Zał 1. Poster

Kiełkowska A., Mačugová L. (2024). Trials on anther cultures in *Vicia faba* L. 59th Croatian & 19th International Symposium on Agriculture, Dubrovnik, February 11 - 16, 2024, Dubrovnik, Croatia, pp. 99

Trials on anther cultures in *Vicia faba* L.

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Abstract

Leguminous species, including *V. faba*, are considered recalcitrant to most *in vitro* approaches including haploidization.

To date there are very few studies dealing with anther (AC) or isolated microspore cultures (IMC) in *V. faba*. Response in anther culture was restricted mainly to anther browning and dying and sporadically to callus development. In isolated microspore cultures rarely microspore division occured and no further development was observed. No plant regeneration from AC nor IMP was reported for *V. faba*. These findings together with the progress in haploidization protocol development in other legumes i.e. pea and chickpea were the assumption to undertake the study on the trials of induction of androgenesis in *V. faba*.

The aim of this study was to analyse the effect of culture media and different temperature treatments applied both on floral buds and on anthers in culture.

Methodology

Two cultivars of *V. faba* – 'Kambos' and 'Bonus' were used in this study. Plants were obtained from the seeds and were grown in the greenhouse. Plants were optimally watered and fertilized.

DAPI (4'6-diamidino-2-phenylindole) staining was applied to determine the developmental stage of the cells in anthers. Anthers containing predominantly microspores were selected for the study.

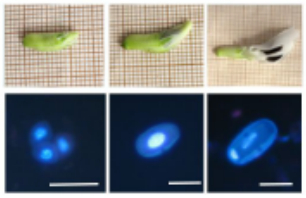
Selected flower buds were pretreated with cold (4°C) for 1 day and were surface disinfected. Anthers were isolated from buds on two solid media (190/8 and 190/10) containing different growth hormones (2,4-dichlorophenoxyacetic acid (2,4-D), 1-napthaleneacetic acid (NAA) and kinetine). Cultured anthers were subjected to temperature treatments (cold, heat) for 1 day. Observations of the development in the cultures were done after 30 days.

Data were analyzed with ANOVA, mean separation was conducted via Tukey's (HSD) test at p≤0.05. Standard error of mean (±SEM) was also shown on graphs.

Plant material: cultivars 'Bonus' and 'Kambos' at flowering stage

Results

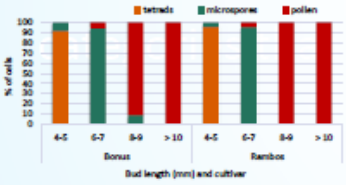
1. Determination of pollen developmental stage in *V. faba* anthers



DAPI-staining of cells collected from anthers excised from floral buds of different size from both cultivars was applied with the aim of determination of appropriate bud size containing prevalence of microspores.

Figure shows exemplary floral buds and respective pollen developmental stage.

Determination of pollen developmental stages in different size floral buds of *V. faba*. From left: tetrad, microspore, bicellular pollen. Scale bar 20 µm.



In both cultivars prevalence of microspores was found in floral buds 6-7 mm in length, and these were selected for further study.

Pollen developmental stages in *V. faba* depending on bud size and cultivar.

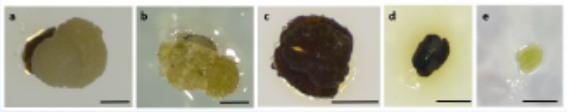
2. Anther culture

Dishes with anthers were placed in dark in temperature 28°C. After 30 days the cultures were subjected to observations.

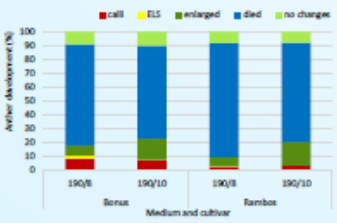
Five major categories of anther development could be distinguished and all of them are shown on the pictures below and they were as follows:

a) anthers with embryo-like structures (ELS),
b) callusing on anthers,
c) swelling and browning of anthers
d) died anthers – did not changed their size, but get dark, and extracted phenolics to the medium
e) anthers with no changes in size, morphology and colour

Scale bar 1 mm.



2.1. Effect of genotype and medium on anther development



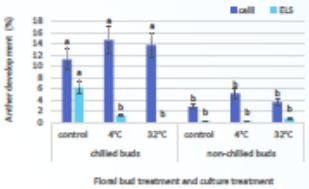
In both cultivars prevalence (70-80%) of cultured anthers died up to 30 days. The frequency of anthers producing callus was 2-9%, and was highest in 'Bonus' cultured on 190/8 medium supplemented with 2,4-D, NAA and kinetin.

ELS were observed in 0,3-2,5% of cultured anthers and were most abundant in 'Bonus' on the same medium, that stimulated callus development.

Remaining anthers (7-30%) showed no changes after 30 days of culture.

2.1. Effect of cold pretreatment of buds on callus and ELS development

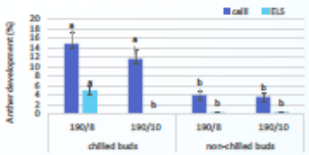
We analyzed the effect of floral bud pretreatment (1 day in 4°C) in combination with temperature treatments applied onto anthers isolated from such buds. Anthers were treated with either cold (4°C) or heat (32°). Untreated cultures were controls.



Anthers were stimulated for increased callus production (11-14%) when they were excised from cold-pretreated buds, independantly from the stress applied into cultured anthers.

The floral bud pretreatment also favored ELS development, however its occurence was highest (6%) when cultured anthers were not subjected to thermal shocks.

2.1. Effect of cold pretreatment of buds and medium on callus and ELS development



The highest efficiency of callus development (12-25%) was observed when anthers were isolated from cold-pretreated buds, irrespectively from the culture medium.

The highest efficiency of ELS development (5%) was observed in anthers isolated from chilled buds and cultured on 190/8 medium supplemented with 2,4-D, NAA and kinetin.

3. Ploidy analysis of obtained callus

Obtained calluses were subjected to ploidy analyses with flow cytometry.

Result showed that majority of samples (39%) were mixoploids 2x+2x and 2x+4x. We also detected tetraploid (15%) and diploids (7%) samples.

Approximately 31% of tested samples have been identified as haploids.

Conclusions:

1. Microspores were found mostly in the anthers of floral buds of 6-7 mm in length, and these were used as explant source.
2. Majority of cultured anthers if *Vicia faba* died up to 30 days of culture; however the development of callus and ELS on cultured anthers was also observed.
3. Callus and ELS production was stimulated predominantly by cold pre-treatment (4°C per 1-3 days) of floral buds and medium supplemented with 2,4-D, NAA and kinetin.
4. Obtained samples of callus tissue developed on cultured anthers were subjected to ploidy analyses, which showed presence of haploid tissue.

Acknowledgements

The research was financed by Polish Ministry of Agriculture and Rural Development (No. DHR.hrn.80214.2023).

Conference

59th Croatian & 19th International Symposium on Agriculture

11-16 February 2024, Dubrovnik, Croatia

2. Poster

Skrzypkowski W, Kiełkowska A. (2024) Próby indukcji gynogenezy u pomidora (*Solanum lycopersicum* L.) z zastosowaniem kultur zalążni oraz izolowanych zalążków. XVI Ogólnopolska Konferencja Kultur In Vitro i Biotechnologii Roślin - Biotechnologia i kultury in vitro roślin w badaniach podstawowych i aplikacyjnych", **23-25 września 2024, Kraków, 242-243**

XVI OGÓLNOPOLSKA KONFERENCJA KULTUR IN VITRO I BIOTECHNOLOGII ROŚLIN:
Biotechnologia i kultury in vitro roślin w badaniach podstawowych i aplikacyjnych
23–25 września 2024, Kraków

Próby indukcji gynogenezy u pomidora (*Solanum lycopersicum* L.) z zastosowaniem kultur zalążni oraz izolowanych zalążków

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Pomidor (*Solanum lycopersicum* L., Solanaceae) jest gatunkiem rośliny warzywnej o dużym znaczeniu handlowym oraz ekonomicznym, o czym świadczy wysoka światowa produkcja owoców tego gatunku, wynosząca w 2022 roku około 186 milionów ton. Tak wysokie znaczenie ekonomiczne determinuje wzmożone zapotrzebowanie na hodowlę twórczą, ukierunkowaną na otrzymywanie nowych odmian uprawnych. W związku z tym, istnieje szczególna potrzeba otrzymywania cennych dla procesu hodowlanego linii homozygotycznych, dla których wysoce obiecującą metodą pozwalającą na znaczne skrócenie czasu ich produkcji (1–2 pokolenia) jest haploidyzacja. Pomimo jednak wysokiej wartości handlowej pomidora, technologia ta wciąż nie ma u tego gatunku praktycznego zastosowania co związane jest z jego opornością na próby haploidyzacji. Celem badań nad indukcją gynogenezy u pomidora było pobudzenie do rozwoju komórek gametofitu żeńskiego, a w rezultacie otrzymanie haploidalnych struktur. W tym celu posłużono się metodą kultur izolowanych zalążni, fragmentów zalążni oraz izolowanych zalążków. Materiał roślinny wykorzystany w badaniach stanowiły obiekty hodowlane płodne i męskosterylne. Zalążnie oraz ich fragmenty wyłożone na pożywki hodowlane w warunkach in vitro ulegały powiększeniu a na ich powierzchni obserwowano rozwój tkanki kalusowej, jednakże, rozwijała się ona wyłącznie z tkanki somatycznej ścian zalążni, co wskazuje na nieskuteczność zastosowanych technik. W innym doświadczeniu, wypreparowane z niezapłodnionych zalążni i oddzielone od tkanki łożyska zalążki wykładano na pożywki indukcyjne. Na ich powierzchni obserwowano rozwój kalusa. Analiza ploidalności otrzymanej tkanki kalusowej pozwoliła określić, że większość grudek kalusa

3. Poster

Kiełkowska A., Gumulak-Wołoszyn N., Skrzypkowski W. (2024). Ovule vultures in *Vicia faba* after foreign pollination. 9th Central European Congress of Life Sciences EUROBIOTECH 2024 26-28 czerwca 2024, Kraków. Pp. 135

Ovule cultures in *Vicia faba* L. after foreign pollination

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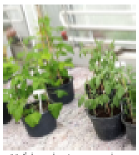
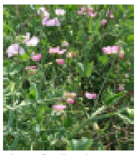
Introduction

Doubled haploids (DH) plants have high potential for shortening the breeding proces of new varieties in plants by producing true homozygous lines in one generation. This technology was successfully applied in breeding of many important crops i.e. rape, wheat, barley, rice, tobacco or triticale (Forster et al. 2007). *V. faba* is a popular vegetable consumed worldwide, and due to its high nutritional content, this species is widely utilized not only in the human diet but also as feed for animals, including pigs, poultry, and ruminants. *V. faba* is very recalcitrant to various tissue culture methods, including micropropagation, agrobacterium transfection, and haploid technology (Ochatt st al. 2009). So far, an efficient procedure for obtaining haploid/DH regenerants in this species has not been developed, even though initial attempts began in the late 1970s. The very few available trials on haploidization in *V. faba* concerned androgenesis and resulted with callus development, but no plant regeneration (Paratasilpin 1978, Hesemann 1980, Shlahi et al. 2012). The study aimed at the stimulation of the development of haploid cells of the female gametophyte of *V. faba* after distant pollination with *Lathyrus odoratus*.

Materials and methods


V. faba plants grown in greenhouse conditions

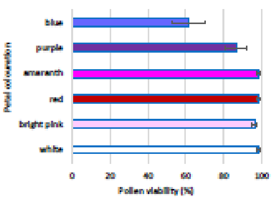

Plants of pollen donor L. odoratus grown in open-field conditions

As a plant material, two commercial cultivars (Bartek, Rambos) of *V. faba* were used.
Before experiments pollen viability of *the pollinator - L. odoratus* - was analyzed with acetocarmine.
Selected flower buds of *V. faba*, in which selfpollination did not occur yet, were emasculated and hand pollinated with pollen of *L. odoratus*. On the next day, the pollinated flowers were sparyed with water solution of gibberelin (GA3). Unpollinated flowers were used as controls. In selected combinations (foreign and control pollinations) the pollen germination, under a fluorescence microscope with aniline blue was analyzed.
Five to seven days after pollination (DAP) pistils of *V. faba* from foreign pollination and control combinations (K1-K4) were collected and disinfected. Dissected ovules were cultured *in vitro* on two solid culture media (MS and B5 based; supplemented with BAP, TDZ, Kin, and 2,4-D). Ovules were cultured in dark or at light at 23±2 °C. The development of explants was monitored after 60 days of culturing.


Tagged *V. faba* flowers pollinated with *L. odoratus* pollen viable (left) and aborted (right) buds 5 DAP

Results

Analyses of pollen viability of *L. odoratus*



Pollen viability in L. odoratus depending from the petal colouration


Variability in L. odoratus petal colouration

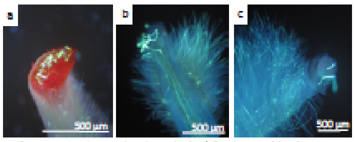
Pollen of L. odoratus stained with acetocarmine

Analyses of flower development in *V. faba* for selection of optimal stage for foreign pollination

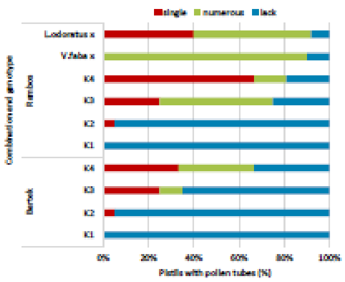
V. Faba is self-pollinated species. The flower is build up five petals, the standard petal white, the wing petals white with a black spot, and the keel petals white. Pollen release from anthers starts in buds (stage III on figure), before flower opening.
It was crucial to spot the timing preceeding pollen release from anthers in *V. faba*, to avoid selfpollination. As optimal, the floral buds in medium phase (stage II on figure) were selected. In that particular phase anther removal (emasculation) and foreign pollinations were performed.

Flower development in *V. faba*. Observations of early (I), medium (II) and late (III) phase of stamen and pistil development. In miniature morphology of flower buds, and on large picture close up on the fertile whorls are shown. On large pictures anthers are marked with (a), hairy stigma is marked with (b).

Analyses of foreign and control pollinations with anilin blue – 24 h after pollination

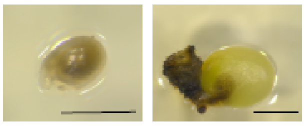


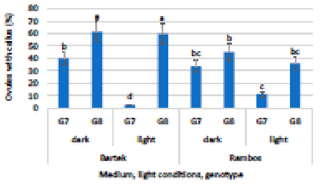
Pollens germination on the stigma in the following combinations: (a) L. odoratus x L. odoratus (control I), (b) V. faba x V. faba (control II), (c) V. faba(♀) x L. odorathus(♂)



Germination of pollens in dependency on genotype (Bartek, Rambos) and combination. Combinations as follows: *L. odoratus x L. odoratus* (control I), *V. faba x V. faba* (control II); K1 - no pollination no gibberelin spraying, K2 - no pollination, gibberelin spraying, K3 - pollination, no gibberelin spraying, K4 - pollination gibberelin spraying

In vitro culture of isolated ovules


Culture of ovules isolated from flowers of *V. faba* pollinated with pollen of *L. odoratus*. a) ovule isolated to *in vitro* conditions 5-7 DAP, b) ovule callusing at the micropylar site, after 60 days of culture with the symptoms of browning (polyphenols extraction). Scale bar 1 mm.



Development of callus in the culture of ovules isolated from flowers of *V. faba* pollinated with pollen of *L. odoratus* and in controls, depending on the media (G7, G8), light conditions (light, dark) and cultivar (Bartek, Rambos). Observations were made after 60 odays of culture. Bars represent means ± SEM. Bars denoted with the same letter are statistically insignificant at p≤0.05, HSD.

Conference

Conclusions

1. Pollen of *L. odoratus* was generally highly viable (80-90%), thus suitable to use as pollinator
2. Emasculation proceeding pollination was done on *V. faba* floral buds at stage II, which refers to floral buds being 1.5-2.0 cm in length in both cultivars
3. Pollen of *L. odoratus* germinated on the stigma of *V. faba* however entering of the pollen tubes into *V. faba* ovules was not observed 24 h after pollination
4. In vitro culture:
   - approximately 50% of cultured ovules produced callus
   - callus developed on the micropylar site of the ovules exclusively
   - callus development was most abundant (36-60%) on G8 medium (B5 supplemented with BAP, Kin and 2,4-D)
   - the light conditions did not affected callus development
   - higher (60%) callus development was observed in cv. Bartek
   - majority of obtained callus tissue get brown during culturing, and future efforts will be directed towards finding a proper medium for callus proliferation with minimized browning

Acknowledgments

The research was financed by Polish Ministry of Agriculture and Rural Development (Biological advancement in plant production 2021-2026).

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