TWO WAYS OF PLANT REGENERATION FROM IMMATURE COTYLEDONS OF PEA

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ABSTRACT

Two different systems of plant regeneration via organogenesis and embryogenesis from immature cotyledons of pea (*Pisum sativum* L.) were developed. The first system was direct multiple shoot regeneration from the proximal to the embryo axis, injured part of cotyledon. The ability of six Polish cultivars to shoot formation was very high. The percent of regenerating cotyledons was from 73 to 86 and mean number of shoots from 3.5 to 9.9 after seven weeks of culture. This multiplication could be prolonged for next several months. The second system was somatic embryogenesis, initiating from the same part of cotyledon simultaneously with slowly proliferating callus. Only three out of six cultivars formed embryoids. The differences of ability to embryo formation ranged between 43% of responding explants for Heiga cultivar to only 6% for Cud Ameryki. The mean number of embryoids was from 4.2 to 2.3.

KEY WORDS: organogenesis, somatic embryogenesis, pea, *Pisum sativum* L., in vitro regeneration.

INTRODUCTION

Pea (*Pisum sativum* L.) is an important grain legume crop and similar to the other legumes have been recalcitrant in tissue culture. However, in last few years significant progress of plant regeneration and transformation have been made.

Plant regeneration via organogenesis was obtained from immature cotyledons (Nadolska-Orczyk, 1991; Ozcan et al., 1992), nodal thin cell layer segments (Nauerby et al., 1991), long term callus (Hussey and Gunn, 1984), hypocotyl explants (Nielsen et al., 1991), shoot apices and axillary buds of seedlings (Griga et al., 1986) and from protoplast culture (Puonti-Kaerlas and Eriksson, 1988). There are also a few papers concerning somatic embryogenesis in pea. The development of somatic embryos was achieved from immature embryos and shoot apex segments (Kysely et al., 1987; Kysely and Jacobsen, 1990). Some of these techniques are good base for plant transformation but could only work with certain genotypes. Possibility of obtaining transgenic plants of pea using *Agrobacterium tumefaciens* mediated transformation was just been confirmed in three papers (De Kathen and Jacobsen, 1990; Schroeder et al., 1993; Davies et al., 1993). GUS expressing shoots were obtained by Nauerby et al., (1991).

We described two new procedures of plant regeneration from immature cotyledons via organogenesis and embryogenesis suitable for *Agrobacterium* transformation. They are based on injured part of explant, short time of culture and appropriate, Polish cultivars for this aid.

MATERIALS AND METHODS

Six cultivars of pea: Diament (D), Ramir (R), Heiga (H), Laser (L), Cud Ameryki (Ca) and Hamil (Ha) were used. The seeds were kindly provided by Prof. J. Szyrmer from Plant

Breeding and Acclimatization Institute and by Division of Plant Breeding, Radzików. The source of explant were immature cotyledons 3-7 mm long, excised from 9-11 day-old pods. The pods were surface sterilized by immersing in sodium hypochlorite (7.5% v/v). Next the immature seeds were isolated and the embryo axis removed. Four cotyledons per Petri dish (8 mm in diameter) were placed their down side on culture media.

The basal media were: MS (Murashige and Skoog, 1962), half strength of MS or MS modified medium (10 mM NH_4NO_3 + 30 mM KNO_3). They contained different growth regulators composition, sucrose level and solidified agent. Composition of media used for organogenesis, embryogenesis and rooting of shoots is showed in Table 1. The experiments of ability of different pea cultivars to regeneration were repeated three times. For better illustrating of this ability (including percent of regenerated cotyledons and mean number

TABLE 1. Composition of media used in plant regeneration systems of pea.

Symbol	Base medium	Composition of growth regul. [mg/l]	Others [g/l]
P1	MS	5 IBA + 0.5 BAP	2 gelrite=30 sucrose
P2	MS	0.25 NAA+1 BAP	as above
P3	MS	1 2,4-D	as above
P4	MS	2 IBA+1 BAP	as above
M1	MS modif.*	0.1 ABA+0.1 IBA +0.1 zeat	as above
K1	1/2 MS	0.1 IAA	2 gelrite + sucrose
K2	1/2 MS	–	5 agar + 15 sucrose

* – 10 mM NH_4NO_3 + mM KNO_3

Figs 1A-C.

A – Initiation of buds on the cotyledon, cut surface of the embryo attachment stalk after 6 weeks of culture.

B – Multiple shoot formation after 8 weeks of culture.

C – Growth and rooting of shoots (12 weeks of culture).

of shoots or embryoids per explant) we estimated the coefficient of response according to the equation:

$$\frac{\text{percent of regenerated cotyledons} \times \text{mean number of embryos}}{100\%}$$

RESULTS AND DISCUSSION

Micropropagation

The model of direct organogenesis from immature cotyledons of six Polish cultivars of pea is showed in Table 2a. The induction of buds required high concentration of IBA (5 mg/l) and low of BAP (0.5 mg/l) (Fig. 1A). The differentiation took place in the proximal to the embryo axis, injured part of the cotyledon. Preliminary results on immature cotyledons as a good source of pea multiplication was presented by us in poster (Nadolska-Orczyk, 1991). Next, similar results were reported by Ozcan et al., (1992). However, authors found that NAA influenced higher regeneration ability than IBA. Subsequent development as well as growth and rooting of shoots can be achieved on medium containing half strength

TABLE 2a. Model of pea plant regeneration via micropropagation

Medium	Period of culture	Observations
P1 5 IBA 0.5 BAP	6 weeks	morphogenic tissue with buds (Fig. 1A)
K1 –	4 weeks	development of small shoots, multiplication of shoots (Fig. 1B)
P1 5 IBA 0.5 BAP	several weeks	continuous multiplication
P2 0.25NAA 1 BAP	2-3 weeks	growth of shoots
K1 – K2 –	2-6 weeks	growth and rooting of shoots (Fig. 1C)

of MS salts (Fig. 1B). Continuous multiplication was obtained after replacing morphogenic tissue on P1 medium.

The percentage of regenerating explants for different cultivars was between 73 and 86% (Table 2b) and number of shoots developing from one explant ranged from 3.5 to 9.9

Figs 2A-C. A – Embryo formation and callus proliferation on the cut surface of the cotyledon after 10 weeks of culture.
B – Development of shoot and root apex of embryo.
C – Mature, flowering and setting seed plants in vitro developed via organogenesis (left) and embryogenesis (right).

TABLE 2b. Ability of different pea cultivars to shoot formation after 7 weeks of culture.

Cultivar	No. of expl.	No. (%) of resp. explants	No. (%) of shoots	Coef. of resp.
D	33	24 (73)	80 (3.5)	2.56
R	51	43 (84)	281.5 (6.5)	5.46
H	36	30 (83)	99 (9.9)	8.22
L	39	29 (74%)	243 (8.4)	6.22
Ca	27	24 (86%)	164.8 (6.9)	5.93
Ha	24	20 (83)	65 (8.1)	6.72

shoots per explant. According to these results, main factor differing ability to plant regeneration among cultivars is number of developing shoots, but not percentage of regenerating explants. Similar, minor effect of cultivar dependency on shooting frequency from nodal thin cell layer segments for both, percentage of regenerating explants (88-97%) and number of shoots (3.3-4.1) was showed by Nauerby et al. (1991). Coefficients of response ranged between 8.22 for Heiga cultivar to 2.56 for Diamant and were several times higher comparing these for embryogenic ability.

Only small part of shoots, from 15 to 39% was rooting on the K1 and K2 media. The results were strongly depended on genotype and length of in vitro culture (data not showed).

The problem of rooting could be avoided by grafting of shoots on germinating seedlings.

Somatic embryogenesis

Plant regeneration via direct somatic embryogenesis from immature cotyledons in three out of six cultivars of pea was achieved using model of plant regeneration showed in Table 3a. Likewise in case of organogenesis, the differentiation took place in the proximal, injured part of the cotyledons. However, the growth regulators used in this system were different. The first embryoids were initiated on medium containing 1mg/12.4-D after 6 weeks of culture and this process could be prolonged for next 4-6 weeks by transferring explants on medium with 2 mg/l IBA and 1 mg/l BAP (Fig. 2A). Development of root and shoot apex appeared on MS medium with modified nitrogen ions and small content of IBA, ABA and zeatin (Fig. 2B). We found, that this medium has general application for developing of grain legume somatic embryos, since we were able to obtain precocious germination of embryoids from lupin (Nadolska-Orczyk, 1992) and soybean (Nadolska-Orczyk and Orczyk, 1994).

TABLE 3a. Model of pea regeneration via somatic embryogenesis.

Medium	Period of culture	Observations
P3 1 2.4-D	6 weeks	callus and first embryoids
P4 2 IBA 1 BAP	4-6 weeks	additional forming of embryoids and callus growth (Fig. 2A)
M1 0.1 IBA 0.1 ABA 0.1 zeat.	4-8 weeks	development of shoot and root apex (Fig. 2B)

The ability of different pea cultivars to embryoid formation differed considerably (Table 3b). Only three out of six cultivars were able to form embryoids. This ability ranged from 6 to 43% of responding explants and from 2.3 to 4.2 of embryos per explant. Coefficient of response was between 1.81 and 0.2. The best responding cultivar was Heiga, showing the highest percent of regenerated explants, number of embryoids per explant as well as coefficient of response. Low (0.46) and very low (0.2) coefficient of response was observed in Ca and L cultivars. These cultivars, showing ability to form embryoids, were also found to be among the best regenerating

TABLE 3b Ability of different pea cultivars to embryoid formation after 7 weeks of culture

Cultivar	No. of expl.	No. (%) of resp. explants	No. (%) of shoots	Coef. of response
D	29	0	0	0
R	66	0	0	0
H	67	29 (43%)	121 (4.2)	1.81
L	49	3 (6%)	10 (3.3)	0.2
Ca	50	10 (20%)	23 (2.3)	0.46
Ha	32	0	0	0

ones via organogenesis. However, coefficients of response of embryogenic ability were several times lower than organogenic ability.

In the other papers concerning somatic embryogenesis in pea, initiation of somatic embryos was achieved from immature embryos or shoot apices using picrolam (1 mg/l) or 2.4-D (0.5 mg/l) and maturation on very small content of NAA, BA and kinetin (Kysely et al., 1987; Kysely and Jacobsen, 1990). However, the number of somatic embryos per explant was even lower than in our case and for the best regenerating genotype equaled 2.9 per zygotic embryo. Similar to our results, authors of these papers observed differences in frequency of somatic embryos among the genotypes used.

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DWA SPOSOBY REGENERACJI ROŚLIN Z NIEDOJRZAŁYCH LIŚCIENI GROCHU

STRESZCZENIE

Opracowano dwa systemy regeneracji roślin grochu (*Pisum sativum* L.) z niedojrzałych liścieni poprzez embriogenezę i organogenezę. Pierwszy jest systemem bezpośredniego namnażania pędów z bazalnej, skaleczonej części liścienia. Zdolność do namnażania pędów dla sześciu badanych odmian była bardzo wysoka. Procentowy udział regenerujących liścieni po siedmiu tygodniach kultury wahał się od 73 do 86 a średnia liczba pędów od 3,5 do 9,9. Następnym systemem była somatyczna embriogeneza. Inicjacja zarodków somatycznych z jednoczesnym słabym namnażaniem kalusa zachodziła w tej samej części liścienia. Otrzymano ją tylko u trzech z sześciu testowanych odmian. Różnice zdolności do formowania zarodków somatycznych sięgały od 43% regenerujących eksplantatów u odmiany Heiga do 6% u odmiany Cud Ameryki. Średnia liczba zarodków wahała się od 4,2 do 2,3.

SOWA KLUCZOWE: organogeneza, somatyczna embriogeneza, groch, *Pisum sativum* L., regeneracja in vitro.