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Somatic embryogenesis and plant regeneration in *Pisum sativum* L.

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Abstract

Somatic embryogenesis was induced in immature zygotic embryos of pea (*Pisum sativum* L.), synthetic auxins α-naphthalene acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and 4-amino-3,5,6-trichloropicolinic acid (picloram, PIC) being used. Only one (line HM-6) of 46 genotypes tested exhibited good potential for somatic embryogenesis. 2,4-D was found as the best somatic embryo inductor. Three different ways of somatic embryo conversion have been described. Plantlets from individual somatic embryos were micropropagated as somaclones and subsequently rooted. A sterile morphological mutant has been found within a group of fertile plants of T_0-generation. Sufficient amount of T_1-seeds is available for somaclonal variation studies.

Introduction

Recent progress in somatic embryogenesis induction in grain legume crops includes *Glycine* (Lazzeri *et al.* 1985, Barwale *et al.* 1986, Hammat and Davey 1987, Tétu *et al.* 1987, Komatsuda and Ohyama 1988, Christou and Yang 1989), *Vicia* (Griga *et al.* 1987, Pickardt *et al.* 1989, Albrecht and Kohlenbach 1989), *Phaseolus* (Martins and Sondahl 1984, Allavena 1984), *Lens* (Saxena and King 1987), *Vigna* (Kumar *et al.* 1988, Eapen and George 1990) and *Arachis* (Ozias-Akins 1989, Hazra *et al.* 1989).

First successful plant regeneration via somatic embryogenesis in pea was reported by Kysely *et al.* (1987). In the same laboratory regeneration of pea plants through somatic embryogenesis was achieved from cultured protoplasts (Lehminger-Mertens and Jacobsen 1989). Shoot apices and immature zygotic embryos are routinely used as initial explants (Kysely and Jacobsen 1990).

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In this paper, somatic embryogenesis from immature zygotic embryos and a complete plant regeneration in a further pea genotype is reported.

Material and methods

The screening of 46 pea genotypes for somatic embryogenesis potential was carried out in 1988: twenty of them were *Pisum sativum* L. and five *Pisum arvense* L. cultivars from pea collection of Research Institute of Technical Crops and Legumes in Šumperk. The remaining 21 genotypes were *P. sativum* and *P. arvense* breeding lines from Plant Breeding Station Horní Moštěnice.

Immature pods from field- or greenhouse-grown plants were surface sterilized with 70 % ethanol (2 min), 5 % m/v commercial Chloramine B (30 min) and then rinsed with sterile deionised water. Embryos 3 to 8 mm long were excised and placed on the solid MSB medium (0.7 % Difco agar, 3 % sucrose, MS salts and B_5 vitamins) supplemented with either 50 μM NAA, or 10 μM 2,4-D, or 10 μM PIC. In the subsequent series of experiments (1989) we used lowered concentrations of auxins (2.26 μM 2,4-D or 4.14 μM PIC) according to Kysely *et al.* (1987). Cultures were kept under 16 h photoperiod and at 26 °C at day 20 °C at night. Cultured zygotic embryos (*i.e.* zygotic embryos with developing somatic embryos accompanied by callus formation) were transferred to fresh medium monthly. Developing somatic embryos were mechanically isolated from the original explants and placed directly on BKZ medium (Lazzeri *et al.* 1987) containing 0.15 μM benzylaminopurine (BAP), 0.15 μM kinetin, 0.15 μM zeatin and 0.05 μM NAA (medium for conversion), or firstly (2 to 4 weeks) on modified medium after Lehminger-Mertens and Jacobsen (1989) supplemented with 0.1 μM NAA and 1.5 μM gibberelic acid (GA_3), and subsequently on BKZ medium. The pH of culture media was adjusted to 5.8 prior to autoclaving (121 °C, 15 min). Filter sterilized GA_3 and zeatin were added to culture media.

When a single shoot emerged from a somatic embryo, it was cut into individual nodes with axillary buds of leaves and transferred to MSB medium with 0.1 μM NAA and 20 μM BAP (Griga *et al.* 1986), where micropropagation was carried out. If multiple buds appeared on somatic embryos, the whole clusters of them were transferred to this medium. Multiplied shoots were rooted on 1/2 MS medium with B_5 vitamins, 4 % sucrose and 1 μM NAA or IBA (indolylbutyric acid) and transplanted to sterile soil in the greenhouse.

For histological studies, different developmental stages of the somatic embryos were fixed in Navashin's fluid for 24 h and then rinsed in running tap water for 24 h. After ethanol/xylene dehydration objects were embedded into paraffin. Sections 10 μm were cut and stained with basic fuchsin and picroindigocarmine (Němec 1962).

Each experiment in this work was repeated at least twice.

Results and discussion

Effect of auxin and genotype: A general response of cultured immature zygotic embryos to the auxin media was callus formation in the presence of 2,4-D and PIC,

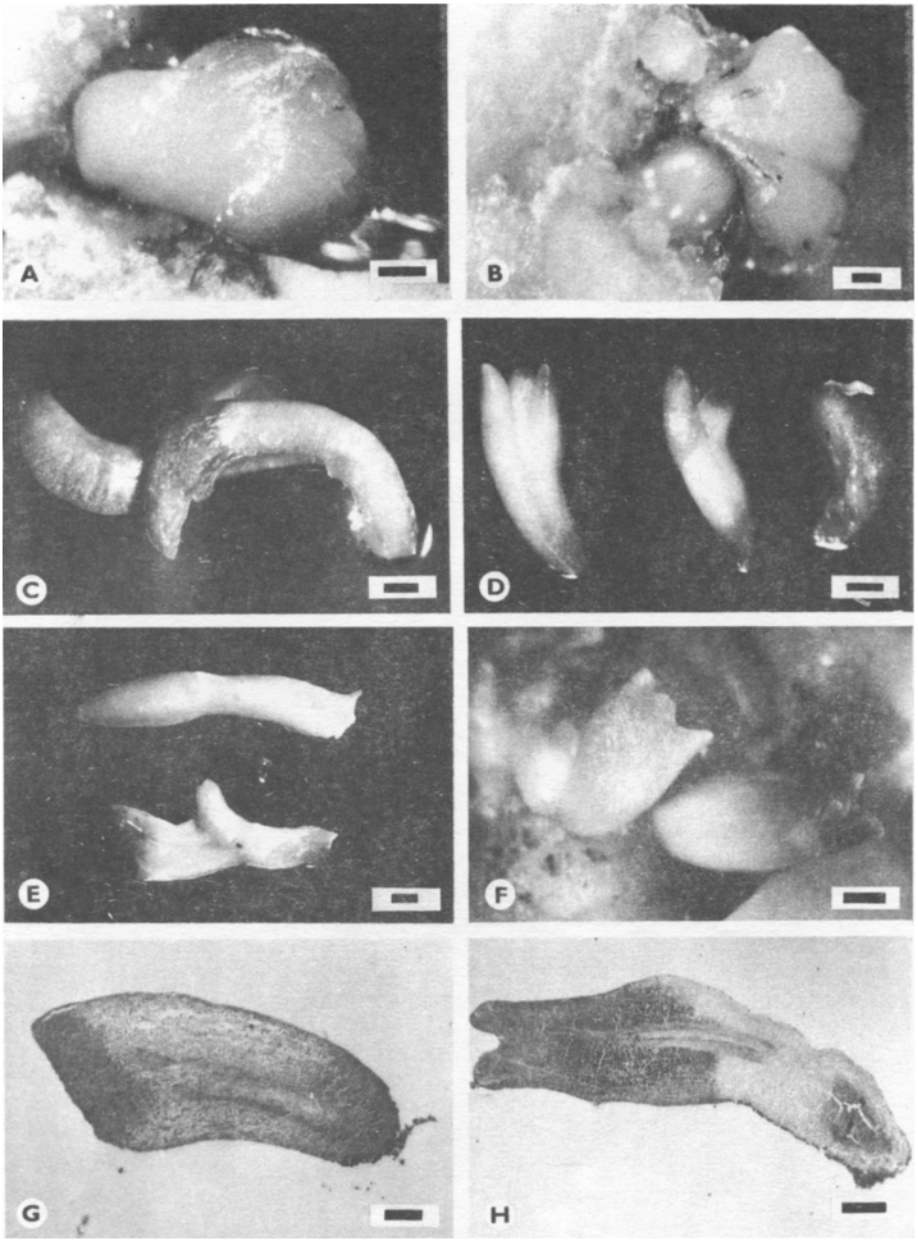

Fig. 1. Somatic embryogenesis in *Pisum sativum*. Single somatic embryo (A) and cluster of somatic embryos (B) developing on cultured immature zygotic embryos. Fully developed morphologically normal somatic embryo at cotyledonary stage (C), embryos with 1 to 3 cotyledons (D), abnormal types (E). Secondary somatic embryogenesis (F). Histology of torpedo (G) and early cotyledonary developmental stage (H). The bar represents 0.25 mm (A, B, G) and 0.5 mm (C, D, E, F).

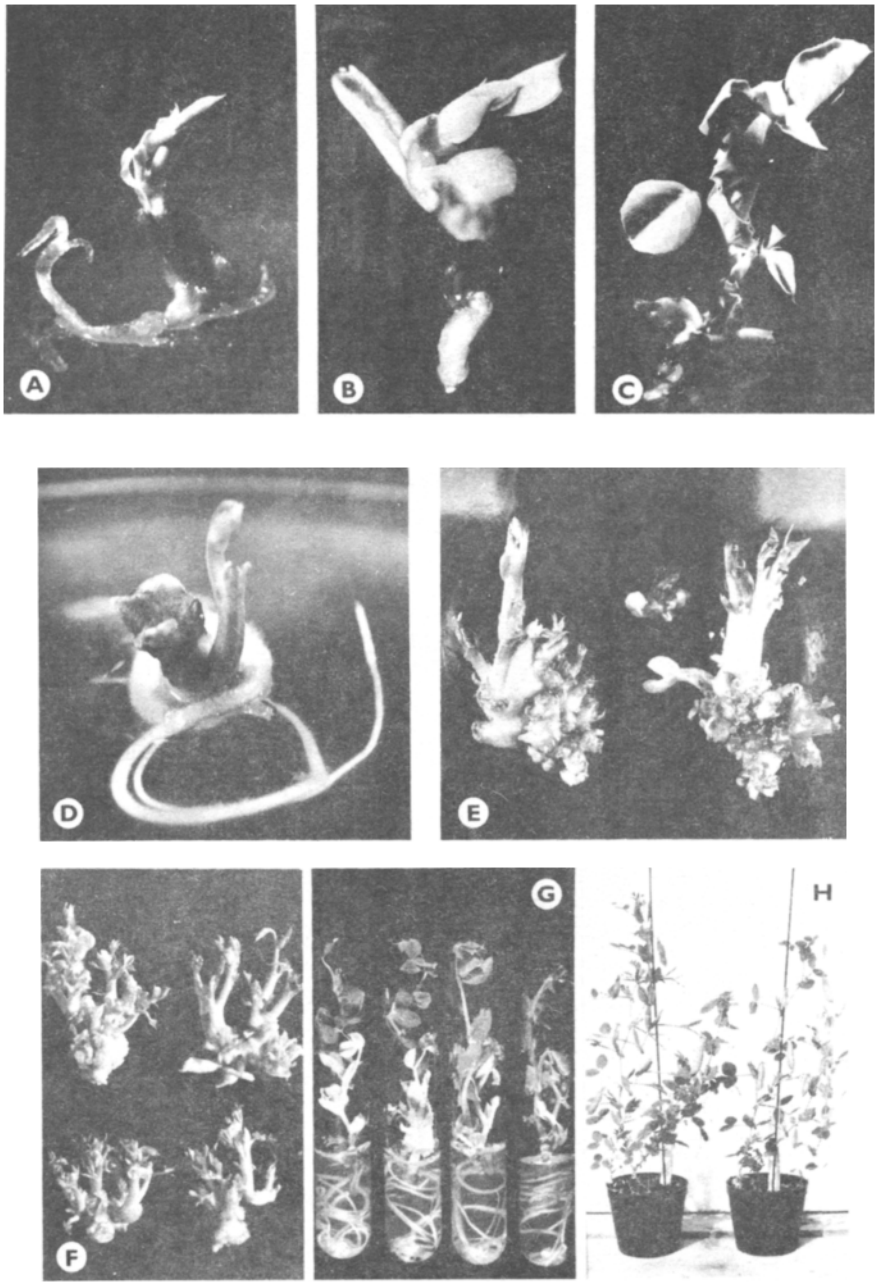

Fig. 2. Conversion of pea somatic embryos. Plantlet development *via* regular germination (A, B, C). Irregular germination (D). Shoot bud differentiation on swollen somatic embryos (E). Micropropagation of regenerated material (F). Root formation (G) and fertile plants of T_0-generation in the greenhouse (H).

or normal shoot and root proliferation in the presence of NAA. This is in agreement with the data of Kysely *et al.* (1987). Of 46 pea genotypes tested, the *P. sativum* cv. Odeon and breeding line HM-6 and *P. arvense* cv. Tyla were chosen as the best ones. These genotypes exhibited well-growing green or yellow-green callus with compact globular structure. Only one genotype (line HM-6) gave rise to several somatic embryos in the first series of experiments, interestingly on medium with NAA. NAA was found to be an effective auxin with respect to the induction of somatic embryogenesis in other grain legumes, *e.g.* soybean (Lazzeri *et al.* 1985, Tétu *et al.* 1987, Barwale *et al.* 1986, Komatsuda and Ohyama 1988), recently even in pea (Tétu *et al.* 1990). However, we were not able to reproduce our positive preliminary result with NAA in pea.

More detailed experiments were started with three selected genotypes and two media with slightly decreased level of auxins (2.26 μM 2,4-D or 4.14 μM PIC). Only one of the six variants tested (genotype × medium), *i.e.* HM-6 on 2,4-D gave positive response with mean frequency 14.6 % embryogenic explants (Table 1). This strict dependence of pea somatic embryogenesis potential on genotype is in agreement with recent results of Lehminger-Mertens and Jacobsen (1989) and Tétu *et al.* (1990) who demonstrated significant differences in several commercial pea cultivars for this character.

Table 1. Induction of somatic embryos from immature zygotic embryos of *Pisum sativum* line HM-6 on MSB-medium with 2.26 μM 2,4-D (evaluated after five weeks of culture).

Experiment	Zygotic embryos plated	embryogenic	Frequency [%]	Somatic embryo / explant*
1	50	8	16.0	0.42 (2.63)
2	50	10	20.0	n.r.
3	100	18	18.0	n.r.
4	190	21	11.8	0.27 (2.48)
total	390	57	14.6	0.30 (2.52)

*mean of all explants in experiment (mean of embryogenic explants only); n.r. - not recorded

Somatic embryo development: Both single somatic embryos and clusters of somatic embryos developed on the embryogenic explants (Fig. 1A, B). The number of somatic embryos per explant varied from 1 to 6. Different somatic embryos were obtained, both two-cotyledonary ones, resembling zygotic embryos (Fig. 1C), and somatic embryos with 1 or 3 cotyledons (Fig. 1D) or abnormal morphology (Fig. 1E). The frequency of the embryogenesis was comparable with that in experiments of Kysely *et al.* (1987), but only after using 2,4-D in place of PIC. In our experiments with genotype HM-6 only one somatic embryo appeared on PIC-medium. Similar results were obtained with soybean, where 2,4-D was the best

inductor of somatic embryogenesis, while PIC stimulated strong formation of non-embryogenic callus (Novák *et al.* 1987, Griga 1990).

In agreement with other authors we found the whole zygotic embryos to be the best explant for pea somatic embryo induction. If only cotyledons without organized meristematic zones were placed on induction media, no embryogenesis, but browning of the explants was observed. This fact suggests that meristematic tissues are involved in somatic embryogenesis. Moreover, if only embryonic axes containing meristematic zones (shoot apex) were cultured, embryogenesis also occured. The whole process starting embryo initiation to fully developed structures could be completely realized on auxin induction medium and lasted from 3 to 6 weeks. Transfer of somatic embryos to media with cytokinins or GA_3 slightly supported their development and maturation. Somatic embryos started gradually to germinate on these media without any remarkable dormancy phase. Histology of somatic embryos is shown on Figs. 1G and 1H.

Conversion of somatic embryos, secondary embryogenesis: After isolation of somatic embryos and placing them on BKZ medium, strong rhizogenesis appeared on most of them. Some of the embryos did not exhibit further development. When isolated somatic embryos were firstly placed on medium with GA_3, they swelled and rooted too. Generally we have observed three different ways of conversion in pea somatic embryos: (1) regular germination - the shoot developed between the cotyledons paralelly with root proliferation (Fig. 2A, 2B, 2C), (2) irregular germination - the shoot emerged outside cotyledons from the swollen hypocotyl with or without parallel proliferation of the root (Fig. 2D) and (3) shoot bud differentiation on swollen somatic embryos (Fig. 2E). Similar types of conversion have been reported in pea (Kysely *et al.* 1987) and in soybean (Griga 1990). In order to complete the process of whole plant regeneration (in cases 2 and 3) the shoots were rooted on medium with auxin (1 μM NAA or IBA). The crucial point of somatic embryo conversion seems to be the morphology of fully developed somatic embryos as demonstrated *e.g.* in soybean (Lazzeri *et al.* 1987, Finer and Nagasawa 1988). Also in pea morphologically normal embryos with well developed shoot apical meristem germinate significantly better than abnormal ones.

Sometimes, secondary embryogenesis appeared on media used for conversion. In this case, new somatic embryos arose on the surface of swollen non-germinated or germinated primary somatic embryos taken from induction medium (Fig. 1F).

Micropropagation of somaclones, plant regeneration: In order to obtain more plant material, we micropropagated plantlets from individual somatic embryos as somaclones in several subcultures (see Material and Methods) (Fig. 2F). Multiplicated shoots were cut (minimal length 2 cm) and routinely rooted in test-tubes as mentioned above (Fig. 2G). The frequency of rooting was approximately 60 %. Transfer of well-rooted plants into soil and obtaining fertile plants was without problems. Survival of regenerants in the greenhouse reached 100 % (Fig. 2H). A sterile mutant with altered stipule and leaflet morphology and aborted flowers has been found among 17 T_0-regenerants. Shoot apices and axillary buds of mutant plant were isolated and transferred back to *in vitro* culture. This material is maintained as

shoot-culture. Presently, sufficient amount of T_1-seeds from fertile regenerants is available in our laboratory for field evaluation as well as for biochemical analyses of genetic stability or variability of somatic embryo seed progenies. The regeneration system described here could be used for pea somaclonal variation studies and pea transformation experiments.

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