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RESEARCH NOTE

Comparison of in vitro haploid induction in balm (*Melissa officinalis*)

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Abstract The generation of haploids in lemon balm (*Melissa officinalis* L.) is the prerequisite for accelerated development of homozygous lines. Wide experiments to induce sporophytic development were arranged. Donor plants of lemon balm were established in a greenhouse, climate chambers and in vitro. For haploid induction, different variations of media and culture conditions e.g. pretreatment with cold and heat were tested. Sporophytic development of pollen was induced in anther and microspore culture. Callus developed sporadically in anther culture. Multi-cellular structures and microcallus generated from microspores. In vitro cultivated ovules became dark brown and somatic callus generated at the cut surface. Cultivation of ovaries maintained swelling on medium, but undeveloped embryos died.

Keywords Haploid culture · Sporophytic division · Lemon balm · Lamiaceae · *Melissa officinalis*

Introduction

Lemon balm (*Melissa officinalis* L.) has been used since the time of the ancient Greeks or longer for medicinal and aromatic purposes. Actually, lemon balm is used as a medicinal plant, as well as an aromatic plant and in folk medicine. The species *M. officinalis* belongs to the family Lamiaceae (syn. Labiatae) and has a wide spectrum of secondary metabolites (Tonelli et al. 2015). All grown lemon balm conforming to pharmacopoeia belong to diploid *M. officinalis* ssp. *officinalis* and have $2n = 2x = 32$ chromosomes whereas the tetraploid ssp. *altissima* has $2n = 4x = 64$ chromosomes (Kittler et al. 2015) and no lemon fragrance (balm). Success in development of homozygous plants via haploid induction, instead of classical inbred line production by self pollination steps, can accelerate the breeding process of lemon balm. Because of the knowledge gap in haploid production of species belonging to the Lamiaceae family and especially of lemon balm (Ferrie 2007), the efficiency of isolated microspore, anther, ovule and ovary culture had to be tested for adaptation. The present study reports the first evidence of successful sporophytic development stages in cultures of anthers and microspores exclusively of lemon balm.

Materials and methods

A total number of 46 accessions of balm were involved in the experiments for haploid production, 24 (BLBP) provided by Bavarian State Research Center for Agriculture (LfL), Freising, Germany, 20 (Meli) provided by the Leibniz Institute of Plant Genetics and Crop Plant Research—Federal ex situ Collection of Agricultural and Horticultural Plants (IPK), Gatersleben, Germany and two provided by the company N. L. Chrestensen (NLC), Erfurt, Germany. Among the 24 LfL accessions 20 were diploid and four triploid. Fourteen accessions of IPK were diploid, five tetraploid and one aneuploid. The two cultivars

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'Lorelei' and 'Erfurter Aufrechte' were diploid. Accessions are from different countries of origin: Armenia (1), France (6), Georgia (3), Germany (8), Hungary (1), Italy (5), Spain (1), Switzerland (1), Turkey (1) and unknown (19).

Balm plants were naturally in flower from June to August and after harvesting of fresh material from September to October. They were grown in field, greenhouse and in climate chambers to get buds all over the year. The primarily absent willingness of donor plants to flower in the climate chamber could be overcome by vernalization of 12 weeks with temperature of 4 °C. Plants were cultivated during flowering in the greenhouse and climate chamber under a 16 h photoperiod (greenhouse: 150 µmol m^{-2} s^{-1}, SONT-Agro 400 in MGR400 lamp Philips, climate chamber: EYE MT250DL/E40 in reflector lamps without glass cover) with temperatures of 22–26 °C in the day period and 18–22 °C during the night period.

For haploid induction the calyx with the bud was surface sterilized in 3 % sodium hypochlorite (NaOCl) solution with a drop of Tween 20 for 15 min and then rinsed three times with sterilized distilled water. Anthers, ovules or ovaries were prepared in sterile 100 × 20 mm plastic Petri dishes using a dissection microscope (Stemi SV6 Zeiss Germany) with a cold light source (KL2500, Schott) and cultivated in 35 × 10 mm Petri dishes with 2 ml medium. The dishes were sealed with Parafilm or placed in 145 × 20 mm dishes and then closed with Parafilm.

Different media, B5 (Gamborg et al. 1968), N6 (Chu 1978) and PTC1 starvation medium (Kim et al. 2008) were used for haploid production. Amino acids [G (L-glutamine) and S (L-serine)], phytohormones [2,4-D (2,4-dichlorophenoxyacetic acid), BAP (6-benzylaminopurine), Kin (kinetin), NAA and Zea], a carbohydrate source (sucrose, maltose or D-mannitol) and solidifying agents [Ag (bactoagar) or F (Ficoll PM 400)] were added to the induction media. Pretreatment with high (30 and 35 °C) and low (6 °C) temperatures respectively for 1–8 days was tested in vitro for the initiation of the sporophytic development. After pretreatment, all cultures were maintained in climate chamber at 27 °C. Haploid structures were cultivated in darkness or with 16 h light period. For regeneration B5 without hormones or supplemented with 1 mg l^{-1} NAA, 1 mg l^{-1} 2,4-D and 2 % sucrose was used.

The viability of microspores or pollen was tested with fluorescein diacetate (FDA). 25 mg FDA were dissolved in 5 ml acetone (Heslop-Harrison and Heslop-Harrison 1970; Kison 1979). Of FDA solution 2 µl were added to 100 µl suspension and assessed under an inverted microscope (Axiovert 200 M, Zeiss, Germany) with bright and fluorescent light.

For culling of flowers it is necessary to know how they look when they contain late uninucleate pollen. Late uninucleated microspores were described aging as optimal stadium for anther and microspore culture (Wenzel and Thomas 1974; Sunderland and Dunwell 1977; Chiang et al. 1985). For characterization of pollen development stage proportional to evolvement of flower anthers of buds of different length were squashed in N6 (vacuoles in microspores were visible) or stained with carminic acid (3 %) for 2 h (nuclei were visible). Length of sepals (4–9 mm) and petals (1–5 mm) were measured with scale paper under the Petri dish during preparation with a dissection microscope. After sterilization and preparation anthers were pretreated and cultivated on medium in chambers with 6, 30 or 35 °C or combinations of these temperatures for 1–8 days. Modified B5, EPM (modified N6: one-half-strength of macro- and micro elements of N6, B5 vitamins, 440 mg l^{-1} G, 90 g l^{-1} maltose monohydrate, 0.2 mg l^{-1} 2,4-D), K0 medium (Keller et al. 1975): modified B5 with 750 mg l^{-1} $CaCl_2 \cdot H_2O$, 800 mg l^{-1} G, 100 g l^{-1} sucrose) or N6 were used for haploid production (Online Resource 1). Medium was liquid or solidified with Ag or F. Growth regulators were tested in different combinations. Sucrose or maltose was added. In K0 only 100 g l^{-1} sucrose was used whereas in N6 and EPM concentrations of 100 g l^{-1} sucrose or 90 g l^{-1} maltose were tested. Either 20 or 50 anthers were placed in a Petri dish. Anther culture was established in 13 single experiments (Online Resource 1). Cultivated anthers were evaluated in 14 day intervals with a binocular microscope and fallen out microspores with an inverted microscope (Axiovert 135, Zeiss, Germany). Samples were biweekly observed in squash preparations of carminic acid (3 %) stained anthers.

Microspore culture was performed according to Kim et al. (2008) and Lantos et al. (2009). Anthers of different genotypes were prepared as described for anther culture. The anthers were precultivated (50 per Petri dish) for 3–8 days at 6 °C in PTC1 with maltose monohydrate or microspores were directly isolated. For isolation, anthers were transferred in PTC1 with D-mannitol and blended at high speed with a mag hot plate stirrer (IKA Labortechnik) for 2 min. Anther debris were removed by filtering through sieves (Wilson sieve, CLF PlantClimatics) with mesh sizes of 40 µm. The resulting suspension was centrifuged (Hettich Universal 30RF) at 500 rpm at 4 °C for 10 min and the microspore pellet was washed with PTC1 and centrifuged again. Microspores were resuspended in culture medium (Online Resource 2) or for pretreatment of isolated microspores in PTC1 with maltose monohydrate. The microspore density of 1.5 × 10^5 ml^{-1} in culture medium was regulated by estimating with a counting cell chamber (Fuchs and Rosenthal). Timentin (GlaxoSmithKline, 93.75 % ticarcillin disodium and 6.25 % clavulanic acid) was used to inhibit infections in microspore culture (300 mg l^{-1}). The plates were incubated in darkness at 27 °C for 14 days and then with a 16/8 h day/night regime.

In microspore culture, ovaries of wheat were applied for medium conditioning. Two bisected ovaries of surface sterilized wheat spikes of the cultivar 'Quattro' were added to every Petri dish (35 × 10 mm). Cultures were weekly evaluated by binocular and inverse microscope. Microspores were cultivated in five experiments (Online Resource 2).

From surface sterilized closed buds with light green to light yellow petals, ovaries were prepared and whole ovaries or the four prepared ovules were transferred to agar solidified medium in five different experiments (Online Resource 3). Either 20 or 50 ovaries or 50 ovules were placed in a Petri dish (35 × 10 mm). Modified B5 and N6 with 2 or 10 % sucrose were used for gynogenetic haploid production. In some experiments, ovules were treated with heat (1 day 35 °C, 3–6 days 30 °C) or cold (3–6 days 6 °C). Then they were cultivated at 27 °C with a 16/8 h light regime. Enlarged ovules were dissected after 3 weeks. Outer integuments were eliminated and dissected embryo sacs cultivated on B5 medium with 2 or 10 % sucrose, respectively.

Results

A different number of anthers and ovaries of plants were prepared for haploid induction from field (Fig. 1a), greenhouse and climate chamber within 3 years. A total of 69.760 anthers were prepared in 13 experiments for anther culture with different accessions, culture conditions of donor plants, preatreatments, media and numbers of anthers per dish (Online Resource 1). The pollen development stage in buds of the same length was different in several accessions. Length of always two anthers of four in a bud was the same, which corresponded to flower morphology in Lamiaceae (Fig. 1b, c). Buds of 2.5–3.5 mm length with anthers of about 1 mm depending on accession have late uninucleated microspores and vacuoles within cells (Fig. 1d, e).

Pretreatment of anthers took place on medium. Cold or heat treatment of buds was not possible because tissue had already degenerated after 6 h. Treatment of whole plants was ineffective due to the successive development of new buds on the balm plant. Anthers became brown after 7 days on agar solidified media but not before 14 days on liquid media. Callus derived from somatic tissue of the anther filament grew mainly on media with 2,4-D and BAP (Fig. 1h). Sporophytic divisions were found in cultivated pollen on liquid medium after 4–6 weeks. They were observed between microspores which had fallen out of anthers. The multi-cellular structures were a little larger than microspores (Fig. 1m, n). They developed into small white or light brown globular calli (Fig. 1o). The small calli were put on regeneration medium with lower sucrose concentration (B5 without hormones or supplemented with 1 mg l^{-1} NAA, 1 mg l^{-1} 2,4-D and 2 % sucrose). Because of degeneration of most multi-cellular structures in liquid medium, they were also transferred to regeneration medium. Neither multi-cellular structures nor calli developed into plants.

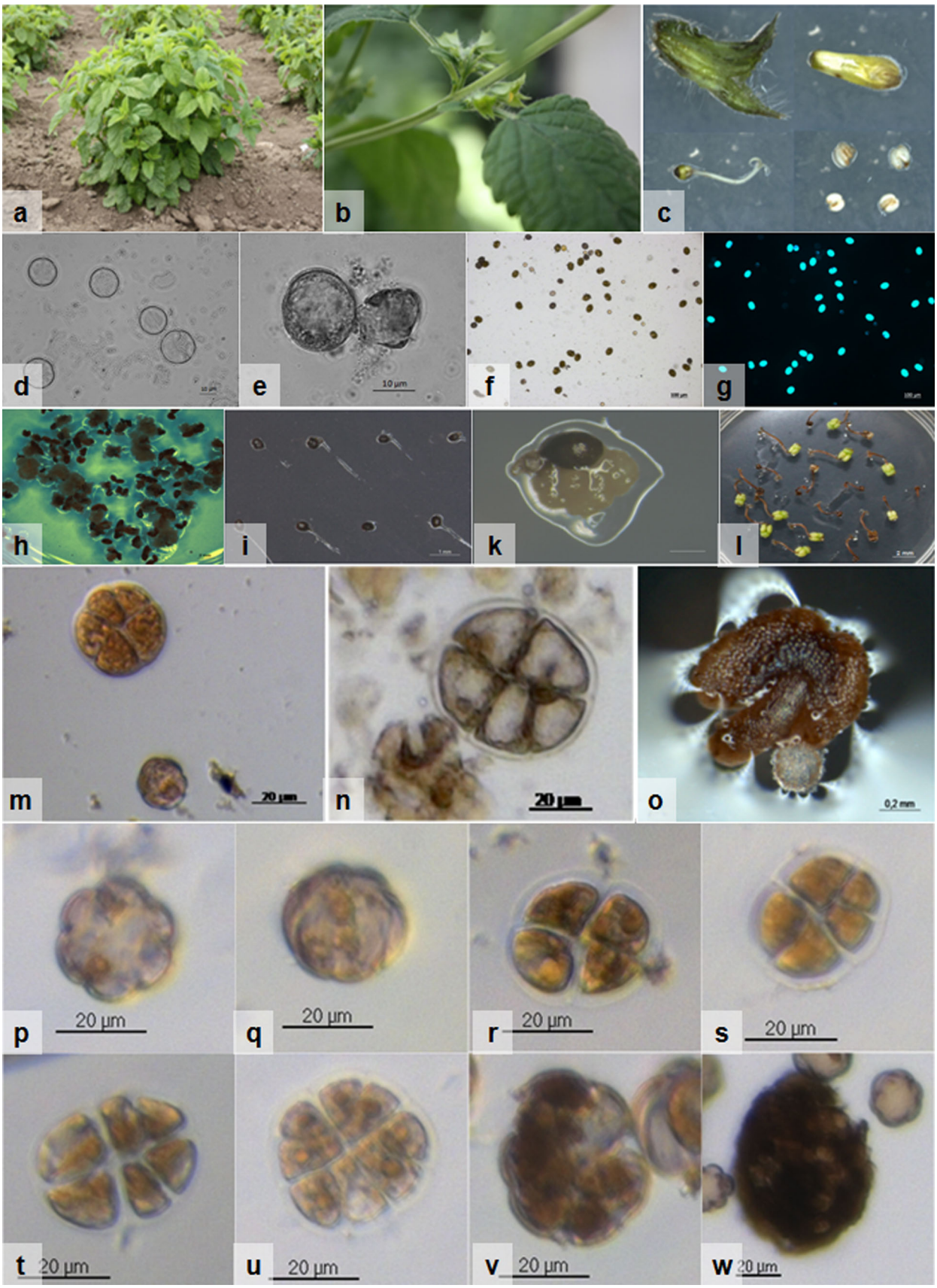
Sporophytic developmental stages and callus were found sporadically in 15 cases in anther cultures of ten accessions of lemon balm. They could be observed in different liquid culture media and after different numbers of days of cold or heat pretreatment (Table 1). Statistics were not performed, because results were fragmented in different experiments (Online Resource 1). There were no structures on agar solidified medium. Multi-cellular structures were not found in acetocarmin stained and squashed anthers.

For microspore culture a total of 24.020 anthers were prepared in five different experiments (Online Resource 2). The viability of microspores was tested after the isolation procedure (Fig. 1f, g). From FDA stained microspores 70–85 % were viable dependent on accession. Differentiation from the late uninucleated via binucleated and multicellular stages to microcalli could be observed in isolated microspore culture (Fig. 1p–w). But in most microspores, only an accumulation of starch was visible. Microcalli did not regenerate to haploid plants. Sporophytic development was found sporadically in microspore cultures of six lemon balm accessions and under different culture conditions (Table 1).

A total of 7.100 ovules and 3.685 ovaries were cultivated under different conditions in five experiments (Online Resource 3). All separated ovules showed dark browning in ovule culture (Fig. 1i). Somatic callus developed occasionally at the cut surface of ovule (Fig. 1k). But a few ovules enlarged (Fig. 1l) during the first week of ovary culture. The whole ovaries stopped development and died after about 4 weeks. Dissected and cultivated embryo sacs from enlarged ovules also died.

Discussion

In vitro culture and propagation of *Melissa officinalis* succeeded in vitro without problems. However the regeneration of haploid plants in balm and Lamiaceae is problematic. Bugara et al. (1986) reported embryoids in *Salvia sclarea*, but they could not regenerate any plant. The review article of Ferrie (2007) about doubled haploid production in nutraceutical species informs about swelling of microspores in three of 14 analyzed Lamiaceae. Hadian et al. (2012) referred about embryoids in microspore culture of *Satureja khuzistanica* and *S. rechingeri*. They found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◀**Fig. 1** **a** Lemon balm plants in field. **b** Inflorescence with buds. **c** Prepared flower organs—calyx, corolla, ovary, four different developed anthers. **d** Late uninucleated stage of microspore development in lemon balm with vacuoles. **e** Vacuole and nucleus are visible. **f** Viability of microspores of lemon balm (71 %). Microscopic field with FDA stained microspores in bright light. **g** Viable microspores under fluorescent light. **h** 4 weeks cultivated anthers with filament callus. **i** *Dark brown* cultivated ovules of lemon balm on agar solidified medium. **k** Somatic callus obtained at cut surface of ovule after 3 weeks. **l** Enlarged *green* ovules on ovaries of lemon balm in a culture dish after 3 weeks. **m** Sporophytic structure in anther culture, larger than microspore. **n** Multi-cellular structure within the pollen wall. **o** *White* callus besides a *brown* anther. **p**–**w** Different sporophytic stages from binuclear pollen via multi-cellular structures to micro callus in microspore culture of lemon balm. (Color figure online)

that it was not possible to generate plants. The present study reports the first evidence of successful sporophytic developmental stages in cultures of microspores and multi-cellular structures between detached microspores in the culture medium of anther culture. However a recipe cannot given, because the results were fragmented in different experiments.

Kim et al. (2008) pointed out several advantages of isolated microspore culture over anther culture, such as allowing direct in vivo observation in the microscope, because squashing preparation is not necessary. In our experiments there were no microcalli in squashed anthers or dehisced anthers.

Germanà (2011) recapitulated that model species do not require the addition of an auxin in the induction medium but the presence of growth regulators is crucial in the majority of plant species. Supplementing phytohormones, particularly 2,4-D and BAP or 2,4-D and Kin induced growth of somatic callus in lemon balm, but not sporophytic division of nuclei. As well Germanà (2011) discussed the temperature shock as most effective to induce pollen embrygenesis. Pretreatment with 30–35 °C and 4 °C was successful in several species. Heat (30 °C) or cold (6 °C) was used when androgenetic divisions took place in our experiments. Many media combinations and pretreatment variations were tested but no response to special nutritional requirements or pretreatment conditions was observed.

Ovary co-culture was tested in microspore culture of lemon balm. But the growth of structures stopped with and without longitudinally bisected wheat ovaries. Lantos et al. (2009) described positive effects of wheat ovaries in microspore culture of pepper. Multi-cellular structures

Table 1 Accessions of lemon balm with sporophytic divisions in anther culture (AC) and microspore culture (MC) after different pretreatment conditions and media (Experiments listed for AC in Online recources1 and MC in Online recources2)

Accessions	Pretreatment conditions, media	Results	Experiment
Anther culture			
LfL BLBP5	5 days 30 °C, K0 + 10 g l^{-1} F	2 multi-cellular structures	AC2
LfL BLBP19	3 days 30 °C, K0 + 1 mg l^{-1} 2,4-D	1 callus	AC13
LfL BLBP26	5 days 30 °C, N6 + 1 mg l^{-1} 2,4-D	2 multi-cellular structures	AC12
LfL BLBP29	3 days 30 °C, N6 + 1 mg l^{-1} 2,4-D	1 callus	AC13
LfL BLBP48	3 days 30 °C, N6	2 calli	AC10
LfL BLBP49	4 days 6 °C, K0 + 10 g l^{-1} F	3 calli	AC9
	5 days 6 °C, N6	1 callus	AC10
LfL BLBP50	5 days 6 °C, K0 + 10 g l^{-1} F	1 callus	AC9
LfL BLBP65	5 days 6 °C, K0 + 10 g l^{-1} F + 100 mg l^{-1} S	1 callus	AC7
	5 days 6 °C, N6	3 calli	AC7
LfL BLBP87	5 days 30 °C, N6	1 multi-cellular structure, 2 calli	AC10
NLC 'Erfurter Aufrechte'	5 days 30 °C, K0 + 10 g l^{-1} F + 1 mg l^{-1} 2,4-D	2 multi-cellular structures	AC12
Microspore culture			
LfL BLBP5	4 days 6 °C anthers, EPM	1 multi-cellular structure	MC2
	3 days 6 °C microspores, EPM	1 multi-cellular structure	MC4
	5 days 6 °C microspores, EPM	1 multi-cellular structure	MC4
LfL BLBP27	6 days 6 °C anthers, EPM	2 multi-cellular structures	MC2
LfL BLBP34	5 days 6 °C anthers, EPM	1 multi-cellular structure	MC2
LfL BLBP49	8 days 6 °C anthers, EPM	3 multi-cellular structures	MC2
IPK Meli10	4 days 6 °C anthers, EPM	20 multi-cellular structures,	MC1
		1 microcallus	MC1
NLC 'Erfurter Aufrechte'	5 days 6 °C anthers, EPM	1 four-cellular structure	MC2

continued to develop in the presence of ovaries in the induction medium. Ovaries seem to send nurse agents for development (Broughton 2008). Lippmann et al. (2015) pointed out the effect of cutting pistils in half. They obtained a five-fold increase in the number of embryogenic calli of barley. However knowledge about this mechanism is relatively limited and remains far from being fully understood.

Cultivated ovules and ovaries in described experiments showed only somatic calli. Growth of ovules on cultivated ovaries of balm and preparation of embryo-like structures was unsuccessful. An elicitor for gynogenesis is missing. Chen et al. (2011) summarized that the female gametophyte can be developed from a wide range of developmental stages. Young uninuclear to mature embryo sacs should be observed in ratio to the length of ovules or the days to anthesis.

There can be a small window for reprogramming the pathway from gametophytic to sporophytic in lemon balm. The regeneration of plants could not be obtained in anther and microspore culture nor in ovule and ovary culture. But the induction of sporophytic development is of scientific interest and closes the knowledge gap in haploid production of lemon balm within the series of Lamiaceae.

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References

Broughton S (2008) Ovary co-culture improves embryo and green plant production in anther culture of Australian spring wheat (*Triticum aestivum* L.). Plant Cell Tissue Organ 95:185–195

Bugara AM, Rusina LW, Resnikowa SA (1986) Embryoidogenesis in anther culture of *Salvia sclarea*. Fiziol Biokhim Kult 18:381–386

Chen J-F, Cui L, Malik AA, Mbira KG (2011) In vitro haploid and dihaploid production via unfertilized ovule culture. Plant Cell Tissue Organ 104:311–319

Chiang MS, Frechette S, Kuo CG, Chong C (1985) Delafield SJ (1985), Embryogenesis and haploid plant production from anther culture of cabbage (*Brassica oleracea* var. capitata L.). Can J Plant Sci 65:1033–1037

Chu CC (1978) The N6 medium and its applications to anther culture of cereal crops. In: Proceedings of symposiym on plant tissue culture. Science Press, Peking, pp 43–50

Ferrie AMR (2007) Doubled haploid production in nutraceutical species: a review. Euphytica 158:347–357

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirement suspension cultures of soybean root cells. Exp Cell Res 50:151–158

Germanà MA (2011) Anther culture for haploid and doubled haploid production. Plant Cell Tissue Organ 104:283–300

Hadian J, Afzalifar M, Mirjalili MH, Shariatpanahi ME, Esmaili A (2012) Embryogenesis from isolated microspore cultures of *Satureja khuzistanica* and *Satureja rechingeri* (Lamiaceae). In: Breedmap 5. international symposium breeding research on medicinal and aromatic plants, Vienna, 18–20 June p 37

Heslop-Harrison J, Heslop-Harrison Y (1970) Evaluation of pollen viability by enzymatically induced fluorescence; intracellular hydrolysis of fluorescein diacetate. Stain Technol 45:115–120

Keller WA, Rajathy T, Lacapra J (1975) In vitro production of plants from pollen in *Brassica campestris*. Can J Genet Cytol 17:655–666

Kim M, Jang I-C, Kim J-A, Park E-J, Yoon M, Lee Y (2008) Embryogenesis and plant regeneration of hot pepper (*Capsicum annuum* L.) through isolated microspore culture. Plant Cell Rep 27:425–434

Kison HU (1979) Pollenlebensfähigkeit und Reproduktionsgrad bei Weizen/Roggen-Allopolyploiden. PhD thesis, Akademie der Landwirtschaftswissenschaften, Berlin

Kittler J, Schrader O, Kästner U, Marthe F (2015) Chromosome number and ploidy level of balm (*Melissa officinalis*). Mol Cytogenet 8:61. doi:10.1186/s13039-015-0166-z

Lantos C, Juhász AG, Somogyi G, Ötvös K, Vági P, Milhály R, Kristóf Z, Somogyi N, Pauk J (2009) Improvement of isolated microspore culture of pepper (*Capsicum annuum* L.) via co-culture with ovary tissues of pepper or wheat. Plant Cell Tissue Organ 97:285–293

Lippmann R, Friedel S, Mock HP, Kumlehn J (2015) The low molecular weight fraction of compounds released from immature wheat pistils supports barley pollen embryogenesis. Front Plant Sci 6:498

Sunderland N, Dunwell JM (1977) Anther and pollen culture. In: Street HE (ed) Plant tissue and cell culture, 2nd edn. Blackwell Sci, Oxford, pp 223–265

Tonelli M, Pellegrini E, D'Angiolillo F, Petersen M, Nali C, Pistelli L, Lorenzini G (2015) Ozone-elicited secondary metabolites in shoot cultures of *Melissa officinalis* L. Plant Cell Tissue Organ 120:617–629

Wenzel G, Thomas E (1974) Observation on the growth in culture of anthers of *Secale cereale*. Z Pflanzenzüchtung 72:89–94