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# Protein patterns associated with *Pisum sativum* somatic embryogenesis

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## Abstract

Total protein patterns were studied in the course of development of pea somatic embryos using simple protocol of direct regeneration from shoot apical meristems on auxin supplemented medium. Protein content and total protein spectra (SDS-PAGE) of somatic embryos in particular developmental stages were analysed in *Pisum sativum*, *P. arvense, P. elatius* and *P. jomardi*. Expression of seed storage proteins in somatic embryos was compared with their accumulation in zygotic embryos of selected developmental stages. Pea vegetative tissues, namely leaf and root, were used as a negative control not expressing typical seed storage proteins. The biosynthesis and accumulation of seed storage proteins was observed during somatic embryo development (since globular stage), despite of the fact that no special maturation treatment was applied. Major storage proteins typical for pea seed (globulins legumin, vicilin, convicilin and their subunits) were detected in somatic embryos. In general, the biosynthesis of storage proteins in somatic embryos was lower as compared to mature dry seed. However, in some cases the cotyledonary somatic embryos exhibited comparatively high expression of vicilin, convicilin and pea seed lectin, which was even higher than those in immature but morphologically fully developed zygotic embryos. Desiccation treatments did not affect the protein content of somatic embryos. The transfer of desiccated somatic embryos on hormone-free germination medium led to progressive storage protein degradation. The expression of true seed storage proteins may serve as an explicit marker of somatic embryogenesis pathway of regeneration as well as a measure of maturation degree of somatic embryos in pea.

*Additional key words*: embryo development, pea, seed proteins, storage substances, zygotic embryogenesis.

## Introduction

Morphological and physiological quality of mature somatic embryos affects their germination and subsequent seedling growth and development, and thus the efficiency of somatic embryogenesis-derived technologies. Among others, the accumulation of storage substances typical for sexual seed is guarantee of proper physiological state. This process may be significantly influenced by previous *in vitro* treatments during induction, development and maturation phase (Lai and McKersie 1994a,b). In legumes, these storage substances are represented mainly by proteins and starch (Casey *et al*. 1993, Vitale and Bollini 1995), in some legume species (soybean, peanut) also by lipids. Expression of storage proteins is under strong regulation control. It is restricted to specific storage tissue in the seed as well as to precise developmental stage. There is no evidence that any genes for true seed storage proteins (those with no other biological role) are expressed in any other plant tissue (Shewry and Casey 1999). Thus, the deposition of specific seed-storage proteins (mainly globulins in legumes) is considered as a biochemical marker of somatic embryo quality and maturity. In addition, specific seed-storage proteins may also serve as a clear marker of embryogenic pathway of regeneration (Stuart *et al*. 1988, Komatsuda *et al*. 1992, Krochko *et al*. 1992, Lai *et al*. 1992), because vegetative structures formed during *de novo* organogenesis (shoot, root) do not synthetize true seed storage proteins.

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*Abbreviations*: MSB-medium - medium with mineral salts after Murashige and Skoog (1962) and vitamins after Gamborg *et al*. (1968); SDS-PAGE - sodium dodecylsulphate polyacrylamide gel electrophoresis; SE - somatic embryo; ZE - zygotic embryo.
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There are several reports on proteins in leguminous plants associated with somatic embryogenesis, referring primarily to the improvement of somatic embryos (based on similarity with their zygotic counterparts) for their better regeneration (review Griga 1999). The main portion of knowledge accumulated so far has been obtained with alfalfa (Stuart *et al*. 1988, Krochko *et al*. 1992, 1994, Lai *et al*. 1992, Lecouteux *et al*. 1993, Lai and Mckersie 1994a,b) and soybean (Christou and Yang 1989, Slawinska and Obendorf 1991, Komatsuda *et al*. 1992, Dahmer *et al*. 1992, Stejskal and Griga 1995, Chanprame and Widholm 1996), which represent traditional experimental models for somatic embryogenesis in forage and grain legumes.

Despite the fact that first report on pea somatic embryogenesis was published more than twenty years ago (Jacobsen and Kysely 1984), and complete plant regeneration in pea *via* embryogenic pathway three years later (Kysely *et al*. 1987), there is a lack of information on biochemical aspects of the process. The same is true of its comparison with zygotic embryogenesis and sexual seed development. Stirn and Jacobsen (1987, 1990) searched for protein markers associated with embryogenic differentiation in pea callus cultures studying also the potential role of soluble auxin receptors (proteins binding specifically active auxins) in auxin-induced pea somatic embryogenesis (Jacobsen 1991). Only a conference abstract is available on pea somatic embryos storage proteins (Lehminger-Mertens *et al*. 1990). The authors found roughly the same protein profiles for both somatic and zygotic embryos deducing almost analogous development of both embryo types. Some preliminary data on storage substances accumulation/metabolism during pea somatic embryogenesis were presented by our laboratory (Griga *et al*. 2001, 2002).

Recently, the protocol of direct pea somatic embryogenesis is relatively well elaborated (Griga 1998). This has enabled to produce sufficient amounts of somatic embryos in morphologically well characterized developmental stages (Griga 2002). In this report we describe the total protein patterns associated with pea somatic embryogenesis and their partial comparison with zygotic embryogenesis.

## Materials and methods

**Plants:** Five dry-seed pea (*Pisum sativum* L.) cultivars (HM-6, Menhir, Junák, Tolar, Komet) and five canning pea cultivars (Oskar, Citrina, Countess, Progreta, Vladan) with previously recorded good embryogenic competence (Griga 1998) were used in the experiments (Table 1). More detailed analyses were additionaly done with two highly embryogenic ones – HM-6 and Oskar, mainly because of their good production of somatic embryos of particular developmental stages necessary for sampling. To study storage protein biosynthesis in zygotic embryos, the additional *Pisum* cvs./taxons were included, namely dry-seed pea cv. Solara, canning pea cv. Moravan, fodder peas (*Pisum arvense* L.) cvs. Algera and Andrea, and wild peas (*P. jomardi* Schrank, *P. elatius* Stev.) (Table 1).

**Tissue culture, somatic embryo production, desiccation treatments:** Direct somatic embryos were induced from shoot apical meristems on MSB-medium with 2.5 μM picloram (2 weeks) and then subcultured on MSB-medium without phytohormones for further development (2 weeks) as reported previously (Griga 1998). No special maturation treatments were applied. Somatic embryos in morphologically well characterized developmental stages (globular, heart-shaped, torpedo, cotyledonary stage; Griga 2002) were sampled, measured and weighed. In addition, the following tissues/samples were collected for analysis: *1*) the callus accompanying direct somatic embryogenesis (usually formed on the basal, cut part of an apical meristem), *2*) swollen apical meristematic dome after separation of callus and somatic embryos, and *3*) morphologically abnormal cotyledonary somatic embryos. The samples were analysed either immediately or frozen in liquid nitrogen and then stored (-80 °C) before analysis. The leaves and roots from greenhouse-grown pea plants of a particular cultivar were analysed for comparison as vegetative tissue samples.

Simple desiccation treatments of torpedo/cotyledonary stage somatic embryos were carried out. First approach (rapid desiccation) consisted of placing isolated somatic embryos in a sterile Petri dish for one week at 20 °C either in the dark or in 16-h photoperiod (irradiance 20.4 μmol $m^{-2}$ $s^{-1}$) (Griga 1990). In the second approach (slow desiccation), SEs were cultured 2 weeks on basal MSB-medium supplemented with 10 % m/v sucrose (Griga and Stejskal 1994, Stejskal 1994) in similar light regimes as mentioned above. The FM of somatic embryos before and after desiccation was recorded. Desiccated somatic embryos were sampled for analysis immediately after desiccation treatment and after further 2 weeks-culture on basal MSB-medium with 3 % m/v sucrose (germination test).

**Plant cultivation, zygotic embryo isolation:** Seeds of studied pea cvs. were sown in a greenhouse in April. The plants were grown in Mitscherlich vessels (8 - 10 plants per vessel, 16 vessels per cv.) with commercial cultivation substrate (*Agro CS a.s.*, Česká Skalice, CR). Zygotic embryos in four developmental stages corresponding to stage 21 - 22 (early to late heart stage, 0.6 - 1.0 mm; designation I), stage 22 - 23 (early cotyledon expansion, 2.0 - 2.5 mm; II), stage 23 (mid maturation stage, 4.0 - 4.5 mm; III) and stage 24 (late-maturation stage, 6.0 - 6.5 mm; IV) (Marinos *et al.* 1970, Liu *et al*. 1996) were isolated during June and July according to flowering/fruiting time of particular pea cultivars. Size and fresh mass of particular classes of

zygotic embryos were recorded and samples were analysed immediately or frozen in liquid nitrogen and then stored (-80 °C) before analysis.

**Protein content:** The protein content of developing somatic embryos was determined according to Bradford (1976). Samples of somatic embryos (10 mg) were homogenized with 0.1 M phosphate-buffered saline (total amount 0.2 $cm^3$, pH 7.2), frozen for 2 h (-80 °C), and after melting centrifuged for 10 min at 15 000 *g*. 0.1 $cm^3$ of supernatant was pipetted into test tube and 5 $cm^3$ of protein reagent was added and mixed by gentle vortexing. Then, absorbance ($A_{595}$) was measured by spectrophotometer (*Eppendorf AG*, Hamburg, Germany) against the reagent blank between 2 min and 1 h after mixing. The amount of protein was calculated using the calibration curve, prepared with a solution of BSA (bovine serum albumin; *Reanal*, Budapest, Hungary) in the range from 0.1 to 1.0 mg $cm^{-3}$. Data were statistically analysed using *UNISTAT* (London, England) software by LSD method (Tukey´s honestly significant difference test).

**Total protein electrophoresis:** Samples of somatic and zygotic embryos as well as other analysed tissues (10 mg) were homogenized with 0.05 $cm^3$ of Tris-HCl buffer (pH 6.8) containing 5 % 2-mercaptoethanol and 2 % sodium dodecylsulphate (SDS). The samples were boiled for 5 min in water and then centrifuged for 10 min at 15 000 *g*. Electrophoretic analyses were carried out according to Laemmli (1970). Proteins were separated using discontinuous SDS-PAGE (12 % running gel, pH 8.8 and 5 % stacking gel, pH 6.8) at 8 °C. Tris-glycin buffer (pH 8.3) containing 0.1 % SDS was used as an electrode solution. Electrophoretic separations were performed at constant current 25 mA until tracking dye (bromophenol blue) had reached the end of the gel. After electrophoresis, the gels were stained overnight in solution of 0.25 % Coomassie Brilliant blue R250. Finally, gels were de-stained, fixed and scanned or photographed.

Table 1. Characterization of peas used in the experiments. Embryogenic competence of particular cultivars is expressed as a mean frequency of somatic embryogenesis based on previously published extensive experiments (Griga 1998).

| Species and types | Cultivars | Embryogenic competence [%] | Seed shape, cotyledon colour | Origin |
|---|---|---|---|---|
| *Pisum sativum* | HM-6 | 50.4 | smooth, yellow | Oseva Praha, CR |
| dry-seed pea | Junák | 17.8 | smooth, yellow | ŠS Horná Streda, SR |
| | Komet | 10.0 | smooth, yellow | Selgen Praha, CR |
| | Menhir | 23.3 | smooth, yellow | Selgen Praha, CR |
| | Solara | 4.2 | smooth, green | Cebeco, Netherlands |
| | Tolar | 32.2 | smooth, yellow | Oseva Praha, CR |
| *Pisum sativum* | Citrina | 29.6 | wrinkled, green | DDR |
| canning pea | Countess | 14.2 | faveolate, yellow | GBR HURST |
| | Moravan | 0 | wrinkled, green | Semo Smržice, CR |
| | Oskar | 20.8 | wrinkled, green | Semo Smržice, CR |
| | Progreta | 12.6 | faveolate, green | GBR |
| | Vladan | 5.0 | wrinkled, green | Semo Smržice, CR |
| *P. arvense* | Algera | 4.2 | smooth, yellow | Selgen Praha, CR |
| fodder pea | Andrea | 22.5 | superficially wrinkled, yellow | Selgen Praha, CR |
| *P. elatius* | wild form | 6.7 | smooth, yellow | Agritec Pea Collection |
| *P. jomardi* | wild form | 10.0 | superficially wrinkled, yellow | Agritec Pea Collection |

## Results and discussion

**Morphological parameters of developing somatic embryos:** Despite the fact that morphology, anatomy and histology of pea somatic embryos was described in detail (Kysely and Jacobsen 1990, Loiseau *et al*. 1998, Griga 2002) and basic developmental stages (globular, heart-shaped, torpedo and cotyledonary stage) may be distinguished, a number of both intermediate morphological types and morphological abnormalities have occured in extensive experiments (Griga 2002). The selection of embryos of strictly morphologically defined stages would drastically limit an amount of samples for analyses. Thus, only morphological extremes were excluded from analyses or they were evaluated as a separate category (abnormal or irregular cotyledonary stage SEs). Besides of a typical morphology, the size and fresh mass were used as descriptors which relatively well characterize particular developmental stages (Fig. 1*A*,*B*).

**Protein content of developing somatic embryos:** The protein content of somatic embryos varied between 10 to 25 μg $mg^{-1}$(f.m.). The mean values of protein content of SEs in particular developmental stages from two independent experiments with line HM-6 are illustrated in Fig. 2. This range is similar to the protein content of

developing alfalfa (Lai *et al*. 1992) or soybean (Stejskal 1994) somatic embryos. The protein content of callus accompanying embryo formation on shoot apical

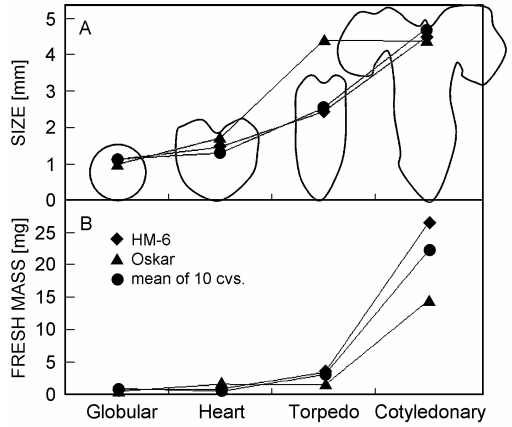


Fig. 1. Size (*A*) and fresh mass (*B*) of somatic embryos in different developmental stages. The graphs represent means for dry-seed pea line HM-6, canning pea cv. Oskar and pooled data for ten pea cultivars. The particular developmental stages were determined and collected based on typical morphology illustrated in *A*.

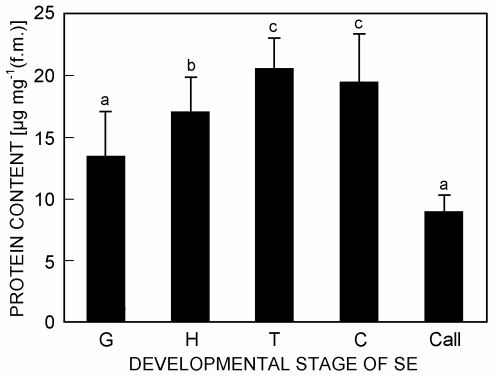


Fig. 2. Protein content in developing somatic embryos of dry-seed pea line HM-6. G - globular, H - heart–shaped, T - torpedo, C - cotyledonary stage, Call - callus (swollen meristematic explant after SE isolation or non-embryogenic explant with callus). Means ± SE of two independent experiments. Different letters indicate statistically different values ($P < 0.05$) according to Tukey´s HSD interval test.

meristem explants never exceeded 10 µg $mg^{-1}$(f.m.) and was significantly different from heart-stage, torpedo and cotyledonary stage SEs, but not from globular SEs (Fig. 2). Expected continual increase of protein accumulation starting with globular stage and culminating with cotyledonary stage SEs was recorded only in one experiment (line HM-6; data not shown). However, the most frequent situation was represented by the maximum and similar protein accumulation in both torpedo and cotyledonary stage SEs (Fig. 2). Nevertheless, there was repeatedly observed trend for a decrease (statistically non-significant) of protein level in cotyledonary stage SEs (Fig. 2). The explanation of such phenomenon may be based on the comparison to storage compounds biosynthesis during pea zygotic embryo development. The main pea seed storage organ is a cotyledon which grows first by cell division (connected with organ formation) and later by cell expansion (connected with maturation). Storage compounds are accumulated in parenchymatous storage cells of the cotyledons. According to Pate (1975), storage product synthesis is not initiated in the cotyledons until the phase of cell division is completed. Accumulation of globulins (key pea storage proteins) in the Pate´s (1975) scheme (in Wang and Hedley 1993) is a relatively late event starting at the stage of maximum pod fresh mass and before maximum seed fresh mass, and culminating with maximum seed dry mass accumulation. Finally the seeds go through drying process and enter a quiescent phase. There is a strong influence of maternal tissues (mediated mainly by testa) on the whole embryo development and final seed size (expressed also by number of cells; Wang and Hedley 1993). In contrast, in pea somatic embryos (as well as in another leguminous species), the cotyledons do not represent major part of fully morphologically developed individual – this is represented by robust hypocotyl (Krochko *et al*. 1992, Griga 1999, 2002). In addition, the absence of maternal tissue (mainly testa) function cannot be simply replaced by culture medium – thus, by the analogy, the presence of storage proteins in globular SEs (see below and Figs. 4, 5) may be probably a sign that cell division is going to be almost completed and storage substances start to form inside expanding cells. However, in the absence of maturation/desiccation conditions, the cotyledonary SEs (sometimes already torpedo SEs) enter continually germination phase (precocious germination). Thus, the protein biosynthesis *in vitro* is probably terminated at torpedo stage (this stage is absent in pea zygotic embryogenesis *in planta* – Liu *et al*. 1995, 1996) and the cotyledonary somatic embryos start in fact to germinate (it is still not visible morphologically as a root or shoot development), which is characterized by storage substances breakdown (Griga *et al*. 2001). Also according to Stuart *et al*. (1988), SEs do not show dormancy, but usually germinate precociously – storage protein breakdown acts as an objective diagnostic for the onset of germination. The trends in protein accumulation/ expression in developing pea somatic embryos may be also deduced from the electrophoreograms (Figs. 4, 5, 6, and unpublished data). Decrease of protein content during development of soybean somatic embryos as related to both FM and DM was reported by Stejskal (1994) and Chanprame *et al*. (1998).

Somatic embryos desiccated by both approaches exhibited a trend to slight increase of the protein content as related to non-desiccated control; nevertheless, this increase was not statistically significant (Fig. 3). In a rapid drying approach, only loss of water content and no further protein biosynthesis was expected. In contrast, culture of torpedo/cotyledonary SEs on medium with elevated sucrose level (slow osmotic desiccation) may

lead to prolonged protein biosynthesis (Komatsuda *et al*. 1992). Stejskal (1994) reported a significant increase in protein content in soybean somatic embryos from long-term repetitive culture matured/desiccated on medium with 10 % sucrose. We did not observed differences in protein content of pea SEs at the end of desiccation between particular treatments (Fig. 3). The germination of desiccated SEs (medium with 3 % sucrose) was connected with the breakdown of proteins; rapid drying approach in the dark followed by germination resulted in the most dramatic decrease of protein content as compared to both rapid and slow light treatments as well as to control, *i.e*. non-desiccated germinating SEs. Protein content data (Fig. 3) were related to key storage proteins expression in particular electrophoreograms (see text below and Figs. 8 and 9).

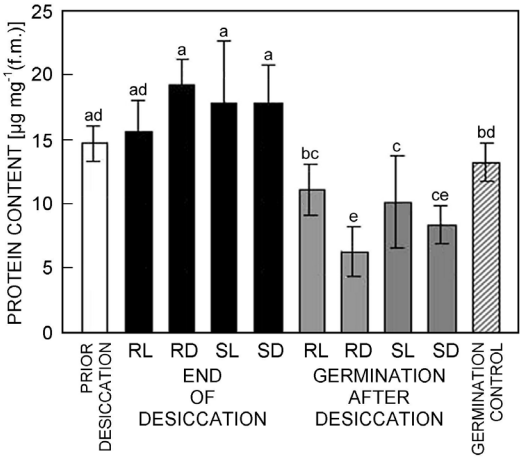


Fig. 3. Protein content changes in SEs during desiccation treatments and following germination. Torpedo/cotyledonary stage SEs were treated by rapid or slow desiccation procedure. RL - rapid desiccation, light; RD - rapid desiccation, dark; SL - slow desiccation, light; SD - slow desiccation, dark. Means ± SE for two pea genotypes (line HM-6 and cv. Oskar). Different letters indicate statistically different values ($P < 0.05$) according to Tukey´s HSD interval test.

**Composition of total proteins in developing somatic and zygotic embryos:** While analyzing total proteins, the key storage proteins (or their subunits/polypeptides) of pea seed could be detected in somatic embryos (Fig. 4, 5). They included mainly 7S and 11S class globulins - vicilin (major subunits $M_r$ ~ 47 000 - 50 000; minor subunits $M_r$ ~ 25 000; 30 000; 34 000), convicilin ($M_r$ ~ 70 000 - 75 000), acidic legumin α ($M_r$ ~ 40 000) and basic legumin β ($M_r$ ~ 20 000) (Casey *et al*. 1986, 1993, Vitale and Bollini 1995, Casey and Domoney 1999). Other proteins abundantly located in storage parenchyma cells of cotyledons, which may also behave as storage proteins are lectin β ($M_r$ < 20 000) and lipoxygenase ($M_r$ ~ 97 000) – (Casey *et al*. 1993, Casey 1999, Peumans and Van Damme 1999). In general, the expression of storage proteins in pea somatic embryos was usually lower as compared to mature dry seed; however, there were quantitative differences between particular experiments (compare Fig. 4, 5). Comparably lower protein expression in somatic embryos than in mature seeds was reported also in soybean (Dahmer *et al*. 1992) and alfalfa (Stuart *et al*. 1988, Krochko *et al*. 1992, 1994). In contrast to other reports comparing total proteins of leguminous SE and seed, where the qualitative differences in protein patterns were observed (Christou and Yang 1989, Dahmer *et al*. 1992, Stejskal and Griga 1995), the protein patterns of both pea SE and seed exhibited relatively high degree of similarity/identity. The differences observed between SE and seed proteins could be caused by, *e.g*., comparing fully matured (dry) seeds with cotyledonary stage (non-dried) SEs. This speculation may be supported by comparing SEs with immature ZEs (Fig. 5), where some similarities could be observed. In vicilins, major subunits in the $M_r$ ~ 47 000 - 50 000 correspond to the first products of synthesis, while all the smaller subunits originate from the precursor polypeptides *via* extensive posttranslational fragmentation (proteolytic processing) (Vitale and Bollini 1995). Thus hypothetically, the extent and the course of posttranslational modifications undergone *in vitro* may led to minor changes/differences in size and quality (*e.g*. glycosylation) of resulting polypeptides as compared to seed *in planta*.

A strict difference between embryogenic and somatic (root, leaf) tissues was quite evident (Figs. 4, 5). There was an absence of practically all key storage proteins/polypeptides in somatic tissues and these tissues expressed some polypeptides not recorded in somatic embryos and seed. Callus accompanying direct somatic embryogenesis exhibited lower expression of total proteins and a little bit altered protein spectrum as compared to embryogenic or somatic tissues. Protein profile of original explant (*i.e*. shoot apical meristem after removing somatic embryos) exhibited character of somatic tissue, but shared many protein bands typical for embryogenic tissues (Fig. 4).

From the point of view of developmental expression of main storage proteins in somatic embryos, there was not recorded a dramatic increase from globular to cotyledonary stage (Fig. 4). Interestingly, globular SEs of cv. Oskar expressed comparable or even higher levels (vicilin subunits $M_r$ ~ 47 000 - 50 000 and 30 000, convicilin, lectin β) of proteins (Fig. 4*A*) as compared to more progressed developmental stages. Polypeptide with $M_r$ ~ 52 000 - 55 000 was displayed practically in all samples, both embryogenic and somatic ones. In another independent experiment (Fig. 5), ZEs of three developmental stages (II, III and IV) and also morphologically normal and abnormal cotyledonary stage somatic embryos were included. In contrast to previous analyses (Fig. 4), the expression of convicilin, vicilin and lectin β was highest in cotyledonary stage SEs (both normal and abnormal; Fig. 5). In vicilin, major subunit with $M_r$ ~ 47 000 is more strongly expressed both in seed and in SE as compared to polypeptide with $M_r$ ~ 50 000. It is interesting, that stage II ZEs exhibited higher expression of total proteins that ZEs of developmentally

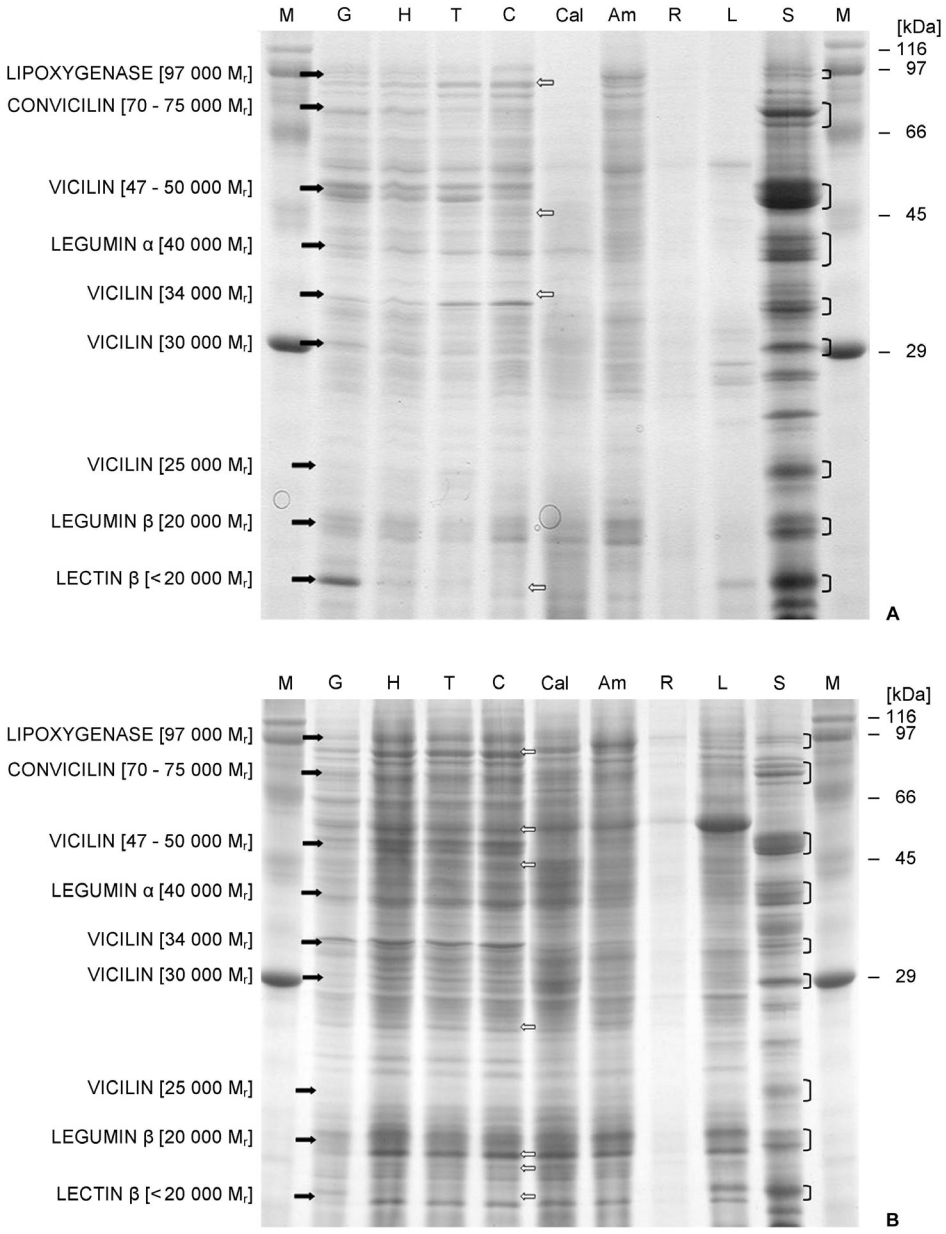


Fig. 4. Protein patterns (SDS-PAGE) of somatic embryos of canning pea cv. Oskar (*A*) and dry-seed pea line HM-6 (*B*). G - globular, H - heart-shaped, T - torpedo, C - cotyledonary stage, Cal - callus accompanying embryogenic explants, Am - primary explant (apical meristem) after SEs and callus isolation, R - root, L - leaf, S - mature sexual seed, M - molecular mass marker. *Bars* and *black arrows* mark the position of main storage proteins. *White arrows* indicate differences (absence × presence) between somatic embryos and mature sexual seed.

more advanced stages (III, IV). Vicilin subunit with $M_r$ ~ 47 000 is surprisingly less expressed in zygotic embryos as compared to somatic embryos. Also lectin β is weakly expressed in zygotic embryos. Polypeptide with $M_r$ ~ 34 000 (minor vicilin subunit) is stably expressed in both somatic and zygotic embryos. The above mentioned data were confirmed by comparison of ZEs in stages I to IV in model genotypes Oskar and HM-6 (Fig. 6), where the expression of total proteins including storage ones was higher in ZEs of stage II, or even in stage I (HM-6) as compared to more developmentally progressed ZEs of stages III and IV. A critical moment was a low expression of vicilin major subunit with $M_r$ ~ 47 000. A more detailed view on the vicilin biosynthesis in a spectrum of garden peas (*Pisum sativum*), field peas (*P. arvense*) and wild *P. jomardi* and *P. elatius* showed differences in timing of biosynthesis (Fig. 7). While vicilin subunit with $M_r$ ~ 50 000 is stably expressed in ZEs of all studied stages, the subunit with $M_r$ ~ 47 000 appeared usually in the stage III ZEs and regularly in the stage IV ZEs (strong

signal). The exception was cv. Oskar with negligible expression in ZEs of all stages, and cv. Tolar with strong expression starting with the stage II ZEs. Thus, the fact that SEs of all developmental stages distinctly express both vicilin major subunits (and most of other storage proteins) may signify that they are physiologically "more mature" as compared to their "morphologically relevant" zygotic counterparts (as classified by Marinos *et al.* 1970, Liu *et al*. 1996).

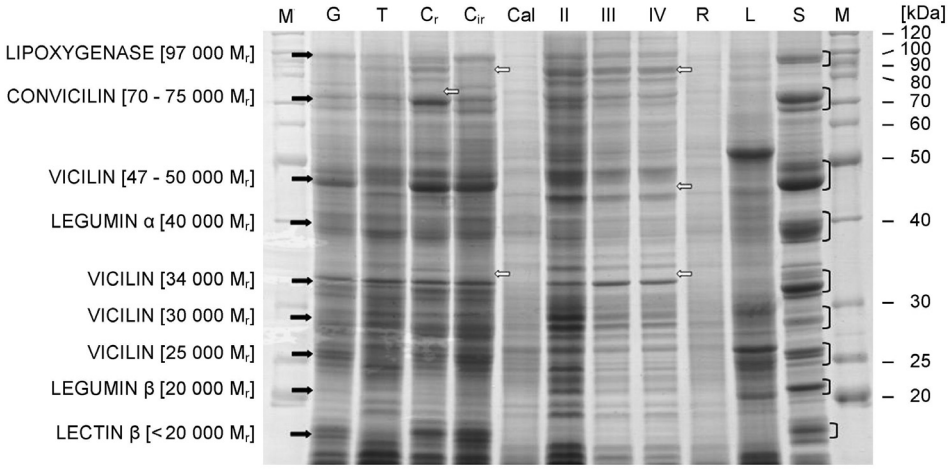


Fig. 5. Protein patterns (SDS-PAGE) of somatic embryos of canning pea cv. Oskar. G - globular, T - torpedo, $C_r$ - cotyledonary stage with regular morphology, $C_{ir}$ - cotyledonary stage with iregular (abnormal) morphology, Cal - callus accompanying embryogenic explants, II, III, IV - stages of zygotic embryos (ZE), R - root, L - leaf, S - mature sexual seed, M - molecular mass marker. *Bars* and *black arrows* mark the position of main storage proteins. *White arrows* indicate differences (absence × presence) between somatic embryos, immature ZEs and mature sexual seed.

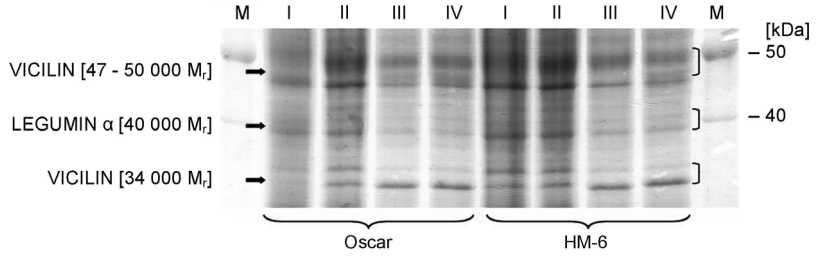


Fig. 6. SDS-PAGE patterns of vicilin ($M_r$ ~ 47 000 - 50 000; 34 000) and legumin α ($M_r$ ~ 40 000) polypeptides of immature zygotic embryos of canning pea cv. Oskar and dry-seed pea line HM-6. I, II, III and IV - stages of zygotic embryos, M - molecular mass marker.

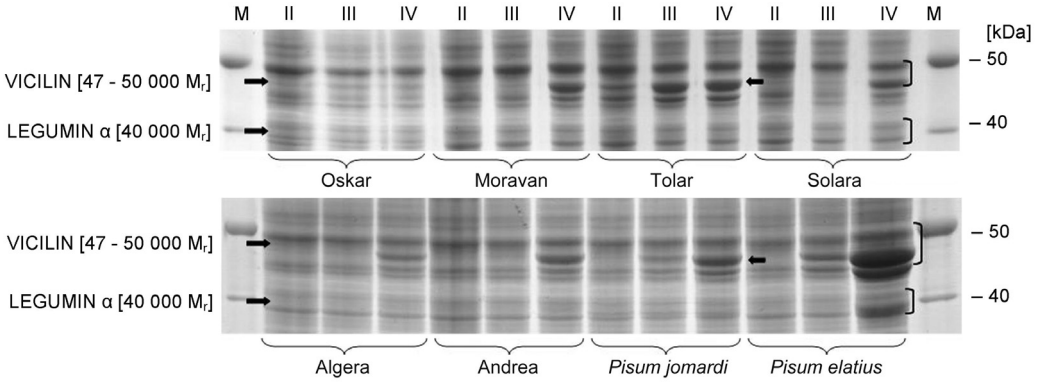


Fig. 7. SDS-PAGE patterns of vicilin ($M_r$ ~ 47 000 - 50 000) and legumin α ($M_r$ ~ 40 000) polypeptides of immature zygotic embryos (ZE) of *Pisum sativum* (cv. Oskar, Moravan, Tolar, Solara), *P. arvense* (Algera, Andrea) and wild pea forms *P. jomardi* and *P. elatius*. II, III and IV - stages of ZE, M - molecular mass marker. See developmental expression of 47 kDa vicilin subunit.

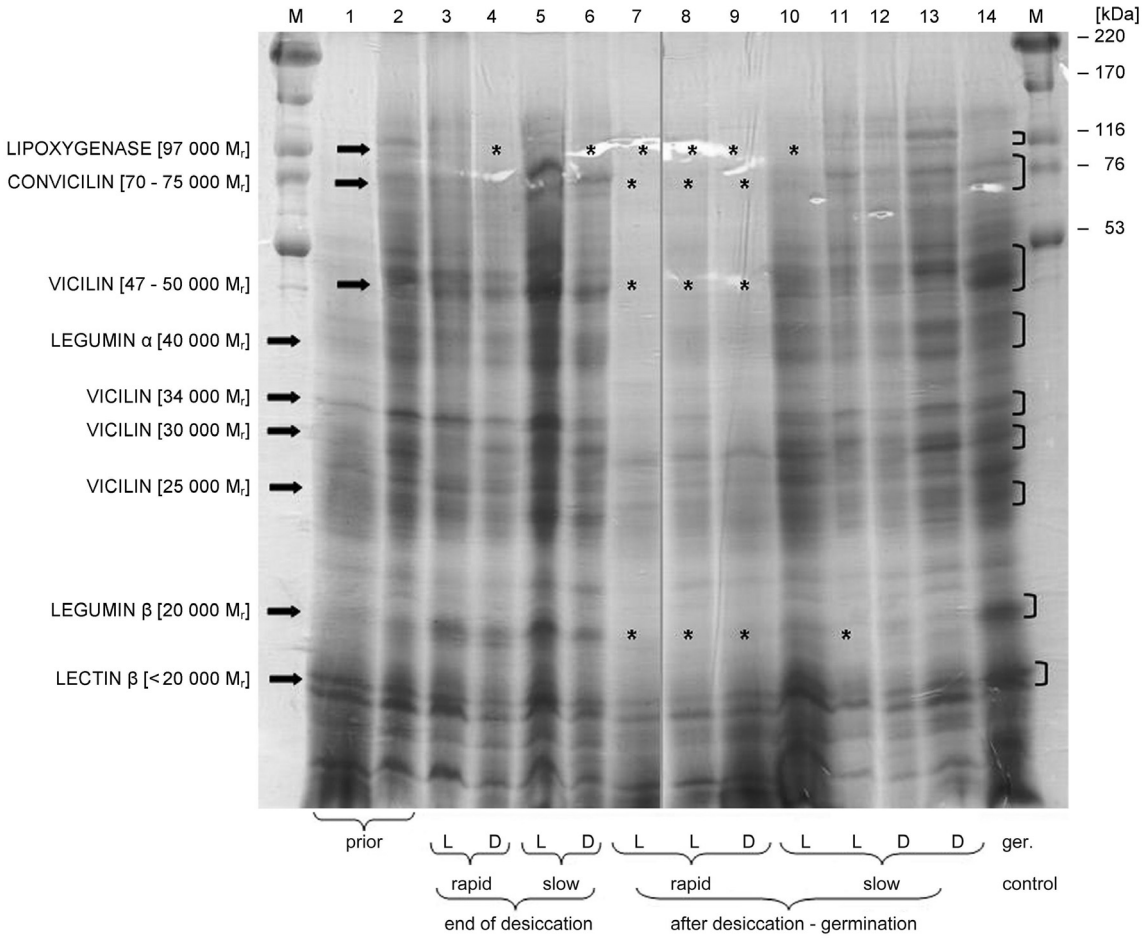


Fig. 8. Protein patterns (SDS-PAGE) of desiccated and germinating somatic embryos of canning pea cv. Oskar. 1,2 - torpedo/cotyledonary SEs prior desiccation; 3 to 6 - SEs at the end of desiccation; 7 to13 - germination of desiccated SEs; 14 - germination of non-desiccated SE (control), M - molecular mass marker. 1, 7, 8, 10, 12, 14 - SE samples with signs of root pole elongation; 2, 3, 4, 5, 6, 9, 11, 13 - SE samples without signs of root pole elongation. *Asterisks* show absence of polypeptides.

Cotyledonary somatic embryos prior desiccation contained all key storage proteins (Figs. 8, 9), the cotyledonary stage SEs with more developed root pole (beginning of root development) had less expressed all proteins (except of lectin β) (Fig. 8). This difference was more evident in cv. Oskar (Fig. 8) than in line HM-6 (Fig. 9). The strongest expression of total proteins at the end of desiccation was observed in a slow desiccation in light (Figs. 8, 9) and in rapid drying in dark (Fig. 9). Lipoxygenase polypeptides disappeared during certain desiccation treatments in cv. Oskar (Fig. 8). The transfer of desiccated embryos to germination medium resulted in dramatic decrease of all proteins expression, namely after rapid drying (Fig. 8). In cv. Oskar, lipoxygenase, convicilin, major vicilin subunits and legumin β were completely degraded (absent) and expression of legumin α and minor vicilin subunits was substantially lower (Fig. 8). Similar situation was observed also in line HM-6 (Fig. 9). In contrast, slowly-desiccated embryos, when transferred to germination medium retained better expression of majority of proteins, particularly with strong expression after slow desiccation in dark (Figs. 8, 9). In embryos with visually evident signs of conversion (elongation of root pole), there was a strong degradation of proteins (typical in Fig. 9). Alternating presence/ absence of polypeptides with $M_r$ ~ 30 000 - 34 000 (minor vicilin subunits) was observed in both pea genotypes (Fig. 8, 9). Progresively germinating embryos (in fact with distinct root and shoot) exhibited – despite of certain storage protein degradation (lipoxygenase, convicilin, vicilin, legumin β – a stronger expression of proteins with $M_r$ ~ 28 000, $M_r$ ~ 60 000 never seen in samples 1 to 13 (Fig. 9).

The detection of key storage proteins (typical for pea seed) in pea somatic embryos revealed that these propagules are close to their zygotic counterparts not only morphologically (Griga 2002), but physiologically, too. Thus, the genes responsible for biosynthesis of these respective seed proteins are fully active also in somatic embryos. The presence of typical storage proteins may serve as an explicit marker of somatic embryogenesis pathway of regeneration in cases where embryo-like structures are not quite distinct (quick reversion of originally initiated somatic embryos into shoots or roots;

the parallel presence of embryos and shoots within one explant due to insufficient embryogenic auxin-induction stimulus).

On the other hand, it is necessary to underline differences resulting from different environments for development of pea somatic embryos *in vitro* and zygotic embryos *in planta* (absence of endosperm and testa in somatic embryos, and thus the absence of mutual interactions of these structures which as a consequence leads to the final shape and size of the embryo within the seed). ZEs of particular developmental stages cannot be simply compared to those of somatic embryos – it is only certain analogy derived from morphological similarities and generally accepted terminology (Griga 2002) – *e.g.* globular SE (size 1 mm in diameter, FM *ca*. 1 - 2 mg) has got substantially higher size and FM than true ZE globular stage, it differs by cell number and degree of cell differentiation. SEs of alfalfa lack developed cotyledons and appear to be enlarged torpedo-shaped embryos (Krochko *et al*. 1992), which is also typical for pea (Griga 2002).

Somatic embryos differ from zygotic embryos primarily in the late stages of development and often completely lack a maturation phase (Lai *et al*. 1994b). From the point of physiological maturity, the pea globular somatic embryo may be considered as mature, because it contains all key storage proteins (and also starch; Griga *et al*. 2001) which is not a case of relatively progressed cotyledonary stages of pea ZEs (incomplete vicilin expression in ZEs of stages I to III or even IV). Small differences in protein patterns detected between SEs and mature seed may be hypothetically caused by posttranslational modifications or these polypeptides may be induced by tissue culture (stress) conditions. The exact detection/characterization (Western blot, 2D electrophoresis) of pea SE proteins will be aim of our further studies.

It is of great significance a high amount of storage substances in synthetic seeds (encapsulated SEs) sown directly into the soil, which affects viability, germination and subsequent plant development. In contrast, in order to obtain the transformants *via* SE, there is no need for fully morphologically and physiologically matured SEs. Even precocious germination of early stages SEs (globular, heart-shaped, torpedo) does not represent negative feature, if the aim is to obtain at least one fertile transformed plant. Thus, maturation of pea SEs *in vitro* represents more theoretical than practical question.

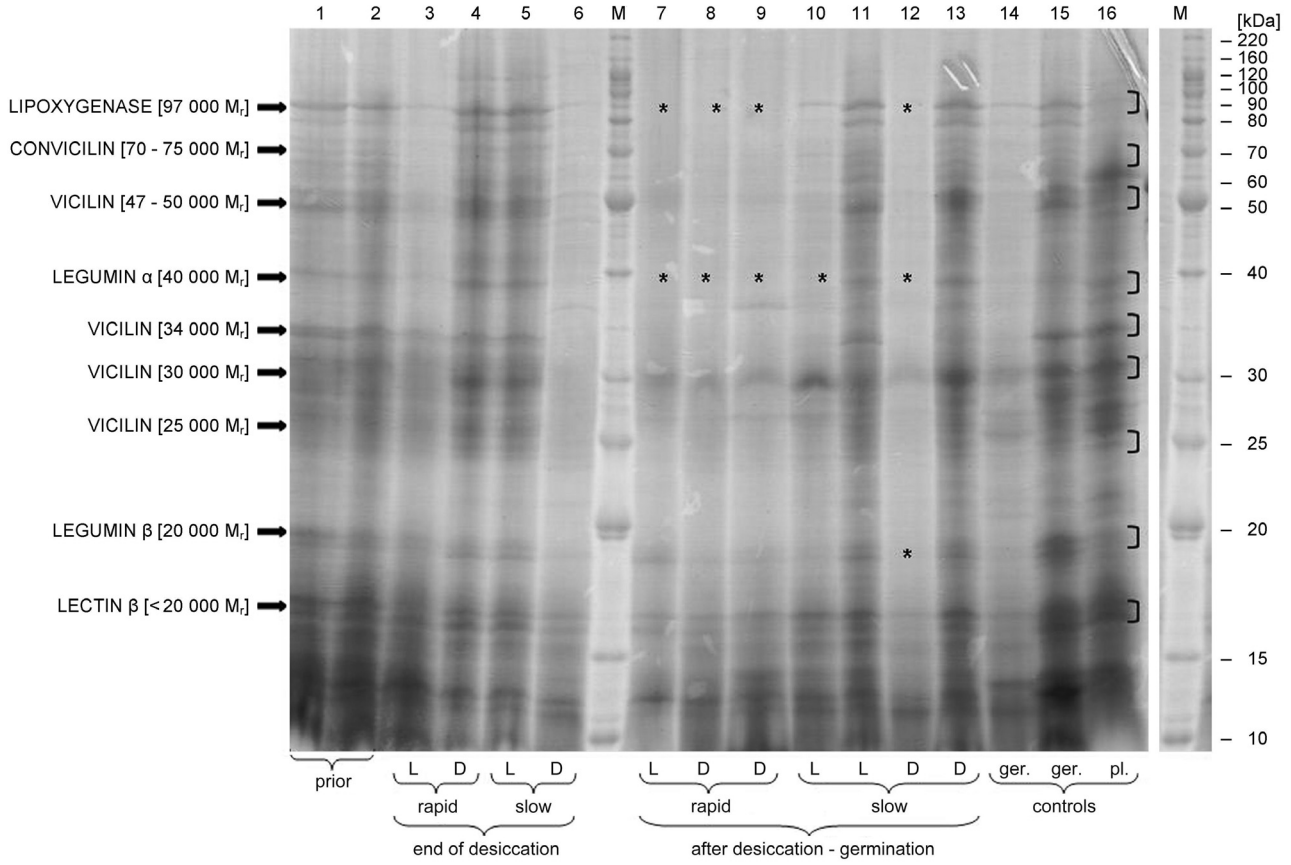


Fig. 9. Protein patterns (SDS-PAGE) of desiccated and germinating somatic embryos of dry-seed pea line HM-6. 1,2 - torpedo/cotyledonary SEs prior desiccation; 3 to 6 - SEs at the end of desiccation; 7 to13 - germination of desiccated SEs; 14, 15 - germination of non-desiccated SE (control), 16 - converted plantlet (embling), M - molecular mass marker. 1, 8, 10, 12, 14 - SE samples with signs of root pole elongation; 2, 3, 4, 5, 6, 9, 11, 13 - SE samples without signs of root pole elongation. *Asterisks* show absence of polypeptides.

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Singh, R.J., Jahuar, P.P. (ed.): **Genetic Resources, Chromosome Engineering, and Crop Improvement. Cereals. Vol. 2. -** CRC Press, Taylor and Francis Group, Boca Raton - London - New York 2006. 442 pp. USD 159.95. ISBN 0-8493-1432-1.

Chromosome engineering is one from recent sphere of biology applicable in crop improvement. Volume 2 of the book series Genetic Resources, Chromosome Engineering and Crop Improvement evaluates its possibilities in cereals. The editors and authors are prominent authorities in particular fields included in this book.

The book is divided into 13 chapters. Each chapter provides a comprehensive account of the crop from its origin through breeding and cytogenetic manipulation to molecular genetics and biotechnology. The wheat, rice, maize, oat, barley, pearl millet, sorghum, rye and *Triticale* are described.

The Chapter 1 deals with cytogenetic architecture of cereal crops and principles and strategies of cytogenetic and breeding manipulations. Chapters 2 to 4 are aimed at wheat. Chapter 2 describes chromosome engineering of durum wheat genome, Chapter 3 treats of utilization of genetic resources for bread wheat improvement and Chapter 4 is aimed at molecular markers, genomics and genetic engineering in wheat. Chapter 5 evaluates cytogenetic manipulations and germplasm enhancement of rice. Chapter 6 describes genetic enhancement of maize by cytogenetic manipulation and breeding for yield, stress tolerance and high protein quality. Chapter 7 describes cytogenetic manipulation in oat improvement. Chapters 8 and 9 are aimed at barley. Utilization of genetic resources for barley improvement and chromosome mapping are described. Primary to tertiary gene pool of barley, haploid, tetraploid and mutation breeding and role of somaclonal variation are discussed. Chapter 10 treats of genetic improvement of pearl millet with respect to cytogenetic manipulation and heterosis breeding. Chapter 11 is aimed at cytogenetic improvement and genetic resources in sorghum. The rye (*Secale cereale*) is described in Chapter 12 and *Triticale* in Chapter 13.

The ploidy levels and germplasm enhancement through interspecific hybridization were especially studied. In the most chapters the *in vitro* techniques, chromosome manipulations, transformation, direct transfer of genes, and other molecular genetic methods are described. The text is adequately supplemented by tables and figures. The chapters include a comprehensive list of references down-to-date 2005. Excellent subject index saves labour with this excellent scientific book.

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