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Isozyme Analysis of Seedling Samples in some species of *Hyoscyamus* from Iran

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Abstract: In this research seedling samples, taken from seed germination, have been studied. Polyacrylamide gel electrophoresis of some enzyme systems have been done. The resulted bands were analyzed by computer. These analyses have been performed by NTSYS-pc software, using UPGMA (Unweighted Paired Group Method Arithmetic average) method and on the basis of Jaccard correlation coefficient. Results show that in the first step the species *H. pusillus* and in the second step *H. insanus* are separated from the other species. The species *H. arachnoideus* and *H. niger* are very heterogenous. We observe that species relationships depend on the locality of plant samples. Therefore, the study of interspecific relationships depends on the study of intraspecific variation.

Key words: *Solanaceae*, *hyoscyamus*, isozyme, electrophoresis, dendrogram

INTRODUCTION

The *Solanaceae* family consists of some plants such as *Atropa*, *Datura*, *Duboisia*, *Scopolia* and *Hyoscyamus* that are important from medicinal point of view. This importance is due to their tropane alkaloid content. Hyoscyamine and its derivatives are the major tropane alkaloids found in the *Solanaceae* family (Evans, 1979).

As mentioned above *Hyoscyamus* is one of these important genera which contains hyoscyamine, scopolamine and to a lesser extent atropine.

According to Khatamsaz (1998), there are 13 species and two varieties of this genus in Iran. Therefore, Iran with three endemic species (*H. malekianus*, *H. tenuicaulis* and *H. bornmulleri*) is one of the most important diversification centers of this genus.

Because of the importance of this genus, actually, numerous studies from different aspects have been done and reported on it. The genus has been studied in regard to anatomy and morphology (Ganj-Karimy, 1997). Scanning Electron Microscopy (SEM) of pollen grains (Khatamsaz and Zangirian, 1998) has also been described. Karyological studies have also been made on 11 species of this genus collected from Iran and have shown two different basic chromosome number, that is x = 14 and x = 17 (Sheidai *et al*., 1999). Interrelationship of these species have been studied through seed storage proteins and numerical taxonomy (Sheidai *et al*., 2000).

Isozymes are practical and useful genetic and biochemical markers as well as good estimators of genetic variability in plant populations (Hamrick and Godt, 1997). Electrophoretic technology facilitated the recognition that enzymes appear in multiple forms (Buth and Murphy, 1999). However, currently the use of molecular techniques (isoenzymes, PCR) has become widespread in the study of biochemical characters (Crawford, 1990; Weising *et al*., 1995). Today isozymes have been used to study genetic variation between and within different species of plants like *Oryza* spp. (Second, 1982), *Oryza sativa* L. (Glaszmann, 1987), Portuguese Tronchuda cabbage and Galega kale (Dias *et al*., 1994), *Tylosema esculentum* (Monaghan, 1995), *Stylosanthes* (Gillies and Abbott, 1999), *Pistacia* L. (Fasihi Harandi *et al*., 2000), *Lathyrus sativus* L. (Tadesse and Bekele, 2001) and *Avena* (Benchacho *et al*., 2002).

In this study, we have tried to describe six isoenzyme systems of seedling samples in some species of *Hyoscyamus*. The objective is to survey the usefulness of such methods to separate taxa and grouping within this genus.

MATERIALS AND METHODS

Plant materials: Different samples of *Hyoscyamus* species used in this study are shown in Table 1. Voucher specimens are deposited either in the central herbarium of Iran (TARI) or Tehran University Herbarium (TUH). Seed samples were planted in the soil separately. Germination started in about a week.

Enzyme extraction: The enzymes were extracted from the seedlings. Crude extracts were prepared by macerating 1g of seedlings in 1.5 mL of extraction buffer (Tris-Glycine, 0.05 M, pH:8.3). The extract was centrifuged at 19000 rpm

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Table 1: Plant materials

No.	Species	Seed source	Date of collection	Distribution	Code
1	*Hyoscyamus arachnoideus* 1	Tehran, Rineh	Aug.1996	Iran, Iraq	14
2	*Hyoscyamus arachnoideus* 2	Tabriz-mishodagh road	Jun.1999	Iran, Iraq	10
3	*Hyoscyamus arachnoideus* 3	Tabriz	Jun.1999	Iran, Iraq	9
4	*Hyoscyamus arachnoideus* 4	Ardebil	Jun.1999	Iran, Iraq	8
5	*Hyoscyamus arachnoideus* 5	Zanjan	Jun.1999	Iran, Iraq	7
6	*Hyoscyamus arachnoideus* 6	Hamadan	Aug.1996	Iran, Iraq	6
7	*Hyoscyamus arachnoideus* 7	Ardebil, Namin	Jun.1999	Iran, Iraq	1
8	*Hyoscyamus niger* 1	Tehran, Kandovan	Jul.1999	Iran, Iraq, middle Asia, north Africa, Europe, Turkey, Russia, Caucasia, Siberia, Afghanistan, Pakistan	15
9	*Hyoscyamus niger* 2	Tehran, Rineh	Jul.1996	Iran. Iraq, middle Asia, north Africa, Europe, Turkey, Russia, Caucasia, Siberia, Afghanistan, Pakistan	12
10	*Hyoscyamus niger* 3	Tehran, Gachsar	Aug.1996	Iran, Iraq, middle Asia, north Africa, Europe, Turkey, Russia, Caucasia, Siberia, Afghanistan, Pakistan	2
11	*Hyoscyamus squarrosus*	Mazandaran	Aug.1996	Iran, Afghanistan, Pakistan, middle Asia	11
12	*Hyoscyamus reticulatus*	Marand-Jolfa road	Jul.1999	Iran, Iraq, Syria, Egypt, Turkey, Europe.	13
13	*Hyoscyamus pusillus*	Markazi, Arak	Aug.1996	Iran, Turkey, Caucasia, middle Asia, Siberia, Afghanistan, Pakistan, north Africa	4
14	*Hyoscyamus insanus*	Bushehr	Aug.1996	Iran, Afghanistan, Pakistan	3
15	*Hyoscyamus turcomanicus*	Golestan	Jun.1996	Iran, Turkmenistan	5

for 30 min at 4°C. The supernatant (enzyme extract) was filtered through cheese– cloth and stored at -20°C for electrophoresis.

Enzyme electrophoresis: Polyacrylamide gel electrophoresis (PAGE) was performed in a vertical, discontinuous system using 12.5% gel. Electrophoresis was done at 4°C and a constant current of 5 mA per gel during 24 h. For all the enzymes, 90 μL extract per well was loaded. Staining procedures followed Desborough and Peloquin (1967), Van Loon (1971) and Wendel and Weeden (1990). The enzyme systems analyzed were: esterase (EST, E.C.3.1.1.1.), peroxidase (PRX, E.C.1.11.1.7.), polyphenoloxidase (PPO, E.C. 1.10.3.2), superoxide dismutase (SOD, E.C.1.15.1.1.), malate dehydrogenase (MDH, E.C.1.1.1.37.) and malic enzyme (ME, E.C.1.1.1.40).

Data analysis: The presence (coded as 1)/absence (coded as 0) data matrix was prepared from the zymograms. The bands which are present in all taxa were removed. Relationships among these taxa were inferred with a UPGMA dendrogram from Jaccard's similarity matrix of the bands. Data were analysed using the SAHN (Sequential Agglomerative Hierarchical and Nested) option of NTSYS-pc (Version 1.60) system of Rohlf (1990).

This research was being done from June 2000 to July 2001 in Laboratory of Plant Physiology, Department of Botany, School of Biology, University College of Sciences, University of Tehran.

RESULTS

Fifty eight electrophoretic bands were detected from the six seedling isozyme systems analyzed.

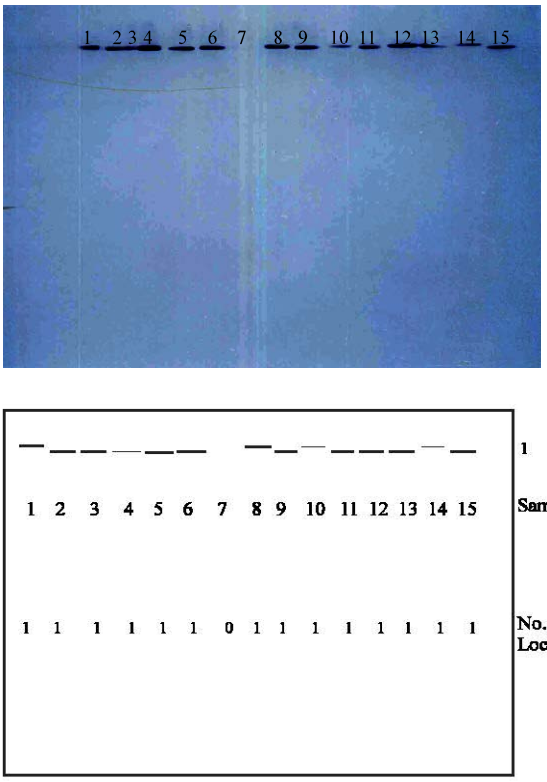

Fig. 1: Photograph and diagram showing isozyme banding pattern for ME

Isozyme malic enzyme (ME) exhibited single band on the gel for each taxon of *Hyoscyamus* which is a typical pattern of a monomeric enzyme. It is coded by one gene at locus ME-1 (Fig. 1). ME-1 has two types of alleles $ME\text{-}1^1$ and $ME\text{-}1^2$ corresponding to bands which differs in their relative mobility. Therefore a total of two bands with malic enzyme were observed.

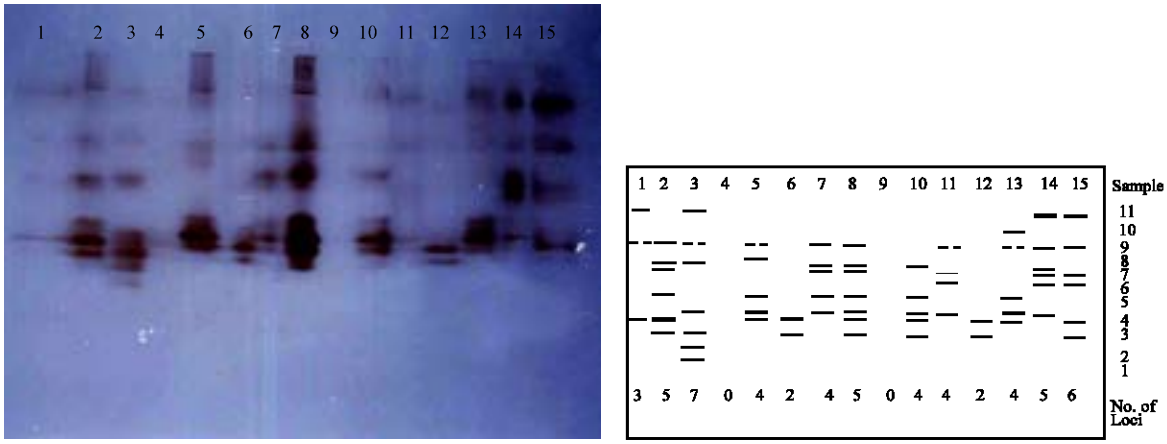

Fig. 2: Photograph and diagram showing isozyme banding pattern for PRX

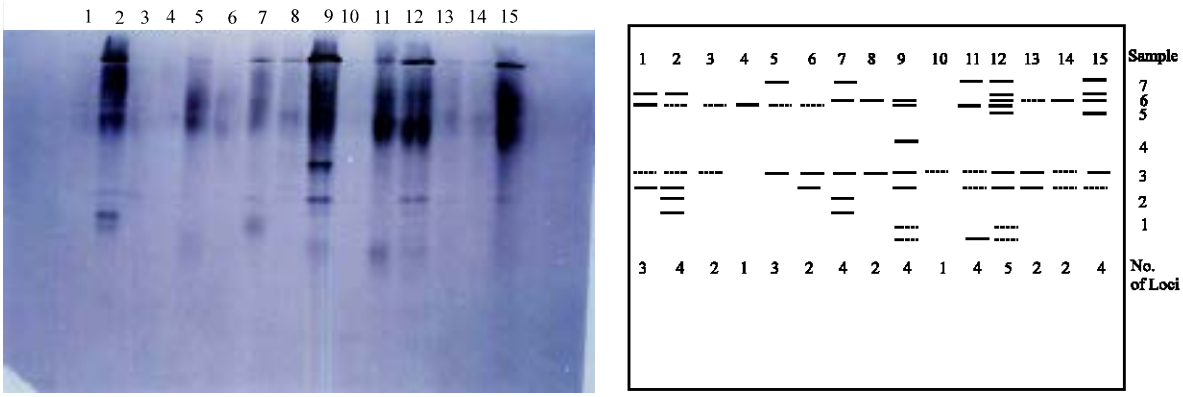

Fig. 3: Photograph and diagram showing isozyme banding pattern for EST

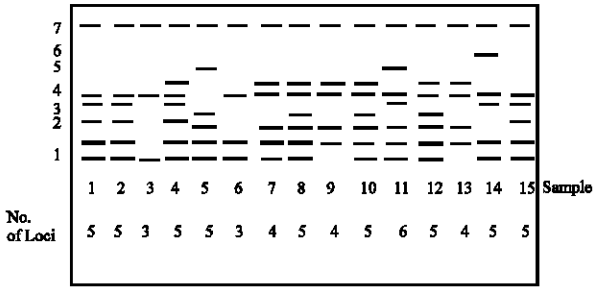

Fig. 4: Schematic illustration of zymogram for PPO

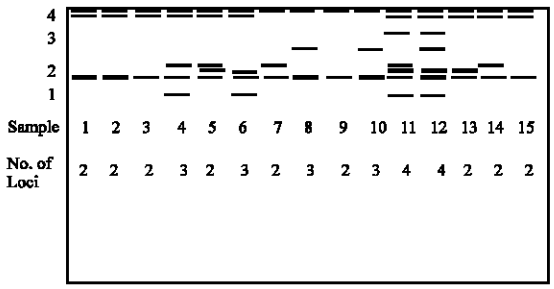

Fig. 5: Schematic illustration of zymogram for MDH

Isozyme peroxidase (PRX) exhibited the most complicated zymogram as it appeared to be controlled by seven loci in *H. insanus* (sample 3). Many of loci are represented by one allele except for locus four and locus seven exhibiting two alleles per locus. All the alleles do not express simultaneously in one sample. Locus one and locus two only expressed in *H. insanus* (sample 3). Locus 8 is restricted to *H. turcomanicus* (sample 5) and locus 10 only appeared in *H. reticulatus* (sample 13). A total of 13 bands that expressed by 11 loci were observed for this isozyme system and diagrammatically represented in Fig.2.

Isozyme esterase (EST) appeared as a monomeric enzyme coded by seven loci. The banding pattern for most of the samples was different and it was possible to

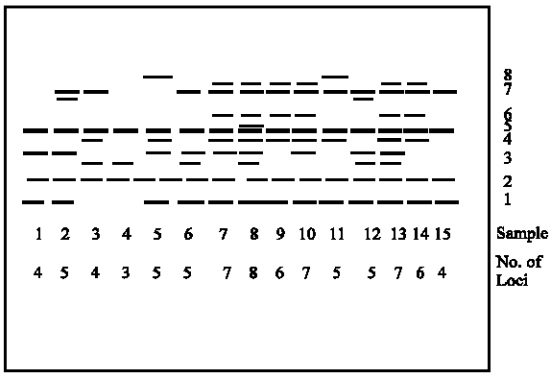

Fig. 6: Schematic illustration of zymogram for SOD

differentiate them from each other (Fig. 3). Locus four only exists in *H. arachnoideus* 3 (sample 9).

Isozyme polyphenoloxidase (PPO) exhibited 11 bands on the gel which appeared to be coded by six genes at loci PPO-1 to PPO-6 (Fig. 4). The loci detected in the present study clearly exhibited monomeric pattern.

Isozyme malate dehydrogenase (MDH) showed eight bands coded by four genes at loci one to four. In this study it appeared as a mixture of monomeric and dimeric activity (Fig. 5).

Isozyme superoxide dismutase (SOD) appeared as 12 bands expressed by eight loci (Fig. 6). It seems that it is a mixture of monomer and dimer. Locus one and locus two are monomorphic.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1.000														
0.560	1.000													
0.280	0.345	1.000												
0.333	0.296	0.273	1.000											
0.321	0.333	0.276	0.222	1.000										
0.476	0.520	0.409	0.421	0.333	1.000									
0.226	0.406	0.267	0.172	0.387	0.233	1.000								
0.281	0.371	0.322	0.156	0.353	0.333	0.621	1.000							
0.222	0.250	0.222	0.208	0.226	0.333	0.393	0.355	1.000						
0.333	0.387	0.286	0.185	0.322	0.346	0.555	0.731	0.370	1.000					
0.322	0.371	0.322	0.321	0.533	0.379	0.382	0.282	0.355	0.250	1.000				
0.375	0.457	0.257	0.333	0.324	0.536	0.282	0.359	0.406	0.371	0.432	1.000			
0.310	0.364	0.310	0.214	0.433	0.423	0.517	0.567	0.500	0.500	0.382	0.428	1.000		
0.407	0.364	0.357	0.214	0.265	0.276	0.517	0.469	0.345	0.400	0.469	0.219	0.419	1.000	
0.542	0.571	0.321	0.269	0.273	0.440	0.303	0.314	0.267	0.281	0.437	0.485	0.344	0.483	1.000

Fig. 7: Similarity matrix calculated using 'Jaccard' correlation coefficient

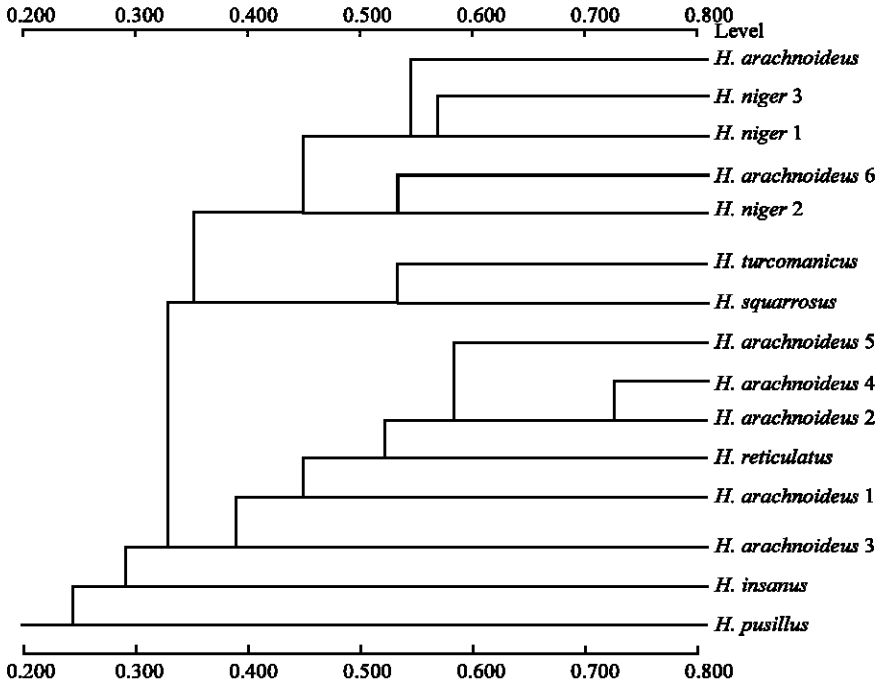

Fig. 8: Dendrogram based on UPGMA analysis of genetic similarity estimates (Jaccard's similarity coefficient)

Table 2: Presence/absence data matrix of the isozyme study

Band	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
EST															
1	0	0	0	0	1	0	1	0	0	0	1	1	0	0	1
2	1	1	0	0	0	0	0	0	0	0	0	1	0	0	1
3	0	0	0	0	0	0	1	1	1	0	0	1	1	1	1
4	1	1	1	1	1	1	0	0	1	0	1	1	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1
6	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
7	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1
8	1	1	0	0	0	1	0	0	1	0	1	1	1	1	1
9	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0
10	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0
12	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0
ME															
1	1	0	0	0	0	0	0	1	0	1	0	0	0	1	0
2	0	1	1	1	1	1	0	0	1	0	1	1	1	0	1
MDH															
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
2	1	1	1	1	1	1	0	0	0	0	1	1	1	1	1
3	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0
4	0	0	0	0	0	0	0	1	0	1	0	1	0	0	0
5	0	0	0	1	1	0	1	0	0	0	1	0	0	1	0
6	0	0	0	0	1	0	0	0	0	0	1	1	1	0	0
7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
8	0	0	0	1	0	1	0	0	0	0	1	1	0	0	0
PRX															
1	1	0	1	0	0	0	0	0	0	0	0	0	0	1	1
2	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
3	1	1	1	0	1	0	1	1	0	0	1	0	1	1	1
4	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
5	0	1	1	0	0	0	1	1	0	1	0	0	0	1	0
6	0	1	0	0	0	0	1	1	0	0	1	0	0	1	1
7	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1
8	0	1	0	0	1	0	1	1	0	1	0	0	1	0	0
9	0	0	1	0	1	0	1	1	0	1	1	0	1	1	0
10	1	1	0	0	1	1	0	1	0	1	0	1	1	0	1
11	0	1	1	0	0	1	0	1	0	1	0	1	0	0	1
12	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
13	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
PPO															
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
2	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
3	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0
4	0	0	0	1	0	0	1	1	1	1	0	1	1	0	0
5	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1
6	1	1	0	1	0	0	0	0	0	0	1	0	0	1	1
7	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0
8	1	1	0	1	0	0	0	0	0	1	0	1	0	0	1
9	0	0	0	0	1	0	1	1	1	1	1	1	1	0	0
10	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1
11	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1
SOD															
1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0
2	0	0	0	0	0	0	1	1	1	1	0	0	1	1	0
3	0	1	1	0	0	1	1	1	1	1	1	1	1	1	1
4	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0
5	0	0	0	0	0	1	1	1	1	0	0	1	1	1	0
6	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
7	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
8	0	0	1	0	1	0	1	1	1	1	1	0	1	1	0
9	1	1	0	0	1	1	1	1	0	1	0	1	1	0	0
10	0	0	1	1	0	1	0	1	0	0	0	1	1	0	0
11	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
12	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1

The presence or absence of each band was treated as a binary character in data matrix (Table 2). Based on the data matrix, Jaccard's similarity index was calculated (Fig. 7). The lowest similarity value (0.156) was observed between *H. pusillus* and *H. arachnoideus* 4, while the highest value (0.731) was between *H. arachnoideus* 4 and *H. arachnoideus* 2.

Dendrogram was produced from the results of similarity matrix using the UPGMA method (Fig. 8). Two main branches are observed within different taxa of genus *Hyoscyamus*. One includes *H. pusillus*, which presents the lowest similarity with the rest taxa (about 0.25). The second node of the tree separates two clusters: The first one includes *H. insanus* while the second one divides into two parts, with *Hyoscyamus arachnoideus* 1, 2, 3, 4, 5 and *H. reticulatus* in the first branch. The second branch has two clusters, one of them consists of *H. squarrosus* and *H. turcomanicus* and the other includes *H. niger* 1, 2, 3 and *H. arachnoideus* 6,7.

DISCUSSION

Malic enzyme (ME) has been studied in many plants including *Pinus halepensis* L. (Loukas *et al*., 1983), *Citrullus* (Navot and Zamir 1987), *Vigna* (Vaillancourt and Weeden 1993; Sonnante *et al*., 1997; Pasquet and Vanderborght, 1999). In all of these studies it is reported that it is expressed by one locus and it displays one zone of activities on the gel.

Peroxidase has been studied in different plants such as *Nicotiana* (Bhatia *et al*., 1967), maize (Brewbaker *et al*., 1985), *Musa* (Jarret and Litz, 1986), *Citrullus* (Navot and Zamir, 1987), Watermelon (Navot *et al*., 1990), Potato (Nieto *et al*., 1990), Garlic (Pooler and Simon, 1993), *Allium* (Abdul Wahab, 1994), *Beta* (Reamon-Buttner *et al*., 1996), *pyrola* (Huh *et al*., 1998), cotton (Farooq *et al*., 1999). Various numbers of loci from one to thirteen has been reported for this enzyme. For example, one locus has been reported for genus *Citrullus* while thirteen loci have been reported for maize (Brewbaker *et al*., 1985).

Esterase has also been studied in different plants such as *Nicotiana* (Bhatia *et al*., 1967), *Solanum* (Desborough and Peloquin, 1967), *Musa* (Jarret and Litz, 1986), Watermelon (Navot *et al*., 1987), *Citrullus* (Navot and Zamir, 1987), *Allium* (Abdul Wahab, 1994), *Lythrum salicaria* (Strefeler *et al*., 1996), *Amaranthus* (Chan and Sun, 1997), *Pinus* (González-Andrés *et al*., 1999), *Vigna* (Pasquet and Vanderborght, 1999), Cotton (Farooq *et al*., 1999). It is reported to be either a monomer such as in *Solanum* (Desborough and Peloquin, 1967) or a dimer like cotton (Farooq and Sayyed, 1999). In some species like

Musa acuminata Colla, EST is useful as a diagnostic tool for cultivar identification in view of the extensive polymorphism for this enzyme (Jarret and Litz, 1986).

Polyphenoloxidase has also been studied in other plant systems to a lesser extent. *Allium* is one of the examples that have been reported for this enzyme (Abdul Wahab, 1994). Sixteen bands have been reported for *Allium* and most of the species have the same band pattern.

Malate dehydrogenase has been extensively studied in plants such as Peach (Arulsekar *et al*., 1986), *Musa acuminata* Colla (Jarret and Litz, 1986), *Citrullus* (Navot and Zamir, 1987), *Festuca* (Aiken *et al*., 1993), *Vigna* (Vaillancourt and Weeden, 1993), *Allium* (Abdul Wahab 1994), Beta (Reamon-Buttner *et al*., 1996), *Lythrum salicaria* (Strefeler *et al*., 1996), *Vigna luteola* and *Vigna marina* (Sonnante *et al*., 1997), *Amaranthus* (Chan and Sun, 1997), *Pyrola* (Huh *et al*., 1998), *Vigna* (Pasquet and Vanderborght, 1999). It has been reported to be a dimer in *Festuca* (Aiken *et al*., 1993), Corn (Yang *et al*., 1977) and Celery (Orton, 1983), *Musa acuminata* Colla (Jarret and Litz, 1986). In the study on *Lythrum salicaria* (Strefeler *et al*., 1996) eight bands were observed for MDH.

Superoxide dismutase has been studied in different plants such as *Citrullus* (Navot and Zamir, 1987), Watermelon (Navot *et al*., 1990), Beta (Reamon-Buttner *et al*., 1996), Cotton (Farooq *et al*., 1999), *Pinus* (González-Andrés *et al*., 1999) and *Vigna* (Pasquet and Vanderborght, 1999). In cotton it has been reported to be monomer (Farooq *et al*., 1999). In most of the other plant species like maize (Baum and Scandalios, 1981), barely (Jaaska and Jaaska, 1982), *Beta* (Reamon-Buttner *et al*., 1996), it has been reported to have dimeric or tetrameric structure.

Separation of the species *H. pusillus* is confirmed by numerical taxonomy (Based on Single- Linkage method) (Sheidai *et al*., 2000). On the other hand chromosome number of this species is 2n = 68 which differs from the others (Sheidai *et al*., 1999). Vegetative form of this species is restricted to annual form (Khatamsaz, 1998). In the second branch the species *H. insanus* separates from the other ones. According to morphological studies (Khatamsaz, 1998), three subgenera for this genus have been reported:

- Subgenus *Para hyoscyamus* includes *H. malekianus* and *H. leptocalyx*.
- Subgenus *Hyoscyamus* includes *H. reticulatus*, *H. kurdicus*, *H. turcomanicus*, *H. senecionis*, *H. arachnoideus*, *H.squarrosus*, *H.pusillus* and *H. niger*.
- Subgenus *Dendrotrichon* includes *H. insanus*, *H. tenuicaulis* and *H. bornmulleri*.

The members of subgenus *Dendrotrichon* have chromosome number that is 2n = 28 (Sheidai *et al*., 1999). The studies mentioned above as well as numerical taxonomy studies (Based on Single-Linkage method) (Sheidai *et al*., 2000) confirm the separation of this species from the others.

Among the other species *H. arachnoideus* is very heterogenous and we observe that seven samples of this species that have been collected from different localities don't have similar isozyme banding patterns. We have a similar manner for the species *H. niger* with three different samples. This heterogeneity shows the high variation of isozyme banding pattern within species, thus for the study of interspecific relationship, we need to survey intraspecific variation.

The close relationship between the species *H. turcomanicus* and *H. squarrosus* and also between *H. reticulatus* and *H. arachnoideus* is in accordance with numerical taxonomy studies (Based on UPGMA method) (Mosallanejad, 1996).

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