Effect of Environmental and Genetic Factors on the Stability of Pea (*Pisum sativum* L.) Isozyme and DNA Markers

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Abstract: Environmental (geographic location, year-to-year effect) and genetic (intravarietal variation) effects on the stability of the isozyme patterns of esterase (EST), acid phosphatase (ACP), alcohol dehydrogenase (ADH), leucine aminopeptidase (LAP), shikimate dehydrogenase (SDH) and glucose-6-phosphate isomerase (PGI) used for pea cultivar genotyping were studied. In addition, selected DNA markers (RAPD, SSR, ISSR, IRAP) were used to study intravarietal genetic homogeneity/variation at a DNA level. Five commercial dry-seed pea (*Pisum sativum* L.) cultivars Canis, Gotik, Komet, Sonet and Zekon were grown during four years (2001 – 2004) in three locations in the Czech Republic (Šumperk, Čáslav, Uherský Ostroh) that differed in soil and climatic characteristics. Mature dry seeds were used as a standard input sample for analyses. No variation in isozyme spectra was found within particular cultivars between years, locations and fruiting nodes in enzymes providing in general a low polymorphism in pea cultivars (ACP, ADH, LAP, SDH, PGI); similarly, these enzymes also exhibited high stability as related to intravarietal variation. In contrast, EST – highly polymorphic in pea cultivars – showed certain qualitative variation within particular cultivars as related to both environmental and genetic factors. The intravarietal variation detected by selected DNA markers was negligible and mostly quantitative. Possible reasons for the instability/variation of isozyme markers are discussed from the aspect of cultivar genotyping used in pea breeding and seed production.

Keywords: intravarietal variation; isozymes; microsatellites; *Pisum sativum*; retrotransposons; seed proteins

Protein and DNA markers are widely used in plant genetic research (phylogenetic and evolution studies, germplasm collections description, QTL mapping) as well as in practical plant breeding and seed production (cultivar genotyping) even though they have not been accepted yet by UPOV as the official (basic) but only alternative descriptors of plant varieties (Guiard 2007). Thus, morphological, physiological and yield characteristics still represent the main and officially used parameters. Protein markers – mainly isozymes – are low abundant, but locus-specific, exhibit a low level of polymorphism and high reproducibility; finally, they are not labour intensive and expensive. In fact, they represent phenotype markers and they may be subjected to changes/modifications caused by environmental influences. Thus, their (environmental) stability is only moderate (Gepts 1993, 1995). Most of recently used DNA markers (based on polymorphism in the primary DNA structure) exhibit a high abundance in genome (RFLP, SSR, ISSR, AFLP), medium (RFLP, RAPD, ISSR) to high (RAPD, SSR, IRAP, AFLP) level of polymorphism, and – with some exceptions (RAPD) – high reproducibility. DNA markers (as genetic markers *per se*) would not be substantially subjected to gross changes caused by environmental factors – they are highly environmentally stable

(Gepts 1993, 1995). Nevertheless, environmental stress may result in methylation changes (Liu *et al*. 2004; Smýkal *et al.* 2007); the same (methylation) changes may occur during ontogenesis. An important point is that DNA markers can reveal very subtle genomic changes (mainly in repetitive non-coding DNA regions) which may be considered as intravarietal variation on the one hand, however, usually with no practical breeder's impact on the other hand (Wiesner *et al.* 2001). Intravarietal variation in isozyme phenotypes has been studied with great interest because of their significance in plant breeding rights. Such variation arises when several morphologically similar lines take part in the development of a cultivar. Since these lines are mostly selected for physiological and morphological uniformity and not for isozymic uniformity, thus these lines often exhibit significant intravarietal variation in their isozymic profiles (Bailey 1983).

In the last two decades we studied the possibilities of utilizing protein markers (mainly isozymes) for pea and flax/linseed cultivar genotyping (Šuška & Stejskal 1992; Šuška 1993; Stejskal *et al.* 1996; Samec *et al.* 1998; Pošvec & Griga 2000; Krulíčková *et al.* 2002). We have found that by combination of various sample types (seed, leaf), various electrophoretic techniques (IEF, NATIVE-PAGE) and various enzyme systems we were able to distinguish the studied sets of pea or flax/linseed varieties relatively precisely (Pošvec & Griga 2000; Krulíčková *et al.* 2002). In the experiments, we generally used the elite seeds from breeders or from germplasm collections (Czech National Pea and Flax Collections), however, without their detailed characterization as related to year-to-year effect, geographical location of trials etc. Based on the fact that isozymes are phenotypic markers, it would be interesting if there existed any environmental effects or an intravarietal variation in cultivars declared as either single-line ones or those composed of several lines. Thus, in contrast to most reports using genetic markers for studying (phylo)genetic relations within the *Pisum* genus (Przybylska 1986; Bagheri *et al.* 1995; Hoey *et al*. 1996; Lu *et al*. 1996; Samec & Našinec 1996; Ellis *et al.* 1998; Samec *et al.* 1998; Burstin *et al.* 2001; Jha & Ohri 2002; Simioniuc *et al.* 2002; Ford *et al.* 2002; Baranger *et al.* 2004; Smýkal *et al.* 2008a), core collection characterization (Swiecicki *et al*. 2000; Smýkal *et al.* 2008a) or *P. sativum* cultivar identification (Šašek *et al.* 1983; Swiecicki & Wolko 1987; Wolko & Swiecicki 1987; Samec *et al.* 1998; Wolko *et al*. 2000; Weder 2002a, b; Smýkal *et al.* 2008b), we stress more practical aspects, namely potential environmental effects and subtle intravarietal protein and DNA variation effects on the stability and reproducibility of protein and DNA markers used for pea cultivar genotyping as these data were not sufficiently provided in the literature dealing with pea biochemical/molecular markers (e.g. Hussain *et al*. 1988; Wolko *et al*. 2000). All analyses were done with mature dry pea seeds as the most easily available sample. The final aim is a recommendation to pea breeders/seed producers about the stability and suitability of selected genetic markers for genotyping of pea cultivars.

MATERIALS AND METHODS

Plant material and design of field experiments. Five commercially grown cultivars of dry-seed pea (*Pisum sativum* L. ssp. *sativum* var. *sativum*) of afila and normal leaf type from the Czech gene bank held in Agritec were used in the experiments, namely Canis, Gotik, Komet, Sonet and Zekon (Table 1). Field trials were planted in a randomised complete block design with three replicates. Each block represented a single plot of 5 m^2. Field trials were established in three locations in the Czech

Table 1. Characterization of dry-seed pea (*Pisum sativum* L.) cultivars used in experiments

Cultivar	Type	Seed shape	Seed colour	Origin
Canis	white flowering/afila	smooth	yellow	Sweden
Gotik	white flowering/afila	smooth	yellow	Czech Republic
Komet	white flowering/normal	smooth	yellow	Czech Republic
Sonet	white flowering/normal	smooth	yellow	Czech Republic
Zekon	white flowering/afila	smooth	green	Czech Republic

Table 2. Characterization of locations of field experiments – climatic and soil conditions

Location	Altitude (m a.s.l.)	Long-term average annual temperature (°C)	Long-term average rainfall (mm per year)	Soil type*	Soil pH
Šumperk (North Moravia)	315	7.45	693	Orthic Luvisol	6.2
Čáslav (Central Bohemia)	260	8.90	555	Luvi-haplic Chernozem	6.9
Uherský Ostroh (South Moravia)	196	9.10	521	Eutric Cambisol	6.7

*According to FAO (1988)

Republic differing in climatic conditions, as well as in soil parameters – Šumperk, Čáslav and Uherský Ostroh, during the period 2001–2004 (Table 2). Trials were established at all locations and each year and maintained according to the same methodology, no fertilization was applied. 1.5 l/ha Sumithion Super (fenitrothion) and 0.6 l/ha Nurelle D (chlorpyriphos and cypermethrin) were used for insect protection. Preemergent application of 5 l/ha Stomp 330 E (pendimethalin) was used for the weed control; manual weeding was also carried out during the vegetation period.

The effect of geographical location and year-to-year effect on isozyme markers. Seeds of 10 plants of each cultivar (5 cvs.) grown in a respective location (3 locations) were mixed and represented one sample, thus the final number of samples was 15 in a respective year (2001). Samples were analyzed on the same electrophoretic gel. Next, electrophoretic analyses of five pea cultivars grown in Šumperk location in the four subsequent years (2001, 2002, 2003 and 2004; Table 3) were performed. Again, the seeds of 10 plants of each variety from a particular year were mixed and represented the tested sample.

Based on our previous experience (Pošvec & Griga 2000), six enzyme systems exhibiting the highest isozyme polymorphism were analysed, namely esterase – EST (E.C.3.1.1.2), acid phosphatase – ACP (E.C.3.1.3.2), alcohol dehydrogenase – ADH (E.C.1.1.1.1), leucine aminopeptidase – LAP (E.C.3.4.11.1), shikimate dehydrogenase – SDH (E.C.1.1.1.25) and glucose-6-phosphate isomerase – PGI (E.C.5.3.1.9).

Intravarietal variability of isozyme and DNA markers. To estimate the level of intravarietal variation of isozyme and DNA markers, seeds from 10 randomly selected plants of each tested pea cultivar grown in Šumperk location (2002) were analyzed separately by six isozyme systems (EST, ACP, ADH, LAP, SDH, PGI) and four different types of DNA-based markers (RAPD, ISSR, SSR and IRAP).

Extraction of enzymes. Seed samples were ground to fine powder on the Cyclotec (Tecator, Hoganas, Sweden) laboratory mill. Seed flour was extracted immediately before analysis in the ratio 1:10 with loading buffer (0.15 mmol/dm^3 Tris-HCl, pH 6.8 containing 12.5% 2-mercaptoethanol, 25% glycerol and 0.01% bromophenol blue) for 30 min in a freezer at –20°C. Then, samples were centrifuged at 30 000 g (4°C) for 10 min and loaded onto an electrophoretic gel.

Isolation of genomic DNA. Seed samples were ground to fine powder on a Cyclotec laboratory

Table 3. Average temperature during vegetation (April–August), average annual temperature, average rainfall during vegetation (April–August) and average annual rainfall in Šumperk location in the period 2001–2004

Year	Average temperature during vegetation (°C)	Average annual temperature (°C)	Average rainfall during vegetation (mm)	Average annual rainfall (mm)
2001	14.4	7.4	421	823
2002	13.9	7.6	325	790
2003	15.6	6.0	224	523
2004	14.1	7.1	231	618

mill. DNA from this flour was isolated using a commercial kit Invisorb Spin Plant Mini Kit (Invitec, Berlin, Germany). The quality and quantity of isolated DNA were then checked spectrophotometrically (Eppendorf, Hamburg, Germany) and DNA was diluted to a concentration of 30 µg/ml and finally stored at –20°C.

Isozyme analysis. Isozymes were separated using native discontinuous vertical electrophoresis on the polyacrylamide gel (NATIVE-PAGE) (7.5% running gel, pH 6.4 and 5% stacking gel, pH 6.0) at 4°C. Tris-tricine buffer (pH 7.1) was used as a cathode electrode solution, Tris-acetate buffer (pH 6.4) was used as an anode electrode one. Electrophoretic separations were performed at a constant current of 50 mA for 4 hours on a VE-2U vertical unit (Laboratory Services, Postřelmov, Czech Republic). Detection of enzymatic activities was done according to Vallejos (1983). Then, stained gels were fixed, dried, scanned and saved as electronic files.

RAPD analysis. Ten RAPD primers (OPW08, P14, OPW02, P9, P10, OPW01, UBC741, UBC561, UBC556, OPAB4) that earlier showed a high polymorphism in pea (Samec *et al.* 1998; Wiesner *et al.* 2001; Simioniuc *et al.* 2002) were used. PCR amplification was done in a 20 µl reaction mixture containing 25 ng genomic DNA, 1.5 µmol/dm^3 primer, 100 µmol/dm^3 of each dNTP, 2.5 mmol/dm^3 $MgCl_2$ and 0.75 U recombinant Taq polymerase (TaKaRa, Shiga, Japan) in PCR reaction buffer. The thermal cycler Mastercycler Gradient (Eppendorf, Hamburg, Germany) was programmed as follows: 5 min denaturation step at 94°C was followed by 40 cycles of 10 s at 94°C, 1 min at 37°C and 1.5 min at 72°C. The product was resolved on 1.5% agarose (Serva, Heidelberg, Germany) gels and visualised by EtBr/UV.

ISSR analysis. ISSR assay was performed as described in Baranger *et al*. (2004) using the following primers based on di/tri-nucleotide repeats, anchored on their 3´ or 5´ end by partially degenerated nucleotides: $(GA)_9$-C, AAG-6, 5´-GCV$(TC)_7$. PCR amplifications were performed in a 15 µl reaction mixture containing: 25 ng genomic DNA, 4 pmol each primer, 100 µmol/dm^3 of each dNTP, 2.5 mmol/dm^3 $MgCl_2$ and 0.7 U Taq polymerase (BioTools, Madrid, Spain) in PCR reaction buffer (75 mmol/dm^3 Tris-HCl, pH 8.0, 20 mmol/dm^3 $(NH_4)_2SO_4$, 0.01% Tween 20). Amplification was performed in a thermal cycler Mastercycler Gradient. Cycling parameters were as follows: 4 min denaturation step at 94°C was followed by 35 cycles of 30 s at 94°C, 30 s at 51°C and 2.5 min at 72°C. The product was resolved on 1.5% agarose gels containing ethidium bromide.

SSR analysis. Microsatellite SSRs analyses were performed as described in Ford *et al*. (2002) using a selected primer pair (PSMPAD-141), which previously showed to be highly polymorphic and informative in pea germplasm genotyping (Smýkal *et al.* 2008a). PCR amplifications were performed in a 15 µl reaction mixture containing: 25 ng genomic DNA, 4 pmol each primer, 100 µmol/dm^3 of each dNTP, 2.5 mmol/dm^3 $MgCl_2$ and 0.7 U Taq polymerase (BioTools, Madrid, Spain) in PCR reaction buffer (75 mmol/dm^3 Tris-HCl, pH 8.0, 20 mmol/dm^3 $(NH_4)_2SO_4$, 0.01% Tween 20). Amplification was performed in a thermal cycler Mastercycler Gradient. Cycling parameters were as follows: 4 min denaturation step at 94°C was followed by 35 cycles of 30 s at 94°C, 30 s at 55°C and 3 min at 72°C. Products were resolved on 10% non-denaturing polyacrylamide gel on a Protean II vertical electrophoresis unit (Bio-Rad, Hercules, USA) and subsequently stained with ethidium bromide and UV visualized.

Analysis of retrotransposons. In the case of IRAP analysis, primers were designed to match close to the 5´ and 3´ end of a long terminal repeat (LTR) sequence of *Ogre* (Y299398), belonging to *Ty3-gypsy* group of retroelements (Smýkal 2006). PCR amplification was performed in a 20 µl reaction mixture containing: 25 ng genomic DNA, 10 pmol each primer, 100 µmol/dm^3 of each dNTP, 2.5 mmol/dm^3 $MgCl_2$ and 0.7 U Taq polymerase (BioTools, Madrid, Spain) in PCR reaction buffer (75 mmol/dm^3 Tris-HCl, pH 8.0, 20 mmol/dm^3 $(NH_4)_2SO_4$, 0.01% Tween 20). Cycling parameters were as follows: 4 min denaturation step at 94°C was followed by 35 cycles of 30 s at 94°C, 30 s at 58°C and 3 min at 72°C. Products were resolved on 10% non-denaturing polyacrylamide gel on a Protean II vertical electrophoresis unit (Bio-Rad, Hercules, USA) and subsequently stained with ethidium bromide and UV visualized.

Data processing. The visualized electrophoretic gels (enzyme/protein-specific staining; EtBr staining of DNA in agarose gels with subsequent visualization on a UV transilluminator) were digitally photographed. Electrophoregrams of individual samples were then compared and qualitative (presence/absence of bands) and quantitative changes (band intensity) were recorded. In order to detect

also subtle changes in the isozyme/DNA patterns, not only robust bands but also weak ones (frequently neglected or not considered in practice) were taken into consideration. Both isozyme and DNA analyses were performed twice at least.

RESULTS

Influence of geographical location on isozyme markers

Isozyme spectra of all six tested enzymes (ACP, ADH, EST, LAP, SDH, PGI) were first analyzed in cv. Komet in all three locations (Čáslav, Šumperk, Uherský Ostroh) in 2001. Except of EST, the isozyme spectra had a low level of polymorphism (usually 1–4 bands) and exhibited no variation between three locations (data not shown). EST – as related to its higher number of polymorphic bands – was then chosen for further analyses of five pea cultivars (mixed sample of 10 randomly selected plants per cultivar). Based on the analysis carried out simultaneously (Figure 1), the high uniformity of EST spectra is evident within particular cultivars and between locations. The cultivars kept their typical spectra (mainly the anodic region) and detected differences usually had a quantitative character. Nevertheless, in cv. Zekon, there was an absence of isozyme in the anodic region in a sample from Šumperk location. Absence or extremely low expression of this isozyme was also recorded in a sample from Čáslav location (Figure 1).

Year-to-year effect on isozyme markers

Similarly like in the location effect, the year-to-year effect on the stability of low-polymorphic enzymes (ACP, ADH, LAP, SDH, PGI) was not recorded either (data not shown). Again, the changes were found only in EST isozymes (Figure 2). In cv. Zekon, there was a doublet instead of triplet in the cathodic region of the gel (Figure 2A, positions 1, 2, 3) in 2001 as compared to the following years 2002–2004. In position 4 (central part of the gel) and position 5 (anodic region of the gel), there was an absence or extremely low expression of isozymes in 2001 as compared to the other years.

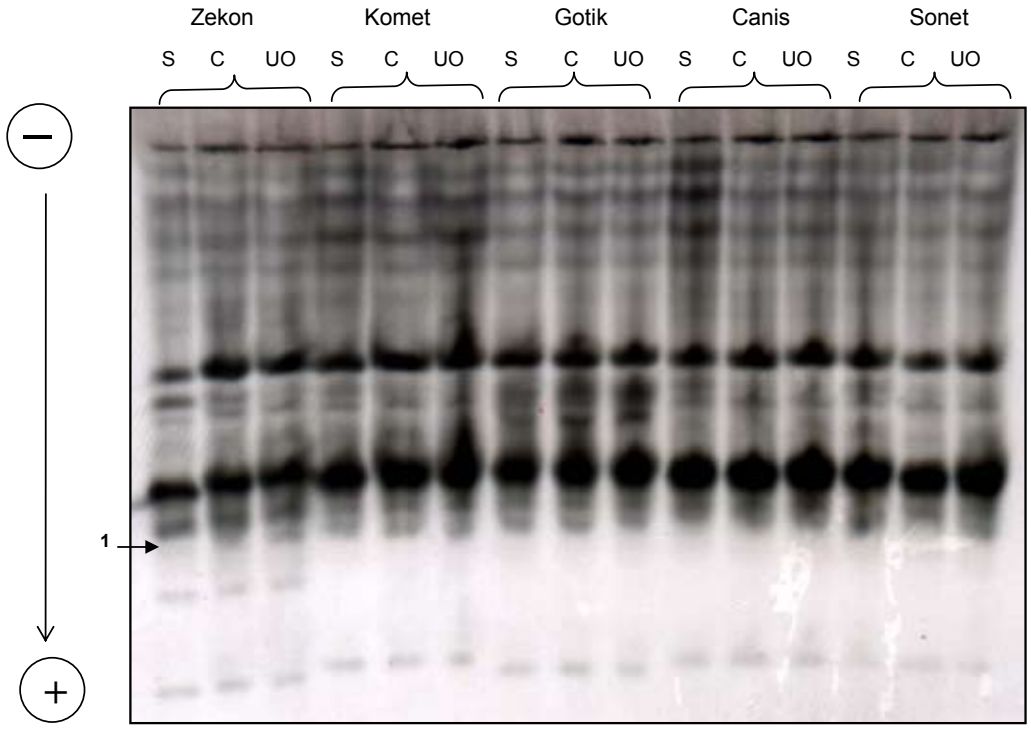

Figure 1. The influence of geographic location on the stability of esterase (EST) isozymes in five dry-seed pea cultivars; field experiment 2001; S – Šumperk, C – Čáslav, UO – Uherský Ostroh; arrow indicates alternating isozymes in position 1 in cv. Zekon

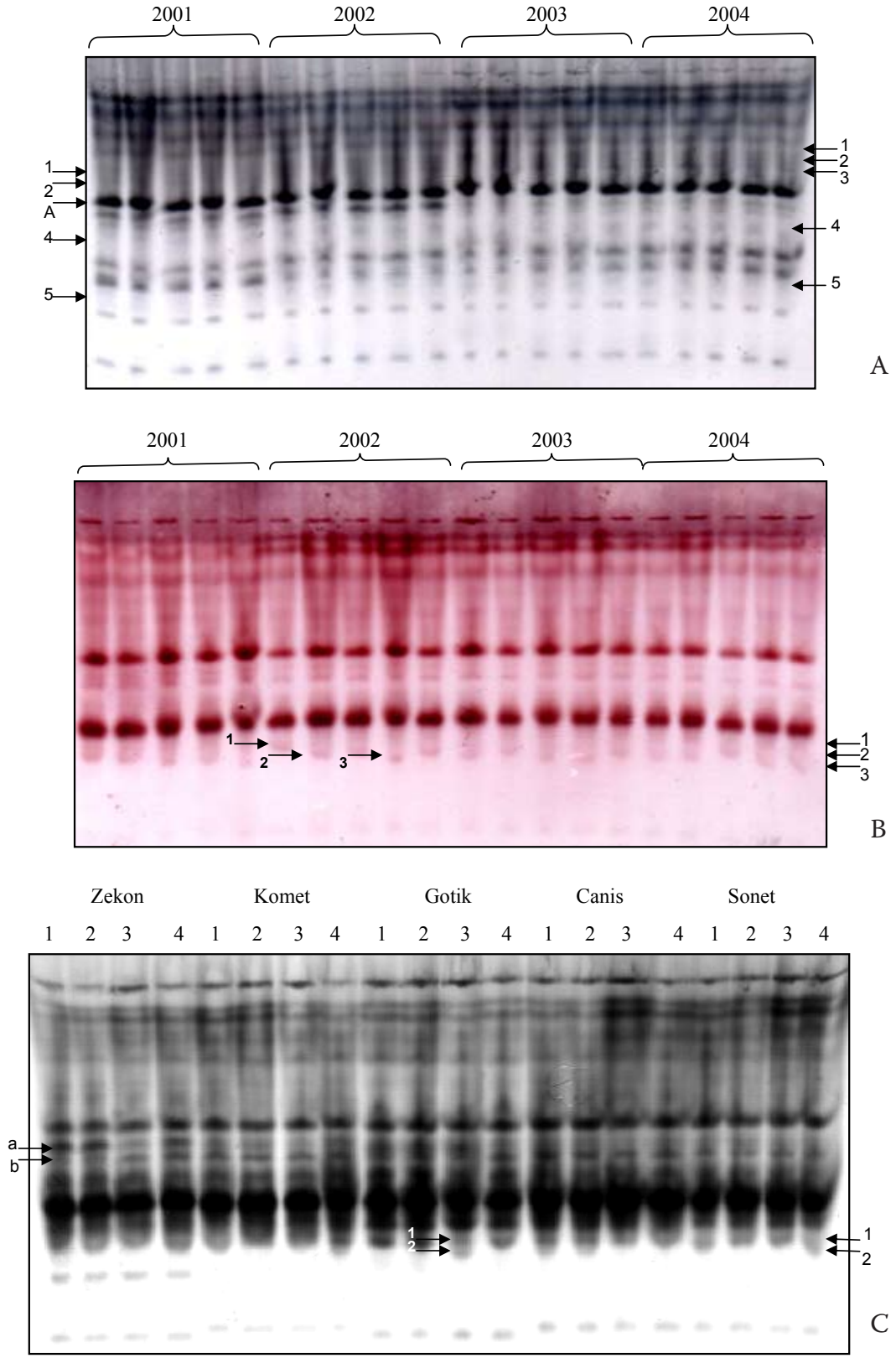

Figure 2. Year-to-year effect on the stability of esterase (EST) isozymes; five pea cultivars were grown in Šumperk location in 2001, 2002, 2003 and 2004; A – Cv. Zekon; each run in the respective year represents one replicate of a mixed sample of 10 randomly selected plants; B – Cv. Komet; sample as in A; C – particular pea cultivars in the respective years analyzed in one gel; arrows indicate positions (1–5, A, a, b) of alternating isozymes

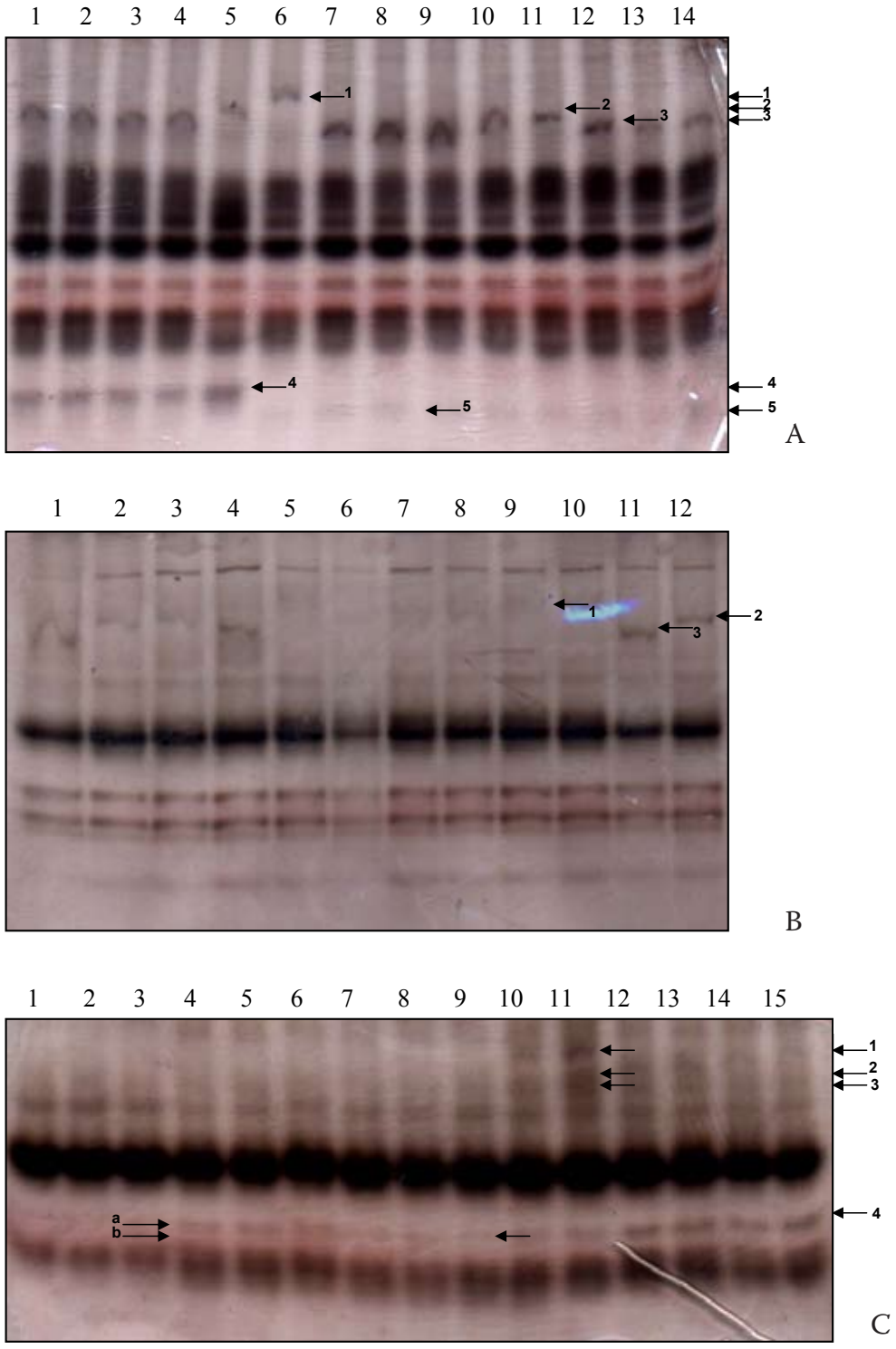

Figure 3. Intravarietal variation of esterase isozymes (EST); twenty selected plants of cvs. Gotik (A), Komet (B) and Zekon (C) grown at Šumperk location in 2001 were analyzed separately by NATIVE-PAGE; arrows (1–4) and letters (a, b) indicate alternating isozymes

There was a very strong expression (quantitative difference) of isoenzyme in position A (central part of the gel) in 2001 and 2002 as compared to 2003 and 2004 (Figure 2A). In cv. Komet (Figure 2B), there is an evident alteration of isoenzymes 1, 2, 3, which, however, occurred also within a single year (2002), which might rather show intravarietal variation or other environmental/physi-

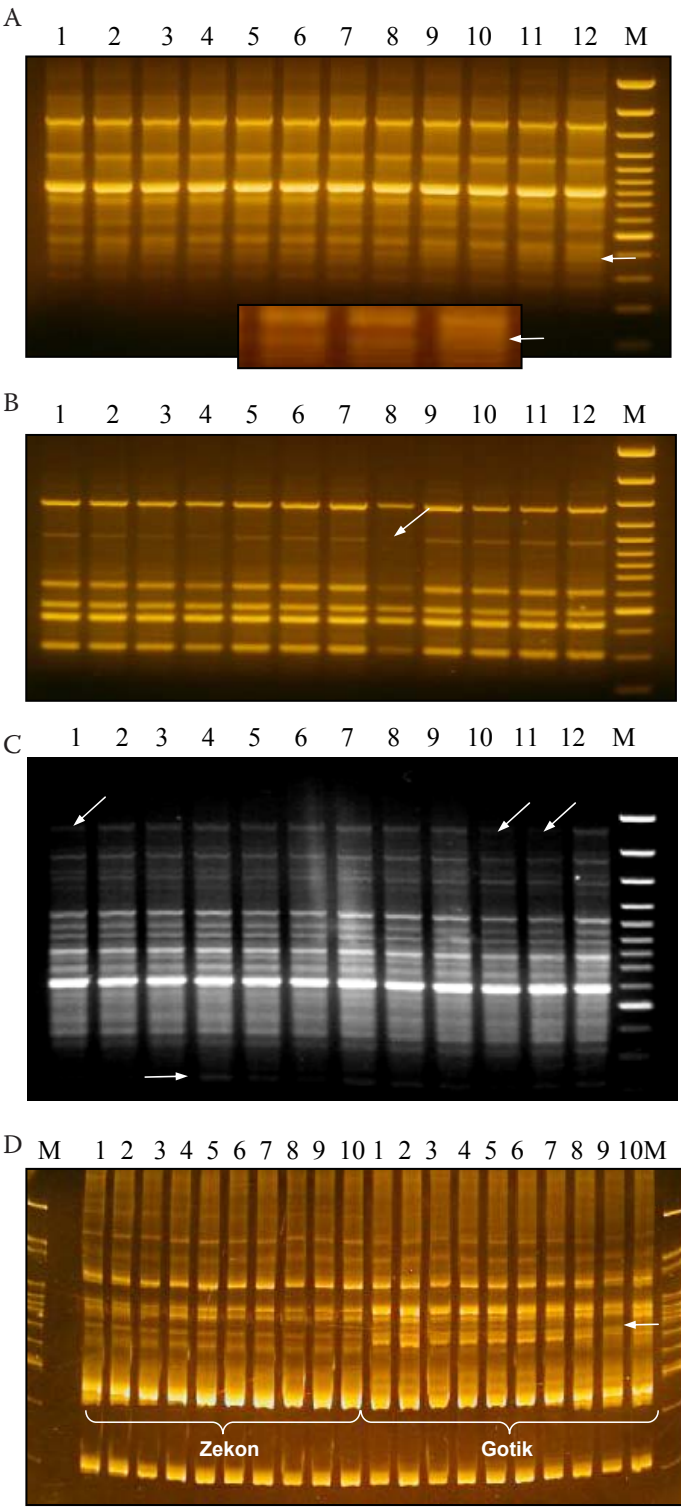

Figure 4. Intravarietal variation of RAPD markers; A – cv. Canis, primer OPW02; B – cv. Canis, primer UBC741; C – cv. Sonet, primer UBC556; D – cvs. Zekon (left) and Gotik (right), primer UBC741; ten selected plants of each cultivar grown at Šumperk location in 2001 were analyzed separately; arrows indicate qualitative/quantitative changes in RAPD markers

ological effects. Quantitative differences in cv. Komet were negligible both between years and within particular years (Figure 2B). Figure 2C shows all studied cultivars in particular years on one gel (cultivar × year samples). In cvs. Gotik, Canis and Sonet, qualitative alterations between years were detected in the anodic region (position 1 and 2). There were evident quantitative differences in the expression of two isozymes (a, b) in the central part of the gel, mainly in cvs. Zekon and Komet. Thus, it may be concluded that it was possible to find certain qualitative changes – though relatively minor – in EST spectra as related to the year of cultivation.

Intravarietal variability of isozyme markers

ACP: Isozyme spectra of ACP of all tested pea cultivars consisted of four well detectable bands. No intravarietal variation was detected within the tested cultivars.

LAP: Isozyme spectra of LAP consisted of two bands representing two isozymes LAP1 (cathodic) and LAP2 (anodic). Nevertheless, both bands were monomorphic among 20 samples of each tested cultivar.

PGI: Seed PGI was represented only by one hardly detectable band. No intravarietal polymorphism was detected within all tested cultivars.

SDH: Isozyme spectra of SDH in seeds of tested cultivars consisted of two bands. Both bands were monomorphic among 20 samples of each tested cultivar.

ADH: Isozyme spectra of ADH of tested cultivars consisted of two bands. Both bands were monomorphic among 20 samples of each tested cultivar.

EST: The most polymorphic enzyme EST showed a certain degree of intravarietal variation in three tested cultivars, namely Gotik, Komet and Zekon (Figure 3). The isozyme spectrum of cv. Gotik consisted of nine scorable (well expressed) bands (Figure 3A) with alterations (i.e. presence/absence of bands) of isozymes in position 1, 2 and 3 in the cathodic region, and in position 4 and 5 in the anodic region. The isozyme spectrum

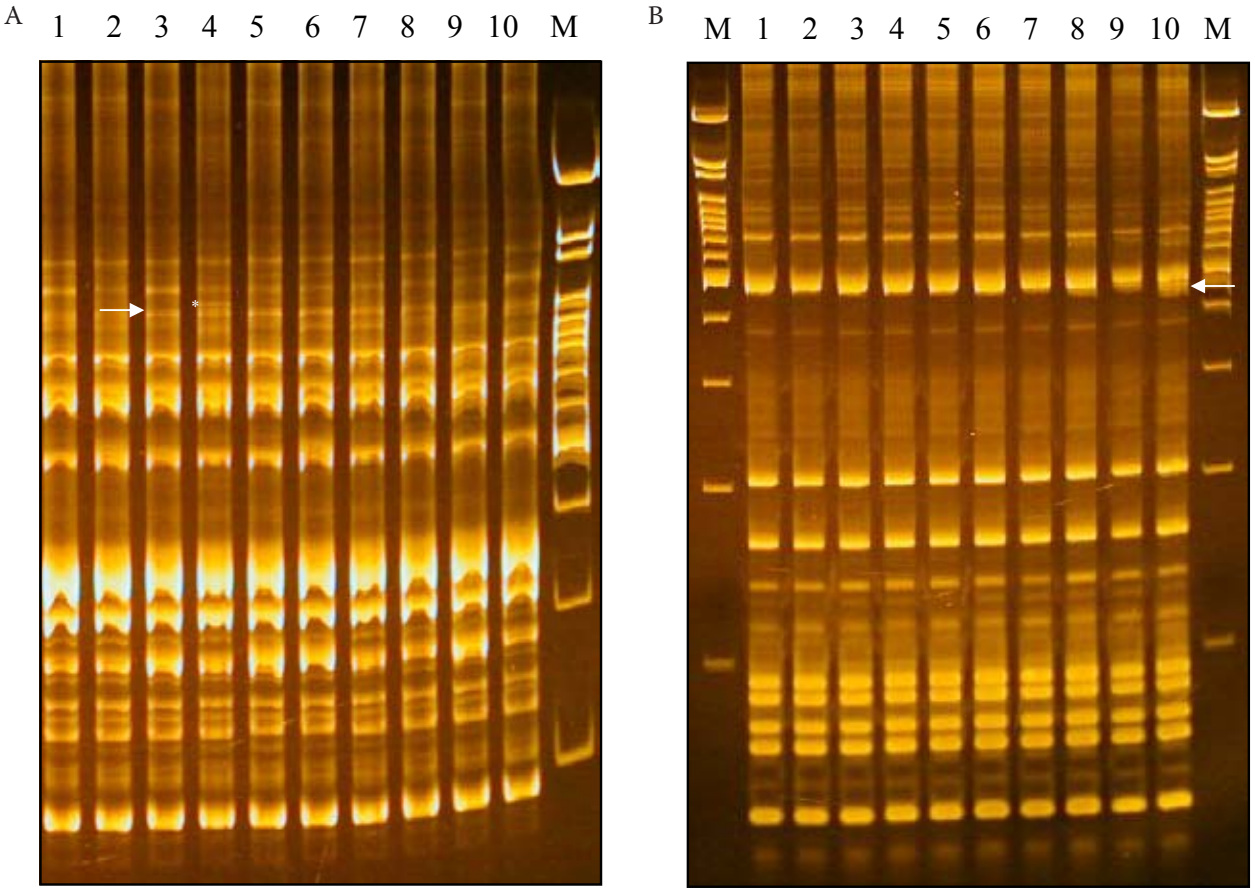

Figure 5. Intravarietal variation of IRAP and ISSR markers; A – cv. Zekon, IRAP-*Ogre* retrotransposon marker; B – cv. Zekon, ISSR-$(GA)_9C$ marker; ten selected plants of each cultivar grown at Šumperk location in 2001 were analyzed separately; arrows indicate qualitative/quantitative changes in DNA markers

in cv. Komet consisted of nine bands; similarly like in cv. Gotik, the variation in the cathodic region in position 1, 2, 3 was detected (Figure 3B). In cv. Zekon, the variation was observed in the cathodic region again (position 1, 2, 3). In position 4 (central part of the gel), the alteration of singlet and doublet was observed (Figure 3C).

Intravarietal variability of DNA markers

RAPD: The studied pea cultivars showed a high degree of intravarietal stability with the utilization of selected RAPD primers (Figure 4). Only very small qualitative differences were recorded in cv. Canis (primers OPW02 and UBC741; Figure 4A, B), Sonet (primer UBC556; Figure 4C) and Gotik (primer UBC741; Figure 4D).

ISSR: ISSR analyses did not practically show any intravarietal variation within the samples of tested cultivars. Detailed scoring of gels may reveal extremely subtle changes as shown e.g. in cv. Zekon (Figure 5A).

SSR: The selected primer pair for highly polymorphic loci did not show any intravarietal variation (data not shown).

IRAP: Retrotransposon IRAP analyses with selected primer combinations did not reveal any intravarietal variation. Similarly like in ISSR analyses, detailed scoring of gels may reveal extremely subtle changes as shown e.g. in cv. Zekon (Figure 5B).

DISCUSSION

Suitability of used enzymes for pea cultivar identification

Despite of the various electrophoresis approaches (lactate-PAGE, starch ELFO, PAGE ELFO) used by various authors, EST seems to be the most polymorphic and thus the best utilizable enzyme

for discrimination of plant cultivars both in grain legumes (Parzysz *et al.* 1985; Pošvec & Griga 2000) and other crops. Out of total 45 commercial pea varieties, the use of EST alone enabled to distinguish unambiguously 19 (Pošvec & Griga 2000). Bernardo *et al.* (1997) found EST to be the most polymorphic and informative enzyme in barley with a high discrimination potential (as compared to PRX, ACP, GOT, MDH). The combination of three EST loci and three hordein (endosperm storage protein) loci practically covered a great part of the studied barley collection. The combination of EST and LAP was successfully used for discrimination of bulked seed samples of closely related varieties (synthetic populations) of allogamous chicory (Baes & Van Cutsem 1992). Based on their experiments, Kumar *et al*. (2007) proposed seed esterases in pearl millet as a complementary characteristic for DUS tests, because they are highly polymorphic among pearl millet cultivars, stable over generations, unaffected by environment and not associated with any morphological characters.

Environmental and year-to-year effect on protein stability/variability

Environmental factors such as temperature, rainfall or nutrient availability/deficiency may immediately alter the seed protein composition and quantity in legumes, cereals or oilseed crops (Singh *et al*. 1983; Comfort 1998; Pritchard *et al.* 2000; Mandal & Mandal 2000). Nutrient deficiency, particularly of sulphur, selectively and markedly depresses sulphur-rich proteins compared to other seed proteins in rape, legumes and cereals (Mandal & Mandal 2000; Nikolova *et al.* 2000). However, the information about such short-term environmental effects on changes in seed isozyme patterns is limited.

From the long-term aspect, it was reported in some species (*Avena barbata*, *Hordeum vulgare*, *H. spontaneum*, *Triticum dicoccoides*, *Abies alba*) that both the temperature and the rainfall are significantly associated with specific allelic combinations of allozymes (e.g. EST, PGD, LAP PRX, MDH, GOT) in the process of the plant adaptedness to climatic and edaphic environment (Pérez de la Vega 1996 and references herein). It was demonstrated e.g. in *Hordeum spontaneum, Triticum dicoccoides* or *Aegilops peregrina* that isozyme polymorphisms are distributed non-randomly, that at least a part of this variation should be adaptive in response to environmental challenges, and correlated with a range of ecogeographical factors (Nevo *et al.* 1981, 1983, 1994 and other papers of the team). Later, the same team demonstrated that also DNA polymorphisms (RAPD, SSRs; *H. spontaneum*, *T. dicoccoides*) might be environmentally adaptive and driven by natural selection (Owuor *et al*. 1999; Li *et al.* 2002 and other papers of the team). Vaz *et al.* (2004) speculated that polypeptide patterns (globulins and glutelins) in white lupin seeds could reflect microclimatic specificities related with altitude and temperature. Nevertheless, the direct relationship between isozymes (and other molecular genetic markers) and adaptation has been an uneasy hypothesis. All effects mentioned above are connected with the long-term adaptation process of wild plant species populations in contrasting environments, not with an immediate reaction to climatic, edaphic or biotic factors.

Farooq and Sayyed (1999), while analyzing leaf peroxidases in cotton (self-pollinated, facultatively cross-pollinated species) found significant differences in PRX activity (= band presence/absence or intensity) within 10 analyzed plants of a particular variety grown at a particular location and within the plants of the same variety grown at different locations. As PRXs participate in a number of physiological processes including defence reactions to biotic and abiotic stresses, the authors speculated about the effect of temperature as well as of disease infection (cotton leaf curl virus) effect on the recorded intravarietal isozyme variation (unfortunately, the seed peroxidases were not studied). Gates and Boulter (1979) concluded that – except of small quantitative differences – EST and GOT zymograms from faba bean cotyledons were identical for the material grown in the field, greenhouse and growth chamber. After 3-year seed storage at 4°C seed cotyledon EST and GOT zymograms were identical with those of freshly harvested material. This absence of sensitivity to environmental fluctuations is essential in any biochemical marker intended for use in a discrimination programme, and in this respect the seed tissue is generally superior to the vegetative tissue. Oleo *et al*. (1992) also questioned the stability of sugar beet seed enzyme (ADH, MDH, ACO) fingerprints across various environments (Denmark, Italy, USA; 1982) and various years of cultivation (two successive years 1981, 1982; Denmark). A very small

impact of tested environments on the stability of isozyme spectra was recorded; in addition, tested lines (isozyme spectra) remained stable across the years studied. Hussain *et al.* (1988) was probably the first to study the effect of location (environment) and year of cultivation on the stability of seed protein patterns in pea (cotyledon proteins; lactate PAGE). Two cultivars of field pea Tipu and Triumph were grown at four different locations in Canada (Portage, Morden, Saskatoon and Melfort) in two years (1986, 1987). The authors concluded that the location and year of cultivation had no effect on the electrophoretic patterns.

The Šumperk location is characterized by the highest altitude, the lowest long-term average annual temperature, the highest long-term average rainfall and the most acidic soil (Table 2). Nevertheless, except of a small change in the EST spectrum in cv. Zekon (absence of a band in position 2, Figure 1), no evident changes were found between Šumperk and the other two locations. Environmental differences between tested locations in the Czech Republic are probably negligible as compared to the above-mentioned reports (Hussain *et al.* 1988, Oleo *et al*. 1992). The years 2001 and 2002 were richer in rainfall and warmer than the years 2003 and 2004 (Table 3), which may be connected with a lower expression of EST isozyme in the central part of the gel (position A) in cv. Zekon (Figure 2A). 2003 was the driest year – additional band in cv. Gotik in position 2 (Figure 2C), weak band in position "A" in Zekon and Komet (Figure 2).

Causes of intravarietal variability in isozyme patterns and DNA markers

Heterogeneity, or intravarietal diversity, is a negative factor affecting the purity of a cultivar. This intravarietal heterogeneity is much higher in cross-pollinated crops as compared to self-pollinated ones. Cultivars of outbreeding/outcrossing crops are usually heterogeneous, which can be a barrier to their accurate identification. A bulk sample is a composite seed sample that represents the mixture of electrophoretic phenotypes present in a cultivar. For example, the comparison between single seed and bulk electrophoregrams in buckwheat showed that a substantial part of differences among cultivars in bulk electrophoregrams can be explained by differences in the band frequencies revealed by single seed analysis and thus variation was much higher within cultivars than between them (Rogl & Javornik 1996). Similarly, Oleo *et al*. (1992) studied intravarietal (intrapopulation) variability in sugar beet (outbreeding crop) by means of ADH, MDH, ACO enzyme analysis and observed intravarietal variation while analyzing single seeds, however, using bulk samples (20 seeds) almost completely masked the individual genotypic differences.

In self-pollinated crops (frequently composed of one line), the opposite situation should be expected, i.e. high intravarietal uniformity and distinct intervarietal variation. Detected heterogeneity (e.g. for elite seeds of barley cultivars or germplasm resources samples) may be then explained by: (1) physical mixing of different genotypes, either at a collection site or methodological errors during later seed handling, (2) genotypic segregation of spontaneous intergenotypic hybrids (up to 5% in winter barley), (3) maintenance of different homozygotic genotypes produced by spontaneous variation via mutation (Bernardo *et al.* 1997, Van Treuren & Van Hintum 2001). Similarly, Echart-Almeida and Cavalli-Mollina (2000) considered the high degree of intravarietal variation in the seed storage protein hordein and isoenzymes in Brazilian barley cultivars as a consequence of the selection process when a new barley cultivar is finished in the F_5 or F_6 generation – thus, a part of polymorphism may be explained by residual heterozygosity. The samples in our experiments were obtained directly from breeders (or from the germplasm collection), and thus we could not exclude the above-mentioned causes of cultivar heterogeneity.

Parzysz *et al.* (1985) studied EST spectra in cotyledons of pea dry seeds from plants grown in the greenhouse in the same year (elimination of year-to-year effect). The analysis of single seeds from plants showed intraline or intrapopulation variation of EST spectra. Using leaf isozymes for pea cultivar discrimination Swiecicki and Wolko (1987) stated "that it should not be surprising that in pea, diploid and self-pollinating species, individuals with different enzyme alleles of one locus exist in the population (even in the theoretically pure line), because these concern genes are not considered during selection". The authors also studied pea line Wt 4367 polymorphic in LAP, GOT and PGD, with a certain frequency of alleles of polymorphic genes in two consecutive years – the analysis confirmed a stable frequency of alleles of

the studied genes. Thus, pea varieties, being pure lines with regard to the number of characters/genes considered during the breeding process, can be polymorphic from the aspect of the isozyme loci (unfortunately no data dealing with seed isozymes were provided). The same authors (Wolko *et al.* 2000) tried to discriminate 33 pea cultivars via the isoenzyme analysis (15 enzyme systems, namely AAT, ACP, ADH, ALDO, DIA, EST, GAL, GPI, LAP, MDH, PGM, PGD, SKDH, SOD, and TPI) of extracts of young leaves. Intravarietal variation (separate analysis of ten individual plants) was observed in 9 cultivars in PGD, ALDO, AAT, LAP, GAL, PGM; in some cultivars the isozyme phenotypes typical of heterozygous genotypes were recorded for EST and GAL (no data dealing with seed isozymes were provided again).

Smýkal (2006) tested eight pea varieties for intra-accession variation by IRAP markers. Five individual plants per variety (leaves) were sampled and further cultivated to obtain a subsequent seed generation in controlled greenhouse conditions. No alteration of the major scorable band patterns was observed among 5 individual plants of the given variety and in two subsequent generations. The only detected variation was in minor low-intensity fragments of less than 150 bp and more than 800 bp, corresponding to the reproducibility level of IRAP technique.

In our latest paper (Smýkal *et al*. 2008b) we have tested intra-accession variability in 5 greenhouse-grown pea varieties (Merkur, Jackpot, Grana, Garde and Zekon) by analysing 16 plants (leaf samples) from each variety. Using 3 SSR loci and IRAP markers system (*Cyclop*, *Ogre*), no alterations in polymorphic patterns were found, thus indicating a high stability of tested varieties. Given the fact that plant breeders have to breed for genetic uniformity during cultivar development, the subtle intravarietal variation detected e.g. by AFLP might be considered a background noise, possibly resulting from small changes in DNA or minor methodological errors (Van Treuren & Van Hintum 2001).

CONCLUSION

Out of the six studied enzymes, five (ACP, ADH, LAP, SDH, PGI) exhibited a low level of polymorphism and they were not affected either by location or by the year of cultivation. EST spectra were negligibly affected by geographic location, but they were affected by the year of cultivation to a larger extent. Intravarietal variation of EST spectra was recorded. Qualitative changes were usually connected with less expressed isozymes, while robust ones were not practically affected. Intravarietal variation studied by selected DNA markers was negligible. Thus, with the use of isoenzyme markers and dry seeds as samples for analysis, it is necessary to take into account certain intravarietal variation caused by sample non-homogeneity (mixture of genotypes/lines within a particular variety) and particularly by year-to-year effect.

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