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Morphological and enzymatic variability and adaptation of zig-zag clover (*Trifolium medium* L.)

Zofia BULIŃSKA-RADOMSKA

Institute of Plant Breeding and Acclimatization, Radzików, Poland

Abstract. Thirty-four populations of *Trifolium medium* collected in central, northern and southern Poland were used in a morphological and enzymatic study. Twenty-two characters of leaf, stem and inflorescence were scored. The enzymatic survey included aspartate aminotransferase (AAT), esterase (EST), glucoso 6-phosphate isomerase (PGI), leucine aminopeptidase (LAP), 6-phosphogluconate dehydrogenase (PGD), peroxidase (PRX), and shikimate dehydrogenase (SKD) assayed in two buffer systems. The populations showed a high intra- and inter populational variability both on morphological and enzymatic levels. The data demonstrate that morphological and enzymatic variability is regionally dependent in the same manner. Morphological traits, such as pedicel length, peduncle length, terminal leaf length and width and stipule length were correlated with the majority of climatic variables. PGI, PRX and LAP phenotypes were correlated with a set of climatic variables of high altitude areas. AAT, EST and PGD phenotypes were good predictors of more balanced climatic conditions. The altitude factor had a significant effect on all phenotypes but AAT. This study also showed that the same climatic variables did not always play a significant role in the morphological and enzymatic differentiation of *T. medium*. In general, enzymes were much more responsive to the variety of climatic factors than morphological characters.

Key words: adaptation, climate, enzymatic variability, morphological variability, *Trifolium medium*.

Introduction

Zig-zag clover (*Trifolium medium* L.) is one of 22 *Trifolium* species occurring in Poland (SZAFER et al. 1969). It is a high polyploid with a chromosome number ranging from 2n = 48 to 126 (ZOHARY, HELLER 1984). It has a perennial habit of

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Correspondence: Z. BULIŃSKA-RADOMSKA, Institute of Plant Breeding and Acclimatization, Radzików, 05-870 Błonie, Poland.

growth and reproduces both sexually and vegetatively by underground stolons. It is broadly distributed in both New and Old Worlds. It occurs in forested and mountainous areas throughout Europe. In Poland it is very common in a wide range of habitats.

In Canada and USA research was undertaken to evaluate the possibility of utilizing zig-zag clover in a forage improvement programme for a high altitude meadow areas. ROBERTSON and ARMSTRONG (1964) noted that zig-zag clover closely resembles red clover in foliage and floral characteristics and under favourable soil conditions almost equalled that species in production of high quality forage. KOWNACKA (1958) reported that *T. medium* had a somewhat higher protein content than white and red clovers growing in the same location. However, zig-zag clover has limited use as cultivated legume most likely due to the low seed yield. Frequently the seeds show poor germination due to chalcid fly damage. Its persistency and winter hardiness can, however, make it a potential competitor of *T. pratense* as a forage crop if the aforementioned limitations are overcome.

The present study was undertaken to investigate the range and level of morphological and enzymatic variation in populations of *T. medium* in association with regional variables. This study is a part of a broad survey of morphological and genetic variability, and phylogenetic relationships in the genus *Trifolium*.

Material and methods

Thirty-four populations of zig-zag clover (*Trifolium medium*) collected as seed samples in central, northern and southern Poland were used in this morphological and enzymatic study (Figure1).

Morphological measurements were made on plants from 28 populations grown under uniform field conditions. In each population, ten plants were selected for character measurements. Records were taken from fully developed plants at the flowering stage. Twenty-two characters of leaf, stem and inflorescence were scored. Selection of characters was based on their importance and diagnostic value in delimitation of this species (Table 1). Continuously variable characters were scored in absolute terms (e.g. mm), the meristic character was counted and other characters were coded. Numerical values of continuously variable characters were expressed as means and standard deviations for each population.

In the enzymatic study, nineteen populations were surveyed with regard to the following enzyme systems: aspartate aminotransferase – AAT (E.C. 2.6.1.1.), esterase – EST (E.C. 3.1.1.), glucoso 6-phosphate isomerase PGI (E.C. 5.3.1.9), leucine aminopeptidase – LAP (E.C. 3.4.11.1), 6-phosphogluconate dehydrogenase – PGD (E.C. 1.1.1.44), peroxidase – PRX (E.C. 1.11.1.7) phosphoglucomutase – PGM (E.C. 5.4.2.2), and shikimate dehydrogenase – SKD (E.C. 1.1.1.25). Thirty to fifty 6-week-old plants per population were examined. The enzyme extracts were made from young leaves using two standard procedures of WEEDEN and EMMO (1985).

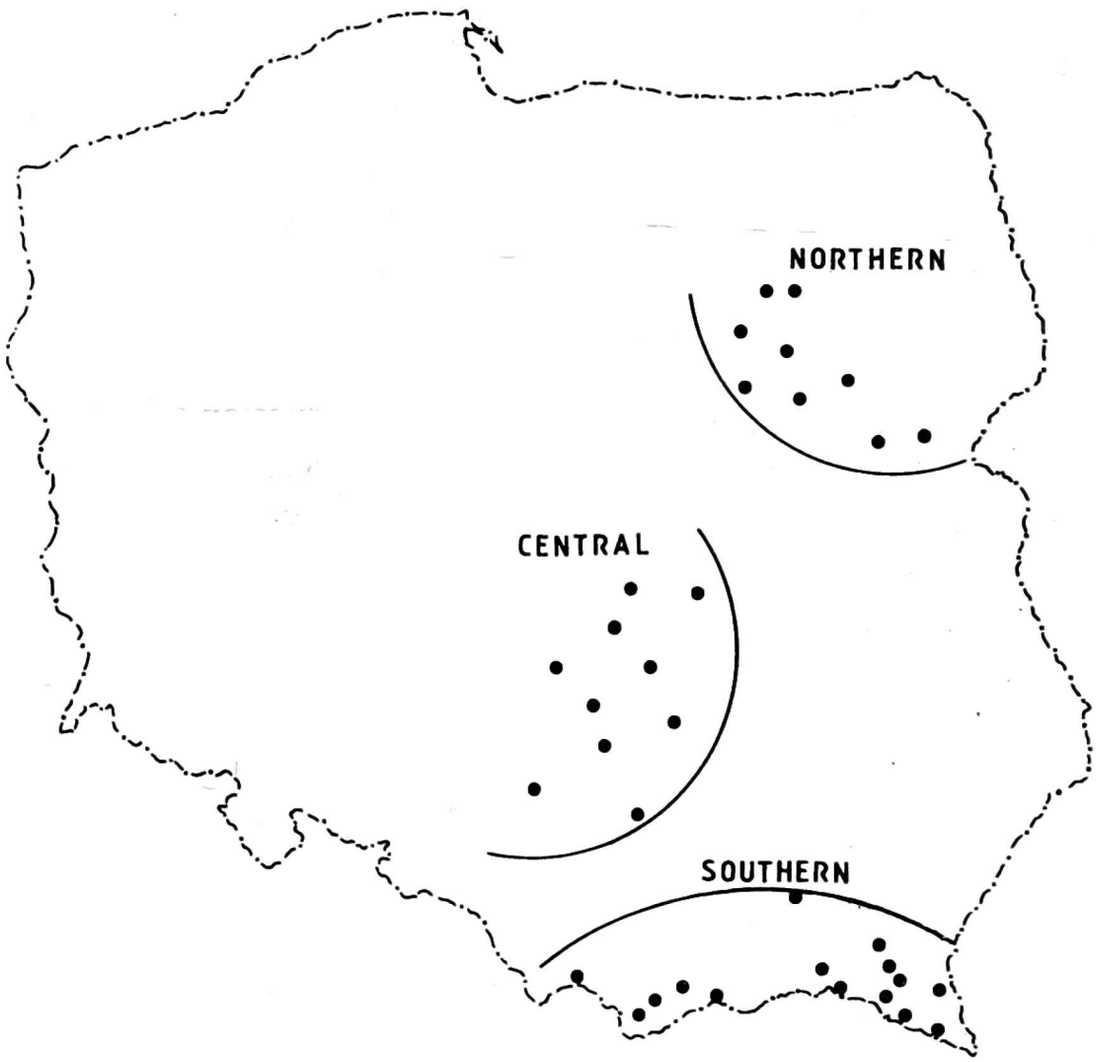

Figure 1. Regional distribution of *T. medium* populations

Table 1. Plan morphological characters scored in *T. medium* populations

Character type		
Continuously variable	Meristic	Other
Inflorescence length	Number of calyx veins	Inflorescence shape
Inflorescence width		Leaf shape
Terminal leaflet length		Type of leaf margin
Terminal leaflet width		Calyx tube pubescence
Stipule length		Stem glabrescence
Stipule width		Teeth/calyx tube ratio
Pedicel length		
Peduncle length		
Petiole length		
Terminal petiolule length		
Flower length		
Calyx tube length		
Length of short calycine teeth		
Length of long calycine teeth		

Electrophoresis was performed on 11% and 12% starch gels in two gel and buffer systems (0.03 M Lithium-borate pH 8.1 and 0.005 M L-histidine pH 6.1). Electrophoretic procedures, buffers, gel recipes and the methods for enzyme visualization were those of WEEDEN and EMMO (1985). LAP, PRX, AAT and ADH were developed in Lithium-borate pH 8.1 running buffer, whereas for PGM, SKD, MDH and EST histidine pH 6.1 running buffer was used.

Regional characteristics

The three regions differ with respect to the altitude range and climatic variables such as summer and winter temperatures, annual precipitation, persistency of snow cover, length of the summer and winter periods and the amount of solar radiation (Table 2).

Table 2. Climatic variables[1]

Variable	Region of Poland		
	Northern	Central	Southern
Days of winter	85-97	92-98	90-120
Days of summer	91-98	88-97	45-80
January mean temperature (°C)	–2.8	–3.2	–6.0
July mean temperature (°C)	18.0	17.0	15.0
Sunny days	55- 65	55-70	50-60
Annual precipitation (mm)	510-540	610-650	750-1200
Snow cover (days)	74-78	80-93	85-200
Cloudy days	110-120	110-120	105-115
Altitude (m)	60-200	160-310	300-2000

[1] After National Atlas of Poland 1973-1978.

Data analysis

Morphological data were standardized prior to calculation of Euclidean Distance (D) – dissimilarity coefficients which were subsequently used in cluster analysis by UPGM using the TAXAL-2 software (BATKO, MORACZEWSKI 1993).

For the comparison of enzyme banding patterns, cluster analysis and principal component analysis (PCA) were performed using Statistica for Windows (1995). Clustering was performed with the WARD algorithm using Euclidean Distance matrices. Jaccard similarity coefficients were computed to determine similarities between pairs of populations.

One-way ANOVA for the morphological characteristics and the most discriminating enzyme phenotypes of PGI, PRX, AAT, LAP, EST and PGD was per-

formed to determine whether populations of those three regions differed significantly. Subsequently Pearson correlation coefficients were calculated to examine the association among ten environmental variables and the aforementioned morphological traits and enzyme phenotypes.

Results

Morphological pattern of diversity

The populations of zig-zag clover showed a high intra- and interpopulational variability. The most variable morphological traits within the populations were: peduncle length, pedicel length, inflorescence length and width, terminal leaflet length and width and stipule length. The populations differed with respect to pedicel and peduncle length, terminal leaflet length and width, stipule length and the number of veins on calyx. One-way ANOVA performed for the aforementioned traits revealed their regional significance.

Cluster analysis divided all the populations into two major groups joined together at a very high level of dissimilarities = 1.0 (Figure 2). The first group contained populations of southern Poland intermixed with two populations of northern and central Poland at the level of clustering D = 0.4. The other major group was composed of two subgroups, one of central and the other of northern populations.

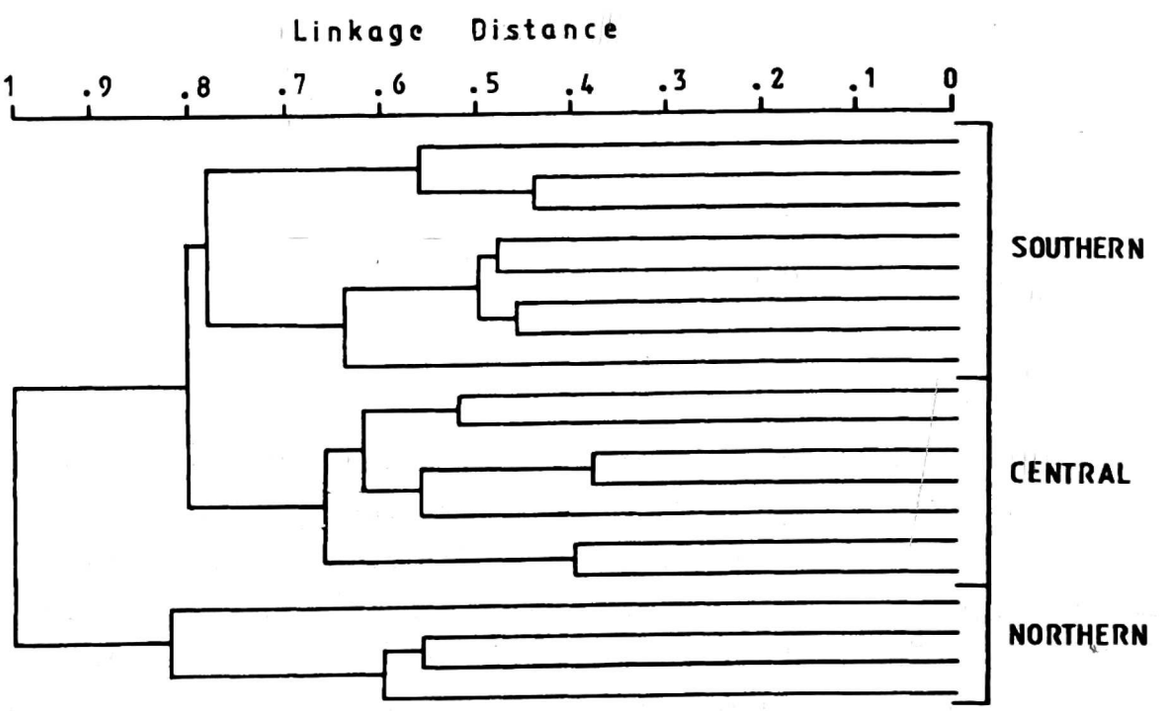

Figure 2. *T. medium* population relationships obtained after the clustering of morphological data

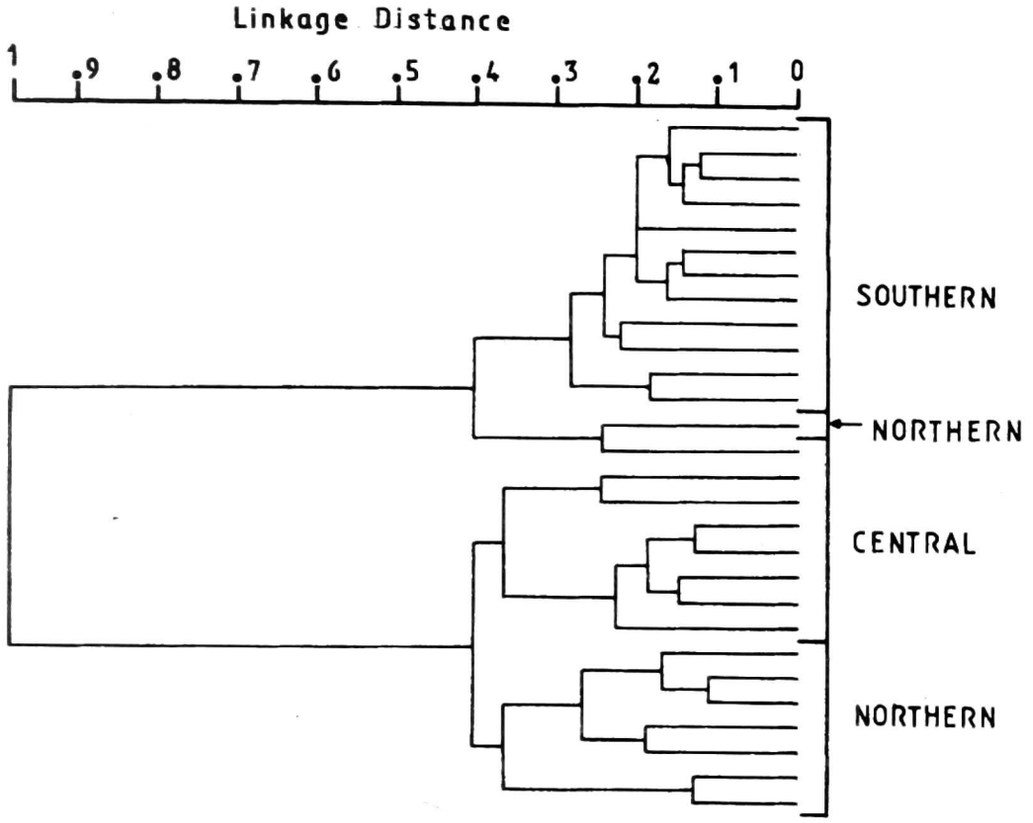

Figure 3. *T. medium* population relationships obtained after the clustering of enzyme data

Electrophoretic pattern of diversity

The number of phenotypes observed in each of the enzymes was as follow: 64 PGI, 16 AAT, 23 LAP, 12 EST, 12 PGD, 10 PGM, 8 PRX and 6 SKD. All the populations were polymorphic at all loci. Table 3 shows mean frequencies

Table 3. Most frequent enzyme phenotypes of *T. medium* in three regions of Poland

Enzyme phenotype	Region		
	Central	Northern	Southern
PGI 2	0.14	0.23	0.89**
PGI 5	0.23	0.32	0.70
PGI 37	0.09**	0.42	0.65**
PGI 47	0.46	0.15 **	0.52
PGI 52	0.32**	0.16	0.62**
PRX 3	0.23	0.32**	0.54**
PRX 9	0.13	0.12	0.34
AAT 1	0.67	0.45	0.23
AAT 2	0.52	0.68	0.07**
LAP 7	0.21	0.65*	0.89**
LAP 12	0.87	0.73	0.35**
EST 3	0.64**	0.23	0.37
EST 8	0.77*	0.59	0.16*
PGD 7	0.49**	0.09	0.06

* $P < 0.05$, ** $P < 0.01$

of the most discriminating phenotypes in the three regions. Distribution of AAT, PGI, LAP, EST, PGD and PRX phenotypes across populations within regions was uneven, unlike that of PGM and SKD.

Statistical analysis of electrophoretic data yielded similar results to those of the analysis of morphological data. In cluster analysis populations of the same region were grouped together prior to clustering with other populations (Figure 3). The level of dissimilarity at which populations clustered together both within and between regions was much higher than in the case of morphological data. In PCA populations of central and northern regions formed one loose group with central populations on one side and northern on the other. Southern populations were separated from others on the first factor responsible for 61% of variance, to which PGI and PRX contributed the most. Significance of the second factor on account of LAP and EST, which was responsible for 6% of the total variance, was much less evident (Figure 4). Jaccard similarity coefficients averaged by region confirmed that populations were most similar to those of the same region, but also revealed that populations of central and northern regions were less distant from each other than from populations of southern Poland (Table 4).

Table 4. Matrix of Jaccard similarity coefficients for enzyme data averaged by region

Region of Poland	Southern	Central	Northern
Southern	**0.897** (0.820-0.905)[1]		
Central	**0.810** (0.759-0.869)	**0.876** (0.812-0.896)	
Northern	**0.798** (0.723-0.802)	**0.832** (0.801-0.865)	**0.868** (0.802-0.879)

[1] Ranges are shown in parentheses.

Correlation among environmental and both morphological and isozyme characteristics

The Pearson correlation coefficient computed for regional variables and morphological traits revealed significant positive correlations between annual precipitation, number of sunny days, number of summer days, collection site and pedicel length, peduncle length, terminal leaflet length and width, and stipule length (Table 5). A negative significant correlation between altitude and both terminal leaflet length and peduncle length was observed. The number of calyx veins was positively correlated with the number of days of winter, mean temperature of January amount of annual precipitation and the nature of collection site (whether meadow, pasture, roadside or waste place). Out of all climatic variables the mean temperature of July, duration of snow cover and the number of cloudy days had no effect on the investigated characters.

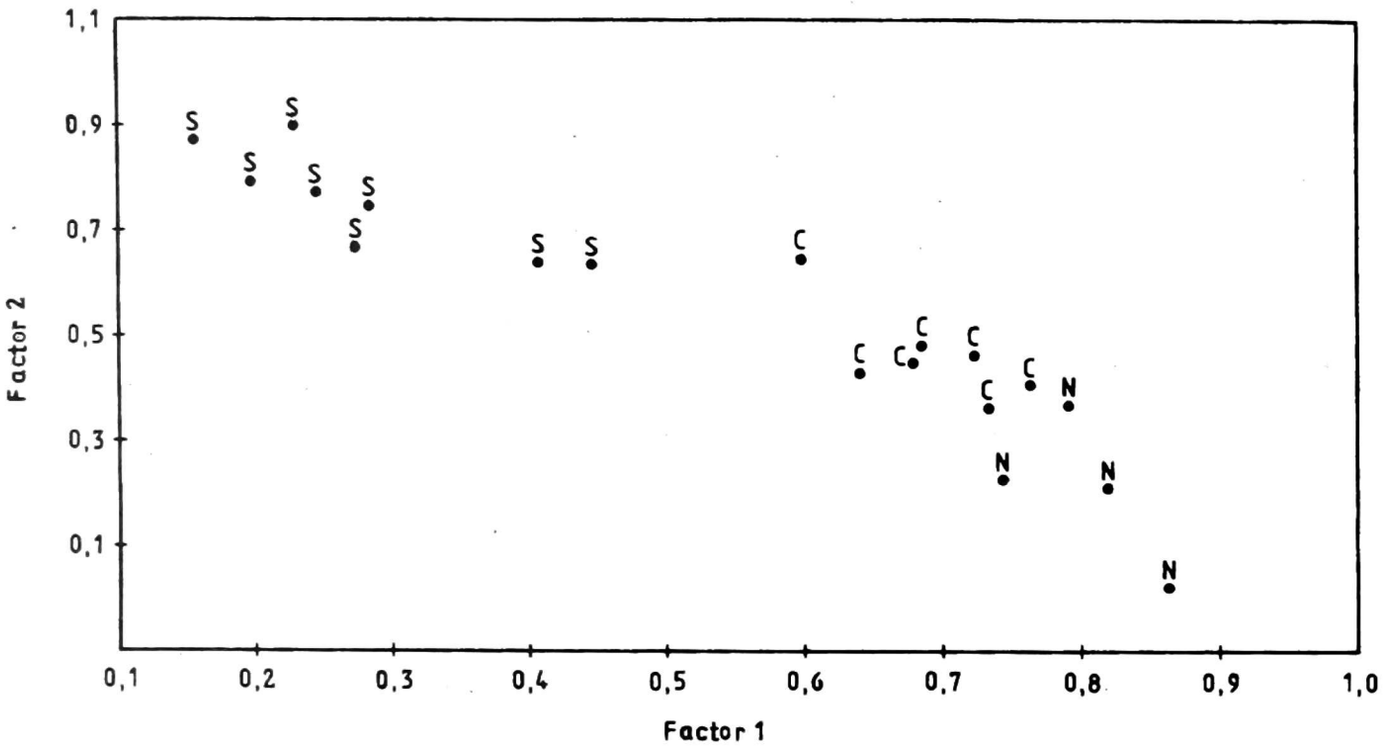

Figure 4. Relationships among *T. medium* populations from three regions of Poland (E – eastern, N – northern, C – central) obtained from PCA of enzyme data

Table 5. Pearson coefficients of correlation between six morphological characters of *T. medium* plants and environmental variables

Environmental variable	Correlation coefficient					
	leaflet length	leaflet width	pedicel length	peduncle length	stipule length	no. of calyx veins
Days of winter	0.321	0.129	0.432	0.032	0.213	0.743**
Days of summer	0.623*	0.846**	0.721**	0.471*	0.487*	0.321
January mean temp.	0.412	0.231	0.321	0.114	0.054	0.896**
July mean temp.	0.364	0.164	0.174	0.132	0.405	0.097
Sunny days	0.841**	0.629**	0.834**	0.865**	0.564*	0.143
Annual precipitation	0.756**	0.743**	0.456*	0.628**	0.820**	0.887**
Snow cover	0.312	0.406	0.212	0.096	0.215	0.074
Cloudy days	0.024	0.138	0.364	0.174	0.048	0.359
Altitude	–0.445*	0.352	0.143	–0.489*	0.089	0.444
Collection site	0.754**	0.523*	0.467*	0.468*	0.698**	0.849**

* $P < 0.05$, ** $P < 0.01$

Table 6. Pearson correlation coefficients of electrophoretic enzyme phenotypes of *T. medium* with environmental variables

Environ-mental variable[1]	Electrophoretic enzyme phenotype													
	PGI2	PGI5	PGI37	PGI47	PGI52	PRX3	PRX9	AAT1	AAT2	LAP7	LAP12	EST3	EST8	PGD7
DW	0.498*	0.492*	0.829**	0.675**	0.641**	0.552**	0.629**	–0.438*	–0.825**	0.852**	0.636**	–0.678**	–0.598**	–0.446*
DS	–0.672**	–0.749**	–0.583**	0.389	–0.525*	–0.493*	0.213	0.612**	0.675**	0.213	0.343	0.920**	0.620**	0.521*
JA	0.667**	0.443	0.648**	0.519**	0.820**	0.511*	0.521*	0.533*	–0.765**	0.813**	0.495*	–0.862**	–0.732**	–0.812**
JU	0.276	0.324	0.143	0.247	0.368	0.356	0.097	0.372	0.289	0.037	0.216	0.411	0.063	0.243
SD	–0.587**	0.461*	–0.729**	0.421	0.513**	–0.479*	–0.412*	0.512*	0.487*	–0.621**	–0.452*	0.632**	0.580*	0.489*
AP	0.725**	0.531*	0.625**	0.472*	0.432	0.531*	0.341	0.213	–0.722**	0.725**	0.464*	–0.842**	–0.874**	0.411
SC	0.450*	0.486*	0.643**	0.829**	0.652**	0.538*	0.523**	–0.668**	–0.512*	0.638**	0.459*	–0.623**	–0.513*	–0.746**
CL	0.648**	0.769**	0.548*	0.482*	0.428	0.617**	0.396	–0.483*	–0.729**	0.584**	0.318	–0.715**	–0.543*	–0.546*
AL	0.502*	0.513*	0.613**	0.374	0.742**	0.470*	0.616**	0.012	0.020	0.893**	0.913**	–0.612**	–0.480*	–0.495*
CS	0.332	0.417	0.405	0.316	0.111	0.006	0.122	0.164	0.187	0.301	0.221	0.354	0.065	0.033

* $P < 0.05$, ** $P < 0.01$

DW – days of winter, DS – days of summer, JA – January mean temp., JU – July mean temp., SD – sunny days, AP – annual precipitation, SC – snow cover, CL – cloudy days, AL – altitude, CS – collection site.

The number of PGI, LAP and PRX phenotypes was significantly positively correlated with the number of days of winter, January mean temperature, number of days with snow cover, number of cloudy days, annual precipitation and altitude (Table 6). Some phenotypes of those enzymes were negatively correlated with the number of summer days and sunny days. AAT, PGD7 and EST were positively correlated with these two variables. Phenotypes of EST, PGD and AAT showed a negative significant correlation with almost all the remaining variables, except for a mean temperature of July and collection site. AAT, unlike phenotypes of other enzymes were not correlated with altitude.

Discussion

Morphological and enzymatic patterns of variability

The present data demonstrate that morphological traits and allelic characteristics are regionally dependent in the same manner. Populations of northern and central regions, whose climatic variables are less distinct than those of the southern region, are both morphologically and enzymatically more similar to each other than either of them to southern populations. The magnitude of variability both within and among regions is much greater with respect to enzyme phenotypes than to morphological characteristics, which prove that enzyme data provide a more sensitive tool for the study of population variability in zig-zag clover.

The effect of the regional differentiation is also reflected in differential frequencies of certain enzyme phenotypes. Similarly, morphological characters such as those of leaf, stipule, pedicel, peduncle, as well as the number of calyx veins and stem glabrescence, showed strong associations with regional variables. The two latter traits occur exclusively in populations of southern Poland and are evidently linked with ecological characteristics of the region.

Climatic variables and differentiation of populations

The effect of climate on morphological and enzyme differentiation at the regional level is reflected in the sequence and the level of clustering among the populations and is evidenced by the high values of coefficient of correlation between climatic variables and both morphological and enzymatic traits. The amount of high temperatures expressed by the high number of summer days, the amount of solar radiation expressed by the number of sunny days, as well as annual precipitation play the most significant role in the formation of relatively robust genotypes which prevail in central and northern regions. Populations of southern areas, in addition to being vegetatively inferior, are distinct from other populations by two unique traits: a higher number of calyx veins (15-20), and a stem covered with spreading hairs. These two characters occur exclusively in the areas with long winters, low temperatures in January and a high annual precipitation.

Enzymatic variability related to climatic conditions was earlier demonstrated in *T. fragiferum* by BULIŃSKA-RADOMSKA (1999). Like in *T. medium*, the differentiation of *T. fragiferum* populations had also a regional pattern. Populations of *T. fragiferum* from the eastern region, where climate is continental, showed a higher variability and a greater genetic distance from populations from regions with more balanced climatic conditions. In *T. medium* severe climatic conditions of mountainous areas of southern Poland were also responsible for enzymatic divergence of southern populations from populations of the other two regions as all the other environmental factors were equal.

The effect of climate on differentiation of populations was investigated by several researchers. ARCONI et al. (1988), and CHARMET et al. (1993) found a clear correspondence between frequencies of several alleles at different loci in *Lolium perenne* and a set of climatic variables from the collection site. Arconi, unlike Charmet, reported correlation between *Pgi* alleles and both temperature and summer rainfall. NEVO et al. (1979) found that different alleles in *Hordeum spontaneum* were correlated with different climatic factors. He noted that temperature, rainfall and humidity were significant predictors of variation. They also found that polymorphism and diversity values are maximal for the rainfall range of 200-600 mm. PEREZ DELA VEGA et al. (1994) established that specific single locus and multilocus allelic combinations in *Avena sativa*, differ widely in different ecogeographical regions, often in correlation with environmental variables. Some locus associations were associated with the warmer temperature class, whereas the complementary association with the colder temperature class.

The enzyme data, like morphological results, confirm the adaptive value of specific phenotypes in relation to climatic conditions. This is evidenced by the correlations between the most frequent PGI, PRX and LAP phenotypes, and a set of severe climatic conditions of high altitude area, as well as between certain phenotypes of AAT, EST and PGD, and variables of a more balanced climate.

This study also showed that the same climatic variables do not always play significant role in morphological and enzymatic differentiation of *T. medium*. In general, enzymes were more responsive to differences in the climatic factors than morphological characters. For instance: the length of the period with snow cover and the number of cloudy days had no significant effect on the development of variability of morphological traits, which is not the case for variability at the enzyme level. Likewise, the mean temperature of January, duration of winter and altitude were only important with regard to one or two morphological characters, which is contrary to the significance of these variables with respect to enzymatic differentiation between the populations.

The results of this study support the notion that information concerning the adaptive properties of specific enzyme phenotypes and interphenotypic associations can facilitate developing strategies for collecting, evaluating, managing and utilizing genetic variability.

Discriminating phenotypes of aforementioned enzymes can serve as regional indicators; whereas climatic variables, which are significant for the regional differentiation of populations, could be used as predictors of variation in *T. medium*.

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