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Preliminary results of in vitro culture of pea and lupin embryos for the reduction of generation cycles in single seed descent technique

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Abstract

The aim of the studies was to establish in vitro conditions for the culture of pea and lupin embryos as the first step in the development of an in vitro assisted single seed descent technique for the attainment of homozygous populations. Materials for the study included of pea, and narrow-leafed and yellow lupin cultivars. Embryos dissected from mature but still-green seeds were cultured in vitro on two modified MS media and under three temperature regimes. Shoot and root lengths of regenerated plants were measured after 7, 14 and 21 days of culture. For pea plants full-strength MS medium with 4 g l^{-1} agar and temperature 22/ 20°C (day/night) appeared to be the most conducive to shoot and root development, whereas for lupin plants lower temperatures were more propitious: 12°C in the dark for narrow-leafed lupin and 16/ 12°C (day/night) for yellow lupin. Almost all the cultured embryos developed into plants, but not all the regenerated plants survived acclimation to ex vitro conditions.

Keywords: *Lupinus angustifolius*, *Lupinus luteus*, *Pisum sativum*, embryo culture

Introduction

Pea and lupin plants have been potentially rich sources of protein for human and animal diets. Currently, in Poland the main source of protein is soya cake, imported to Poland in amount of 2 mln tons per year. For substitution of soya with domestic protein plants breeding methods for higher and stable yield of grain legumes should be developed. About 12 generations are needed to develop a new cultivar and under central or north European climate conditions only one generation per year is feasible in the field. For that reason the development of new tools is needed to accelerate obtaining homozygosity in breeding of pea and lupins. Homozygosity can be attained by selfing successive generations or by hapoidization of hybrids using different in vitro techniques and doubling chromosomes in haploid plants (for review see, e.g., [1–3]). *Pisum* and *Lupinus* species are known to be recalcitrant to in vitro culture [4–7]. In spite of numerous studies focused on the production of doubled haploids (DH), no important results have been noted to date [8–11]. For shortening the breeding cycle, the single seed descent technique (SSD) in combination with in vitro culture of immature embryos may be an alternative to the DH system. Currently, SSD populations are frequently used as alternatives to DH populations in genetic and genomic studies (see, for example, [12,13]). The SSD technique connected with embryo in vitro culture allows the attainment of homozygous lines in a relatively short time. Such an approach applied to the development of winter barley SSD lines enabled the shortening of the generation cycle to ca. 4 months (including verbalization) [14]. Ochatt et al. [15] proposed the reduction of pea generation cycles based on in vitro culture of embryos and a greenhouse strategy, but no information was found on embryo culture for accelerating breeding process in lupins.

The aim of the present studies was to establish in vitro conditions for the culture of pea, and narrow-leafed and yellow lupin embryos as the first step in research aimed at shortening generation cycles in the SSD technique used to accelerate the development of homozygous populations.

Material and methods

Material for the studies consisted of two pea cultivars (*Pisum sativum* L.) – Cysterski and Akord, two narrow-leafed lupin cultivars (*Lupinus angustifolius* L.) – Kadryl and Sonet, and two yellow lupin cultivars (*L. luteus* L.) – Mister and Taper. Pea and lupin cultivar seeds were sown in the experimental field at Wiatrowo near Poznań. Pods from each pea and lupin cultivar were detached 21–28 days after flowering, when seeds were still-green but fully developed. Seeds taken out of pods were sterilized by successive dips into ethanol 70% (3 min) and

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Javel solution containing 1.5% active chlorine (5 min), and next rinsed three times with sterilized water.

For each pea and lupin cultivar 252 mature embryos were cultured in six treatments: two media and three temperature regimes, each in 3 replications. Dissected embryos were cultured on MS medium [16] with some modifications: MS-I that contained full-strength MS compounds and 4 g l^{-1} agar (pH 5.7), and MS-II that contained half-strength MS compounds and agar 6 g l^{-1} (pH 6.0). Three temperature conditions were used for culture: K-I – 22/ 20°C (day/night), 16/ 8 h photoperiod; K-II – 16/ 12°C (day/night), 16/ 8 h photoperiod for 2 weeks, then 22/ 20°C (day/night) and 16/ 8 h photoperiod; K-III – 12°C in the dark for 2 weeks, then 22/ 20°C (day/night), 16/ 8 h photoperiod. Embryos were cultured in tubes (2 embryos in one tube).

After 7, 14 and 21 days of culture shoot and root length were measured. Plants 3–5 cm in height with well-developed roots were transferred ex vitro into pots and they continued their growth in a greenhouse.

Statistics

The data for shoot and root length of pea and lupin plants were statistically processed using 4-way analysis of variance. For each species culture duration (term of measurement), medium, temperature regime and cultivars, as well as corresponding interactions were sources of variation. The Newman–Keuls method was used for testing differences between mean values [17].

Tab. 1 Mean shoot and root length of *P. sativum* (mm) plants developed from embryos cultured in vitro on different media and temperature conditions after 7, 14 and 21 days of culture.

Cultivar	Medium and temperature combination	7 days		14 days		21 days	
		Shoot	Root	Shoot	Root	Shoot	Root
Cysterski	MSI, K-I	4.08bc	44.2f	11.9d	66.7g	24.9e	83.5f
	MSI, K-II	3.1ab	25.7d	3.4a	47.8f	11.0ab	88.9f
	MSI, K-III	2.6ab	14.0b	3.8ab	30.9e	8.8a	71.7e
	MSII, K-I	7.9c	14.8bc	16.3e	26.01cb	38.3g	50.8d
	MSII, K-II	3.6bc	12.8b	9.3cd	20.1b	21.1d	45.1c
	MSII, K-III	1.9ab	7.1a	8.5c	11.8a	16.8c	22.4b
Akord	MSI, K-I	2.7ab	28.5d	6.0b	44.6f	19.2d	86.9f
	MSI, K-II	2.2ab	15.7bc	2.5a	23.8b	11.4b	71.1e
	MSI, K-III	1.5ab	6.9a	2.3a	28.4c	9.8ab	73.3e
	MSII, K-I	5.7c	34.2e	14.8e	45.2f	27.7f	71.4e
	MSII, K-II	1.8ab	19.1c	6.0b	31.4e	15.6c	44.9c
	MSII, K-III	1.0a	2.7a	2.6a	12.4a	11.7b	16.5a

Within columns means followed by the same letter are not significantly different at $P = 0.05$.

Tab. 2 Mean shoot and root length of *L. angustifolius* plants (mm) developed from embryos cultured in vitro on different media and temperature conditions after 7, 14 and 21 days of culture.

Cultivar	Medium and temperature combination	7 days		14 days		21 days	
		Shoot	Root	Shoot	Root	Shoot	Root
Kadryl	MSI, K-I	4.5	4.8ab	6.8ab	11.1ab	12.0bc	17.9b
	MSI, K-II	8.3a	12.4b	8.3ab	16.4b	16.5c	36.3c
	MSI, K-III	7.0a	18.1bc	7.1ab	26.8c	26.3d	57.1e
	MSII, K-I	3.6a	0.8a	4.1a	2.6a	3.6a	5.6a
	MSII, K-II	5.9a	10.8b	6.6ab	17.1b	9.4b	44.1cd
	MSII, K-III	8.6a	13.1b	9.7b	22.6b	22.1d	42.4cd
Sonet	MSI, K-I	8.3a	0.8a	10.6b	2.2a	12.5bc	3.0a
	MSI, K-II	15.7b	18.9bc	22.5c	32.0cd	36.0e	48.6de
	MSI, K-III	18.4b	38.9d	32.7d	66.4e	64.1g	90.0f
	MSII, K-I	5.6a	2.0ab	5.2ab	3.0a	10.1b	4.7a
	MSII, K-II	15.3b	10.5ab	21.4c	14.7b	32.3e	17.2b
	MSII, K-III	18.0b	24.2c	25.3c	36.9d	41.9f	46.4

Within columns means followed by the same letter are not significantly different at $P = 0.05$.

Results

Mean values for shoots and roots length of pea and lupins measured after one, two and three weeks of culture duration depending on media and temperature regimes are presented in Tab. 1–Tab. 3, and summarized results are given in Tab. 4. Analysis of variance revealed the significant influence of all the applied factors on plant development, i.e. medium, temperature and genotype (Tab. 5). Highly significant differences in the length of roots and shoots in successive weeks of culture indicate a relatively rapid rate of plant development, but some differences may be seen between species (Fig. 1a,b). In pea, roots developed faster than shoots and after 3 weeks of culture the mean length of roots was about 6 cm, with the range from 1.65 cm for cv. Akord on MS-II – K-III to 8.89 cm for cv. Cysterski on MS-I – K-II, whereas mean length of shoots it was only ca. 2 cm with the range from 0.98 cm (cv. Akord on MS-I – K-III) to 3.83 cm (cv. Cysterski on MS-II – K-I; Tab. 1). In the case of lupins, the development rate of shoots and roots was similar, especially in yellow lupins. After 21 days of culture the mean length of shoots of both lupin species was similar (about 2.3 cm), but roots were slightly longer in narrow-leafed lupin than in yellow ones (Tab. 4).

MS-I medium containing full-strength MS and a reduced amount of agar appeared to be more propitious to lupin than to pea plant development, especially narrow-leafed lupins. In peas, plants cultured on MS-I developed longer roots than on MS-II, but on MS-II medium the development of shoots and roots was more balanced.

The studied species differed in their reaction to temperature regimes during in vitro culture. For pea plants the K-I combination, i.e. 22/ 20°C (day/night), was most propitious for shoot and root development. Lupin plants generally grew better in lower temperatures; the narrow-leafed lupin was characterized by the best development at the lowest temperature (12°C in the dark for the first 2 weeks), whereas yellow lupin plants developed better in the K-II combination, i.e. 16/ 12°C (day/night) for first 2 weeks, although in the case of yellow lupins differences between K-II and K-III temperature regimes were not so high (Tab. 2, Tab. 3).

Tab. 3 Mean shoot and root length of *L. luteus* plants (mm) developed from embryos cultured in vitro on different media and temperature conditions after 7, 14 and 21 days of culture.

Cultivar	Medium and temperature combination	7 days		14 days		21 days	
		Shoot	Root	Shoot	Root	Shoot	Root
Taper	MSI, K-I	9.6ab	12.2ab	11.3ab	20.1b	31.1d	29.2bc
	MSI, K-II	9.3ab	17.0b	33.0c	32.8c	42.1e	54.2d
	MSI, K-III	5.4ab	11.8ab	18.8b	30.1bc	24.4c	35.8c
	MSII, K-I	3.8ab	14.7ab	10.2a	23.0bc	21.4bc	29.1bc
	MSII, K-II	10.0b	14.7ab	31.3c	27.3bc	45.8e	44.5cd
	MSII, K-III	8.3ab	9.5ab	23.9b	19.6b	24.8cd	22.0b
Mister	MSI, K-I	8.3ab	6.4ab	11.1ab	14.1ab	17.6b	20.8b
	MSI, K-II	5.0ab	5.5a	5.1a	7.9a	12.6ab	8.4a
	MSI, K-III	3.2a	6.7ab	10.2a	15.1ab	15.4ab	21.9b
	MSII, K-I	4.4ab	6.7ab	6.7a	11.6ab	9.2a	12.4ab
	MSII, K-II	5.1ab	8.7ab	6.6a	11.4ab	14.3ab	13.5ab
	MSII, K-III	7.2ab	5.5a	17.4b	14.6ab	20.6bc	22.6b

Within columns means followed by the same letter are not significantly different at $P = 0.05$.

Tab. 4 Shoot and root length of pea and lupin plants (mm) developed after 21 days of in in vitro culture – mean values for media, temperature conditions and cultivars.

Factor		*P. sativum*		*L. angustifolius*		*L. luteus*	
		Shoot length	Root length	Shoot length	Root length	Shoot length	Root length
Medium	MS-I	14.2a	79.2b	27.9b	42.1,ba	23.9a	28.4a
	MS-II	21.9b	41.8a	19.9a	26.7a	22.7a	24.0a
Temperature	K-I	27.5b	73.1c	9.5a	7.7a	19.8a	22.9a
	K-II	14.8a	62.5b	23.5b	36.5b	28.7b	30.1b
	K-III	11.8a	46.0a	38.6c	59.0c	21.3a	25.6a
Cultivar	1	20.1b	60.4a	15.0a	33.9a	31.6a	35.8b
	2	15.9a	60.7a	32.8b	35.0a	14.9b	16.6a

Within columns means followed by the same letter are not significantly different at $P = 0.05$.

Tab. 5 *F*-statistics from four-way analysis of variance for shoot and root length of pea and lupin plants (mm) cultured in vitro.

Source of variation	DF	*P. sativum*		*L. angustifolius*		*L. luteus*		$F_{0.05}$	$F_{0.01}$
		Shoot length	Root length	Shoot length	Root length	Shoot length	Root length		
Term (A)	2	**258.94**	**230.99**	**64.62**	**90.50**	**87.64**	**100.66**	3.12	4.91
Medium (B)	1	**64.11**	**139.12**	**13.07**	**47.70**	0.02	**5.19**	3.97	7.00
A × B	2	**12.50**	**26.23**	**3.59**	2.75	0.43	1.87	3.12	4.91
Temperature (C)	2	**101.11**	**70.86**	**121.08**	**217.90**	**12.53**	**5.64**	3.12	4.91
A × C	4	**16.54**	0.20	**25.53**	**12.14**	**3.77**	1.81	2.50	3.59
B × C	2	**6.28**	1.93	0.87	**13.92**	**7.77**	1.74	3.12	4.91
A × B × C	4	0.20	**3.10**	2.04	2.02	0.60	0.17	2.50	3.59
Genotype (D)	1	**28.58**	0.52	**160.52**	**19.74**	**99.99**	**192.13**	3.97	7.00
A × D	2	2.33	1.00	**6.04**	2.64	**17.19**	**14.83**	3.12	4.91
B × D	1	3.38	**31.66**	0.69	**12.17**	0.20	**5.37**	3.97	7.00
C × D	2	2.19	3.08	**39.99**	**28.83**	**24.56**	**26.34**	3.12	4.91
A × B × D	2	2.29	1.28	0.25	1.18	0.01	0.74	3.12	4.91
A × C × D	4	1.20	1.21	**6.75**	1.33	**4.60**	**7.78**	2.50	3.59
B × C × D	2	0.75	6.39	**5.40**	**19.16**	0.18	**6.70**	3.12	4.91
A × B × C × D	4	0.63	0.52	2.21	2.26	0.24	0.86	2.50	3.59
Error	72								

Bold characters show significant variation.

For all the studied species significant differences between cultivars were observed, with the exception being root length in peas. In addition, genotype × medium interaction was revealed for the root length of all the studied species, and genotype × temperature interaction for shoot and root length in lupins. Both cultivars of narrow-leafed lupin cultured under K-III temperature regime (12°C) developed significantly longer shoots and roots than in K-II and K-I treatments (Tab. 2). In the case of yellow lupine the dependence of plant development on temperature regimes was not so clear because of significant genotype × temperature × medium interaction (Tab. 5) – for cultivar Taper the most conductive was K-II treatment (16/12°C) regardless of medium, whereas for Mister K-III (12°C) on MS-II medium (Tab. 3).

Almost all the cultured embryos (90–100%) developed into plants. Germination rates of pea and lupin cultured embryos were close to 90% within 2–5 days of culture. After about 3–4 weeks of culture (Fig. 2–Fig. 4) 100 plants of each species were transferred ex vitro into 4 l pots filled with peat substrate and put in a greenhouse. In spite of good root development, not all the potted plants continued their growth – about 10% of pea and 30% of lupin plants did not survive acclimation to ex vitro conditions. Moreover, during the first 3 weeks the potted plants grew slowly, especially pea plants, and they started to flower 1.5–2.5 months after the beginning of in vitro embryo culture. Pea and lupin plants that survived transfer to ex vitro conditions set at least 2 pods with 1–3 seeds.

Discussion

The present results demonstrate that pea and lupin embryos cultured in vitro develop in plants, which are ready for transfer into pots after ca. 4 weeks. Embryos were dissected from green but fully developed seeds and such approach resulted in rapid germination of cultured embryos.

In the genus *Lupinus* some experiments were performed to establish requirements for in vitro vegetative propagation and embryo rescue culture and they were focused mainly on kind of media and their chemical components, especially growth hormones [18–21]. The present studies were aimed on establishing medium and physical conditions for pea and lupin

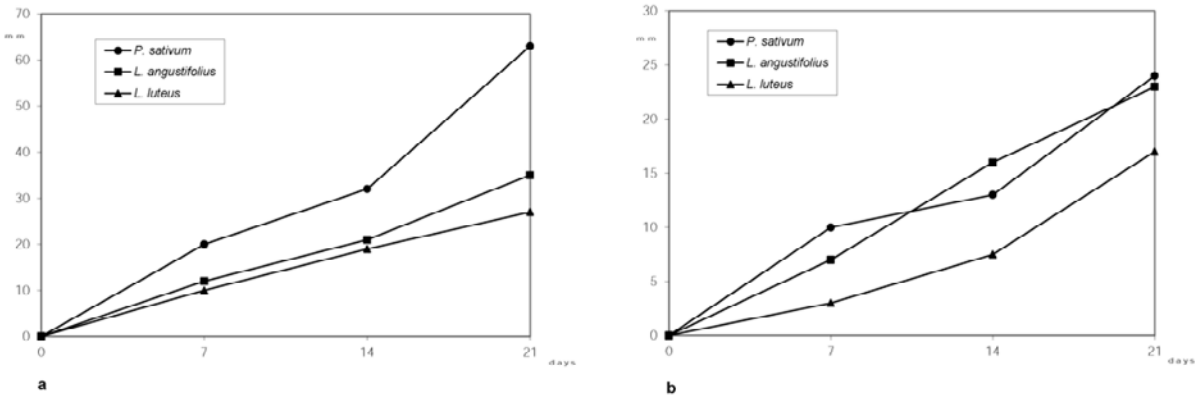

Fig. 1 Increase of shoots (**a**) and roots (**b**) of pea and lupin plants during in vitro culture (averaged over cultivars, media and temperature conditions).

Fig. 2 Embryo-regenerated pea plants cv. Cysterski after 24 days of culture before transfer ex vitro into pots.

Fig. 3 Embryo-regenerated yellow lupin plants cv. Taper after 28 days of culture before transfer ex vitro into pots.

embryo culture. It was found that the rate of plant development was dependent on the medium and – in a higher degree – on temperature regime during in vitro culture. For pea plants full-strength MS medium with reduced agar and a temperature of 22/ 20°C (day/night) appeared to be the most conducive to shoot and root development, whereas for lupin plants lower temperatures were more propitious; plants of the narrow-leafed lupin developed markedly faster at a low temperature (12°C) in the dark, but development of yellow lupin plants at a temperature of 16/ 12°C and 16/ 8 h photoperiod (day/night) was somewhat better than at 12°C in the dark. Stafford and Davies [22], who cultured in vitro immature pea embryos on MS medium, showed that growth of embryos was dependent on sucrose concentrations. They observed a substantial increase in embryo weight in medium with 10–18% sucrose in comparison to 4% concentration. In our studies concentration of sucrose was differentiated in a low degree: 3% in MS-I and 1.5% in MS-II, and substantial for plant development seems to be the amount of agar. In MS-I the amount of agar was reduced from 8 in the original MS [16] to 4 g l^{-1} and this resulted in better development of roots of all the studied species than on original MS medium, what was observed in our earlier studies (data not shown). Ochatt et al. [15] performed excellent studies on in vitro culture of pea embryos in the context of integrating it with the SSD technique. They obtained the best results culturing pea embryos on medium containing half-strength MS macro-elements, full strength MS micro-elements, 15 g l^{-1} sucrose and 6 g l^{-1} agar and at the temperature of 24/ 22°C and 16/ 8 h photoperiod (day/night). In our studies MS-I medium containing full-strength MS compounds and reduced agar appeared to be propitious both for pea and lupins – lupin plants developed longer shoots in comparison to pea and while their roots were about two times shorter than those in peas, development of the whole plants was generally better balanced, i.e. shoots and roots were similar in length, in contrast to pea plants, where roots were several times longer than shoots.

In the present studies genotype × medium and genotype × temperature interaction was revealed, which indicates that we can expect different plant development rates of grain legume cultivars depending on medium and temperature regime. Kasten and Kunert [20] also reported that response of *L. luteus* immature embryos cultured in vitro varied depending on cultivar and medium used. Further experiment should be performed to examine the share of genotype × culture condition interaction and its influence on in vitro development of grain legume plants. Because not all the regenerated lupin plants survive acclimation to ex vitro conditions, soil substrate, fertilization and temperature regimes in a greenhouse should be modified to be more propitious for plant growth.

In the present experiments, which were performed in the summer season, pea and lupin plants started to flower 1.5–2.5 months after the beginning of in vitro embryo culture, but – as it was observed in our earlier studies [23] – in autumn and winter this period may be lengthened to 3–3.5 months.

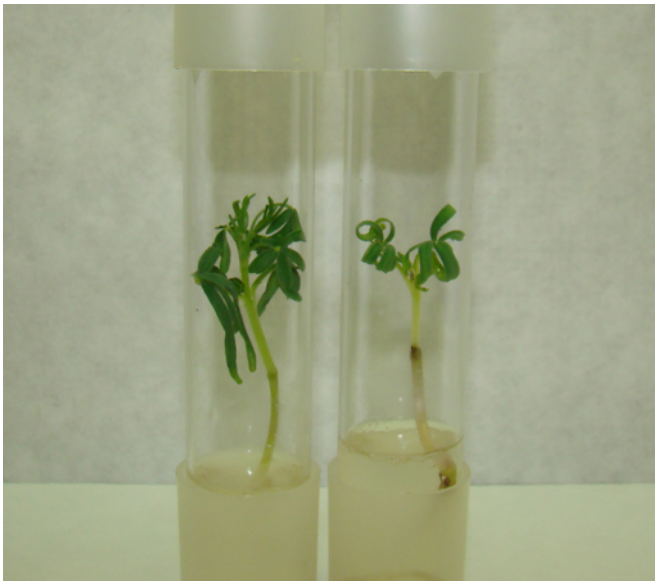

Fig. 4 Embryo-regenerated narrow-leafed lupin plants cv. Sonet after 28 days of culture before transfer ex vitro into pots.

Embryo-regenerated pea and lupin plants that survived transfer to ex vitro conditions set at least 2 pods with 1–3 seeds each – that is enough in SSD technique, in which only one seed per plant is required. These results indicate that in vitro culture of lupin and pea embryos can be applied for attainment of successive generations in the single seed descent technique.

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Authors' contributions

The following declarations about authors' contributions to the research have been made: research designing, writing the manuscript, conducting field and greenhouse experiments, conducting in vitro experiments, statistical calculations: MS, TA, WŚ, PB, ZK, AK, KK, KM, PO.

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