. olympicum* and lowest for *V. lagurus* when all organs considered. This result indicates that the amount of the nitrate supply can be high in the habitat of *V. olympicum* collected from around Hotels area and Kirazlıyayla picnic area, while it is low in the habitat of *V. lagurus* collected among forest plantations in the Campus site of Uludağ University. Nitrate reductase activity is used as an indicator in ecological studies on nitrogen assimilation and nutrition of plants from different habitats. For example, the very low NRA had been reported for bog species, especially shrub species belonging to *Ericaceae* family and species from poor grasslands (3, 14). In addition, it had been reported that the highest NRA is found in plants (*Urtica dioica* L. and *Lamium album* L.) growing on nitrogen rich ruderal sites (3, 11).

V. olympicum and *V. bombyciferum* are endemics to Uludağ Mountain, and they are widespread around wastelands. For example, we can see that *V. bombyciferum* spread on roadsides, public gardens and archaeological sites in Bursa City and *V. olympicum* widespread around Hotels and roadsides in the high altitudes (especially around winter sports centre) of Uludağ Mountain. Also, the NRA values of *V. sinuatum* are similar to that of these endemic species. Therefore, these endemics have more ruderal character because of the high NRA caused by nitrate supply power in their habitats. In addition, according to Ellenberg (22), the mineralization rates of organic compounds are variable and the mineralization rate in the soils of wastelands is the highest under natural conditions.

A correlation between altitudes and NRA in aboveground organs of *Verbascum* species is significant. In our opinion, this correlation is possible a result of the changeable atmospheric conditions. Because, the climate of Bursa city and alpine zone in Uludağ Mountain are different from each other. Bursa city has a typical Mediterranean climate which is arid during summer period, whereas Uludağ alpine zone has an oceanic climate which is rainfall and humid during summer period (16). Thus nitrate reductase activity in the aboveground organs of *Verbascum* species serves as an important ecological indicator.

This study showed that the nitrogen assimilation rates vary (1) among the *Verbascum* species, (2) among habitats, (3) among organs. In addition, we obtained pioneer information about the nitrogen metabolism and ecology of endemic species (*V. olympicum* and *V. bombyciferum*).

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