INTRAPOPULATIONAL VARIABILITY OF ENDANGERED SPECIES *Centaurea pontica* Prodan & Nyar USING ISOZYMES ELECTROPHORETIC SPECTRA

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Abstract. *Centaurea pontica* Prodan & Nyar is an endangered, endemic species, growing in the South – East of Romania, in the area of Sulina town, on ruderal sands. This taxon belongs to Asteraceae family, being a biannual species. For our study, unlignified young shoots with flower buds were collected. The aim was the evaluation of intrapopulational variability using the analysis of electrophoretic spectra of isozymes, as genetic diversity markers. The isozymes spectra for peroxidases, catalases and alkaline phosphatases detected intrapopulational variability; the analysed individuals from this population showed polymorphism concerning the electrophoretic spectra. These enzymes are suitable as markers for assessing the variability of this species.

Keywords: *Centaurea pontica*, intrapopulational variability, isozymes, electrophoretic spectra.

Rezumat. Variabilitatea intrapopulațională a speciei periclitate *Centaurea pontica* Prodan & Nyar evidențiată prin analiza spectrului enzimatic. *Centaurea pontica* este o specie endemică, periclitată răspândită în sud-estul României, în zona orașului Sulina, pe nisipuri ruderalizate. Taxonul face parte din familia Asteraceea, fiind o specie bianuală. Pentru studiul nostru s-au recoltat lăstari tineri nelignificați cu muguri florali. Scopul studiului a fost evaluarea variabilității intrapopulaționale a speciei pe baza analizei electroforetice ale unor izoenzime ca markeri ai diversității genetice. Spectrele peroxidazei, catalazei și fosfatazei alcaline au evidențiat variabilitate intrapopulațională, indivizii analizați prezentând polimorfism în ceea ce privește spectrele electroforetice. Aceste enzime pot fi utilizate ca markeri pentru evaluarea variabilității acestei specii.

Cuvinte cheie: *Centaurea pontica*, variabilitate intrapopulațională, izoenzime, spectre electroforetice.

INTRODUCTION

Centaurea pontica, named knapweed, is an endemic and endangered species, which grows on sands and is exclusively present in the South –East of Romania, Sulina town and the sands from its surroundings and in the south of Sfântu Gheorghe, in sunny areas. This biannual plant from Asteraceae family grows on marine sandy areas (sometimes nude) or on clay and sandy soil. The species presents a thickened root, branched to the base; the leaves have coil trichomes on both parts (DIHORU & NEGREAN, 2009). The plant is about 30-40 cm high. It is easy to recognise it by the thorns which surround the flower.

The name of the genus comes from the corn flower, valuable medicinal plant, which is believed to have been shown to man by Chiron Centaur and the name of species comes from Pontos, which means sea, from the Black Sea (http://dev.adworks.ro/natura/specii/157/Pesma-Deltei-Dunării-vinetele-dioc-zglavoc.html).

The species is protected by law, being included in different red lists and books (OPREA, 2005; DIHORU & NEGREAN, 2009). There is necessary a strategy to protect better *in situ* this taxon. For *ex situ* conservation, several measures have to be taken, such as: the cultivation in botanical gardens, harvesting the seeds for the maintenance in gene banks, biotechnological approaches and the repopulation of the natural habitat with preserved and multiplied plant material.

In our study concerning *C. pontica*, we tested the most affordable biochemical markers, represented by isozymes to characterize several individuals from a small population from Sulina area.

We analysed peroxidases, isoesterases, alkaline phosphatases and catalases spectra, which are used as biochemical markers for intrapopulational diversity (VOICHIȚĂ et al., 2013).

Some isozymes are described as biochemical markers for the characterization of the intrapopulational variability such as peroxidases and esterases (HALMAGY & BUTIUC-KEUL, 2007).

Isozymes were the first employed molecular markers from 1950 (HAMRICK & GODT, 1989). They are multiple molecular forms of an enzyme, which has electrophoretic mobility and the same substrate. Sometime, the terms allozyme and isozyme are similar but, allozyme are enzyme forms and are products of different alleles and not the product of different genes with similar enzymatic activity (VALGIMIGLI, 2004-2005). Allozymes have been extensively used as genetic markers for: identifying cultivars, species and hybrids, measuring genetic diversity of plant populations, etc. (JASIENIUK & MAXWELL, 2001). The main advantage of isozymes is the simplicity of the method and the low cost, individuals can be scored for several allozymes in the same time, allozymes are codominant and, in this respect, heterozygotes can be distinguished from homozygotes. The disadvantages are the reduced number of loci from genome corresponding to alloenzymes, the necessity of use of fresh biological material and sometimes a weak variation of pattern (BUTIUC-KEUL, 2006).

These isozymes are useful as biochemical markers in forestry species (ŞOFLETEA et al., 2009). The domains of using isozymes in genetic forestry and breeding are: evaluation of intra and interpopulational genetic diversity, the identification of linkage isoenzymatic loci for genetic maps establishment, the obtaining of valuable populations for conservation and the use in the breeding programs.

Figure 1. Detail of *C. pontica* with flowers (original).

Figure 2. Ruderal area with *C. pontica* located at the periphery of Sulina town (original).

Figure 3. Population of *C. pontica* (original).

MATERIALS AND METHODS

1. Plant material

Plant material was harvested from a population close to Sulina town. Young shoots with leaves and flowers buds were collected from 10 individuals (1-10) located at a distance of at least 5 m one from each other.

The fragments of leaves were grounded with quartz sand for obtaining the homogenate of the total protein extract. The extraction of enzymes was carried out in 0.05M phosphate buffer pH 7, 2mM EDTA, PVP 4%, at 4°C for 4 h. After centrifugation at 18000 rpm, for 20 min, the supernatant was used for electrophoresis. It was prepared the running polyacrylamide gel 7% (for catalases) and 10 % (for esterases, phosphatases and peroxidases) and stacking gel; as buffer we used 0.05M Tris-Gly, pH 8.3. The running marker was bromophenol blue.

2. Evidentiation of electrophoretic bands

a) For peroxidases detection, a solution of benzidine in acetate buffer and H_2O_2 as substrate was used. The bands were stained in brown (WANG & WANG, 1989).

b) For esterases detection, the substrate alpha, beta naphthyl acetate and Fast Blue RR dissolved in phosphate buffer 0.1 M, pH=6.5 was used. The bands were stained in red-violet (BACH, 1989 modified method).

c) For catalases, as substrate 0.003% H_2O_2 prepared in 0.01 M phosphate buffer, pH=7, 2% $K_3(Fe(CN)_6)$ and 2 % $FeCl_3$ was used. The bands were stained in yellow with a green-blue background (IORDĂCHESCU & DUMITRU, 1988).

d) For phosphatases alkaline, as substrate Na alpha, beta-naphthyl phosphate, 0.5M $MgCl_2$ and 0.25 M $MnCl_2$ and Fast Blue RR in Tris-citrate, pH=8.3 was used. The bands were stained in brown.

RESULTS AND DISSCUSIONS

The analyses of electrophoretic **peroxidases spectra** showed the presence of 5 common isozymes bands in the case of individuals 1, 2, 3, 5, 7, 9, 10. In 4 and 8 individuals, a supplementary band was observed. In the case of individual 6, the presence of 4 isoperoxidases electrophoretic bands was detected. The electrophoretic spectrum of peroxidases revealed differences among individuals.

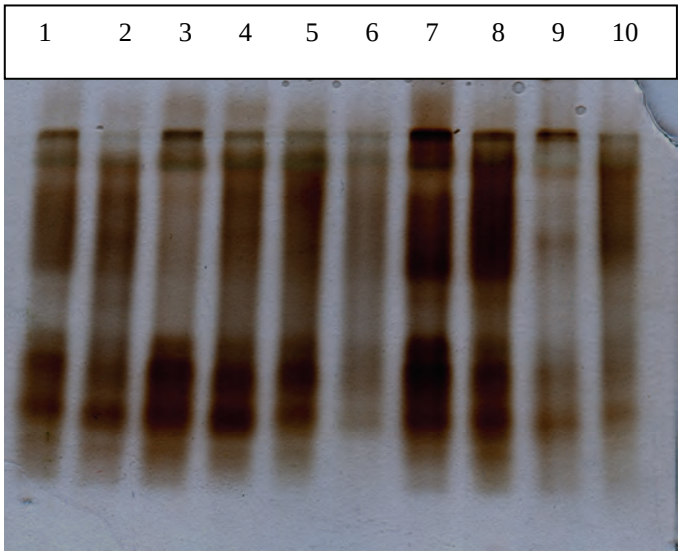

Figure 4. The peroxidases electrophoretic spectrum in 10 individuals of *C. pontica* (original).

The **phosphatases alkaline** spectrum showed that 1 and 3 individuals did not express electrophoretic bands. In the case of individuals 2, 4, 5, 6, 7, a varied number of electrophoretic bands (between 1 and 4) were detected. This result proved the existence of an intrapopulational variability concerning this biochemical marker.

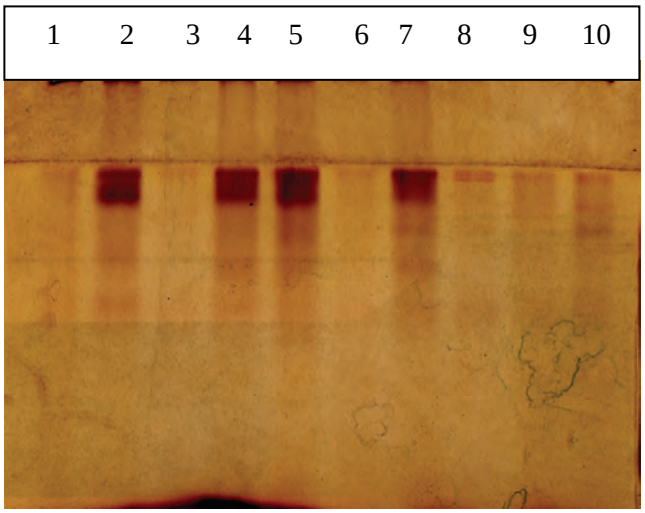

Figure 5. The alkaline phosphatases electrophoretic spectrum in 10 individuals of *C.pontica* (original).

The electrophoretic spectrum of **esterases** was identical all tested individuals, showing only two bands. This biochemical marker did not reveal any variability.

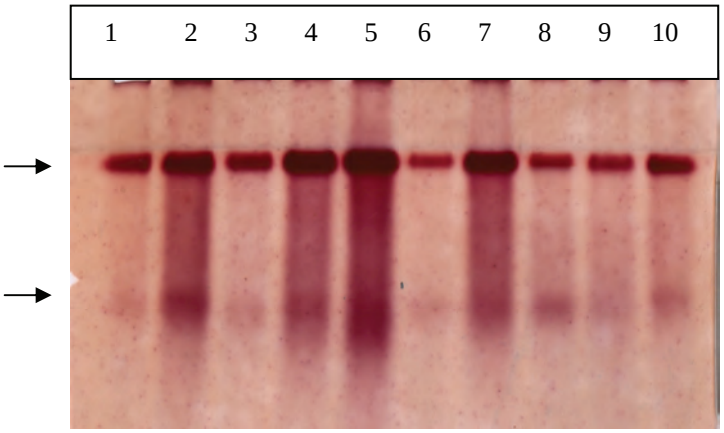

Figure 6. The esterase electrophoretic spectrum in 10 individuals of *C. pontica* (original).

BUTIUC- KEUL & DELIU (2001) also used esterase isozymes spectrum to analyse the individuals from different *in vitro* clones of *Arnica montana*. In this case, also the pattern of several individuals from different clones was the same.

The electrophoretic spectrum of **catalases** was different for each individual, proving a higher polymorphism concerning this biochemical marker. A common catalase band was observed in 1, 2 ,5, 7, 9 individuals. The 3, 6, 9 and 10 individuals showed different numbers of electrophoretic bands.

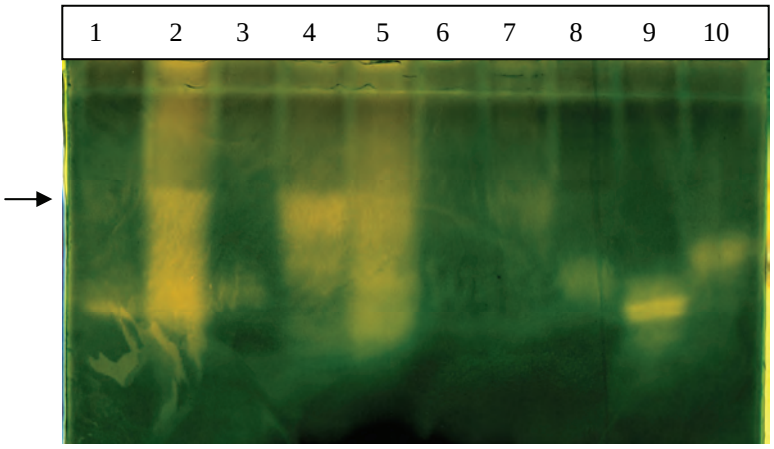

Figure 7. The catalases electrophoretic spectrum in 10 individuals of *C. pontica* (original).

Our results show that, in the case of *C. pontica* from Sulina, despite the fact that there is a small population with relative low number of individuals, there is genetic variation taking into account the alkaline phosphatases, peroxidases and catalases spectra.

There are studies regarding the intrapopulational genetic polymorphism using biochemical markers as izoymes (KRZAKOVA & CELKA, 2007). The authors investigated the intrapopulational variation of *Calamagrostis arundinaceae* (L) Roth (small reeds or Reed Grass) by electrophoretic spectra of 10 isozymes (aconitase, alcohol dehydrogenase, diaphorase, formic acid dehydrogenase, glutamate dehydrogenase, glutamate oxaloacetate transaminase, malate dehydrogenase, phosphoglucomutase, shikimate dehydrogenase, peroxidases) and found individual differences in respect of 16 loci for 30 individuals analysed. The results indicated that the population is polymorphic in respect with all enzyme system.

Research on the population genetics of *Arabidopsis thaliana* from Poland has concerned variability detected by enzymatic electrophoresis. The materials of this study were samples from each of the 18 populations (20 individuals per population). The authors report a polymorphism of *A. thaliana* populations with regard to 8 enzyme systems: esterase, acid phosphatase, leucine aminopeptidase, glutamate-oxaloacetate-transaminase, catalase, malate dehydrogenase, glutamate dehydrogenase, isocitrate dehydrogenase. Considerable differences between the enzyme systems were detected for the levels of polymorphism. The most variable enzyme system was esterase. There was low level of variation for glutamate dehydrogenase; the relatively high level of polymorphism was observed for the leucine aminopeptidase system (FUGLEWICZ & KILIAN, 1985).

Other studies regarding the intrapopulational variability in *Dianthus giganteus* used isoesterases and isoperoxidases. Unfortunately, the patterns of peroxidases and esterases were identical in all individuals (10) and were not suitable to characterize the variation in this taxon. The use of DNA markers as RAPD was more relevant (HALMAGY & BUTIUC-KEUL, 2007).

CONCLUSIONS

The biochemical markers for assessing the intrapopulational variability represent a useful tool for the characterization of the polymorphism of individuals from a population of *C. pontica*, an endemic and endangered species from the Danube Delta.

These analyses represent a novelty for this endemic species concerning variation among individuals.

The electrophoretic spectra of four enzymes were analysed; two of them, esterases and peroxidases, are known in the literature as biochemical markers for intrapopulational diversity, and the other two, catalases and phosphatases showed a polymorphism of individuals for this species.

Our results showed that not all enzymes are relevant for the characterization of the genetic diversity. Esterase electrophoretic pattern did not reveal any variation among the individuals while using alkaline phosphatases, isoperoxidase and catalases spectra detected high intrapopulational variability.

Being affordable, the biochemical markers besides other methods can help to the evaluation of the natural population genetic variability in the case of endangered, endemic taxa, especially with reduced area and low number of individuals and to establish of an appropriate strategy of conservation.

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