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PRODUCTION OF BREEDING MATERIALS FROM *IN VITRO* SOMATIC AND MERISTEMATIC CULTURES OF FABA BEAN

Thesis of PhD Dissertation

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1. PRELIMINARIES AND OBJECTS

Faba bean (*Vicia faba* L.) is one of the most ancient cultivated crops. It is not so important crop in the world pulse production that is represented only about 5 % of the total area of world grain legumes. That means the physiological and genetical knowledge, which can be useful in plant biotechnology, is not enough comparing to other important crops, such as wheat, rice, maize, etc. The faba bean is one of the most favourite plants used in cytology, because of its chromosome number (n=6) and size. The increasing knowledge in cytogenetics of *Vicia faba* can help in the use of biotechnology in faba bean breeding. The partial lack of plant biotechnological knowledge and its peripherical importance in crop production led to few recent applied results in the biotechnology of faba bean. There is not a ready-made *in vitro* plant-cell-plant system of this crop in any ploidity level.

Molecular markers getting more and more important not only in basic research, but in applied sciences, like plant breeding, too. The importance of PCR-based techniques is increasing compared to the previous isozymes and RFLPs. Several genes have identified in *Vicia faba* genome (like legumin, vicilin) and successful transformations were made with them. Identification of genetic diversity in European and Mediterranean faba bean germplasms and genetic linkages have revealed by RFLP and PCR markers. There is not any information about the Hungarian varieties in this field.

According to the above mentioned characteristics of biotechnology of *Vicia faba* L. our *in vitro* experiments were done to reach the following goals:

- examination the effects of different culture conditions on meristem cultures (genotypes, isolates, content of plant hormones in culture media);
- study of *in vitro* vegetative micropropagation with different explants;
- initiation of somatic cell cultures (callus cultures);
- genome comparison in some Hungarian genotypes based on PCR techniques.

2. MATERIALS AND METHODS

2.1 Meristem cultures

Several faba bean genotypes were tested such as 'Minor', 'Vica', 'Erna', 'Alfred', 'Óvári-137', 'Dino', 'KU-22', 'Lippói', 'Karola', 'VF1 809682', 'Jasny II'. The are derived from the Plant Breeding and Agronomy Station of the Faculty of Agriculture and Food Sciences at

University of West Hungary. Shoot meristems were isolated from *in vitro* grown seedlings. Before germination seed coat were removed to aviod phenolic compounds. Solidified Knop solution was used for germination. Seedlings were grown at 24±1 °C in 16 hours light with 125 M/m^2/s intensity for 5-6 days. Culture medium was B5 (Gamborg-1968) supplemented with 0.5 μM/l BAP, 2 % sucrose and 0.8 % agar-agar. The pH-value was set to 5.8 prior to autoclaving. Meristems with 1-1.5 mm size were put on the surface of culture media in glass tubes. Incubation took place in the same conditions as described earlier during germination. After culturing growth of shoots and calli were evaluated and fresh weight were measured.

The most suitable genotype ('Lippói') for *in vitro* cultures was used in the following experiments. Shoot, cataphyll and cotyledon meristems from aseptic grown 5-6 day-old seedlings were isolated. MS (Murashige-Skoog-1962) culture medium was used supplemented with different combination and concentration (0-0.1-1.0-10 mg/l) of plant hormones, such as BAP and auxins (IAA, NAA, 2,4-D). The concentration of sucrose and agar-agar were 3 % and 0.8 %, respectively. Auxins were combined with BAP and 46 different media were examined as a sum. Meristems with 1-1.5 mm size were put on the surface of culture media in glass tubes. Incubation took place in the same conditions as described earlier during germination. After culturing different parameters were evaluated: fresh weight, shoot number, culture development. The optimum combination of BAP and auxins was determined.

Shoot regeneration occurred on adventive meristems derived from mature cotyledons were used in one of our experiments. Seeds of 'Lippói' variety were surface sterilized and soaked in sterile distilled water for 24 hours. After peeling of seed coat the two cotyledons were separated to each other and embryo was removed. Explants were isolated on the surface of B5 medium supplemented with various concentrations of BAP and IBA, and 2 % sucrose and 0.8 agar-agar. The culture media were autoclaved, pH value was 5.8 prior to autoclaving. Cultures were incubated for 6-8 weeks depending on their development under the above mentioned conditions. Shoot root and callus development, shoot and root numbers were evaluated after measuring the fresh weight.

In all three experiments described above regenerated shoots were rooted on hormone-free MS or B5 media. Plantlets were acclimatized on perlite moistened with Knop solution in 3-4 weeks at 20-22 °C and 16 hours light. Data were analyzed with ANOVA.

2.2 Shoot cultures

Three genotypes ('Lippói', 'Kornberger', 'Óvári-137') were used. Stem of aseptic grown (see Chapter 2.1) 5-6 day-old seedling were cut below the second node. Shoot tips and stems with two nodes were transfered to rooting medium supplemented with MS macro and Heller micro elements, 0.186 mg/ l NAA (1 M) 10 g/l active carbon, 3 % sucrose and 0.8 agar-agar. MS medium with the same plant hormones was also examined. Culture media were solidified in glass tubes and Erlenmeyer flasks. The pH was set to 5.8 prior to autoclaving. Cultures were incubated at 24±1 °C in 16 hours light with 125 $M/m^2/s$ intensity. After 7-8 weeks root numbers were scored.

2.3 Callus cultures

Different isolates and culture media were tested. First surface sterilized and peeled seeds were germinated *in vitro* on solution contained 1. MS macro and micro elements, 2. MS macro and micro elements + 3 mg/l 2,4-D, 3. Knop nutrients, 4. sterile tap water. All four were solidified with 1 % agar-agar. Mesocotyl segments and shoot meristems were isolated after 5-6 days on hormone-free media, and after 12-14 days on auxin containing medium. Explant were isolated on the surface of MS and B5 media supplemented with 0.2 mg/l BAP and 1.0 mg/l 2,4-D, 10 g/l active carbon, 0.8 % agar-agar. The pH value was calibrated to 5.8 prior to autoclaving. Callus development was continuously evaluated in 4 weeks.

Immature embryos derived from seeds of glass-house grown 'Lippói' and 'Kornberger' varieties were isolated in the next experiment. Green pods were surface sterilized and embryos were prepared for *in vitro* culture on MS medium with 3 % sucrose and 0.8 % agar-agar. Plant hormones were BAP and IAA in concentrations of 0-0.5-1.0-2.5-2.0 mg/l. All concentrations were combined to each other and 25 media were tested. The pH value was calibrated to 5.8 prior to autoclaving. Embryos were cultured at 24±1 °C in dark. After 6 weeks fresh weight and callus development were scored.

The effects of polyvynil-pirrolydon (PVP) were studied in the third experiment. Shoot tip, cataphyll, cotyledon meristems and epicotyl, mesocotyl, hipocotyl segments of *in vitro* grown seedlings were isolated on the surface of MS medium supplemented with 3 % sucrose, 0.8 % agar-agar, 0.175 mg/l IAA and 2.25 mg/l BAP. The amount of PVP was 0-10-100-500-1000-2000 mg/l. After 8 weeks of incubation fresh weight, shoot and callus development were evaluated. Data were analyzed with ANOVA.

3.4 Identification of microsatellite and RAPD markers

Genomic DNA from 'Jasny II', 'Lippói', 'Minor', 'Óvári-137', 'Vica' and 'Fehérvirágú' faba bean genotypes were isolated from squeezed 1 g of fresh green leaves with 2-mercapto-ethanol. DNA precipitates were treated with RNAse in TE-buffer solution and 4 µl DNA was used in PCR reaction. The components of PCR reactions in 25 µl volumes were:

(1) 15.625 µM dNTP mixture (dATP : dGTP : dCTP : dTTP = 1:1:1:1),

(2) 0.05 µM $MgCl_2$,

(3) 0.3 µl (1,5 U) Taq-polimerase (Promega Co.),

(4) 20 pM primer,

(5) 4 µl (20 nM) sample DNA.

Oligonucleotide primers were with length of 16-18 bp $(GACA)_4$, $CA(GACA)_4$, $(GACA)_4CA$, $(ACTG)_4$ and with 10 bp OP/A-11, OP/B-1, OP/B-3, OP/B-5, OP/B-7, OP/B-11, OP/B-12 (OPERON Sci.). Samples were separeted on agarose gels by electrophoresis after PCR reactions. Characteristic DNA bands were visually evaluated.

3. RESULTS & DISCUSSION

3.1 The effect of genotype on *in vitro* development of shoots

Most of the shoots (2,0±0,9) were obtained in 'Lippói' while 'KU-22' produced less (1,0±0,3). The frequency of shoot development was not significantly different in all genotypes (92,8-100 %). Well organized shoots arised in 'Lippói' and 'Alfred', while weak shoot development occurred in 'Minor', 'Vica', 'Erna' and 'KU-22'. The highest fresh weight was measured in 'Lippói' (288,5 mg). The genotypes 'Lippói' and 'Alfred' are members of large-seeded ("ancient") group of faba bean varieties. They could contain some "wild" genes responsible for *in vitro* reactions.

3.2 The effect of BAP and auxins on the development of different meristems

3.2.1 The effect of BAP in the presence of 2,4-D

The highest fresh weight (424.3 mg) was obtained on culture medium with 1 mg/l BAP in cataphyll meristems. Hormone-free medium gave the lowest weight (1.8 mg). Significant difference was found among combinations and between the two plant hormones at 0.1 % level. Most of the shoots were counted in shoot meristems developed on medium with 0.1

mg/l BAP. No shoot multiplication was occurred on hormone-free and 2,4-D containing media. Significant difference was found among combinations and between the two plant hormones at 0.1 % level. Regenerated shoots were weakly developed only shoots from 10 mg/l BAP medium showed proper phenotype (value 3,0 in a scale 1-3). There was a significant difference among treatments at 0.1 % level. Evaluation based on all three meristems 1.0 mg/l BAP was the most effective in combination with 2,4-D.

3.2.2 The effect of BAP in the presence of NAA

Fresh weight of cultures from 0.1 mg/l BAP and 1.0 mg/l NAA containing medium was the highest in cataphyll meristems (631.7 mg). The least fresh weight was measured on hormone-free medium (1.0 mg/l). Significant difference was found among combinations and between the two plant hormones at 0.1 % level. The most shoot (4.5) developed on medium with 0.1 mg/l BAP from cataphyll meristems. Significant difference was found among combinations and between the two plant hormones at 0.1 % level. Most frequently multiplied shoots was counted on medium with 0.1 and 1.0 mg/l NAA. Medium sized shoots were developed which are better than in the previous experiments. Significant difference was found among combinations at 0.1 % level but between the two plant hormones was at 10 % level. Evaluation based on all three meristems 1.0 mg/l BAP was the most effective in combination with NAA.

3.2.3The effect of BAP in combination with IAA

The highest fresh weight (784.8 mg) was obtained in cotyledon meristems on medium supplemented with 1.0 mg/l BAP and 1.0 mg/l IAA. While hormone-free medium produced the least value (0.7 mg). Significant difference was found among combinations and between the two plant hormones at 0.1 % level. Most of the shoot (6.5) were multiplied on culture medium with 1.0 mg/l BAP and 10 mg/l IAA in shoot meristems. No shoots were at low (0.1 mg/l) auxin concentration. Also significant difference was found among combinations and between the two plant hormones at 0.1 % level. Medium sized shoots were developed from each meristem type. Tipical shoots were grown on medium with 1.0 mg/l BAP and different IAA level. Significant difference was found among combinations at 0.1 % level but between the two plant hormones was at 5 % level. Evaluation based on all three meristems 1.0 mg/l BAP was the most effective in combination with IAA.

3.2.4 Development of shoot tip, cataphyll and cotyledon meristems in vitro

Previously the effects of plant hormone combinations and concentrations were evaluated in details. However, it is important to know: is there any difference among the *in vitro* reactions of meristems derived from different plant sections? Shoot, root and callus development was observed because of the plant hormones (BAP and auxins) used. Shoot organogenesis and multiplication were resulted on medium with BAP alone, while auxins resulted root and callus development. Enhanced shoot regeneration was at high BAP and low auxin levels compared to the control. The opposite ratios (low BAP and high auxin levels) served enhanced callus induction and occasionally root development occurred. High BAP and high auxin levels produced more shoots but callus development on the base of shoots. The BAP and IAA combination gave the most multiplicated shoots in all explants: 10 events at each one. Root organogenesis occurred in mediums with BAP and NAA, 4 events. There was not any difference among hormone combinations and explants based on callus development, but 2,4-D treatments resulted more deviations than the others did.

The origin of *in vitro* cultures has an effect of shoot regeneration. Most of the shoots derived from shoot tip and cataphyll meristems, while cotyledon meristems were not capable to regenerate enough plants. This is an opposite result according to the literature mentioned. Regenerated shoots were rooted on hormone-free MS medium.

3.3 Induction of callus development and shoot organogenesis in cultures derived from mature cotyledons

Most of the shoots (42.2 %) were derived from 1.0 mg/l BAP and 1.0 mg/l IBA combination. Less of them (8.0 %) were from culture medium with 1.5 mg/l IBA alone. The most intensive root induction (92.7 %) occurred in 1.0 mg/l IBA while least (2.0 %) derived from 0.5 mg/l BAP treatment. Cytokinin alone (1.0 mg/l and 1.5 mg/l, 3.0 % and 3.0 %) resulted weakly developed roots. Callus development was the best (88.7 %) in the combination of 1.5 mg/l BAP and 1.0 mg/l IBA. Least frequent callus induction (7.0 %) was in the 0.5 mg/l IBA treatment. Highest number of shoots (5,5±2,6) were multiplicated in 1.5 mg/l BAP and 0.5 mg/l IBA combination, while small amount of them (1,3±0,5) developed with 1.5 mg/l IBA. There was a significant difference according to the fresh weight at 0.1 % level. The highest fresh weight (3.55 g) was with 0.5 mg/l IBA, the lowest (2.29 g) was with 1.5 mg/l BAP. The high regeneration potential of cotyledon regio *in vitro* was justified using BAP and IAA plant growth regulators.

3.4 Cloning based on nodal segments

Callus development was occurred on the base of both shoot tips and nodal segments after 12-14 days in culture. Shoots were alongated and rooted. Cataphyll buds on nodal segments developed into shoots nearly in all cases. Phenolic compounds produced black necrosis frequently. There was not any significant difference between MS and modified MS media. Rooting frequency was also not differed among varieties studied (45.9-54.9 %). Earliest rooted shoots derived from 'Lippói' than 'Kornberger' and 'Óvári-137' variety.

Rooted shoots were acclimatized before potting to the soil. Plantlets were put into perlite moistened with half strength MS medium supplemented with thiamine inositol. They were grown in glass-house after 2-3 weeks of treatment. Survival rate was low because of necrosis caused by phenolic compounds.

Addition of active carbon or PVP should enhance the 50 % efficiency of *in vitro* cloning based on nodal segments. Nodal segments derived from 'Lippói' variety were more suitable than the other genotypes.

3.5 The effect of explant origin on callus induction and development

Different isolates and culture media were tested in our callus cultures. Nearly all of the experiments based on *in vitro* grown seedlings so germination media were evaluated first. Vital and healthy plants were grown on hormone-free medium. The highest germination rate (98.0 %) was observed on Knop nutrient medium. External auxin treatment (2,4-D) caused strongly deformed plants with callus development on mesocotyl and shoot tip explants within 10-14 days. Callus induction on mesocotyl segments was higher than on shoot tips. Significant difference was observed after evaluation of fresh weight at P= 0.1 % level. Calli developed from 2,4-D treated seedlings were friable compared to the compact tissue structure from hormone-free seedlings. More roots were obtained on cultures with synthetic auxin pre-treatment.

In vitro cultures were initiated from immature embryos 'Lippói' and 'Kornberger' varieties on media with BAP and IAA. Embryos developed on cytokinin containing media alone were enlarged thus auxins produced calli without any growth phase. Low BAP and high IAA levels caused friable callus tissue, while high BAP and low IAA levels caused compact ones. Embryo-like structures were obtained with 2:1 BAP and IAA ratio.

After evaluation of different plant hormones an adsorbent was tested. Phenolic compounds in faba bean tissue cultures are major disadvanteges for biotechnological applications. Several concentrations of PVP (~40000 molecular weight) were tested. Most

of the shoots were developed on all three meristems with 100 mg/1 PVP: shoot tip meristem- 2.6±1.3; cataphyll meristem- 1.6±1.1; cotyledon meristem- 1.8±1.8. Significant difference was obtained among treatments and PVP concentrations at p= 5 % level.

In vitro grown seedling of faba bean is suitable for biotechnological purposes. They germinated more frequently on simple Knop-solution than on other media. External auxin (2,4-D) supply resulted higher rate of callus development on mesocotyl and shoot tip cultures. Calli from shoot tip meristems were green and friable showing higher regeneration capacity than the yellow, compact calli originated from auxin pretreated cultures. Embryo-like structures were developed on cultures derived from immature embryos. Higher regeneration potential was observed using 'Lippói' genotype in this case, too. Chemical adsorbents with high molecular weight, such as PVP, in 100 mg/l concentration have an enhancing effect on the viability of *in vitro* cultures of faba bean.

3.6 RAPD patterns of faba bean varieties using 3' and 5' anchored SSR/ISSR and OPERON primers

Primers, namely OP/A-11, OP/B-1, OP/B-3, OP/B-5, OP/B-7, OP/B-11 and OP/B-12, were used. Characteristic bands (DNA fragments) were separated by gel electrophoresis. Genetic polimorphism among different faba bean varieties was observed after evaluation of patterns of PCR fragments. The length of DNA fragments varied from 250 bp to 1400 bp, as it was visible using OP/A-11 primer. Three fragments obtained with OP/B-11 primer showed monomorphic pattern. Primers OP/A-11 and OP/B-5 produced high number of fragments and can be used as reliable polimorphic primers. Using microsatellite primers resulted similar fragment numbers as it was with the RAPD ones. These primers were sensitive because of theirs divergency. OPERON and microsatellite primers produced the most variable patterns in the case of 'Jasny II', 'Lippói', 'Minor', 'Óvári-137' varieties. The $CA(GACA)_4$ ISSR microsatellite primer produced monomorphic, while the $(GACA)_4CA$ primer produced polymorphic patterns. The fragment length in RAPD primers ranged from 2-300 bp until 1400 bp. Microsatellite primers produced smaller DNA fragments, about 1000 bp. Individual variability of plants was observed because of the difference among the samples.

3.7 New scientific results

1. Connection between genetic background and *in vitro* culture:

In vitro cultures can be sucessfully initiated and maintained from 'Lippói' and 'Alfred' varieties among the tested 11 genotypes.

2. The effect of hormone combinations on meristem development of faba bean with different origin:

The combination of BAP and IAA is suitable for *in vitro* plant regeneration in cultures derived from shoot tip and cataphyll meristems. The optimum concentrations are 1.0 mg/l in BAP and 1.0-10 mg/l in IAA. Regenerated shoots can be rooted on hormone-free MS medium.

After evaluation of the effect of auxins in combination of BAP on meristematic cultures we state: 2,4-D is for callus induction, NAA for root induction and development, while IAA is for shoot regeneration.

3. Organogenesis in mature cotyledon cultures:

The combination of BAP and IBA can be useful in micropropagation and genetic transformation studies.

4. The potential of *in vitro* cloning based on nodal segments:

The 50 % efficiency of *in vitro* cloning based on nodal segments can be enhanced by modification of culture medium.

5. Callus induction on different isolates:

Auxins in germination media produced intensive callus development. The combination of BAP and IAA in 2:1 ratio contributes to the development of embryo-like structures.

The addition of maximum 100 mg/l PVP in culture media enhanced the viability of meristematic cultures of faba bean by absorbing of poliphenolic compounds.

6. Comparative molecular analysis of faba bean genotypes:

After the molecular analysis of six faba bean genotypes ('Jasny II', 'Lippói', 'Minor', 'Óvári-137', 'Vica' and 'Fehérvirágú') with oligonucleotid primers (SSR/ISSR: $(GACA)_4$, $CA(GACA)_4$, $(GACA)_4CA$, $(ACTG)_4$; RAPD: OP/A-11, OP/B-1, OP/B-3, OP/B-5, OP/B-7, OP/B-11 és OP/B-12) we have observed:

- the variability of molecular weight in amplified DNA fragments;
- PCR reactions with OP/A-11, $(GACA)_4$ and $(GACA)_4CA$ primers produced polimorphic patterns and they can be useful for identification of genotypes and

molecular markers;

- the primer $CA(GACA)_4$ with its monomorphic pattern can be useful for the determination of similarities and differences in *Vicia* genus;

- sometimes the individual variability among the samples was higher than among the tested varieties. It is a sign of high plasticity of genetic background in faba bean.

5. PUBLICATIONS RELATED TO DISSERTATION

5.1 Papers

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