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In vitro multiplication of *Lepidium meyenii* Walp.

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Summary

The multiplication protocol of the medicinal plant *Lepidium meyenii* was established through axillary bud proliferation. Multiplication was initiated on modified MS supplemented with a combination of zeatin (2 μM) and gibberellic acid 3 (0.3 μM). The average number of proliferated shoots was 5.3 per segment. Shoots were rooted on modified MS medium with 9.8 μM of indole-3-butyric acid and 3 μM of α-naphthalene acetic acid. *In vivo* rooting was also successful and 94 % regenerated plantlets were acclimatized.

Introduction

Lepidium meyenii Walp. (2n=8x=64, CHENG et al., 2004) or Maca belongs to the *Brassicaceae* family and it is also known as *L. peruvianum* Chacón (TAILOR, 2005). *L. meyenii* grows at altitudes of 4000 - 4500 m elevation and it is well adapted to poor agricultural soils and cold climate (RUBIO et al., 2007). Maca was used in Peru from before the time of the Incas. Maca has been grown together with potatoes in Andean regions. It is known that *L. meyenii* itself naturally repels most root crop pests (GONZALES et al., 2009) and furthermore, it has the defensive gene *Lm-def* with activity against the pathogen *Phytophthora infestans* responsible for late blight potato and tomato (SOJIS et al., 2007).

Maca is grown above all for its fleshy hypocotyls which are used as a root vegetable and medicinal raw material. The hypocotyls have potential therapeutic value for increasing physical energy, strengthening endurance and for immunostimulation. In folk medicine, Maca hypocotyls are used for alleviating hormonal dysfunction, as an antidepressant and for enhancing wound-healing (DINI et al., 1994). The biological activity of Maca is attributed to its phytosterol content, (DINI et al., 1994), alkaloids (MUHAMMAD et al., 2002; CUI et al., 2003), isothiocyanates (DINI et al., 2002), macaenes and macamides (MUHAMMAD et al., 2002). The biochemical and toxicological activity of Maca has been studied extensively in recent years (VALENTOVÁ et al., 2004; VALENTOVÁ et al., 2006; VALENTOVÁ et al., 2008). Besides the pharmaceutical effects, Maca has significant nutritional value due to its content of proteins, carbohydrates, whole fibre, lipids, vitamins and minerals (DINI et al., 1994). Maca is ingredient in some dietary supplements (VALENTOVÁ, 2006).

The growing conditions for *L. meyenii* however are not favourable in Middle Europe and thus yield and quality remain problematic (LEBEDA et al., 2003) and no hypocotyls are produced at low altitudes (REA, 1994; VALENTOVÁ et al., 2001). Apropos the above, Maca has been becoming a subject for tissue culture, cell engineering and gene transformation; e.g. Maca (*L. meyenii*) has been used for the creation of somatic hybrids with *Brassica oleracea* (RYSCHKA et al., 2003). Many of the biotechnological methods do not manage without skills of *in vitro* multiplication. Micropropagation shoots derived from calli of *L. meyenii* was described by CHENG et al. (2004) and WANG et al. (2007). This paper describes the multiplication protocol of *L. meyenii* through meristematic axillary buds. This method of multiplication preserves the original materials and produces large numbers of uniform plants.

Materials and methods

Plant material

Experiments were done with seed collection of *Lepidium meyenii* No. 314 morphotype „Raku chupa“. This collection originated from the region Ayacucho (Peru) and was collected in 1999. The genotype was kept by reseeding. Two seed size fractions were used. For the fraction f2 the weight of 1000 seeds was 0.186 g and for the fraction f3 0.416 g.

Seed surface sterilization

The seeds were sterilised in 2% solution of Chloramin BS (Bochemie, CZ) with soaking agent. After 15 minutes, the seeds were washed with sterile distilled water and then dipped in 70% ethanol for 1 minute. The seeds were properly washed with sterile distilled water and were put on the surface of MS medium (MURASHIGE and SKOOG, 1962).

In vitro plant cultivation

In vitro seed cultivation was carried out in a culture chamber with a 16 h photoperiod (light conditions: fluorescent tubes, daylight, 60 μmol m^{-2} s^{-1} and 15 μmol m^{-2} s^{-1}), at 22 °C for about 2 months without subcultivation.

The seedlings at the stage of minimally 5 true leaves were the source of the material for initiating the regeneration of dormant axillary buds. The root and growth apex were removed and discarded in these plants. The obtained segments (i.e. epicotyl and shortened stem; size of segments was approximately 5 mm) were placed horizontally on multiplication medium. The shoots (minimally 5 mm) originating from axillary buds were planted to the rooting medium. The total time for the rooting experiment was 51 days.

The young rooted plants were planted in pots (4 cm) in a mixture of peat, earth and sand 3:3:1 (V/V). The plants were acclimatized under controlled conditions with a 16 h photoperiod (light conditions: fluorescent tubes, daylight, 60 μmol m^{-2} s^{-1}), at 22 °C for 3 weeks.

Multiplication media

All media (Z1, Z2, Z+GA, BAP+GA, mT+GA) contained MS basal medium (MURASHIGE and SKOOG, 1962) without NH_4NO_3 with 3 % sucrose (w/v) and 0.7 % agar (w/v) and they were supplemented with different variants of plant growth regulators:

1 μM of zeatin (Z1), 2 μM of zeatin (Z2), 2 μM of zeatin and 0.3 μM of gibberellic acid 3 (Z+GA), 2 μM of benzylaminopurine and 0.3 μM of gibberellic acid 3 (BAP+GA), 2 μM of meta-topolin and 0.3 μM of gibberellic acid 3 (mT+GA).

The growth regulator concentrations and combinations were designed according preliminary experiments published by LEBEDA et al. (2003).

Rooting media

Rooting media (IBA1, IBA2, IBA+NAA) contained MS medium (MURASHIGE and SKOOG, 1962) without NH_4NO_3 supplemented 1.5 % sucrose (w/v) and 0.7 % agar (w/v). These media contained

different variants of plant growth regulators:
12.25 μM of indole-3-butyric acid (IBA1), 24.5 μM of indole-3-butyric acid (IBA2), 9.8 μM of indole-3-butyric acid and 3 μM of α-naphthalene acetic acid (IBA+NAA).

Statistical evaluation

24 segments were put on each multiplication medium (12 segments originated from f2 seed fraction and 12 segments originated from f3 seed fraction). The obtained shoots were equally distributed to three rooting media. The experiment was repeated twice. The statistical evaluation included data from both repetitions.
The influence of seed size fractions on multiplication, the multiplication coefficient of media (the number of shoots from one segment), labour intensity (i.e. average shoot harvesting number), the influence of multiplication medium on rooting, the rooting efficiency of media (RE; calculated as percentage of successful rooting, i.e. percentage of rooting shoots to total number of planted shoots) and average time needed for rooting of shoots were evaluated. The data were statistically evaluated using ANOVA and the Tukey method. All statistical determinations were made at the $P \leq 0.05$ significance level using the statistical programme STATISTICA 7.1 (Statsoft, Inc., CZ).

Fig. 1: Shoot proliferation on segment of *L. meyenii* in multiplication medium Z2 (MS with 2 μM of zeatin) after 32 days. Length of segment was 8 mm.

Results

The germination capacity after surface sterilization was 91 % at fraction f2 and 98 % at fraction f3. The influence of seed size fraction was not statistically significant.
In vitro seed cultivation in low light intensity (15 μmol m^{-2} s^{-1}) led to prolongation of shortened stem and appeared better with regard to easier harvesting of regenerated shoots.
The average number of shoots per segment, labour intensity and the earliest possible time for the shoot harvesting are shown in Tab. 1, Fig. 1.

Tab. 1: Effect of multiplication media on both average number of shoots from one segment of *L. meyenii* and average number of shoot harvestings; "time" means the least period needed to obtain shoots for rooting.

multiplication medium	average number of shoots per segment	average number of shoot harvestings	time (day)
Z1	3.7	2.2	20
Z2	4.1	1.5	10
Z+GA	5.3	2.4	10
BAP+GA	5.1	2	15
mT+GA	5.9	2.3	20

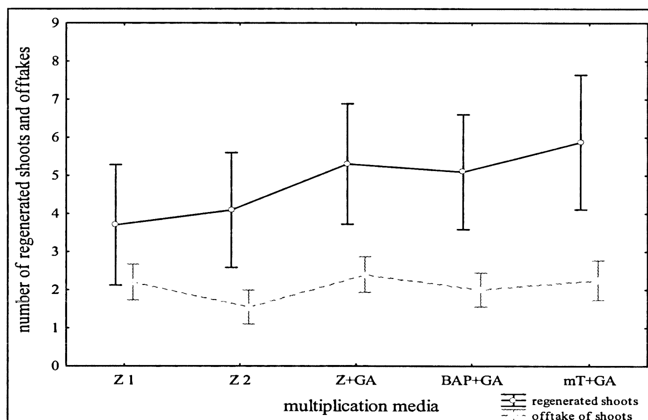

Fig. 2: Graphical visualization of ANOVA analyses – influence of multiplication media (x-axis) on both average number of shoots from one segment of *L. meyenii* and average number of shoot harvestings (y-axis); average number of shoots per segment $F(4.135) = 1.378$, $p = 0.245$; average number of shoot harvestings $F(4.135) = 1.679$, $p = 0.159$. Vertical columns sign 0.95 confidence interval.

Tab. 2: Effect of rooting media on rooting efficiency (RE, percentage of rooted shoots to planted shoots) with regards to origin multiplication media; "time" in days is average period needed for rooting.

multiplication medium	IBA1 RE	time	IBA2 RE	time	IBA+NAA RE	time
Z1	0	51¬	21	35.5	70	24
Z2	0	51¬	23	32	74	23.7
Z+GA	20	27	43	27.5	78	24.7
BAP+GA	0	51¬	14	47.5	73	21.7
mT+GA	0	51¬	50	36	78	22
overview	**3**	**46.2**	**28**	**35.6**	**75***	**23.2***

¬ after 51 days the rooting experiment was finished
* significantly different ($p < 0.05$)

The media with a mixture of growth regulators appeared to be better, the best multiplication coefficient was obtained on medium mT+GA but the shoot initiation from dormant axillary buds was quite time consuming (Tab. 1). Vitrification was observed at regenerated shoots that originated from medium BAP+GA. The medium Z+GA offered a reliable yield of vigorous shoots in an acceptable time. With regards to laboriousness of multiplication, the media Z2 and BAP+GA were less time-consuming. However, none of the multiplication coefficients of tested media or labour intensity was statistically significant (Fig. 2).
The rooting capacity of media and average time needed for rooting of shoots are summarised in Tab. 2. The influence of multiplication medium on rooting was not statistically significant. Most shoots apart from shoots originating from Z+GA did not root in medium IBA1. The rooting efficiency in medium IBA2 (without regard to multiplication media) did not reach 30 %. The medium for rooting IBA+NAA was statistically significantly better than IBA1 and IBA2 in efficiency of rooting and time needed (Fig. 3). The rooting efficiency was 75 % and average time needed for rooting was 23 days in this medium.
The rooted shoots were easy transferred to *ex vitro* conditions and efficiency of acclimatization was 94 % (Fig. 4).
The multiplication process is designed as a scheme (Fig. 5).

Discussion

Maca naturally reproduces sexually by self pollination; one plant can produce about 14 g seeds (QUIRÓS and ALIAGA, 1997). The germination capacity of seeds *in vitro* is high and independent of size fraction.
To obtain a homology line, the multiplication protocol of *L. meyenii* was designed. The principle of this method is initiation of regeneration of dormant axillary buds on segments. The creation of a multiplication protocol of the chosen plant species can be difficult due to individual reactions to both in vitro cultivation and plant growth regulators. The situation is well described by a number of researchers also at *Brassicaceae*. OSUNA et al. (2006) described the multiplication protocol of medicinal plant *Lepidium virginicum* L. *In vitro*-germinated seeds were used to obtain pathogen free cotyledons, hypocotyls and apical bud explants. The best multiplication rate was obtained on MS medium supplemented with 0.57 µM of indole-3-acetic acid and 13.94 µM of kinetin from apical bud

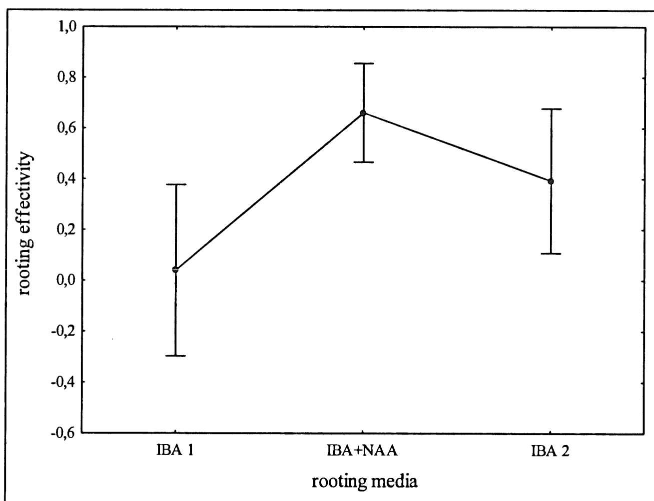

Fig. 3: Graphical visualization of ANOVA analyses - influence of rooting media (x-axis) on rooting efficiency of *L. meyenii* (y-axis); $F(2. 24) = 5.6740$, $p = 0.00960$. Vertical columns sign 0.95 confidence interval.

Fig. 4: Plants of *L. meyenii* obtained from *in vitro* multiplication process. Plants were planted to mixture of peat, earth and sand in 4 cm pots. Age of plants was 24 days.

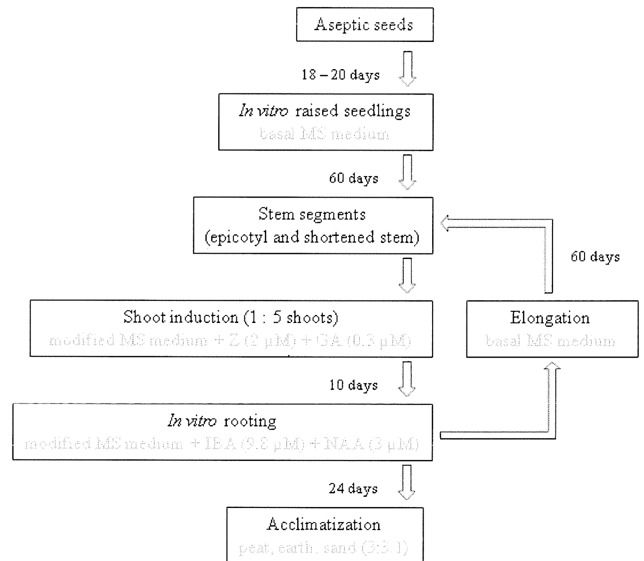

Fig. 5: Schema of multiplication protocol of *L. meyenii* using segments (i.e. epicotyl and shortened stem; Z – zeatin, GA – gibberellic acid 3, IBA – indole-3-butyric acid, NAA – α-naphthalene acetic acid).

explants after 15 day cultivation. PANDE et al. (2002) used two multiplication strategies at *Lepidium sativum* Linn. – multiplication via calli and via nodal segments of adult plants. At the first strategy, the highest regeneration frequency with the highest number of shoots (25.8 per 100 mg of calli) were obtained on MS medium with 2.85 µM of indole-3-acetic acid and 46.4 µM of kinetin. At the second strategy, the regeneration frequency reached 75 % and 5 shoots per explant were obtained on MS medium with 1.14 µM of indole-3-acetic acid and 23.2 µM of kinetin. LEAL et al. (2009) obtained the best multiplication rate (3.4 shoots per nodal segment) in B5 medium (GAMBORG et al., 1968) supplemented with 4 µM of benzylaminopurine at *Isatis tinctoria* L. GUO et al. (2000) used a high dose of 2,4-dichlorophenoxyacetic acid (22.6 µM) and benzylaminopurine (17.7 µM) for multiplication of *Brassica rapa* L. The best direct shoot organogenesis from leaf segments of *Brassica campestris* ssp. *chinensis* was achieved on MS medium supplemented

with 9 μM of thidiazuron, 2.6 μM of α-naphthalene acetic acid and 44.1 μM of $AgNO_3$ (highest multiplication coefficient was 66 shoots per explant; MEMON et al., 2009). For multiplication of *Juncus effusus* L., different concentrations of benzylaminopurine, kinetin and indole-3-acetic acid and their combination were tested at five genotypes but only two of them had good tissue culture ability. The calli shoot proliferation required two steps involving successive replacement of medium without and with indole-3-acetic acid (benzylaminopurine 2.2 μM; kinetin 4.6 μM; indole-3-acetic acid 17.1 μM), under these conditions the multiplication rate was 8.25 shoots (XU et al., 2009). The combination of α-naphthalene acetic acid (3.3 μM), gibberellic acid 3 (0.058 μM) and isopentenyl adenine (4.9 μM) contributed to the shoot proliferation on calli at *Brassica oleracea* L. and *Brassica napus* L. (KAUR et al., 2006).

In this paper, the best multiplication medium for *L. meyenii* was MS medium supplemented with 2 μM of zeatin and 0.3 μM of gibberellic acid 3 (Z+GA) and multiplication coefficient was 5.3 shoots per segment (Fig. 2). The multiplication of *L. meyenii* was solved for example by CHENG et al. (2004) and WANG et al. (2007) but via calli organogenesis. The organogenesis from calli was induced in MS medium supplemented with 8 μM of benzylaminopurine and 1.5 μM of α-naphthalene acetic acid but hyperhydricity was frequent. Hyperhydricity was reduced by employing 0.1 mM La^{3+}, Ce^{3+} and Nd^{3+} (WANG et al., 2007). In our experiment, the hyperhydricity was observed at shoots on medium with benzylaminopurine too. The shoots that originated from the other media (Z1, Z2, Z+GA and mT+GA) grew without vitrification. There was no significant difference in multiplication rate for any of the media. For this reason, it was not necessary to consider hyperhydricity in our case.

The rooting of regenerated shoots is realized in media with auxins especially with indole-3-butyric acid or their combination and it is species dependent. OSUNA et al. (2006) obtained the highest rate of rooting for *Lepidium virginicum* L. using MS medium with 14.76 μM of indole-3-butyric acid. PANDE et al. (2002) achieved 100% rooting at *Lepidium sativum* Linn using MS medium supplemented with 684 μM of glutamine. The concentration 1 μM of α-naphthalene acetic acid proved effective in rooting initiation for *Brassica campestris* ssp. Chinensis (MEMON et al., 2009). KAUR et al. (2006) used half-strength medium without plant growth regulators for rooting *Brassica oleracea* L. and *Brassica napus* L. In our case, the reliable rooting medium for shoots of *L. meyenii* was MS with 9.8 μM of indole-3-butyric acid and 3 μM of α-naphthalene acetic acid (IBA+NAA). The rooting efficiency of this medium was 75 % (Fig. 3). No desirable rooting effect was found in media IBA1 and IBA2 with indole-3-acetic acid. These results are in contrast to the experiences of CHENG et al. (2004) who obtained 66.7% rooting effect of shoots from Maca calli on half-strength MS medium supplemented with 2.45 μM of indole-3-acetic acid. We can speculate that these different results could be due to different concentrations of basal media.

L. meyenii can be transferred and established *in vivo* to good effect. In our case it was 94 %. A common rate of surviving at *Brassicaceae* varies from 62 to 100 % (PANDE et al., 2002; KAUR et al., 2006; OSUNA et al., 2006; MEMON et al., 2009; XU et al., 2009).

In conclusion, we established an effective multiplication system of *L. meyenii*. This system is able to produce large numbers of uniform plants.

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