Variability of *PSPAL1* (Phenylalanine Ammonia-lyase Gene-1) Proximal Promoter Sequence and Expression in Pea Challenged with *Mycosphaerella pinodes*

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Abstract

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Part of the *PSPAL1* gene (corresponding to the proximal promoter, exon 1 and intron) from eight pea varieties was sequenced and compared to the published sequence of *PSPAL1* gene from Midoriusui cultivar (GenBank: D10002.1). The sequences showed a very high level of identity (96–99%), except in five varieties there occurred a motif TTATTACAAAATATTA close to the Goldberg-Hogness (TATA) box, and it was not detected in the other four varieties, including Midoriusui. Plants of eight pea varieties were subjected to controlled infection with *Mycosphaerella pinodes* and the disease index was determined (it ranged from 5.2 to 42.3%). The *PSPAL1* gene of the most resistant cultivar (Walor) contained the above-mentioned motif and that of the most susceptible (Polar) did not. However, the relationship was not clear in varieties with intermediate levels of resistance. In four varieties (Walor, Ezop, Ramrod and Polar) the expression level of *PSPAL1* gene in leaves was analysed (1, 3, 6, 9, 12 and 15 h post inoculation) and it showed a weak negative correlation with disease severity ($R = -0.53$). The activation of *PSPAL1* gene occurred not only in infected pea leaves but also in stems and – to a much lower degree – in roots (with the relative level of *PSPAL1* transcripts amounting to 0.15 in roots and 38.75 in leaves), indicating some kind of signal transmission beyond the infected plant tissues.

Keywords: expression; *Mycosphaerella pinodes*; phenylalanine ammonia-lyase gene-1; *Pisum sativum*; polymorphism; resistance

Plants respond to attacks by pathogens or pests with diverse measures of active defence, e.g. oxidative burst, cell wall reinforcement, accumulation of pathogenesis-related proteins, phytoalexin production or programmed cell death (Romero *et al.* 2008). It seems that various subsets of these defence systems are used depending on the type of invader – whether it is a herbivorous animal or a necrotrophic pathogen, or it is biotrophic (Antico *et al.* 2012). Moreover, the efficiency of these reactions may depend on genotypes of both the plant host and its antagonist (Salvaudon *et al.* 2005). A necrotrophic fungus *Mycosphaerella pinodes* causes significant losses in pea production and only pea genotypes with a limited resistance to this pathogen have been found so far (Boros & Wawer 2009). Rather typically for plant interactions with necrotrophic phytopathogens, the genetic determinants of resistance/pathogenicity in pea/*M. pinodes* interactions are weak, numerous and complex. A microarray analysis suggests that in pea responding to infection with *M. pinodes* the difference between a cultivar highly susceptible to the pathogen and the one partially resistant consists in the expression of approximately 350 genes, some of them – particularly

those related to primary metabolism – suppressed in the resistant variety, others – like those controlled by jasmonic acid and ethylene signals – upregulated (Fondevilla *et al.* 2011). Among the pathways induced in infected or stressed plants the biosynthesis of phenylpropanoids has long been given great attention, as it is strongly activated by elicitors and provides antimicrobial phytoalexins, lignin monomers and precursors of salicylic acid. PAL (phenylalanine ammonia-lyase) seems to be one of the critical elements of this system, catalyzing deamination of L-phenylalanine to *trans*-cinnamic acid and thus linking primary metabolism to the conversions of plant phenolics (Mauch-Mani & Slusarenko 1996; Mac Donald & D'Cunha 2007). Fondevilla *et al.* (2011) found that the *PAL* gene is among those most relevantly upregulated in a pea variety developing partial resistance reactions to *M. pinodes*.

PAL genes from many plant species have been cloned so far – GenBank search for *PAL* sequences of seed plants currently retrieves over 1600 entries from 245 species. *PAL* genes commonly occur in a few nonallelic copies, forming gene families. The number of members of these families varies from a few (four in *Arabidopsis*) to 13 in tomato and 50 in potato (Joos & Hahlbrock 1992; Chang *et al.* 2008; Huang *et al.* 2010). Two *PAL* genes from pea have been described so far, however based on Southern hybridization experiments it was hypothesized that there are at least three copies of *PSPAL* gene in the *Pisum sativum* genome (Kawamata *et al.* 1992, 1997). The structure and expression patterns of specific members of *PAL* gene families have been a subject of many detailed analyses. Both cases of functional specialization (with specific members of a gene family responding to different stimuli and activated in different tissues) and redundancy (with overlapping expression patterns or gene suppression) were reported (Seki *et al.* 1999; Imura *et al.* 2000; Kumar & Ellis 2001; Pluskota *et al.* 2005; Chang *et al.* 2008). So far, little – if any – attention has been given to sequence polymorphisms of *PAL* genes, to the comparisons of sequences of the same member of *PAL* gene family in different cultivars. In this paper we try to address this question with reference to the *PSPAL1* gene (*PAL1* gene of *Pisum sativum*).

MATERIAL AND METHODS

Plant material and growth conditions. Seeds of pea varieties used in this study are readily available in Poland and were purchased from these seed companies: Piast, Spójnia, CNOS Garden, Plantico, Torseed.

Seeds were surface sterilized with 1% bleach (15 min), washed with autoclaved water and germinated on water agar. After a week contaminated seedlings were discarded, material for gene cloning (roots) was collected and the other seedlings were transferred to flower pots (with capacity of approx. 400 ml) filled with autoclaved perlite.

Twenty-one days old seedlings of pea (*Pisum sativum* L.) of cultivars with normal leaf type: Polar, Walor, 6-tygodniowy ("6-week"), Telefon, Kiler, Cud Kelvedonu (Kelvedon Wonder) and of two "afila" leaf type cultivars – Ezop, Ramrod – were used for the infection experiments. These seedlings were grown in a greenhouse under natural daylight supplemented for 16 h daily by sodium lamps. The maximum temperature was 24°C during day and it fell to a minimum of 10°C at night. A twofold diluted solution of macro- and microelements of Murashige-Skoog medium was used as fertilizer once a week.

***PSPAL1* gene fragment cloning and sequencing**. DNA was isolated with 2% CTAB essentially according to Doyle and Doyle (1987). The *PSPAL1* gene was amplified using Phusion polymerase (Thermo Scientific, Waltham, USA) according to the manufacturer's recommendations. The primers used were: PsPAL-ENHANC and PsPAL-EGZ2 (Table 1, Figure 1) and the annealing temperature was 58°C. The DNA fragment was cloned using the CloneJET PCR Cloning Kit (Thermo Scientific) and chemically competent DH5a *E. coli* cells. Sequencing was carried out in a commercial facility (Genomed), using vector specific primers pJET1.2Forw and pJET1.2Rev. The sequences were assembled into contigs and compared and analysed using the DNA Baser, Blast, ClustalW and Signal Scan programs.

Fungal inoculum preparation. The strain 165/T of *M. pinodes* was isolated from leaves of cv. Piast with ascochyta blight symptoms (Okorski & Majchrzak 2009). A single spore culture of this fungus was obtained on a PDA medium. Spore suspensions were prepared from newly grown (at 21°C) 7-days-old *M. pinodes* cultures. The spores were harvested by flooding plates with sterile distilled water and by rubbing with a sterile glass rod. The concentration of spores was determined with a Thoma chamber and adjusted with sterile distilled water to 1×10^6 spores/ml.

Plant infection. Approximately 21-days-old seedlings (with leaves and bracts of the second and third internode fully expanded; 14/33 stage in the BBCH

Table 1. Primers used for gene cloning and expression analyses

Gene	Primers	Primer sequence	Amplicon size	Location within *PSPAL1*
PSPAL1	PsPAL-ENHANC*	5'-CAATATTGTGATTTGAGACACTC-3'	~1600	–459 ~ –481
	PsPAL-EGZ2*	5'-TGGTAGTGTATGGCTTGACTC-3'		1146 ~ 1125 (rev. primer)
PSPAL1	PsPAL1-Forward**	5'- CACTGACGATTTCTCAGGTGGC-3'	235	322 ~ 344
	PsPAL1-Reverse**	5'-TATTCCAGCATTCAAAAACCTGATG-3'		1110 ~ 1088...539 ~ 536 (rev. primer spanning the intron; for RT-PCR)
PSEF1a	PsEF1a-Forward**	5'-TTCCCTTCGTTCCCATCTCTG-3'	230	–
	PsEF1a-Reverse**	5'-TACAAGCATACCGGGCTTCAC-3'		–

*primers used for *PSPAL1* gene cloning; **primers used for real-time PCR analyses; amplicon size in bp

scale) were inoculated with spore suspension (2 ml per plant), using a brush (Tu & Poysa 1990). Clear plastic propagator tops were placed over the plants to maintain a high humidity during the first 24 h. Control plants were treated with distilled water in the same way. The experiment consisted of two sets of plants with each set including three replicated pots for each cultivar with four plants/pot.

The health status of pea plants was estimated in 14 days after infection, using the modified Hillstrand and Auld scale (1982): 0 – no disease symptoms, 1 – infection rate (IR) of 1–10%, 2 – IR of 11–20%, 3 – IR of 21–30%, 4 – IR of 31–40%, 5 – IR of 41–60%, 6 – IR of 61–80%, 7 – IR of 81–90%, 8–9 – IR of 91–100%. The results provided a basis for calculating the disease index (DI), as described by McKinney (Lacicowa 1969). The results were processed statistically (Data Analysis Software System, ver. 8.1 Statsoft Inc., 2007; www.statsoft.com). The significance of differences between mean values was estimated by the Newman-Keuls test ($P = 0.01$).

RNA extraction and gene expression analysis. Based on results of plant infection experiments four varieties were selected for gene expression analyses: the most resistant (Walor), the most sensitive (Polar) and two cultivars with intermediate levels of disease severity (Ramrod and Ezop, see below). Leaves, stems and roots were collected from inoculated and uninoculated seedlings (control plants). Samples were harvested after 0, 3, 6, 9, 12, and 15 h post inoculation (14/33 BBCH). Total cellular RNA was prepared using a method similar to that described by Chomczyński and Sacchi (1987) and dissolved in 25 µl of DEPC-treated water.

Total RNA concentrations and purity were determined by spectrophotometry and by staining ribosomal RNA with ethidium bromide, respectively. RNA preparations were analysed according to Masek *et al*. (2005).

The extracted total RNA (1 µg) was used to obtain the first strand cDNA by reverse transcription (RT-PCR) using the ImProm II Reverse Transcription System (Promega, Madison, USA) according to the manufacturer's instructions in a Mastercycler (Eppendorf, Hamburg, Germany).

The levels of *PSPAL1* transcripts were analysed by real-time PCR using SYBR Green fluorescent dye. Primers were designed based on sequences from the

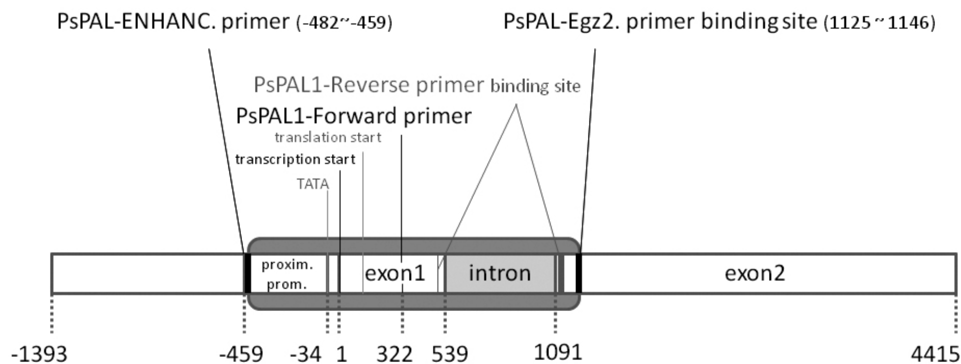

Figure 1. The position of the studied region (grey rounded rectangle) and applied primers in relation to the reference sequence of *PSPAL1* from pea cv. Midoriusui; all values are given in bp and expressed relative to the transcription start; proxim. prom. – proximal promoter

NCBI database (GenBank, www.ncbi.nml.gov) using Primer Express (Applied Biosystems Inc., South San Francisco, USA). PsPAL1-forward and PsPAL1-reverse primers were designed so as to span the exon-exon junction in the *PSPAL1* gene (Figure 1). Primers PsEF1a-forward and PsEF1a-reverse were used to amplify the alpha elongation factor gene (*EF1 alfa*) as internal control (Table 1). All analysed samples were normalized to the *EF1 alfa* gene, which was first validated as showing stable expression in the analysed tissues.

The PCR reaction was carried out as follows: initial 3 min of denaturation at 95°C; followed by 40 cycles of denaturing at 95°C for 15 s, annealing at 60°C for 15 s and extension at 72°C for 1 min. Immediately after the final PCR cycle, a melting curve analysis was performed to determine the specificity of the reaction. All the reactions were also analysed by agarose gel electrophoresis. Each sample was loaded in duplicate or triplicate onto a single PCR plate. The real-time PCR reaction was performed in a reaction mixture (25 µl total volume) with 0.625 U Taq DNA polymerase (recombinant, 1× buffer, 3mM $MgCl_2$) (Invitrogen, Grand Island, USA), 400µM dNTPS (Roth, Karlsruhe, Germany), 400nM primers, 1:40000–1:10000 SYBR Green I (Molecular Probes, Grand Island, USA), 1× ROX (Molecular Probes), 0.5 ng/µl Ac-BSA (Sigma-Aldrich, St. Louis, USA), 5% DMSO (Sigma-Aldrich), Glycerol (POCH, Gliwice, Poland), 0.01% Tween 20 (POCH) PCR Master Mix (Applied Biosystems, South San Francisco, USA), water to 25 µl. The PCR was monitored on an ABI Prism 7500 Sequence Detection System (Applied Biosystems). Quantification of the gene expression was done by the comparative CT method (Livak & Schmittgen 2001).

RESULTS AND DISCUSSION

The primer pair used in this work enabled the amplification of a fragment of *PSPAL1* gene in all the varieties studied. According to the functional characteristics of *PSPAL1* carried out by Yamada *et al.* (1992), in Midoriusui cultivar the PCR product should start 459 bp upstream of the transcription initiation site and should extend over the length of 1605 bp down to the very beginning of exon2. Indeed, products of approx. 1600 bp were obtained with all varieties studied here (Figure 1). The sequences of these products showed remarkable similarity to *PSPAL1* of Midoriusui, differing from it by no more than 5% in the intron and 1% in the coding region (Table 2). Based on the *PSPAL1* promoter sequence the varieties studied could be clearly divided into two categories: those containing a string of 16 nucleotides (TTATTACAAAATATTA) located 14 bp upstream of the Goldberg-Hogness box, and those like Midoriusui which do not contain this block (Figure 2A). Within each of these two groups of varieties the sequences of all analysed *PSPAL1* gene regions, including intron, were practically identical (Figure 2B). This means that the very high observed similarities between studied cultivars in *PSPAL1* sequence reflect very small genetic distances between these cultivars rather than the function-related evolutionary sequence conservation. This was surprising, as the cultivars chosen for this work differ in morphology (normal versus narrow leaf) and application range (garden vs. field peas). Simioniuc *et al.* (2002) used AFLP and RAPD markers to estimate overall genetic diversity among 21 pea cultivars used in Germany. They concluded that pea cultivars used in Germany

Table 2. The level of identity of *PSPAL1* partial sequences from different pea cultivars with the published sequence (GenBank) of *PSPAL1* from Midoriusui cultivar

Cultivar	Level of sequence identity with the corresponding fragment of *PSPAL1* from Midoriusui				
	the whole region 1613*	Promoter 459*	Exon1 539*	5'UTR 114*	Intron 552*
Percent sequence identity:number of SNIPs					
Walor	97:53	96:17	99:8	95:6	95:27
Polar	99:11	100:0	99:1	99:1	98:10
Ezop	99:12	100:0	99:1	99:1	98:11
Ramrod	99:12	100:0	99:1	99:1	98:11
6-tygodniowy	99:11	100:0	99:1	99:1	98:10
Telefon	96:53	94:17	99:8	95:6	95:27
Cud Kelvedonu	97:49	96:17	99:8	95:6	95:27
Kiler	97:53	96:17	99:8	95:6	95:27

*length of the region in PSPAL1 Midoriusui sequence

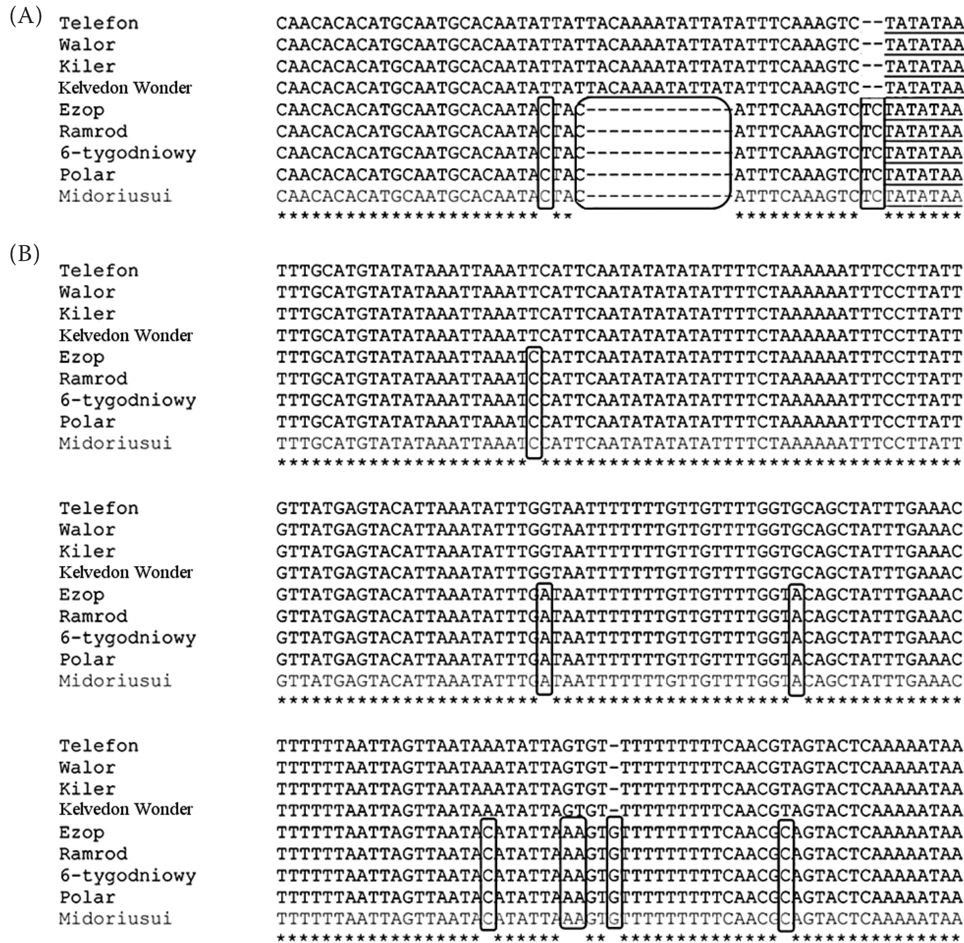

Figure 2. Comparison of partial sequences of *PSPAL1* promoter and intron in different pea varieties: (A) – promoter region –74 bp to –36 bp; shown polymorphisms at positions –51, –48 (1 transition, 13 bp indel), –35, –30 to –28 bp; (B) – intron region 656 to 833 bp; shown polymorphisms at positions 678, 738, 760, 793, 799, 800, 803 and 818 bp, relative to the transcription start

are quite closely related (genetic similarity was found to be 0.84 to 0.94). On the other hand, even if the variation of pea PAL genes is quite small, the presence of several additional nucleotides in the area which is very close to the TATA box may significantly affect the rate of transcription and gene activity level. A search for transcription factors binding the above-mentioned sequence was carried out by the SIGNAL SCAN program (Prestidge 1991; Higo *et al.* 1999). It suggests that the sequence could be related to light and morphogenetic regulation. The described 16 nucleotide region contains a TTATTA motif and its imperfect repeat (ATATTA). The TTATTA motif was first described as a human cis-element bound by AIRE DNA-binding proteins and involved in autoimmune reactions (Kumar *et al.* 2001). It is also highly represented in *A. thaliana* genes, particularly those related to shade avoidance (88% frequency – Devlin *et al.* 2003). Further experiments should verify any significance of this characteristic 16 bp element (or its 6 bp components) in PAL genes of some pea varieties and possibly their responses to pathogens or other stimuli. The 16 bp element described here has not been observed in PAL genes of any other plant species (as revealed by BLAST searches).

In all of the four pea varieties analysed for *PSPAL1* gene expression, the maximum enhancement of *PSPAL1* transcript accumulation as a result of the inoculation of *M. pinodes* spores was observed in leaves (i.e. at the site of primary infection), however the rate of changes in the relative mRNA level and its maximum value differed depending on the cultivar (Figure 3). In Ezop and Ramrod cultivars the level of *PSPAL1* transcripts significantly increased in leaves within six hours after infection and remained very low in roots and stems in the entire analysed period (15 h). However, in cultivar Ramrod the maximum relative expression level of *PSPAL1* gene was much

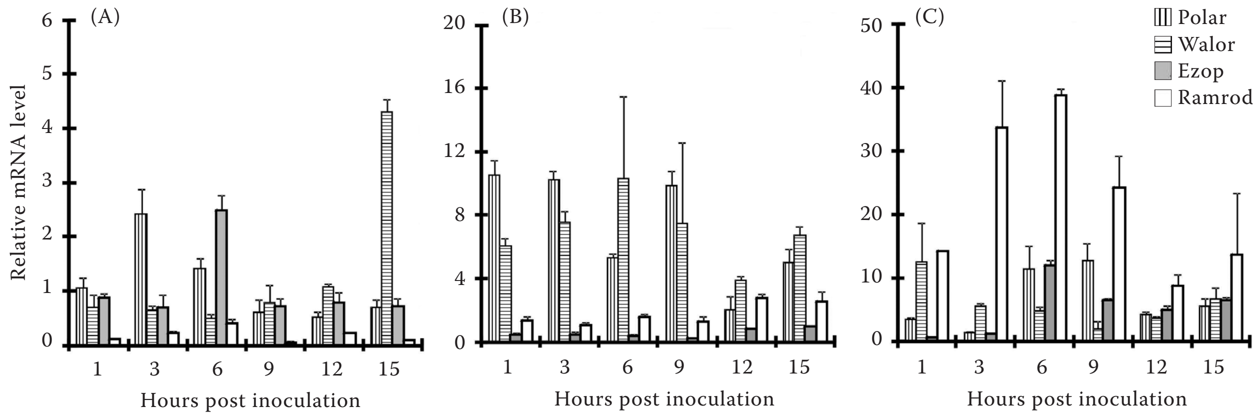

Figure 3. The relative mRNA level (the ratio of *PSPAL1* transcripts in infected plants/PSPAL1 transcript level in untreated plants) in roots (A), stems (B), and leaves (C) in pea cv. Polar, Walor, Ezop and Ramrod infected with *M. pinodes*

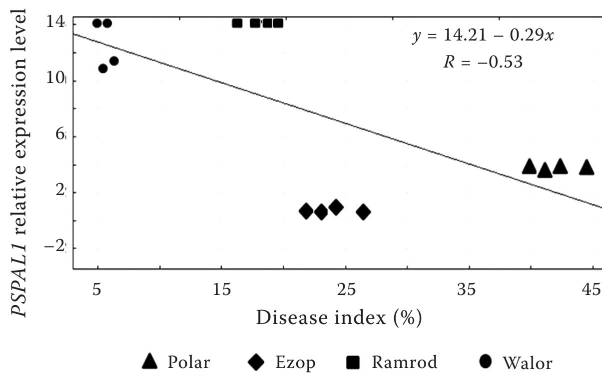

Figure 4. The relationship between *PSPAL1* expression in pea leaves and infection index

higher than in any other cultivar. Walor cv. was characterised by a high level of relative *PSPAL1* expression in leaves at the earliest time after inoculation. It also showed a fairly strong stimulation of *PSPAL1* expression in stems, similarly like cultivar Polar, which however was characterised by delayed activation of *PSPAL1* transcription in leaves.

The rate of stimulation of *PSPAL1* transcription in leaves showed a weak negative correlation with the degree of plant infection (Figure 4). Based on disease severity the analysed pea varieties could be divided into four categories (Figure 5), with Walor cv. as the most resistant, and Polar as the most susceptible. Among the five most resistant cultivars three have the longer promoter and among the three most susceptible two have the shorter version of *PSPAL1* promoter. The difference in resistance between Walor, Polar and Ramrod cultivars seems to confirm assertions made by some authors – Yamada *et al.* (1992), Montesano *et al.* (2003) – that it is rather the rate of activation than the maximum level of defence gene expression that determines the level of plant resistance to phytopathogens. Fujita *et al.* (2004) reported that plant's defence responses can occur as early as thirty minutes after its contact with fungal spores. Our results suggest that the rate of *PSPAL1* gene activation in pea leaves is much more important in this regard than in roots or stems. More experiments will be needed to see if the polymorphisms found in the *PSPAL1 gene* promoter determine the rate of *PSPAL1* gene activation and how important these differences are for the variation of pea cultivars in sensitivity to phytopathogens.

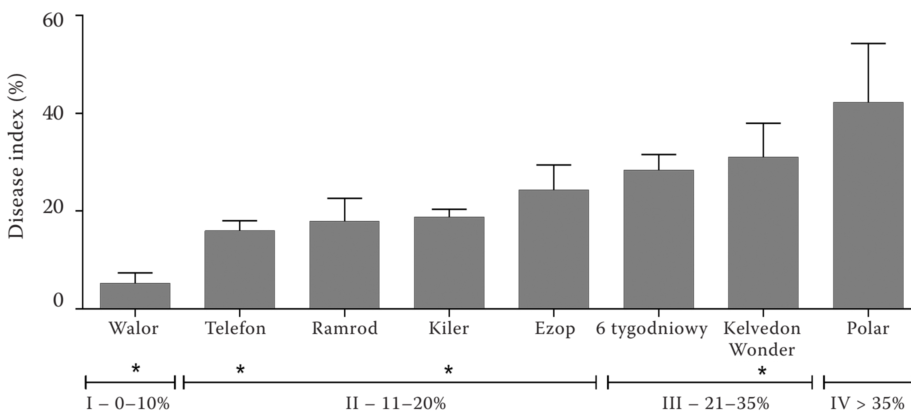

Figure 5. Disease index for eight pea varieties infected with *M. pinodes* (asterisk denotes cultivars with extended *PSPAL1* promoter)

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