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In vitro culture of white lupin embryos as a stage for SSD technique

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Abstract. White lupin is an important source of proteins, the content of which amounts to 36–40% in its seeds. Breeding of new varieties usually takes several years. The present study aimed to shorten the breeding cycle by applying the single-seed descent technique together with *in vitro* culture of embryos dissected from immature seeds. The effects of temperature on embryo development were studied in indeterminate vs. determinate varieties in *in vitro* culture using the following temperature regimes: (1) 8 °C; (2) 16 °C in the first week, then 8 °C; (3) 20 °C in the first week, then 20/18 °C; and (4) 16 °C throughout the culture period. The embryos were cultured on the standard Murashige and Skoog medium. The observed growth rate of plantlets as well as the survival of plants *ex vitro* showed that at lower temperatures (8–16 °C) the plants developed slowly, but their *ex vitro* survival rate was higher compared to those grown at a higher temperature (20 °C). In addition, the results indicated that the *in vitro* embryo development rate of determinate varieties is significantly lower than that of indeterminate varieties.

Keywords: embryo development, single seed descent technique, survival rate, temperature regimes, type of growth, white lupin

INTRODUCTION

Grain legumes are important crops in Central and Northern Europe because they are a great source of fat, protein, and carbohydrates. White lupin has high protein content in seeds, varying from 36% to 40% based on genotype and location, and hence is considered a valuable feed ingredient (Annicchiarico et al., 2010; Frankowski et al., 2015; Huyghe, 1997; Lucas et al., 2015). It is predominantly self-pollinating – outcrossing occurs in 5–10% of this species, depending on the environment. In the breeding of new varieties, the primary aim of selection is the development of homozygous lines, mainly by selfing in subsequent generations (Berger et al., 2012; Williams, 1987). This procedure is usually applied under field conditions, which allows obtaining only one generation per year in the temperate climate. However, the breeding cycle can be accelerated by shortening the generation time. Watson et al. (2018) proposed "speed breeding" methods, which enable achieving up to six generations per year. It is difficult to shorten the breeding cycle by using the doubled haploid (DH) system, which has been attempted, for example, in cereals (Adamski et al., 2014; Forster, Powell, 1997; Hu, 1997; Kaczmarek et al., 1999) because *Lupinus* spp. are known to be recalcitrant to *in vitro* culture (Kozak et al., 2012; Ochatt et al., 2002; Ribalta et al., 2017; Skrzypek et al., 2008; Wędzony et al., 2009). The single-seed descent (SSD) technique (Goulden, 1941) may be used as an alternative to the abovementioned DH system. The SSD approach was successfully used, among others, to study the resistance of cereals to abiotic stress (Janiak et al., 2019; Ogrodowicz et al., 2017; Wyka et al., 2019). It has also been employed in other species (Snape, Riggs, 1975). The SSD technique involves the random selection of one seed from an individual plant in each generation (e.g., from F_2 to F_6, or a more advanced generation). In the target generation, the seeds that were selected from each plant are harvested separately and used as an SSD line. The SSD approach can be modified by dissecting embryos from immature seeds and culturing them *in vitro*. This significantly reduces the duration of one generation and consequently allows obtaining more than one generation per year (Surma et al., 2013).

The present study was performed to establish the *in vitro* conditions for culturing white lupin embryos as the

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first step in the experiment aimed at shortening generation time in the development of white lupin homozygous lines. The influence of temperature on embryo development was evaluated by applying different temperature treatments. Additionally, the differences in growth rate between the indeterminate vs. determinate genotypes were investigated.

MATERIALS AND METHODS

Plant material and experiments

The study tested 10 white lupin (*Lupinus albus* L.) genotypes – two characterized by the normal type of growth (cultivar Butan and N-BAC accession) and eight showing self-completing (restricted branching) type of growth (cultivar Boros and accessions no. 95144, 95177, 95179, 95180, 95181, 95442, and 95673). The seeds of all these genotypes were obtained from the National Lupinus Cat Wiatrowo.

Two experiments were conducted in the study, in which the first one was aimed at establishing the optimal temperature conditions for the embryo *in vitro* culture. Butan and Boros cultivars were used in this experiment.

The second experiment was performed to verify whether the development of plantlets under *in vitro* culture can be attributed to the type of growth. In this experiment, besides Butan and Boros cultivars, accessions N-BAC, 95144, 95177, 95179, 95180, 95181, 95442, and 95673 were examined.

Donor plants were grown under greenhouse conditions in pots filled with a mixture of peat substrate (Klasmann-Deilmann TS1) and sand in a ratio of 3:1. The seeds were sown several times a year, starting at the end of February, to ensure that the material was still available for the *in vitro* embryo culture.

Seed preparation

Immature seeds were collected from the donor plants at stage 80 on the BBCH scale. Before embryo dissection, all the seeds were sterilized by successively dipping them into 70% ethanol solution (3 min) and Javel solution containing 1.5% active chlorine (5 min), followed by rinsing three times with sterilized water. The embryos were excised from the seeds – seed coat and cotyledons were removed. Then, they were transferred to Murashige and Skoog (MS) medium (Murashige, Skoog, 1962) containing macronutrients, micronutrients, and vitamins of the original classic formulation (Sigma Aldrich, cat. no. M5519) supplemented with 3-indoleacetic acid 1.0 mg L^{-1} (Sigma- Aldrich, cat. no. I2886), kinetin crystalline 0.1 mg L^{-1} (Sigma-Aldrich, cat. no. K3378), sucrose 30 g L^{-1} (SERVA, cat. no. 35580), and agar 4 g L^{-1} (SERVA, cat. no. 11396), which was adjusted to pH 6.5. The embryos were cultured in tubes (Ø25 × 150 mm; two embryos per tube). After transplanting, the plants were grown in a greenhouse.

In vitro temperature regimes

In the first experiment, which was carried out for establishing the optimal physical conditions of embryo culture, the embryos of Boros and Butan cultivars were cultured in a phytotron. Four temperature treatments were applied as follows: K1 – 8 °C; K2 – in the first week 16 °C, then 8 °C; K3 – in the first week 20 °C, then 20/18 °C (light/dark); K4 – 16 °C throughout the culture period. In each treatment, the culture was conducted in dark in the first week and in the next weeks a photoperiod of 8 h (dark) and 16 h (light with 234 μmol m^{-2} s^{-1} PAR irradiance Apollo 8 LED Grow Light) was maintained. Low-temperature treatments were applied in the *in vitro* embryo culture taking into account the requirements of this species grown under field conditions and the results of our earlier experiments with lupins (Surma et al., 2013).

In the second experiment, the embryos of the examined cultivars and the accessions were cultured on the same MS medium as in the first experiment, but only the K4 temperature treatment was applied (16 °C throughout the culture period).

Both experiments were performed in three replications. In the first experiment 240 embryos dissected from Boros and Butan cultivars were cultured: in each replication 10 tubes each with 2 embryos were assigned for each genotype and each temperature treatment. In the second experiment 60 embryos of each examined genotype were cultured – each replication covered 10 tubes, each with 2 embryos of each genotype.

After 7, 14, and 21 days of culture, the shoot and root formation was observed and quantified by measuring the shoot and root lengths. Plants with a shoot length of approximately 5 cm and developed roots were transferred *ex vitro* to pots filled with a mixture of peat substrate (Klasmann-Deilmann TS1) and sand in a ratio of 3:1. These plants were grown further in a greenhouse. All the developed plants of each variety and subjected to each treatment were planted in the pots.

After 30 days of planting plant survival was observed and estimated as a ratio of the number of plants growing to the number of all plants planted.

Statistical analysis

For the data collected in the first experiment, a three-way analysis of variance (ANOVA) was used to assess the effects of genotype, temperature treatment, and the term of observation, and the interaction effects for the shoot and root lengths of cvs. Boros and Butan which were measured three times during the *in vitro* culture. Pairwise contrasts between the treatments were assessed using Tukey's test.

For the data obtained in the second experiment, ANOVA was performed, and the mean values and confidence intervals of the observed traits were estimated.

Plant survival was excluded from statistical calculations because observations were made without replications for this trait.

Table 1. F statistic from ANOVA analysis of variance for shoot and root length of Boros and Butan plantlets measured in 7, 14 and 21 days of *in vitro* culture (experiment 1).

Source of variation	DF	Stat. F		$F_{0.05}$	$F_{0.01}$
		shoot	root		
Variety	1	580.38	520.51	4.40	7.20
Treatment	3	73.10	36.77	2.80	4.22
Variety × treatment	3	43.02	36.67	2.80	4.22
Term	2	48.36	26.04	3.19	5.08
Variety × term	2	15.88	7.42	3.19	5.08
Treatment × term	6	7.90	0.95	2.29	3.20
Variety × treatment × term	6	6.34	1.33	2.29	3.20

Table 2. Mean length [mm] of shoots and roots of Boros and Butan plantlets after 3 weeks of embryo in vitro culture under different temperature regimes (K1–K4), and ex vitro survival of plants after 30 days of planting (experiment 1).

Cultivar	Trait	K1	K2	K3	K4	Mean
Butan	shoot length	9.38	26.07	36.86	38.66	27.74
	root length	13.61	18.75	34.18	30.00	24,13
Ex vitro survival [%]		58.3	75.0	41.7	66.7	60.42
Boros	shoot length	3.59	7.48	7.28	8.47	6.71
	root length	7.26	5.79	8.45	5.38	6.72
Ex vitro survival [%]		50.0	33.3	27.8	10.0	30.27

Table 3. Results of testing the differences between temperature treatments – averaged for Boros and Butan cultivars (experiment 1).

Contrast	Shoot		Root	
	score	F statistic	score	F statistic
K1 vs. K2	-6.22	50.89	-2.16	5.64
K1 vs. K3	-11.05	160.94	-9.12	99.93
K1 vs. K4	-11.15	163.83	-4.57	25.16
K2 vs. K3	-4.84	30.83	-6.95	58.09
K2 vs. K4	-4.94	32.10	-2.41	6.97
K3 vs. K4	-0.10	0.01	4.54	24.81

$F_{0.05} = 4.04$; $F_{0.01} = 7.20$

RESULTS

The ANOVA performed for the shoot and root lengths of Boros and Butan plantlets measured on 7, 14, and 21 days of *in vitro* culture revealed the significant effects of all the variation sources (exceptions were treatment × term and variety × treatment × term interactions for root length) (Table 1).

Table 2 presents the shoot and root lengths of both cultivars measured after 21 days of the *in vitro* culture. The embryo development of Butan cultivar examined in the first experiment was found to be better under higher temperature (K3, K4) than low temperature (K1, K2), whereas in the case of cv. Boros differences between temperature treatments in shoot and root lengths were not so important.

The differences between the temperature treatments in shoot and root lengths were significant in all the cases, with an exception of the difference in shoot length between K3 and K4 treatments (Table 3).

The lengths of shoots and roots of Boros and Butan plantlets measured during the *in vitro* culture are presented in Fig. 1. It was observed that the plantlets of cv. Boros developed slowly in comparison to cv. Butan.

Plants developed under the K1 and K2 regimes were indeed smaller than those grown under K3 and K4 treatments, but their *ex vitro* survival was higher. In general, the *ex vitro* survival

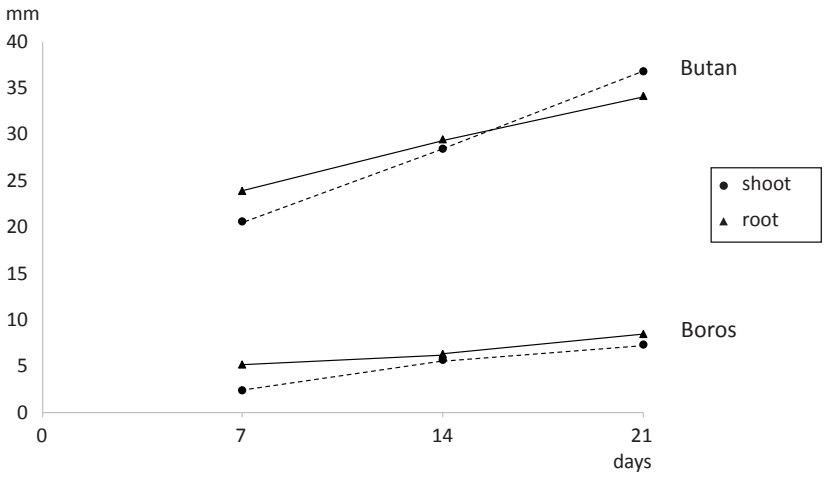

Figure 1. Shoot and root length of cvs. Butan and Boros plantlets during 21 days of *in vitro* culture at K3 temperature treatment (experiment 1).

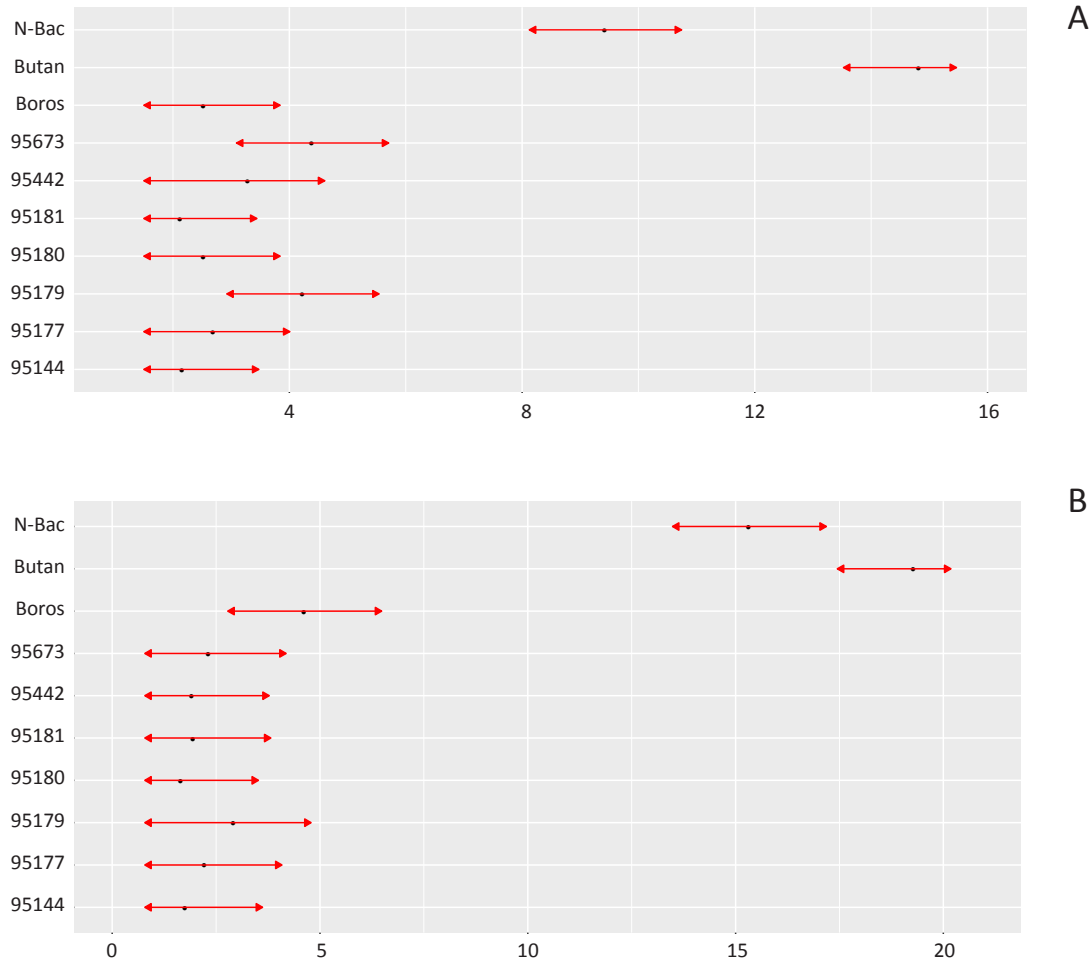

Figure 2. Estimated mean values and confidence intervals of root (A) and shoot (B) length (mm) of white lupin plantlets after 3-weeks of *in vitro* culture. Overlapping red arrows indicate an insignificant difference between the mean values (experiment 2).

of cv. Butan plants was better than that of cv. Boros (cv. Butan: on average 60.42%, cv. Boros: 30.27). All the plants growing in pots were characterized by a low height (about 12–20 cm at the flowering stage) and developed 1–3 pods.

The results of the second experiment conducted with ten genotypes confirmed the slow development of self-completing plantlets in the *in vitro* culture. Two genotypes (N-Bac and Butan) of normal growth type were characterized by significantly longer shoots and roots compared to the remaining seven accessions and cv. Boros (Fig. 2).

DISCUSSION

The plant material used in the two experiments was Boros and Butan, which are the only cultivars of white lupin registered in Poland. Of these, Boros is a determinate cultivar, and due to the lack of branching, its seeds ripen very evenly, with a short growing period. On the other hand, Butan is an indeterminate type – the plants have short side shoots that do not show excessive lushness.

The results of this study revealed significant differences in *in vitro* embryo development between Boros and Butan cultivars. The plantlets of cv. Boros developed significantly slower than those of cv. Butan.

To verify whether this effect can be attributed to the type of growth, an additional experiment was conducted, in which, besides Boros and Butan, eight other white lupin genotypes were tested. The results obtained from this experiment confirmed the earlier observations and revealed the better *in vitro* growth of plantlets of the indeterminate type.

In the present study, embryo culture was carried out on MS medium with the amount of agar reduced to 4 g l^{1} and pH adjusted to 6.5 instead of 5.7–5.8. Due to these modifications, the medium was less solid than the original. In our earlier pilot test, it was observed that such a medium is more conducive to the development of white lupin embryos compared to the original solid medium.

The results of the experiments indicated that in the breeding of white lupin, the *in vitro* embryo culture can

complement the SSD technique. Although the plants growing *ex vitro* in a greenhouse develop only a few pods, one seed from a plant is theoretically sufficient for the SSD method. As not all plants survive *ex vitro*, 2–3 seeds should be taken from each plant to ensure obtaining the next generation by *in vitro* embryo culture, which can finally provide the established number of lines.

In our approach, a full cycle of one generation lasts about 3–4 month, which means that within a year, 3–4 generations of white lupin can be produced, using greenhouse cultivation and *in vitro* culture of embryos taken from immature seeds. The greenhouse conditions were not fully controlled in our experiments. As outlined by Watson et al. (2018), installing additional lighting in a greenhouse (e.g. light-emitting diode) could increase the number of generations obtained per year and significantly shorten the breeding cycle of white lupin.

CONCLUSIONS

1. White lupin embryos that were dissected from immature seeds can be cultured *in vitro* under different temperature regimes.
2. The plantlets develop slowly at lower temperatures, but their *ex vitro* survival rate is higher than that of plantlets cultured at room temperature.
3. Determinate plants show a lower rate of development *in vitro* compared to the indeterminates. Further research is required to explain this phenomenon.

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