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Biotechnology of Flax (*Linum usitatissimum*)

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ABSTRACT

Biotechnology can offer useful tools to complement conventional breeding programs. The techniques of *in vitro* selection, that exploit phenomenon, called somaclonal variation, by screening cell cultures for resistance to herbicides, different types of stress or diseases, already helped to obtain tolerant lines of flax (*Linum usitatissimum*), which were used in further commercial breeding programs. The other important techniques in flax biotechnology are anther and immature embryo cultures. Development of haploid or dihaploid lines based on a regeneration capacity of microspores in immature anthers can speed up conventional breeding. Embryo rescue helps to overcome the post-zygotic incompatibility mechanisms after wide crosses for the transfer of desired traits. One of the main objectives of tissue culture studies is to obtain high-frequency shoot regeneration or somatic embryo formation. Age and viability of the explant, the tissue source and genotype of donor plant from which the explant was excised, composition of the culture medium and plant growth regulator supplementation, are very important for the effectiveness and the direction of morphogenic responses. This review highlights main studies devoted to flax biotechnology and the main factors controlling morphogenesis of *L. usitatissimum*.

Keywords: explants, fibre flax, morphogenesis, oilseed, regeneration, somatic embryogenesis
Abbreviations: **ABA**, abscisic acid; **BAP**, 6-benzylaminopurine; **IAA**, indole-3-acetic acid; **IBA**, indole-3-butyric acid; **NAA**, α-naphthalene acetic acid; **TIBA**, 2,3,5-triiodobenzoic acid

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INTRODUCTION

Flax (*Linum usitatissimum* L.) is an ancient but important crop cultivated for its seeds and fibres. It has a long history of cultivation in warm and cool temperate climate regions. Fibre obtained from the stem of the plant is widely used in the textile industry and its high quality drying oil is used in the production of paints, varnishes, inks, linoleum and patent leathers. Moreover, since it has a small nuclear genome, it has been used as a model system for genetic engineering techniques. However flax improvement has not developed at the same rate as in other crops, and advances in conventional breeding of this crop are not sufficient. Biotechnology offers useful tools to complement conventional breeding programs. Genetic engineering, cellular selection, mutagenesis, haploidisation, immature embryos culture and protoplast techniques – genetic progress is expected from those technologies both by saving time and increasing genetic variation. Therefore, improvements in biotechnological techniques used for this crop are of great importance.

One of the main objectives of tissue culture studies is to obtain high-frequency shoot regeneration. Viability, age of

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explant and the tissue source from which the explant was excised are the main factors determining regeneration capacity. Flax can easily be regenerated from hypocotyl segments and less easily from callus, protoplasts and cotyledon explants. The other major factors, which regulate growth of callus, frequency of organogenesis and somatic embryogenesis induced from explants of *in vitro*, are the effects of genotype and plant growth regulators supplementation of culture medium.

The cultivation of tissue and cells *in vitro* causes changes in heredity, called somaclonal variation. This variation depends on the duration of cultivation and usually occurs during the callus stage. It can cause problems in obtaining genetically stable lines regenerated from callus. On the other hand, this phenomenon has been used as a source of genetic variability in the breeding programs.

Although tissue culture of *L. usitatissimum* has been carried out for 30 years (Rybczynsky 1975; Gamborg and Shyluk 1976; Mathews and Narrayanaswamy 1976; Murray *et al*. 1977; Lane 1979; McHughen and Swartz 1984; Pret'ova and Williams 1986; Nichterlein and Fredt 1993; Poliakov *et al*. 1998; Dedičova *et al*. 2000; Mudhara and Rashid 2002; Yildiz and Ozgen 2004), the knowledge of factors that control organogenesis and induction of somatic embryogenesis in this species is still incomplete and contradictory. In this paper we attempted to classify data that have been reported by different researchers in the field of flax biotechnology and to take up the main subjects controlling morphogenesis of *L. usitatissimum*. Because of the great number of studies devoted to flax genetic engineering we decided to examine them separately in our next digest.

L. usitatissimum is a dicotyledonous plant from the *Linaceae* family pertaining to the genus *Linum*. The genus *Linum* includes more than 200 species of annual and perennial herbaceous plants, disseminated in temperate and subtropical areas all over the world. The most economically important within these species is the cultural flax (*L. usitatissimum* L.). There are two distinguishable groups within *L. usitatissimum*, fibre flax and oilseed flax (linseed). In Europe however, the term "flax" is used for varieties grown for fibre production. Rather confusingly, there are also varieties grown for both seed and fibre. The linseed form of *L. usitatissimum* is a significant oilseed crop in many regions of the world, particularly in cool temperate environments, with an annual world production of around three million metric tones. The principal growing areas are Argentina, India, China, Canada, USA and Russia (Milliam *et al*. 2005).

These two groups differ much in agronomic characters. Linseed has reduced height, more branching and a later harvest time compared with fibre flax; moreover these two groups differ much in their behavior *in vitro*, the topic which we are going to consider further.

MORPHOGENIC RESPONSE OF *L. USITATISSIMUM*

One of the main features of biotechnological methods appears to be the ability to regenerate a fully differentiated organism from a single cell. This ability to generate any cells from such starting tissue is the property of "totipotency".

Generally, organogenesis *in vitro* and indirect somatic embryogenesis have several common structural features (Thorpe 1984). Organogenesis and somatic embryogenesis require a certain degree of cell dedifferentiation, reinitiation of cell division, and morphogenic control over cell expansion under appropriate inductive and permissive conditions. In a wider sense, somatic embryogenesis can be considered as an extreme case of adaptation that is based on the phenotypic plasticity of individual somatic cells. Phenotypic plasticity, a significant characteristic of plants, allows individuals to adapt or acclimate themselves to a wide range of environments – in this case to *in vitro* conditions (Dedičova *et al*. 2000).

For morphogenesis *in vitro* in general, several cellular aspects play a crucial role. The previously mentioned reinitiation of cell division, considered one of the key factors during regeneration, appears to be controlled in different ways depending on the various model systems. The type of first division under inductive conditions can differ, often depending on growth regulators in the culture medium and the type of the primary explant used (Pret'ova 1995). It seems that the nature of the first cell division might be important during the early induction, leading to a particular developmental and regeneration pathway. Other important cytological features of early morphogenesis are the changes in the plane of early cell divisions leading to formation of a group of cells with increased regeneration potential. These changes were universal for organogenic and embryogenic regeneration and they represent a structural consequence of undergoing molecular and biochemical changes necessary for obtaining new regeneration competence (Dedičova *et al*. 2000).

The predominant role of growth regulators in plant growth and development suggest that their involvement in tissue culture procedures can cause inductive and permissive conditions for realization of particular morphogenic pathway (Pret'ova 1998). A complex view of the effect of various growth regulators on flax calli induction, organogenesis, and somatic embryogenesis was precisely described by Gomes *et al*. (1996) and Pret'ova and Williams (1986). In both cases the strong influence of growth regulators on the initiation of somatic embryos in flax was stated. Auxins are the most important factor for embryogenesis induction and development. It can be hypothesized that genes involved in the phytohormone signals are involved in plant regeneration. The duration of 2,4-D pretreatment in combination with sucrose type strongly influenced the number of shoots, roots and somatic embryos regenerated in flax cultures (Dedičova *et al*. 2000).

Type of explants and regeneration capacity

Studies, carried out by different scientists, showed that flax organs and tissues significantly differ in their tissue culture competence and the capability for the morphogenetic response; in particular, root segments and cotyledons were described by many authors as non-morphogenic explants. Root segments were inclined to form abundant friable and non-morphogenic callus and roots. On cotyledonal explants, in most cases, small thin rootlets, or the weakly-growing non-morphogenic callus, or, in the single cases, adventitious buds were formed. The segments of hypocotyls, which most frequently have been used as the source of explants for obtaining the morphogenesis in different species of *Linum*, as it was shown by many authors, demonstrated the highest capability for morphogenesis (**Table 1**).

Evidently the lack of a tissue's capacity for regeneration is associated with block of the morphogenic response, which can be caused according to Vasil (1986) by the following reasons: 1) This block can be genetic, including absence of totipotency. It was shown that there is high degree of heritability in inability to organogenesis *in vitro*. 2) This block can be epigenetic, meaning stable but potentially converse functioning of genes that control growth and morphogenesis. These cells can be designated as "incompetent", i.e., incapable of the perception of hormonal as well as any other signals converting cells to the way of organogenesis. 3) This block can be physiologic, i.e., cells are genetically capable of reacting to the signal, and there is no epigenetical block either, but there are no signals of environment (for example, the absence of special substances in the necessary concentration and proportion or interaction of such signals with the inhibitors, being present in the culture media or the tissue). It is suggested that in the case of physiologic block all explants are capable of the organogenesis and it is possible to find chemical or physical means, in order to force them to appear this ability (Thorpe and Patel 1984).

The success in overcoming the physiologic block of

Table 1 Morphogenetic response of different types of explants of *Linum* species.

Species	Explants	Growth regulators (mg/l)	Type of morphogenesis	References
L. usitatissimum L. stricktum, *L. altaicum*, *L. narbonense*, *L. grandiflorum*, *L. alpinum*, *L. lewissii*	protoplasts	BAP 0.5; NAA 0.05-2.0	shoots, somatic embryogenesis	Barakat and Cocking 1983, 1985
L. usitatissimum	immature embryos	BAP 0.05	somatic embryogenesis	Pret'ova and Williams 1986
L. usitatissimum	hypocotyl segments	BAP 0.05	shoots, roots	Kaul and Williams 1987
L. alpinum, *L. amurense*, *L. hologynum*, *L. perenne*, *L. salsoides*, *L. usitatissimum*	protoplasts	BAP 0.5-0.7; NAA 0; 0.02; 0.1	shoots, roots	Ling and Binding 1987
L. usitatissimum	hypocotyl segments	BAP 1.0; NAA 0.1	shoots	Basiran *et al*. 1987
L. marginale	segments of: roots, hypocotyl, cotyledon, leaves and protoplasts	NAA 0.02; BAP 1; 2; 5; zeatin 1; 2; 5 kinetin 0.1-2.0; NAA 0.5	shoots	Zhan *et al*. 1989
L. usitatissimum	hypocotyl segments	BAP 1.0; NAA 0.02	shoots	Jordan and McHughen 1988; Dong and McHughen 1993
L. usitatissimum	hypocotyl segments	BAP 1.0; NAA 0.05	shoots	McHughen *et al.* 1989
L. usitatissimum	hypocotyl segments	BAP 1.0; NAA 0.05	shoots	Mlynarova *et al.* 1994
L. usitatissimum	hypocotyl segments	IBA 0.4-0.6; zeatin 0.5-1.6; 2,4-D 0.05-3.2	somatic embryogenesis	Cunha and Ferreira 1996, 1997
L. usitatissimum	hypocotyl segments	kinetin 0.1-0.2; BAP 0.25-0.5; NAA 0.02-0.5; IAA 0.25-0.5; IBA 0. 37-0.7	shoots	Koronfel and McHughen 1998
L. usitatissimum	anthers	BAP 1; 2; NAA 0.05; 1	shoots	Tejklova 1998
L. usitatissimum	anthers	BAP 1; NAA, 0.1	shoots	Rutkowska-Krause *et al*. 1998
L. usitatissimum	anthers	zeatin 0.9; TDZ	shoots	Chen *et al*. 1998, 1999
L. usitatissimum	hypocotyl segments	BAP 1.0; NAA 0.05	shoots	Poliakov *et al*. 1998
L. usitatissimum	hypocotyl segments	BAP 0.025; NAA 0.001	shoots	Rakousky *et al*. 1999
L. usitatissimum	hypocotyl segments	BAP 0.3; NAA 0.02	shoots	Laine *et al*. 2000
L. usitatissimum	hypocotyl segments	BAP 0.5; NAA 0.5-1; ABA	somatic embryogenesis, roots	Tejavathi *et al*. 2000
L. usitatissimum	hypocotyl segments, cotyledon segments	BAP 0-1.0; NAA 0-1.0; TDZ 0.25	Only on the hypocotyl segments shoots and embryo-like structures formation	Dedičova *et al*. 2000
L. usitatissimum	anthers	BAP 1.0-2.0; NAA 0.05-1.0	Haploid and diploid shoots, somatic embryogenesis	Poliakov 2000
L. usitatissimum	hypocotyl segments	BAP 1.0-2.0; NAA 0.05-1.0; kinetin 1.0; IBA 1.0	shoots	Poliakov 2000
L. usitatissimum	hypocotyl segments	BAP 1.0; NAA 0.05	shoots	Kalyaeva *et al*. 2000
L. usitatissimum	immature embryos, anthers	BAP 0.05; 2,4-D, 2.0-5.0	shoots, somatic embryogenesis	Pret'ova *et al*. 2000
L. usitatissimum	hypocotyls of intact and decapitated seedlings *in vitro*	ABA 1.7	shoots	Mundhara and Rashid 2001
L. usitatissimum	hypocotyl segments	without growth regulators; TDZ	shoots	Jain and Rashid 2001
L. usitatissimum	hypocotyl segments	BAP 0.5-2.0; TDZ	shoots	Mundhara and Rashid 2002
L. usitatissimum	stem segments	BAP 0.01	shoots	Bonell and Lassaga 2002
L. usitatissimum	hypocotyl segments	BAP 1.0; NAA 0.2	shoots	Yildiz and Er 2002
L. usitatissimum	anthers	zeatin, 1	shoots	Chen and Dribnenki 2002
L. usitatissimum	hypocotyl segments	BAP 1.5; NAA 0.075	shoots	Hjordis *et al*. 2003
L. usitatissimum	anthers	BAP 0.1; 1.0; NAA 0.001-0.5	shoots, somatic embryogenesis	Rutkowska-Krause *et al*. 2003
L. usitatissimum	hypocotyl segments	BAP 1.0; NAA 0.02	shoots	Yildiz, Özgen 2004
L. usitatissimum	cotyledons	BAP 1-4; NAA 0-0.5	shoots	Belonogova *et al.* 2003, 2005, 2006

kinetin 1 mg/l corresponds to 5 μM
zeatin 1 mg/l corresponds to 5 μM
BAP 1 mg/l corresponds to 4.44 μM
NAA 1 mg/l corresponds to 5.3 μM
IAA 1 mg/l corresponds to 5 μM
IBA 1 mg/l corresponds to 7.4 μM
ABA 1 mg/l corresponds to 5.8 μM

regeneration and in obtaining of organogenesis depends on three basic factors and the interactions between them: 1) the plant organ used as an explant; 2) the composition of culture medium; 3) the genotype and the physiologic status of donor plant.

The physiologic status of donor tissue is included: the physiologic and ontogenetic age of organ; season, in which explant was excised; from the quality of donor plants. George and Sherrington (1984) note still several important properties: explant pretreatments; explant orientation and the density of inoculation in the Petri dishes. Manipulation with these factors leads to the organized development initiation and to the structural changes. These changes depend on a number of biochemical, physiological and molecular events, caused by activity of corresponding genes in the population of cells that finally brings to the morphogenesis initiation (Thorpe and Patel 1984).

Phenomenal nature of shoot-bud regeneration on hypocotyls of Linum seedlings

Occurrence of shoot-buds on hypocotyl of *Linum* seedlings, as an unusual developmental response, first time was recorded already a century ago (Burns and Hedden 1906). This finding was corroborated a year later, on decapitated seedlings (Tammes and Flachsstengel 1907). Later it was rediscovered and confirmed (Adams 1924).

The hypocotyl represents a complex explant composed of several cell types elongated along their longitudinal axis, namely epidermis, subepidermal layer, cortex, stellar tissue with tracheary and sieve elements, and procambium as well as pith parenchyma in the apical part close to cotyledons. Within this complex structure, certain cell types, e.g. epidermis, subepidermis, and cortical cells, can respond to inductive culture conditions by the reactivation of cell division, while the cells in the vascular tissue do not divide (Dedičova *et al*. 2000).

Adventitious shoots on the flax hypocotyls form exogenously without an intervening callus phase. Histological studies suggest that the most of the tissue in an adventitious shoot is derived from the parent epidermal cells (Crooks 1933). The flax hypocotyl epidermis consists of several distinct types of cells: guard cells, stomatal accessory cells, pavement cells and gland cells (also called pseudohairs or "fat" cells). The pavement cells – are the cells that cover most of the hypocotyls surface, they are about 200 μm long by 15 μm wide. The gland cells are approximately 80 μm long and 40 μm wide. It is suggested that most meristems are derived from pavement cells, while the gland cells are rarely involved (Shepard 2000).

The first sign of epidermal reaction can be observed after 1 day in culture when the nuclei of the epidermal cells increased in size and were positioned in the middle of the cells. Such reactivated cells could very often be located close to stomata (Dedičova *et al*. 2000). In the earliest stages of shoot formation, 3 to 5 competent pavement cells undergo periclinal and anticlinal division yielding a meristemoidal dome of small, densely cytoplasmic cells. It is suggested that this domed structure (called the "bulge" stage by Link and Eggers 1946) has functional properties of a shoot apical meristem, as it was showed experimentally, that the bisection of the bulge structure by transverse incision causes two shoot meristems formation. Recruitment in the subepidermal tissues of the hypocotyl occurs relatively late in adventitious meristem development, and the shoots connect with the vasculature of the hypocotyl only after several leaf primordia have formed. Differentiation of vascular tissues begins in the adventitious shoot and proceeds basipetally through the hypocotyl cortex to the stele (Crooks 1933; Shepard 2000). Shoot primordia become fully differentiated after 9 days in culture. Mature shoot primordia are characterized by apical shoot meristems with leaf primordia, procambial and vascular strands (Dedičova *et al*. 2000). They are connected to the mother explant with a broad basal part and vascular tissue Sometimes the meristematic apical dome is wide and subsequently gives rise to two or three apical meristems.

Information on factors controlling the distribution of adventitious meristems on the flax hypocotyl is scare. No clear pattern of radial distribution has been reported. In the longitudinal direction, most studies agree that following the decapitation, adventitious shoots first emerge near the base of hypocotyl but subsequently arise on the other regions of the hypocotyl (Link and Egers 1946). It was suggested that this was due to an inherent gradient within the hypocotyl tissue. This polarity is also reflected in the production of shoot primordia on the hypocotyl segments in the presence of the cytokinin BAP. As it was noted by many researchers, the frequency of shoot buds regeneration on the segments of hypocotyls of germinated seedlings of flax was influenced by place of explant's initiation (Kaul and Williams 1987; Kalyaeva *et al*. 2000; Jain and Rashid 2001). For example, in the experiments of Slovak scientists the regeneration gradient along the hypocotyl explants of the fibre flax cultivars 'Caroline' and 'Alex' increased basipetally (Dedičova *et al*. 2000). Thus, with the cultivation of the basal part of the hypocotyl the number of regenerated shoots for the fibre flax reached 9.6 shoots per explant and it decreased to 7.4 shoots when the apical part was cultivated (Kalyaeva *et al*. 2000). Furthermore, shoot regeneration was observed along the length of hypocotyl explants, but with a higher incidence near the cut end of explant (Gamborg and Shyluk 1976).

A similar gradient was also observed by Kaul and Williams (1987) on the hypocotyls of germinating mature flax embryos. When the hypocotyl of germinating mature flax embryos is severed just below the cotyledons, the tissues on either side of the cut show a marked ability to regenerate. On a basal nutrient medium without growth regulators, adventitious shoot primordia are formed around the top of the lower embryo segment, and root primordia are formed around the base of the upper segment. Such a pattern of reciprocal differentiation from formerly adjacent cells implies a strong polarity of internal growth regulation within the hypocotyl. However, the casual nature of this phenomenon has not been investigated.

Furthermore, for adventitious bud regeneration on hypocotyl segments of significant importance for induction of organogenesis *in vitro* on the hypocotyls had the size of the used segments. In several cases regeneration occurred only on the segments of the size of 1-2 mm (Basiran *et al*. 1987), or 2-3 mm (Mlynarova *et al*. 1994) which were excised from the 2-5 days old seedling *L. usitatissimum*. But in most of studies 5-10 mm segments of hypocotyls that were excised from 5-8 days old seedlings possessed the greatest regeneration ability (McHughen 1987; Jordan and McHughen 1988; Dong and McHughen 1993a; Koronfel and McHughen 1998; Dedičova *et al*. 2000; Poliakov 2000; Jain and Rashid 2001).

As hypocotyls mature and undergo secondary growth, their ability to initiate adventitious shoots decreases and particularly in the apical regions of the axis (Crooks 1933; Link and Eggers 1946).

The adventitious shoot meristems on the decapitated flax seedlings develop asynchronously and show a variety of fates. Complete and fully differentiated flax plants can be regenerated only from the shoot primordia. However, more leaf primordia can also be observed (Dedičova *et al*. 2000). A lot of shoot primordia never make leaves, while others stop developing after several small leaves have been formed. One to two shoots per hypocotyls continue to grow and flower within the two months of decapitation. Removal of the dominant shoot permits resumption of growth for some arrested ones (Shepard 2000).

The phenomenon of direct shoot bud formation from hypocotyls is rare, but occurs in at least two species of the genus (in *L. marginale*, shoot buds arise also directly from epidermal cells of the hypocotyl (Zhan *et al*. 1989)). Most of the biotechnological works on flax and linseed has focused on the ways to capitalize on this phenomenon. Moreover, as this meristems form on the hypocotyl's surface, meristem and leaf primordium are easily observed and accessible to experimental manipulations making this adventitious shoots an excellent system in which to study organogenesis.

This direct shoot regeneration might be useful for genetic transformation techniques, especially that are based on particle acceleration, because they allow to take advantage of the phenomenon, that the adventitious buds develop from the epidermal cells on the whole explant surface. One potential problem is that after the transformation via *Agrobacterium tumefaciens* transformation and proliferation of transformed cells occur only around the wound area. Thus, the majority of shoots is formed from non-transformed cells, and, when the shoot arises from more than one cell, some regenerated shoots are composed of both transformed and non-transformed cells (chimeric shoots) (Jordan and McHughen 1988). And as it was shown by Dong and

McHughen (1993b), that even after significant improvement of transformations protocols by extension of the wound area by partial peeling of the epidermis before transformation, preliminary pretreatments of explants to increase cell competence and prolonged cocultivation with agrobacteria (Dong and McHughen 1993a), the number of chimeric regenerants still remain very high – 45%.

Occurrence of shoot-buds on hypocotyl of Linum seedlings is a stress related response

Induction of shoot-buds on hypocotyl of *Linum* seedlings is a stress-related response. Transient stress of different types effective in this process are heat-, salt-, mineral-stress; treatment with abscisic acid (ABA) or proline resulted in shoot formation on intact non-hormone treated seedlings of *Linum in vivo* (Mundhara and Rashid 2001). Decapitation of the seedling just below the cotyledons, however, yields the most dramatic formation of adventitious shoot meristems. All the seedlings, on decapitation formed shoot-buds within 15 days. The production of shoots on decapitated plants inhibited by darkness and stimulated by red and blue light. Adventitious root meristems typically do not form on the flax hypocotyls.

Precise study of the individual effects and interaction between different types of stress in promoting shoot-bud regeneration showed that the highest frequency of shoot regeneration was recorded, when mineral deprivation was combined with decapitation. The stress-causing chemical – ABA – was also effective only combined with mineral stress. Although in the case of ABA treatment, there was no difference in terms of average number of shoot-buds per responding seedling but there was a substantial increase in percent responding seedlings (Mundhara and Rashid 2001).

A comparison of two types of stress, physical and chemical, for example as heat and salt treatments, indicates that an increase in frequency of shoot formation is dose dependent. On salt treatment (NaCl, 0.5%), there was a corresponding increase in the number of shoot-buds, with an increase in duration of stress treatment. Maximum number of shoot-buds (3.8-4, shoot-buds/seedling) were recorded when seedlings were subjected to 2-3 days of salt treatment. Just a day of heat treatment (at 35°C) resulted in an increase in frequency of shoot formation as compared to control (from 1 to 2, shoot-buds/seedlings). The number of shoot-buds per responding seedling also increased with an increase in duration of heat treatment. For example, the number of buds per seedling with 2 days of heat treatment was 2.8 and it was enhanced to 4 shoot-buds after 3 days of treatment. However, there was no significant increase in percent responding seedlings up to 2 days of heat treatment. Instead there was a sharp increase (from 30% to 60%) in the percent of responding seedlings, when treatment was continued for 3 days. This was not there in salt treatment. Identically, 50% of seedlings responded to form shoots and it was irrespective of different durations of salt treatment (Mundhara and Rashid 2001).

One of the metabolic effects of salt treatment to plants is the accumulation of proline. Interestingly when seedlings were subjected to proline treatment there was increase in regeneration frequency and the percent of responding plants was of the same order as that of salt treatment (Mundhara and Rashid 2001).

It has been shown, that the induction of shoot-buds formation on intact seedlings by transient withdrawal of calcium is possible. Prior to appearance of shoot-buds, calcium-deprived seedlings are characterized by a swelling of the hypocotyl. Moreover, these seedlings were characterized by the restriction of shoot-buds to the basal region, whereas in different types of stress, the regenerants were distributed all over the seedlings. The other difference that characterized these seedlings was the absence of wilting in case of transient calcium deprived seedlings, whereas spontaneous wilting was always seen in seedlings subjected to different types of stress. Yet another difference that marked these seedlings was the time taken for regeneration. In transient calcium deprived seedlings the regeneration was noticed in about a month whereas in most of other types of stress the regeneration occurred within 15 days, except that of heat stress (Mundhara and Rashid 2001).

Although it is implied that shoot formation occurs, on intact seedlings, without any growth regulator but it is difficult to comprehend the formation of meristems without an involvement of growth regulators. So it still remains to be resolved as to how transient withdrawal of calcium results in shoots formation. Another important distinction between calcium-stress and other stress treatments is that in case of a transient calcium stress shoot-buds develop on intact seedlings, whereas in other stress treatments, there was break-up of correlative influence, emanating from apex (decapitation or apex wilting), that results in shoot formation.

Furthermore Indian scientists studied the role of calcium in morphogenesis using lanthanum (La^{3+}), which inhibits an influx of calcium from the medium to plant. It was showed that La^{3+} inhibited shoot formation on calcium-deprived seedlings, when they were resupplied with calcium. The use of inhibitors of calmodulin, chlorpromazine and trifluoperazine, which inhibit calmodulin-dependent processes in animal systems, resulted in a reduction in number of shoots, and only 30% seedlings responded to form shoot-buds (in comparison with control on the calcium–fortified medium without calmodulin inhibitors after the transient withdrawal of calcium – 80% of seedlings produced adventitious shoot-buds). These inhibitors of calmodulin were effective at very low level (0.1 μM), this is comparable to the concentration employed in animal systems. For animal cells the distribution of intracellular calcium (second messenger) is involved in a signal transduction chain, a cascade, which activates protein kinases controlling genes acting on differentiation. Moreover, calcium is associated in *Fucus* with the maintenance of polarized growth of the embryo. This is in conformity to calcium, a key element, in control of a diverse array of cellular and morphological responses. It is a reasonable working hypothesis that comparable transduction chains may be operative in plant embryogenesis and shoot regeneration *in vitro* as well as *in situ* (Mundhara and Rashid 2002).

Somatic embryogenesis on flax hypocotyls

Indirect somatic embryogenesis

In some other cases under certain conditions of culture flax hypocotyl explants are capable to form the morphogenic or embryogenic callus with the subsequent induction of regeneration or somatic embryos formation. For example, somatic embryos differentiated from callus initialed from 0.5 cm hypocotyl explants excised from 7 to 15 days-old seedlings of three elite Indian dual-purpose flax varieties on Murashige and Skoog (MS) medium supplemented with NAA at various concentrations (Tejavathi *et al*. 2000). The most effective concentrations varied with variety. The embryos arose as shiny, globular structures from two-weeks-old callus and after 4 weeks of culture individual embryos could be separated from the callus mass. The origin of embryos from a single cell and subsequent developmental stages was traced. Histological studies confirmed that these embryos were somatic embryos which passed through all the stages of normal embryo development. A single peripheral cell of the callus differentiated into the initial of the somatic embryo. This cell followed the normal pattern of development as a zygotic embryo. It passed through all the stages from the 2-celled to torpedo stage of a typical dicotyledonous embryo. Further studies revealed that after the torpedo stage the embryo callused to give secondary embryos.

However, the results indicated that the rate of shoot tip growth was very slow and there was an underlying problem which blocked the complete development of the embryo. Since root development occurred in the embryo at a very early stage this precocity might have inhibited the develop-

ment of the shoot pole. Inhibition of shoot pole development by early root formation is a problem encountered in several other plants (Tejavathi *et al*. 2000).

To induce somatic embryo formation from flax zygotic embryos, it was necessary to culture the immature zygotic embryos on medium supplemented with 2,4-D (2-5 mg/l) and to do several subcultures (3-4 times) at 2-week intervals. After transfer to auxin-free medium during the second subculture, mass formation of somatic embryos could be observed (Pret'ova *et al*. 2000).

Direct somatic embryogenesis

It is known, that the hypocotyl tissues of immature zygotic embryos of flax under certain conditions of culture are capable of producing somatic embryos without foregoing callus formation. A direct morphogenic response was obtained from flax embryos isolated at the late heart-shape to torpedo stages and cultured under specific conditions. According to Pret'ova and Williams (1986) this process appears to involve three main factors: a) inhibition of the main embryo shoot-root axis; b) non-disorganization of the internal pre-embryogenic determined state of the initiating cells; c) a continuing stimulus for mitotic divisions.

It is suggested that initiation involves a weakening of the cell-cell interaction gradient which coordinates normal bipolar development of the embryo. In the presence of a continuing stimulus for mitotic divisions, cells which are relatively undifferentiated and retain their internal predetermination for embryo morphogenesis may escape from overall group control to re-initiate the embryogenic pathway independently as somatic embryoids (Pret'ova and Williams 1986).

It is believed that the formation of accessory cotyledons and root poles on the zygotic embryos is a process, which might be described as embryo proliferation or «cleavage», homologous with the production of discrete, complete embryos. Embryo cleavage and somatic embryos formation produce a continuous spectrum of morphogenic structures and appear to be manifestations of the same phenomenon. The production of accessory cotyledons and embryos at the top of the hypocotyl and accessory root poles near the base presumably reflects a gradient along the hypocotyl, either with respect to early stages of differentiation of the hypocotyl cells initiating morphogenic structures, or to some other determinative factor affecting cell-cell coordination (Pret'ova and Williams 1986).

The process of somatic embryo formation began with the thickening of the hypocotyl tissue and its conspicuous striation. Later on, cotyledonary somatic embryos were observed on the hypocotyl tissue of the zygotic embryo without callus formation. Embryos and accessory cotyledons usually were formed in a ring around the top of the hypocotyl, and on some embryos 1-2 accessory root poles were also initiated lower down near the original root pole. This response was particularly marked for embryos dissected at late torpedo or very early cotyledonary stages, but did not occur for some younger and older embryos. Normal plants with viable pollen were obtained from those embryos (Pret'ova and Williams 1986).

Thus, on the zygotic embryos of flax somatic embryos can be formed through both direct and indirect pathways. Cell predetermination and proper application of growth regulators at the proper stage and concentrations are the key factors in both procedures. In the described system of direct somatic embryo formation the cells of the young zygotic embryos were proembryogenic determined cells, and BAP was sufficient for induction of somatic embryo development. In the system of indirect somatic embryo formation the cells of older zygotic embryos were induced in embryogenically-determined cells after 2,4-D treatment (Pret'ova *et al*. 2000).

Although histological studies were not done, the initial appearance of embryogenic structures as broadly based mounds on the hypocotyl surface strongly suggests multicellular initiation (Pret'ova and Williams 1986). This contrasts with the epidermal origin of adventitious shoots formed on the hypocotyls of decapitated seedlings *in vivo*, on hypocotyl and on stem segments *in vitro* (Murray *et al*. 1977). In the study of Murray and co-workers, some shoots were also differentiated indirectly from callus derived from conical cells and the fascicular and interfascicular cambium. Direct shooting, however, appeared to involve only superficial cells.

Development of a somatic embryogenesis system may provide a significant tool not only for research and breeding, but also, ultimately, for production of linseed oil lipids in a continuous mass culture system. Furthermore, flax embryos have proved a particularly favorable material for studies of morphogenesis *in vitro*. Responses are rapid and, depending on the state of maturity, provide clear examples of either embryogenesis or adventive organogenesis.

Shoot regeneration on the flax cotyledon explants

The striking example of physiologic block of morphogenesis was the absence or seldom morphogenic ability in flax cotyledons, so most of researchers characterized cotyledon explants of *L. usitatissimum* as low-morphogenic (Bretagne *et al*. 1994, 1996; Dedičova *et al*. 2000; Poliakov 2000). This physiologic block of organogenesis of the cotyledon explants was overcome in the studies of the Russian researchers by changing culturing conditions (Belonogova and Raldugina 2006). Moreover, in this case, the optimal phytohormonal balance of the medium and culturing conditions varied depending on physiologic age and origin of explants. Significant improvement of regeneration frequency showed the explants pretreatment during 2 days in the dark on the special medium. This medium contained NAA (2 mg/l), kinetin (4 mg/l) and 2,4-D (0.1 mg/l). It was suggested, that pretreatment on this medium, containing higher auxins concentration, increases the level of cells dedifferentiation. And as it was mentioned before cells division reinitiation requires a certain degree of cell dedifferentiation, therefore, the regeneration frequency in the pretreated explants subsequently transferred on the regeneration medium significantly increases because the cells become more competent for the regeneration (Belonogova and Raldugina 2005). The regeneration medium contained BAP (1-4 mg/l), and it was or in some cases it was not supplemented with NAA (0.05-0.5). The greatest callus formation was observed on the media with high auxin content. Callus formation occurred rapidly (within 10-20 days), and its color varied from light yellow to dark green. The higher was the BAP level in the medium, the more calli were formed. No shoot regeneration was observed in treatments with the most intensive callus formation. Regeneration commonly started on the 4^{th} week of the first subculture or the 1^{st} week of the second subculture. In all cases the regeneration of shoots was observed on the cut surface of the explant where a small callus was first formed.

Shoot regeneration was primarily genotype-dependent. In this study the cotyledons of 5-10 days-old seedlings of four fibre flax cultivar of domestic breeding ('Belosnezhka', 'Lenok', 'Alexim', and 'A-29') were used. Among the tested cultivars explants of 'A-29' showed the highest morphogenic ability. They produced the highest number of shoots (up to 105-145% from number of explants that have been used) under the same conditions. Furthermore, even the explants from 10-days-old seedlings of this cultivar possessed low morphogenic ability (3-8%), whereas no shoots formed on the 10 days-old explants of other genotypes. Cultivar 'Alexim' had the lowest morphogenic potential under the used conditions. Only the explants from 5 days-old seedlings of this cultivar formed shoots (3-13%). In this study it was assumed that the reduction in regeneration frequency in the explants of the physiologic age older than five days is associated with the assimilation and reduction of internal growth regulators and reserve nutrient components in cotyledons. By the 5^{th}-6^{th} day, the apical buds of the seedlings usually start to develop. Therefore,

for regeneration on cotyledon explants, it was necessary to utilize cotyledons before first true leaves developed. This confirms the observations have made on the other plants. For example, the best regeneration of *Swainsona salsula* was obtained on cotyledons excised from 3 days-old seedlings (Yang *et al*. 2001), in *Brassica napus and B*. *campestris*, the best age of seedlings was 5 days (Raldugina and Sobolkova 1995; Malishenko *et al*. 2003) and the limitation of the optimal age of explants was also associated with the apical bud development. Furthermore, this supposition confirms also the fact that the regeneration frequency on the physiologically younger explants (5 days-old) increased on the medium containing less concentrations of cytokinin (BAP 1 mg/l) and in the absence of auxin, whereas in the older explants (8 days-old) higher concentrations of cytokinin (BAP 2 mg/l) and the presence of auxin NAA (0.05 mg/l) was needed (Belonogova and Raldugina 2006).

During the optimization of regeneration medium by the content of macro- and micronutrients in this study, it was showed that the enrichment of nutrient media with Zn^{2+}, B^{+} and Mn^{2+} positively influenced on the regeneration frequency and, and in combination with heightened thiamine concentration and supplementation with asparagine, serine, glutamine, and glycine, significantly increased the regeneration frequency (up to 180%). This also confirms the observations have made by other scientists on the hypocotyl explants of flax (Poliakov 2000).

Thus, in this study the factors affecting shoot formation from cotyledon explants of fibre flax as the basis for the new regeneration system were discussed. In the majority of studies on flax genetic transformation as the primary explants the hypocotyl segments were used. But as it was mentioned in our paper before, despite of easy plant regeneration from hypocotyls segments, these explants have an essential disadvantage because after genetical transformation via *Agrobacterium tumefaciens*, they develop significant number of chimeric shoots (up to 45%) (Dong and McHughen 1993). The developed method for shoot regeneration from cotyledon explants of fibre flax is highly efficient and can be used as alternative protocol for flax transformation. This method showed good results in *Agrobacterium*-mediated fibre flax transformation (Belonogova *et al*. 2003). Although histological studies were not done, the coloration of transgenic shoots containing reporter GUS genes and segregation of transgenic plants in the generation T_1 allow to assume, that adventitious shoots, formed on the cotyledon explants, arise from single primary cells (Belonogova 2006).

Anther culture

Other explants, which have been most frequently used in the tissue culture of different *Linum* species, are anthers. The regeneration ratio in the anther culture is far lower than for somatic tissues (the percentage of plants regenerated from anthers ranged from 0.32–7-10% (Rutkovska-Krause *et al*. 2003)), and the ploidy level of callus cells derived from anthers varied much strongly than for somatically derived callus, but despite all these findings, it is believed that anther culture technique can help accelerate breeding programs through avoidance of repeated, time-consuming cycles of inbreeding. Microspore culture may become an alternative to the production of doubled haploid lines, but it still needs further improvement before routine application in the flax breeding program. The microspore-derived populations and doubled haploid populations have been used to study the inheritance patterns of molecular markers and disease-resistance genes. Furthermore, between the practical results obtained in flax anther culture for the crop it is important to note that the plant material obtained in anther culture in comparison to initial forms and best commercial cultivars characterized by higher level of the main agricultural traits which allows to create initial material for flax breeding. Thus, Russian scientists have studied 357 regenerants obtained in anther culture of different fiber flax genotypes: ‘R-736’, ‘Belinka’, ‘Dashkovsky 2’ and others (Poliakov *et al*. 1998). Testing plants of the second to the third generations showed that some regenerants according to the one or a few main agronomical traits surpassed significantly initial genotypes and standard cultivar. It was found that the most often regenerants formed in anther culture surpass initial cultivars by seed productivity (number of bolls on a plant (7.3-28.3%), number of seeds on a plant (21.8-29.0%)), dry mass of fibre (12.4-13.0%) and dry mass of stems (7.2-17.1%). The obtaining of regenerants which surpassed initial genotypes by content of fibre (0.7-1.0%) and length of stems (0.5-4.3%) were much rare (Poliakov *et al*. 1998). Anther culture allowed obtaining regenerants which surpassed initial genotypes according to two and more traits, by: length of plant – dry mass of stems; dry mass of stems – number of seeds on a plant; length of plants – dry mass of stems – number of seeds on a plant and some others. Regenerants which surpassed significantly initial genotypes according to length of plants and dry matter of stems composed 4.7-6.5%. Regenerants which surpassed initial genotypes according to dry matter of stems and number of seeds on a plant consisted of 6.5-11.9% and according to length of plants-dry matter of stem-number of seed on a plant – 3.6-4.1% (Poliakov *et al*. 1998). Furthermore, the assessment of lines obtained on the basis of anther culture regenerants in breeding nursery allowed to select high seed productive lines which were also resistant to *Fusarium oxysporum* (Rutkowska-Krause *et al*. 2003).

Development of haploid or dihaploid lines is based on a regeneration capacity of microspores in immature anthers. They can be able to produce either pollen embryos (direct regeneration) or callus-regenerating buds (indirect regeneration). At the basis of the anther culture method is the phenomenon of androclinia – switching the development of sporogenic cells from the common for them gametophytic way on the fundamentally different – the sporophytic way of development. Changes of generations, that is typical for the conditions *in vivo*, it does not occur, microspores or cells of pollen grain behave similarly to the zygotes.

Anther calli can be haploid or with higher ploidy or even aneuploid (Rutkowska-Krause *et al*. 1998), however plants regenerated from the anther calli were predominantly diploid and, in less frequency, haploid (Nichterlein *et al*. 1991; Rutkowska-Krause *et al*. 1996; Tejklova 1996). In most cases only indirect plant regeneration has been described in flax anther culture.

The flax anther culture has been studied since the early 1980s in China (Sun and Fu 1981). They were followed by Nichterlein in Germany (Nichterlein *et al*. 1991), Poliakov in Russia (Poliakov 1991; Poliakov *et al*. 1998; Poliakov 2000), Tejklova in the Czech Republic (Tejklova 1998), Rutkowska-Krause with co-workers in Poland (Poliakov *et al*. 1998; Rutkowska-Krause *et al*. 2003), Chen in Canada (Chen *et al*. 1998) and Pret’ova with co-workers in the Slovak Republic (Pret’ova *et al*. 2000). The studies indicated high genotypic dependence in anther response and the influence of culture conditions. Another considerable problem in both anther and isolated microspore cultures in flax is the low number of plants regenerated.

It was shown by authors mentioned above, that for organogenesis induction in the anther culture of flax the specified conditions of the cultivation several factors had a value: genotype and the age of donor plant and their culture conditions; stage of the pollen development and pretreatment of isolated anthers, exogenous growth regulators and other cultural factors (basal media, carbohydrates) (Chen *et al*. 1998; Tejklova 1998; Poliakov 2000; Rutkowska-Krause *et al*. 2003).

Thus, in the researches of Slovak scientists (Pret’ova *et al*. 2000) androgenic response was obtained only if the cultured anthers contained microspores in late uninucleate stages. The first calli generally appeared within 3 weeks of the start of culture. Callogenesis was not synchronous, and callus formation occurred at different intensities. From one responding anther mostly 1-2 microspore-derived calli were

obtained. The calli were of variable size and color (white-yellow, yellow or yellow-green, green). Percentage of anthers producing calli ranged from 0% to 12% depending on the composition of the nutrient media, however the optimal composition depended on the genotype. Calli more than 1 mm diameter (after 30-35 days of culture) were transferred to regeneration medium by Nichterlein *et al*. (1991) containing 1 mg/l BAP. From the calli derived on media by Nitsch (1974) either shoots or embryolike structures were obtained. Embryo-like structures did not develop beyond widened globular stages. In those experiments yielded plants formed via shoot formation. The microspore origin of the regenerants was confirmed using karyological analysis (Pret'ova *et al*. 2000).

In the experiments of Czech scientists genotype differences on three media differing in nitrogen content in basal medium and in levels of NAA and BAP were noted. Between the two genotypes that were testes, explants of 'Areco' cultivar were more responsible than explants of 'Marina' cultivar on all media. Callogenesis on the explants of 'Marina' cultivar was the same on all media, that have been tested, but it was more intensive on the explants of cultivar 'Areco', the highest shoot bud regeneration was obtained on the lowest level of NAA (0.05% mg/l) (Tejklova 1998).

Between the donor plant culture conditions the importance of the season, temperature and photoperiod, where donor plants were cultivated, the importance of sowing density and additional fertilization were noted (Poliakov 2000; Rutkowska-Krause *et al*. 2003). For example, better results in the plant regeneration in the anther culture were obtained in the spring and the autumn when the donor plants were grown in lower temperature in the field, as compared to the summer. Callus formation in anthers harvested from plants grown either in vegetation hall or in greenhouse was higher in anthers from the plants cultivated at the lower temperature (7-13°C /13-25°C day/night). It was suggested that mainly low night temperature of donor plant growing can positively influence anther responsiveness *in vitro* (Nichterlein *et al*. 1991; Tejklova 1998). Anthers harvested from plants grown in short day (8 h) conditions were more responsible than those from plants grown in a 16-h day. Lower sowing density and additional fertilization caused better state of donor plants and higher callogenesis in the anthers cultured *in vitro*. The highest callogenesis in anthers was obtained in the anthers from plants grown in 5 × 5 cm and additionally fertilized (Tejklova 1998; Poliakov 2000).

Between the anther culture conditions it was shown, that organogenesis induction is significantly influenced by explants pretreatment with high (Chen *et al*. 1998; Tejklova 1998) or low temperatures (Tejklova 1998; Poliakov 2000; Rutkowska-Krause *et al*. 2003), dark period (Tejklova 1998; Chen *et al*. 1998; Poliakov 2000; Rutkowska-Krause *et al*. 2003). For example, pretreatment of explants 16-72 h with the higher culture temperature (35 and 38°C) showed an insignificant increase of callogenesis in the anthers, but showed a decreasing of bud induction in anther calli (Tejklova 1998). However, the low temperature pre-treatment effect displayed the differences between the flax cultivars. On the number of genotypes (cultivars: 'Agreco', 'Nike') 1-5 days pretreatment with low temperature (4°C) decreased callogenesis in anthers (Tejklova 1998; Rutkowska-Krause *et al*. 2003), whereas other fibre flax cultivars ('Alba', 'Orshansky 2') showed a slight stimulation of callusing rate after 12 h and 24 h incubation at 4°C, respectively, but the activation of callus formation was not accomplished by an increase in regeneration rate in these plants (Rutkowska-Krause *et al*. 2003). Callus formation was influenced with the light conditions of the anther culture. It was higher on the explants pretreated or cultivated in the dark than in the 16-h photoperiod (Chen *et al*. 1998; Rutkowska-Krause *et al*. 1998; Tejklova 1998; Poliakov 2000). Furthermore, 4-weeks dark period at 21°C gave higher callogenesis than at 30°C. However, callogenesis on the explants pretreated in the dark was higher, but in some cases shoot bud regeneration was higher in calli cultivated in a 16-h photoperiod (Rutkowska-Krause *et al*. 1998; Tejklova 1998).

Between plant growth regulators that in anther culture were tested in the study of Czech scientists (Tejklova 1998), following auxins (1 mg/l each): 2,4-D, NAA and Picloram induced more intensive callus formation than Benzolinon, Dicamba and IBA. IAA or TIBA in the culture media in the same concentrations appeared to be the most ineffective. Zeatin was more effective for the callus and shoot bud formation in anthers than BAP in the same concentrations (only the culture media supplemented with NAA 1 mg/l in the combination with 2 mg/l of cytokinin was tested). Furthermore, effectiveness of BAP was compared to the effectiveness of the cytokinin meta-topalin (2 mg/l). Meta-topalin was more convenient for callus induction than BAP. However, evaluation of induced calli had to be done very carefully because callus sometimes arose from the rest of filament. Culture of anthers on the medium with meta-topolin instead of BAP with subsequent bud regeneration on medium, complemented with BAP as a source of cytokinin, gave better results than using only BAP or meta-topolin in both induction and regeneration media. Anthers, which were placed onto the surface of culture medium, solidified with agar, produced calli in higher frequency, than those explants, that were immersed into the medium.

Sun *et al*. (1991) reported about the interaction of KNO_3 and NaH_2P0_4 as a very significantly influencing factor of variability in callogenesis in anthers. Furthermore, influence of different levels of nitrogen in anthers culture medium on callus formation and bud regeneration depended on the genotype. Anthers were cultured on MS medium which was modified by NH_4NO_3 and growth regulators contents. Induced calli were transferred to three different media for bud regeneration. Regeneration of buds was the higher on media with the highest level of NH_4NO_3 regardless of initiation medium. The highest bud regeneration was obtained when medium with lower NH_4NO_3 content was used as the initiation medium and medium with higher NH_4NO_3 content was used as a regeneration medium (Tejklova 1998).

Carbohydrate content in culture medium is also often reported as a factor affecting plant regeneration. In the researches of Czech scientists the influence of sucrose content was determined by plant growth regulators. There were no differences in the anther callogenesis and organogenesis between media (developed by Nichterlein *et al*. (1991)) containing 6 and/or 10% sucrose, but they occurred when NAA in culture medium was replaced with 2,4-D. The best variant was medium contained 3% sucrose + 3% maltose and the worst one with 6% glucose (Tejklova 1998). Poliakov prefers combination glucose-sucrose to sucrose alone (Poliakov 2000). Chen *et al*. (1998) found 6-9% maltose in induction medium more convenient for bud regeneration than other tested concentrations (3-15%). Calli developed on medium with 6% sucrose gave the higher bud regeneration than calli from 9-15% sucrose media (Chen *et al*. 1998). Whereas modified optimized culture medium for the low linolenic oilseed flax cultivars in the more recent studies contained 9% sucrose (Chen and Dribnenki 2002).

Callogenesis in anthers can be influenced with the level of all components of the culture medium. The influence of the strength of culture medium on the callus formation was noted. Reduction of all components of the culture medium to one half increased the callogenesis in anthers (Tejklova 1998).

In spite of the production of doubled haploid plants in anther culture of flax has been improved significantly, however, plant regeneration is highly dependent upon the genotype of the donor plants; therefore the second approach to improve anther culture response and to speed up an effective doubled haploid production is to identify highly responsive genotypes. It was found out that the anther culture response could be improved dramatically when the F_1, hybrids of recalcitrant genotypes with responsive genotype were used as the donor plants (Chen *et al*. 1999; Chen and

Dribnenki 2002).

Thus, object of the study of Canadian scientists was to evaluate the response of a wide range of flax germplasm to a standard anther culture protocol (Chen *et al*. 1999). 44 flax genotypes, consisting of 32 cultivars or advanced breeding lines and 12 F_1 hybrids were evaluated. These genotypes were chosen on the basis of their important quality and agronomic performance, including low cadmium content, low cyanogenic glycosides content, high oil content, high linolenic acid content, yellow seed coat color, low linolenic acid content (Linola™ quality), early maturity and high yield potential. A strong genotype effect on callus induction and shoot regeneration in anther culture was confirmed. A number of genotypes, including two low cadmium content lines 96-11785 and 96-1 1826, a high oil content line 96-22109 and a high linolenic acid content line M 4919 were identified as highly responsive. Calluses were produced from all genotypes evaluated except 94-5358-6. The percentage of anthers producing calluses ranged from 0.3 to 44.5. Shoots were regenerated from all responding genotypes, except 94-5356-5, M 4669 and 96-22199. The overall efficiency of regeneration varied from 0.1% to 42.3% depending on genotype. In most cases, more than one callus regenerated into shoots for a given responding anther. Most F_1 hybrids appeared to have higher anther culture efficiency than their respective parents (Chen *et al*. 1999).

The strong genotypic effect in anther culture of flax observed in this study further confirms the importance of genetic background in determining callus induction and shoot regeneration found previously. The highly responsive cultivars or advanced breeding lines identified in this study have been used as parents to cross with agriculturally desirable genotypes to produce F_1 hybrids for routine double-haploid production. Preliminary data have shown that F_1 hybrids with improved efficiency of regeneration could be obtained by crossing one parent having a good callus induction with another parent having relatively high shoot regeneration. This was verified by comparing the overall efficiency of regeneration of pure lines (Chen *et al*. 1999).

The average frequency of microspore-derived plants for AC McDuff/94-5355-5 and AC McDuff/94-5356-5 FI hybrids was estimated as about 55% and the frequency of spontaneous chromosome doubling in microspore derived plants as approximately 38% (Chen *et al*. 1999).

In more recent studies of this scientific group, 16 genotypes/populations consisting of the economically important low linolenic linseed cultivars, advanced breeding lines and nursery populations with important agronomic and quality characteristics were evaluated (Chen and Dribneniki 2002). Differences in callus induction and shoot regeneration were obvious. A number of genotypes, such as 96-3-F. and 94-72 had a higher overall efficiency of regeneration and higher anther efficiency than others. The overall efficiency of regeneration was generally higher than anther efficiency since more than one callus from each anther regenerated shoots. Five of the six genotypes that did not produce any shoots in this study had a common parent in its pedigree. The anther culture response could be improved dramatically when the F, hybrids of recalcitrant genotypes with responsive genotype were used as the donor plants. The second approach to improve anther culture response of the recalcitrant genotypes is to identify physiological and environmental factors, particularly medium components that influence the response of anthers in culture (Chen and Dribnenki 2002).

In order to use anther culture as a viable breeding tool, regeneration from most genotypes of interest with a reasonable rate of success would be essential. Therefore, the current efforts should be focused on the following directions: to improve the regeneration from the recalcitrant genotypes: to reduce the regeneration from somatic tissues of anther: to increase the frequency of spontaneous chromosome doubling in microspore-derived plants; to develop a system to induce direct embryogenesis from microspore using anther/or isolated microspore culture and investigating the molecular genetics determining callus induction and shoot regeneration and for the subsequent suitable molecular marker screening methods developing. In spite of the production of doubled haploid plants in anther culture of flax has been improved significantly, however, there are still some unknown factors influencing negatively plant regeneration yet and modifications of the anther culture protocols for particular breeding material are still needed.

Plant protoplasts as the source of the new forms of Linum

The protoplasts of plant cells can be used also as the source of obtaining the new forms of plants. Protoplasts of almost all forms of plants are the good objects for works with mutagens and the somaclonal variants obtaining. Protoplasts can also be used for cytoplasmic genetic determinants transfer by protoplasts fusion and obtaining from the products of their fusion the new forms of plants.

It is known that some wild species of *Linum* (2n=18) possess genes of agriculturally useful traits: disease resistance, for example, to the flax rust (*Melampsora lini* Lev.), drought resistance, high quality of oil in seeds (Seetharam 1972). However, the attempts to obtain hybrid seeds by the cross pollination of cultural flax (2n=30) with wild species from the genus *Linum* were not successful (Beard and Comstock 1980). Somatic hybridization using the protoplasts allows the potential possibility of obtaining the hybrids between the closely-related and phylogenetically diverse species of plants, which is not possible to cross by usual methods (Butenko 1999). Protoplasts are ideal recipients for the alien DNA. It is possible to obtain the genetically modified plants via the genetic transformation of the isolated protoplasts. Furthermore, in protoplasts culture manipulations at a level of an exchange of extranuclear genetic elements (organellas) are possible. Besides that the protoplasts irradiation for the nucleus inactivation allows to create more effective system of organelle transfer.

One of the first successful works for *L. usitatissimum* was establishing conditions for enzymatic protoplast isolation, culture and low frequency plant regeneration obtaining, has been made by Barakat and Cocking (1983). Later they developed techniques of protoplast isolation from the roots, hypocotyls, cotyledons of seedlings, and also from their *in vitro*-grown shoots and cell suspension cu1tures and the subsequent regeneration of plants for a number of wild flax species: *L. stricktum*, *L. altaicum*, *L. narbonense*, *L. grandiflorum* (Barakat and Cocking 1985). Protoplasts were isolated from all the sources as well as all species investigated. Highest yield of protoplasts was from cotyledons with significantly lower numbers of protoplasts from hypocotyl and cell suspensions. First divisions occurred within 48 h in the case of cotyledon, hypocotyl and cell suspension protoplasts and within 48-72 h in those from roots and shoots. Colonies grew into calli and produced masses of green nodules in the case of all the species and in most of the media which were surveyed for their regeneration capability. Nodules sometimes failed to regenerate into shoots even after three subcultures on fresh aliquots of the same medium from which they had developed, and rhizogenesis was more common. This was observed in the case of *L. alpinum*, *L. narbonense*, *L. grandiflorum* and *L. altaicum*. However shoot regeneration was obtained from cotyledon protoplasts of *L. strictum* in MS agar medium containing 1.13 mg/l BAP and 0.02 mg/l NAA, but only a low percentage of calli (1%) formed shoots. This medium had been found suitable for shoot regeneration in stem and callus explants of flax (Murray *et al*. 1977) and suitable for shoot regeneration from protoplast-derived tissues of root and cotyledon of *L. usitatissimum* (Barakat and Cocking 1983). High frequency (70%) shoot regeneration was obtained in the case of *L. lewissii* from either *in vitro*-grown shoot protoplasts or from cell suspension protoplasts. To obtain a high frequency of shoot regeneration from *in vitro*-grown shoot protoplasts, colonies were transferred to MS

containing 0.05 mg/l NAA and 0.5 mg/l BAP. To obtain high frequency of shoot regeneration from cell suspension protoplasts, of *L. lewissii*, either colonies or calli which had been produced on solidified MS containing 2.0 mg/l NAA and 0.5 mg/l BAP were transferred to the same but liquid medium. The success of shake cultures may be related to the more efficient washing away or diluting out of endogenously produced auxins or other substances that may otherwise suppress regeneration. It has been suggested that the use of root, hypocotyl and etiolated cotyledon protoplasts could be of advantage in flax somatic hybridization assessments (Barakat and Cocking 1985). The high plant regeneration capacity of *L. lewissii* from protoplast derived tissues of *in vitro* shoots and cell suspension cultures makes this species an attractive experimental system for somatic hybridization with *L. usitatissimum*.

In order to realize the transfer genes in *L. usitatissimum* by protoplasts fusion more investigations on the possibility of the regeneration of plants from the isolated protoplasts of fourteen cultural flax genotypes and several wild forms (*L. alpinum*, *L. arboretum*, *L. amurense*, *L. catharticum*, *L. hologynum*, *L. leonii*, *L. perenne*, *L. salsoides*) have been done by other scientists (Binding 1986; Ling and Binding 1987). Different parts of the seedlings and shoot apices were used as the donor material. Good protoplasts preparation of all species and genotypes, except *L. arboretum*, were obtained. It was shown that the frequency of regeneration and capacity for callus formation depended on the genotype of the donor-plants. This corresponds to the findings of Barakat and Cocking (1983, 1985). Five wild *Linum* species and eight genotypes of *L. usitatissimum* regenerated shoots (Ling and Binding 1987). Best callus formation and the subsequent regeneration of plants were obtained from protoplasts which has been isolated from apex of the seedlings. Protoplasts of *L. salsoides* showed the highest regeneration frequency of calli, approximately 20-40%. Good plant regeneration capacities were shown in *L. amurense* and *L. hologynum*. However, the regeneration frequency for all the responsible genotypes of *L. usitatissimum* was very low. A single adventitious embryo was regenerated from a protoplast- derived callus of cotyledons in *L. alpinum* on medium MS with 2.5 μM 6-BAP and 0.1 μM NAA 48 days after the isolation of protoplasts. Differences in the capacity to regenerate probably occurred due to different demands of the culture conditions rather than to the loss of the totipotency of the cells. This conclusion stems from the observation that the susceptible species showed different responses to the organogenetic culture media and that meristem protoplasts were present in the preparations of all species. The most universal medium appeared to be the MS media with 6-BAP (2.5 μM). Addition of coconut milk (5%) was beneficial for shoot formation and for callus proliferation of young regenerants (Ling and Binding 1987).

Interestingly, in flax protoplast culture under identical culture conditions and independent of the genotype, significantly different types of flax callus appeared. Two types of callus were found in each of the fourteen genotypes on identical culture media. Type 1 callus was bright green and compact and the inner structure of this callus was compact; many vascular tissues were differentiated; most intracellular spaces were filled with air. Type 2 callus was dark green and soft and looked like sponge parenchyma; fewer vascular tissues appeared and all intracellular tissues were filled with water. Both types were able to differentiate shoots. Roots were organized only from dark green, soft callus type 2. This callus differences may be traced back to the original protoplasts donor cell type. Differences between the species were also found with respect to root formation. This was indicated by different pathways of root development as well as by different response to IAA and NAA, respectively (Ling and Binding 1987).

The possibility of regeneration from the protoplasts, isolated from shoots of *L. marginale* was studied by Zhan *et al*. (1989). Plants regeneration of high efficiency was obtained and the possibility of genetic transformation *L. marginale* was discussed.

One more attempt to improve the regeneration protocol from protoplasts of *L. usitatissimum* was made by Chinese scientists (Yuan *et al*. 2000). Four fibre flax cultivars ('7309', '948', 'Belinka' and 'Viking') were used. Plant regeneration was obtained in protoplast-derived calluses of '7309' (regeneration frequency = 0.8%) and 'Belinka' (regeneration frequency = 0.4%) on solidified media containing 0.6 mg/l 6-BAP and 0.1 mg/l NAA. Root and leaf regeneration was observed in Viking and 948, respectively (Yuan *et al*. 2000).

Resuming the results of all reported researches mentioned above, nine out of 14 *Linum* species are capable of regenerating plants from isolated protoplasts. High plant regeneration capacities were shown in *L. lewissii*, and in *L. salsoides*, *L. amurense*, *L. marginale* and *L. hologynum*. However the regeneration frequency in protoplasts-derived calli of *L. usitatissimum* remains relatively low. Because of these works the possibility of obtaining the protoplasts of different forms of flax was opened. Furthermore, protoplasts transformation via electroporation, microinjection and polyethylene glycol-induced uptake or protoplasts fusion techniques, with subsequent plants regeneration, can be used for the creation of genetic variety and the forms of fibre flax with the new traits obtaining.

Embryo culture as a source of genetic diversity

Embryos culture of interspecies hybrids and self pollinated genotypes can have a value for creating the new forms of the plants of flax with the valuable combination of traits. In the studies of Russian scientists, culture of immature embryos of hybrids that were obtained after interbreeding of different fibre flax cultivars, the lines of plants, surpassing the initial genotype in the morphological traits, and in the basic agricultural traits, such as: the height of plants, the mass of stem and the mass of fibre, were obtained (Poliakov 2000).

Moreover, culture of the isolated embryos, as anther culture, gives the possibility to obtain haploid flax plants. For example, rescue of hybrid embryos after pollination of *L. usitatissimum* with pollen of *L. grandiflorum* allowed up to 18% of haploid flax plants to be obtained (Poliakov 2000).

Although attempts to cross cultural flax with the wild forms were carried out (Seetharam 1972; Egorova 1999) actual results, in fact, remain ineffective; therefore interspecific hybrid embryo rescue and culture makes it possible to solve the problems of postgamic incompatibility. Additionally, valuable flax genotypes for initial breeding material, characterized by higher levels of main agricultural traits, can be obtained via regeneration or somatic embryogenesis following the culture flax embryos, obtained after interbreeding of different flax cultivars and even after interspecies crosses. Thus, Russian scientists studied plant regeneration after the culture of immature embryos obtained after hybridization with *L. usitatissimum* (fibre flax) with *L. grandiflorum* (Egorova 1999; Poliakov 2000). Most regenerants had the maternal (*L. usitatissimum*) phenotype and were highly viable. Regenerants with the paternal phenotype (*L. grandiflorum*) was much more rare (0.1-0.2%); furthermore, viability of those regenerants was very low. Precise analyze of those plants showed that they were doubled haploids formed after spontaneous doubling number of chromosomes. Testing plants of the 2^{nd}-4^{th} generations of 87 regenerants showed that some regenerants according to one or a few traits surpassed significantly initial genotypes and the standard cultivar, 'Alexim'. It was found that the most often regenerants formed in anther culture surpass initial cultivars by seed productivity. But several regenerants surpassed initial genotypes by length of stems and by content of fibre. Thus those regenerants surpassed initial genotypes and the standard cultivar in the mass of seeds per m^2 on 30% and 69% respectively; in the length of stems on 2% and 9% respectively. Moreover several lines

surpassed the standard cultivar in the mass of technical part of the stems –11-32%; in the number of bolls on a plant – 15-207%; in the number of seeds on a plant –16-136%; in mass of fibre –26-31%; and in the content of fibre –2.5-14%. Thus, the results obtained in those experiments showed that immature embryos culture of interspecies hybrids can be a new source of genetic diversity and plants with the traits of great agricultural importance obtaining. Lines, which were obtained in this study, were involved into practical process of fibre flax breeding (Egorova 1999).

Dependence of morphogenesis on the genotype of donor plant

One of the important factors of the explants morphogenetic answer, which is associated with the presence physiologic block of regeneration, is the plant's genotype. The dependence of morphogenesis on different types of explants from different genotypes in flax tissue culture was shown by many researchers (Barakat and Cocking 1985; Ling and Binding 1987; Koronfel and McHughen 1998; Tejklova 1998; Dedičova *et al*. 2000; Kalyaeva *et al*. 2000; Poliakov 2000; Chen *et al*. 2002).

Practically all experiments designed to optimize regeneration from *L. usitatissimum* tissues *in vitro*, including surveys of different basal media and different phytohormone types, concentrations and combinations, had been conducted, using mainly Canadian oilseed and some European genotypes. However, as was noted by many researches, the protocols for the regeneration of oilseed flax gives low frequency regeneration for fibre flax genotypes, thus, special protocols for the fibre flax genotypes were developed (Kalyaeva *et al*. 2000).

Furthermore, higher morphogenic activity of hypocotyl explants of oilseed flax in the comparison with fibre flax was noted. It was shown (Kalyaeva *et al*. 2000) that effectiveness of regeneration on the segments of hypocotyl for the oilseed flax cultivars is significantly higher, in a number of cases up to 20-40% in comparison with the fibre flax cultivars. The process of regeneration on the explants of oilseed cultivars occurs more rapidly by approximately 1 week. Among the tested Russian fibre flax cultivars, hypocotyl and cotyledonal explants of cv. 'A-29' possessed the greatest capacity for regeneration (Kalyaeva *et al*. 2000; Belonogova and Raldugina 2006).

Furthermore, Russian scientists studied the interaction between the effectiveness of regeneration, size of explant and genotype of donor plants (Kalyaeva *et al*. 2000). For cvs. 'Belinka', 'Dashkovskiy-2' (fibre flax), 'Norlin' (oilseed flax) a 7-10 mm size explant was the most efficient, and for cvs. 'A-29' and 'Aleksim' (fibre flax), 4-6 mm. It was also shown that the capacity for regeneration on hypocotyl segments and cotyledonal explants of fibre flax significantly depended on the age of seedlings, from which explants were excised (Kalyaeva *et al*. 2000; Belonogova and Raldugina 2006). Maximum regeneration for hypocotyls was observed on the explants which were obtained from 14 day-old seedlings, and an increase in age considerably decreased the regeneration capacity, completely disappearing in explants excised from 21 day-old seedlings (Kalyaeva *et al*. 2000); for the cotyledons maximum regeneration frequency was observed on explants excised from 5 day-old seedlings for the all genotypes and completely disappeared on explants older than 10 days.

The influence of genotype on the ability of plants to regenerated shoots *in vitro* and the poor response of some genotypes can be compensated to some extent by varying the composition of culture media and culture conditions of either source of explants.

Dependence of morphogenesis from the composition of the culture medium

The composition of the culture medium is the determining factor in the initiation of morphogenesis and the further development of formed structures. At present, there are a number of nutrient media which are used for *in vitro* cultivation and induction of morphogenesis (White 1939; Murashige and Skoog 1962; Gamborg and Evelegh 1968; Nitsch 1974). However, for many plant species introduced into *in vitro* culture, the developed protocols do not fit due to the fact that different plant species and organs, evidently, have their individual internal growth regulators and mineral element composition. Therefore very often it is necessary to adapt the composition of nutrient media for each new genotype and type of explants introduced into the culture *in vitro*. Many researchers optimized the cultural medium by the content of the macro- and micro-elements, vitamins, amino acids and other components, but mainly, by the content, combination and correlation of plant growth regulators.

In order to obtain regeneration in different *Linum* species the cytokinin BAP and auxin NAA were mostly used. The concentrations depended on the plant organs, which were exploited as the source of the primary explants, and the direction of the morphogenesis which was needed to obtain. For the majority of oilseed flax genotypes, it was shown that for shoot bud regeneration the optimum concentrations were: BAP at 1-2 mg/l and NAA at 0.02-0.1 mg/l (Basiran *et al*. 1987; Jordan and McHughen 1988; Dong and McHughen 1993; Dedičova *et al*. 2000; Yildiz and Er 2002; Hjordis *et al*. 2003; Yildiz and Ozgen 2004). Although for some genotypes the highest effectiveness of regeneration was with lower concentrations of growth regulators: BAP at 0.025; NAA at 0.001 (Rakousky *et al*. 1999), and also without auxins in the culture medium (Kaul and Williams 1987; Koronfel and McHughen 1998; Chen *et al*. 1998; Dedičova *et al*. 2000; Chen and Dribnenki 2002) or even without any growth regulators in the nutrient medium (Koronfel and McHughen 1998; Jain and Rashid 2001). The regeneration frequency varied on average from 5.5-9 depending on the plant genotype.

For shoot bud regeneration on hypocotyl segments of fibre flax the optimum concentrations were: BAP at 1 mg/l and NAA from 0.05 to 0.1-0.2 mg/l (Kalyaeva *et al*. 1998; Poliakov 2000), depending of the genotype. In this case the complete absence of NAA in the nutrient medium led to a considerable reduction in regeneration (Kalyaeva *et al*. 1998; Poliakov 2000). Good results on the hypocotyl segments of flax were obtained by using kinetin (1 mg/l) as the source of cytokinin, alone or in combination with NAA (Koronfel and McHughen 1998; Poliakov 2000). Those protocols improved the regeneration frequency (on average up to 9-10 shoots per explant for oilseed cultivars). However the effectiveness of type of cytokinin, which has been used, significantly depended on the plant genotype.

TDZ possessed cytokinin activity at 0.25 mg/l (Bretagne *et al*. 1994; Dedičova *et al*. 2000; Jain and Rashid 2001). Bud initials began to appear 6-7 days after planting. Knowing that light is an essential requirement for shoot bud regeneration, the most significant finding was that TDZ could induce shoots in the dark, where BAP was ineffective. This effect of TDZ was dose-dependent. High concentration (0.5 mg/l) increased frequency of responding seedlings (from 40 up to 70%) (Jain and Rashid 2001). Furthermore, it was shown that fibre flax cultivars were more sensitive to TDZ treatments than oilseed cultivars (Dedičova *et al*. 2000).

TDZ, a synthetic phenylurea derivative is a non-purine that simulates cytokinin action (Mok *et al*. 1982). It promotes synthesis and accumulation of purines, as well as alter cytokinin metabolism. It stimulates the conversion of cytokinin nucleotides to nucleosides. Furthermore, it is known that phenylurea inhibits cytokinin oxidase. TDZ is more effective in stress-ethylene production than cytokinin. Therefore, it remains to be seen that efficacy of TDZ in induction of shoot-buds in the dark is effected on production of ethylene. It is a morphogen that supports diverse array of morphogenic responses in flax, ranging from tissue proliferation to induction of shoot-buds or somatic embryos

(Bretagne *et al*. 1994; Chen *et al*. 1998; Dedičova *et al*. 2000; Jain and Rashid 2001).

For embryogenic callus and subsequent somatic embryo formation, other correlations and concentration of growth regulators were necessary. Thus, embryogenic callus was formed on hypocotyl segments or on hypocotyls of immature embryos, which were sub-cultured 3-4 times on media containing 2,4-D at 0.4-2.5 mg/l. Subsequent transfer of explants to medium containing only BAP at 1 mg/l (Pret'ova *et al*. 2000) or zeatin at 1.6 mg/l (Cunha and Ferreira 1997), induced somatic embryo formation. Development of somatic embryos into plantlets involves several important steps, which are crucial at each stage. A number of experiments were carried out to induce the growth of the shoot apex. In several plant systems, the transfer to a growth regulator-free medium or reduction in auxin concentration, favors embryo development. However, for flax in these conditions the flax somatic embryos failed to develop (Tejavathi *et al*. 2000). On full strength or ¾-strength basal medium only extensive root growth was observed. On ½-strength basal medium secondary somatic embryos were observed. On ¼-strength medium, 1-2% of the somatic embryos grew into plantlets, but remained achlorophyllous. When embryogenic callus was transferred to MS + NAA (5.37 μM) + BAP (2.22 μM), green nodular structures developed into tiny plantlets but showed stunted growth and fused leaves. Somatic embryos developed into normal plantlets on the MS medium supplemented with NAA (2.69 μM) and BAP (2.22 μM). The survival rate on transfer to soil, was however only 2-3% (Tejavathi *et al*. 2000).

Slovak scientists (Dedičova *et al*. 2000) obtained embryo-like structures (ELS) on oilseed flax hypocotyl explants excised from 5-day-old seedlings. These explants were pretreated during 24 h on Mo medium (Monnier 1978) supplemented with 2,4-D (5 mg/l). Subsequent cultivation on medium supplemented with zeatin (2 mg/l) induced ELS formation. Replacing zeatin with BAP in combination with NAA inhibited the formation of ELS completely. Experiments also showed the correlation between 2,4-D and the duration of the treatment. The longer (72 h) the pretreatment, the lower the concentrations the greater the effectiveness of 2,4-D (2 mg/l) in morphogenenic induction. On regeneration medium supplemented with 5 mg/l 2,4-D hypocotyl explants of oilseed cv. Szegedi-30 produced white granular callus. With extended cultivation period, the callus produced white or light green parts with dark green globular embryo-like structures which later developed into more mature stages. Longitudinal semi-thin sections showed globular and differentiated bipolar stages. The globular and heart-shaped ELSs contained epidermises on their surfaces, two or three subepidermal layers of parenchyma cells, and meristematic tissues in their inner parts. The bipolar stages had elongated hypocotyls and two small polar located parts interconnected with vascular tissue which showed a deviation on the top of the structure typical for the entering of vascular strands to cotyledons. Very often the organization of both root and shoot poles was abnormal. Occasionally the root meristem did not develop a normal root cap. The shoot meristem was not located on the top of the structures, and normal leaf primordia failed to develop. The ELSs formed had poorly defined or fused cotyledons and no apparent shoot apices. As a consequence of the structural abnormalities, these arrested ELSs were unable to produce mature embryos and complete plants (Dedičova *et al*. 2000). It was assumed in this study that in this system of somatic embryo production, strong disturbance of the polar auxin transport occurred.

It is known that auxin polar transport strictly controls the initiation of the shoot apical meristem. The correct auxin transport influences the final initiation of cotyledons and the right bilateral symmetry of the early embryos. Even in globular zygotic flax embryos cultured *in vitro*, the formation of cotyledons was a crucial stage in their development (Pret'ova and Williams 1986). Root pole formation seemed to be less influenced by auxin transport since fewer abnormalities were observed there. With the auxin 2,4-D, some evidence of abnormal development was already apparent. A relatively high concentration of 2,4-D in the induction medium inhibited the further development of somatic embryos.

As was already mentioned above, during zygotic embryo maturation the hypocotyl can be induced to form somatic embryos directly, by explanting onto a medium supplemented with the cytokinin BAP (0.05 mg/l) together with yeast extract (YE) at 1 mg/l and glutamine (Glut) at 0.4 g/l. Cultivation on media containing the same concentrations of BAP and glutamine but without YE induced only intense callus formation (Pret'ova and Williams 1986; Kaul and Williams 1987; Pret'ova *et al*. 2000). However, cultivation of hypocotyl segments of mature flax embryos on media supplemented only with BAP stimulated the production of more shoot primordia on the lower hypocotyl, but prevented their elongation, and also suppressed elongation of the original shoot in upper embryo segments. The effect is similar to that of the epicotyl and cotyledons in growing seedlings (Link and Eggers 1946).

The specific roles of YE and BAP, which have been used for induction of embryos have not been defined. It is suggested that cytokinin provides the required mitotic stimulus, while one or more components of YE appear to be involved in both the suppression of the parent zygotic embryo axis, and the preservation of the embryogenic state of hypocotyl cells which allows continuing division to produce organized embryos rather than disorganized callus. BAP alone stimulates disorganized abnormal shoot growth and proliferation, and the formation of callus from cotyledon and hypocotyl tissues, while yeast extract alone does not stimulate cell divisions. That YE simply enhanced an intrinsic cellular response and was not determinative for somatic embryogenesis, was shown by the occurrence of occasional accessory root poles, accessory cotyledons or embryoids in treatments containing BAP but lacking YE. Addition of YE alone strongly inhibited the growth of the embryo shoot-root axis. Glutamine has been found necessary for growth and differentiation of very immature embryos (Pret'ova and Williams 1986). Basal medium without glutamine gave a similar response with slightly weaker growth. The lack of a full embryogenic response to the treatment (Glut + YE + BAP) may be due to a similar requirement for glutamine during early stages of somatic embryogeny. In the absence of glutamine, cultures showed slightly less overall growth than that observed in the corresponding treatments with glutamine. Treatments with BAP showed preservation of the green color of embryos, whereas in treatments lacking BAP, particularly (Glut+ YE), the embryo color faded during the culture period. This action of BAP in preserving the intense green color of embryos is similar to the preservation of chlorophyll by kinetin (Pret'ova and Williams 1986).

For obtaining morphogenesis in anther cultures of oilseed flax, according to Nichterlein and Friedt (1993), BAP at 2 mg/l and NAA at 0.1 mg/l were necessary; in contrast, Russian (Poliakov 2000; Proletova 2003) and Polish scientists (Rutkowska-Krause *et al*. 2003) claimed that the induction of embryogenic calli in anther culture of Polish and Russian fibre flax cultivars required lower plant growth regulator concentrations: 1 mg/l BAP and 0.05 mg/l NAA. Zeatin was also efficient in the induction of morphogenesis in anther culture, whereas kinetin was ineffective (Proletova 2000). BAP at 0.1-1 mg/l, NAA at 0.5 mg/l and IAA at 0.3 mg/l increased the intensity of callus formation, however, no regeneration was detected (Proletova 2003; Rutkowska-Krause *et al*. 2003). Thus, high auxin concentrations resulted in stimulation of callus formation and the inhibition of plant regeneration.

Abscisic acid is one of the plant growth regulators which influences different processes in plant cells. It is assumed, that the majority of general answers of ABA is growth retardation or inhibition. However, it also signifi-

cantly stimulates embryogenesis and organogenesis in the callus of several species, specifically, *Brassica oleracea* (Sharma *et al*. 1991), *Brassica rapa* (Sharma 1989), *Brassica napus* (Raldugina and Sobolkova 1995), *Pennisetum americanum* (Vasil and Vasil 1981), and *Pinus taeda* (Sen *et al*. 1989). A similar effect was observed on the hypocotyls of fibre flax seedlings, when they were cultured under conditions of insufficient mineral nutrition and transient ABA treatment. A significant increase in the quantity of plants forming adventitious shoot buds on hypocotyls was observed (Mundhara and Rashid 2001). However, for the somatic embryogenesis in flax callus cultures (Tejavathi *et al*. 2000) and for the induction of shoot regeneration on cotyledon explants of flax (Belonogova and Raldugina 2006) a transient ABA treatment was ineffective.

The direction of morphogenetic processes depends not only on the content of plant growth regulators in the nutrient medium. Other factors, such as the mineral composition of the nutrient medium, the concentration of sucrose, vitamins and amino acids, the presence of selective agents and other components can have an essential effect on morphogenesis. Optimal composition for regeneration of fiber flax hypocotyls occurred on medium containing the full strength MS or medium containing MS macronutrients and Gamborg and Shyluk (1976) micronutrients. Other researchers (Pret'ova and Williams 1986; Kaul and Williams 1987; Dedičova *et al*. 2000; Pret'ova *et al*. 2000) used the medium designed by Monnier (1978) for culture and morphogenic induction of mainly oilseed flax. Summarizing the published data, we assume that the optimal nutrient composition depends on the type of explant and on the direction of morphogenesis that was needed. Thus, for the somatic embryogenesis induction mainly the medium by Monnier has been used, whereas for the shoot regeneration more often the MS or sometimes in combination with micro-nutrients by Gamborg and Shyluk was used.

Another important factor affecting plant regeneration is the carbohydrate content in the culture medium. Sucrose at 2-3% was optimal for regeneration of hypocotyl segments (Koronfel and McHughen 1998; Dedičova *et al*. 2000; Kalyaeva *et al*. 2000; Poliakov 2000). For somatic embryo formation higher concentrations of sucrose (5%) were needed (Pret'ova and Williams 1986; Kaul and Williams 1987). However, an increase in sucrose concentration above 5% caused necrosis of the embryogenic calli and embryos.

The variation in sucrose concentration in flax anther culture media is wide: from 2% (Rutkowska-Krause *et al*. 2003) to 3-6% (Proletova 2000) to 12-13% (Nichterlein and Friedt 1993; Chen *et al*. 2002). The concentration and the type of carbon sources were determined on the type of morphogenesis needed. Thus, high concentrations of sucrose (from 6% and higher) in media for callus induction were most effective, whereas for the subsequent shoot regeneration, only sucrose at lower concentrations. According to Chen *et al*. (1998) calli developed on medium with 6% sucrose resulted in higher bud regeneration than calli from 9-15% sucrose-supplemented media. As already mentioned above good results were obtained on medium containing 3% sucrose + 3% maltose (Tejklova 1998) or on medium containing a glucose-sucrose combination (Poliakov 2000; Rutkowska-Krause *et al*. 2003). Chen *et al*. (1998) found that 6-9% maltose in the induction medium was better for bud regeneration than other tested concentrations (3-6% and 9-15%).

Furthermore, it appeared that the optimal carbohydrate content and concentration in flax anther culture is genotype-dependent. Therefore, to optimize the culture media for low linolenic oilseed genotypes, in the experiments three F_1 hybrids (99-179- F_1, 99-181- F_1, and 99-182- F_1) were used to investigate the effect of different carbohydrates on the response of anther culture. In this experiment, media containing 9% sucrose, maltose or lactose were compared (Chen and Dribnenki 2002). Calli induced from all three media were brown. The calli induced from 9% lactose were smaller than those from 9% sucrose or maltose. In comparison with sucrose, lactose increased the percentage of anthers producing calli for all three genotypes tested. Lactose significantly increased shoot regeneration for 99-182- F_1, but significantly decreased shoot regeneration for 99-179- F_1. In comparison with 9% sucrose, medium containing 9% maltose increased callus induction for two of the three genotypes tested. However, maltose did not increase shoot regeneration for any of the three genotypes (Chen and Dribneniki 2002).

Therefore, for the economically important low linolenic oilseed flax genotypes, but which are poorly responsive in anther culture, more efforts have been directed towards improving and modifying the culture medium. Preliminary studies with Linola^{TM} genotypes indicated that the overall efficiency of regeneration from anther culture was less than 3% (Chen *et al*. 1999). Linola® is the trademark name of low linolenic flax developed by the Commonwealth Scientific and Industrial Research Organization (CSIRO) (Green 1986) and currently produced commercially by a joint venture between CSIRO in Australia and Agricore United in Canada. Linola oil is considered a high quality edible oil as it comprises high polyunsaturated and low saturated fat. Therefore, the objectives of the study of Canadian scientists were to evaluate the anther culture response of 16 genotypes/populations from our Linola Breeding Program and to determine the effect of medium composition on callus induction and shoot regeneration in Linola anther culture (Chen *et al*. 2002). Two F_1 hybrids (96-3-F_1 and 96-45-F_1) were chosen to determine the effect of medium type on anther culture response. Two culture media were used: the first contained modified MS (165 mg/l ammonium nitrate), modified vitamins (10 mg/l thiamin hydrochloride) and was supplemented with 2 mg/l 2,4-D, 1 mg/l BAP, and 9% sucrose; the second contained 2 mg/l 2,4-D (Chen and Dribnenki 2002). On the first culture medium calli induced from both genotypes were brown and on the second medium were golden. After culture on shoot regeneration medium for 14-21 days, calli from the first medium became compact, shiny-looking and dark green. In contrast, calli from the second medium were friable, dull-looking and light green. However, there was no significant difference between the two media in shoot regeneration. The main difference between the media was in the salt concentration and in the presence of cytokinin. The first has approximately 10 times higher K^+ and 3 times higher Mg^{2+} than the second medium. Therefore, the concentration of these ions as well as the presence of cytokinin may affect chlorophyll synthesis and other physiological functions related to *in vitro* morphogenesis.

It is well known that morphogenesis in flax tissue culture could be regulated by the action of other components of the culture medium. Thus, the addition to the culture medium of different amino acids increased the frequency of regeneration in hypocotyl and in anther cultures. The induction of morphogenesis in the anther culture of fibre flax was increased by the addition of glutamine, asparagine, and serine (Poliakov 2000; Proletova 2000; Chen *et al*. 2002, respectively). The positive effect of the use of amino acids on adventitious bud formation, as well as on the rooting of developed shoots, was significantly improved by increasing the concentrations of boron (H_3BO_3 from 6.2 to 17-19 mg/l), zinc ($ZnSO_4 \cdot 7H_2O$ from 8.6 to 12.3 mg/l) molybdenum ($Na_2MoO_4 \cdot 2H_2O$ from 0.25 to 0.75 mg/l) and thiamine (from 1 to 10-15 mg/l) (Poliakov *et al*. 2000; Belonogova and Raldugina 2006).

As it was shown on the hypocotyl explants of flax, the concentration of calcium in the culture medium had considerable influence on the shoot buds regeneration (Jain and Rashid 2001; Mudhara and Rashid 2002). By the data of Indian researches, the best results were obtained on the hormone-free basal medium containing Ca at the concentration of 166 mg/ml (Jain and Rashid 2001). Furthermore, one of the usually neglected components of the nutrient medium, such as the gelling agent that is used for the me-

dium solidification, significantly influences the availability of calcium from the nutrient medium, as explained next (Laine *et al*. 2000).

The two most commonly used gelling agents are agar (or its purified derivate, agarose) extracted from the algae *Laminaria* and gellan gum, also known as Gelrite or Phytagel, an extracellular polysaccharide produced by *Pseudomonas elodea*. Gelling with gellan gum (Phytagel) involves Ca^{2+} ions, thus affecting Ca^{2+} availability in the medium after solidification. Phytagel requires Ca^{2+} ions to create its net, thus decreasing the remaining quantity of free Ca^{2+} ions. On the other hand, Phytagel contains 0.85% Ca (= 213 mmol/kg) (data provided by Sigma), that may, at least in part, counterbalance this decrease in Ca^{2+} concentration. As it was mentioned before, calcium is a key element in the control of a diverse array of cellular and morphological responses. Agar binds a very large proportion of the Ca^{2+} ions in the medium: more than one third of the quantity supplied by MS salts (Laine *et al*. 2000). The quantity of calcium ions provided in the medium along with agar only slightly counterbalances this reduction of available calcium, since Bacto Agar contains only 34 mmol kg/l of Ca^{2+} (Scholten and Pierik 1998). Mineral salt availability is also affected by the nature of the gelling agent and can account for the difference in behaviour of plant material placed on gelled media. The salts present as impurities in the gelling agents are quantitatively and qualitatively very different. Furthermore, it has been demonstrated that the calcium concentration in the medium can inhibit the effect of aminoglycoside antibiotics such as kanamycin, probably due to interference between the aminoglycoside effect and a particular gelling agent (Laine *et al*. 2000).

In order to combine the advantages of both gelling products or minimize their disadvantages, and reduce the occurrence of vitrification (hyperhydricity) in susceptible species, a mixture of agar and gellan gum is also commercially available, sold under the trade name Agargel (Sigma). Species subjected to vitrification sometimes require the use of agar, one of its components, probably an oligosaccharide or an "agaroid-type xylogalactan" preventing such hyperhydricity from occurring (Laine *et al*. 2000).

Transformation protocols generally involve the use of a selectable marker gene for the screening of transgenic material. This gene, at least for academic studies, is often the bacterial gene *npt*II, coding for a neomycin phosphotransferase. This enzyme detoxifies arninoglycoside antibiotics by phosphorylation thereby permitting cell growth in the presence of antibiotics in the same family, such as kanamycin, paromomycin and gentamycin. These antibiotics inhibit plant regeneration to varying degrees in wild (non-transgenic) material via inhibition of protein synthesis in the chloroplast. Kanamycin (and other aminoglycosides) is therefore commonly used as selective agents in transformation protocols for a wide range of species. Nevertheless, the screening for transgenic regenerated shoots is often partial, as many 'escape' shoots may be generated as a result of detoxification by adjacent transformed cells, e.g. in flax (Jordan and McHughen 1988). When such 'escapes' occur, additional screening steps on selective medium are necessary.

According to the data of Laine *et al*. (2000) selection efficiency varies greatly with the gelling agent used. Fresh weight of explants cultivated on media containing concentrations of kanamycin ranging from 50 to 200 mg/l was significantly better when the medium was solidified with Phytagel (Sigma). Growth was reduced 2-5–fold on media solidified by the two other gelling agents (Agargel (Sigma) and Difco Bacto Agar (Difko)); the strongest inhibitory effect was observed with Bacto Agar.

Therefore, to produce a similar efficiency of selection, the concentration of the selective agent has to be adapted to the nature of the gelling agent. These results indicate that the concentrations of kanamycin that can ensure convenient selection in the presence of agar, will fail to select transgenic buds if used with a Phytagel containing medium, even if the detoxification by transgenic cells is not taken in account. Thus, the kanamycin concentration has to be modulated when the nature of the gelling agent needs to be changed during the selection steps of a transgenic shoot regeneration protocol. Consequently, one has to be cautious when comparing protocols from different authors, or different steps within the same protocol, that require the use of different gelling agents, such as shoot regeneration or rooting (Laine *et al*. 2000).

To explain this phenomenon of the better behaviour of explants cultured on Phytagel in the presence of kanamycin several hypotheses can be suggested. This could result from different availabilities of the aminoglycoside due to precipitation, but no visible precipitation occurred. It could also be due to differences in uptake of the antibiotic resulting from competition for membrane transporters between Ca^{2+} ions and aminoglycosides as suggested by Joersbo and Okkels (1996), who observed an increase in the toxic effect of aminoglycoside antibiotics under a low calcium concentration. Calcium has also been reported to bind to kanamycin and to inhibit its antibacterial activity (Crawford 1972), and calcium gluconate is used medically to reverse the toxic effect of kanamycin (Noble 1976). Such a phenomenon could play an important role. Therefore the observed effect of gelling agent on the selection efficiency suggests that one should be cautious when choosing the gelling agent for a given selection step, and that the aminoglycoside concentration must be adapted as a function of the gelling agent (Laine *et al*. 2000).

Thus, summarizing the published data about the factors, controlling morphogenesis of plants in the genus *Linum*, it is possible to say that all the information is extremely contradictory.

Thus, most researchers starting their experiments have to optimize the existing protocols according to the genotype and type of explant used and the purpose of their experiments. Probably, one of the most important genotypic differences between the plants appears to lie in the content and the correlation of endogenous plant hormones and mineral elements in their organs, associated with the climatic and specific soil characteristics of the place of origin and growth of plants. Then, it would be possible to explain precisely why for the same genotype in each specific case it is necessary to find out the specified conditions for morphogenic induction.

Explant viability and regeneration capacity

Of significant importance to morphogenetic activity is explant viability. The most important treatment prior to culture initiation, which has a direct influence on the viability of the explant, is perhaps its surface sterilization. Since *in vitro* conditions provide bacteria and fungi with an optimal growth environment, unsuccessful surface sterilization hinders the progress of tissue culture studies. On the one hand, surface sterilization aims to eliminate all microorganisms that can easily grow in *in vitro* conditions; on the other hand, it should guarantee the explant's viability and regeneration capacity, which are known to be affected by the disinfectant concentration and sterilization period. It is well known that direct contact with a disinfectant during the sterilization process can have a severe effect on regeneration frequency. Therefore, the use of aseptic seedlings as a source of explants is highly recommended (Yildiz and Er 2002). A wide range of surface disinfectants, such as ethanol, hydrogen peroxide, bromine water, mercuric chloride, silver nitrate, and antibiotics are used for surface sterilization. However in routine tissue culture studies of flax, ethanol and sodium hypochlorite (commercial bleach) solutions in different concentrations have been most widely used for surface sterilization of seeds. Sodium hypochlorite (NaOCl) is highly effective against all kinds of bacteria, fungi, and viruses. It kills microbes by oxidizing biological molecules such as proteins and nucleic acids.

The negative effect of increasing disinfectant concen-

tration on shoot regeneration has been well known for many years. It significantly reduced seed germination, seedling growth, hypocotyl and root lengths of flax seedlings. It was observed that seed germination *in vitro*, seedling growth and shoot regeneration frequency of hypocotyl explants varied excessively between summer and winter seasons. All these parameters, which were not affected by surface sterilization regime in winter, were reduced significantly when carried out at ambient temperature on hot summer days. Results clearly show that in the surface-sterilization process, the concentration and temperature of sodium hypochlorite solutions are closely related to each other and they should be considered together. In the research by Turkish scientists it was experimentally confirmed that the regeneration capacity of explants was negatively affected by increasing the concentration of the disinfectant (the seeds of an ecotype (Diyarbakir) commonly cultivated in Turkey have been used) (Yildiz and Er 2002). This negative effect became also more severe with increasing temperature.

The surface-sterilization regime should aim to use the lowest concentration of disinfectant for the smallest amount of time. The best results in seedling growth and shoot regeneration were obtained when 40% (v/v) disinfectant (commercial bleach) at 10°C was used. Increased temperatures resulted in substantial decreases in shoot regeneration. It was also found that higher disinfectant temperatures resulted in morphologically abnormal seedlings with stunted hypocotyls and roots (Yildiz and Er 2002). These results could be related to the fact that the disinfection activity of sodium hypochlorite increases and that the disinfectant penetrates more easily through the seed coat (Schull 1920) at higher temperatures. Every 10°C increase in disinfectant temperature decreases the required time for sterilization by 50% (Racoppi 1990; Yildiz and Er 2002).

Explant pretreatments and the initiation of morphogenesis

Explant pretreatment has significant importance in inducing morphogenesis on the different types of explants. As was already mentioned, such a pretreatment significantly increases the morphogenetic response of anther and cotyledon explants and, interestingly, can also improve the regeneration frequency of hypocotyl explants (Chen *et al*. 1998; Tejklova 1998; Poliakov 2000; Yildiz and Özgen 2004; Belonogova 2006; Belonogova and Raldugina 2006).

For example, treatment of explants with water before culture initiation increased the morphogenic response. In the experiments of Turkish scientists (Yildiz and Özgen 2004) water-treated and non-water-treated hypocotyl explants of three flax cultivars ('Madaras', '1886 Sel.' and 'Clarck') were compared with regard to fresh weight, dry weight, shoot regeneration percentage, shoot number per hypocotyl, shoot length and total shoot number per Petri dish. Hypocotyl segments of 5 mm in length were excised from 7-day-old seedlings. Some hypocotyls were submerged in sterile distilled water with gentle shaking for 20 min before placing on regeneration medium, while others were directly cultured on MS medium supplemented with 1 mg/l BAP and 0.02 mg/l NAA for regeneration.

The results showed sharp and statistically significant differences in all cultivars between water-treated and non-water-treated explants concerning fresh and dry weights. Water-treated explants had a higher fresh weight than non-water-treated ones in all cultivars used. The water-treated explants had also a larger dry weight. The explants to which water-treatment was applied were more vital and grew well. In the parameters of shoot regeneration, shoot number per explant and total shoot number per Petri dish, in all cultivars, the highest results were obtained from pretreated hypocotyl explants.

To explain this phenomenon it was suggested that water pretreatment softened the epidermis layer and increased its permeability which caused higher tissue metabolic activity. Thus, increases in the fresh and dry weights of water-treated hypocotyl explants at the end of culture were due to an increase in the absorption of water and other components from the basal medium via a highly permeable epidermal membrane. All solutes and plant growth regulators transferred into the tissue more easily, provided all cells with a high regeneration capacity and consequently increasing explant tissue culture response. It was reported by other researches that cuticular water permeability increases significantly by increasing air humidity and that the fresh weight increase is mainly due to cell enlargement by water absorption, cell vacuolation, and turgor-deriven wall expansion (Dale 1988). Osmotic water absorption affects cell elongation. It has been suggested that osmotic stress modifies the biochemical changes in the cell wall during growth and that water stress alters the level of plant hormones (Morgan 1990). Dry weight increase of water-treated explants, probably, was closely related to cell division and was due to an increase in carbohydrate metabolism and new material synthesis resulting from increased water uptake (Yildiz and Özgen 2004).

Similar effects of water treatment were noted in the rooting stage. This means that shoots regenerated from water-treated explants were more capable of establishing new plantlets than the ones grown from non-water-treated explants. Finally, from the results presented in this study, it could be recommended that water pretreatment of explants before culture initiation could increase the success of *in vitro* studies and the procedures to obtain high frequency shoot regeneration *in vitro*.

SOMACLONAL VARIATION OF FLAX AND SELECTION *IN VITRO*

The cultivation of tissue and cells *in vitro* causes changes in heredity, termed somaclonal or gametoclonal variation, depending on the tissue that is cultivated, somatic or gametophytic, respectively. This variation depends on the duration of cultivation, and usually occurs during the "unorganized" growth phase - callus phase. It can cause problems in obtaining genetically stable lines regenerated from callus. On the other hand, this phenomenon has been used as a source of genetic variability in flax breeding programs and it was shown that some lines (regenerants of cvs. 'Torzhoksky 4', 'Belinka', 'Dashkovski 2' and 'Slavni 82'), that were tested during 3-4 generations, surpassed their parents in respect of the main important agronomic traits (Rutkowska-Krause *et al*. 1998).

Genetic diversity occurring in callus can be derived from several factors such as stress of *in vitro* culture, and such events as single gene mutation, transposable element activation, quantitative trait variation, chromosome breakage and preexisting cell's dissimilarity in explants and selection for specific genotypes during plant regeneration (Evans *et al*. 1984). All those factors can be the reason for somaclonal variation. The frequency of somaclonal variants is related to the cultured plant genotype, initial genotype uniformity and can run in flax up to 20% (Rutkowska-Krause *et al*. 1998).

Gametoclonal variation which is associated with variation among regenerants obtained from generative organs of plants, i.e. anthers (pollen grains) and ovules, has been investigated much less intensively than somaclonal variation. Nevertheless, this phenomenon is very interesting because of dominant and recessive mutations which can occur among regenerants R_0. Recombinant processes are result of meiotic crossing-over, lethal and semi-lethal recessive mutations can be demonstrated by dying haploid cells (Evans *et al*. 1984; Rutkowska-Krause *et al*. 1998).

It was shown that the cultivation of explants obtained in anther culture of flax increased the spectrum of variation. The types of spontaneous alterations cover the range from changes of monogenic traits, to changes in ploidy level. Variability of callus ploidy is much higher in long-term

cultures *in vitro*. After 6 months of anther callus cultivation only 6% of analyzed explants remained haploid, 22% were diploid, 24% tetraploid, 4% octaploid and 43% mixoploid in nature. However most plantlets regenerated on the explants contained cells with different ploidy level were diploid. Only 2% of regenerants turned out to be haploid, but they were too weak, and were not able to survive a process of chromosome doubling using colchicine. This indicates that preferentially diploid cells produce buds and shoots while the haploid level of callus ploidy is very rare. It was supposed that a part of flax regenerants obtained in anther culture were double haploids formed in the process of spontaneous diploidisation of originally haploid microspore tissue (Rutkowska-Krause *et al*. 1998).

The colour of flax petals is a monogenic trait and therefore any changes in segregation can be easily observed. Emergence of single blue plants among the R_1 generation of regenerants from anther explants of initially white petals genotypes can indicate the process of mutation of diploid tissue originating from microspores. Chromosome doubling led to the formation of regenerants, which consistently demonstrated this new feature. Research by Polish scientists in collaboration with the scientists from the Russian Flax Institute showed that up to about 47% of regenerants arising from microspores had other (blue) petal colors (Rutkowska-Krause *et al*. 1998).

Study of polygenic agricultural traits in R_3 and R_4 showed that somaclonal variation increases flax genetic diversity. It was shown that cultivation of somatic tissues allows the heredity of some flax genotypes to change with respect to the main agricultural traits. Most lines were not stable from year to year. However as it was also already mentioned above that some lines of regenerants stably surpassed the initial cultivar (with respect to the number of balls by 20-180%, the number of seeds per plant by 39-93%, and the content of fibre by 11-19.5%). It is especially important that such characteristics as productivity of seeds and fibre can be improved by this method (Poliakov *et. al*. 1998). Moreover it is possible to obtain lines from regenerated plants whose resistance to diseases is improved, for example, the line regenerated from a flax cultivar ('Torzhoksky 4') sensitive to *Fusarium oxysporum* turned out to be resistant to this disease (Rutkowska-Krause *et al*. 1998). High frequency of somaclonal variants with good agricultural traits could be explained by *in vitro* conditions which are unfavorable, unusual and only the most adaptive genotypes (cells) can produce buds, shoots and regenerate into plants.

The possibility of applying this novel source of genetic variability to crop breeding programs became more feasible with the development of cellular selection systems. Here, a chemical stress (in the form of herbicide, salts, disease toxin, etc.) is applied to the population of cells growing *in vitro*. Only those cells exhibiting tolerance to the stress agent are selected, regenerated to whole plants and advanced for further testing. The advantages of the cellular selection system are that only those cell lines with observed potential for herbicide resistance, salt-tolerance, etc., will be regenerated; most of the other types of variants will be eliminated.

Salt-tolerance *in vitro* selection

Excessive salt in soil is a major agronomic problem in many parts of the world, and traditional breeding methods have met with limited success solving this problem. The nature of salt tolerance would be clearer if the nature of salinity stress were better understood. Salinity stress has at least two components: one is that of an osmotic stress from an increased water potential from saline ions present in abundance in the soil; the other is ion toxicity from an overabundance of a particular types of ions. A plant can be salt tolerant due to one or more several possible mechanisms. These can range from fairly specific, simple genetic means such as chloride-excluded genes or increased levels of certain substances though to protect cells from osmotic damage such as glycine betaine or proline, to more complex genetic systems involving morphological mechanisms. Other mechanisms of salt tolerance include those bearing no direct relationships to salt metabolism. These include those in plants which grow only in the wet season, thus avoiding salinity stress imposed in the same soil during the dry season, or plants which have increased vigor and are thus better able to withstand any kind of stress imposed (McHughen 1987).

Cellular selection schemes are thought to provide the possibility of identifying some forms of salt tolerance which are active at the cellular level. At the Canadian Crop Development Centre, a population of cells derived from a single seed of the flax cv. 'McGregor' was plated onto a highly saline culture medium. One cell colony survived the treatment, was rescued, regenerated and gave rise to the STS-II (salt tolerant selection II) strain (McHughen and Swartz 1984). In greenhouse tests, STS-II performed better than its parent 'McGregor' in both saline and non-saline soils. Cells from STS-II plants reintroduced to the saline selection medium still survived better than cells from 'McGregor' plants.

But non-saline and saline-affected field evaluations of STS-II, 'McGregor' and other cultivars, showed that the seed yield of 'McGregor' was greater than that of STS in both saline and non-saline conditions and was significantly less affected by salinity than STS. However, when equal numbers of seeds were sown in the saline-affected field more STS plants survived to maturity than 'McGregor' and other cultivars ('Norlin', 'Noralta'). And the decrease in seed yield per plant with increasing salinity was usually greater for 'McGregor' than STS, but not always significantly. The results of the germination test indicated that the STS-II line was more vigorous in every soil, but the differential was most pronounced in the most saline soil, where almost twice as STS-II seeds germinated as the 'McGregor' (50% and 27%, respectively) (McHughen 1987; Rowland *et al*. 1987, 1988).

After 70 days of growth, this difference in vigor was more noticeable in the height of the plants. Under non-stressed soil condition, the STS-II plants were about approximately 50% taller than 'McGregor' plants (34.5 cm and 24.5 cm, respectively). In moderately/highly saline soil, 'McGregor' and STS-II plants both retarded by the salinity, the differential, however, between the controls and the STS-II remains 50% (23.5 cm and 16.7 cm, respectively, similar to the results received under non-stressed soil conditions). Under the highest level of salinity, the 'McGregor' plants average only 11.4cm, while the STS-II plants have grown only about 17 cm, again showing about 50% differential over the control plants under a similar level of saline stress. The relative performance of 'McGregor' and STS-II plants remained the same, and so it was presumed that the two lines respond to salinity in the same way: that is, an increase in saline stress has a similar depressing effect, or regression, on both lines. This indicates that the reason for superiority of the STS-2 line over 'McGregor', and its survival as a cell line in lethally saline culture medium, is not due to any specific salt metabolism, but rather more due to a general increase in vigor. Earlier flowering of STS-2 line under non-saline soil conditions also supports this interpretation. The apparent salt tolerance of STS is part of a more general phenomenon, affecting several traits. STS yielded less, was earlier to bolt, flower and mature, and had larger seeds with lower oil content than 'McGregor'. The yield of STS was similar to other early-maturing cultivars under test. Unfortunately, there was no consistent pattern in the results to suggest that STS was more saline-tolerant than were flax cultivars developed using conventional breeding procedures (McHughen 1987; Rowland *et al*. 1988).

Wilt-resistance *in vitro* selection and screening

Fusarium oxysporum f. sp. *lini*, a ubiquitous and highly persistent soil fungal pathogen, causes a vascular wilt in linseed and fibre flax. Chlamydospores of the fungus are difficult to destroy by agrochemicals and resistant cultivars are the only effective way to control the disease. Screening for *Fusarium* resistance is included in breeding programs for flax and linseed the world over (Liu *et al*. 1993; Kenaschuk and Rashid 1993, 1994; Popescu *et al*. 1994).

Conventional methods for screening of resistance in flax and linseed consist of field trials at infested sites or flax wilt nurseries, with visual assessment of wilt development. Such trials give highly variable results due to interactions between pathogenic and non-pathogenic *Fusaria*, existence of races of *F. oxysporum* f. sp. *lini*, and because the severity of the disease is influenced by soil type, and therefore those trials require large numbers of replications, both in space and time. The genetic system of *Fusarium* resistance in flax and linseed is complex and so a simple and reliable greenhouse *in vivo* and *in vitro* screening tests for detecting resistance to different biotypes of the fungus are highly desirable (Kroes *et al*. 1998).

Scottish scientists attempted to devise an *in vivo* screening assay for assessing the resistance of linseed lines which had been produced via tissue culture incorporating *in vitro* selection with culture filtrates from *F. oxysporum* f. sp. *lini*. Callus derived from hypocotyl explants of the linseed cultivar 'Norlin' was plated onto culture media which incorporated sterile crude culture filtrates of *F. oxysporum* f. sp. *lini*. Calli were treated with a range of culture filtrate concentrations and over different periods of *in vitro* selection (1-3 cycles of 20 days). Finally, surviving calli were regenerated and the R_o putative *F. oxysporum* f. sp. *lini*-resistant lines were screened. Seeds of the somaclonal lines plus control varieties (Norlin, Norlin R_o and Regina, a susceptible cultivar) were sown in pots with vermiculite where highly infectious *F. oxysporum* (strains A 10 and F60) inoculum (10^6 /ml) was added (Marshall and Courduries 1994).

These somaclonal lines were difficult to screen accurately since the genetic material in the R_o generation was very variable. There was a significant difference in disease development between the two runs of this experiment and none of these somaclonal lines showed a consistent improvement in *Fusarium* resistance. This screening test was not reproducible due to fluctuations in the temperature under greenhouse conditions which influence the rate and severity of disease development (Marshall and Courduries 1994).

Still, greenhouse screening for assessing resistance could be usefully applied in a controlled environment facility. Moreover, further work to optimize a callus-based regeneration system *in vitro* is needed, and the improvement of the frequency of genetic variation *in vitro* by incorporating mutagens in the culture medium is also possible.

The genetic basis for resistance has not been definitively characterized and no major genes have so far been mapped to chromosomal locations. Several studies involving field assessment of resistance in segregating generations of crosses between resistant and susceptible parents failed to identify major gene effects and instead concluded that resistance was due to polygenic effects. Furthermore, it is possible that conclusive identification was hindered by factors such as segregation of many genes affecting resistance due to choice of genetically diverse parental lines, and difficulties in classification of resistance status in early generations (e.g. F_2, or F_3) due to the presence of high levels of heterozygosity and genetic heterogeneity within families. Inheritance studies that avoided some of potentially complicating factors frequently resulted in simpler patterns of inheritance. For example, Indian studies of mainly indigenous germplasm conducted in one field nursery postulated that resistance was determined by either one or two dominant genes (Jeswani and Upadhyaya 1970; Kamthan *et al*. 1981; Spielmeyer *et al*. 1998). A profounder understanding of the molecular genetics of wilt disease and identifying DNA markers tightly linked to resistance genes in flax are highly desirable for improvement of the methods of selection for wilt resistance. Moreover, the identification of major genes and their linkage associations with DNA markers is a prerequisite and for the eventual cloning and manipulation of such genes.

The relevance of major genes of durable flax wilt resistance depends to a significant degree on the race-specificity of the resistance phenotype. Some studies that have identified either monogenic or digenic inheritance of flax wilt resistance have also to examined the race-specificity of the response. In contrast, when plants were grown in a sterile soil medium under glasshouse conditions, they demonstrated specific resistance to one particular isolate of flax wilt fungus (Knowles *et al*. 1956). Absence of competition among isolates or races and a greater potential for infection in the glasshouse due to improved control over temperature and inoculum levels could explain the detection of race specific interactions in such studies. However, race-specificity may also play a role under field conditions. For example, it was reported that some varieties resistant at one field location proved susceptible when challenged with fungal populations at another location (Spielmeyer *et al*. 1998).

Australian researchers tried to identify differences attributable to major gene effects between parents with contrasting resistance status and to establish linkage relationships between any identified resistance genes and other economically important genes in flax. To maximize the opportunity to detect major gene effects the study analyzed resistance under glasshouse conditions in a set of homozygous (doubled-haploid) recombinant lines grown in wilt nursery soil which had been super-inoculated with a mixture of fungal isolates. Field assessment of resistance was also undertaken to examine the value of glasshouse screening in predicting field response in the experimental population (Spielmeyer *et al*. 1998).

A recombinant doubled haploid (DH) population was derived from the haploid component of polyembryonic F_2, seeds originating from a cross between a wilt resistant line and the wilt susceptible Australian flax cultivars. The frequency distribution of disease severity amongst the DH lines suggested that three phenotypic classes exist. The observed frequencies were tested against the expected ratio of 1:2:1, representing the resistant, intermediate and susceptible category respectively, for the segregation of two independent genes in homozygous F_2,-derived recombinant lines. The segregation ratio indicated that *fusarium* wilt resistance was likely to be determined by two major genes with additive effects. The wilt score data from the field nursery supported the hypothesis of a 1:2:1 segregation in the appearance of three phenotypic classes (Spielmeyer *et al*. 1998).

Glasshouse scores for resistance to *Fusarium* wilt and field data showed a positive correlation. Three data groups apparently corresponded to the resistant, intermediate and susceptible phenotypic classes. The segregation ratio suggests that resistance to the population of *fusarium* wilt in this particular cross between the wilt-resistant and the wilt-susceptible flax cultivars is predominantly controlled by two major genes. However, a significant number of resistant DH lines produced a more extreme phenotype than the resistant parent, suggesting that factors other than environmental influences contributed to the observed effect. It is likely that major gene effects are superimposed on transgressive segregation of additional minor genes controlling resistance to fusarium wilt. Minor resistance genes may have also contributed by modifying the resistance response, if a suitable single dominant gene for wilt resistance is absent, a polygenic complement would determine the response to infection by the pathogen (Spielmeyer *et al*. 1998).

Elucidating the mode of inheritance has provided the basis for investigating the molecular genetics of the corresponding wilt resistance mechanism and for the subsequent

suitable molecular marker screening methods developing, such as the AFLP technique to identify molecular markers linked to resistance loci. These markers constitute the first step in a series of strategies aimed at developing marker - assisted selection for a significant proportion of the resistance to an important fungal disease of this crop species.

LINUM BIOREACTOR TISSUES AND CELL SUSPENSION CULTURES

Another approach of biotechnology is not associated with regeneration and morphogenesis; it is related to cell and tissue *in vitro* culture for obtaining secondary metabolites. Medical, perfumery and food industries can profitably use secondary metabolites produced by cells of biomass that are identical to the metabolites produced by intact plants. For example plants of the family *Liniaceae*, mainly of the species *L. album*, *L. fiavum* and *L. nodiflorum* accumulate considerable amounts of podophyllotoxin-1 and/or 6-methoxypodophyllotoxin-2 (Kranz and Petersen 2003). Podophyllotoxin is a lignan compound with anticancer properties. At present, podophyllotoxin is produced commercially by extraction from the rhizomes of plants of the *Podophyllum* genus. However, because of the limited supply of these plants, other production methods are of great importance. Therefore, high-yielding tissue cultures can serve as a suitable system for lignan biosynthesis (**Fig. 1**).

The suspension cell line of *L. nodiflorum* was characterized and the activity of the enzymes of podophyllotoxin biosynthesis pathway was studied (Kranz and Petersen 2003). In another study the hairy root cultures of *L. flavum* producing coniferin, an effective metabolic precursor of podophyllotoxin were obtained. Root biomass showed significant coniferin accumulation (58 mg/g dry weight) (Lin *et al*. 2003).

Besides those studies, flax suspensions are a valuable tool for biochemical and molecular studies, due to their homogenous nature and rapid multiplication rates. They have also been used as model of plant cell wall, lignin and pectin studies (Schaumann *et al*. 1993). The study of activity of pectin methyltransferase (PMT) in microsomes, and pectin methylesterase (PME) were studied in flax cell suspension culture, and further in transgenic cell suspension culture (Lacoux *et al*. 2003).

CONCLUDING REMARKS

The improvement of such an important fibre and oilseed culture as flax has not developed at the same rate as in other crops. Modern industry lays high claims to the present flax cultivars, which suffer from the absence of genetic diversity, and advances in conventional breeding of this crop are insufficient. Biotechnological techniques such as genetic engineering, cellular selection, mutagenesis, haploidisation, immature embryos and protoplasts culture and other techniques can complement conventional breeding programs by saving time and increasing genetic variation.

Studies carried out by different scientists showed that flax organs and tissues significantly differ in their tissue culture competence and the capacity for morphogenetic response. The highest capacity for morphogenesis was demonstrated by hypocotyl segments. The occurrence of shoot-buds on *L. usitatissimum* hypocotyls is an unusual, stress-related developmental response. Adventitious shoots on flax hypocotyls form exogenously without an intervening callus phase. In some other cases the hypocotyl explants are capable of direct embryogenesis or of embryogenic and morphogenic callus formation. Major factors controlling the particular developmental and regeneration pathways are the effects of genotype and plant growth regulators supplementing the culture medium. The lack of a tissue's capacity to regenerate is usually associated with a genetic or physiological block of the morphogenic response, which is regulated by the following factors: physiological status of donor plant, type of explant, explant pretreatment and culture medium composition.

Most biotechnological studies on fibre flax and linseed have focused on ways to capitalize the phenomenon of

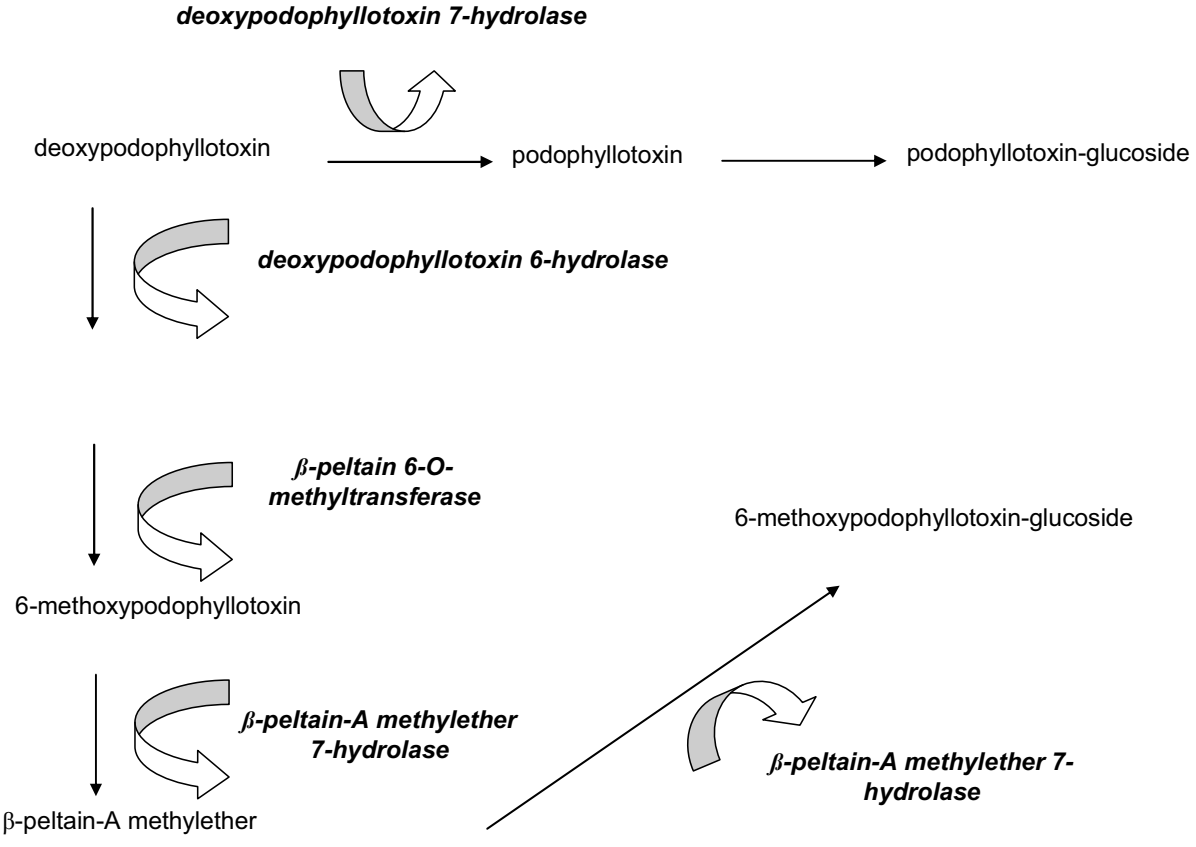

Fig. 1 Hypothetical scheme for the biosynthesis of the aryltetralin lignans podophyllotoxin and 6-methoxypodophyllotoxin from deoxypodophyllotoxin.

direct shoot formation. This might be useful for genetic transformation techniques especially that are based on particle acceleration. However, after transformation via *A. tumefaciens* the majority of shoots are formed from non-transformed cells and a significant number of regenerated shoots are chimeric (up to 45%). The possibility of avoiding the problem of chimeric shoot formation could provide a regeneration system based on another type of explant, probably, a cotyledon-based regeneration system. Development of a somatic embryogenesis system may provide a significant tool not only for research and breeding, but also, ultimately, for production of linseed oil lipids in a continuous mass culture system.

Immature embryo culture showed good results as a source of genetic diversity. Via embryo culture new lines surpassed parental genotypes in main agricultural traits and is, together with anther culture, a perspective tool in the production of haploid material, homozygous lines for plant breeding, and for molecular marker studies in flax. Somaclonal and gametoclonal variation as a source of new plant lines for breeding programs on high quality traits and for pathogen resistance have been demonstrated. However, a more profound understanding of the molecular genetics of disease and different types of stress resistance identifying DNA markers tightly linked to resistance genes in flax are of great importance. Moreover, the identification of major genes and their linkage associations with DNA markers is a prerequisite for the eventual cloning and manipulation of such genes. Additionally we could assume that all the advantages of the methods that have been illustrated are not sufficient. It is clear that further strategies for improvement of this ancient, but still highly relevant crop could be achieved via technologies of gene transfer and expression by genetic transformation, where more targeted and certain traits in plants can be incorporated. A number of studies devoted to this part of biotechnology are going to be discussed in our following review.

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