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In vitro embryo rescue of interspecific hybrids of *Lepidium*

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Utveckling av in vitro metod för interspecifik hybridisering av *Lepidium*

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Estefany Reyes Estévez,

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Abstract

With the ongoing climate change and growing world population, there is an increasing demand for vegetable oils for industrial and food purposes. *Lepidium campestre* (field cress) is a wild cruciferous, which is currently under domestication as a new potential high-yielding oil crop for cold climate conditions. Because of problems with early shattering silicles and low oilseed content, there is an initiative to cross the crop with relatives *L. draba* and *L. graminifolium*, to achieve a hybrid with shattering resistance and a higher oilseed content. Because of hybrid incompatibility, the hybridization of these species is difficult to achieve with traditional breeding methods. In an attempt to produce a protocol for the successful interspecific crossings of *L. campestre* x *L. draba* and *L. campestre* x *L. graminifolium*, ovary culture and direct embryo culture, were performed, and various maturity-degrees at the date of harvest were tested. A self-pollinated *L. campestre* control produced an embryo which developed in vitro, but no hybrid embryos were achieved in the interspecific crosses. It was concluded that experiments with pre-fertilization methods, reciprocal crossing, and more culture media compositions are needed to successfully develop a method for embryo rescuing to obtain interspecific hybrids of *L. campestre* x *L. draba* and *L. campestre* x *L. graminifolium*.

Sammanfattning

På grund av pågående klimatförändringar och den växande världsbefolkningen finns det en ökande efterfrågan på vegetabiliska oljor för industriella- och livsmedelsändamål. *Lepidium campestre* (fältkrassing) är en vild kålväxt som just nu är under domesticering som ny potentiellt högavkastande oljegröda för kalla klimatförhållanden. På grund av problem med dråsning och lågt oljeinnehåll har ett initiativ tagits för att korsa fältkrassingen med släktingarna *L. draba* och *L. graminifolium*. Målet är att uppnå en hybrid som är dråsfast och har frön med ett högre oljeinnehåll. Hybridinkompatibilitet gör dock hybridiseringen av dessa arter svår att uppnå med traditionella förädlingsmetoder. Därför utfördes två olika in vitro-metoder samt test av olika mognadsnivåer vid skörd i ett försök att utveckla ett protokoll för lyckade interspecifika korsningar av *L. campestre* och *L. draba* samt *L. campestre* och *L. graminifolium*. En självpollinerad *L. campestre*-kontroll bildade ett embryo som påvisade tillväxt in vitro men inga hybridembryon åstadkoms i de interspecifika korsningarna. Slutsatsen drogs att experiment med förbefruktningsmetoder, reciproka korsningar samt flera olika sammansättningar av odlingsmedium är nödvändiga för att kunna utveckla en framgångsrik metod och uppnå interspecifika hybrider av *L. campestre* x *L. draba* och *L. campestre* x *L. graminifolium*.

Table of contents

Abbreviations

BAP	6-Benzylaminopurine (synthetic cytokinin)
CH	Casein hydrolysate
DAP	Days after pollination
IAA	Indole-3-acetic acid (naturally occurring auxin)
MS	Murashige and Skoog basal salt mixture
NAA	1-Naphthaleneacetic acid (synthetic auxin)

Introduction

Domestication of a novel oil crop

In this day and age of global warming, fossil fuels are an overexploited limited resource contributing to climate change. Therefore, it is no news that renewable oils of vegetable origin are of great interest as a replacement for petroleum oils in industries and biofuel (Merker et al., 2010). What is more, the demand for vegetable oils is increasing because of a steadily growing world population.

Palm and soybean are the most extensively produced oil types (Colombo et al., 2018), but they cannot be grown in Scandinavia for various reasons, for example, both species need a warmer climate to thrive. Nevertheless, palm and soy cultivations are controversial because of the considerable biodiversity loss that deforestation and monocultures have caused in tropical forests in Asia and South America (Hospes, 2014; WWF, 2020).

In Sweden, winter oilseed rape (*Brassica napus*) dominates the plant oil crop production (97 %), along with a minor contribution from turnip rape and spring turnip rape (*B. rapa*) (Lind, 2020). However, because of their Mediterranean origins, these well-established *Brassica* oil crops are not adapted to withstand the colder climate and short growing season of the northern parts of Sweden (Eriksson, 2009), currently limiting the production of plant-derived oils to the

southern part of the country (Sandelius, 2017). By the same token, the domestic alternatives for oilseed and feed crop are also limited.

Where it is not possible to sow winter oilseed crops in the autumn, such as in the north of Sweden, the cultivation of annual spring crops like spring oilseed rape and turnip rape, means that fields are left bare after the growing season is over. Furthermore, this creates prime conditions for nutrients to run off or percolate down to the groundwater as there are no plants to catch them (Eriksson, 2009; Sandelius, 2017), contributing to already severe problems with eutrophication and a disturbed balance of species diversity (Andersson et al., 1999; Bohman, 2018; Jordbruksverket, 2020). Moreover, these crops depend on chemical pesticides as there are issues with fungal diseases and the pollen beetle (Merker et al., 2010).

Lepidium campestre

Lepidium campestre, commonly known as field cress, is a wild cruciferous plant indigenous to Scandinavia (Merker et al., 2010; Merker and Nilsson, 1995). Even though it is often regarded as a weed, domestication work has been ongoing during the last 30 years and has intensified over the last ten years, for example, in the research project Mistra Biotech, funded by MISTRA and SLU. This is because the plant has the potential of being developed into a new, high-yielding oil crop, suitable for cold climate conditions (Ivarson et al., 2013; SLU, 2020).

Field cress is a self-fertilized diploid with a chromosome number of 2n = 16 (Geleta et al., 2020; Ivarson et al., 2016). Unlike rapeseed and turnip rape, field cress was not introduced as an exotic species to Sweden, meaning that it does not have to suffer adaptation barriers to withstand Nordic weather conditions (Eriksson, 2009). Moreover, a study has demonstrated its superior cold hardiness to rapeseed (Merker et al., 2010) and its higher-yielding potential compared to that of winter oilseed rape and turnip rape (Eriksson, 2009; Ivarson et al., 2013). What is more, field cress has a natural resistance to the pollen beetle that makes it a suitable option for organic cropping systems, which currently lack a profitable option for oilseed crop production (Merker et al., 2010; Nilsson et al., 1998).

Field cress has an upright stature and synchronous flowering, which are both desirable agronomical traits (figure 1). The crop produces white flowers that later turn into silicles (the fruit, also referred to as ovaries in the text) that encase two seeds each. Today, the seeds of field

cress only have an oil content of about 15 – 20 % (Sandelius, 2017), which is low compared to the widely grown winter oilseed rape (canola), containing 45 % oil (Canola council, n.d. a). Moreover, the fatty acid profile of the seeds is a good starting point to breed field cress for industrial/chemotechnical and nutritional purposes (Merker and Nilsson, 1995). Because of the complexity of the genes that code for cold hardiness, yield, and adaptation to the cropping system and environment, it is significantly easier to domesticate field cress for traits that are dictated by single or a few genes, like higher oil content, than to breed the established oil crops for the more complex characteristics (Merker et al., 2010).

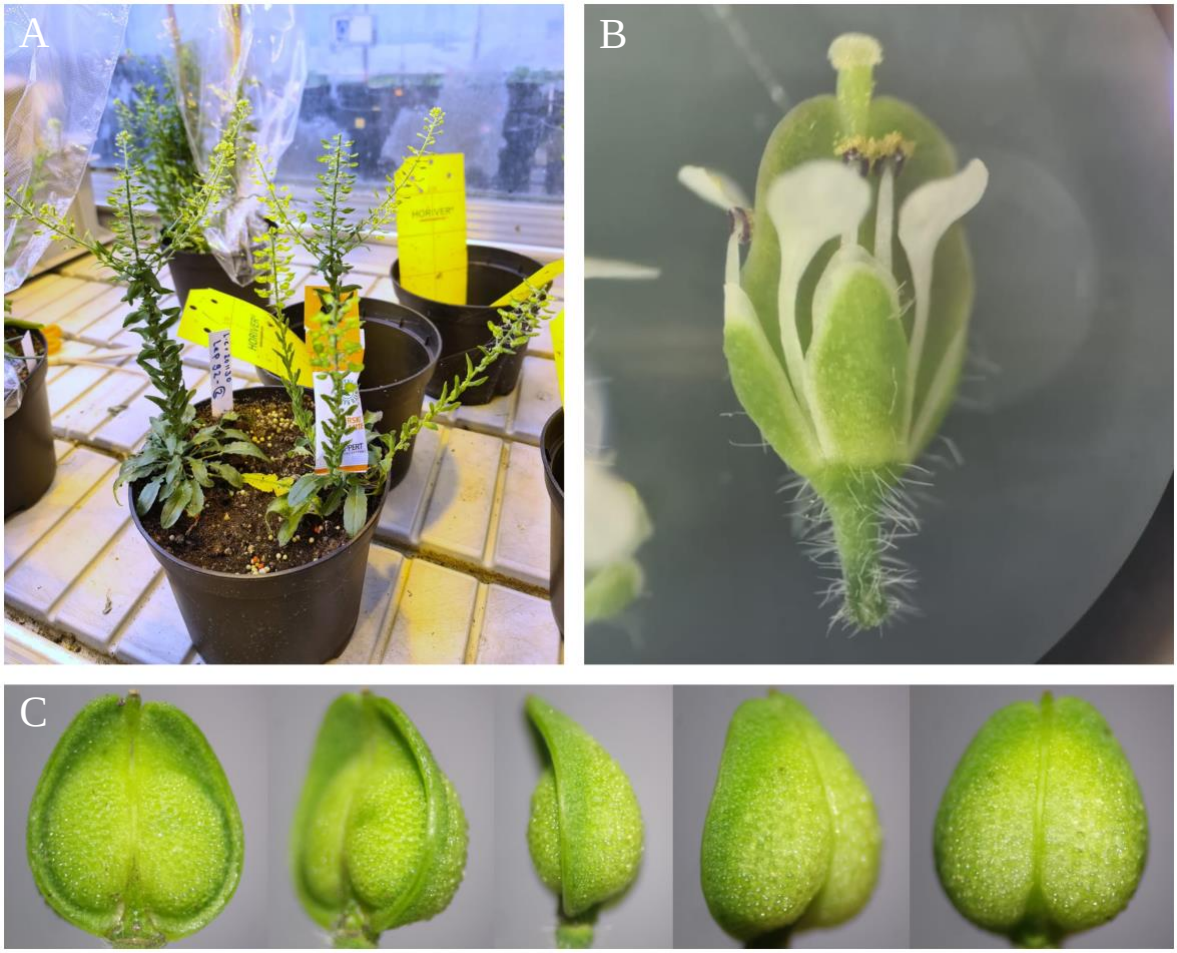

Figure 1. Pictures of *L. campestre*. (A) Potted plants in the greenhouse of the Department of plant breeding, SLU, Alnarp (Reyes). (B) Pollinated flower viewed through a stereoscopic microscope (Reyes). (C) A silicle from different angles ("Lepidium campestre" by Stefan.lefnaer / CC BY-SA 4.0)

The fatty acids dominating the oil content of field cress are mainly alpha-linolenic acid (33 – 39 %) followed by, erucic acid (22 – 25 %), while the composition of canola oil, which is the

result of extensive breeding of native rapeseed for food oil purposes, is mainly dominated by oleic acid (61 %) and linoleic acid (20 %) (table 1) (Andersson et al., 1999; Madawalaa et al., 2012; Nilsson et al., 1998).

Table 1. The fatty acid composition of oil from field cress and canola.

Oilseed crop	Alpha-linolenic (18:3)	Erucic acid (22:1)	Oleic acid (18:1)	Linoleic acid (18:2)	Eicosenoic acid (20:1)	Palmitic acid (16:0)
Field cress	33 – 39 % [a]	22 – 25 % [b]	12 – 16 % [b]	8 – 11 % [b]	5 – 6 % [b]	4 – 5 % [b]
Canola [c]	10 %	< 0.5 %	61 %	20 %	No value found	4 %

[a](Nilsson et al., 1998),
[b](Andersson et al., 1999),
[c](Madawalaa et al., 2012).

The composition of fatty acids determines what a vegetable oil can be utilized for (Nilsson et al., 1998). For example, a high content of oleic acid (18:1) is good for food, biofuel, and other industrial uses (Sandelius, 2017), erucic acid (22:1) can be used as a slipping agent and is suitable as a technical oil because of its high heat tolerance, while alpha-linolenic acid (18:3) is fast-oxidizing and can be used in paint and drying oils (Andersson et al., 1999). In Europe it is especially common to grow rapeseed cultivars that are so called “double-zero”, lines which contain close to zero levels of glucosinolates and erucic acid, for food and feed purposes (Canola council, n.d. b). For field cress to be classified as an oil suitable for human consumption in the European Union, the content of erucic acid, a non-nutritional fatty acid, must be lowered to 5 % or under (Kumar et al., 2010; Sandelius, 2017). Furthermore, the high contents of polyunsaturated fatty acids in field cress, such as linoleic acid and alpha-linolenic acid, limits the oil’s functionality in the food industry, as it makes it prone to oxidation and thus unsuitable for food processing (Ivarson et al., 2016; Sandelius, 2017). Another significant aspect is that field cress can be processed into a seed cake that could, along with rapeseed cake, increase the domestic production of animal feed and decrease the import of soybean meal, to improve the sustainability of animal production in Sweden (Arefaine et al., 2019; Johansson & Nadeau, 2007). It is, however, important to consider the levels of glucosinolates when breeding field cress for animal consumption, as high levels in the feed can exhibit harmful effects on livestock (Bischoff, 2021).

Because field cress is biennial, it can be intercropped and undersown with, for example, spring cereals (Nilsson et al., 1998). The crop also has close perennial relatives, which means that

perennial hybrids could be a possibility in the future (Gustafsson et al., 2018). What is more, undersown field cress has demonstrated the potential to enhance the yield of its fellow intercropped crop. As Merker et al. (2010) reported, growing barley with undersown field cress could improve the productivity of barley. Additionally, when the undersowing of biennials or perennials is incorporated into a cropping system, it results in both energy and labor savings for the producer. Because both crops are sown simultaneously, there is no need for tilling or sowing in between harvests. Importantly, no management between the crops is beneficial for soil structure and soil organisms, as there is minor disturbance (Kladivko, 2001). Furthermore, a reduced need for management in the cropping system also spares the surrounding environment from the pollution and ecological damage that nutrient leaching can cause during the off-season (Jensen, 1991; Jordbruksverket, 2020). Instead, the overwintering oil crop functions as a catch crop, accumulating nutrients in its biomass, more efficiently than in production systems with spring- or autumn-sown annuals (Merker et al., 2010), while also preventing soil erosion (Sandelius, 2017).

Interspecific hybridization

For *L. campestre* to be successfully established as an oil crop, there are two main traits that pose an issue. The first one is the low seed oil content, which needs to be increased (Nilsson et al., 1998), and the second one being early shattering silicles. Silicle-shattering may be a favorable trait for a wild plant to spread its seeds. On the contrary, from an agricultural point of view, early shattering silicles can cause between 10 – 50 % seed loss, as harvesting the seeds becomes impossible once they are dispersed (Eriksson, 2009). Furthermore, their germination in the field could bring more problems for the farmer (Sandelius, 2017). Desirable traits could be introgressed (transferred) through interspecific hybridization where *L. campestre* is crossed with relatives *L. draba*, which has shattering resistance (Bon et al., 2005; Mulligan and Frankton, 1962; Zinhari et al., 2017), and *L. graminifolium*, which possesses a higher seed oil content (Nilsson et al., 1998). Gustafsson et al. (2018) demonstrated in a study how *L. campestre* could successfully be hybridized with close relatives *L. heterophyllum* and *L. hirtum*, respectively. However, *L. campestre* is not as closely related to *L. draba* and *L. graminifolium* as the subjects were in Gustafsson et al. (2018)'s study. Moreover, because both are polyploid and do not share the same ploidy level as diploid *L. campestre*, they can be a challenge to cross with traditional breeding methods (Tonguç & Griffiths, 2004a). A key point

to mention is that the more distantly related plants are to one another, the greater the barrier to hybridization becomes, and could lead to disruption of fertilization or embryogenesis, and abortion of hybrid embryos (Bajaj et al., 1986; Singh et al., 2021). Thus, there is a likely risk for complications with incompatibility that calls for a new, alternative, way of hybridizing *L. campestre* x *L. draba* and *L. campestre* x *L. graminifolium* (Brown et al., 2014; Singh et al., 2021).

Successful interspecific hybridization using embryo rescuing within the *Brassicaceae* (cabbage) family, has been documented (Bajaj et al., 1986; Ripa et al., 2020; Wen et al., 2008; Zhang et al., 2003). The research regarding embryo rescuing of interspecific hybrids of *Lepidium* is, however, very limited, and only one previous experiment has been performed. In the study, Fahlgren (2014) used *L. campestre* as a female parent in the crossings with *L. draba* and *L. graminifolium* to attempt embryo rescuing on different media containing Murashige and Skoog basal salt mixture (MS) and supplements in different combinations and concentrations. The low success rate in Fahlgren's study (2014) coincided with previous studies by Ripa et al. (2020) and Tonguç & Griffiths (2004a), which indicate that higher regeneration rates are obtained when the parent with the highest ploidy is utilized as the female.

A key aspect to consider for the hybridization of *Lepidium* is that plant cells have three different types of DNA: nuclear-DNA (chromosomes), chloroplast-DNA, and mitochondrial-DNA. In addition, DNA-containing organelles, chloroplasts, and mitochondria, are mainly inherited maternally, while the primary source of paternal DNA is the nucleus (Greiner et al., 2015; Mogensen, 1996). This means that in order to produce a hybrid that possesses the majority of the *L. campestre* genome, it should be used as the mother plant, as sexual progenies (offspring) would inherit its organellar genome, while important nuclear genes (traits), could be obtained from *L. draba* and *L. graminifolium* as pollen donors. However, as seen in the previous studies by Ripa et al. (2020) and Tonguç & Griffiths (2004a), using the parent with the lowest ploidy level as the mother plant, could lead to a risk with incompatibility and a lower success rate.

Culturing ovaries and embryos at the correct developmental stage is another important factor to increase the chances of succeeding with embryo rescuing. In other words, harvesting and culturing after the adequate number of days after pollination, makes a significant impact on the success rate, and should be done before ovule degeneration (Zhang et al., 2003).

The present study aimed to generate valuable data that can be used in a protocol for the successful interspecific crossings of *L. campestre* x *L. draba* and *L. campestre* x *L. graminifolium*, through two methods of embryo rescuing on two types of media, according to previous studies by Ripa et al. (2020) and Wen et al. (2008) on other cruciferous oilseed crops.

Aim

To assess different methods to regenerate embryos of different interspecific crossings of *Lepidium*.

Research question

Is it possible to develop a method for embryo rescuing to obtain interspecific hybrids of different *Lepidium* species?

Limitations

The experiment was performed by testing three *Lepidium* species, on two culture media, in a limited number of treatments depending on the plant material that was available after the crossings were performed. This prioritized the testing of as many explants as possible given the limited time available.

Material and methods

Literature studies

While embryo rescue is not a novel tool used in plant breeding, it is a new method when attempting to create interspecific hybrids of *Lepidium*. Previous research on other commercially

established crops from the *Brassicaceae* family (Bajaj et al., 1986; Momotaz et al., 1998; Ripa et al., 2020; Sharma et al., 2017; Tonguç & Griffiths, 2004a; Tonguç & Griffiths, 2004b; Wen et al., 2008; Zhang et al., 2003) along with one study by Fahlgren (2014) where embryo rescue was attempted on *L. campestre*, provided the background to initiate this study (table 2).

Table 2. Materials and methods used to study embryo rescue in the literature.

Explants	Species	Basal medium	Supplemented compounds	Harvest	Reference
Ovaries and ovules	*Lepidium campestre* x *L. draba* and *L. campestre* x *L. graminifolium*	4.9 mg/L MS	30 g/L sucrose 0.1, 0.3, 0.5 mg/L Thidiazuron 0.01, 0.1, 0.5 mg/L 1-Naphthaleneacetic acid (NAA) 1.5 mg/L 6-Benzylaminopurine (BAP)	6 – 9 DAP	Fahlgren, 2014
Ovaries and ovules	*Brassica rapa* x *B. napus* and *B. juncea* x *B. rapa*	4.9 mg/L MS 2.45 mg/L MS		14 DAP	Ripa et al., 2020
Ovaries and ovules	*B. oleracea* x *B. juncea*	4.9 mg/L MS	50, 30 g/L sucrose 500 mg/L casein hydrolysate (CH)	Ovaries 5 – 7 DAP Ovules 5 – 10 DAP	Tonguç & Griffiths, 2004b
Ovaries and embryos	*B. rapa* ssp. *Oleifera* x *B. oleracea* var. *Acephala* and *B. oleracea* x *B. rapa*	4.9 mg/L MS 2.45 mg/L MS	Ovaries 30 g/L sucrose, 0.2, 3 mg/L BAP, 0.1 mg/L NAA Organic components of NLN	Ovaries 4, 7, 10, 14 DAP Embryos 10 – 25 DAP	Wen et al., 2008
Ovaries and ovules	39 cross combinations of *Brassica* and *Sinapis* (*arvensis, turgida, alba*)	White's Gamborg's B5	Ovaries 300 mg/L CH Ovules 2.5 mg/L NAA 2.5 mg/L kinetin 150 ml/L coconut milk	Ovaries 7 – 10 DAP Ovules 15 – 20 DAP	Momotaz et al., 1998
Ovaries and ovules	*B. juncea* x *B. hirta* ("syn. *Sinapis alba*)	4.9 mg/L MS White's	2.0 mg/L Indole-3-acetic acid (IAA) 0.5, 2.5 mg/L kinetin 0.5 mg/L NAA 150 mg/L coconut milk 300, 500 mg/L CH	Ovaries 4 – 9 DAP Ovules 10 – 15 DAP	Mohapatra & Bajaj, 1985
Ovaries and ovules	*B. napus* x *B. juncea* and *B. juncea* x *B. napus*	4.9 mg/L MS White's	IAA Kinetin CH Coconut water	Ovaries 2 – 9 DAP Ovules 7 – 12 DAP Embryos 10 – 14 DAP	Bajaj et al., 1986
Embryos	*B. napus* x *B. juncea* and their reciprocals	4.9 mg/L MS Gamborg's B5	30 g/L sucrose 0.1, 0.2, 0.3 mg/L NAA 1.0, 1.5, 2.0 mg/L BAP	10 – 15 DAP	Zhang et al., 2003

Plant material

The plant material included in the experiment was provided by Associate Professor Mulatu Geleta Dida from the department of plant breeding at SLU Alnarp and consisted of research/breeding lines of *L. campestre* (collected or gene bank accessions), gene bank accessions of *L. graminifolium*, and collected accessions of *L. draba*.

Crosses

Flower buds, ready to bloom within 1 – 2 days, were emasculated and manually cross pollinated by Associate Professor Mulatu Geleta Dida, under a stereoscopic microscope, and covered with a bag to avoid unwanted pollination. Caution was taken to not self-pollinate the flowers during emasculation.

Ovary culture following Wen et al. (2008)

Because of the risk of incompatibility in the crosses, degeneration could occur at some point during the seed-development, making the success rate unpredictable. Therefore, silicles of different developmental stages were chosen for the experiment (Bajaj et al., 1986). Silicles were harvested 6 – 7 days after pollination and surface sterilized in Falcon, 50 mL tube with 70 % ethanol for 30 seconds, then rinsed with 0.3 % calcium hypochlorite for 7 minutes and rinsed 5 times with autoclaved Milli-Q water. The harvest of the silicles was opted for different developmental grades, thus harvesting on 3 different days, as the success rate may vary. The ovaries were cut transversely above the base of the stalk on autoclaved filter paper in a laminar flow hood under a stereoscopic microscope and placed with the basal cut end on Petri dishes with medium E (figure 2; table 3). The ovaries were then placed in a growth chamber with a photoperiod (light period) of 16 hours at 33 μmol m^{-2} s^{-1} and a temperature of 21/18 °C (day/night). The cultures were regularly observed and Petri dishes with signs of contamination were removed to avoid further contamination. 35 days after the start of the ovary culture, the ovaries were taken out from the media and were aseptically excised on autoclaved filter paper, in a laminar flow hood under a stereoscopic microscope.

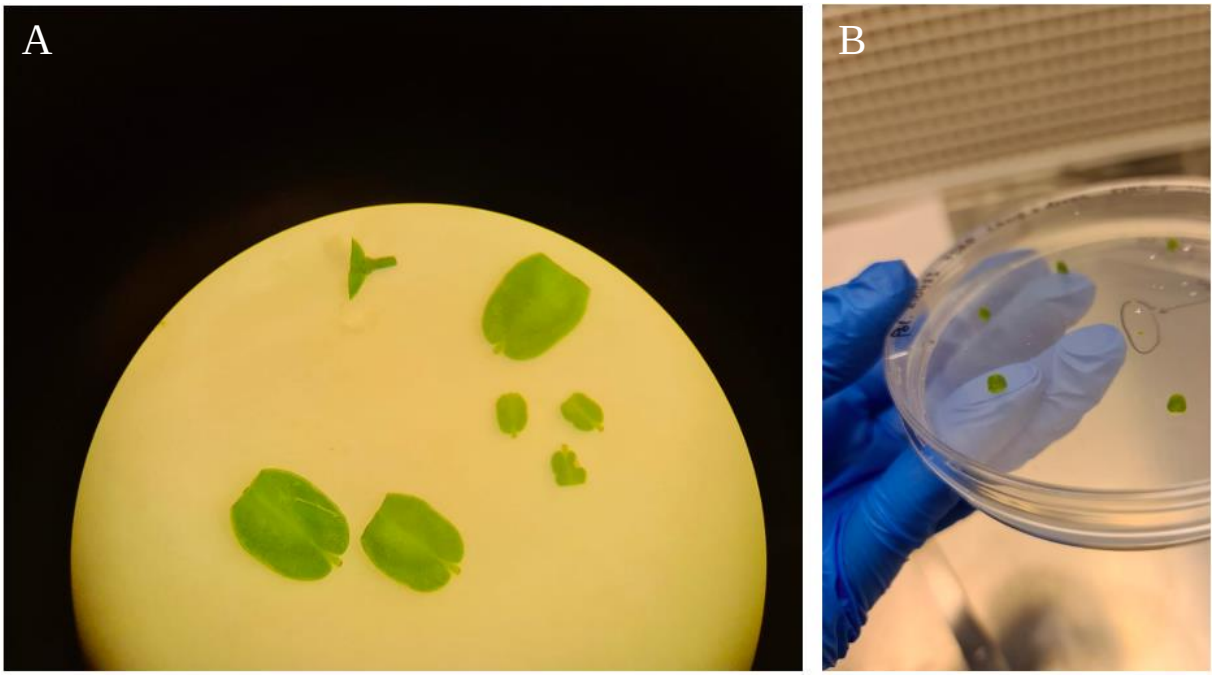

Figure 2. Harvested silicles (ovaries). (A) Ovaries with the bases of the stalks cut off, viewed through a stereoscopic microscope. (B) Ovaries placed on medium E with the basal cut end down (Reyes).

Table 3. The compositions of the different media tested. Medium E was used for ovary culture while Embryo culture medium was used for direct embryo culture (Wen et al., 2008).

Composition	Medium E	Embryo culture medium
MS (g/L)	2.45	4.9
Sucrose (g/L)	30	30
BAP (mg/L)	-	0.2
Agar (g/L)	8	8

Direct embryo culture (control experiment)

A control experiment was performed to test the efficiency of Wen et al. (2008)'s embryo culture medium (table 3), as well as to practice embryo excision and culture at 14 DAP, before attempting direct embryo culture on the crossed plant material.

Flowers from a branch on a *L. campestre* plant were self-pollinated manually and flowers from a second branch were plucked off with forceps, only leaving flowers around the same developmental stage as the manually self-pollinated flowers.

Silicles from *L. campestre* were harvested circa 14 days after self-pollination and surface sterilized in a Falcon, 50 mL tube with 70 % ethanol for 30 seconds, then in 0.3 % calcium

hypochlorite for 7 minutes, and rinsed 5 times with autoclaved Milli-Q water. Ovules were aseptically excised and naked embryos were taken out on autoclaved filter paper, in a laminar flow hood under a stereoscopic microscope. They were then cultured in a Petri dish with embryo culture medium and maintained in a growth chamber with a photoperiod (light period) of 16 hours at 33 μmol m^{-2} s^{-1} and a temperature of 21/18°C (day/night) (figure 3).

Direct embryo culture following Ripa et al. (2020) and Wen et al. (2008)

Silicles were harvested at 11, 12, 14, and 17 DAP and surface sterilized in Falcon, 50 mL tubes as above. The harvest of the silicles was done on different days to investigate if the developmental stage would influence the success rate. Ovules were aseptically dissected on autoclaved filter paper in a laminar flow hood under a stereoscopic microscope.

Collection of data

The effect of embryo rescuing was observed through the number of embryos obtained after ovary collection and/or cultivation.

Results

Ovary culture

A total of 69 ovaries were harvested from *L. campestre* x *L. draba* crosses and cultured at 6, 7, and 8 DAP (table 4). 27 ovaries were removed from the cultures due to fungal infections, thus only 42 ovaries were cultured for at least 35 days. A variation in ovary-sizes, and developmental stages, was observed within each day of harvest. The ovaries harvested 6 DAP were overall small in size, while the ones harvested 7 and 8 DAP included a notable number of ovaries that were bigger and showed swellings due to ovule-growth.

Throughout the cultivation, all ovaries swelled up, turned yellow and started browning. When the ovaries were taken out of the petri dishes, on day 35 of cultivation, they had a leather-like texture which tended to crumble when cut through. However, most ovaries were hollow, and only a small amount contained ovules that had started to develop but degenerated (figure 3). No embryos were obtained.

Table 4. All crosses made using *L. campestre* as mother plant and *L. draba* as pollen donor for ovary culture.

Crosses	DAP	Harvested ovaries (excl. infections)	Days of incubation after harvest	Developing ovules
L. campestre x *L. draba*	6	6	35	0
L. campestre x *L. draba*	6	2	35	4
L. campestre x *L. draba*	7	8	35	0
L. campestre x *L. draba*	7	2	38	0
L. campestre x *L. draba*	7	15	35	0
L. campestre x *L. draba*	8	9	38	5
	Total:	**42**		**9**

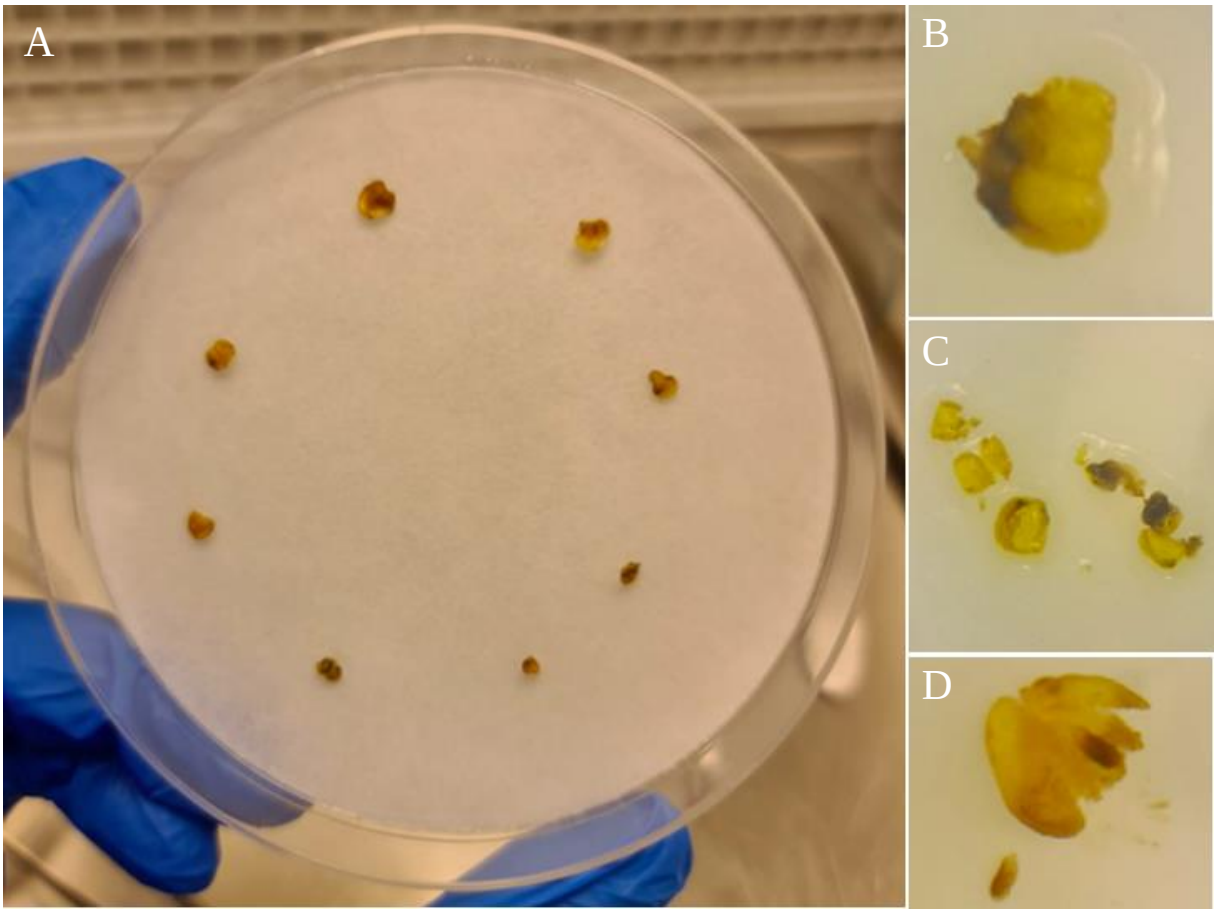

Figure 3. Ovaries of *L. campestre* x *L. draba* after 35 days of culture. (A) Ovaries before examination. (B) Swollen ovary appearing to contain grown ovules. (C) Ovary B after incision showed no signs of developing ovules. (D) Ovary which contained two ovules that seemed to have initiated development but degenerated (Reyes).

Direct embryo culture (control experiment)

The manually self-pollinated flowers from *L. campestre* produced seven ovaries whereof three ovules grew, but only one contained an embryo, which was at cotyledonary stage (table 5). Furthermore, only one embryo developed from the ovaries of the naturally self-pollinated flowers (numbers not recorded). The embryo was also in the cotyledonary stage of development, and started elongating and the cotyledons grew, when cultured on embryo culture medium (figure 4, figure 5).

Table 5. Self-pollinated *L. campestre* used as control for direct embryo culture on embryo culture medium.

Mother-plant	DAP	Harvested ovaries	Developing ovules	Embryos
L. campestre (manually self-pollinated)	14	7	3	1
L. campestre (naturally self-pollinated)	14	-	1	1
	Total:	**7**	**4**	**2**

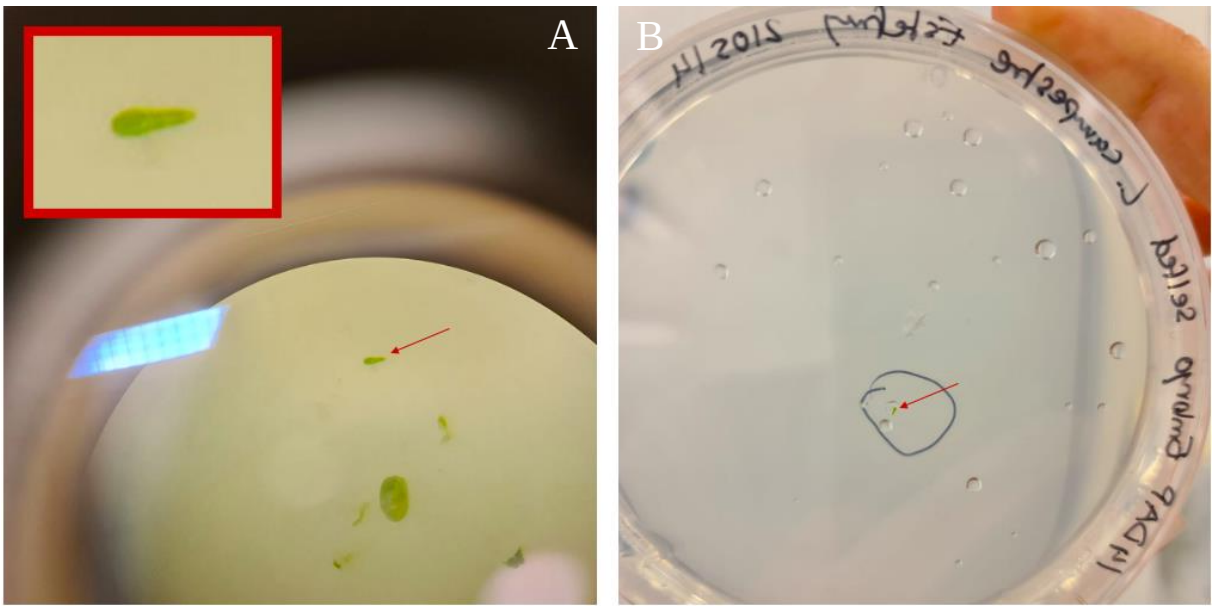

Figure 4. Embryo from the self-pollinated *L. campestre* control. (A) Excised cotyledonary embryo viewed through a stereoscopic microscope. (B) Cotyledonary embryo A on embryo culture medium (Reyes).

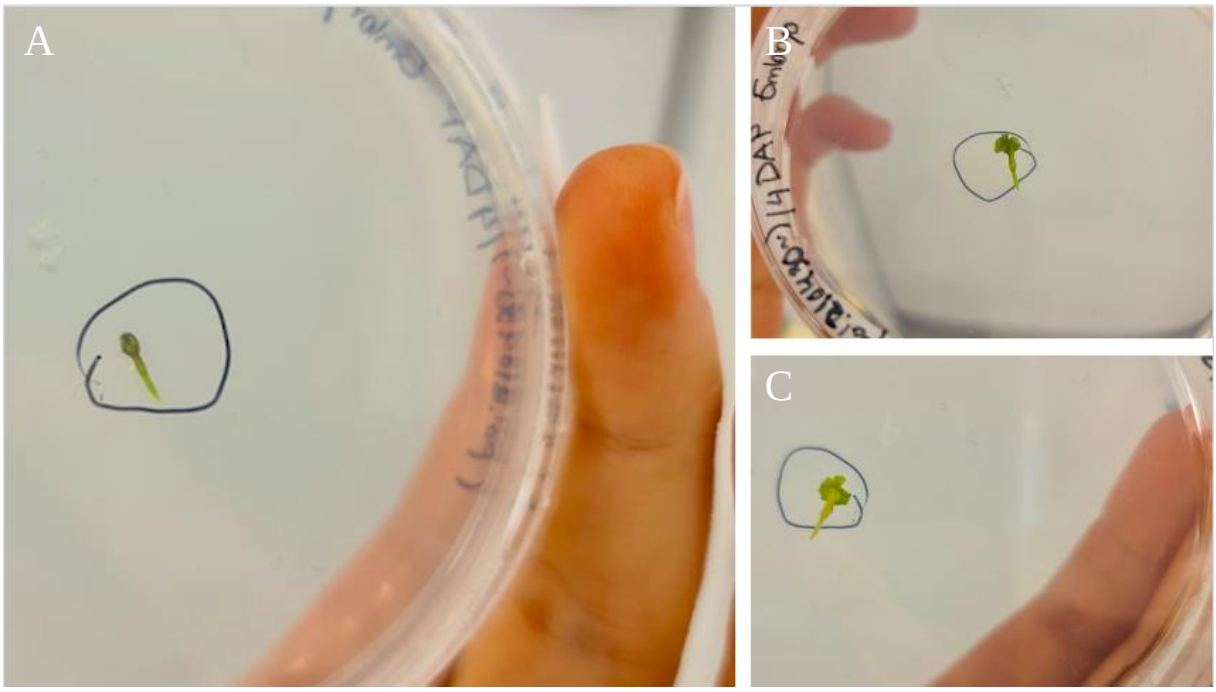

Figure 5. Growing embryo from the self-pollinated *L. campestre* control. (A) Embryo at 6 days of culture. (B) Embryo at 12 days of culture. (C) Embryo at 26 days of culture (Reyes).

Direct embryo culture

Eight crosses were made between *L. campestre* and *L. draba* from which a total of 75 ovaries were obtained. Two additional crosses were carried out between *L. campestre* and *L. graminifolium*, and 50 ovaries were obtained. The ovaries were harvested and excised at 11, 12, 14, and 17 DAP (table 6).

All crosses looked healthy and seemed to develop properly as they grew normally on the mother plants. However, no embryos were obtained when the ovules were excised and examined. The ovules produced by the *L. campestre* x *L. draba* crosses were mainly yellow or brown and dry, while a few were turgid (plump due to being full of liquid) and displayed green contents but proved to only contain a clear liquid with an indistinguishable green mass. On the other hand, only a handful of ovules from the *L. campestre* and *L. graminifolium* crosses were yellow, brown, and dry, while the majority of the ovules were turgid and contained a clear liquid (figure 6).

Table 6. All crosses made using *L. campestre* as mother plant and either *L. draba* or *L. graminifolium* as pollen donor for direct embryo culture.

Crosses	DAP	Harvested ovaries
L. campestre PL-52-1 x *L. draba*	11	4
L. campestre Trelleborg 7-1-52-1 x *L. draba*	11	15
L. campestre AA x *L. graminifolium*	12	20
L. campestre Trelleborg 7-1-52-1 x *L. draba*	14	11
L. campestre PL-52-2 x *L. draba*	14	10
L. campestre Xx x *L. draba*	14	14
L. campestre 94-2 x *L. draba*	14	4
L. campestre 94-2 x *L. draba*	14	1
L. campestre 92-2 x *L. draba*	17	16
L. campestre AA-02 x *L. graminifolium*	17	30
	Total:	**125**

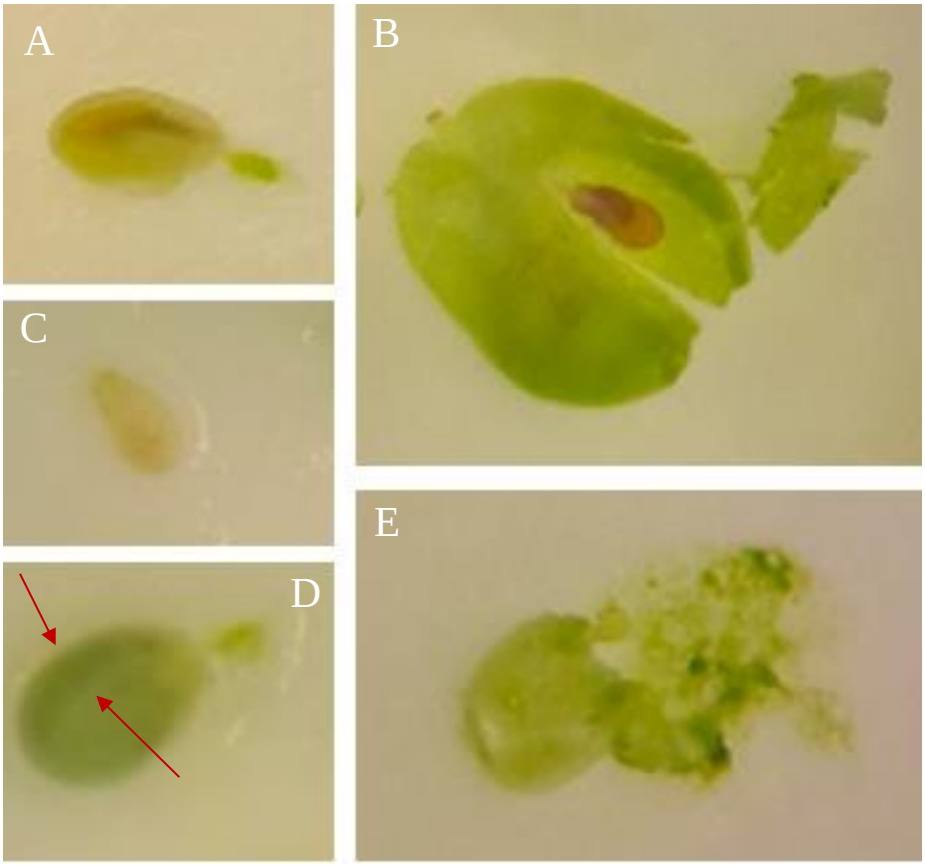

Figure 6. Harvested ovules. (A) Ovule that appears to contain a browned embryo. (B) Ovary containing browned ovules which had initiated development. (C) Yellowed, dry ovule. (D) Plump, green ovule with green contents that have the shape of an embryo. (E) Ovule D with indistinguishable green mass leaking out after an incision was made (Reyes).

Discussion

Because of the ongoing climate change, there is a strong interest for renewable vegetable oils to replace oils and fuels of fossil origin (Merker et al., 2010). What is more, with a growing world population and significant ecological damage caused by extensive palm and soybean oil production, there is a demand for more sustainable food oil alternatives (Colombo et al., 2018; Hospes, 2014; WWF, 2020). *L. campestre* is currently under domestication as a new potential high-yielding oil crop for cold climate conditions (Eriksson, 2009; Ivarson et al., 2013; Merker and Nilsson, 1995; SLU, 2020). However, problems with early shattering silicles and low oilseed content have sparked the search for ways to hybridize it in order to transgress shattering resistance and higher oilseed levels from *L. draba* and *L. graminifolium*, respectively (Bon et al., 2005; Mulligan and Frankton, 1962; Nilsson et al., 1998; Zinhari et al., 2017). Moreover, because they are not closely related, and have different ploidy levels, they have a high risk of being incompatible, which makes them difficult to cross with traditional breeding methods (Brown et al., 2014; Ripa et al., 2020; Singh et al., 2021; Tonguç & Griffiths, 2004a). Embryo rescuing is a method that has proven to be successful in facilitating interspecific hybridization within the *Brassicaceae* family (Ripa et al., 2020), however, attempts at embryo rescuing *Lepidium* have been limited and unsuccessful (Fahlgren, 2014; Merker et al., 2010).

In this experiment, two in vitro techniques to mitigate post-fertilization factors that could lead to a failure to obtain interspecific hybrids of *L. campestre* x *L. draba* and *L. campestre* x *L. graminifolium*, were tested. Ovary culture of 6 – 8 DAP ovaries was performed on hormone-free half-MS medium (medium E). Additionally, direct embryo culture at 11, 12, 14, and 17 DAP embryos was attempted on full-MS medium enriched with BAP (embryo culture medium), but no embryos were obtained.

In addition to studying hybrid ovaries and embryos, a control was performed on naturally self-pollinated 14 DAP *L. campestre* ovules, where one of two embryos found was cultured on embryo culture medium, and growth was observed. While medium E did not appear to promote the growth of interspecific hybrids of *L. campestre*, the results suggested that embryo culture medium stimulated the normal development of naturally self-pollinated *L. campestre* embryos. Furthermore, the successful excision and culture of the control embryo, indicated that 14 DAP was an appropriate number of days for the development of a viable embryo, although, the time may be different for an interspecific hybrid embryo. Additionally, the control demonstrated that an embryo could survive being excised and transferred to a culture vessel after 14 DAP, but

also that the correct organ (an embryo) could be accurately identified and cultured without damage.

Because of its lower ploidy level, it could be speculated that the use of *L. campestre* as a mother plant was one of the causes for the failure of the regeneration of interspecific hybrids of *L. campestre* x *L. draba* and *L. campestre* x *L. graminifolium*. Compared to *L. draba* and *L. graminifolium*, which had higher ploidies, *L. campestre* had lower prospects of success as a pollen receiver in the crosses (Ripa et al., 2020; Tonguç & Griffiths, 2004a). Furthermore, it may therefore be argued that the chances of producing successful embryos would have been higher if reciprocal crosses had been performed. In other words, a higher regeneration rate could have been obtained if the crosses had been made with the sexes reversed as well (Gai & He, 2013), adding following crosses to the study: *L. draba* x *L. campestre* and *L. graminifolium* x *L. campestre*. This was, however, not practical with the available plant material, as a larger number of *L. campestre* plants were available and flowered more than *L. draba* and *L. graminifolium*. Additionally, the flowering of the plants was not as predictable as expected, resulting in *L. draba* and *L. graminifolium* flowering very late on in the experiment, underlining their impracticability as mother plants for this experiment. Nonetheless, because no organelle-DNA is transferred from pollen donors, achieving a hybrid with predominantly traits from *L. campestre*, without utilizing it as a mother plant in the cross would have been a difficult task (Greiner et al., 2015; Mogensen, 1996). Most significantly, the very first obstacle to overcome before introgression, expression, and selection of desirable traits could be considered for a cross, is obtaining a viable hybrid.

As far as the difficulties with hybrid incompatibility are concerned in this experiment, they could have been external and caused by cross incompatibility. On the other hand, they could also have been internal, due to hybrid inviability (Singh et al., 2021). Other, limiting factors could have been the use of old pollen and/or flowers, which could have caused further incompatibility. Harvesting too early or too late could also have been determinant factors contributing to the embryo rescue being unsuccessful, as embryo development might not have started or have already degenerated. Conversely, this hypothesis clashes with the results from the self-pollinated *L. campestre* controls. This is because, although most of the control-ovules contained an undistinguishable green mass, some of them had developed embryos at cotyledonary stage at 14 DAP, which, according to Ripa et al. (2020), is the developmental stage at which embryo regeneration has the best chance of succeeding. However, the Ripa et al. (2020) study was performed on *Brassica rapa*, *B. juncea* and *B. napus*, hence the optimum

time could be different for the *Lepidium* hybrids in this experiment. Since the success rate of the self-pollinated controls were proven to be low at 14 DAP it may be indicating just that, or that the natural success rate of embryo/seed generation in *L. campestre* is naturally low (Singh et al., 2021).

Even though all seeds failed embryo development and/or to reach maturity, the way in which the two interspecific hybrids failed, seemed to differ. When making the crosses, false indicators of positive development were seen in the growth of the ovaries. Bigger ovaries with swollen ovules appeared to be developing but when extracted, ovules that had not dried up, had turned yellow or brown, which coincided with previous studies by Fahlgren (2014). Other ovules looked either empty or appeared as if their contents were green and embryo shaped. Yet, when incisions were made, liquid with an indistinguishable green mass, leaked out, and no solid embryos were found. The results showed that many ovules with *L. draba* as pollen donor contained, the mentioned, indistinguishable mass which indicated to that either embryo or endosperm had started developing but had been aborted or failed at some point. On the contrary, the clear liquid contents of the ovules which had *L. graminifolium* as pollen donor, suggested that they had failed before the onset of embryo or endosperm development. By the same token, the failure of embryo development could lie in pre-fertilization factors, such as pollen grains not germinating on the stigma of *L. campestre*, pollen tubes not growing down the style or not being directed towards the ovule. Moreover, it could also depend on an inability of male gametes to fuse with the egg, or a failure in the fusion of the nuclei from pollen and egg (Brown et al., 2014). Therefore, methods of interspecific hybridization that can be implemented pre-fertilization, would be good to include in future studies. Examples of such are reciprocal crosses with *L. draba* and *L. graminifolium*, respectively, and doubling the ploidy level of *L. campestre* using colchicine before performing the interspecific hybridization (Singh et al., 2021). Another suggestion is to reduce the ploidy level of the related pollen donors (making them di-haploids if they are tetraploids) and doubling the ploidy level of the hybrid cross using colchicine or spontaneous doubling resulting from in vitro culture. Finally, if sexual crossing proves to be unachievable, creating somatic hybrids by fusing protoplasts, could be attempted (Brown et al., 2014).

Another significant aspect is that most of the ovaries from *L. campestre* x *L. draba* crosses that were collected at a later stage, aimed for direct embryo culture, contained underdeveloped ovules, however, some with signs of an initiated development. Notably, the fact that some ovules had started to develop, could be a positive indication of fertilization having been

successful. Hypothetically, it could be that these ovules might have had a chance to survive if they had been excised earlier. For this reason, it could be suggested that ovary culture has the potential to rescue embryos that would have been too small for extraction right before their degeneration. It is, however, important to emphasize that if the crosses failed the initial fertilization, neither ovary culture, nor a longer growth in situ (on the mother plant) would have helped the promotion of embryo growth.

Because embryo rescue works by replacing the natural endosperm which may have been aborted or not developed due to incompatibility in a cross (Brown et al., 2014), it could be that the attempted ovary culture failed due to the media composition being unsuitable for embryo rescue of interspecific hybrids of *L. campestre* and *L. draba*. For example, it might have created unfavorable osmotic potential conditions for the development of the embryos (CABI, 2018). Nevertheless, the fact that all ovules failed to produce embryos when the ovaries were cultured suggests that ovary culture should be tested other media-formulations, to increase the chances of succeeding in future research. Furthermore, because the embryo culture medium had a positive effect on the control embryo culture, possibly because of its contents of BAP (a synthetic growth promoting plant hormone) (Miller et al., 1956; Wong et al., 2016), its composition should be considered as a possible starting point for further experiments on ovary culture.

Conclusions

The use of a culture medium with full-MS, 30 g/L sucrose and 0.2 mg/L BAP proved to be successful for direct embryo culture of self-pollinated *L. campestre* and should be regarded as a good starting point for future experiments.

More extensive documentation of the number of pollinated flowers, developing ovaries and ovules, as well as plump ovules, brown, dried, and non-developing ovules, should be carried out in order to assess the results more thoroughly.

It was not possible to determine what the cause of unsuccessful seed development was in this study. Therefore, experiments with a variety of media compositions, reciprocal crosses, and pre-fertilization methods, are recommended for future research.

In conclusion, more research is needed to successfully develop a method for embryo rescuing to obtain interspecific hybrids of *L. campestre* x *L. draba* and *L. campestre* x *L. graminifolium*.

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