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Chitosan is involved in elicitation of vestitol production in *Lotus japonicus*

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Abstract

Leaves of *Lotus japonicus* infected by powdery mildew accumulate isoflavonoids, especially vestitol and sativan as protective compounds, whereas their flavonol-glycosides or phenylpropanoic acids content were rather decreased. For a better understanding of the induction of phytoalexin production in *L. japonicus*, the influence of important signalling molecules, namely methyl jasmonate and salicylic acid, was tested by leaf application, up to 15 d. No effects after spraying the leaves with 0.5 mM salicylic acid were observed regarding the plant isoflavonoid content, whereas methyl jasmonate showed moderate effect on vestitol accumulation. Chitosan application led to a strong increase in vestitol content, sativan and vestitol derivative also increased. In a similar way to the effect of powdery mildew infection, the content of ferulic acid and kaempferol glycosides decreased, except for one compound, identified as kaempferol-6-deoxyhexose, that is the result of acetylation of kaempferol glycosides. The application of chitosan also led to an approximately 2.5- to 3.5-fold increase in hydrogen peroxide content, indicating the involvement of H_2O_2 in the chitosan signalling pathway. The activity of key enzyme of the phenolic biosynthesis, phenylalanine amonia lyase (PAL), was stimulated as well as the expression of 3 *PAL*-isogenes (*LjPAL4*, *LjPAL6*, and *LjPAL9*), whereas *LjPAL8* decreased. Moreover, the expression of two key enzymes of vestitol and sativan biosythesis: pterocarpan reductase and isoflavone synthase were also strongly induced. The data suggest that chitosan acts as an elicitor of the fungal attack in *Lotus japonicus* that do not lead to a general stimulation of the metabolism of phenols, but rather to a specific induction of isoflavonoid-phytoalexine production, especially for production of vestitol.

Keywords: biotic stress, chitosan, *Lotus japonicus*, phytoalexins, vestitol.

Introduction

Plants are often exposed to different abiotic and biotic stresses that induce multigene response, resulting in specific modulations in primary and secondary metabolite accumulation (Zhao *et al.* 2005). Low molecular mass compounds absent in healthy tissues or present only in insignificant amounts are produced by plants in significant quantities as a response to biotic stresses (mainly to attack by fungi or bacteria) are collectively called phytoalexins. They take part in an intricate defence system which enables plants to control invading patogens (Jeandet *et al.* 2014). Phytoalexins have been described in number of plants belonging to different families including those of economic importance such as *Brassicaceae*, *Fabaceae*, *Solanaceae*, *Vitaceae*, and *Poaceae* (Ahuja *et al.* 2012).

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Abbreviations: BI - Bayasian inference method; DAD - diode array detector; ESI - electrospray ionization; IFS - isoflavone synthase; ML - maximum-likelihood method; MS - mass spectrometry; PAL - phenylalanine ammonia lyase; PTR - pterocarpane reductase.
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Most phytoalexins produced by the *Fabaceae* belong to six isoflavonoid classes: isoflavones, isoflavanones, pterocarpans, pterocarpenes, isoflavans, and coumestans (Jeandet *et al.* 2014). In *Lotus* species, the main phytoalexin biosynthesised is vestitol, which belong to the class of isoflavans. Vestitol, and its methylated/demethylated derivatives; sativan or demethylvestitol, are induced in several *Lotus* species, such as *L. corniculatus*, *L. uligonus*, *L. hipidus*, *L. edulis* or *L. angustissimus* leaves following treatment with fungal pathogens (Ingham and Dewick 1980, Robbins *et al.* 1995).

One of the main elicitors for the plant defence response is chitosan (Ramachandra Rao and Ravishankar 2002), a natural non-toxic polymer of β-1,4-linked glucosamine that is a product of chitin deacetylation, which is also a main component of the shell of shrimps and crabs (crustaceans shell), mollusks (endoskeleton of cephalopods), fungi cell walls, and insects (exoskeleton) (Elieh-Ali-Komi and Hamblin 2016). Chitosan treatment has a considerable effect on plants as it stimulates photosynthetic rate (Farouk and Amany 2012) and stomatal closure through ABA synthesis (Iriti and Faoro 2008, Khokon *et al.* 2010), it also enhances the production of antioxidant enzymes *via* nitric oxide and hydrogen peroxide signalling pathways (Rakwal *et al.* 2002, Lin *et al.* 2005, Hidangmayum *et al.* 2019), and induces biosynthesis of organic acids, sugars, amino acids, and other metabolites which are required for the osmotic adjustment, stress signalling, and energy metabolism under stresses (Bistgani *et al.* 2017, Li *et al.* 2017). Besides, it is used as antitranspirant compound through foliar application in many plants thus reducing water use and ensures protection from other negative effects of abiotic stress. Based on such beneficial properties, chitosan can be utilized in sustainable agricultural practices in the current context of climate change (Hidangmayum *et al.* 2019).

Chitosan activates the expression of genes related to the biosynthesis of phytoalexins as described in different plant species (Côté and Hahn 1994, Ebel and Mithöfer 1998, Iriti and Faoro 2008, Mejía-Teniente *et al.* 2013, Malerba and Cerana 2015, Jiao *et al.* 2018). However, the action mechanism of chitosan inducing phytoalexin biosynthesis is still largely unknown.

Isoflavonoids are typical legume phytoalexins that are produced in the response to phytopathogens (Stafford 1997). Most of the studies in this field focused on the identification and characterisation of the genes involved in the biosynthesis of the isoflavonoid phytoalexin vestitol in *Lotus japonicus* (Shimada *et al.* 2000, 2003, 2006, 2007; Akashi *et al.* 2003, 2006; Shimamura *et al.* 2007), however there have been few studies focusing on the interaction between leguminous plant defences and symbioses with compatible and incompatible fungi through isoflavonoids (Masunaka *et al.* 2011). Generally, isoflavones are considered as phytoalexins in leguminous plants that are strongly inducible and especially sensitive to pathogen and insect attack (Ahuja *et al.* 2012). It is well known that chitin is a typical pathogen-associated molecular pattern (PAMP) present in fungal cell walls (Zhang *et al.* 2015). For this reason, chitosan (the water-soluble derivative of chitin) is able to elicit plant defence response *via* simulating the attacks of fungal pathogen, which can eventually result in the enhancement of phytoalexin biosynthesis (Isah *et al.* 2018). In the present study, the effect of chitosan on plant phytochemical responses, and on the key steps of phenolic biosynthesis and vestitol production was investigated. The aim of this work was to study the action of chitosan to get a better understanding of the elicitation of *Lotus* phytoalexin accumulation.

Materials and methods

Plants, growth, and stress treatments: The model legume plant *Lotus japonicus* (Regel) K. Larsen ecotype Gifu (B-129-S9) was used for all the experiments. Seedlings were propagated by 8 cm shoot cuttings transferred to pots filled with *Vermiculite*. Plants were grown for 30 d at a photon flux density at the plant level of about 150 μmol m^{-2} s^{-1}, a 16-h photoperiod, a temperature of 22°C, and a relative humidity of 70%. Standard Hornum nutrient solution was used for watering as described by Handberg and Stougaard (1992). The leaves of the plants spontaneously infected by powdery mildew were collected and the pathogen was identified as *Erisiphe* sp. using a light microscope (Moreira *et al.* 2014, Ellis 2022).

Chitosan (CAS no. 9012-76-4; degree of deacetylation 75%, *Sigma-Aldrich*, St. Louis, USA) was dissolved in 5% (m/v) acetic acid, the solution was then diluted with distilled water. The final concentration of chitosan was 100 or 400 mg dm^{-3} in 0.7% acetic acid and 0.05% *Tween 20* was added as a surfactant. The pH of the chitosan solutions was adjusted to 6.5 with 2 M NaOH. These solutions were applied on the shoots using a hand sprayer until complete wetness, approximately 20 cm^3 per pot (1 pot contained 4 plants). Control plants were sprayed with chitosan-free solution. The treatment was carried out three times a week for 15 d. Methyl jasmonate (112.2 mg dm^{-3}) and salicylic acid (69.1 mg dm^{-3}) were also applied on the shoots similarly to chitosan up to 15 d. Plants treated with methyl jasmonate were covered by glass bell-jar. Alternatively, whole plants were immersed to the solution containing 7.5 mM methyl jasmonate or salicylic acid and 0.05% *Tween 20* for 24 h.

HPLC analysis: For phenolic compound extraction, the harvested *L. japonicus* samples were dried at 60°C for 24 h. For each biological replicate, all the leaves (about 10) of one individual plant were used. Dry tissue (50 mg) was extracted with 1.5 cm^3 of 80% (v/v) methanol for analysis of flavonoid glycosides, phenylpropanoids, and isoflavonoids. The extracts were centrifuged at 716 *g* for 5 min and filtered using a 0.2 μm nylon membrane. Samples were analysed by gradient reversed phase HPLC using an *Agilent 1260 Infinity Quaternaly LC* (Santa Clara, USA) system with *1260 Infinity DAD* detector and *Kromasil* (Bohus, Sweden) C_{18} 250 × 4.6 mm × 5 μm column. The solvent was delivered to the column at a 0.7 cm^3 min^{-1} flow rate. A gradient system with two mobile phases was used; 5% (v/v) acetonitrile with 3% (v/v) trifluoroacetic acid (A) and 80% (v/v) acetonitrile (B).

The gradient programme utilised for determination of flavonoids, phenylpropanoids, and isoflavonoids was as follows: 0 min A/B (90/10); 0 - 5 min A/B (84/16); 5 - 30 min A/B (80/20); 30 - 35 min A/B (71/29); 52 - 56 min A/B (0/100); 56 - 60 min A/B (90/10). Peak identities were determined based on their retention time, UV-absorption spectra, and by comparison with standards of vestitol (*Biorbit*, Cambridge, UK), biochanin A, formononetin, *p*-coumaric acid, *p*-ferulic acid, kaempferol-3-O-glucoside, kaempferol-3-O-rhamnoside (*Sigma Aldrich*), hyperoside (*Extrasynthese*, Genay, France), medicarpin (*Plantech*, Reading, UK), or using the standards isolated and identified by NMR methods (García-Calderón *et al.* 2015). The identities of some peaks were also verified according the MS data obtained by LC-DAD-ESI-MS/MS analyses on *Diodex Ultimate 3000 Quaternary Analytical LC System* (Germering, Germany) interfaced to *Varian 310 MS*, triple quadrupole mass spectrometer with electrospray ionisation source (Walnut Creek, CA, USA). Chromatographic separation was performed on the same column as above under the slightly modified conditions. Mobile phases A and B contained 0.1% (v/v) formic acid both in 5 and 80% (v/v) acetonitrile, respectively, while the same gradient was used as described above. The 0.7 cm^3 min^{-1} mobile phase flow exiting the HPLC column was split allowing approximately 0.2 cm^3 min^{-1} inside the ESI interface. ESI parameters were as follows: positive-ion mode; capillary voltage of 4.5 kV; nitrogen nebulising and drying gas flow rate of 50 and 30 psi, respectively; desolvation temperature of 300°C. Mass spectra were recorded in the *m/z* range of 250 - 1 000. MS/MS fragmentation was carried out using argon as a collision gas with the collision energy of 10 eV. All flavonols were evaluated at λ=350 nm, phenylpropanoids at λ=280 nm, vestitol, sativan, and biochanin A at λ=220 nm. Kaempferol-3-O-glucoside was used for evaluation of MS identified kaempferol-6-deoxyhexose, -hexose, -hexose-acetyl, kaempferol-7-O-glucoside for kaempferol-7-O-6-deoxyhexose, quercetin-3-O-rhamnoside for quercetin-6-deoxyhexose, -hexose, and quercetin-6-deoxyhexose, -6-deoxyhexose. Vestitol was used as the reference for sativan. The content of the compounds was expressed as μmol g^{-1}(dry mass).

PAL enzyme activity: The activity of phenylalanine ammonia-lyase was quantified according to the production of *trans*-cinnamic acid from phenylalanine (dos Santos *et al.* 2004). Fresh leaves were extracted with 0.1 M borate buffer (pH 8.6). The reaction was started by addition of phenylalanine. Samples were incubated for 1 h at 38°C and then the reaction was stopped by addition of hydrochloric acid. Samples were centrifuged shortly and the accumulation of *t*-cinnamic acid was quantified by isocratic HPLC (*Agilent 1260 Infinity Quaternaly LC*, Santa Clara, USA) with 55% acetonitrile as a mobile phase, using a *Kromasil* C_{18} 150 × 4.6 mm × 7 μm column, flow rate 0.5 cm^3 min^{-1} and detection at 275 nm.

Superoxide anion scavenging activity: The superoxide anion scavenging activity was measured according to the Nishikimi (1975) with slight modifications. The leaf fresh mass (100 mg) was homogenised in a mortar in 10 cm^3 5% (m/v) DMSO and centrifuged at 10 000 *g*. A portion (0.1 cm^3) of each supernatant was mixed with 1 cm^3 of NADH (468 μM of nicotinamide adenine dinucleotide solution in 0.1 M phosphate buffer at pH 7.4) and 1 cm^3 of NBT (156 μM of nitroblue tetrazolium solution in 0.1 M phosphate buffer at pH 7.4). The reaction was started by adding of 0.1 cm^3 of PMS (60 μM of phenazine methosulfate solution dissolved in 0.1 M phosphate buffer at pH 7.4). The mixture was incubated at room temperature in the dark for 5 min, then the absorbance was measured at 560 nm in a spectrophotometer (multi-detection microplate reader; *Synergy HT*, *BioTek*, Vermont, USA). The superoxide anion content was expressed as a percentage by the following equation: Superoxide anion scavenging activity [%] = $[(A_0 - A_1/A_0) \times 100]$, where A_0 was the absorbance of negative control, and A_1 was the absorbance of reaction mixture of plant samples.

Chlorophyll and hydrogen peroxide content: For chlorophyll content determination, 0.1 g of fresh leaves from the shoot tip (2nd and 3rd leaves) were homogenised in a mortar with 8 cm^3 of absolute methanol and 1 mg of $MgCO_3$ in dark. The extracts were centrifuged for 10 min at 2 800 *g* to remove the insoluble material. The absorbance was measured at 666, 653, and 750 nm (*Jenway 7310*, Stone, Staffordshire, UK) and chlorophyll *a* or *b* content was calculated according to Wellburn (1994). The content of hydrogen peroxide was determined by monitoring the formation of titanium-peroxide complex as described Zhang *et al.* (2019): 0.5 g of whole shoot fresh mass was homogenized in a mortar with 3 cm^3 of 50 mM K-phosphate buffer (pH 6.5) and centrifuged at 2 800 *g* for 20 min at 4°C (*Hettich Mikro 200R*, Tuttlingen, Germany). 1 cm^3 of 0.1% titanium tetrachloride in 20% sulphuric acid (v/v) was added to 3 cm^3 of supernatant. The intensity of the yellow colour was determined at 410 nm (*Jenway 7310*, Stone, Staffordshire, UK) and the peroxide content was calculated as the difference of samples with and without titanium addition.

RNA extraction and real-time PCR: Leaves from 2 individual plants from the same pot were collected, pooled together as a one biological sample and quickly frozen in liquid nitrogen and stored at -80°C. Total RNA was isolated using the protocol described by Kistner and Matamoros (2005). The integrity and concentration of the RNA preparations were checked by electrophoresis (*Biorad Power Pack Basic*, Gémenos, France) and using a fluorescent microplate reader *Biotek Synergy HT* (*Bio-Rad*, Hercules, USA) respectively. RNA extraction was carried out using three independent biological samples for each condition. For RT-qPCR analysis, total RNA was treated with the *TURBO* DNA free DNAse (*ThermoFischer*, Waltham, MA, USA). Reverse transcription was carried out using *Thermo Scientific RevertAid* reverse transcriptase (*ThermoFischer*, Waltham, MA, USA) using 1 μmol of total RNA extract and oligo dt: (dt)12GN, (dt)12CN, (dt)12AN-(1:1:1) (*Sigma*). DNA

contamination and RNA integrity were checked by performing RT-qPCR reaction with oligonucleotides that amplified an intron in the *L. japonicus* hypernodulation aberrant root formation (*LjHAR1*) gene and the 5' and 3' ends of the *L. japonicus* glyceraldehyde-3-phosphate dehydrogenase (using the oligonucleotide pairs LjGAPDH5' and LjGAPDH3'). RT-qPCR reactions were carried out in a 20 mm^3 final volume with *Light Cycler® Nano Roche* using a *FastStart Essential DNA Green Master* kit (*Roche*, Basel, Switzerland). $2^{-\Delta\Delta Ct}$ values were standardized by dividing them by the geometric mean of 2^{-Ct} values of the genes encoding for *L. japonicus* polyubiquitin4 (*LjUBQ4*) and protein phosphatase 2A reg. subunit (*LjPP2A*), which were selected from the most stably expressed genes in the plants (Czechowski *et al.* 2005, Sánchez *et al.* 2008).

Oligonucleotide pairs specific for isogenes encoding phenylalanine ammonia lyase (PAL), the key enzyme of the biosynthesis of phenolic compounds were designed. After new *Lotus* genome updates, 12 isogenes were found in *Lotus* database (*Lotus BASE*; Mun *et al.* 2016). Previously designed oligonucleotides specific for 3 genes for isoflavone synthase (IFS), the key enzyme of isoflavonoids synthesis (Kaducová *et al.* 2019) and 4 genes for pterocarpane reductase (PTR), the enzyme that produces vestitol (Kaducová *et al.* 2022), were also employed. A list of all the oligonucleotides used is provided in Table 1S.

Phylogenetic analyses: A phylogenetic tree was constructed using the protein-coding nucleotide sequences of 4 *AtPAL*s, 6 *MtPAL*s, 8 *GmPAL*s, and 11*LjPALs* as well as *NtPAL* and *OsPAL*, the latter used as outgroup (Table 3S, Fig. 1S). *LjPAL5* was not considered due to short coding sequence available for this gene in the *LotusBASE*. The nucleotide sequences were aligned using *MUSCLE* multiple alignment method in *MEGA11 v. 11.0.13* (Tamura *et al.* 2021), then checked by eye for positions of indels, and finally trimmed. Substitution model GTR+I+G was determined as the best fitted the data based on the *Akaike* information criterion in *jModeltest v. 2.1.9* (Darriba *et al.* 2012). Phylogenetic relationships between sequences and tree reconstruction were conducted applying Maximum-likelihood (ML) and Bayasian inference (BI) methods performed in *Garli v. 2.01* (Zwickl 2006) and *MrBayes v. 3.2.7* (Ronquist *et al.* 2012), respectively. Both analyses were run for 5 000 000 generations. The BI analysis with Markov chain Monte Carlo algorithm was performed with 2 runs, each with four chains, and with trees sampled every 500 generations. Finally, a majority-rule consensus tree was computed excluding those found in the burn-in phase (25% of all sampled trees). The results of ML and BI analyses were complemented with distance-based sequence similarities between accessions as inferred from network constructed in *SplitsTree4 v. 4.18.3* (Huson and Bryant 2006) using the NeighborNet split algorithm (Bryant and Moulton 2004) based on the uncorrected p-distance.

Results

The leaves of plant infected with powdery mildew were collected and the pathogen was identified as *Erysiphe* sp., most likely *Erysiphe trifoliorum*, but as only anamorphs were observed, it is difficult to distinguish it to *Eryphe pisi* that might also occur on *Lotus*. Telemorphic stage with the chasmothecia was not found. The infected leaves were analysed for flavonoid profile and compared with the leaves of healthy plants (Table 1). In leaves affected by powdery mildew, a decrease in content of most of flavonol-glycosides was observed, namely of

Table 1. Effect of infestation *Erysiphe* sp. on content of phenolic compounds [µmol g^{-1}(d.m.)] in *Lotus japonicus* leaves. Means ± SEs of 3 biological replicates. *Asterisks* indicate significant difference ($P < 0.05$) between treatments according to *Student*'s test. **bold** - significant differences between control and treated plants, N.D. - not detectable, 6DH - 6-deoxyhexose, Hex - hexose.

Compound	Control	*Erysiphe* sp.
Kaempferol-3-O-glucosyl (1-2)-glucoside-7-O-rhamnoside	0.8474 ± 0.0577	**0.5060** ± 0.0597*
Kaempferol-3-O-glucosyl (1-2)-galactoside-7-O-rhamnoside	1.5274 ± 0.0574	**0.9457** ± 0.1363*
Quercetin 6 DH, -Hex	0.3922 ± 0.0288	**0.1985** ± 0.0288*
Kaempferol-3-O-galactoside-7-O-rhamnoside	0.1080 ± 0.0269	0.0270 ± 0.0113
Kaempferol-3-O-glucoside-7-O-rhamnoside	0.9232 ± 0.0621	1.0621 ± 0.1607
Quercetin 6DH, -6DH	1.8294 ± 0.4863	1.5024 ± 0.1797
Kaempferol-3,7-di-O-rhamnoside	16.693 ± 0.6368	**6.6130** ± 0.8127*
Kaempferol-3-O-glucoside	0.0995 ± 0.0173	0.1401 ± 0.0188
Kaempferol-7-O-6DH	0.0189 ± 0.0024	**0.0545** ± 0.0050*
p-Ferulic acid	1.7510 ± 0.1549	N.D.*
p-Coumaric acid	2.7431 ± 0.1711	N.D.*
Vestitol	0.2011 ± 0.0240	**8.6640** ± 1.3428*
Vestitol derivative	0.0860 ± 0.0324	0.1176 ± 0.0388
Biochanin A	N.D.	N.D.
Sativan	0.0477 ± 0.0164	**0.4096** ± 0.0861*

kaempferol-3-O-glucosyl (1-2)-glucoside-7-O-rhamnoside, kaempferol-3-O-glucosyl (1-2)-galactoside-7-O-rhamnoside, kaempferol-3,7-di-O-rhamnoside, and quercetin 6-deoxyhexose, -hexose were significantly reduced. Ferulic acid and coumaric acids that were present in control samples in considerable amounts [1.75 and 2.74 μmol g^{-1}(d.m.), respectively], were not detected in leaves affected by the disease. The amount of vestitol, the main isoflavane phytoalexin in *Lotus japonicus*, increased almost 10-fold after infection with *Erysiphe* sp. A similar increase was also observed for sativan, a 2' methoxy-derivative of vestitol.

For a better understanding of the induction of phytoalexine production in *L. japonicus*, several signalling molecules were tested by application to leaves of uninfected plants. The possible increases of isoflavonoids content were focused, with a special interest on vestitol content. No effects after spraying the leaves with 0.5 mM salicylic acid were observed on plant isoflavonoids content up to 15 d of treatment (Table 2S). Significant increases of isoflavonoids were observed after 5 d of methyl jasmonate treatment that slightly decreased after 15 d. Submersion of plant to relatively high concentration (7.5 mM) of metyljasmonate or salicylic acid led to some increase of vestitol accumulation that was significant only for salicylic acid (Table 2S).

In contrast to metyljasmonate and salicylic acid, the tested plants showed much more sensitivity to chitosan application. Chitosan is a linear polysaccharide produced by deacetylation of chitin, which is a structural component of fungal cell walls and insect and crustacean exoskeletons. The plant biochemical response to chitosan was similar to those observed in leaves infected by powdery mildew (Table 2). Decrease in content of ferulic acid and kaempferol glycosides was observed, namely the content of kaempferol-3-O-glucosyl (1-2)-glucoside-7-O-rhamnoside, kaempferol-3-O-glucosyl (1-2)-galactoside-7-O-rhamnoside, kaempferol-7-O-deoxyhexose, and kaempferol-3-O-glucose, at least after application of 400 mg dm^{-3} of chitosan. The content of one previously unidentified kaempferol glycoside strongly increased. This compound was analysed by LC-ESI^+-MS/MS. The compound showed the $[M+H]^+$ ion peak at m/z 799. Peaks at m/z 653, 595, 433, and 287 were detected as the main fragmentation product ions. Ions at m/z 653 suggested the loss of 6-deoxyhexose ($[M+H-146]^+$), ions at m/z 595 suggested the loss of hexose and acetyl ($[M+H-162-42]^+$), and ions at m/z 433 suggested the loss of two hexose units and acetyl ($[M+H-162-162-42]^+$). The fragment ions at m/z 287 indicated the kaempferol aglycone. On the other hand, the ion peak at m/z 449 ($[M+H-146-162-42]^+$) was not observed, suggesting the lack of kaempferol fragment with a single hexose unit. Thus, the compound was tentatively identified as kaempferol-6-deoxyhexose, -hexose-hexose-acetyl. It was present in control only in small amounts but increased after chitosan treatment in leaves. Chitosan could enhance the acetylation of the triple kaempferol glycosides - mainly kaempferol-3-O-glucosyl (1-2)-galactoside-7-O-rhamnoside that strongly decreased after chitosan treatment. The amount of the dominant kaempferol glycoside, kaempherol-3,7-di-O-rhamnoside, was unchanged in the cases with different concentrations of chitosan. Quercetin glycosides did not accumulate after chitosan treatment, and their content was low both before and after the treatment. The amount of ferulic acid decreased, and at chitosan content of 400 mg dm^{-3} was undetectable.

Vestitol and its derivative were present in control in low amounts, while sativan and biochanin A were not

Table 2. Effect of different chitosan concentration on content of phenolic compounds [μmol g^{-1}(d.m.)] in *Lotus japonicus* leaves. Means ± SEs of 3 biological replicates. **bold** - significant differences between control and treated plants, *different letters* marked significant differences among treatments (*ANOVA* + *Tukey*'s test, $P < 0.05$), N.D. - not detectable, 6DH - 6-deoxyhexose, Hex – hexose.

Compound	Control	Chitosan (100 mg dm^{-3})	Chitosan (400 mg dm^{-3})
Kaempferol-3-O-glucosyl (1-2)-glucoside- 7-O-rhamnoside	0.3481 ± 0.0174^a	**0.2188** ± 0.0363^b	**0.1755** ± 0.0097^b
Kaempferol-3-O-glucosyl (1-2)-galactoside-7-O-rhamnoside	0.7819 ± 0.0775^a	**0.4866** ± 0.0277^b	**0.4677** ± 0.0277^b
Quercetin 6DH, -Hex	0.1971 ± 0.0286^a	0.1658 ± 0.0156^a	0.1747 ± 0.0074^a
Kaempferol -6DH, -Hex, -Hex-acetyl	0.0730 ± 0.0258^b	**0.6011** ± 0.1854^a	**0.6006** ± 0.0699^a
Kaempferol-3-O-galactoside-7-O-rhamnoside	0.0751 ± 0.0017^a	0.0693 ± 0.0065^a	0.0736 ± 0.0053^a
Kaempferol-3-O-glucoside-7-O-rhamnoside	0.7148 ± 0.1264^a	0.7299 ± 0.0414^a	0.5712 ± 0.0362^a
Quercetin 6DH, -6DH	0.1791 ± 0.0139^a	0.1610 ± 0.0043^a	0.1575 ± 0.0016^a
Kaempferol-3,7-di-O-rhamnoside	7.9613 ± 0.3907^a	7.6057 ± 0.3888^a	7.2538 ± 0.3122^a
Kaempferol-3-O-glucoside	0.0855 ± 0.0310^a	0.0313 ± 0.0145^a	0.0165 ± 0.0020^a
Kaempferol-7-O-6DH	0.0071 ± 0.0004^a	0.0054 ± 0.0006^{ab}	**0.0026** ± 0.0015^b
p-Ferulic acid	1.4575 ± 0.0610^a	0.8869 ± 0.4436^a	N.D.^{b}
p-Coumaric acid	1.8517 ± 0.4016^a	1.8909 ± 0.3832^a	1.5303 ± 0.0938^a
Vestitol	0.0243 ± 0.0009^c	**0.5550** ± 0.0377^b	**1.2607** ± 0.0710^a
Vestitol derivative	0.0141 ± 0.0040^b	0.1457 ± 0.0276^a	0.2013 ± 0.0273^a
Biochanin A	N.D.^{c}	**0.0910** ± 0.0028^a	**0.0400** ± 0.0101^b
Sativan	N.D.^{b}	**0.3277** ± 0.0536^a	**0.3192** ± 0.0570^a

Table 3. Effect of chitosan treatment on some physiological parameters in *Lotus japonicus* leaves. Data are means ± SEs of 3 biological replicates, *different letters* marked significant differences among treatments (*ANOVA* + *Tukey*'s test $P < 0.05$).

	Chl *a* [mg g^{-1}(f.m.)]	Chl *b* [mg g^{-1}(f.m.)]	Chl *a*/*b* ratio	Carotenoids [mg g^{-1}(f.m.)]	H_2O_2 [µmol g^{-1}(f.m.)]
Control	2.286 ± 0.166^{a}	1.040 ± 0.142^{a}	2.229 ± 0.569^{b}	0.9791 ± 0.019^{a}	6.461 ± 0.322^{b}
CHS 100 mg dm^{-3}	2.248 ± 0.089^{a}	0.649 ± 0.030^{b}	3.463 ± 0.032^{a}	0.8898 ± 0.035^{a}	13.034 ± 2.700^{ab}
CHS 400 mg dm^{-3}	2.208 ± 0.024^{a}	0.633 ± 0.004^{b}	3.487 ± 0.018^{a}	0.8153 ± 0.053^{a}	16.321 ± 0.675^{a}

detected. After chitosan application, vestitol content in leaves strongly increased. Increased accumulation was also determined for vestitol derivative and sativan, and some trace amount of biochanin A was also detected. The vestitol derivative ($[M+H]^+$ m/z 303) is identical to be the compound previously described in *L. corniculatus* (Kaducová *et al*. 2022).

The foliar application of chitosan had no significant effect on the amount of chlorophyll *a*, but the amount of chlorophyll *b* significantly decreased at a chitosan concentration of 400 mg dm^{-3} (Table 3). No significant changes in carotenoid content were found although their content tended to decrease slightly. Chitosan treatment caused a significant, approximately 2.5-fold and 3.5-fold increase on the H_2O_2 content after spraying with 100 and 400 mg dm^{-3} chitosan solution, respectively, indicating a change in metabolism in leaf tissues as a result of oxidative stress. The superoxide anion radical scavenging activity of *L. japonicus* extract assayed by the PMS-NADH system is shown in Fig. 1. The superoxide scavenging activity of leaf extracts increased markedly with increasing concentrations of chitosan. Thus, the inhibitory effect of the extracts on the formation of superoxide anions shown here supports the idea of the possible use of chitosan as a plant protective agent to oxidative stress.

As the treatment with 100 mg dm^{-3} chitosan was found to be sufficient for the induction of vestitol accumulation, this concentration was chosen for a further analysis of gene expressions. The transcriptional profiles of the genes encoding for phenylalanine ammonia lyase (PAL), the key enzyme of the biosynthesis of phenolic compounds were determined by RT-qPCR. At least 10 different genes are present in the *L. japonicus* genome according to the whole-genome sequence and previously published data, which were numbered from *LjPAL1* - *LjPAL10* (Chen *et al*. 2017). After a careful analysis of the gene sequences, a new set of primers was designed for the present work, keeping the previous gene numbering. Three extra pairs of primers were designed for the sequences Lj0g3v0121549.1, Lj1g3v4590760.2, and Lj1g3v4590760.3 (*Lotus BASE*; Mun *et al*. 2016) that were not used before (Table 1S).

Among the 12 tested isogenes, for three of them no expression was detected in leaves. In control samples *LjPAL8* was the most expressed gene, followed by *LjPAL9*. The expression of the rest of genes was very low (Fig. 2). In *L. japonicus* leaves exposed to chitosan treatment a significant induction in the expression of *LjPAL9*, *LjPAL6, and LjPAL4* was observed. In the case of *LjPAL5*, *LjPAL10*, and *LjPAL2* genes, no change in expression was observed. On the other hand, *LjPAL8*, that was the most

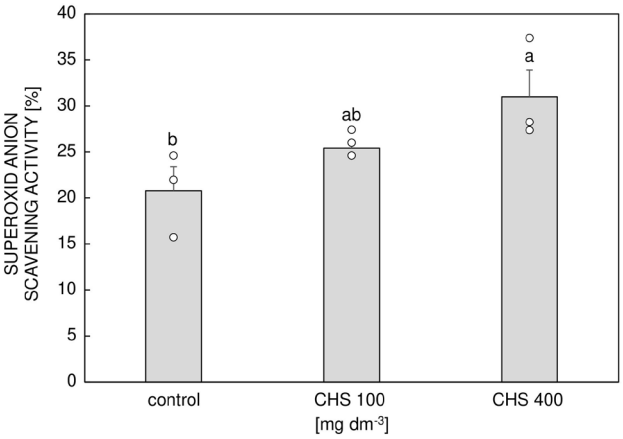

Fig. 1. Effect of different concentrations of chitosan on superoxide anion scavenging activity in leaves of *Lotus japonicus*. Means ± SEs, $n = 3$. *Different letters* indicate significant difference between treatments based on *ANOVA* with post-hoc *Tukey*'s HSD test ($P < 0.05$). *Open circles* represent individual data points of each biological replicate.

expressed in control, significantly decreased after chitosan treatment. The increased expression of some *LjPAL* genes was also paralleled by an increase in the activity of the PAL enzyme (Fig. 3).

A phylogenetic tree was constructed using the protein-coding sequences of 4 *AtPALs*, 6 *MtPALs*, 8 *GmPALs*, *11LjPALs*, and *NtPAL* as well as *OsPAL* used for rooting. *OsPAL* was found to be suitable root-inducer and it represents a different group of PAL-genes present in monocots. *LjPAL5* was not considered due to short coding sequence available for this gene in the *Lotus BASE*. ML and BI analyses gave similar results regarding sequence grouping and tree topology and differed only slightly. BI phylogenetic majority-rule consensus tree (Fig. 1S) showed six well recognized and highly supported (100% posterior probability) clades: C-I (*AtPAL1* and *AtPAL2*), C-II (*AtPAL3* and *AtPAL4*), C-III (*LjPAL9*, *MtPAL2*, *GmPAL2.2*, and *GmPAL2.4*), C-IV (*LjPAL6*, *MtPAL4*, and *GmPAL3.1*), C-V (*LjPAL8*, *MtPAL1*, *GmPAL2.1*, and *GmPAL2.3*), and C-VI (*LjPAL1*, *LjPAL2*, *LjPAL3*, *LjPAL4*, *LjPAL7*, *LjPAL10*, *LjPAL11*, *LjPAL12*, *MtPAL5*, *MtPAL6*, *GmPAL1.1*, *GmPAL1.2*, and *GmPAL1.3*). ML tree differed from BI tree only in weakly supported internal subgroupings of clade C-VI (ML contained a clade consisting of two *LjPAL7* and *LjPAL10* sequences. The *NeighbotNet* analysis resolved the groupings which perfectly fit to both ML and BI trees.

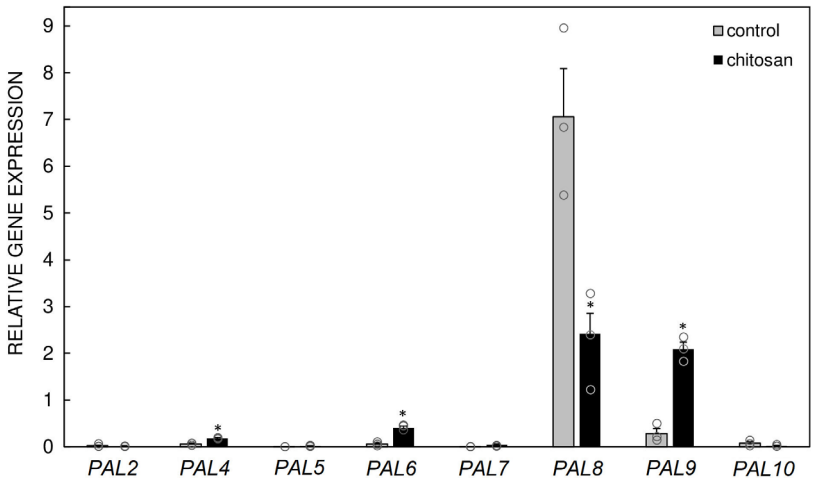

Fig. 2. Relative expressions of phenylalanine ammonia lyase (*PAL*) genes. Means ± SEs of the genes expressions relative to the combined housekeeping genes *LjPP2A* and *LjUBQ4* from three biological replicates. *Asterisks* indicate significant difference ($P < 0.05$) between treatments according to *Student*'s test. *Open circles* represent individual data points of each biological replicate.

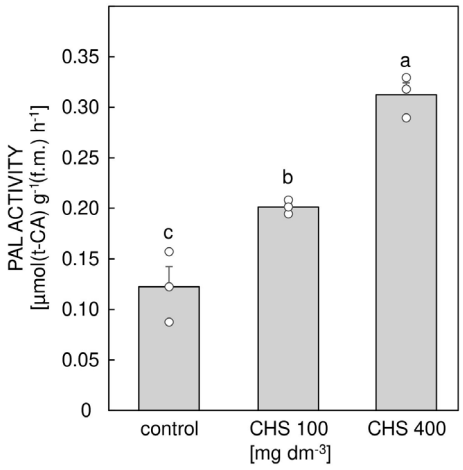

Fig. 3. Effects of different concentrations of chitosan on PAL activity in leaves of *Lotus japonicus*. Means ± SEs, $n = 3$. *Different letters* indicate significant difference between treatments based on *ANOVA* with post-hoc *Tukey*'s HSD test ($P < 0.05$). *Open circles* represent individual data points of each biological replicate.

Isoflavone synthase (IFS) is a key enzyme in the synthesis of isoflavones and defence-inducible phytoalexins. The expression of the genes encoding for isoflavone synthase isoform 1 and 3 (*LjIFS1*, *LjIFS3*), significantly increased in chitosan-treated leaves (Fig. 4), whereas the expression of *LjIFS2* was undetectable. The synthesis of vestitol is catalysed by pterocarpan reductase (PTR). Four genes (*LjPTR1-4*) encode PTR in the *L. japonicus* genome. PTR enzyme activity converts medicarpin to vestitol. In the case of chitosan treatment, an increase of *LjPTR2* gene expression was observed, while *LjPTR1*, *LjPTR3*, and *LjPTR4* expression did not changed significantly.

Discussion

Powdery mildews (*Erysiphales*, *Erysiphaceae*) are an important group of plant pathogenic fungi, consisting of about 873 species in 17 genera (Braun and Cook 2012), which affect about 10 000 species worldwide and cause serious diseases on numerous economically important plants such as cereals, legumes, vegetables, flowers, fruit trees, and ornamental plants, *etc*. (Amano 1986, Braun and Cook 2012). Most often, plants of *Lotus* genus are affected by *Erysiphe pisi* or *Erysiphe trifolii*. Pathogen damage induces certain stress response pathways in plants, including accumulation of phytoalexins. In this work, leaves of *L. japonicus* infected by powdery mildew accumulated isoflavonoids, particulary vestitol and sativan as protective compounds. In alfalfa seedlings after infection with *Colletotrichum trifolii* an increase in expression of genes involved in flavonoid biosynthesis, and in production of the phytoalexins medicarpin and sativan was described (Saunders and O'Neill 2004). Profiles of isoflavones were found to be altered in the leaves of narrow-leafed lupine plants after application of a *Colletotrichum lupini* spore suspension (Muth *et al.* 2009). Vestitol accumulation was found to be a defence mechanism of *Lotus* sp. to different biotic stressors such as the intrusion of roots by the parasitic plant *Striga hermonthica* (Ueda and Sugimoto 2010) or in response to the parasitic fungi *Calonectoria ilicola, Fusarium equiseti*, or *Penicillium simplicissimum*. On the contary, *Trichoderma koningi* is able to colonize *Lotus* roots by supression of vestitol production (Masunaka *et al.* 2011) and neither the compatible parasitic plant *Orobranche aegyptiaca* induced vestitol accumulation (Ueda and Sugimoto 2010). Thus, the ability to supress production of plant defence compounds and/or remaining unrecognized seems to be an important strategy of parasitic organisms. Moreover, that can be important also for the establishment of symbiotic interactions. Inoculation with *Mesorhizobium*

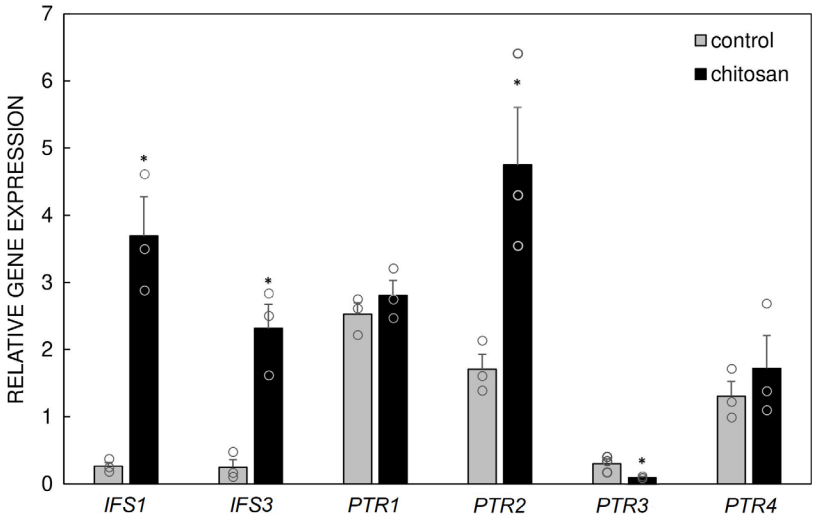

Fig. 4. Relative expressions of key genes encoding for enzymes for isoflavonoid biosynthesis in leaves of *Lotus japonicus* after chitosan treatment. *IFS* - 2-hydroxyisoflavanone synthase; *PTR* - pterocarpan reductase. Means ± SEs of gene expressions relative to the combined housekeeping genes *LjPP2A* and *LjUBQ4* from three biological replicates. *Asterisks* indicate significant difference ($P < 0.05$) between treatments according to *Student*'s test. *Open circles* represent individual data points of each biological replicate.

loti does not induce vestitol production in *L. japonicus* that is likely surpressed by interaction of ethylene-signalling pathway (Okazaki *et al.* 2004, Masunaka *et al.* 2011, Nascimento *et al.* 2012).

Elicitors are the chemicals or bio-factors from various sources that can trigger physiological and morphological responses and also phytoalexin accumulation in the target living organisms (Thakur and Sohal 2013). Elicitor perception is able to enhance the synthesis of signal compounds such as jasmonic acid, methyl jasmonate, salicylic acid, NO, and others and the subsequent stimulation of secondary metabolite production (Giri and Zaheer 2016, Narayani and Srivastava 2017). Induction of isoflavonoid production with methyl jasmonate was described in some legumes such as *Astragalus membranaceus* (Gai *et al.* 2016), *Genista tinctoria* (Łuczkiewicz and Kokotkiewicz 2012) or *Pueraria candollei* (Udomsuk *et al.* 2011). Induction by salicylic acid was described in *Pueraria candollei* (Udomsuk *et al.* 2011) and *Psoralea corylifolia* (Shinde *et al.* 2009). In all these studies the induction was carried out in cell suspension, or hairy root cultures.

Little is known about the elicitation and signalisation pathways for phytoalexine production in *Lotus.* In this work, the possible involvement of metyl jasmonate and salicylic acid in the induction of isoflavonoid production, especially vestitol was tested. A moderate increase in vestitol content was determined after 5 d of spraying with 500 µM methyl jasmonate. Salicylic acid caused a significant increase in vestitol content only after submersion of plants in a higher (7.5 mM) concentration of this compound. Such concentration induced the isoflavonoid accumulation also in bean cotyledones (Durango *et al.* 2013). Similar treatment with reduced glutathione was previously used for vestitol induction that resulted in significant vestitol accumulation and paralleled by the induction of the genes that encode for the enzymes of the biosynthetic pathway of this compound (Robbins *et al.* 1995, Lanot and Morris 2005, Shelton *et al.* 2012). However, due to diverse effect of signalling molecules, such concentrations might be excessively high, and the specificity of their effect could be questionable. It cannot be excluded, that sensitivity of the signalling pathways may differ somewhat among different tissues or species.

Chitosan that is considered to be a potent elicitor of defence responses, showed a strong induction capacity on phytoalexine production and it was comparable to the response to powdery mildew infection. Chitosan is a biopolymer obtained from chitin, which is the second most common natural polysaccharide after cellulose (Hidangmayum *et al.* 2019, Muthu *et al.* 2021). The fraction of the repeating units with free amino-groups in the polysaccharides is defined as the degree of deacetylation. Degree of deacetylation is an important parameter that impact water solubility and biological activity. Only chitosan with more than 50% degree of deacetylation can be soluble in water of diluted aqueous acids (Raafat and Sahl 2009, Kou *et al.* 2022). The chemical nature of chitosan differs from chitin because of the exposed amino groups that can bind positively charged protein molecules. Consequently, chitosan shares inductive properties with polycationic proteins such as protamine, histones, and synthetic polymers of ornithine, arginine, and lysine, but without being digested by proteases (Hadwiger 2013). Thus, treatment of plant with chitosan is able to induce plant defence response by simulating fungal pathogen attacks, which can eventually result to an increase in phytoalexin biosynthesis.

One of the earliest cellular responses to elicitation by chitosan that simulate fungal pathogen attack is an oxidative burst including a strong increase of H_2O_2 content (Kamalipourazad *et al.* 2016, Gai *et al.* 2019).

Hydrogen peroxide and/or other reactive oxygen species can act as signal molecules and cause the enhanced expression of genes related to the production of secondary metabolites (Baxter *et al.* 2014) *via* protein kinase signalling cascades (Gai *et al.* 2019). Increased content of H_2O_2 in response to chitosan elicitation, determined in present work was also paralleled by an increase in superoxide scavenging capacity of the enzymes present in leaf extracts. The chloroplast is considered the primary organelle for the action of chitosan and its signalling pathway. Signalling molecules such as NO and H_2O_2 are produced in the chloroplast (Schaller and Stintzi 2009, Zhang *et al.* 2011, Pichyangkura and Chadchawan 2015) and also superoxide (O_2^-) on photosystem II, which together with singlet oxygen causes photoinhibition (Zulfugarov *et al.* 2014).

Activation of signalling pathways by chitosan led to stimulation of the production of phenolic compounds through the phenylpropanoid pathway, which in turn increases tolerance to pathogens (Sharif *et al.* 2018). Exposure of *L. japonicus* leaves to chitosan treatment significantly increased the activity of phenylalanine amonia lyase (PAL, EC 4.3.1.5), the key enzyme catalysing the first rate-limiting step of the phenylpropanoid pathway that is affected by abiotic or biotic stress factors (Hyun *et al.* 2011) including chitosan application (Mejía-Teniente *et al.* 2013). PAL overexpression in tobacco reduced its susceptibility to *Cercospora nicotianae* (Shadle *et al.* 2003), whereas PAL-specific inhibition in oat increased its susceptibility to *Erysiphe graminis* infection (Carver *et al.* 1992). In *Medicago sativa*, infection with fungal pathogen *Colletotrichum trifolii* led to a strong increase of PAL activity and relative transcription rate of respective gene (Saunders and O'Neill 2004).

Four genes encoding PAL were reported in *Arabidopsis thaliana* (Cochrane *et al.* 2004), which provide about 90% of PAL (Huang *et al.* 2010). *AtPAL1* and *AtPAL2* are related to the flavonoid biosynthesis (Huang *et al.* 2010). PAL enzyme is encoded by several genes also in *L. japonicus* genome. Among them expression of *LjPAL9* was found to be the most induced by chitosan. The expression of *LjPAL4* and *LjPAL6* genes also significantly increased but was still much lower than *LjPAL9*. Overexpression and RNA interference analysis demonstrated that *GmPAL2.1* enhanced resistance to *Pseudomonas sojae* in transgenic soybean plants (Zhang *et al.* 2017). A phylogenetic tree constructed using the protein-coding sequences from legumes and *Arabidopsis* depicted that *GmPAL2.1* sequence is in the same clade with *LjPAL8*, which was, however, downregulated with chitosan treatment (Fig. 1S). This suggests different regulatory pattern of the 2 genes. On the other hand, the *Pseudomonas*-induced *GmPAL1.1* and *GmPAL1.3* in soybean (Shine *et al.* 2016) fell to the same clade with *LjPAL4* that was moderately induced by chitosan. The closest ortholog gene to *LjPAL6* in *M. truncatula* is the *MtPAL4* (called *MtPAL2* in Thalineau *et al.* 2018), which was induced significantly by the pathogen *Aphanomyces euteiches* and its expression was correlated with plants resistance. Four *AtPAL*s fell in the 2 distinct clades separated from all the legume *PAL*s.

The expression of *LjIFS1* and *LjIFS3*, two of the genes encoding for IFS, the key enzyme of isoflavonoid pathway, was also strongly induced. Similarly, the expression of *LjPTR2*, a gene encoding for PTR was also significantly increased, suggesting that chitosan induced the isoflavonoid pathway and vestitol production. Expression of *LjIFS1-3* was determined after UV-B irradiation. This also strongly stimulate vestitol production together with induction of PAL activity and gene expression (Kaducová *et al.* 2019). In soybean, chitosan-induced expression of *GmIFS1* and *GmIFS2* led to the enhanced accumulation of genistin and genistein isoflavones (Jia *et al.* 2019).

Conclusions

Based on the data obtained in this work we can conclude that the induction of isoflavonoid-phytoalexine production, especially vestitol, in *L. japonicus* in response to fungal attack is likely induced by chitosan. Chitosan elicitation of leaves activated the hydrogen peroxide related signalling pathways, which led to stimulation of isoflavonoid biosynthesis and accumulation of vestitol and other isoflavane phytoalexins. Further research is needed to test the role of metabolites produced by these pathways in the defence responses of *Lotus japonicus* against diseases.

This work also demonstrated an effective and safe method of increasing vestitol content in *Lotus japonicus*, that is an adaptive compound to various types of stress in plants and seems to be a perspective bioactive molecule.

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