Direct somatic embryogenesis from shoot apical meristems of pea, and thidiazuron-induced high conversion rate of somatic embryos

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Abstract

Direct somatic embryogenesis from shoot apical meristems of pea is described. Somatic embryos were induced directly (without callus intervention) from meristematic tissues grown on a medium supplemented with 2.5 μM picloram. Within 4 to 5 weeks, fully morphologically developed somatic embryos were obtained. Somatic embryos originated from apical as well as from basal parts of meristem explants. The initiation and development of somatic embryos was asynchronous, basal somatic embryos developed more quickly than apical ones. Abundant secondary embryogenesis was observed after isolation of primary somatic embryos and culturing them on media for germination. Morphologically normal somatic embryos germinated on medium without growth regulators; the conversion rate was increased by application of 10 μM thidiazuron (TDZ). TDZ was also able to induce shoot bud regeneration on embryos without differentiated a shoot apex, allowing to germinate up to 78 % of all harvested somatic embryos with various morphology. The protocol was successfully tested in 47 out of 48 *P. sativum* and *P. arvense* cultivars as well as in two wild peas (*P. elatius*, *P. jomardi*).

Additional key words: embryogenic competence, picloram, *Pisum sativum*, regeneration, secondary embryogenesis.

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Abbreviations: BAP - 6-benzylaminopurine; 4-CPA - 4-chlorophenoxyacetic acid; 2,4-D - 2,4-dichlorophenoxyacetic acid; NAA - α-naphthaleneacetic acid; MSB-medium - medium with mineral salts after Murashige and Skoog (1962), vitamins after Gamborg *et al.* (1968); MSF - multiple shoot formation; PIC - picloram [4-amino-3,5,6-trichloropicolinic acid]; SE - somatic embryogenesis; TDZ - thidiazuron [N-phenyl-N´-1,2,3-thiadiazol-5´-yl urea]; 2,4,5-T - trichlorophenoxyacetic acid.

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Introduction

It is now generally accepted that competence for somatic embryogenesis (SE) is genetically controlled (Kielly and Bowley 1992, Henry *et al.* 1994, Crea *et al.* 1995) and is mediated through the actual ontogenetic state of the initial explant (biochemical, physiological and cytological status) which responds to a specific inductive signal. This in many plant species is exogenous auxin. The interaction of these factors leads to acquisition of embryogenic competence and induction of somatic embryos *per se* (Ivanova *et al.* 1994, Murthy *et al.* 1995, Brown *et al.* 1995, McKersie and Brown 1996). Despite the series of results with pea SE that have been obtained in last ten years (Kysely *et al.* 1987, Lehminger-Mertens and Jacobsen 1989, Kysely and Jacobsen 1990, Tétu *et al.* 1990, Stejskal and Griga 1992, Nadolska-Orczyk *et al.* 1994, Van Doorne *et al.* 1995, Loiseau *et al.* 1995), the state of the technology and of understanding individual steps of the protocol, as well as the transfer of this protocol closer to breeding applications, is still far behind the most frequently studied leguminous model species – alfalfa (McKersie and Brown 1996) and soybean (Komatsuda 1995, Brown *et al.* 1995).

The majority of papers reporting SE in pea (*Pisum sativum* L.) used callus-mediated regeneration from immature embryos, shoot apices, nodal segments (Kysely *et al.* 1987, Kysely and Jacobsen 1990, Griga 1990, Stejskal and Griga 1992, Van Doorne *et al.* 1991, 1995) or from protoplast-derived calli (Lehminger-Mertens and Jacobsen 1989). The first report of direct pea SE from immature zygotic embryos without callus intervention was published by Tétu *et al.* (1990). More recently Loiseau *et al.* (1995) reported an efficient system of pea SE from shoot apices which may be considered as a direct SE as well.

Here we bring further evidence of direct pea SE from the shoot apical meristems. We also provide a protocol which was tested with a broad spectrum of *Pisum* genotypes. In addition, the improvement of germination of pea somatic embryos by TDZ application is reported.

Materials and methods

Plants: The crucial steps of the complete protocol were formulated using the model *Pisum sativum* line HM-6 (Stejskal and Griga 1992). 37 cultivars/lines of *P. sativum*, 11 cvs. of *P. arvense* and two wild peas *P. elatius* and *P. jomardi* from the *AGRITEC* Pea Collection were tested for verification of the protocol (Table 5). Seeds were surface sterilized and aseptically germinated in the dark as described earlier (Griga *et al.* 1986). Shoot apices (3 - 5 mm in size) and shoot apical meristems (0.5 - 1 mm in size) were isolated from 3 to 5-d-old etiolated seedlings and placed with the cut surface on induction agar medium in test tubes.

Somatic embryo induction and development: Induction medium MSB contained 3 % sucrose, 0.7 % *Difco* agar and various concentrations of PIC: 0, 0.5, 2.5, 5.0, 10, 50 and 100 μM. The explants were cultured either continuously on PIC induction

medium or transferred to MSB-0 medium (without PIC) after 2 - 3 weeks. No special maturation medium was used. The cultures were grown under 16-h photoperiod (cool-white fluorescent tubes; irradiance 20.4 μmol(photon) m^{-2} s^{-1}) and temperatures of 25 °C at day and 20 °C at night. The frequency of SE was recorded after 4 to 8 weeks. Each experimental treatment usually consisted of 20 explants in two replicates. Extensive experiments were then repeatedly carried out with a selected optimum concentration of PIC.

Somatic embryo germination and conversion into plants: Morphologically well developed somatic embryos as well as those with abnormal morphology were placed on germination media: MSB-0 and MSB with TDZ. The screening of optimum concentrations of TDZ preceded the germination experiments. Growth responses of isolated pea meristems on the following concentrations of TDZ was recorded: 0, 0.5, 2.5, 10, 50, 100 μM. One set of cultures was exposed to TDZ continuously, the second set was transferred to MSB-0 medium after 2 weeks. Final evaluation was done after 8 weeks of culture. Optimum TDZ concentration was then used for conversion experiments. The pH of induction as well as germination media was adjusted to 5.8 prior to autoclaving (121 °C, 15 min). The cultures were grown on the above mentioned conditions. Plants originated either directly by germination of somatic embryos or by rooting of TDZ-induced shoots, and were routinely transferred to the soil in the greenhouse and grown to maturity.

Screening of *Pisum* genotypes for embryogenic competence: Fifty *Pisum* genotypes (mainly Czech, Slovak and West European cultivars) were tested for their capacity for SE with the use of the protocol optimized for the model pea line HM-6. Three experiments were done; each experimental treatment consisted of 20 explants in two replicates, *i.e.* 120 explants per genotype in total.

Results

Effect of auxin concentration and explant type: For induction of SE only one concentration of PIC (2.5 μM) was effective, other concentrations inducing callus formation (Table 1). SE was observed also on MSB medium with 10 and 50 μM PIC after transfer from PIC-medium to MSB-0 medium, but it was combined with intensive callus formation. During the course of our experiments, Loiseau *et al.* (1995) published an optimum concentration of 4.5 μM PIC for the highly embryogenic French genotype Cl 830; thus, we compared in additional experiment 2.5 with 5 μM PIC. The comparison resulted in similar frequency of SE (29.9 % for 2.5 μM PIC and 28.6 % for 5 μM PIC).

The response of two types of initial explants, *i.e.* shoot apices (3 to 5 mm) *versus* apical meristems (0.5 to 1 mm) was compared on medium with 2.5 μM PIC. The highest SE frequency was obtained in shoot apices transferred from PIC to MSB-0 (36.5 %), followed by apical meristems continuously cultured on PIC (34.0 %). Shoot apices tended to show pronounced callus formation as compared to apical meristems,

where clear direct SE without callus intervention was predominantly observed (Figs. 1*A,B,C*). The highest absolute frequency of SE was obtained with apical meristems in line HM-6 cultured continuously on 2.5 μM PIC (61.1 %) in another experiment than that presented in Table 2.

Table 1. Effect of PIC concentrations and media sequence on growth response from shoot apical meristems of pea line HM-6; transfer from MSB-0 to MSB-0 (0 - 0, control) after 4 weeks, transfer from the auxin-induction medium to the same medium (PIC - PIC) after 4 weeks, transfer from the auxin-induction medium to MSB-0 (PIC - 0) after 3 weeks; evaluated after 8 weeks of culture; mean of two experiments ± SD.

Media sequence	PIC [μM]	Growth response	Frequency of SE [%]
0 - 0	0	Shoots, sporadically roots	0
PIC - PIC	0.5	Callus	0
PIC - PIC	2.5	Somatic embryos	41.40 ± 13.39
PIC - PIC	10	Callus	0
PIC - PIC	50	Callus	0
PIC - PIC	100	Callus	0
PIC - 0	0.5	Callus	0
PIC - 0	2.5	Somatic embryos	41.08 ± 17.37
PIC - 0	10	Somatic embryos, callus	30.00 ± 14.10
PIC - 0	50	Somatic embryos, callus	5.00 ± 7.10
PIC - 0	100	Callus	0

Table 2. Effect of two types of initial explants (apical meristems 0.5 - 1 mm, AM, and shoot apices 3 - 5 mm, SA), and two media sequences on SE frequency of pea line HM-6; medium MSB-0 (0) and MSB + 2.5 μM PIC (PIC), evaluated after 8 weeks of culture; mean of three experiments ± S.D. Each experimental treatment consisted of 20 explants in two replicates.

Explant type	Media	SE frequency [%]	SE [cultured explant^{-1}]	SE [responding explants^{-1}]
AM	PIC - PIC	34.00 ± 11.12	0.71 ± 0.37	2.04 ± 0.39
AM	PIC - 0	21.13 ± 11.24	0.35 ± 0.20	1.72 ± 0.53
SA	PIC - PIC	22.43 ± 11.00	0.33 ± 0.24	1.39 ± 0.38
SA	PIC - 0	36.56 ± 10.74	0.66 ± 0.26	1.76 ± 0.29

Somatic embryo induction and development: Somatic embryos originated directly (without callus intervention) from shoot apical meristems on induction medium MSB supplemented with PIC. First visible signs of somatic embryos, *i.e.* globular structures, were evident on meristem explants after 2 weeks of culture (Fig. 1*A*). Further development on the same induction medium was very quick - fully developed cotyledonary stage somatic embryos may be obtained during the next two weeks of culture (Figs. 1*C,D*).

Somatic embryos originated from apical part of meristematic dome as well as from basal part of meristem explant. The initiation and development of somatic embryos was asynchronous, *i.e.* basal somatic embryos developed more quickly than apical ones (Figs. 1*B,C*). A single 4-week-old explant may contain a continuous scale of

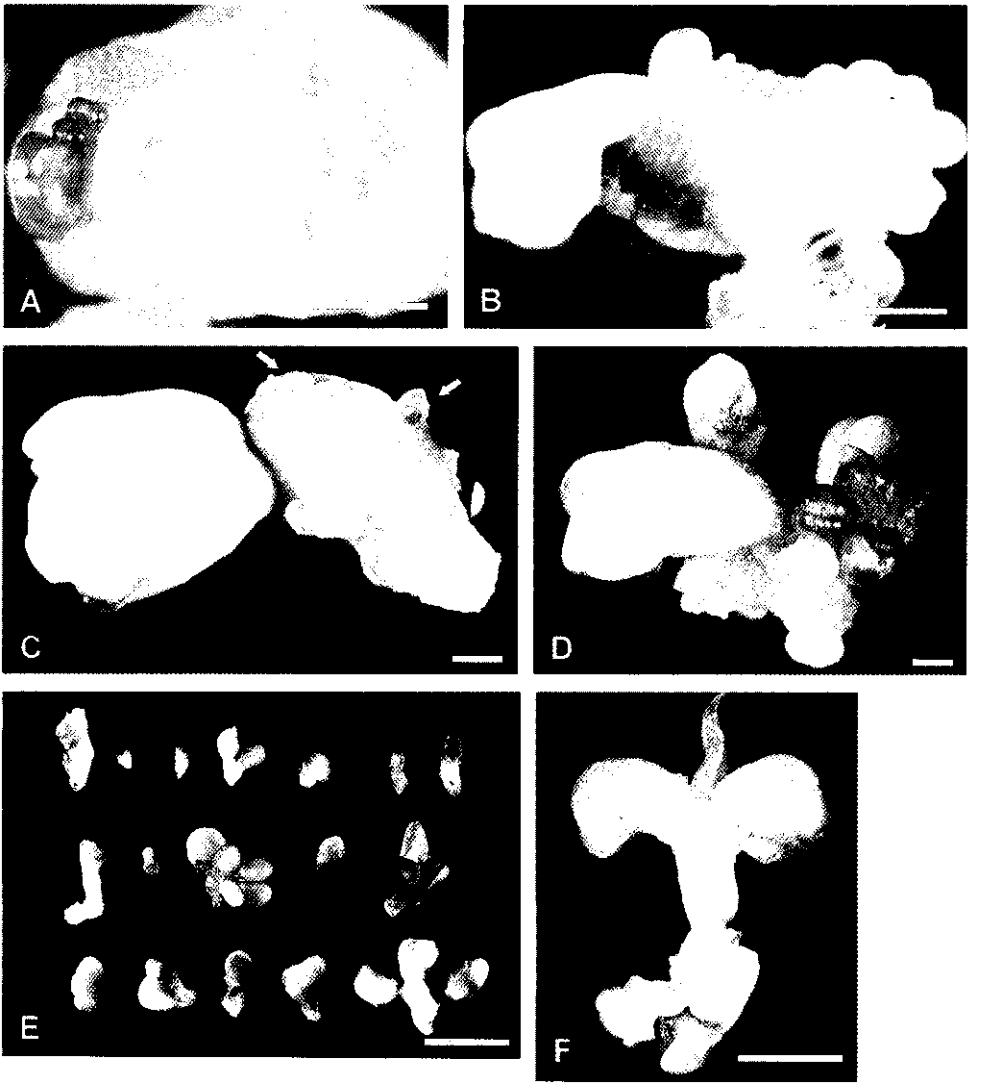

Fig. 1. Direct SE in pea line HM-6. *A* - initiation of globular somatic embryos in leaf primordia area of meristematic dome in 2-week-old culture on PIC-medium. *B,C,D* - asynchronous development of somatic embryos. Practically all developmental stages of embryos could be detected in one individual explant: in 4-week-old explant (*C*) there are mature cotyledonary somatic embryos alongside newly formed globular ones (*arrows*); see no or negligible callus formation. *E* - range of morphological types of somatic embryos harvested from three explants. *F* - precocious germination of morphologically well developed cotyledonary somatic embryo still connected with the initial explant; MSB-0 medium, 5-weeks-old culture. *Bar* represents 1 mm (*A,B,C,D*), 4 mm (*F*) and 5 mm (*E*).

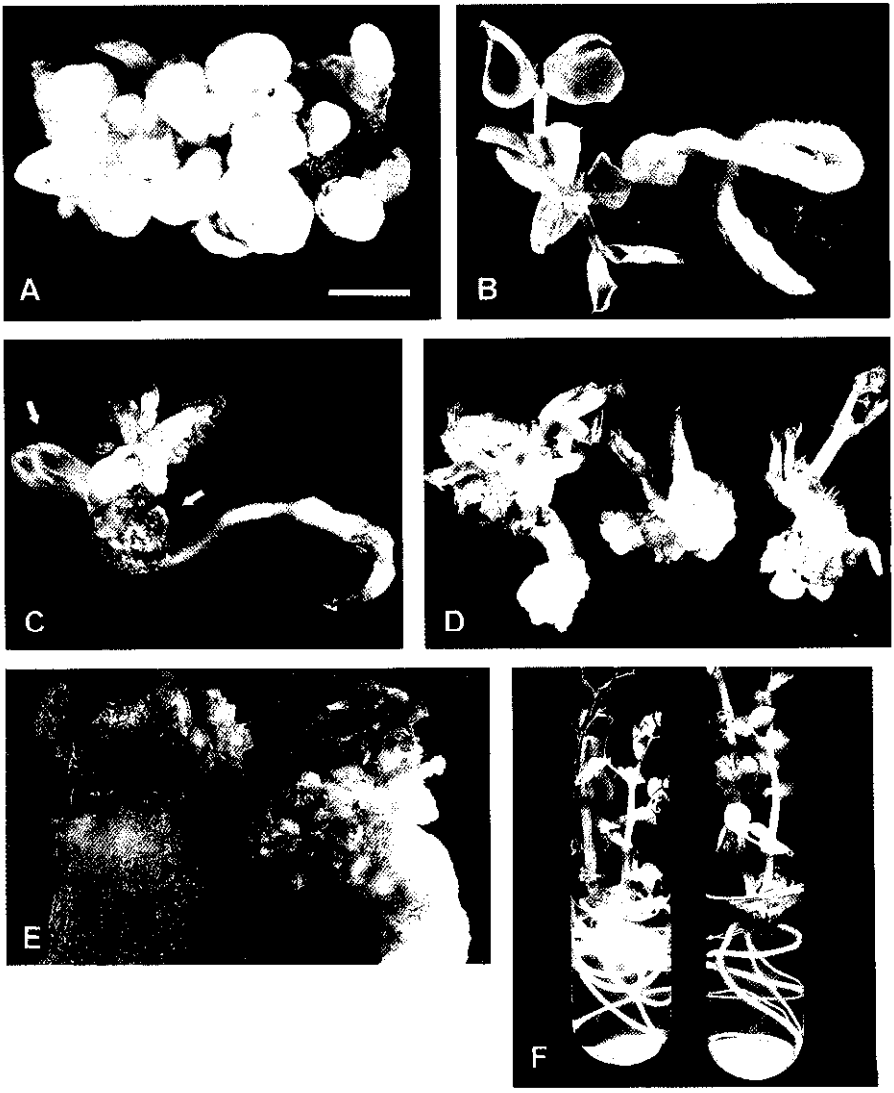

Fig. 2. Direct SE in pea. *A* - secondary SE on medium for germination (MSB-0), cv. Janus. *B* - converted plantlet from morphologically normal somatic embryo on MSB-0 medium, line HM-6. *C* - converted plantlet from morphologically normal somatic embryo on MSB medium with 10 μM TDZ (see well developed cotyledons - *arrows* - and multiple shoot formation in the shoot apex area), line HM-6. *D* - intensive multiple bud and shoot formation (proliferation of axillary buds as well as *de novo* regeneration of adventitious buds) on TDZ-treated somatic embryos with abnormal morphology, line HM-6. *E* - *de novo* adventitious shoot-bud proliferation on TDZ-treated somatic embryos with very abnormal morphology and absence of shoot apical meristem, line HM-6 (detail). *F* - TDZ-induced shoots rooted easily on 1/2 MS medium with 1 μM NAA, line HM-6. *Bar* (*A*) represents 1 mm.

developmental stages from fully developed cotyledonary somatic embryos at the base to newly differentiating globular ones in the apical part (Figs. 1*B,C*). 1 to 10 somatic embryos developed on single explant, but usually 1 to 3 embryos reached the cotyledonary stage relatively quickly (4 - 5 weeks, Figs. 1*C,D,F*), while the remaining ones were gradually overgrown by callus (after 8-week culture of primary explants). There is some morphological and anatomical evidence that somatic embryos initiated preferentially from leaf primordia areas (Fig. 1*A*; anatomical data not shown).

Fully developed somatic embryos were not morphologically uniform - we observed a broad spectrum of morphological types (Figs. 1*E,F*): normal somatic embryos with two cotyledons and a shoot apex, abnormal somatic embryos with one, three or more cotyledons, fused cotyledons - trumpet shaped somatic embryos, several somatic embryos fused at the root and hypocotyl area. There was a shift in the proportions of individual organs in morphologically normal somatic embryos at the cotyledonary stage as compared to pea zygotic embryos, to a more developed hypocotyl and less developed cotyledons.

Germination of somatic embryos, conversion into plants; secondary embryogenesis: Morphologically normal somatic embryos germinated in some cases on the mother explant (Fig. 1*F*) or immediately after their isolation and transfer into MSB-0 medium (Fig. 2*B*); the conversion of morphologically abnormal somatic embryos was significantly increased by the application of 10 μM TDZ (concentration selected in preliminary experiment; Table 3), which induced MSF (proliferation of axillary buds and massive *de novo* regeneration of adventitious buds) on treated embryos (Figs. 2*C,D*). Thus, TDZ was able to induce shoot organogenesis in somatic embryos without a differentiated shoot apex (Fig. 2*E*). Through these procedures we were able to germinate in an individual experiment 78.3 % of the total of somatic embryos harvested.

Table 3. Frequency of pea line HM-6 explants (shoot apical meristems) with multiple shoot formation (MSF), number and length of shoots grown on MSB medium with various TDZ concentrations. The explants subcultured after two weeks on the same medium or MSB-0 medium; evaluated after 9 weeks of culture.

TDZ	TDZ - TDZ			TDZ – MSB-0		
[μM]	MSF [%]	shoot number	length [cm]	MSF [%]	shoot number	length [cm]
0	0	1.0	1.2	0	1.0	1.2
0.5	100	4.1	1.0	33.3	2.3	2.4
2.5	100	8.9	1.1	88.0	3.2	1.5
10	100	13.8	0.9	96.0	7.8	1.9
50	0	0	0	100	5.9	0.3
100	0	0	0	0	0	0

The cultural scheme significantly affected the germination response and frequency (Table 4). Culture on PIC-medium followed by transfer to MSB-0 resulted in very

Table 4. Germination of pea (line HM-6) somatic embryos; all embryos taken from cultures induced on MSB medium with 2.5 μM PIC (PIC) and then transferred either to the same medium or MSB-0 medium (0) followed by medium for germination, *i.e.* MSB-0 again or MSB + 10 μM TDZ (TDZ); subculture interval 4 weeks. Mean ± S.D.; experimental treatment – medium sequence – represents 20 to 60 explants in two replicates. Total germination frequency was calculated from original experimental data.

Media sequence	Normal germination (A) [%]		MSF (B) [%]		Shoot only (C) [%]		Root only [%]		Secondary SE [%]		Total germination[3] (A+B+C) [%]	
	Cult.[1]	Vital[2]	Cult.	Vital	Cult.	Vital	Cult.	Vital	Cult.	Vital	Cult.	Vital
PIC - PIC - 0	0	0	0	0	0	0	0	0	16.7±7.8	50.0±23.7	0	0
PIC - PIC - TDZ	0	0	24.5±11.6	26.7±12.6	3.1±1.5	3.3±1.6	18.4±8.7	20.0±9.5	1.0±1.4	1.0±1.6	27.6±10.1	30.0±11.0
PIC - 0 - 0	8.3±3.9	20.0±7.6	0	0	2.8±4.0	6.7±8.8	13.9±4.0	33.3±6.3	8.3±3.9	20.0±7.6	11.1±7.8	26.7±16.4
PIC - 0 - TDZ	5.4±2.0*	7.1±2.5*	30.2±8.3*	40.2±11.4*	0.7±0.9	0.9±1.3	2.7±1.8	3.6±2.5	0.7±0.9	0.9±1.3	31.5±10.2	42.0±13.9

[1] - calculated for all cultured explants, [2] - calculated only for vital explants at the time of evaluation (necrotic somatic embryos excluded); [3] - only embryos germinating via (A), (B) and (C) were recorded; in such embryos there is a high probability (practically 100 %) of obtaining complete plants; the best values in individual experimental treatment (replicate) were obtained in the media sequence PIC - 0 - TDZ, 56.3 % and 78.3 % in cultured and vital embryos, respectively; * - presence of normal germination and MSF in one explant (see Fig. 2C)

abundant secondary SE (more than 20 secondary embryos per explant - Fig. 2*A*). Replacement of MSB-0 in this sequence by TDZ induced MSF and dramatically decreased secondary SE. Continual culture on PIC led to total absence of normal germination (parallel shoot and root development). Transfer PIC - MSB-0 - MSB-0 induced the highest frequency of normal germination and also of significant secondary SE. The media sequence PIC - MSB-0 - TDZ resulted in maximum MSF but a drop in secondary SE.

Screening of *Pisum* genotypes for embryogenic competence: Of 50 tested *Pisum* genotypes only one was without any embryogenic response (canning pea cv. Moravan). Thus, the frequency of SE ranged from 1.7 % (dry seed pea cv. Montana) to 50.4 % (line HM-6) (Table 5). Both wild forms, *i.e. P. elatius* and *P. jomardi* responded positively, too.

Discussion

Pea explants respond to a variety of exogenous auxins for SE induction: NAA, 2,4-D, 2,4,5-T, PIC, 4-CPA (Kysely *et al.* 1987, Kysely and Jacobsen 1990, Tétu *et al.* 1990, Griga 1990, Stejskal and Griga 1992, Özcan *et al.* 1993, Van Doorne *et al.* 1991, 1995, Loiseau *et al.* 1995); except NAA, all types belong to the chlorinated auxins. The presence of auxin in the induction medium was necessary in all the above-mentioned papers. In contrast to some other grain legumes, in which cytokinins or TDZ alone or in combination with auxins have been reported to induce SE (*e.g. Lupinus* spp. - Nadolska-Orczyk 1992, *Phaseolus* spp. - Malik and Saxena 1992, *Cicer arietinum* - Shri and Davis 1992, Murthy *et al.* 1996, *Psophocarpus tetragonolobus* - Gupta *et al.* 1997), in pea, the cytokinins (BAP, zeatin, kinetin) added to the auxin-induction medium reduced or strongly inhibited SE (Kysely and Jacobsen 1990, Tétu *et al.* 1990, Loiseau *et al.* 1995). In our experiments, exposure of pea shoot apical meristems to PIC resulted in SE, TDZ treatment of the same explants inducing only MSF or callogenesis (Table 3). In addition, TDZ strongly inhibited secondary embryogenesis on pea primary embryos (Table 4). These data confirm the inhibitory effect of cytokinin on induction of pea SE. Unfortunately, there is a lack of exact data in pea on the mutual effect of exogenous growth regulators with endogenous phytohormones leading to acquisition of embryogenic competence, as has been reported recently, *e.g.* in alfalfa (Ivanova *et al.* 1994) and peanut (Murthy *et al.* 1995).

So far, only one report has been published describing direct SE from immature zygotic embryos in pea (Tétu *et al.* 1990), although probably two other papers were dealing with the same phenomenon, despite the fact the authors did not use the term "direct" SE (Kysely and Jacobsen 1990, Loiseau *et al.* 1995). Not only the type of initial explant but its size, the auxin used and its concentration may significantly affect the kind of regeneration, *i.e.* direct/indirect SE. Of four auxins tested, only NAA and 2,4-D were able to induce SE from immature cotyledons; 43 μM NAA induced direct somatic embryogenesis, while concentrations higher than 80 μM

Table 5. Frequency of somatic embryogenesis in 50 cultivars and lines of *Pisum sativum*, *P. arvense* and wild peas *P. elatius* and *P. jomardi*. Mean ± S.D. of 3 experiments; medium MSB with 2.5 μM PIC; each experiment included 20 explants in two replicates for each cultivar.

Species	Type	Cultivar	SE frequency [%]	SE [cult. exp.$^{-1}$]	SE [resp. exp.$^{-1}$]
Pisum sativum	dry seed peas	HM-6	50.4 ± 8.2	0.88 ± 0.46	1.68 ± 0.63
		Tolar	32.2 ± 10.1	0.49 ± 0.23	1.50 ± 0.32
		Menhir	23.3 ± 5.8	0.36 ± 0.14	1.50 ± 0.21
		HM-2689	21.1 ± 6.7	0.30 ± 0.14	1.33 ± 0.32
		Schobi	19.8 ± 8.9	0.31 ± 0.22	1.65 ± 0.61
		Junák	17.8 ± 12.6	0.28 ± 0.26	1.42 ± 0.39
		Romeo	15.8 ± 8.8	0.35 ± 0.34	1.95 ± 0.95
		19/1-O-T	15.2 ± 4.5	0.23 ± 0.04	1.58 ± 0.42
		Janus	14.2 ± 7.6	0.19 ± 0.12	1.32 ± 0.12
		Progreta	12.6 ± 7.5	0.15 ± 0.09	1.25 ± 0.25
		Belman	12.5 ± 15.6	0.19 ± 0.27	1.34 ± 0.47
		Maxi	11.7 ± 16.1	0.14 ± 0.20	1.11 ± 0.16
		Jubilat	10.6 ± 9.6	0.17 ± 0.17	1.45 ± 0.39
		Bohatýr	10.0 ± 0.0	0.13 ± 0.03	1.25 ± 0.25
		Komet	10.0 ± 7.5	0.11 ± 0.08	1.08 ± 0.14
		Renata	10.0 ± 8.6	0.10 ± 0.08	1.00 ± 0.00
		Sandra	9.2 ± 10.1	0.11 ± 0.13	1.13 ± 0.18
		Smaragd	8.3 ± 1.4	0.11 ± 0.04	1.28 ± 0.25
		Amino	7.5 ± 6.6	0.11 ± 0.09	1.44 ± 0.51
		Dukát	5.4 ± 2.6	0.06 ± 0.04	1.11 ± 0.19
		Tyrkys	4.2 ± 2.8	0.08 ± 0.09	1.13 ± 0.23
		Solara	4.2 ± 1.3	0.04 ± 0.01	1.00 ± 0.00
		Odeon	3.5 ± 3.8	0.04 ± 0.04	1.00 ± 0.00
		Olivín	2.8 ± 2.6	0.03 ± 0.03	1.00 ± 0.00
		101-O-T	2.0 ± 1.8	0.05 ± 0.06	1.50 ± 0.71
		Montana	1.7 ± 1.4	0.02 ± 0.02	1.00 ± 0.00
Pisum sativum	canning peas	Citrina	29.6 ± 2.0	0.40 ± 0.09	1.36 ± 0.25
		Oskar	20.8 ± 16.6	0.32 ± 0.27	1.36 ± 0.31
		Countess	14.2 ± 7.6	0.23 ± 0.13	1.62 ± 0.40
		Havel	11.9 ± 1.7	0.17 ± 0.02	1.43 ± 0.06
		Vitiaz	10.0 ± 14.1	0.23 ± 0.13	2.25 ± 0.89
		Puget	9.3 ± 13.7	0.14 ± 0.22	1.30 ± 0.42
		Helka	7.5 ± 4.3	0.11 ± 0.07	1.47 ± 0.50
		Radim	5.8 ± 5.2	0.06 ± 0.05	1.00 ± 0.00
		Vladan	5.0 ± 5.0	0.06 ± 0.07	1.13 ± 0.18
		Colt	2.5 ± 2.5	0.04 ± 0.04	1.25 ± 0.35
		Moravan	0	0	0
Pisum arvense	fodder peas	SGC-32	22.5 ± 2.5	0.29 ± 0.07	1.26 ± 0.16
		Tyla	13.3 ± 1.4	0.20 ± 0.08	1.48 ± 0.43
		56-O-R	10.8 ± 9.5	0.20 ± 0.00	1.24 ± 0.13
		Arvika	10.8 ± 9.5	0.13 ± 0.11	1.17 ± 0.23
		Dora	10.0 ± 2.5	0.13 ± 0.03	1.34 ± 0.15
		Sirius	7.5 ± 6.6	0.13 ± 0.11	1.56 ± 0.51
		Nike	7.5 ± 2.5	0.08 ± 0.03	1.00 ± 0.00
		Ina	6.7 ± 1.4	0.10 ± 0.05	1.33 ± 0.58
		Dundale	5.9 ± 5.3	0.15 ± 0.17	2.46 ± 1.12
		Golf	5.2 ± 7.0	0.05 ± 0.07	1.00 ± 0.00
		Algera	4.2 ± 2.9	0.06 ± 0.02	1.33 ± 0.58
Pisum jomardi	wild forms		10.0 ± 2.5	0.11 ± 0.04	1.07 ± 0.12
Pisum elatius			6.7 ± 6.3	0.11 ± 0.10	1.55 ± 0.07

resulted in callus or root formation (Tétu *et al.* 1990). Similarly, as the embryo size significantly influences the embryogenic response in pea (Kysely and Jacobsen 1990, Tétu *et al.* 1990), the size of shoot apex/apical meristem may affect the final mode of SE. In our work, the use of bigger explant (3 - 5 mm shoot apices; see also Kysely *et al.* 1987) usually resulted in formation of embryogenic callus followed by somatic embryo initiation. We cannot strictly exclude that in these explants somatic embryos also developed in some cases directly, but somatic embryo initiation was masked by abundant callus formation on the basal part of the explant and on the young leaves. A certain degree of tissue differentiation (shift from fully meristematic to parenchymatous cells) of young leaves and explant bases of the initial explant was the probable reason that callus initiation started very early or was parallel with somatic embryo initiation. The isolation of smaller explants (0.5 to 1 mm) with minimaly differentiated structures (composed completely of meristematic cells) led to clearly direct SE with delayed and negligible callus formation (Fig. 1). If the shoot apical meristem is a structure with an organogenic developmental program (shoot and leaf morphogenesis), its isolation together with the auxin signal probably terminated this program and switched it on to embryogenesis (Merkle *et al.* 1995, Yeung 1995). This idea may be supported by the localization of pea somatic embryos preferentially in the leaf primordia area (Kysely *et al.* 1987, Kysely and Jacobsen 1990) and especially by the serial development of somatic embryos from the basis (older leaf primordia) to the apex (younger leaf primordia) of meristematic explants (Figs. 1*B,C*). Even in 4 to 5-week-old explants with fully differentiated cotyledonary embryos (located basally) initiation of new globular ones started in the meristematic dome area (Fig. 1*C*).

Developing pea somatic embryos exhibited clearly distinct globular, heart-shaped, torpedo and cotyledonary stages. In the case of globular and especially heart-shaped embryos we could find absolutely regular and symetrical individuals resembling zygotic embryos in shape, organ proportions (presence of apical meristem, root meristem, cotyledons; absence of hypocotyl) and size (data not shown). In contrast to zygotic embryogenesis in pea, where the torpedo stage is practically absent (Liu *et al.* 1996), we may find this stage during pea SE, as did Kysely and Jacobsen (1990), Stejskal and Griga (1992), and Loiseau *et al.* (1995). In morphologically normal somatic embryos, there was generally a shift in the proportions of individual organs as compared to their zygotic counterparts, *i.e.* reduced size of cotyledons and the presence of a robust hypocotyl (Fig. 1*F*). This is in agreement with the frequently documented fact that cotyledons of somatic embryos seldom reach the same size as in the corresponding zygotic embryos as well as that the most often reported morphological abnormalities of somatic embryos involve the cotyledons (Buchheim *et al.* 1989, Yeung 1995). However, the cotyledons probably markedly affect the germination of somatic embryos; there was found a direct relationship between the amount of cotyledonary tissue and conversion days, *i.e.* time required for conversion of soybean somatic embryos into plants (Buchheim *et al.* 1989). The occurrence of frequent abnormalities in pea somatic embryo morphology is connected predominantly with torpedo and cotyledonary stages, where organ proportions are disrupted by the absence of physiological and mechanical influences of ovular tissues

on the developing embryo in *in vitro* conditions, and by the detrimental effect of continued exposure to auxin on differentiation and following germination capacity (Parrott *et al.* 1988, Merkle *et al.* 1995). Thus, induction and development on a single auxin medium, though quick, simple and seemingly efficient, frequently results in somatic embryos of poor quality and limited germinability. In contrast, a relatively short auxin signal (3 d) was sufficient to induce soybean somatic embryos with increased proportion of morphologically normal individuals easily converting on hormone-free medium (Parrott *et al.* 1988). We could observe a similar situation in pea, where a shorter exposure to auxin was better for normal germination. 8-week-exposure to PIC strongly blocked the normal conversion on hormone-free media.

We did not use any special maturation or dessication treatment for physiological maturation (storage substances accumulation, dessication tolerance); morphologically normal somatic embryos often developed continually from cotyledonary stage to plantlets (even on the mother explant) via typical precocious germination (Fig. 1*F*). First results on the accumulation of starch and proteins in developing pea somatic embryos are recently available in our laboratory (Griga *et al.*, unpublished). In contrast to Loiseau *et al.* (1995), who converted 40 % of somatic embryos previously induced and developed on single auxin medium, we observed no germination (on MSB-0) of somatic embryos after continual culture on PIC. Moreover, the lack of normality in somatic embryos strongly reduced the conversion rate. To overcome these shortcomings, we use TDZ which is known by its strong cytokinin effect (Thomas and Katterman 1986) and which was successfully used to induce shoot organogenesis in pea (Malik and Saxena 1992, Böhmer *et al.* 1995, Sanago *et al.* 1996). In contrast to Böhmer *et al.* (1995) we had no problems with the subsequent rooting of shoots (Fig. 2*F*) originated on somatic embryos, probably due to the relatively short TDZ treatment (4 - 8 weeks). Even on morphologically very abnormal somatic embryos, TDZ was able to induce abundant MSF (Fig. 2*E*). The approach was similar to those suggested by Wright *et al.* (1991) to overcome inability of germination or to increase conversion rates of soybean somatic embryos by BAP treatment.

The protocol presented here was successfully applied to 49 *Pisum* genotypes out of 50 tested. The SE varied from 50.4 % to 1.7 % (the maximum value obtained in individual experiments was 61.1 %, model line HM-6). We did not obtain so high frequency of SE as Loiseau *et al.* (1995) with their highly embryogenic pea line Cl 830, *i.e.* 95 - 100 %. Nevertheless, our results represent the most extensive screening of embryogenic capacity in pea. Van Doorne *et al.* (1995) found 13 embryogenic cultivars of 16 tested, Tétu *et al.* (1990) 6 embryogenic of 9 tested, Kysely and Jacobsen (1990) and Lehminger-Mertens and Jacobsen (1989) found all 5 and 6 genotypes tested to be embryogenic, respectively. Despite the fact that our recent protocol was appropriate for almost all genotypes tested, the frequency in many genotypes was below 10 % and is not probable that under given cultural conditions it will be increased due to evidently strong genotype effect. Thus, it is logical that the highest frequency of SE was repeatedly obtained in model pea line HM-6 for which the protocol was optimized and this level was not overcome by any other of the tested

cultivars. It is evident that protocol still needs special modifications for certain genotypes.

In general, callus mediated regeneration (organogenesis, SE) is considered to increase the probability of producing phenotypically and/or genetically altered plants. Also in pea there is evidence that plants from embryogenic calli may exhibit cytological (Kysely *et al.* 1987) and even dramatic morphological changes, including chimaeric and lethal individuals (Stejskal and Griga 1992, Griga *et al.* 1995). Somaclonal variation potentially useful in pea breeding has been extensively studied in several seed progenies of callus-derived somatic embryos (Griga *et al.* 1995, Griga and Létal 1995, 1996). On the other hand, direct SE should guarantee clonal fidelity and genetic stability with donor plant tissue, necessary in transformation experiments; however, there are so far no available data for this in pea.

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