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In vitro germination and embryo rescue of *Lepidium campestre* hybrids

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Abstract

Lepidium species are members of the Brassicaceae family, in which some commercial oil crops belong to and these species are wild. In an effort for domesticating a new oil crop that started about 20 years ago, *Lepidium campestre* has been chosen as a target as it is cold hardy and has high seed yield potential. However, *L. campestre* contains low oil content and has pod shatter problem. To solve these problems, crosses between different *Lepidium* species have been previously carried out. In this project, a laboratory study with the aim of facilitating the germination of *L. campestre* x *L. draba* hybrid seeds in vitro and embryo rescue of hybrids between *L. campestre x L. graminifolium* was conducted. Different types of medium were tested both for germination and for embryo rescue. There was no significant difference in the germination rate between nine different germination media when using the old storage seeds of *L. campestre*. The hybrid seeds did not germinate on the germination media tested. In the embryo rescue experiment, one of the parental ovule grew on the medium containing 0.1mg/l NAA and 1.5mg/l BAP. Several ovules of parental and hybrid seeds turned brown on the other media including the abovementioned medium. Two hybrid ovules produced callus on the medium containing 0.5 mg/l TDZ and 0.01mg/l NAA and one grew on the medium containing 0.1mg/l TDZ and 0.5mg/l NAA. The hybrid ovaries grew larger after 7 days, four of them become infected with fungi after 16-17 days and were therefor removed, several of the parental ovaries produced callus on their basal end.

Acknowledgements

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Content

Introduction

Background

Development of new oilseed crops is important since the energy is mainly produced from fossil oil, which has a limited resource and will eventually be depleted. Moreover, the world population is increasing that will need more energy and food oils. Therefore, the demand of plant oil production for both industrial uses and food purposes will increase in the future (Carlsson et al., 2011). This increased demand in plant oil will not be possible to meet with today's limited oil crops, which are mainly used for food purpose. Thus, new oil crops need to be developed (Carlsson et al., 2011). Moreover, the six most commonly used oil crops today are palm, soybean, rapeseed, sunflowers, peanuts, and cottonseed and except for rapeseed and none of them are economically viable to grow in Sweden (Mistra Biotech Annual Report 2013). Therefore, it is important for Sweden to develop new oil crops that are cold hardy to be able to increase the production of plant oil (Mistra Biotech Annual Report 2013).

Different *Lepidium* species

Seeds of *Lepidium* species are rich in oil and they are members of the Brassicaceae family. The three different *Lepidium* species used in this work are *L.campestre* (*Field cress*); *L. draba; L. graminifolium. L. campestre* has a morphological form that is similar to that of cultivated crops. It is biennial and cold hardy (Nilsson et al,. 1998). This makes *L. campestre* a suitable candidate to be a new potential oilseed crop in Sweden. Its cold hardy nature will ensure it to grow in the northern parts of Sweden. Its biennial character makes it possible to be sown with cereal crops as a catch crop and cover the soil after the cereal crop has been harvested, since it will produce its seeds the next year (Mistra Biotech Annual Report 2013). Problems with *L. campestre* are that the seed oil content (about 20%) is only about half of that of winter rapeseed and pod shatter that can result in a seed loss of up to 50% (Mistra Biotech Annual Report 2013). Moreover, the seed oil of *L. campestre* is composed of linolenic acid 33%-39% (Nilsson et al., 1998) and 25% erucic acid, while erucic acid

is a non-nutritional fatty acid and the maximum allowed amount of erucic acid in food oil is 5% according to EU regulations (Mistra Biotech Annual Report 2013).

Compared with L. campestre, *L. graminifolium* has more than 37% seed oil content with high linolenic acid content and almost no erucic acid (Nilsson et al., 1998). However, the species has smaller seeds than that of *L. campestre* that makes it less suitable to be used as an oilseed crop.

L. draba is a perennial species with a deep root system and most of its pods do not shatter when the seeds ripen (Mclnnis et al., 2003). In addition, it has a horizontal root system that can produce shoots from buds for reproduction (Mclnnis et al., 2003).

In the ongoing Mistra project, one of the goals of with *L. campestre* is to increase the oil content and decrease the erucic acid content in the seeds (Mistra Biotech Annual Report 2013). Therefore, crosses between *L. campestre and L. graminifolium* have been previously carried out, but the hybrids did not germinate. In addition, crosses between *L. campestre* and *L. draba* were made to try to solve the pod shatter problem of *L. campestre*. However, the hybrid seeds did not germinate either. Therefore, a good method facilitating the germination of the hybrid seeds needs to be developed.

Embryo rescue

Interspecific hybrid embryos often develop poorly or stop growing at an early stage of seed development accompanied by the pod falloff. This often causes the failure of interspecific hybridyzation. Embryo rescue methods could therefore be used to produce plants from such hybrids where the seeds will not grow due to some hinders of embryo development. Embryo rescue has been successfully used in hybrids of *Brassica* species even if the success rates were low (Zhang et al., 2003; Tonguç and Griffiths 2004). There are several different embryo rescue methods. One is to grow ovaries directly on medium (e.g. Mohapatra' and Bajaj 1987; Momotaz et al., 1998). It is also possible to grow excised ovules on medium both from ovaries previously grown on medium and from ovaries taken from the plant to make them germinate or

produce calli, preferably germinating since getting plants from calli needs more additional work (Momotaz et al., 1998; Tonguç and Griffiths 2004).

Aim

The aims of this study were to 1) get seeds from hybrid crosses *L. campestre* and *L. draba* to grow or germinate in vitro, and 2) to help hybrid seeds of *L. campestre* and *L. graminifolium* to grow into plants through embryo rescue techniques.

Abbreviations:

BAP= 6-benzylaminopurine. DAP = Days after pollination. GA3= Gibberellin acid IAA= Indole-3-acetic acid. IBA = Indole-3-butyric acid. MS = Murashige and Skoog basal salt. NAA= Naphthylene acetic acid. Kin =Kinetin. TDZ= Thidiazuron. 2,4-D = 2,4- dichlorophenoxyacetic acid. 2,4,5-T= 2,4,5-Trichlorophenoxyacetic acid.

Materials and methods

Media used for seed germination and embryo rescue

Since there is no report available for growing hybrid seeds and embryo rescue in *Lepidium* species available, we have tested nine different media (named A-F) containing different types of plant growth regulators and combinations. These media contain the same basal medium MS supplemented with different amount of sucrose (Table 1 and Table 2). The media were adjusted to pH 5.6 before addition of agar and autoclaved using standard program. GA_3 was filter-sterilised and added after autoclaving because it is not heat stable. The culture containers were Petri dishes with 50mm diameter.

Table 1 The compositions of different media used for germination test

Composition	1	2	3	4	5	6	7	8	9
MS (mg/l)	2.45	2.45	2.45	2.45	2.45	2.45	2.45	2.45	2.45
GA3 (mg/l)	1	0.5	-	0.5	1	-	0.5	1	-
Sucrose (g/l)	-	-	0.5	0.5	0.5	10	10	10	-
Agar (g/l)	7	7	7	7	7	7	7	7	7

Table 2 The types and amount of plant growth regulators (PGR) used in different media (A-F) in the embryo rescue experiments. The basal medium is half strength of MS

PGR	A	B	C	D	E	F
MS (mg/l)	4.9	4.9	4.9	4.9	4.9	4.9
TDZ(mg/l)	0.1	0.1	0.3	0.5	0.5	-
NAA(mg/l)	0.01	0.5	0.1	0.5	0.01	0.1
BAP(mg/l)	-	-	-	-	-	1.5
Sucrose(g/l)	30	30	30	30	30	30
Agar (g/l)	6.5	6.5	6.5	6.5	6.5	6.5

We have chosen to test ½MS with different concentrations of GA3 and sucrose in this study since positive results with GA3 combined with ½ MS and sucrose have been reported (Table 3).

Table 3 Seed germination media for various species reported in the literature

Species	Basal medium	Compounds supplimented	Reference
Oncidium flexuosum Sims	MS ½	Sucrose 0.088m pH5.7	Galdiano et al., (2013).
Brassica oleracea	MS ½	IBA 0.2mg/l 1% sucrose	Noguchi et al., (1992).
Arabidopsis thaliana	MS+ Arabidopsis vitamin	Sucrose 1%Wt/vol) pH 5.6	Sophia Chen and McCormick (1996)
Annona cherimola Mill	MS and W and DW	GA3 0, 2.89, 5.77, 8.67µM sucrose 8764 mM pH5.8	Padilla and Encina (2003)
Carica papaya L	MS	BAP 1-20µM/l NAA 1-20µM/l TDZ 1-20µM/l 2,4-D 1-20µM/l 2,4,5-T 1-20µM/l pH 5.8	Bhattacharya and Khuspe (2001)
Musa velutina	MS ½	0, 0.1, 10, 100 µM GA3 pH 5.8 2% (wt/vol) sucrose	Pancholi et al., (1995)
Calanthe siebldii	MS and (MSH) and Hyponex medium	2% (wt/vol) sucrose pH5.5	Park et al., (2000)
Gossypium hirsutum	MS	0.1-1.0mg/l BAP 0.02-0.4mg/l GA3 30g/l sucrose pH 5.7–5.8	Ganesan and Jayabalan (2004)
Cymbidiym gigabteum	MS M KS Pytamax	0, 1.0, 2.0mg/l peptone 0, 1.0, 2.0mg/l BAP 0, 1.0, 2.0mg/l 2,4-D	Hossain et al., (2010).

The media chosen in this study were also based on precious studies (Table 4) where MS was often used as a basal medium, supplemented with different PGRs.

Table 4 Media used for embryo rescue studies in the literature

Species	Explants	DAP	Basal medium	Hormones, sucrose and pH	Reference
Hylocereus	ovules	5	0.5 MS	NAA 0.5µM TZD 0.45 µM Sucrose 0.17m pH 5.8	Cisneros and Tel-Zur (2010)
Lilium spp	ovules	3.5 and 10	B5 and 0.5 B5	Sucrose 3% and 9% pH 5.7	Ikeda et al., (2003)
brassica napus and B.juncea	embryo	7 10 and 5	MS	NAA 0.3mg/l BAP 1.5 or 2.0 mg/l Sucrose 3 % pH 5.8	Zhang et al., (2003)
brassica juncea and B.hirta	ovaries and ovules	7-10	Whites and MS	IAA in ms 2mg/l Kin 0.5mg/ CH 300 mg/l in w And in 500 mg/l ms	Mohapatra' and Bajaj (1987).
Seven *brassica* species and three *sinapis*	ovaries and ovules	Ovaries 7-10 Ovules 15-20	Whites and B5	W Caseinhydrolysat 300mg/L B5 kinetin 2.5mg/l Coconut milk 150ml/l	Momotaz et al., (1998).
oleracea L. and Brassica accession	ovaries and ovules	5-7	MS	Caseinhydrolysat 500mg/l sucrose 5 % pH 5.7	Tonguç and Griffiths (2004)

Seed disinfection for the germination test

All the seeds used in this study were disinfected using the standard procedure used in the lab. Briefly, the seeds were put in 3% Ca-hypochlorite solution with Tween20 on a shaker for 15 minutes. They were then handled in a sterile bench by removing the disinfection solution, followed by 5-6 rinses with sterile water to get rid of the chlorine. The chlorine solution was filtered and kept in dark since the active compound in the solution is light sensitive.

Germination test using the old control seeds

The germination medium test was conducted prior to the seed germination trial of on the hybrid crosses of *L. draba and L. campestre* using the old seeds of *L. campstre.* The hybrid seeds used in this experiment are listed in Table 5. As stated earlier that 9 media were used in this test with four Petri dishes for each medium. Twenty seeds were sown on each Petri dish. The Petri plates were sealed with surgeon tape and placed in a growth chamber with 16h photoperiod, 33 µmol light intensity and $21/18^0$C (day/night) temperature.

Germination test on hybrid seeds

For the germination of the hybrid seeds media 5 and 8 were used (Table 1). Six different kinds of hybrid seeds and the control seeds (Table 5) were sown on Petri plates. For the seeds S2-S6, two Petri plates of medium 8 with five seeds in each were used due to seed limitation. Seed S1 had four Petri plates of medium 8 and two of medium 5 with five seeds in each. The control got two plates of both media with six seeds in each. All the Petri plates were sealed with surgeon tape and placed in the growth chamber.

Table 5 The hybrid seeds used in the germination test, Em stand for emasculated and Po for pollinated

Seed	Hybrid	Date for hybrid crosses and collection of the seeds
S1	Norra-varm L x Draba-L	15-06-13 Em po 05-08-13 collected
S2	Gävle-96 x L-p43275-35	06-06-13 Em/po 29-07-13 collected
S3	Hy-6-2-(2) x LS 43275-35-L-a	24-6-13 Em 25-6-133 po 12-6-13 collected
S4	Lepg3-8-7-(L) x L.S 4327-35-8-2B	03/06/13 Em/Po 29/07/13 collected
S5	Lepg2-6-2-(2) x Lp.4327-35-8-2-A	19-06-13 Em/po 08-08-13 collected
S6	Pou 6-6 x L-S 43275-3-(L)	Em/po 08/06/13 51 days
Control	Lepidium N094-7	

Disinfection of developing seeds for the embryo rescue test

All the parental *L. campestre* ovaries and hybrid ovaries used were sterilised in 0.3 % Ca-hypochlorite solution for 5-10 minutes in the same way as for the germination test before ovules were excised.

Parental plant material for the embryo rescue test

Approximately 6-11days old non-hybrid parental ovaries after pollination (DAP) were disinfected for 5 minutes as described above. In total, twenty-four ovules were excised and twelve ovaries were used. The ovules were excised under light microscope by cutting the ovaries longitudinally. For the ovary culture, the ovaries were cut on one side as my supervisor instructed and then placed with that side down onto the medium. They were divided equally on six Petri plates with medium A-F. Four ovules and two ovaries were placed on each of the plates. The same were

performed with parental ovaries, but with 10 min disinfection. The age of the ovaries used were estimate by their size compared to ovules followed on the mother plant for ten days.

Hybrid material for the embryo rescue test

Disinfected 6DAP hybrid ovaries of *L. campestre x L. graminifolium* were excised and one ovule was placed on one Petri plate with medium A and two on medium F, one on medium D. The Petri plates with medium D and F contained one cut ovary each. Disinfected 8DAP old hybrid ovaries were excised and two ovules were placed on Petri plates with medium B, C and E and one cut ovary on each medium/plate. The disinfected 9DAP old ovaries were excised and two ovules were placed on each Petri plates with medium A, D and E and one cut ovary on each medium/plate. All the Petri plates were sealed with surgeon tape and placed in the growth chamber. From each of the 6DAP hybrid ovaries grown for seven days on medium D and F two ovules were excised and placed on the same type of medium. From the 8DAP ovaries grown for 8 days on medium B, C and E two ovules were excised from each and placed on the same type of media. Later twelve disinfected hybrid ovaries 8DAP were cut and placed on Petri plates with medium A-F with two on each plate (Figure 1). In addition four excised ovules were placed on Petri plates with medium A, D and F except for F that only got three ovules and two ovules were placed on Petri plates with medium C, D and E.

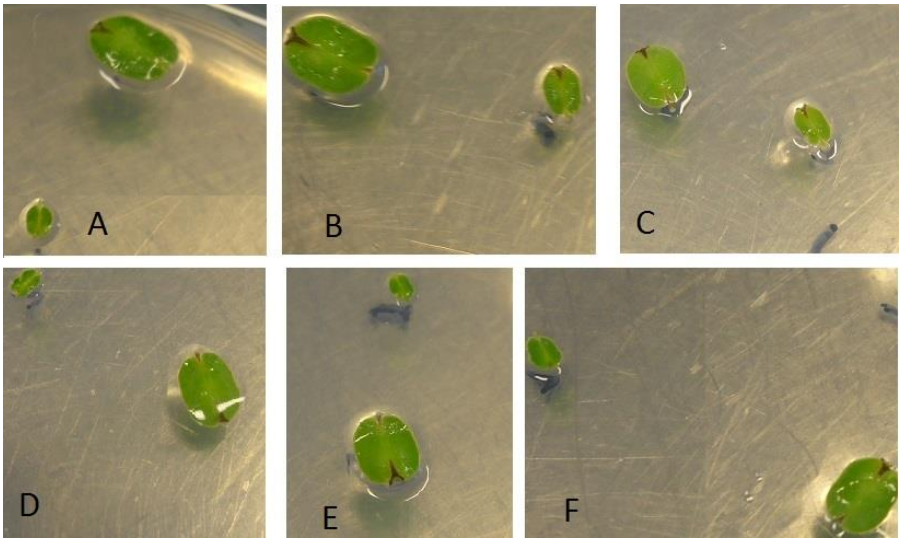
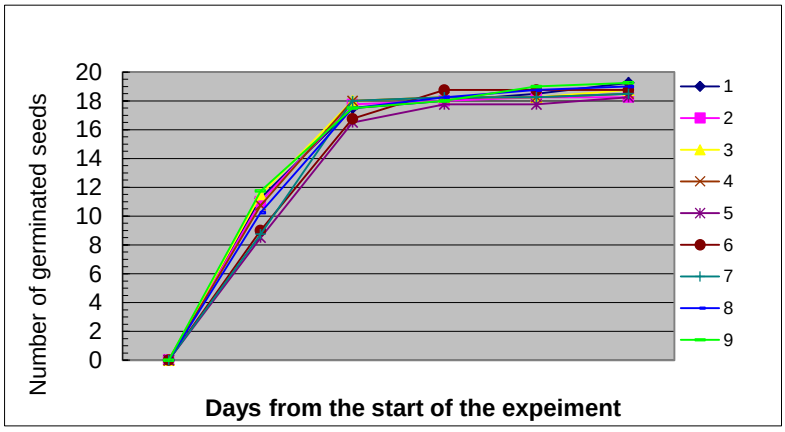


Figure 1 Excised ovaries (8DAP) placed on the 6 different media (A-F) (Fahlgren).

Result and Discussion

Germination medium test



0 3 6 8 10 13

Figure 2 The germination rate of the control seeds on the 9 germination media tested after 3, 6, 8,10 and 13 days.

The result of the germination medium test showed no significant difference in the germination rate among the different media tested (Figure 2). However, the numbers of germinated seeds varied more in the four replicates on the same media in the beginning, but less in the end, probably due to natural variations in how quickly the seeds germinate, probably due to the seed quality. Moreover additions of GA3 and sucrose did not make any difference in the germination rate for the control seeds.

Hybrid seed germination

Since the hybrid seeds were smaller and poorly developed compared to the control seeds, they probably had less stored nutrients than the control seeds. The two media with different contents of sucrose and the highest contents of GA_3 therefore were used for the germination test on the hybrid seeds. The results showed that no seed germinatedafter 19 days (Figure 3). For the control, all but three seeds germinated.

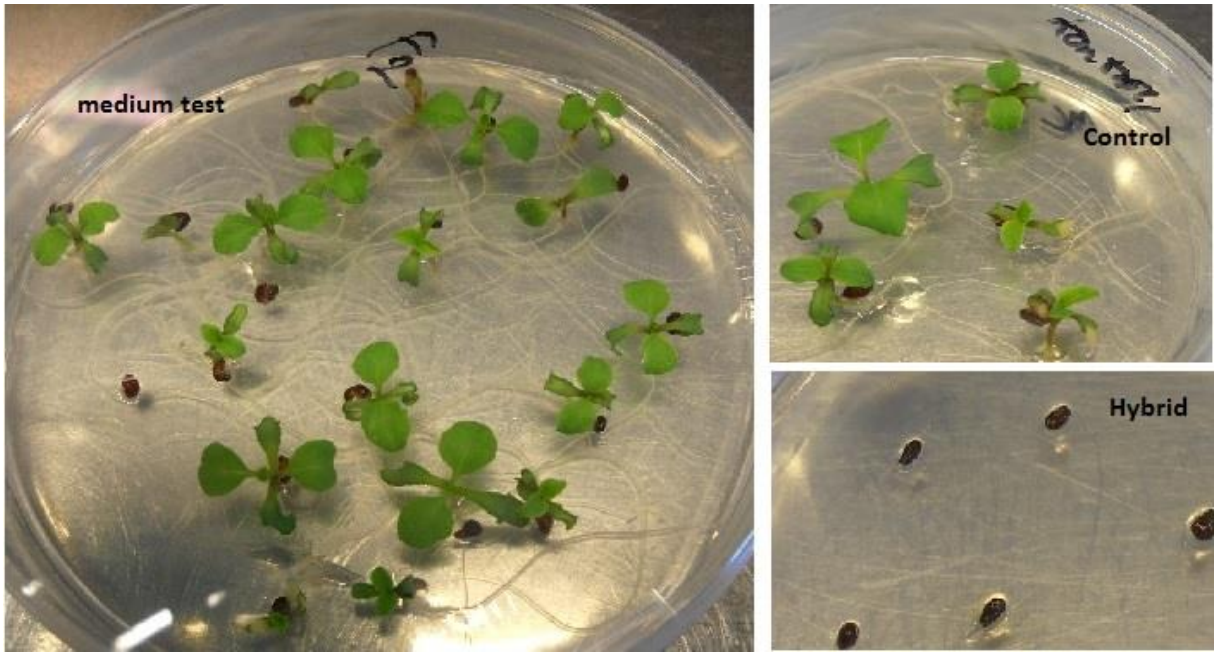


Figure 3 Seed germination from the germination medium test (left) and the hybrid germination test (right): the control seeds germinated well (upper right) and the hybrid seeds did not germinate at all (lower right) after 8 days. (Fahlgren)

Parental Ovules

After 10-11 days on the media, some ovules became darkened and there were some darker spots on otherwise light ovules and some had not changed at all (Figure 4). After approximately 36 days of culture on medium F, one of the parental ovule started to germinate (Figure 5). That it took 36 days for the parental ovule to germinate indicates that it could take even longer time for the hybrid ovules to germinate as according to Mohapatra' and Bajaj (1987) the hybrid ovules took longer time to germinate than the parental ones.


Figure 4 Parental ovules grown on medium B (left) and D (right) for about 11 days where some have darkened. (Fahlgren)

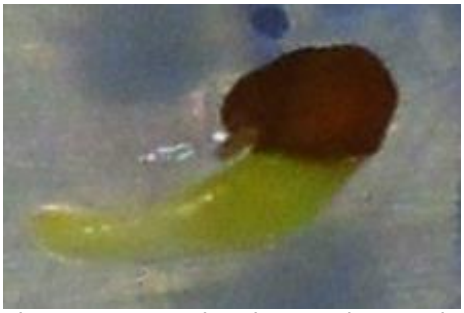


Figure 5 A parental ovule started to germinate after being cultured for 36 days on medium F. (Fahlgren).

Parental ovaries

The parental ovaries started to turn lighter and several of them formed calli on the basal end after they had grown for about 2.5 weeks (Figure 6). Many of the parental ovaries taken 6-11DAP and grown for 27 days started to produce calli on the basal end. All 6-11DAP parental ovaries grown for 27 days were cut open but all the ovules had ceased to grow and died except for one on medium E and two on F which were small and deformed. Tonguç and Griffiths (2004) got similar results from ovaries grown for 15-20 days. That the ovules did not grow is consistent with the result from Mohapatra' and Bajaj (1987) that callus formation on the ovary inhibits the development of the ovules.

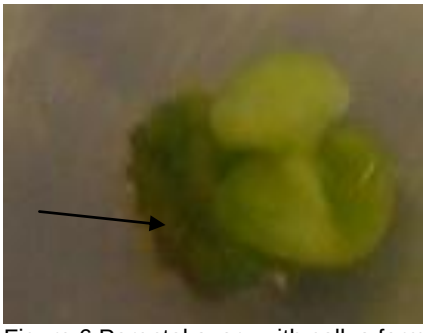


Figure 6 Parental ovary with callus formation on the basal end pointed by an arrow. (Fahlgren)

Hybrid ovules

The ovules excised from 6DAP ovaries grown for 7 days were small but had probably grown a little in size, yet none of them had germinated. Some of the ovules from 8DAP ovaries grown on medium B and D formed calli on their basal end (Figure 7). This result is similar to that of Mohapatra' and Bajaj (1987) where ovules 10DAP tended to form callus more frequently than ovules 15DAP, indicating probably that

ovules around 8-10DAP have higher tendency to form calli than younger and older ovules. The calli formed may possibly be used to grow a full plant, however since it was formed on the basal end, it will most probably have the genes of the mother plant and not from the hybrid. Since the ovule is produced from the mother plant and it is the embryo inside the ovule that contains DNA from both the pollen and mother plant. The ovules grown on medium A, C, E and F did not germinate or produce calli.

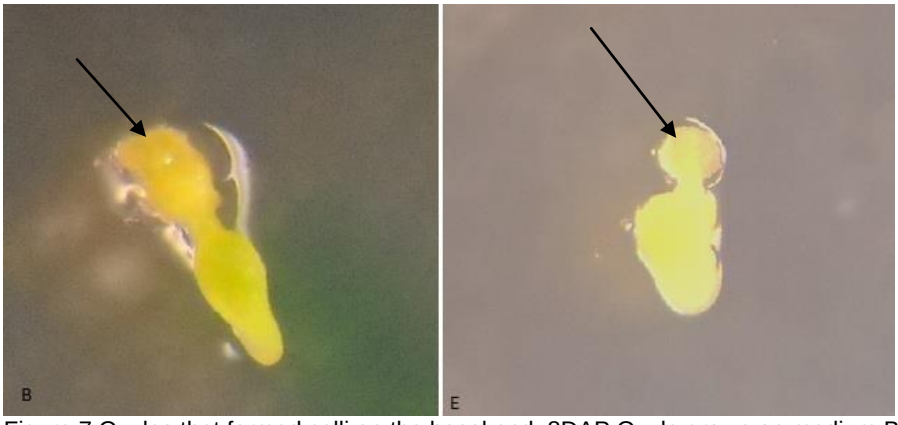


Figure 7 Ovules that formed calli on the basal end. 8DAP Ovule grown on medium B (B) and medium E (E). (Fahlgren)

Hybrid ovaries

Since the hybrid ovaries started to grow after one week of culture (Figure 8), the same as reported by Momotaz et al. (1998), they could probably be cultured for some more days to get better results. Then the ovules should be excised since Tonguç et al., (2004) got fifteen plants from ovules excised from five-hundred and twenty-seven ovaries 5-7DAP grown for 5-7 days and only four from six-hundred and fifty-seven ovules grown for 15-20 days. The 8DAP old ovaries on the media B, C, D, and F began to mould after approximately 16-17 days (Figure 9).

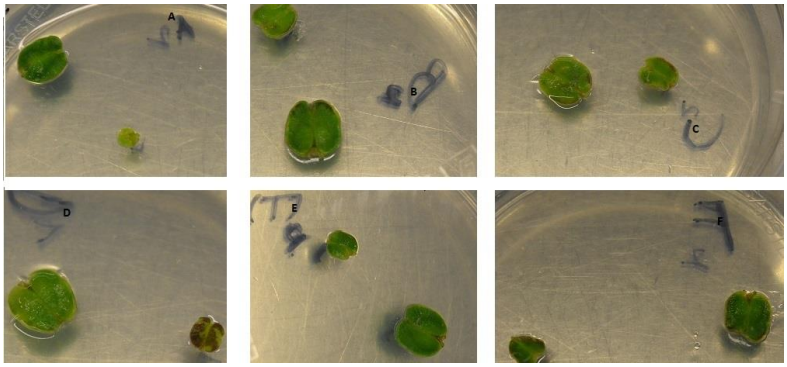


Figure 8. Ovaries grown on the six different embryo rescue media (A-F) for approximately 7 days. (Fahlgren)

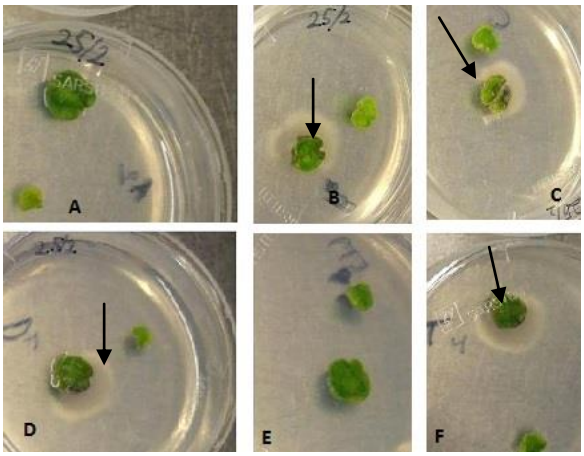


Figure 9 Ovaries grown for 10 days and four of them got infected with fungi (arrows). (Fahlgren)

Conclusions

We did not succeed in germinate the hybrid seeds using the media tested in this study. However, we have successfully shown that it is possible to get growth or to produce calli from either ovlues or ovaries using the media tested in the embryo rescue experiment. Due to the limited time and plant materials, we could not make any conclusion about the best medium for hybrid embryo rescue. Further medium test with different types and combinations of hormones in combination with other compounds or other factors should be conducted to find out the best medium or conditions where hybrid seeds could germinate and grow. More studies on embryo rescue are needed to find a more suitable medium for field cress because only one parental ovule germinated and only some hybrid ovules produced callus in our case. A large number of ovules would be probably needed to produce plants since the germination rate of the hybrid ovules are less than that of the parental according to Mohapatra' and Bajaj (1987).

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Fahlgren D. Figure 1-9 and table 1-5