BIOLOGIA PLANTARUM 43 (2): 161-165, 2000

Morphological alterations in sterile mutant of *Pisum sativum* obtained via somatic embryogenesis

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Abstract

A sterile mutant of pea (*Pisum sativum* L. line HM-6) with a number of morphological alterations was found after plant regeneration via somatic embryogenesis. Embryogenic callus was derived from the whole immature zygotic embryo on medium with 2.26 μM 2,4-dichlorophenoxyacetic acid. Morphological changes included altered leaflet shape, one pair of leaflets only, altered stipule morphology, shortened internodia, irregular or opposite leaf position on the stem, shortened flower stalk, and aborted flowers resulting in complete sterility. If the isolation of the shoot apex and axillary buds from evidently sterile plant and their culture *in vitro* resulted in morphologically normal and fertile regenerated plants, the chimaeric nature of R_0 mutant is considered.

Additional key words: grain legumes, pea, regeneration *in vitro*, somaclonal variation, variant phenotype.

Introduction

Somatic embryogenesis is considered as a means for clonal mass propagation of plants. However, both direct and indirect somatic embryogenesis may generate somaclonal variation. In grain legumes it was demonstrated mainly in soybean (Griga 1999), where a broad spectrum of previously not-reported variant phenotypes was recorded. These included twin seeds, multiple shoots, dwarfs, determinate growth habit, abnormal leaf morphology, abnormal leaf number, wrinkled leaves, curled leaves, yellow edges on cotyledons, lack of unifoliates, yellow-green plants, isozyme variants (malate dehydrogenase and aconitase), chlorophyll deficiency, chlorophyll chimaeras or variegated plants, partial and complete sterility (Barwale and Widholm 1987, 1990, Ranch and Palmer 1987, Shoemaker *et al.* 1991, Amberger *et al.* 1992a,b). Chlorophyll deficiency, sterility, wrinkled leaves and curled leaves appeared to be inherited in a Mendelian fashion; these traits are controlled by single recessive nuclear genes. Other traits were inherited stably, but with aberrant segregation ratios or occurred more randomly and not in all generations and the genetic basis of this random variation is not recently satisfactorily explained (Barwale and Widholm 1987, Amberger *et al.* 1992a). The somaclonal variation generated through organogenesis and indirect (callus mediated) somatic embryogenesis was observed also in pea (Stejskal and Griga 1992, Griga *et al.* 1995, Griga and Létal 1995). In this paper morphological alterations in a sterile pea mutant obtained via somatic embryogenesis from immature zygotic embryo are described.

Received 15 November 1999, *accepted* 25 January 2000.

Abbreviations: BAP - 6-benzylaminopurine; BKZ-medium - medium for somatic embryos germination supplemented with NAA and three cytokinins BAP, kinetin and zeatin after Lazzeri *et al.* (1987); 2,4-D - 2,4-dichlorophenoxyacetic acid; IBA - indolylbutyric acid; MSB-medium - medium with mineral salts after Murashige and Skoog (1962), vitamins after Gamborg *et al.* (1968); NAA - α-naphthaleneacetic acid.

Acknowledgement: The author is grateful to Prof. W. Swiecicki for critical reading the manuscript and valuable comments. This research was supported by the Grant Agency of the Czech Republic (grant. No. 206/96/K188) and INCO-COPERNICUS grant No. IC15-CT96-096.

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Materials and methods

Embryogenic calli were induced from the whole immature zygotic embryos (3 to 6 mm in size) of pea line HM-6 isolated from field-grown pea plants on MSB-medium supplemented with 2.26 μM 2,4-D. The cultures were kept under 16 h photoperiod (cool-white fluorescent tubes; irradiance 20.4 μmol m^{-2} s^{-1}) and temperatures of 26 °C at day and 20 °C at night. The experiment yielded 16 % embryogenic calli. The calli were then subcultured after 4 weeks on the same medium, and after following 10 d the somatic embryos in torpedo and cotyledonary stages were isolated and placed on BKZ-medium (Lazzeri *et al.* 1987) containing 0.15 μM BAP, 0.15 μM kinetin and 0.15 μM zeatin. In the course of 7-week culture on BKZ medium, some somatic embryos germinated regularly (paralel shoot and root development), other developed either shoot or root. In order to increase the number of plants from single germinated embryo, the emerging shoot was cut into individual nodes with axillary buds of leaves (usually two to three cuttings from developing shoot) and micropropagated as described previously (Griga *et al.* 1986). Micro-cloned shoots were rooted on half-strength MSB-medium, 4 % sucrose and 1 μM NAA or IBA and then transplanted to the sterile (autoclaved) soil in the greenhouse. Phenotype of R_0 regenerants was evaluated and compared to control seed-derived plants of the progenitor HM-6 line. R_1 seeds from fertile R_0 plants were harvested and stored for further evaluation.

Results and discussion

A morphological mutant regenerated from the cotyledonary stage somatic embryo with two cotyledons and the absence of shoot apical meristem (shoot emerged outside the cotyledons from the swollen hypocotyl) (Table 1, Figs. 1, 2). The plant was probably chimaeric, because some traits were not distributed throughout the

Table 1. Description of morphological changes observed in R_0 regenerant of pea line HM-6 with variant (mutated) phenotype obtained via somatic embryogenesis.

Trait	Standard phenotype of line HM-6	Altered (mutant) phenotype of R_0 regenerant
Leaflet shape	oval	narrow
Length/width ratio [mean ± S.D.]	1.58 ± 0.02	2.53 ± 0.21
Leaf structure	two or three pairs of leaflets	one pair of leaflets only
Leaf position	alternate	irregular or opposite
Flower	standard type	morphological deformations; sterility
Inflorescence	on long shoots	on short shoots
Internodia	standard - long	significantly shortened
Stipule	standard type, oval, with dentation	narrow, without dentation, the main vein in apical part elongated into tendril

Table 2. Probable genetical status of mutations recorded in R_0 regenerant of pea line HM-6 with variant (mutated) phenotype obtained via somatic embryogenesis (based on Blixt 1974 and Weeden *et al.* 1998). Mutation of stipules recorded in mutated plant described in this paper has not been found in the literature.

Gene symbol	Mutation type	Mutant character	Linkage group	Phenotype	Author of a gene symbol
ad	*apex densifolius*	Shoot	-	Upper internodes shortened, flowers sterile	Gottschalk (1973)
ang	*angustifolia*	Pleiotropic complex	-	Leaflet lanceolate, very narrow; female sterile	Lamprecht (1945)
brev	*brevifilamentosus*	Pleiotropic complex	-	Inflorescences on short shoots; sterile	Lamprecht (1935)
elo	*elongata*	Foliage	-	Reducing foliage width	Lamprecht (1967)
le	*breviinternodium*	Root and shoot	3	Shortening internodes	White (1917)
lm	*breviinternodium*	Root and shoot	6	Short internodes	Rasmusson (1938)
red	*reductus*	Foliage	1	Leaflets very narrow	Lamprecht (1942)
ten	*tenuifolius*	Foliage	-	Narrow leaflets and stipules	Lamprecht (1949)
up	*unipetiolle*	Foliage	-	One pair of leaflets only	Rosen (1944)

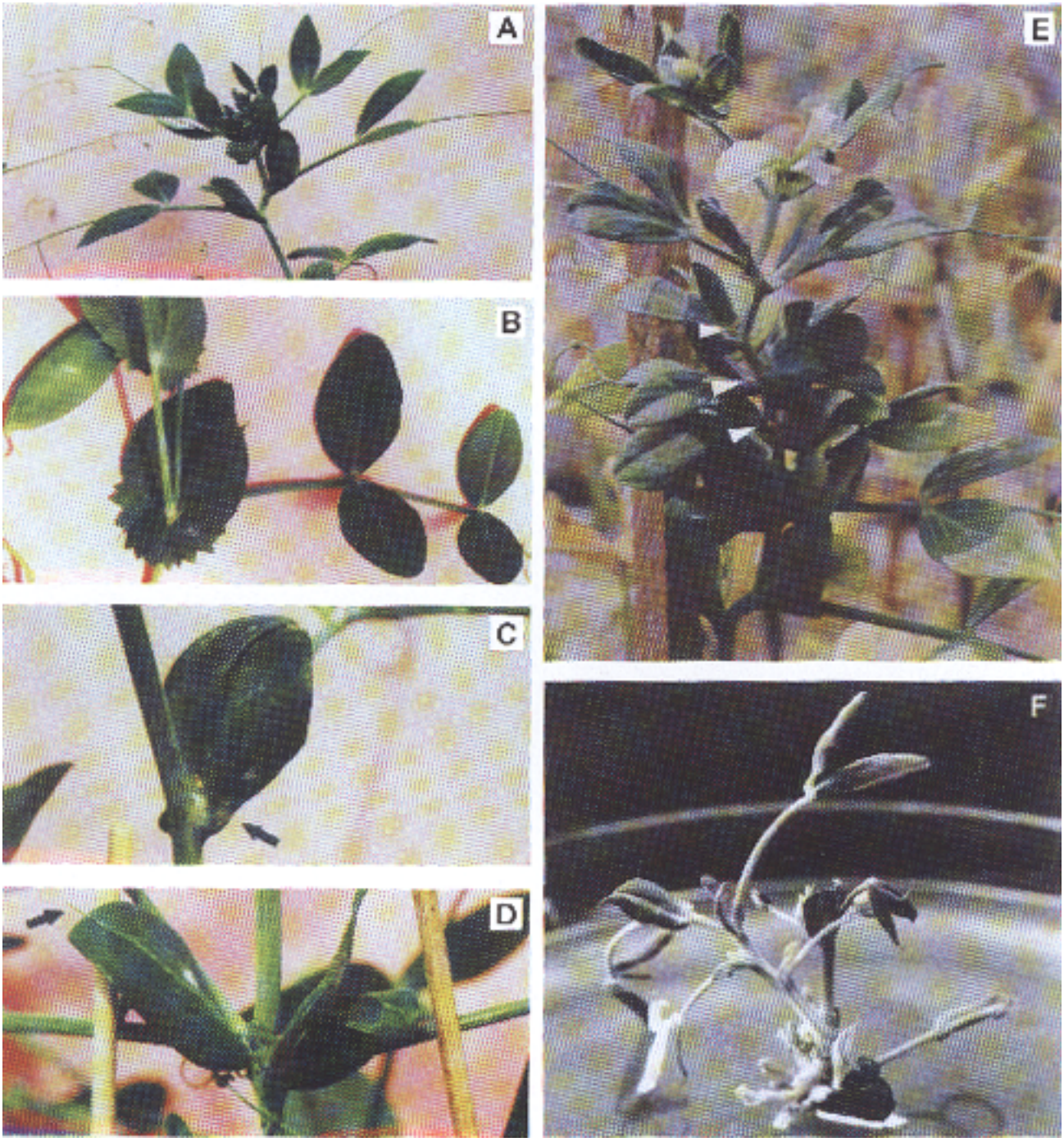

Fig. 1. Morphological alterations recorded in R_0 pea plant (line HM-6) regenerated via somatic embryogenesis: *A* - mutated regenerant in the vegetative phase (internodia start to be shortened, one pair of leaflets only, narrow leaflets, stipules without dentation); *B* - standard phenotype of line HM-6 (oval leaflets, stipules with dentation, standard length of flower/pod stalk); *C, D* - details of altered stipules (absence of dentation - *arrow*, elongated main vein into tendril - *arrow*, and opposite leaf position in *D*); *E* - mutated regenerant in reproductive phase (aborted sterile flowers in the upper part of plant, older flowers in the middle part of plant are shed - *arrows*, strongly reduced length of internodia and flower stalk, irregular or opposite leaf position); *F* - mutated regenerant put back to *in vitro* culture derived from the shoot apex of greenhouse-grown plant, mutated phenotype of leaflets still evident, multiple shoot buds arising from the explant basis successively converted to standard phenotype and gave fertile progeny.

whole plant, but these exhibited certain trend. For example, internodes in the basal part of the plant exhibited more or less normal length, but in the upper part there was a dramatic reduction of their length (Fig. 1*A*,*E*). Most of mutations observed were probably those described earlier (Table 2), the changed stipules recorded in our experiment we did not find in the literature. When it was clearly evident (in the phase of flowering), that flowers are aborted and sterile (Fig. 1*E*), we attempted to maintain this plant by vegetative propagation. Vegetative shoot apex and some axillary buds from the upper part of the plant were isolated, surface sterilized and transferred back to *in vitro* culture on medium for micropropagation (Griga *et al.* 1986). Unfortunately, the mutated phenotype of cultures (Fig. 1*F*) were subsequently lost during micropropagation phase and the regenerated and rooted shoots converted to standard phenotype, normally flowered and set seeds.

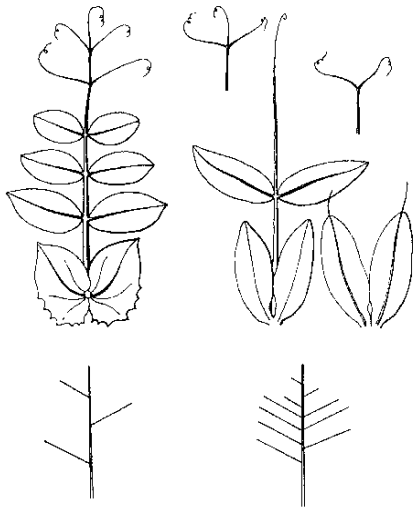

Fig. 2. Schematic representation of alterations of leaflets, stipules and the plant architecture. *Left upper and bottom*: standard phenotype of pea line HM-6. *Right upper and bottom*: variant phenotype of R_0 regenerant.

Our results demonstrate that callus mediated somatic embryogenesis may generate even very dramatic changes in morphology of R_0 regenerants. We suppose the obtained phenotypic variation is culture-induced. Possible factors inducing mutations in our experiment may be 2,4-D as a potential mutagen, genetic heterogeneity of callus cells, and multicellular origin of somatic embryo connected with that state (the last fact may cause a chimaeric character of the regenerated plant and its sterility). Low concentrations of 2,4-D were reported to induce heritable variation in soybean plants regenerated via somatic embryogenesis from immature cotyledons (Shoemaker *et al.* 1991). However, the authors did not find any correlation between the concentration of 2,4-D in the induction medium and the type of variants observed. An interesting fact is that so many mutation cases took place in one single pea plant and was not seen in other regenerants within particular experiment whose all exhibited standard phenotype. The mutated phenotype represented 5.9 % of regenerants successfully transferred to the soil, 12.5 % as related to the recorded embryogenic calli, and 20 % as related to the original number of germinating/converting somatic embryos, which were successively micropropagated by node cuttings. Similarly, Ezhova *et al.* (1989) recorded significant morphological changes (shortened internodia, leaf and flower morphology) in 10 % of pea R_0 regenerants obtained from long-term organogenic calli. R_0 plants with gross morphological alterations either did not flower at all, or they exhibited high percentage of pollen sterility– none of these regenerants set seeds. This fact represents the same situation as in our experiment. In contrast, Amberger *et al.* (1992a) observed only little phenotypic variation in R_0 soybean plants derived via somatic embryogenesis.

Seed progenies of the fertile regenerants (second cycle of R_0) derived from mutated plant via repeated *in vitro* culture did not exhibit in R_1, R_2, R_3 and R_4 generation any of the morphological changes observed in original mutant which fact demonstrates that mutated sectors were completely lost during micropropagation and formation of reproductive structures. Thus, we can only speculate about the nature of observed changes: heritable mutations or epigenetic changes or developmental abnormalities? Despite of the fact, that we were not able to obtain viable solid mutant, our results extend the recent knowledge about the possibility to create new variability via tissue culture in pea (Gostimski *et al.* 1985, Ezhova *et al.* 1989, Lutova and Zabelina 1988).

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