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Utilisation of *in vitro* Techniques in Rescue of Gene Resources of Meadow Vetchling (*Lathyrus pratensis* L.)

Lenka KLČOVÁ and Marcela GUBIŠOVÁ

Research Institute of Plant Production, Piešťany, Slovak Republic

Abstract: In the case of poor germination of seed samples and minimal number of seedlings obtained, *in vitro* methods can be used to revitalise and recover the gene resource. The highest germination of meadow vetchling (*Lathyrus pratensis* L.) seeds was achieved after scarification with H_2SO_4 and cultivation in MS medium. The seedlings were used as a material for micropropagation. Regeneration passed through nodal segments cultivated on basal MS medium solidified with a combination of agar and phytagel. This culture medium was also suitable for the plant maintenance. An addition of cytokinin to the induction medium did not support multiplication and growth. In the basal MS medium rooted 72.5% (gene resource 62) or 42.5% (gene resource 28) of shoots. The rooting of gene resource 28 was increased to 63% by the addition of indolylbutyric acid to the culture medium. The regenerated plants were successfully transferred to the soil. This protocol can be used to rescue gene resources of this species.

Keywords: micropropagation; *in vitro* cultivation; seed germination; revitalisation; gene resources; meadow vetchling; *Lathyrus pratensis* L.

During long-term conservation of seed accessions in gene banks, problems related to the reduction of germinating ability are likely to occur. Moreover, some samples obtained at the collecting expeditions may also be characterised by a low germinative ability. *In vitro* techniques however, can improve the germination of such seeds. There are two basic possibilities of *in vitro* revitalisation of seed samples: (1) germination in the culture medium or (2) cultivation of isolated embryos – embryoculture (Dilday *et al*. 1994). To induce *in vitro* germination, the approach mostly used is the addition of gibberellins to the culture media. The main problem of a low germinative ability in some species is the hard seed coat which, however, can be removed by seed scarification (Acharya *et al*. 1999). To recover the gene resource in the case that the number of the seedlings obtained is minimal (low number of seeds, old or badly-stored seeds), it is possible to multiply the plants using *in vitro* cultures (Sharma *et al*. 1996). For the propagation and *in vitro* conservation of species (e.g., in the case of revitalisation of problematic species out of season), simple and efficient regeneration protocols are needed providing a rapid multiplication of plants. In most cases, micropropagation proceeds by apical and axillary shoot meristem proliferation. In the first stage, shoot development in the cytokinin-containing medium is induced and, in the second stage, rooting in the auxin-containing medium is induced. Different species require appropriate optimisation of the culture conditions.

Many reports describe plant regeneration of legume species but we are not aware of any published results on meadow vetchling micropropagation. Franklin *et al*. (1991) reported regeneration of common bean (*Phaseolus vulgaris* L.) in the medium with BA (3.3 mg/l). Rooting was achieved by the addition of NAA and GA_3. In yellow lupine (*Lupinus luteus* L.), Li *et al*. (2000) regenerated shoots by the cultivation of apical meristems in MS media with B5 vitamins and 1 mg/l BA. The most efficient induction in faba bean (*Vicia faba* L.) shoot was the combination of BA and TDZ. Shoots rooted in half-

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strength MS medium or after the addition of IAA (Khalafalla & Hattori 2000). Gulati *et al*. (2001) evaluated the effect of BA concentration on lentil (*Lens culinaris* MEDIK.) propagation (0–4 mg/l). Shoot induction of adzukibean (*Vigna angularis* [Wild.] Ohwi & Ohashi) in the MS medium with B5 vitamins and 1 mg/l BA was described by Avenido and Hattori (2000). Sinha *et al*. (1983) observed regeneration of one from six tested genotypes of grass pea (*Lathyrus sativus* L.) from stem segments. The highest response was achieved in the medium containing 1 mg/l BA, rooting was induced after NAA addition.

The objective of this study was to revitalise seed samples of two meadow vetchling gene resources, and to develop a simple but efficient micropropagation protocol for their recovering.

MATERIAL AND METHODS

The accessions were used of two gene resources (GR) of meadow vetchling (*Lathyrus pratensis* L.) – GR 28 and GR 62, obtained at the collecting expeditions (in Kysuce, Slovakia, 1997). Their germinative ability in the soil was 0%. Seeds were surface-sterilised with 96% ethanol for 1 min followed by 10 min in 0.1% $HgCl_2$ solution supplemented with 2 drops of surfactant Tween 20 per 100 ml. The seeds were then rinsed 5× with sterile distilled water. Because of a high percentage of hard-coated seed (stated after 24 h imbibition in the water), samples were treated with concentrated H_2SO_4 for 15 min. Germinative ability was tested on filter paper or in the basal MS medium (Murashige & Skoog 1962). The number of seeds per treatment varied from 38 to 51. The seeds were left to germinate for 24 days.

For micropropagation, nodal segments and shoot tips (5–10 mm) of *in vitro* germinated seedlings were used. Thirty to forty explants were plated per variant. Explants were inoculated into MS, B5 (Gamborg *et al*. 1968) or MSB (MS salts + B5 vitamins) media. As a source of carbon, 3% sucrose was added. Agar (7 g/l), phytagel (2.5 g/l), or their combination (3.5/1.25 g/l) were used to solidify the media. The effect of benzylaminopurine (BA: 0.5; 1 mg/l) and thidiazuron (TDZ: 0.1, 0.5 mg/l) on the shoot induction was determined. To improve the rooting of shoots, 0.25/1 mg/l of indolylacetic acid (IAA), indolylbutyric acid (IBA) and naftylacetic acid (NAA) was added to the basal MS medium, or the salt concentration was reduced to the half-strength. All the media were adjusted to pH 5.8 and autoclaved at 121°C for 25 min.

Cultures of nodal segments and rooting explants were all incubated at 25/20°C under 16/8 h light-dark cycle (50 μmol/m^2/s). The subcultivation interval lasted 4 weeks. Rooted plantlets were rinsed with herbicide (0.15% Previcure) and transferred to the soil. They were acclimatised to *ex vitro* conditions in the growth chamber at 15/13°C under 16/8 h light-dark cycle for 3 weeks by gradual lowering the humidity. To maintain high humidity during the first week of acclimation, the plantlets were covered with a plastic foil. During the second week, the plantlets were partially uncovered and the next week plants were cultured without covering.

In the experiments, we evaluated the regeneration efficiency (number of nodal segments per plated explant), shoot height (mm), and rooting frequency (%). Data were subjected to the analysis of variance (ANOVA). Differences between means were tested by least significant difference (LSD, $P \leq 0.05$).

RESULTS AND DISCUSSION

Germination

Seeds of meadow vetchling (two GR) did not germinate in the soil. The germination on the moist filter paper ranged from 0 to 10%. In *in vitro* culture on MS medium without growth regulators there germinated 53.8% (GR 28) and 39.7% (GR 62) of seeds. After the scarification procedure (H_2SO_4 for 15 min), seed germination increased to 89.5% (GR 28) and 71.5% (GR 62) on moist filter paper and to 94.1% (GR 28) and 100% (GR 62) in the basal MS medium. Seeds germinated significantly better after scarification and under *in vitro* conditions.

Micropropagation

To micropropagate the revitalised seed samples, nodal segments and shoot tips of the germinated seedlings were used as explants. We added BA (0.5; 1 mg/l) or TDZ (0.1; 0.5 mg/l) to MS medium to improve regeneration. The effect of cytokinins was significantly negative. The explants grew better in the basal medium (Figure 1). The differences between the gene resources were not significant. The responses obtained on TDZ were lower in comparison with BA, although a positive effect of cytokinins (especially TDZ) was described in lentil, pea and chickpea regeneration (Malik &

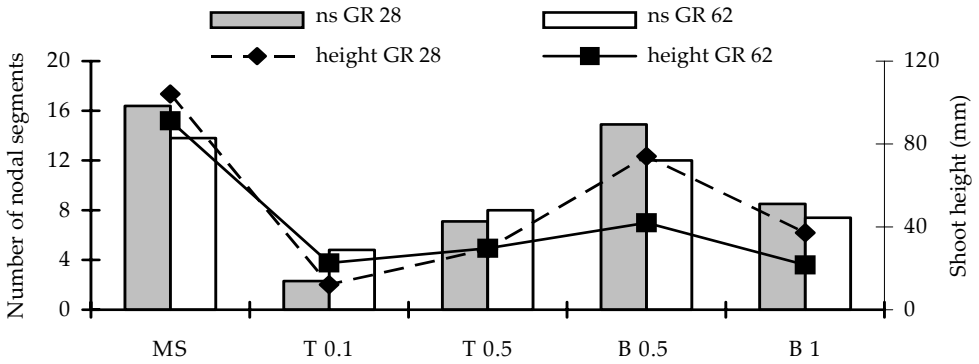

Figure 1. Effect of cytokinins on meadow vetchling propagation from nodal segment explants (MS – basal MS medium, T – thidiazuron 0.1/0.5 mg/l, B – benzylaminopurine 0.5/1 mg/l, GR – gene resource). The height of plants regenerated after 4 weeks and the number of their nodal segments are means of 30 explants

Saxena 1992a,b). BA (1 mg/l) was effective in grass pea regeneration (Sinha *et al*. 1983). The response to the cytokinin treatment varies with the species. Three basal media (MS, B5, MSB) were tested for regeneration. MS medium induced the highest regeneration efficiency (Table 1), we obtained 7.3 and 10.3 nodal segments per explant in GR 28 and 62, respectively, which suggests that the efficiency was genotype-dependent. Medium MS was found to be superior to B5 for the regeneration of peanut (Atreya *et al*. 1984). Genotype-dependent regeneration capacity of grass pea was described by Sinha *et al*. (1983). The results of the use of different gelling agents (agar, phytagel, and the combination of both) indicated advisability of the combination of agar with phytagel. The differences

Table 1. Effect of basal media MS, B5, MSB (MS salts + B5 vitamins) on meadow vetchling regeneration from nodal segments (GR – gene resource) – The height of plants regenerated after 4 weeks and the number of their nodal segments are means of 40 explants

Medium	Number of nodal segments			Shoot height (mm)		
	GR 28		GR 62	GR 28		GR 62
MS	7.3	a	10.3	45.7	a	102.6
MSB	7.0	a	8.6	54.55	a	92.4
B5	6.1	b	7.2	38.95	b	62.1
	6.8 b		8.7 a	46.4 b		85.7 a

Different letters mean significant difference at $P \leq 0.005$

Table 2. Effect of gelling agent on meadow vetchling propagation from nodal segments (GR – gene resource, A – agar, Ph – phytagel) – The height of plants regenerated after 4 weeks and the number of their nodal segments are means of 40 explants

Medium	Number of nodal segments			Shoot height (mm)		
	GR 28		GR 62	GR 28		GR 62
Ph	6.2	a	6.5	29.3	b	28.8
A/Ph	7.8	a	5.3	42.1	a	32.6
A	6.1	a	6.2	28.8	b	26.0
	6.7 a		6.1 a	33.4 a		29.1 a

Different letters mean significant difference at $P \leq 0.005$

were significant only for the shoot height (Table 2). The effect of gelling agents on plant regeneration was reported by Veramendi *et al*. (1997), Nešťáková *et al*. (2000).

Rooting

Root initiation of shoot tip explants occurred in the basal medium in frequency 72.5% (GR 62) and 42.5% (GR 28). Neither the addition of auxin nor the reduction of the salt strength improved rooting of GR 62. In GR 28, the frequency of rooting was increased by IBA addition to the medium, the concentration of 0.25 mg/l was sufficient (Table 3). Sinha *et al*. (1983) used NAA in the concentration of 2 mg/l to induce the rooting of grass pea. In our experiments IBA was superior to NAA. The rooted plantlets were transferred to the soil (Figure 2). Acclimation was successful in 64.3% and 88% in GR 28 and 62, respectively. The acclimatised plants were delivered to the Gene Bank for the recovery of the gene resources.

Table 3. Rooting frequency of meadow vetchling shoots (Percentage of explants rooted after 4 weeks are means of 40 explants)

Medium	Rooting (%)		
	GR 28		GR 62
MS	42.5	a	72.5
½ MS	35.6	c	33.0
MS + IAA	25.8	b, c	59.3
MS + IBA	63.0	a	54.0
MS + NAA	38.8	a, b	55.8
	41.2 b		54.9 a

Different letters mean significant difference at $P \leq 0.005$
½ MS – half-strength MS medium; MS – MS basal medium; GR – gene resource

Figure 2. Meadow vetchling micropropagation from nodal segments (A) and plants after 3 weeks of acclimation (B)

In conclusion, we have developed a simple and efficient revitalisation and micropropagation protocol for meadow vetchling. Seeds after scarification germinate in MS medium, propagation passes through the nodal segments cultivation in the basal MS medium solidified with a combination of agar and phytagel. Rooting may be improved by an addition of IBA to the culture medium. This protocol may be utilised in gene resource recovery, gene resource conservation, and multiplication of the species.

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Abstrakt

Klčová L., Gubišová M. (2003): **Využitie *in vitro* techník pri záchrane genetických zdrojov hrachora lúčneho** (*Lathyrus pratensis* L.). Czech J. Genet. Plant Breed., **39**: 84–88.

Revitalizáciu a obnovu genetického zdroja v prípade nízkej klíčivosti semien a pri minimálnom počte získaných klíčencov je možné zabezpečiť využitím *in vitro* metód. Najlepšia klíčivosť vzoriek genetických zdrojov hrachora lúčneho (*Lathyrus pratensis* L.) bola dosiahnutá po skarifikácii semien kyselinou sírovou a kultiváciou na živnom MS médiu. Klíčence boli použité ako východiskový materiál pre mikropropagáciu. Regenerácia prebiehala kultiváciou nodálnych segmentov na bazálnom MS médiu spevnenom kombináciou agaru a phytagelu. Toto médium bolo tiež vhodné pre udržiavanie rastlín hrachora. Prídavok cytokinínov do indukčného média nepodporil rast a množenie. Na bazálnom MS médiu zakoreňovalo 72,5 % (genetický zdroj 62) a 42,5 % (genetický zdroj 28) výhonkov. Korenenie genetického zdroja 28 bolo zvýšené na 63 % prídavkom kyseliny indolylmaslovej do kultivačného média. Regenerované rastliny boli úspešne aklimatizované na *ex vitro* podmienky. Vypracovaný protokol môže byť využitý pri záchrane genetických zdrojov tohto druhu.

Kľúčové slová: mikropropagácia; *in vitro* kultivácia; klíčenie semien; revitalizácia; genetické zdroje; hrachor lúčny; *Lathyrus pratensis* L.

Corresponding author:

Mgr. Lenka Klčová, Výskumný ústav rastlinnej výroby, Bratislavská cesta 122, 921 68 Piešťany, Slovenská republika
tel.: + 421 337 722 311, fax: + 421 337 726 306, e-mail: klcova_l@vurv.sk