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On the reproductive biology of *Onobrychis pindicola* Hausskn. subsp. *urumovii* Deg. & Dren. (*Fabaceae*)

Abstract

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Onobrychis pindicola Hausskn. subsp. *urumovii* Deg. & Dren. is a Balkan endemic and a glacial relict, rare to Bulgaria. The plant was found self-incompatible, obligately dependent upon bumblebees for pollen transport. The bumblebee pollinator activity increased more or less parallelly to the size of population in the studied sites. According to the results of the pollen analysis, *O. pindicola* subsp. *urumovii* was the only "major" for all bumblebee species collected on its flowers with few exceptions. The recorded high pollinator activity indicates that the possible reason for the restricted number of *O. pindicola* subsp. *urumovii* populations occurring in Bulgaria is not to be connected to pollinator limitation.

Introduction

Onobrychis pindicola Hausskn. subsp. *urumovii* Deg. & Dren. is a Balkan endemic and a glacial relict, which is considered rare to Bulgaria (Velchev & al. 1984). It grows in stony and grassy habitats on carbonate rocks in the coniferous and subalpine belts of Pirin and Slavjanka Mts. This subspecies occurs in the territory of the Balkan peninsula notably in Former Yugoslavia and Greece. The subspecies *O. pindicola* Hausskn. subsp. *pindicola* is also endemic to the Balkans, but it does not occur in Bulgaria (Kozuharov 1976, Velchev & al. 1992).

O. pindicola subsp. *urumovii* is a perennial plant that forms dense tufts. It has almost vertical reddish-brown rhizome. Stems are short or lacking. The leaves are pinnately compound and normally bear four to seven pairs of lancet, hairy leaflets and a similar terminal leaflet. The numerous purple flowers are bore on dense racemes. The legume is round and dentate (Kozuharov 1976).

Genetic erosion occurs in populations with a small number of individuals (Briggs & Walters 1997). Reduced fitness in small and isolated populations occurs, owing to reduced reproductive success and inbreeding (den Nijs & Oostermeijer 1997). Species with restricted ranges and few individuals are more likely to be self-compatible, as proved for two restricted *Astragalus* species (Karron 1989).

The aim of this study is to provide information on the reproductive biology of *Onobrychis pindicola* subsp. *urumovii*. A hypothetical reason for the restricted number of its populations may be connected to some reproductive specific factors.

Material and methods

Study sites

Field observations were carried out in August 1995 and July 1996 in Pirin Mts. The population structure of the investigated species is of mosaic type. In fact, *O. pindicola* subsp. *urumovii* occurs, scattered, in the marble slopes of North Pirin Mts. Five study sites of different size and altitude were chosen (Figs. 1, 2, Table 3). The flower morphology was investigated on 50 fresh flowers. They were collected randomly from different individuals in the natural population.

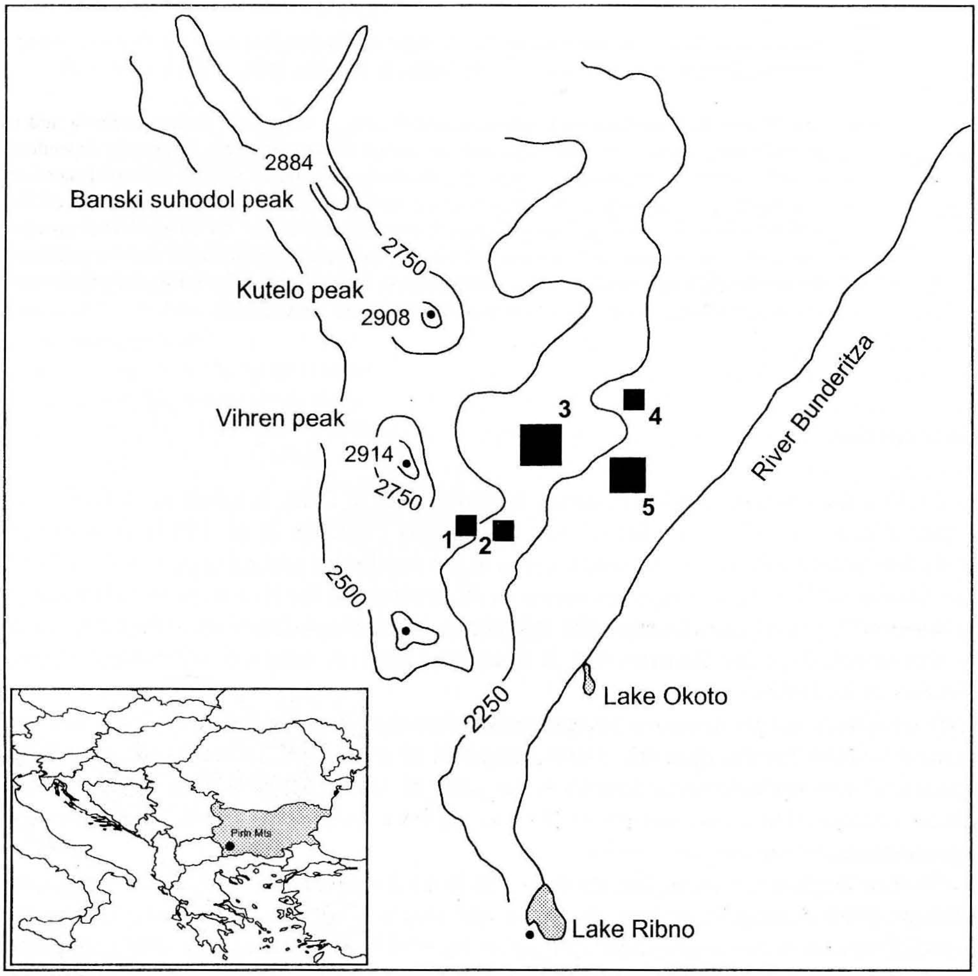

Fig. 1. Map of the Balkan peninsula and a part of North Pirin Mts (enlarged 1 : 55000). Study sites 1, 2, 3, 4, and 5.

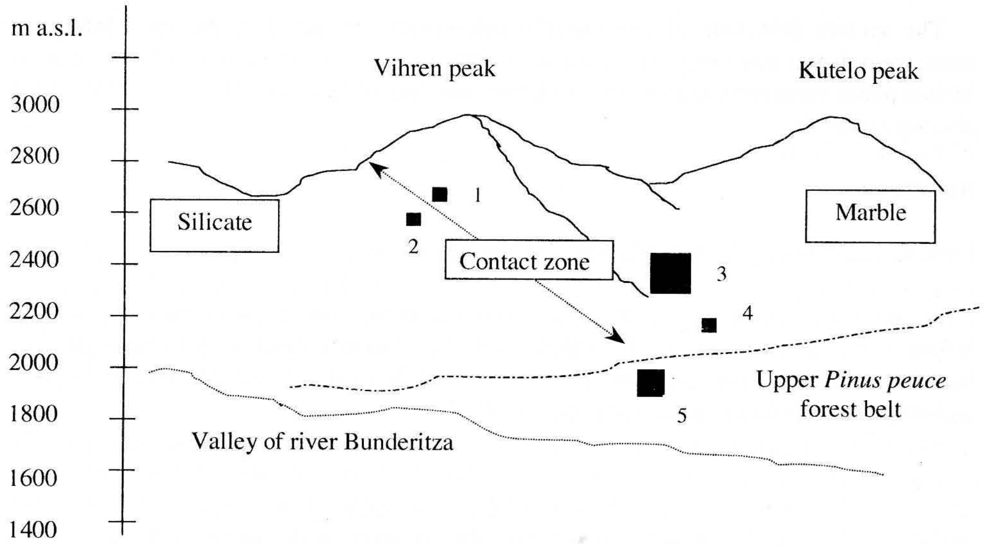

Fig. 2. Scheme of part of North Pirin Mts. Study sites 1, 2, 3, 4, and 5 - the size of the symbol [■] corresponds to the investigated *Onobrychis pindicola* subsp. *urumovii* population patches.

Self-fertility and self-compatibility tests

Spontaneous self-pollination was tested on 6 different individuals in the site 4. Their inflorescences were covered at bud stage with fine mesh insect exclosures. The self-compatibility test was done on 6 flowers of different individuals (excluded at bud stage, hand self-pollinated and immediately covered again). The fruit set of the experimental flowers was examined. The open pollination fruit set was tested by counting the matured fruits versus the empty ones and the wilted flowers as follows: 10 randomly chosen inflorescences from the site 2 in 1995, 10 inflorescences form the site 4 in 1996, and 21 inflorescences from the site 5 in 1996.

Pollinator activity

The insect visitors (in the sense of Faegri & van der Pijl 1971) were investigated after a transect method combined with observation on a plant group (Dafni 1992, Dlusskii, pers. comm.). The pollinator activity was observed during 10 days. Several periods of time (from 60 to 150 minutes) were spent on each study site. All observed visitors were recorded. The insects were identified in the field as strictly as possible. An insect specimen was collected for detailed identification. The behaviour of pollinators was observed to reveal the mechanism of cross-pollination and to evaluate the probability of geitonogamy.

Most of the observed bumblebees were collected and narcotized with ethyl-acetate, and released after extraction of their pollen loads (Heinrich 1979a). Pollen contamination was avoided by catching each bumblebee separately. Pollen identification and counting (at least 200 pollen grains according to Louveaux & al. 1978) was conducted under light microscope "Nikon" and magnification x 320 and x 800.

The species flowering in close neighbourhood simultaneously with the investigated ones were listed according to Jordanov (1963-1995). The pollinators of the actively visited plants were recorded in order to obtain an approximate evaluation of the pollinator sharing (Table 2).

Results

Flower morphology, floral mechanism and breeding systems

In the same inflorescence there are simultaneously buds and mature fruits. The inflorescence development is of acropetal type: first the two basal flowers open more or less simultaneously. The two ones above them open the next day, etc. The terminal flower and several others below it may not open at all.

The bright purple flower of *Onobrychis pindicola* subsp. *urumovii* is typical of the *Fabaceae*. The wing petals are small and only the keel one bears the weight of the pollinator. The anthers of the stamen sheath have more or less equal filaments. The slightly curved pistil is usually longer than the stamens so the stigma extends slightly beyond them (64.3% of the flowers). In some of the flowers (21.4%) the pistil is shorter and the stigma is situated beneath the anthers. Spontaneous self-pollination including geitonogamy is not possible in both cases as the flowers are self-incompatible. The results of the experiment confirmed that the excluded flowers did not set seeds as well as the hand-selfed flowers (Table 1). Flowers with extremely short styles and with stigmas hidden at the bottom of the stamen sheath are 14.3% of the population. These flowers are functionally male and donate pollen only.

The fruit set of open pollinated flowers was different in space and time (Table 1). There are several hypothetical explanations of these contradictory results. Inflorescences at late stage of maturing were sampled in the site 2 in 1995. Some of the flowers/fruits had fallen and were not counted. Inflorescences at early stage of maturing were sampled in the sites 4 and 5 in 1996. Their low fruit set may reflect the early stage of pollinator activity when the bumblebees have not yet discovered *Onobrychis pindicola* (see Heinrich 1976a, b, 1979a). However, additional tests are necessary.

Nectar is secreted at the base of the stamen sheath. It is readily accessible to short tongued insect species since the petals are free and the calyx tube is short (2-2.5 mm).

Pollination ecology

The pollinator activity was high as a whole but varied in space and in time. The highest pollinator activity was recorded in the site 3 in 1996, and the lowest one in the site 1 in 1996 (Tables 2, 3). These differences in pollinator activity are correlated to the number of individuals in each site. The bigger the population is, the higher is the pollinator activity (Fig. 2, Table 3). The pollinator activity in the site 2 was higher in 1995 (Table 2). One explanation of this fact may be the difference in phenological stages: in 1996 the plants were observed at the end of their flowering period, while in 1995 they were in fool blooming. Correlation to the altitude is expressed by reduced activity in the highest site 1, where there is a small number of individuals.

Table 1. Self-fertility, self-compatibility tests and open pollination fruit set in *Onobrychis pindicola* subsp. *urumovii.*

experimental fruit set/1995-1996	
covered inflorescences	6
total number of covered flowers	140
unfertilized covered flowers	140
total number of hand selfed flowers	6
unfertilized hand selfed flowers	6
open pollination fruit set	
1995 / site 2	
number of inflorescences	10
total number of matured fruits	162
total number of unfertilized flowers	4
percent of fertilization	97.6 %
1996 / site 4	
number of inflorescences	10
total number of matured fruits	31
total number of unfertilized flowers	164
percent of fertilization	15.9 %
1996 / site 5	
number of inflorescences	21
total number of matured fruits	136
total number of unfertilized flowers	567
percent of fertilization	19.4 %

The composition of bumblebee species was more or less similar in all sites. Most frequent visitors in all sites were workers *Bombus pyrenaeus* Perez (Table 2). Visitors were also workers *B. lapidarius* L., *B. terrestris* L. and *B. pratorum* L. Honeybee workers (probably a wild race of *Apis mellifera* L.) visited actively *Onobrychis pindicola* subsp. *urumovii* in the site 4 only once during the observations. All bees collected nectar. When they stepped on the keel it bent down and threw out the stamen sheath. This way the thorax and abdomen of the insect were abundantly dusted with the orange pollen of *O. pindicola* subsp. *urumovii* sternotribically. Only one worker *Bombus pyrenaeus* was observed to collect pollen while sucking nectar. The others broomed the pollen from their bodies into the baskets. All of them followed strictly only *Onobrychis pindicola* subsp. *urumovii* except for one worker *Bombus pyrenaeus.* The analysis of the pollen loads confirmed very high flower constancy with few exceptions (Tables 4, 5). All bumblebees tended to visit successively more than one flower per inflorescence.

In the site 2, on silicate rocks, *Onobrychis pindicola* subsp. *urumovii* is growing sympatrically with *Oxytropis campestris* and both taxa flower simultaneously. The pale yellow flowers of *O. campestris* were pollinated by *Bombus lapidarius.* Pollinator activity was high. About two workers visited the plants every ten minutes (Table 2). The bumblebee workers *B. lapidarius* were observed to follow strictly *Oxytropis campestris.* They collected nectar and the pollen was adhering on the hair of their abdominal sternits. Pollen collecting activity was not observed.

Table 2. Plant complex flowering simultaneously with *Onobrychis pindicola* and visitation rate = number of visitors per minute; * = dominant species, # = still not flowering during the observation in 1996, M = male.

plant species	*Bombus lapidarius*	*Bombus terrestris*	*Bombus pratorum*	*Bombus pyrenaeus*	*Apis* sp.	*Syrphidae*	*Empididae*	*Thricops* spp.
site 1								
Onobrychis pindicola				**0.046**				
Rhodax canum *				0.015				
Cerastium alpinum *								
Thymus pyrinica								
Helianthemum nummularium								
Campanula cochlearifolia								
site 2								
Onobrychis pindicola*		**0.010** **0.083**^{1995}		**0.082** **0.133**^{1995}				
*Oxytropis campestris**	0.173 0.160^{1995}			0.027 -^{1995}				
*Cerastium alpinum**								
Gentiana verna								
Thymus pyrinica				0.029				
Genista depressa								
Acynos alpinus			0.013					
Rhodax canum								
Armeria alpina								
Antennaria dioica								
Alyssum cuneifolium								
Aster alpinus								
Achillea ageratifolia								
site 3								
Onobrychis pindicola*	**0.150** **-**^{1995}	**0.050** **-**^{1995}		**0.400** **0.230**^{1995}				
Gentiana verna			0.050					
Anthyllis vulneraria	0.170							
Helianthemum nummularium								
Linum capitatum								

Thymus pyrinica					0.010				
Acynos alpinus									
Teucrium montanum^{1995}		0.021^{1995}		0.064^{1995}					
Scabiosa gr. collina^{1995}					0.064^{1995M}				
Dianthus cruentus^{1995}									
Campanula velebitica $^{1995\#}$								0.400^{1995}	0.100^{1995}
Campanula cochlearifolia$^{1995\#}$								0.100^{1995}	
	site 4								
Onobrychis pindicola*		**0.055**	**0.009**	**0.036**	**0.109**	**0.045**	**0.009**		
Linum capitatum									
Scorzonera rosea									
Polygala major									
Helianthemum nummularium									
Rhodax canum									
	site 5								
Onobrychis pindicola*		**0.089**	**0.022**	**0.067**	**0.222**				
Carduus sp.		0.183							
Centaurea stoebe		0.100							
Clinopodium vulgare									
Dianthus petreus									
Galium gr. Mollugo									
Acynos alpinus		0.033							
Trifolium medium									
Rhinanthus javorkae				0.005					

Table 3. Total pollinator activity and approximate number of plants in each site.

patches of *Onobrychis pindicola*	patch size	approximate number of plants	*Bombus* spp.	*Syrphidae*
site 1	50 m^2	30 plants		
1995				
1996			0.046	
Site 2	70 m^2	300 plants		
1995			0.216	
1996			0.092	
site 3	10000 m^2	50000 plants		
1995			0.230	
1996			0.600	
site 4	30 m^2	120 plants		
1995				
1996			0.254 (incl. *Apis*)	0.009
site 5	200 m^2	1100 plants		
1995				
1996			0.400	

Few individuals visited both *O. campestris* and *Onobrychis pindicola* on the same foraging trip. The pollen analysis revealed very high constancy for *Oxytropis campestris*, and only one of the analyzed pollen loads contained sporadic pollen grains of *Onobrychis pindicola* (Tables 4, 5). Sporadic visits of workers *Bombus pyrenaeus* were observed. They collected nectar and had baskets full of *Oxytropis campestris* pollen (Tables 4, 5).

Discussion

Floral mechanism and breeding systems

The main disadvantage of inbreeding versus outcrossing is a reduction of the genetic variability. One of its negative consequences is the reduced possibility of survival and of successful adaptation to the environmental changes. Plants developed plenty of mechanisms to avoid the inbreeding during the process of evolution (Faegri & van der Pijl 1971, Richards 1990, Handel 1983). Self-incompatibility is also a mechanism to avoid inbreeding (Richards 1990, Handel 1983). On the other hand it has some restrictions: e.g. in self-incompatible plants strongly vegetative-propagated and then existing as clone-populations. According to the optimal foraging theory the pollinator collects its food in the most energetically effective way, visiting flowers and inflorescences in the close neighbourhood (Levin 1978, 1979, Waddington 1983, Richards 1990).

The wide distributed and cultivated *Onobrychis viciifolia* has self-compatible flowers and its automatic self-pollination is mechanically largely prevented (Free 1970).

Table 4. Bumblebee workers and their pollen loads.

Bombus spp.	bumblebee workers with full pollen baskets [%]	number of analysed pollen loads	number of pollen types per pollen load (incl. *O. pindicola*)
Onobrychis pindicola			
B. pyrenaeus	75.5 %	23	mean = 2.57 min = 1 max = 7
B. lapidarius	77.7 %	6	mean = 2.25 min = 1 max = 4
B. terrestris	100.0 %	4	mean = 2 min = 1 max = 4
B. pratorum	50.0%	3	mean = 2.33 min = 1 max = 4
Oxytropis campestris			number of pollen loads with *Onobrychis* pollen
B. pyrenaeus	75.5 %	2	
B. lapidarius	50.0 %	7	1

The flowers of *O. pindicola* subsp. *urumovii* are self-incompatible and do not self-pollinate. In this case, the hypothesis according to which species with restricted ranges and few individuals are more likely to be self-compatible is reversed.

Self-incompatibility may cause high level of inbreeding depression. Such phenomenon has been observed in *Filipendula rubra* (*Rosaceae*) in which the seed set decreases subject to the size of clones, as the chance of self-pollination is higher due to specifics of pollinator behaviour (Aspinwall & Cristian 1992). *Onobrychis pindicola* forms dense tufts with plenty of inflorescences and the pollinators tend to visit successively many of their flowers so that inbreeding depression could be expected. The results of the open pollination fruit set test are not sufficient to reveal inbreeding depression. Further experiments are necessary.

Plant-pollinator interface

Onobrychis viciifolia represents the simplest type of legume floral mechanism. The upper edge of the keel is open, so that when a visitor forces its way into the flower the wing and the keel are pressed down, uncovering the relatively rigid stamens and style which come into contact with the under side of the body of the insect. As the insect leaves the flower the wings and keel spring back into the place again, once more covering the stamens and the stigma (Proctor & al. 1996).

		Onobrychis	*Oxytropis*	*Trifolium*	*Thymus*	*Chamaecytisus*	*Lamiaceae*	*Achillea/Aster*	*Cirsium/Carduus*	*Centaurea*	*Campanula/Jasione*	*Hypericum*	*Scabiosa*	*Dianthus*	*Cerastium*	*Verbascum*	*Veronica*	*Rhynanthus*	*Potentilla*	*Helianthemum*	*Geranium*	*Pinus*	*Poaceae*
Onobrychis																							
B. pyrenaeus	mean [%]	91,5	1,2	0,4		46,1	0,6	0,3	0,1	0,2	0,1	4,8	10,2	0,2				0,7	0,7	27,3	0,2	0,3	0,1
	min [%]	27,5	1,2	0,1		46,1	0,2	0,1	0,1	0,2	0,1	4,8	0,1	0,1				0,7	0,7	0,1	0,1	0,1	0,1
	max [%]	100,0	1,2	0,6		46,1	1,4	0,4	0,1	0,2	0,1	4,8	49,9	0,3				0,7	0,7	72,3	0,2	0,5	0,1
	No of loads	23	1	2		1	6	3	2	1	1	1	5	2				1	1	3	2	3	1
B. terrestris	mean [%]	87,7					0,2			0,6				0,1						38,3			0,1
	min [%]	51,1					0,2			0,6				0,1						38,3			0,1
	max [%]	100,0					0,2			0,6				0,1						38,3			0,1
	No of loads	4					1			1				1						1			1
B. lapidarius	mean [%]	97,6		7,4						3,3								1,9		0,2			
	min [%]	86,0		7,4						3,3								0,4		0,1			
	max [%]	100,0		7,4						3,3								3,3		0,2			
	No of loads	6		1						1								2		2			
B. pratorum	mean [%]	98,7				49,7								2,6					0,1	0,5			
	min [%]	97,4				1,9								2,6					0,1	0,5			
	max [%]	100,0				97,4								2,6					0,1	0,5			
	No of loads	2				2								1					1	1			
Oxytropis																							
B. lapidarius	mean [%]	0,2	98,9					0,2	0,6						0,1	0,1			3,8			0,1	
	min [%]	0,2	95,3					0,2	0,6						0,1	0,1			3,8			0,1	
	max [%]	0,2	100,0					0,2	0,6						0,1	0,1			3,8			0,2	
	No of loads	1	7					1	1						1	1			1			3	
B. pyrenaeus	mean [%]		98,7	1,4	0,7						0,1				0,1		0,4						
	min [%]		97,4	1,4	0,7						0,1				0,1		0,4						
	max [%]		99,9	1,4	0,7						0,1				0,1		0,4						
	No of loads		2	1	1						1				1		1						

Table 5. Composition of the pollen loads of the bumblebee workers, pollinators of *Onobrychis pindicola* subsp. *urumovii* and *Oxytropis urumovii*.

Pollen vectors of *O. viciifolia* are mainly honeybees (Free 1970). In Britain an oligolictic bee, *Mellita* sp. (*Melittidae*) is specialized on *O. viciifolia* (Proctor & al. 1996). In Alberta (Canada) 6 species of bees are pollinators of *O. viciifolia;* among them, *Megachile rotundata* has been observed 56 % of the times, honeybees 40 % and bumblebees 4 % (Richards & Edwards 1988).

Three species of peaflower flowering in early summer on English chalk grassland were all pollinated by bumblebees. Each one was pollinated by different bumblebee species (*Onobrychis viciifolia* by *Bombus lapidarius, Anthyllis vulneraria* by *B. hortorum,* and *Hippocrepis comosa* by *Apis mellifera* and *Bombus lapidarius)* and therefore they may have avoided direct competition (Proctor & al. 1996).

Onobrychis pindicola subsp. *urumovii* is obligately dependent upon bumblebees for pollen transport. In the sites 3 and 4 *Anthyllis vulneraria* and *Hippocrepis comosa* are also members of the plant community. However in this case direct competition is avoided by phenologycal differences between these species. *Hippocrepis comosa* is totally wilted when *Onobrychis pindicola* is blooming, while the flowering of *Anthyllis vulneraria* is close to its end. In addition *Onobrychis pindicola* is mainly pollinated by *Bombus pyrenaeus*, while *Anthyllis vulneraria* is visited by *Bombus lapidarius.* Direct competition with other peaflower, such as *Oxytropis campestris* in the site 2, is reduced by sharing of different bumblebee species.

Very important for pollinator activity and hence pollination success, and seed production is the size of the plant population and the flower density as well. The bigger the size is, the higher is the pollinator activity (Heinrich 1979b, Levin 1978, Handel 1983, Pleasants 1980, Sih & Baltus 1987, Kwak 1987, Richards & Edwards 1988, Petanidou & al. 1995). The biggest groups of *Onobrychis pindicola* with more flowers were visited by more bumblebees per time unit. Thus the effective pollination rate (the number of pollinators per time unit per available flowers) was more or less similar in the different sites. The bumblebee number increased more or less parallelly to the size of a patch (Table 3).

Bumblebees are polylectic, but they express preferences to one or several plant species in the community - their "majors" (Free 1970, Macior 1974, Bauer 1983, Heinrich 1976a, b, 1979a, b). Their choice depends on many factors, including their tongue length (Pouvreau 1984, Brian 1951, 1957, Teras 1976, 1985). The analysis of pollen loads together with flower characteristics of a species may allow some inferences about pollinator sharing (Arroyo & Dafni 1993). According to the results of the pollen analysis, *Onobrychis pindicola* was the only "major" for all bumblebee species collected on its flowers, except for one *B. pyrenaeus* worker, "majoring" on *Chamaecytisus* sp. (*Chamaecytisus* type pollen) too. Another *B. pyrenaeus* worker had been "majoring" on *Helianthemum* sp. "Minors" were species belonging to family *Lamiaceae*, *Scabiosa* sp., etc. These plants often were not in close neighbourhood. Pollen of *Onobrychis pindicola* and *Oxytropis campestris*, two species with similar flower morphology, but different in colour and inflorescence structure, practically was not mixed in the loads (Table 4).

The recorded high pollinator activity indicates that the possible reason for the restricted number of *O. pindicola* subsp. *urumovii* populations is not connected to pollinator limitation. Other reproductive specifics such as seed germination and surviving of the seedlings are necessary to be investigated in the future.

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