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Variability for resistance to *Fusarium solani* culture filtrate and fusaric acid among somaclones in pea

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Abstract

Pea (*Pisum sativum* L.) somaclones of cultivars Adept, Komet and Bohatýr were obtained after selection *in vitro* with *Fusarium solani* filtrate and fusaric acid (FA). R2 regenerants were analyzed by random amplification of polymorphic DNA (RAPD; OPAB4, P-14, UBC-556) and inter-retrotransposon amplification polymorphism (IRAP; Ogre) markers. Marker UBC-556 showed different banding patterns for each cultivar but without specific bands for selected and control plants. Markers OPAB4, P14, and Ogre were useful for clear discrimination between selected and non-selected variants of all three cultivars. Flow cytometry analysis proved the same genome size of selected and non-selected pea lines. Therefore *in vitro* selection by pathogen derived agents could be the efficient method for obtaining pea somaclones with increased resistance to *F. solani*.

Additional key words: in vitro culture, fusarium wilt, IRAP, molecular markers, *Pisum sativum*, RAPD, selection agent.

Introduction

Genetic variation induced *in vitro* is known as "somaclonal variation" (Larkin and Scowcroft 1981) and provides a valuable source of genetic diversity for plant breeders. However, genetically and phenotypically unstable cloned plantlets are undesirable. Culture filtrates containing a mixture of fungal metabolites are able to produce disease-like symptoms and trigger elicitation of various defence responses. Besides, fusaric acid (FA) is probably one of the most widely distributed mycotoxins produced by strains in the genus *Fusarium* spp. It is mildly toxic to animals but is also implicated in plant pathogenesis (Bacon *et al.* 1996). This chemical substance was used for selection focused on improving resistance to *Fusarium* spp. in plants such as *Musa acuminata* (Matsumoto *et al.* 1995), *Gladiolus communis* (Remotti and Löffler 1996) and *Solanum lycopersicum* (Kuzniak 2001).

Randomly amplified polymorphic DNA (RAPD) is based on arbitrarily amplified DNA and characterizes nucleic acids without prior knowledge of nucleotide sequence. Fingerprints are composed mainly of amplification products of varying length. Inter-retrotransposon amplification polymorphism (IRAP) is used in molecular fingerprinting (Kalendar *et al.* 1999, Smýkal 2006). This method relies on the knowledge of retroelement sequences, namely long terminal repeat (LTR) regions, which are used in designing primers. Since Ty3-gypsy type of retroelements is abundant in plant genomes, it can be successfully used in IRAP-PCR analysis (Neumann *et al.* 2003). IRAP method is a fast and efficient method for identification of pea cultivars and provides highly polymorphic information in a single PCR analysis (Smýkal 2006).

Epigenetic variability involves inherited changes in phenotype or gene expression caused by mechanisms other than changes in the underlying DNA sequence. The objective of this study was to determine genetic or epigenetic variability in selected and non-selected pea somaclones derived from *in vitro* selection by fungus culture filtrate and FA by using molecular methods. This experiment aims at improving disease resistance in pea through selection in culture.

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Abbreviations: BAP - benzylaminopurine; FA - fusaric acid; IRAP - inter-retrotransposon amplification polymorphism; ISSR - inter-simple sequence repeat; NAA - naphthaleneacetic acid; RAPD - random amplification of polymorphic DNA.
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Materials and methods

Cultivars of pea (*Pisum sativum* L. cv. Adept, Komet, and Bohatýr) were obtained from the Czech Germplasm Collection maintained by *Agritec Ltd*. and were used for establishing explant cultures *in vitro*. Seeds were surface sterilized and cotyledonary internodes were used for initiation of multiple shoot culture (Griga *et al.* 1986). Fungus culture filtrate of *Fusarium solani* was prepared according to Švábová *et al.* (2011) by filtering through 0.22 μm *Millipore* filter and added to the initiation medium after autoclaving at a concentration 10 % (v/v). The other selective agent was 0.05 mM fusaric acid (*Sigma-Aldrich*, St. Louis, USA) which was filter sterilized and added to the initiation medium [macro and micro elements according to Murashige and Skoog (1962), B5 vitamins (Gamborg *et al*. 1968), 20 μM benzylaminopurine (BAP), 0.1 μM naphthaleneacetic acid (NAA)]. Surviving regenerants from both selections were rooted on medium with the same content of macro and micro elements and vitamins as before but complemented with 1 μM NAA. Plantlets with well developed roots were transferred *ex vitro* and grown in a culture room (a 16-h photoperiod with irradiance of 20.4 μmol m^{-2} s^{-1}, temperature of 22 °C, and relative humidity of 85 - 90 %) to produce R1 seeds. Random plant samples of R2 generation (5 plants each) were examined by RAPD and IRAP methods.

Genomic DNA was isolated from fresh leaves at developmental stage BBCH13 (Hess *et al.* 1997) using the commercial kit *Invisorb Spin* plant mini kit (*Invitec*, Berlin, Germany). The quality and quantity of DNA was measured by spectrophotometry (*Eppendorf*, Hamburg, Germany) and DNA was diluted to the concentration of 30 μg cm^{-3} and stored at -20 °C.

RAPD analysis was conducted according to Samec *et al.* (1998), Wiesner *et al.* (2001), and Simioniuc *et al.* (2002). Polymerase chain reaction (PCR) amplification was performed in a 0.02 cm^3 reaction mixture containing 25 ng of genomic DNA, 1.5 μM primer, 100 μM of each dNTP, 2.5 mM $MgCl_2$ and 0.75 U of recombinant *Taq* polymerase (*BioTools*, Madrid, Spain) in PCR reaction buffer. Thermal cycler (*Mastercycler*, *Eppendorf*) was programmed as follows: 5 min denaturation step at 94 °C was followed by 40 cycles of 10 s at 94 °C, 1 min at 37 °C and 1.5 min at 72 °C. Product was resolved on 1.5 % agarose (*Serva*, Heidelberg, Germany) gels containing ethidium bromide. *Ladder Plus* (*MBI Fermentas*, Vilnius, Lithuania; 100 bp) was used as molecular mass marker.

For IRAP analysis, primers were designed to match close to 5´ and 3‘ ends of long terminal repeat (LTR) sequences of Ogre and Cyclop (Kalendar *et al.* 1999, Smýkal 2006). PCR amplification was performed in a 0.02 cm^3 reaction mixture containing: 25 ng of genomic DNA, 1.5 μM of each primer, 100 μM of each dNTP, 2.5 mM $MgCl_2$, and 0.7 U of *Taq* polymerase (*BioTools*) in PCR reaction buffer (75 mM Tris-HCl, pH 8.0, 20 mM $(NH_4)_2SO_4$, 0.01 % *Tween 20*). Cycling parameters were following: 4 min denaturation step at 94 °C was followed by 35 cycles of 30 s at 94 °C, 30 s at 58 °C and 3 min at 72 °C. Product was resolved on 1.5 % agarose (*Serva*) gels containing ethidium bromide.

The absolute DNA amount of studied plants was estimated by flow cytometry using *CyFlow-ML* (*Partec*, Münster, Germany) equipped with green laser (532 nm). As internal standard was used *Zea mays* CE-777 (2C = 5.46 pg, Lysák and Doležel 1998) for all studied plants. Sample preparation followed Galbraith *et al.* (1983) using LB01 buffer (15 mM TRIS; 2 mM EDTA; 0,5 mM spermine-4HCl; 80 mM KCl; 20 mM NaCl; 0.1 % *Triton X-100*; pH 8,0; Doležel *et al.* 1989) with modifications. Leaf tissues of standard and analyzed peas were co-chopped with razor blade in 1.3 cm^3 of LB01 buffer. The suspension of nuclei was filtered through nylon mesh (42 μm) and incubated in room temperature for 20 min. After incubation, 0.05 cm^3 of propidium iodid (PI) was added to suspension of nuclei and after next 20 min incubation measured on the flow cytometer up to 3 000 particles. The 2C values of the samples were calculated from a linear ratio between G1 peaks position of the standard and the sample. Each sample was measured twice.

Results

In the preliminary experiments, 9 sets of RAPD markers (OPAA3, OPAB4, OPAB8, OPAB12, P10, P14, UBC556, UBC561, UBC741) and 2 IRAP markers (Cyclop, Ogre) were used to investigate genetic differences between somaclones derived from selections *in vitro* by *F. solani* filtrate and fusaric acid. Markers showing polymorphic patterns of electrophoretic bands were used for verification of results on sets of 5 selected and 5 non-selected (control) plants.

RAPD marker UBC556 showed a different electrophoretic pattern for each cultivar (Adept, Bohatýr, Komet) but no difference was recorded between selected and non-selected lines (Fig. 1*A*). Cultivar Adept possessed the largest number of polymorphic bands. The fingerprints of Komet differed in two missing bands with molecular masses (Mr) of about 850 and 750 bp. Bohatýr fingerprints were similar to Komet but one 750 bp band was absent.

RAPD marker OPAB4 showed different polymorphic banding patterns for Adept but was similar for Komet and Bohatýr (Fig. 2*A*). However, a difference was found for Adept lines selected by FA (at Mr about 520 bp) but not for lines selected by *F. solani* filtrate as compared to control plants. Results of RAPD-OPAB4 analysis for

Komet did not exhibit the difference between selected and control plants. In selected lines of Bohatýr, a band of 860 bp was missing compared to the control.

RAPD marker P14 did not exhibit clear fingerprints in Bohatýr (Fig. 3*A*), the clear distinctness was recorded in 980 bp band in Komet that was missing in selected somaclone lines but present in controls.

Analysis of IRAP marker Ogre revealed a different electrophoretic pattern for Adept but a similar for both Komet and Bohatýr (Fig. 4*A*). Adept and Komet did not show differences in banding patterns for selected and non-selected lines. One band at 1050 bp was absent in

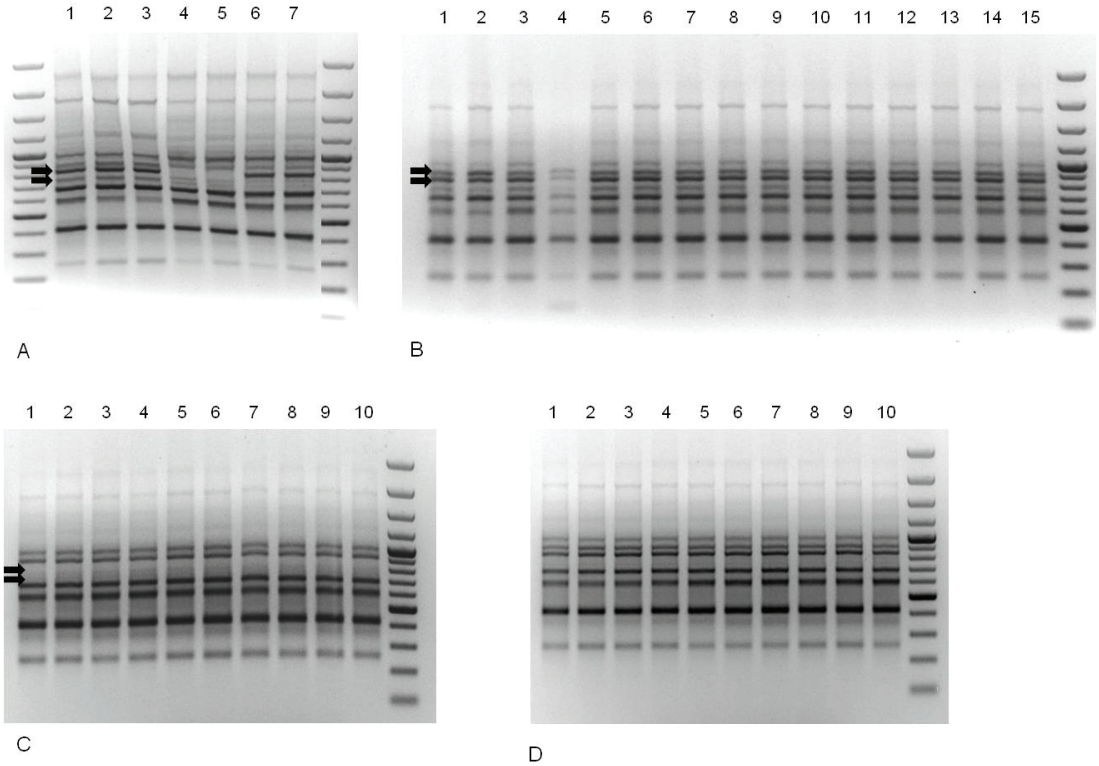

Fig. 1. Banding patterns of RAPD-UBC556. *A*: *lines* 1 - cv. Adept selected by fusaric acid (FA), 2 - cv. Adept selected by FS filtrate, 3 - cv. Adept control; 4 - cv. Komet selected by FA, 5 - cv. Komet control; 6 - cv. Bohatýr selected by FS filtrate, 7 - cv. Bohatýr control. *B*: replicates of cv. Adept, *lines* 1 to 5 - selected by FA, 6 to 10 - selected by FS filtrate, 11 to 15 - control. *C*: replicates of cv. Komet, *lines* 1 to 5 - selected by FA, 6 to 10 - control. *D*: replicates of cv. Bohatýr, *lines* 1 to 5 - selected by FS filtrate, 6 to 10 - control. *Arrows* indicate position of bands with 750 and 850 bp.

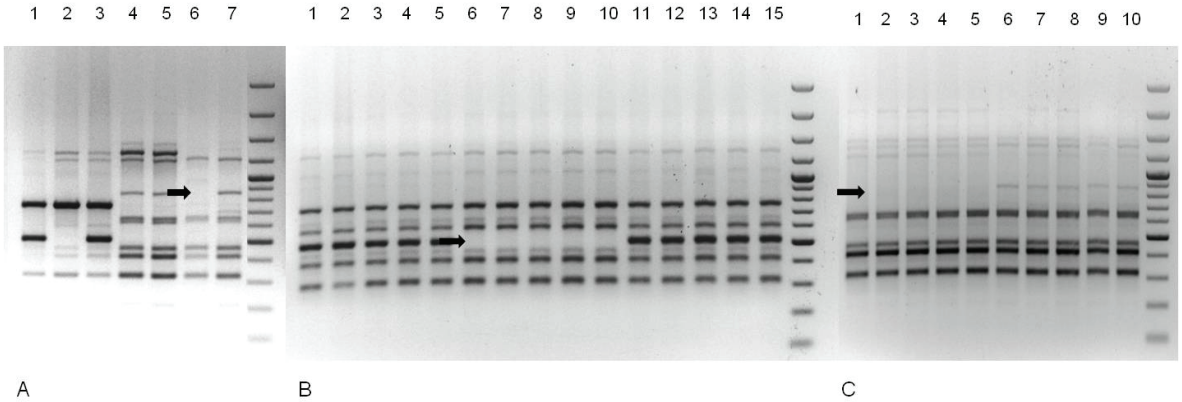

Fig. 2. Banding patterns of RAPD-OPAB4. *A*: *lines* 1 - cv. Adept selected by FA, 2 - cv. Adept selected by FS filtrate, 3 - cv. Adept control; 4 - cv. Komet selected by FA, 5 - cv. Komet control; 6 - cv. Bohatýr selected by FS filtrate, 7 - cv. Bohatýr control. *Arrow* indicates band at 860 bp. *B*: replicates of cv. Adept, lines 1 to 5 - selected by FA, 6 to 10 - selected by FS filtrate, 11 to 15 - control. *Arrow* indicates missing 520 bp band. *C*: replicates of cv. Bohatýr, *lines* 1 to 5 - selected by FS filtrate, 6 to 10 - control. *Arrow* indicates missing 860 bp band.

Bohatýr. Subsequent series of experiments followed with 5 replications of the same somaclone and corresponding cultivars. These analyses verified our results for the four markers mentioned above. Of the four banding patterns, three markers (RAPD-UBC556, RAPD-OPAB4, IRAP-Ogre) showed precisely the same pattern in all 5 replications. Cultivar Komet selected by FA (Fig. 3*B*), showed the same banding pattern as the control. This may be attributed to segregation in R2 progeny or to the incidence of one escape plant (not a somaclonal variant).

DNA size measured by flow cytometry in the samples of pea somaclones possessed uniform results. Total average of all samples was 8.44 pg with min-max values 8.19 - 8.67. Genome size of cvs. Adept and Komet in controls and their selected variants did not differ significantly.

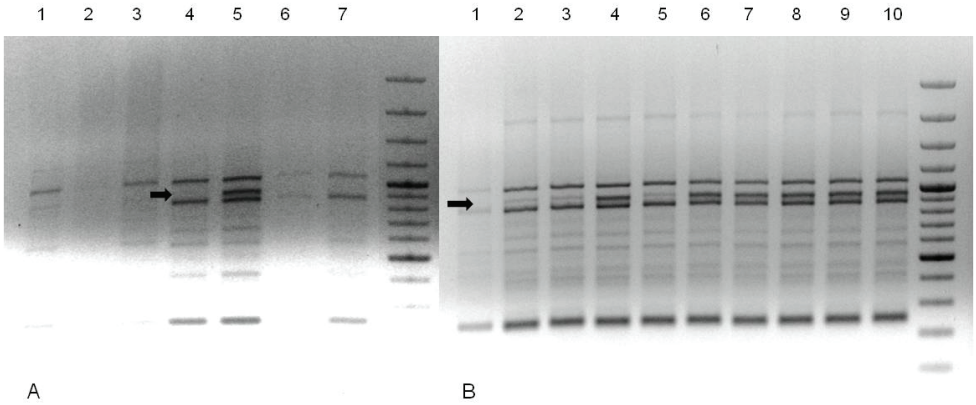

Fig. 3. Banding patterns of RAPD-P14. *A*: *lines* 1 - cv. Adept selected by FA, 2 - cv. Adept selected by FS filtrate, 3 - cv. Adept control; 4 - cv. Komet selected by FA, 5 - cv. Komet control; 6 - cv. Bohatýr selected by FS filtrate, 7 - cv. Bohatýr control. *B*: replicates of cv. Komet, *lines* 1 to 5 - selected by FA, 6 to 10 - control. *Arrows* indicate 980 bp bands.

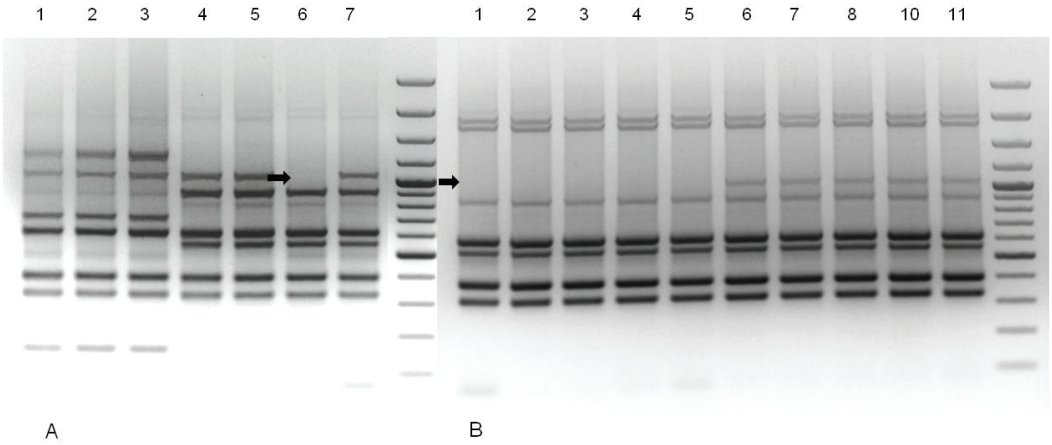

Fig. 4. Banding patterns of IRAP-Ogre. *A*: *lines* 1 - cv. Adept selected by FA, 2 - cv. Adept selected by FS filtrate, 3 - cv. Adept control; 4 - cv. Komet selected by FA, 5 - cv. Komet control; 6 - cv. Bohatýr selected by FS filtrate, 7 - cv. Bohatýr control. *B*: replicates of cv. Bohatýr, lines 1 to 5 - selected by FS filtrate, 6 to 11 - control. *Arrows* indicate 1050 bp band.

Discussion

Larkin and Scowcroft (1981) were the first who stated that plant cell culture generates genetic variability and they termed this phenomenon "somaclonal variation". One possible mechanism suggested for somaclonal variation is the activation of transposable elements (McClintock 1984, Scowcroft 1985). Hirochika (1993) stated that activation of retrotransposons is responsible for somaclonal variation: most tissue culture-induced mutations are stable like the mutations induced by retrotransposons.

DNA markers were studied for the identification of the degree of somaclonal variation in various crops such as date palm (Sakker *et al.* 2006), oil palm (Rival *et al.* 1998), potato (Soniya *et al.* 2001), soybean (Freytag *et al.*

1989, Gesteira *et al.* 2002), and tobacco (Hirochika 1993). We analyzed somaclonal variants of 4 pea cultivars by 8 RAPD primers (OPW08, P14, OPW02, P10, UBC741, UBC561, UBC556, OPAB4) which demonstrated a high polymorphism in pea (Samec *et al.* 1998, Wiesner *et al.* 2001, Simioniuc *et al.* 2002).

The stability of isozyme and DNA markers in pea cultivars was previously studied (Horáček *et al.* 2009). Variation in isozyme esterase banding pattern was found within cv. Zekon. Intracultivar variation detected by RAPD, SSR, ISSR, and IRAP was negligible and mostly quantitative. In cv. Bohatýr, a long-term (24 years) clonaly propagated multiple shoot culture did not show any major alteration in primary genomic DNA structure. Study of retroelement status proved high uniformity and stability of material *in vitro* (Smýkal *et al.* 2007). Similar results were achieved by Mohanty *et al.* (2011) in their experiments with *Zingiber rubens* after 30 months of *in vitro* culture. Given the stability of DNA markers in pea cultivars as well as in micropropagated pea cultures, it can be concluded that the variability of the banding patterns of molecular markers (RAPD-OPAB4, RAPD-P14, IRAP-Ogre) we observed in our somaclonal variants may have resulted from the fungal filtrate and fusaric acid treatments used for *in vitro* selection.

Molecular analysis of somaclones selected by *F. solani* filtrate and fusaric acid detected differences between somaclones and control plants for all three studied cultivars Adept (RAPD-OPAB4), Komet (RAPD-P14), and Bohatýr (RAPD-OPAB4, IRAP-Ogre). Similarities of banding patterns in cultivars Komet and Bohatýr were probably linked to their common genetic origin [Komet is a hybrid of (Tyrkys × Erygel) × Bohatýr].

Range of assigned 2C DNA content varies in *P. sativum* L. from 8.11 to 9.73 pg (Coyne *et al.* 2007, Loureiro *et al.* 2007). The flow cytometric data obtained in our experiments respond to the size of *P. sativum* genome of about 8Gb (2C) after calculation of genome size (bp) = (0.987 × 10^9) DNA content (pg) (Greilhuber *et al.* 2005). After selection, somaclones did not show major genome changes detected by flow cytometry.

Somaclones selected by pathogen derived selection agents can be a valuable source of variance for resistance breeding purposes. Their further involvement in breeding programme depends on their response to pathogens in field conditions and stability of introduced genetic or epigenetic changes. In our work with *P. sativum* L. somaclones, RAPD-OPAB4, RAPD-P14, and IRAP-Ogre render suitable electrophoretic bands for identification of differences in selected somaclones.

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