# Cross-talk interactions of exogenous nitric oxide and sucrose modulates phenylpropanoid metabolism in yellow lupine embryo axes infected with *Fusarium oxysporum*

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## ABSTRACT

The aim of the study was to examine cross-talk of exogenous nitric oxide (NO) and sucrose in the mechanisms of synthesis and accumulation of isoflavonoids in embryo axes of *Lupinus luteus* L. cv. Juno. It was verified whether the interaction of these molecules can modulate the defense response of axes to infection and development of the pathogenic fungus *Fusarium oxysporum* f. sp. *lupini*. Sucrose alone strongly stimulated a high level of genistein glucoside in axes pretreated with exogenous nitric oxide (SNP or GSNO) and non-pretreated axes. As a result of amplification of the signal coming from sucrose and GSNO, high isoflavonoids accumulation was observed (+Sn+GSNO). It needs to be stressed that infection in tissues pretreated with SNP/GSNO and cultured on the medium with sucrose (+Si+SNP/+Si+GSNO) very strongly enhances the accumulation of free isoflavone aglycones. In +Si+SNP axes phenylalanine ammonia-lyase activity was high up to 72 h. As early as at 12 h in +Si+SNP axes an increase was recorded in gene expression level of the specific isoflavonoid synthesis pathway. At 24 h in +Si+SNP axes a very high total antioxidant capacity dependent on the pool of fast antioxidants was noted. Post-infection generation of semiquinone radicals was lower in axes with a high level of sucrose than with a deficit.

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## 1. Introduction

The transcription of defense genes induced by pathogens in plants is regulated by a complex network of signaling pathways [1–4]. In these pathways, metabolites, such as salicylic acid (SA), jasmonates (JA), hydrogen peroxide ($H_2O_2$), ethylene (ET), and nitric oxide (NO) have been identified as secondary signals [5–7]. At different times after pathogen recognition, these secondary signal substances accumulate transiently and transduce the signal into the nucleus where the transcription of specific defense genes is activated. It is likely that convergence of these signaling pathways occurred [8–10].

Nitric oxide is a multifunctional, active signaling molecule which regulates cellular responses, because it can interfere with the functioning of signal transduction pathways or can modify proteins structurally via the direct addition of the NO molecule itself or NO-derived molecules [11–15]. NO can exert its biological function through the modulation of gene expression, the mobilization of second messengers, or interplays with protein kinases. Besides, as already mentioned above, NO can be responsible of the post-translational modifications (PTM) of target proteins [16]. S-nitrosylation [17–21] is NO-dependent direct protein modification involved in plant defense signaling. Although several reports have demonstrated by proteomics approach there are plant proteins specifically nitrated during the plant response to stress conditions [11,16,22–26], and that the nitration is likely dependent on the conditions of nitrosative stress, i.e. increased production of ROS and RNS, however there is no evidence, that PTM has any specific signaling function, in contrast to growing evidence of signaling role of S-nitrosylation. Actually protein nitration can be considered as a marker of nitrosative stress, which means co-localization of target protein and the site of increased ROS and RNS production.

For example, the plant signaling pathway leading to defense responses and impacted by NO through S-nitrosylation involves the nonexpressor of pathogenesis-related gene 1 (NPR1)/Transcription factor TGA1 system. NPR1 is a key regulator of salicylic acid (SA)-dependent signaling that promotes defense responses in plants. Following oxidative changes triggered by SA, NPR1 hexamers dissociate through the reduction of intermolecular disulfide bounds into monomers, and are translocated into the nucleus where they interact with TGA factors including TGA1, allowing the expression of defense related genes. In turn, [24] identified 21 nitrated proteins that are involved in several processes, such as the primary metabolism (photo- and ATP synthesis, carbohydrate and nitrogen metabolism), the proteasome pathway and antioxidant machinery. NO is known to mediate many physiological functions, including as a developmental regulator, it promotes root growth and seed germination, controls stomata movement and delays flowering and senescence [27]. Moreover, in the context of plant–pathogen interactions, NO is involved not only in the modulation of SA, but also JA, ET synthesis and possibly other defense mechanisms in plants [28–30]. It has also been reported that $H_2O_2$ acts together with NO during programmed cell death (PCD) [31]. In turn, experiments by [32] indicated that superoxide anions rather than $H_2O_2$ function as the molecules that synergize with NO to unlock the programmed cell death (PCD) program. Thus, NO appears as an early signaling component, possibly orchestrating a number of downstream signaling pathways. Additionally, it results from literature data that NO induces the expression of several defense genes connected with the biosynthesis of antimicrobial compounds, such as phytoalexins in soybeans, preventing pathogen spread [33,34] and stimulates phytoalexin accumulation in tissues of potato (*Solanum tuberosum*) [35]. The NO molecule activates hypersensitive cell death as the executor signal causing apoptosis in *Kalanchoe daigremontiana*, *Taxus brevifolia* [36] and *Arabidopsis* [37]. Thus these observations suggest that the

signal released by NO indirectly activates secondary metabolites exhibiting antimicrobial action. In turn, the findings reported by [38] showed that there is a close dependence between the activation of the phenylpropanoid pathway and the effect of sodium nitroprusside (SNP), a nitric oxide ($NO^+$) donor, in soy seedlings. Suita et al. [39] also revealed that SNP stimulated all cGMP-induced genes, i.e. several genes encoding flavonoid-biosynthetic enzymes in soybean (*Glycine max* L.) involved in legume-specific isoflavone, phytoalexin and anthocyanin biosynthesis. Synthesis of phytoalexins during pathogenesis is regulated by the activity of phenylalanine ammonia-lyase (PAL)—an enzyme initiating phenylpropanoid metabolism and chalcone synthase (CHS) and chalcone isomerase (CHI) [40]. Moreover, the results of studies conducted by [41] show that the activity of PAL was activated by SNP in the light, not in the dark.

In turn, the participation of carbohydrates in the regulation of gene expression of phenylpropanoid biosynthesis pathways has been revealed in scarce studies. Enhanced expression of anthocyanin biosynthesis pathway genes by sucrose was found in radish by [42] that of anthocyanins and flavonoids in *Arabidopsis* by [43], anthocyanins in *Arabidopsis* by [44] and anthocyanins in grape cells by [45]. Additionally, the sucrose-specific regulation of accumulation of artemisinin [46], anthocyanin [43,47–49], hypericin [50], eleutherosides, phenol, flavonoids and polysaccharide [51], anthraquinone [52], phenolics and flavonoids [53,54], saponin and polysaccharide [55] have also previously been reported for various plant species. Moreover, Vitrac et al. [47] reported that calcium is involved in the sugar signaling pathway inducing anthocyanin biosynthesis in *Vitis vinifera* L. cell cultures. It was suggested that the increase in cytosolic calcium occurs by an influx of $Ca^{2+}$ from extracellular origin across the plasma membrane rather than by an efflux of calcium from intracellular stores.

Research reported by Morkunas et al. [57] revealed that in cells of yellow lupine as a result of amplification of the signal released by sucrose and infection caused by the pathogenic fungus *F. oxysporum* a strong increase occurs in the expression level of flavonoid biosynthetic pathway genes, as well as very high accumulation of these compounds. Moreover, earlier studies showed that sucrose alone stimulated PAL activity in tissues and infection additionally enhanced this activity [58,59]. At the same time a strong accumulation of free isoflavonoid aglycones was recorded in infected tissues with a high sucrose level. Soluble carbohydrates, such as sucrose and its hexoses, i.e. glucose and fructose serving the function of signal molecules, similarly as phytohormones, have the status of both the internal modulator and coordinator of various biochemical pathways through activation or repression of several genes in plant cells [60–66]. The effect of these sugars on the level of gene expression is frequently modified by environmental factors, including biotic factors. However, there are few data concerning a relationship between sugar signaling and stress-activated signal transduction pathways, i.e. pathogenic fungal infection [8,57,67–70]. There is an increasing body of evidence on the crossing, modulation and integration of signal pathways for carbohydrates with other signaling pathways. These complex interactions affect the level of signal transduction and coordination of regulation for gene expression, playing the key role in the source-sink regulation [8].

In research on the regulation of gene expression and metabolism regulated by sugars in higher plants the application of sugar-response mutants as well as the application of *in vitro* cultures of isolated embryo axes are used as auxiliary tools. Through manipulation of the source of organic carbon in the medium changes in sugar level in cells and tissues are made. In this work isolated embryo axes, both pretreated and non-pretreated with nitric oxide, inoculated and non-inoculated with *F. oxysporum*, and cultured *in vitro* on Heller medium with sucrose or without it, were used. Sucrose

plays a particularly important role, as it is the major form of translocated sugars in plants and it is the most frequently used sugar in studies of plant sugar responses in gene regulation and development [71]. However, in many cases the effects of sucrose could be completely substituted by a hexose, such as glucose or fructose [72]. Both sucrose and hexoses have been recognized as important signal molecules in source–sink regulation [73–75]. Changes in concentrations of soluble sugars in sink tissues, which are dependent on the import of carbohydrates from source tissues, initiate changes in gene expression and in metabolic and developmental reactions.

The aim of the study was to examine the effect of exogenous nitric oxide (NO) in terms of the varied level of sucrose, on the mechanisms of synthesis and accumulation of isoflavonoids in embryo axes of *Lupinus luteus* L. cv. Juno. At the same time it was verified whether cross-talk of these molecules can modulate the response of axes to infection and development of the pathogenic fungus *F. oxysporum* f. sp. *lupini*. Apart from the estimation of the level of isoflavonoids, expression of genes encoding PAL, CHS, CHI and IFS as well as PAL activity, total antioxidant capacity (TAC) was also determined. Furthermore, analyses covered postinfection changes in the generation of semiquinone radicals exhibiting high reactivity and cytotoxicity, which are formed during the oxidation of phenols by peroxidase or polyphenol oxidase. Additionally, morphometric measurements were taken on embryo axes of yellow lupine along with symptom diagnostic analyses.

It needs to be stressed that the problem investigated in this study, i.e. cross-talk of the signal induced by nitric oxide, sucrose and the hemibiotrophic fungus *F. oxysporum* and its effect on the mechanism of regulation for isoflavonoid synthesis and accumulation of isoflavonoids, has not been investigated to date.

## 2. Materials and methods

### 2.1. Plant material and growth conditions

Yellow lupine (*L. luteus* L. cv. Juno) seeds of the S-elite class were used in the experiments. Seeds were surface-sterilized, immersed in sterile water and left in an incubator (25 °C). After 6 h of imbibition the seeds were transferred onto filter paper (in Petri dishes) and immersed in a small amount of water in order to support further absorption. After a subsequent 18 h the seed coats were removed from the imbibed seeds and the cotyledons were removed to isolate the embryo axes.

At the beginning of the experiment isolated embryo axes were pretreated with a specific NO donor, i.e. 100 μM SNP, or not pretreated with SNP (pretreated with $H_2O$) for 12 h in the light at 25 °C. SNP releases nitric oxide in the form of nitrosonium cation ($NO^+$) upon light irradiation. Following embryo axes' pretreatment with SNP or with $H_2O$ (time 0 h) they were either inoculated with an *F. oxysporum* f. sp. *lupini* spore suspension (0i+SNP, 0i−SNP), or were not inoculated (0n+SNP, 0n−SNP). These were placed in groups of 4 onto Whatman filter papers, which were subsequently transferred to sterile glass test tubes (diameter 3 cm, height 13.5 cm) containing 14 ml of Heller's mineral medium [76], where they were suspended so that one end of the axis was immersed in the medium. A space was left below the paper to allow better aeration. After removal of the cotyledons the embryo axes were dependent on the carbon source provided by the medium. Eight culture variants were applied: embryo axes pretreated with 100 μM SNP, not inoculated and cultured on Heller's medium supplemented with 60 mM sucrose (+Sn+SNP); embryo axes pretreated with 100 μM SNP, inoculated and cultured on Heller's medium supplemented with 60 mM sucrose (+Si+SNP); embryo axes pretreated with 100 μM SNP, not inoculated and cultured on Heller's medium without sucrose (−Sn+SNP); embryo

axes pretreated with 100 μM SNP, not inoculated and cultured on Heller's medium without sucrose (−Si+SNP); embryo axes not inoculated with 100 μM SNP, not inoculated and cultured on Heller's medium with 60 mM sucrose (+Sn−SNP); embryo axes not pretreated with 100 μM SNP, inoculated and cultured on Heller's medium with 60 mM sucrose (+Si−SNP); embryo axes not pretreated with 100 μM SNP, not inoculated and cultured on Heller's medium without sucrose (−Sn−SNP); embryo axes not pretreated with 100 μM SNP, inoculated and cultured on Heller's medium without sucrose (−Si−SNP). Because from a strictly chemical point of view SNP is an $NO^+$ donor, we also tested the effect of another NO donor, i.e. S-nitrosoglutathione (GSNO), on accumulation of isoflavone glucoside and free isoflavone aglycones in 48 h embryo axes. Isolated embryo axes were pretreated with a specific NO donor, i.e. 250 μM GSNO or non-pretreated GSNO (pretreated with $H_2O$, −GSNO) for 12 h in the light at 25 °C. Then isolated embryo axes, both non-pretreated and pretreated with GSNO and non-inoculated and inoculated with *F. oxysporum*, were cultured in vitro for 48 h on Heller medium with 60 mM sucrose or without it. The applied sucrose concentration was optimal to ensure appropriate growth of embryo axes, fresh and dry weight, as well as the uptake of minerals from the medium. The experimental system developed by our team – embryo axes cultured *in vitro* – is a valuable model system, resembling natural plants much more effectively than cell or protoplast suspension cultures. After removal of the natural sources of nutrients, such as cotyledons, embryo axes of germinating seeds are limited in the collection of sugars to the medium as the only source of nutrients, supplied in a controlled manner by the experimenter. The applied experimental system provides a unique possibility to study the direct effect of sugars on plant defense responses to fungal infection.

Embryo axes were incubated in the dark at 25 °C. Samples were collected for analyses at 0 h and after 24, 48, 72, and 96 h of culture, following which they were frozen at −80 °C to determine isoflavonoid contents, phenylalanine ammonia-lyase activity, total antioxidant capacity, as well as semiquinone radical concentration. In order to determine the level of expression in genes coding the phenylpropanoid pathway enzymes, samples were collected for analyses at 0 h and after 12, 24, 48, and 72 h of culture. Changes of growth of embryo axes were analyzed in the period from 24 to 96 h of culture.

### 2.2. Preparation of spore suspension and inoculation

*F. oxysporum* f. sp. *lupini* strain K-1018 (subsequently referred to as *F. oxysporum*) was obtained from the Collection of Plant Pathogenic Fungi, the Institute of Plant Protection, Poznań. The pathogen was incubated in the dark at 25 °C in Petri dishes (diameter 9 cm) on a potato dextrose agar (PDA) medium (Sigma, pH 5.5). After 3 weeks of growth an *F. oxysporum* spore suspension was prepared. The spore suspension was obtained by washing the mycelium with sterile water and shaking with glass pearls. Then the number of spores was determined using a Bürker hemocytometer chamber. Embryo axes were inoculated with the spore suspension at a concentration of $5 \times 10^6$ spores per 1 ml. Inoculation was performed by injecting 10 μl of spore suspension into the upper part of the embryo axis shoot and additionally also by spraying the upper part of the embryo axis shoot with the inoculum.

### 2.3. Analysis of isoflavonoids

#### 2.3.1. Isolation of phenolic compounds

Prior to LC profiling of isoflavone glucosides, frozen plant tissue was homogenized in 80% methanol (6 ml g$^{-1}$ FW) at 4 °C. After homogenization, 30 μl of luteolin standard (2.78 mg/1 ml MeOH, 0.3 μmol) was added to each analyzed sample as an internal

standard (LC retention time and UV spectral data did not interfere with those of studied compounds). Moreover, 2 ml of 80% methanol were also added to each sample. Samples were vortex-mixed and ultrasonic treatment was applied at room temperature for 30 min. Next samples were centrifuged at $3000 \times g$ for 10 min. Supernatants obtained after centrifugation were evaporated at 45 °C. After evaporation of samples 1 ml of 80% methanol was added. Next samples were vortex-mixed, ultrasonic treatment was applied at room temperature for 15 min, and samples were centrifuged at $8000 \times g$ for 10 min.

### 2.3.2. Liquid chromatography (LC/UV)

Quantitative analyses were performed on a Merck Hitachi HPLC pump Model L-7000, equipped with a diode array detector Model L-7450 (Darmstadt, Germany) and Supersphere 100 RP-18 column ($250\,mm \times 2\,mm$; Merck). Quantification of total isoflavones (in embryo axes pretreated with SNP) was achieved by integration of UV chromatograms at 259 nm, normalization to the peak of an internal standard was performed and concentrations of lupine isoflavone glucosides and free aglycones were expressed in arbitrary units. During LC/UV analyses the elution protocol was carried out with two solvent mixtures: A (95% acetonitrile, 4.5% $H_2O$, 0.5% acetic acid, v/v/v) and B (95% $H_2O$, 4.5% acetonitrile, 0.5% acetic acid, v/v/v). Elution steps were as follows: 0–5 min isocratic at 10% A, 5–40 min linear gradient from 10% to 30% of A, 40–48 min linear gradient up to 100% of A, and 48–60 min isocratic at 100% of A. Free isoflavones and their glucosides were identified by comparing their retention times with data obtained for standards. Genistein 4′,7-O-diglucoside, genistein 7-O-glucoside and genistein, 2′-hydroxygenistein or their prenylated derivatives were run under identical chromatographic conditions. The above mentioned standards were obtained and characterized during earlier studies in our laboratory [77].

### 2.3.3. Liquid chromatography (LC/UV/MS)

Quantitative analyses were performed on a Waters Acquity UPLC system, equipped with a diode array detector and Poroshell 120 RP-18 column ($100\,mm \times 2.1\,mm$, $2.7\,\mu m$; Agilent). The UPLC system was additionally connected to an MS detector (micrOTOF-q, Bruker Daltonics) for the proper identification of particular isoflavones (in embryo axes pretreated with GSNO) based on MS and fragmentation spectra. Quantification of total isoflavones was achieved by integration of UV chromatograms at 259 nm and normalization to the peak of an internal standard (luteolin). The concentrations of lupine isoflavone glucosides and free aglycones were expressed in arbitrary units. During LC/UV/MS analyses the elution protocol was carried out using a 20 min gradient of two solvents: A (95% $H_2O$, 4.5% acetonitrile, 0.5% formic acid, v/v/v) and B (95% acetonitrile, 4.5% $H_2O$, 0.5% formic acid, v/v/v). Elution steps were as follows: 0–5 min gradient from 10% B to 30% B, isocratic to 12 min, 12–13 min linear gradient 95% B, 13–15 min isocratic at 95% of B. Free isoflavones and their glucosides were identified both by MS/MS analysis and by comparing their retention times with data obtained for standards. Genistein 4′,7-O-diglucoside, genistein 7-O-glucoside and genistein, 2′-hydroxygenistein or their prenylated derivatives were run under identical chromatographic conditions. The above-mentioned standards were obtained and characterized during earlier studies in our laboratory [77].

### 2.4. Extraction and assay of phenylalanine ammonia-lyase (PAL) activity

PAL (EC 4.3.1.5) was extracted at 4 °C using 0.1 M Tris–HCl buffer at pH 8.9 (4 ml per 500 mg of frozen tissue) containing 10 mM of mercaptoethanol and 30 mg of Polyclar AT. Samples were ground in a mortar and centrifuged at $15,000 \times g$ for 30 min. PAL

activity was determined using a modified method of [78]. The incubation mixture contained 80 mM borate buffer (pH 8.9), 30 mM L-phenylalanine and 0.5 ml of enzymatic extract in a volume of 1.5 ml. The reaction proceeded for 1 h at 30 °C and was interrupted by the addition of an equal volume of 2N HCl to the incubation mixture. The product of the reaction, trans-cinnamic acid, was determined at 290 nm (the Perkin–Elmer Lambda 11 spectrophotometer). Protein content was assayed according to [79].

### 2.5. Real-time RT-PCR

For the isolation of RNA, lupine embryo axes (0.5 g) were frozen in liquid nitrogen and ground with a mortar and pestle in the presence of liquid nitrogen. Total RNA was isolated from 50 mg of tissue using the SV Total RNA Isolation System (Promega), according to the supplier's recommendations. This protocol is for processing small tissue samples. The RNA level in samples was assayed spectrophotometrically at 260 nm. The $A_{260}/A_{280}$ ratio varied from 1.8 to 2.0 according to the manufacturer's protocol.

The transcript levels of target genes were analyzed by two-step quantitative RT-PCR (qRT-PCR). First-strand cDNA was synthesized from 1 μg of RNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer's protocol. qRT-PCR was performed using a 7900HT Fast Real-Time PCR System (Applied Biosystems) apparatus and the Power SYBR Green Master Mix kit (Applied Biosystems) in a final volume of 10 μl containing 2 μl of three-fold diluted cDNA or digested plasmid standard dilution and 2.5 pmol of each primer. Primers for amplification were designed on the basis of the cDNA sequences encoding genes of phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), chalcone isomerase (CHI), isoflavone synthase (IFS) and actin from yellow lupine (Table S1, supplementary method). This table is shown in supplementary method. In order to minimize inaccuracies due to genomic DNA contamination, amplicons were located in plausible joining regions of exons. In assays of CHI and IFS gene expression the applied thermal cycling conditions consisted of an initial denaturation at 95 °C for 10 min followed by 50 cycles at 95 °C for 15 s, 57 °C for 20 s and 60 °C for 1 min. In assays of CHS and actin gene expression the used program consisted of an initial denaturation at 95 °C for 10 min, followed by 50 cycles at 95 °C for 15 s, 52 °C for 20 s and 65 °C for 45 s. For the PAL gene expression assay thermal cycling conditions consisted of an initial denaturation at 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s, 56 °C for 15 s and 60 °C for 40 s.

The quantification analysis was performed using the standard curve method. In each assay for a specific cDNA target, standard curves were prepared using six 10-fold dilutions of the linear form of plasmid-cloned specific amplicons (pre-amplified PCR products) from 100 to 10,000,000 copies. Standards, cDNA samples, and the no-template control were analyzed in three repeats in each assay. The specificity of products was validated by dissociation curve analyses. The results were analyzed using SDS 2.3 software (Applied Biosystems). The expression level of target genes was normalized to the actin expression value as a constitutively expressed reference gene.

### 2.6. Total antioxidant capacity (TAC)

Total antioxidant capacity was measured using the ability of antioxidants contained in the extract to reduce the cation $ABTS^+$ according to the method described by [80] and modified by [81]. Typical antioxidants (ascorbate, glutathione) react very rapidly with $ABTS^+$; measuring the decrease in absorbance of the solution containing the extract after a very short time (10 s) is a measure of their contents in sample. Other substances (residues of tyrosine and tryptophan in proteins) react more slowly; measuring the

decrease in absorbance of the solution containing the extract after a longer time (30 min) is a measure of their contents. The starting ABTS$^+$ solution was prepared by dissolving 19.5 mg of ABTS in 7 ml of 0.1 M potassium phosphate buffer (pH 7.4), and 3.3 mg of potassium persulfate. After thorough mixing the solution was left in the dark for 12 h. Immediately prior to the determination the starting ABTS$^+$ solution was diluted with 0.1 M potassium phosphate buffer, pH 7.4, so that absorbance at wavelength $\lambda = 414$ nm was 1.0. To determine TAC 500 mg of embryo axes were homogenized in 5% trichloroacetic acid. Next samples were centrifuged at $15,000 \times g$ for 30 min at 4 °C. The cuvette contained 1.9 ml of diluted ABTS$^+$ and absorbance ($A_0$) was measured at a wavelength $\lambda = 414$ nm, and then 100 μl of extract were added. Absorbance was measured again after 10 s ($A_1$). TAC dependent on "fast" antioxidant (ascorbic acid or glutathione) $\Delta A_s = A_1 - A_0$ was calculated. At the same time TAC dependent on "slow" antioxidant (tyrosine and tryptophan residues in proteins) was calculated (unpublished results). The calibration curve was plotted by adding to the diluted ABTS$^+$ followed by 5 μl portions of 1 mM Trolox and measuring the gradual decrease in absorbance. The final result of TAC was expressed as μmol Trolox per gram of fresh weight.

## 2.7. Electron paramagnetic resonance (EPR)

Samples of 1 g fresh weight of embryo axes were frozen in liquid nitrogen and lyophilized in a Jouan LP3 freeze dryer. The lyophilized material was transferred to EPR-type quartz tubes of 4 mm in diameter. Electron paramagnetic resonance measurements were performed at room temperature with a Bruker ELEXSYS X-band spectrometer. The EPR spectra were recorded as first derivatives of microwave absorption. A microwave power of 2 mW and a magnetic field modulation of about 2 G were used for all experiments to avoid signal saturation and deformation. EPR spectra of free radicals were recorded in the magnetic field range of 3330–3400 G and with 4096 data points. In order to determine the number of paramagnetic centers in the samples the spectra were double-integrated and compared with the intensity of the standard Al$_2$O$_3$:Cr$^{3+}$ single crystal with a known spin concentration [82–86]. Before and after the first integration some background corrections of the spectra were made to obtain a reliable absorption signal before the second integration. These corrections were necessary due to the presence of a small amount of paramagnetic Mn$^{2+}$ ions in the samples under investigation. Finally, EPR intensity data were recalculated per 1 g of dry sample.

## 2.8. Statistical analysis

All determinations were performed in three independent experiments. Data shown are means of triplicates for each treatment; standard deviation was calculated and its range is shown in figures. The analysis of variance (ANOVA) was applied and results were compared in order to verify whether means from independent experiments within a given experimental variant were significantly different. Analysis of variance between treatment means was also carried out. The effects of three factors, i.e. sodium nitroprusside (SNP), sucrose and the pathogenic fungus, were investigated in the experiments.

## 3. Results

### 3.1. The effect of nitric oxide donors and sucrose on accumulation of isoflavonoids

Quantitative and qualitative analyses of isoflavonoids were performed by liquid chromatography (LC/UV). The isoflavonoid profile of yellow lupine embryo axes pretreated and non-pretreated with

SNP (NO$^+$ donor), non-inoculated and inoculated with *F. oxysporum*, and cultured on a medium with sucrose or without it, revealed the presence of isoflavone glucoside, i.e. 2′OH genistein 7-O-glucoside and isoflavone aglycones, i.e. genistein, 2′hydroxygenistein, wighteone and luteone. Sucrose alone in non-inoculated embryo axes of yellow lupine, non-pretreated with SNP (+Sn−SNP), in the interval from 12 to 96 h culture caused a very high accumulation of 2′OH genistein 7-O-glucoside (Fig. 1A). The highest level of this glucoside was recorded in these axes at 48 h of culture. Infection of embryo axes cultured on a medium with sucrose, not pretreated with SNP (+Si−SNP), caused a reduction of the level of this glucoside in relation to +Sn−SNP axes at all time points after the *F. oxysporum* infection, being observed with particularly strong intensity from 48 h. In turn, the concentration of this glucoside in non-inoculated embryo axes with a high sucrose level and pretreated with SNP (+Sn+SNP) remained high, although it was lower than in the +Sn−SNP axes. Up to 48 h after inoculation with the pathogenic fungus a slightly higher level of this glucoside was observed in +Si+SNP tissues than in +Sn+SNP ones. At later time points after infection, particularly at 72 h, in +Si+SNP tissues a considerable reduction was found in the level of this compound in relation to +Sn+SNP. Carbohydrate deficit in the medium resulted in a reduced accumulation of 2′OH genistein 7-O-glucoside in SNP-pretreated and non-pretreated embryo axes (−Sn+SNP and −Sn−SNP). In inoculated axes with carbohydrate deficit, both pretreated and not pretreated with SNP (−Si+SNP and −Si−SNP), a general decrease was recorded for the level of 2′OH genistein 7-O-glucoside at 72 and 96 h after infection.

It needs to be stressed that the action of both SNP and sucrose caused a very high postinfection accumulation of free isoflavone aglycones, i.e. genistein, 2′hydroxygenistein, wighteone and luteone, in +Si+SNP axes, being higher than in the other experimental variants (Fig. 1B–E). ANOVA showed that differences in these results were highly significant. However, we also need to focus on the high post-infection level of these isoflavone aglycones in embryo axes not pretreated with SNP with a high sucrose level (+Si−SNP). Significant differences were observed between applied experimental variants, such as +Si+SNP and +Si−SNP using ANOVA.

In order to strengthen our hypothesis on NO-dependent effects, we performed additional experiments using another NO donor (s), S-nitrosoglutathione (GSNO). Therefore, as a result of amplification of the signal coming from another NO donor, i.e. GSNO, and sucrose alone, we observed high accumulation of 2′OH genistein 7-O-glucoside, 2′hydroxygenistein, luteone and wighteone in 48 h +Sn+GSNO axes, significantly higher than in +Sn−GSNO and −Sn−GSNO axes (Fig. 2A, C, D, E). ANOVA showed that differences in these results were highly significant. These analyses of isoflavonoids were performed by liquid chromatography (LC/UV/MS). An example LC–UV (DAD) chromatogram of extracted isoflavone compounds identified is shown in supplementary data (Fig. S1, supplementary data).

Additionally, infection with *F. oxysporum* strongly enhanced the level of isoflavone aglycones, such as genistein, 2′hydroxygenistein, luteone and wighteone in 48 h-embryo axes pretreated with GSNO with a high sucrose level (+Si+GSNO) (Fig. 2B–E). However, the same sucrose and infection (without GSNO) also caused the accumulation of free isoflavone aglycones in 48 h +Si−GSNO tissue; the level these free aglycones was higher than in +Sn−GSNO tissue (Fig. 2B–E).

In turn, in inoculated axes with carbohydrate deficit, both those pretreated and not pretreated with SNP (−Si+SNP, −Si−SNP), the level of free isoflavone aglycones was much lower than in inoculated axes with a high level of sucrose, pretreated and not pretreated with SNP (+Si+SNP, +Si−SNP). Moreover, it was also found that pretreatment of embryo axes with the nitric oxide donor, applied alone, caused post-infection accumulation of

2′hydroxygenistein, genistein, wighteone and luteone in −Si+SNP axes, with their levels being higher than in +Sn+SNP axes. Moreover, at 24 and 72 h axes not pretreated with SNP, i.e. −Si−SNP, a post-infection increase in the levels of wighteone and luteone was observed in comparison to −Sn−SNP axes.

### 3.2. Cross-talk interactions of NO⁺ donor and sucrose in terms of expression levels of isoflavonoid biosynthesis pathway genes

The action of SNP and sucrose in embryo axes of yellow lupine, both non-inoculated and inoculated with the pathogenic fungus *F.* *oxysporum*, on the level of expression in genes encoding enzymes of the phenylpropanoid pathway, i.e. PAL, CHS, CHI and IFS, was analyzed using real-time PCR. At the early stage of infection (12 h) in embryo axes of yellow lupine, pretreated with SNP and cultured on a medium with sucrose (+Si+SNP), the level of expression was observed to increase in genes of the specific isoflavonoid biosynthesis pathway, i.e. CHS, CHI and IFS, in relation to the other experimental variants (Fig. 3). Significant differences were observed between applied experimental variants, i.e. +Sn+SNP and +Si+SNP, +Si+SNP and −Si+SNP, +Si+SNP and −Sn+SNP, +Si+SNP and +Si−SNP revealed by ANOVA.
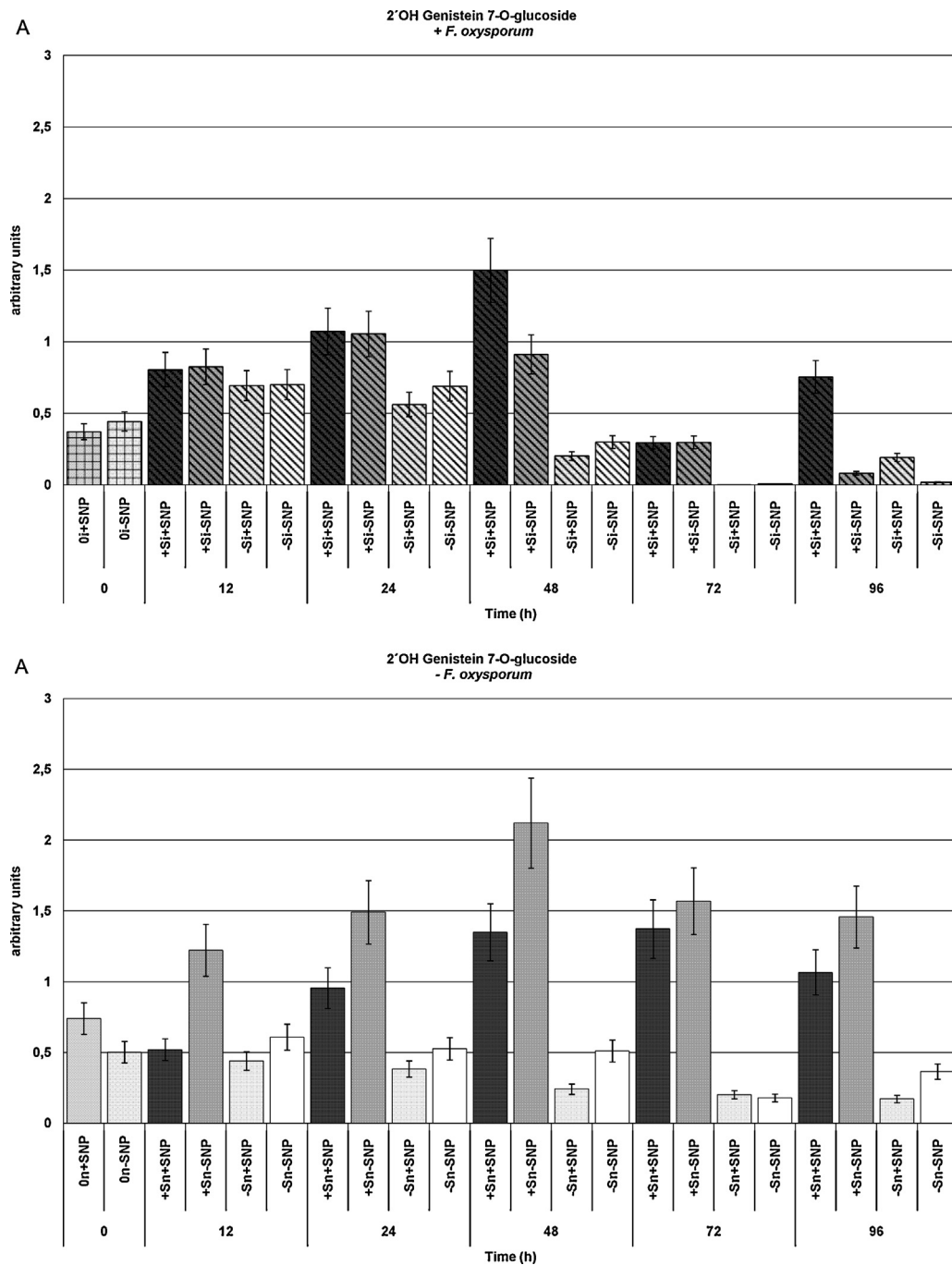


**Fig. 1.** The effect of NO⁺ donor (SNP) and sucrose on accumulation of isoflavone glucoside (A) and free isoflavone aglycones (B–E) in embryo axes of *Lupinus luteus* L. cv. Juno infected with *Fusarium oxysporum* f. sp. *lupini* and cultured *in vitro* on Heller medium. Isolated embryo axes were pretreated with a specific NO⁺ donor, i.e. 100 μM SNP (+SNP), or not pretreated with SNP (pretreated with $H_2O$, −SNP). No significant differences were found between means from experiments within a given experimental variant (ANOVA at the level of significance $p > 0.05$, differences are statistically non-significant). Significant differences were observed between applied experimental variants.
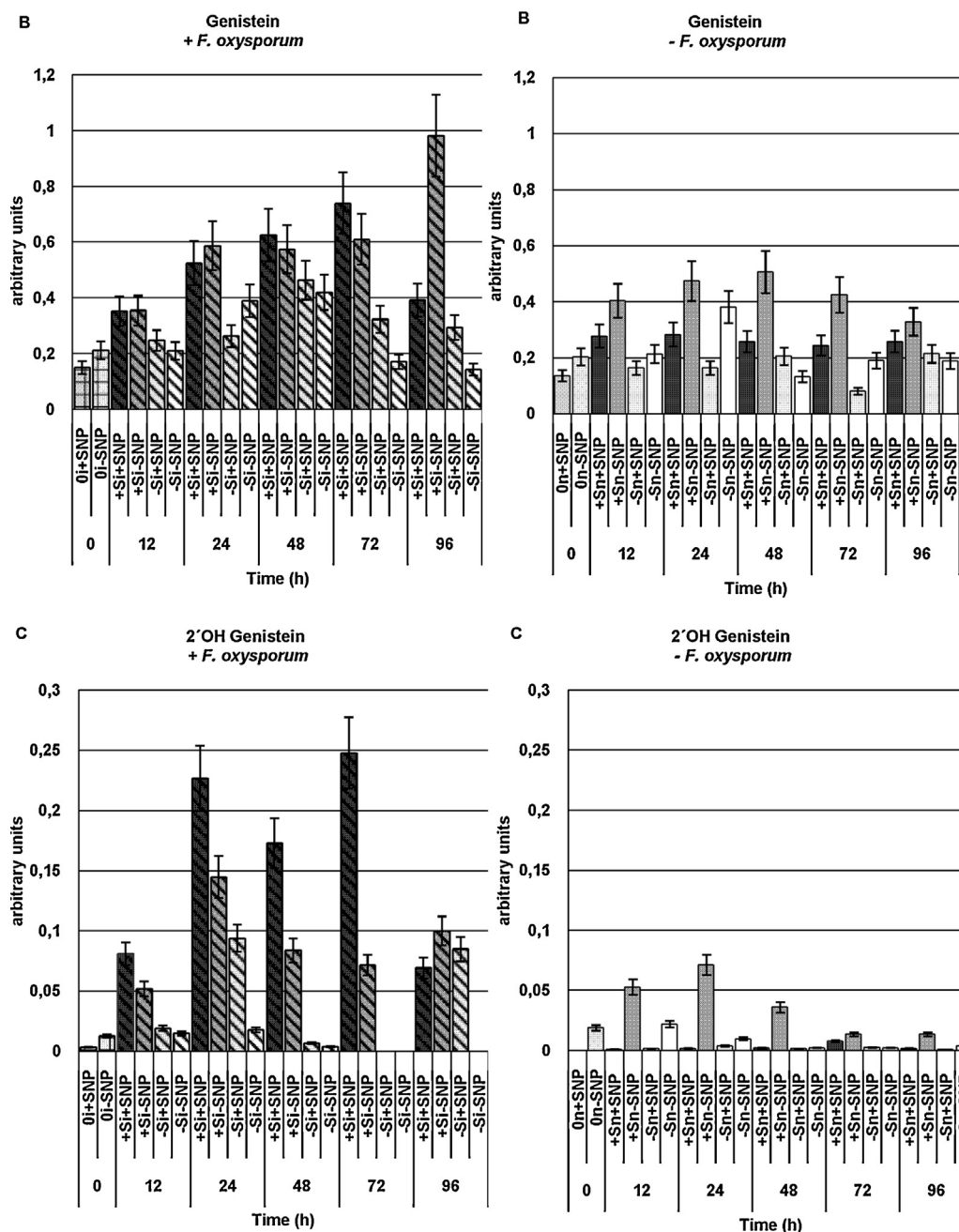
**Fig. 1.** (Continued)

Additionally, at 48 and 72 h after inoculation in +Si+SNP axes a higher level of PAL, CHS, CHI and IFS expression was observed in comparison to non-inoculated axes pretreated with SNP and cultured on a medium with sucrose (+Sn+SNP) and in inoculated axes not pretreated with SNP and cultured on a medium with sucrose (+Si−SNP). It was also found that the SNP pretreatment alone in the case of *F. oxysporum*-inoculated embryo axes, irrespectively of the presence of sucrose, caused enhanced expression of PAL-coding genes at 48 and 72 h.

Moreover, we need to stress here a very high level of expression of the isoflavonoid biosynthesis pathway (PAL, CHS, CHI and IFS) in 72-h *F. oxysporum*-inoculated embryo axes pretreated with SNP and cultured on the medium without sucrose (−Si+SNP).

In embryo axes not inoculated with *F. oxysporum*, pretreated with SNP or not pretreated with SNP, and cultured under sucrose deficit conditions (−Sn+SNP, −Sn−SNP) at 48 and 72 h of culture the level of PAL, CHS, CHI and IFS expression was much lower than in the other experimental variants, i.e. in embryo axes not inoculated and inoculated with *F. oxysporum*, SNP pretreated and non-pretreated, and cultured on the medium with sucrose (+Sn+SNP, +Sn−SNP, +Si+SNP, +Si−SNP) and in embryo axes inoculated with *F. oxysporum*, SNP pretreated and non-pretreated, and cultured under sucrose deficit conditions (−Si+SNP, −Si−SNP).

When analyzing the level of expression in PAL, CHS, CHI and IFS genes it was found that in 48 and 72-h non-inoculated embryo axes, SNP pretreatment as well as exogenous administration of sucrose (+Sn+SNP) generally causes a 1.5–2-fold increase in the level of expression of these genes in relation to non-inoculated embryo axes, pretreated with SNP and cultured under sucrose deficit conditions (−Sn+SNP).
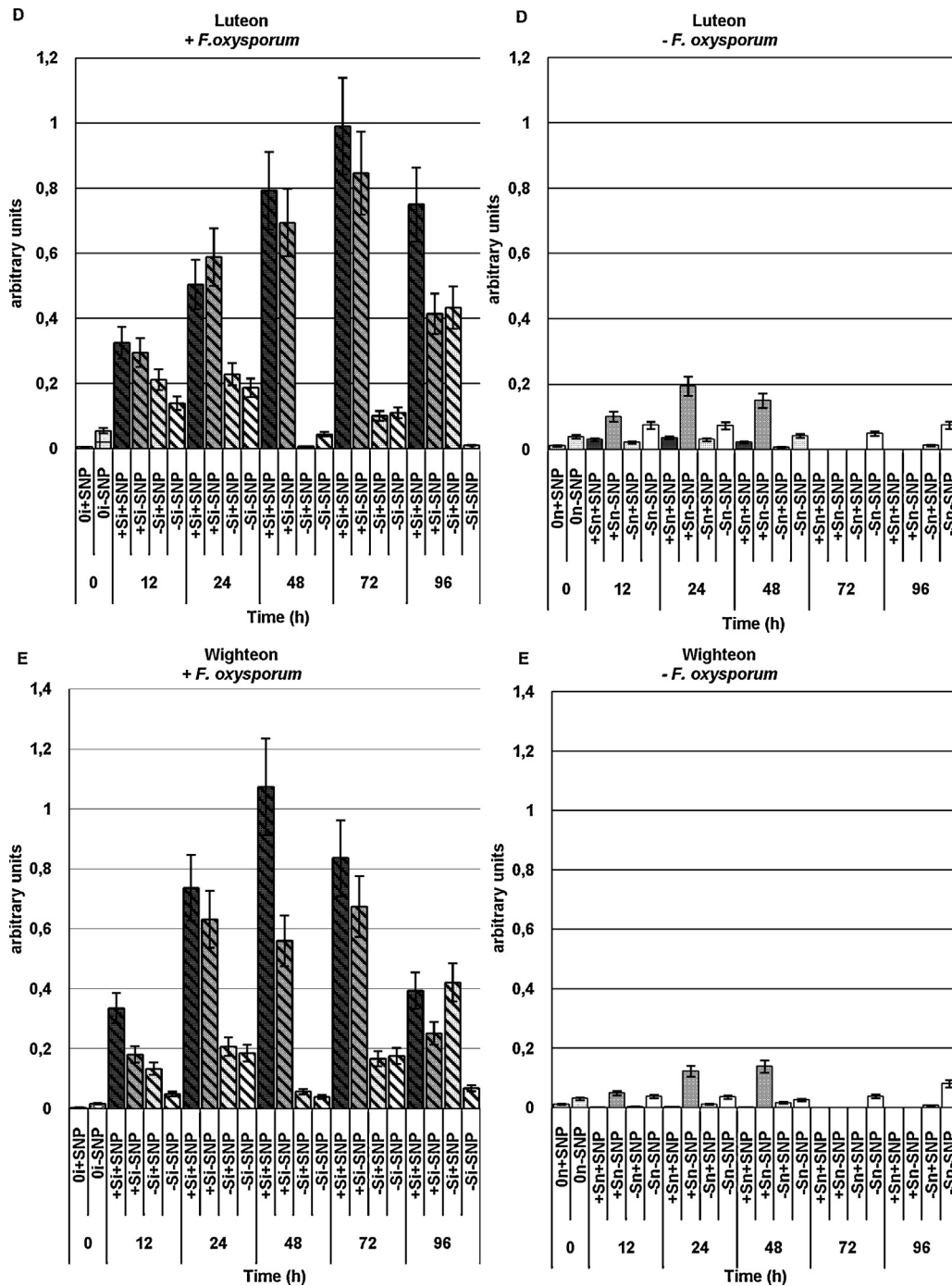
**Fig. 1.** (*Continued*)

## 3.3. The effect of NO[+] donor and sucrose on phenylalanine ammonia-lyase (PAL) activity

Already at 0 h inoculation alone caused an increase in PAL activity (Fig. 4). We also need to stress here the high PAL activity in axes pretreated with SNP and inoculated with *F. oxysporum*, cultured on a medium with sucrose (+Si+SNP). The highest activity of this enzyme in the tissues was recorded at 48 and 72 h after inoculation. Moreover, the high PAL activity was maintained up to 72 h of culture in axes pretreated with SNP and not inoculated, cultured on a medium with sucrose (+Sn+SNP), being higher than in axes not pretreated with SNP and cultured

on a medium with sucrose (+Sn−SNP) and much higher than in axes pretreated with the nitric oxide donor and cultured on the medium without sucrose (−Sn+SNP). ANOVA revealed significant differences between mentioned experimental variants. Moreover, non-inoculated embryo axes with carbohydrate deficit, pretreated with SNP (−Sn+SNP), showed higher PAL activity in relation to non-inoculated axes not pretreated with SNP (−Sn−SNP). In embryo axes with carbohydrate deficit infection alone caused an increase in enzyme activity (−Si+SNP, −Si−SNP), irrespective of whether they were pretreated with SNP; PAL activity was generally higher in −Si−SNP axes than in −Si+SNP embryo axes.

### 3.4. The effect of NO+ donor and sucrose on total antioxidant capacity (TAC)

We need to stress here the very high TAC, dependent on the pool of fast antioxidants in embryo axes cultured on a medium with sucrose, i.e. +Sn+SNP, +Si+SNP, +Sn−SNP and +Si−SNP, being much higher than in axes cultured under sucrose deficit, i.e. −Sn+SNP, −Si+SNP, −Sn−SNP and −Si−SNP (Fig. 5). Significant differences were observed between the above-mentioned applied experimental variants using ANOVA. Infection enhanced TAC levels in SNP-pretreated axes inoculated with *F. oxysporum* and cultured on a medium with sucrose (+Si+SNP). At 24 h in +Si+SNP axes the TAC value, dependent on fast antioxidants, was the highest, higher in relation to the other experimental variants. ANOVA showed that differences in the level of TAC between applied experimental variants were highly statistically significant. Its very high level was also maintained at the successive time points, i.e. 48, 72 and 96 h. Moreover, from 48 h after inoculation a high TAC level was also observed in inoculated axes not pretreated with SNP and cultured on the medium with sucrose (+Si−SNP). From 48 h in embryo axes with carbohydrate deficit infection caused a reduction of TAC dependent on fast antioxidants, both in tissues pretreated with SNP and those not pretreated with SNP.

### 3.5. Effect of NO+ donor and sucrose on semiquinone radical generation in response to infection with F. oxysporum

In the period from 0 to 96 h a post-infection increase was observed in the generation of semiquinone radicals (Fig. 6) with the spectroscopic *g*-factors $g_{\parallel} = 2.0037(\pm 0.0007)$ and $g_{\perp} = 2.0055(\pm 0.0005)$. The concentration of these radicals in *F. oxysporum*-inoculated embryo axes pretreated and not pretreated with SNP, cultured on the medium with sucrose and without it, was higher than in non-inoculated axes. ANOVA results showed that the differences in concentration of semiquinone radicals in non-inoculated axes compared to axes inoculated with *F. oxysporum* at all time points after inoculation were highly statistically significant. Generally the post-infection generation of semiquinone radicals was lower in axes cultured on the medium with sucrose (+Si+SNP and +Si−SNP) than in axes with sugar deficit (−Si−SNP, −Si+SNP). Among the tested experimental variants the highest level of these radicals was recorded at 72 and 96 h in inoculated axes cultured at carbohydrate deficit (−Si−SNP, −Si+SNP). ANOVA showed that differences in these results were highly significant. Moreover, it needs to be added here that inoculated axes pretreated with SNP already at 0 h showed an over 2.5-fold higher concentration of semiquinone radicals than those not pretreated with SNP. This result may indicate that pretreatment of SNP and inoculation with *F. oxysporum* caused strong generation of these radicals, which proves the rapid activation of defense mechanisms involving NO+ donor. In turn, at 48 h in +Si+SNP axes the generation of free radicals was only 17% higher in relation to +Sn+SNP axes, while in +Si−SNP axes it was 154% higher than in +Sn+SNP axes.

### 3.6. The effect of NO+ donor and sucrose on growth in yellow lupine embryo axes

The presence of 60 mM sucrose in the medium in the period from 0 to 96 h culture stimulated growth of yellow lupine embryo axes (+Sn+SNP, +Sn−SNP) (Fig. S2 supplementary data). Embryo axes exogenously pretreated with a nitric oxide donor, i.e. SNP, to a limited extent reduced their elongation or increase in fresh weight, both in axes cultured on the medium with sucrose and without sucrose, i.e. +Sn+SNP and −Sn+SNP, in relation to +Sn−SNP and −Sn−SNP. These results are shown in supplementary data (Fig. S2). Starting at 48 h after inoculation with *F. oxysporum*, growth inhibition was observed in embryo axes, although it was stronger in axes cultured at carbohydrate deficit than in axes cultured on the medium with sucrose. SNP pretreatment of axes did not have an
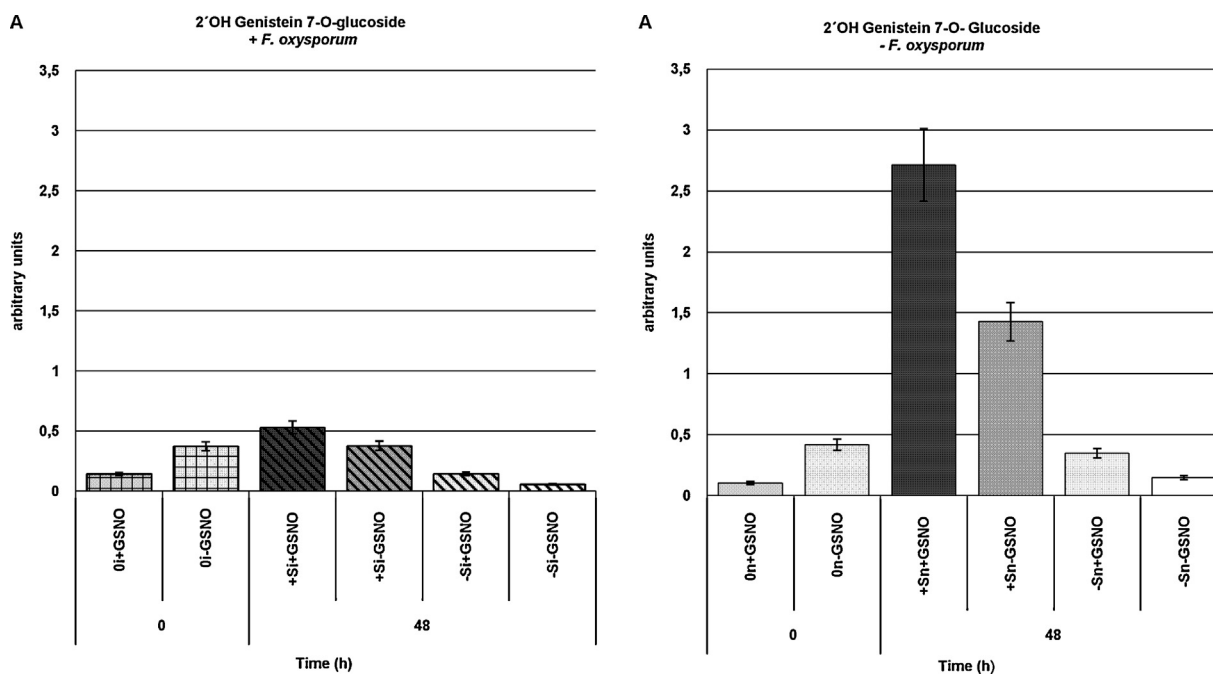


**Fig. 2.** The effect of NO donor (GSNO) and sucrose on accumulation of isoflavone glucoside (A) and free isoflavone aglycones (B–E) in embryo axes of *Lupinus luteus* L. cv. Juno infected with *F. oxysporum* and cultured *in vitro* on Heller medium. Isolated embryo axes were pretreated with a specific NO donor (GSNO) or not pretreated with GSNO (pretreated with H₂O, −GSNO). No significant differences were found between means from experiments within a given experimental variant (ANOVA at the level of significance *p* > 0.05, differences are statistically non-significant). Significant differences were observed between applied experimental variants.
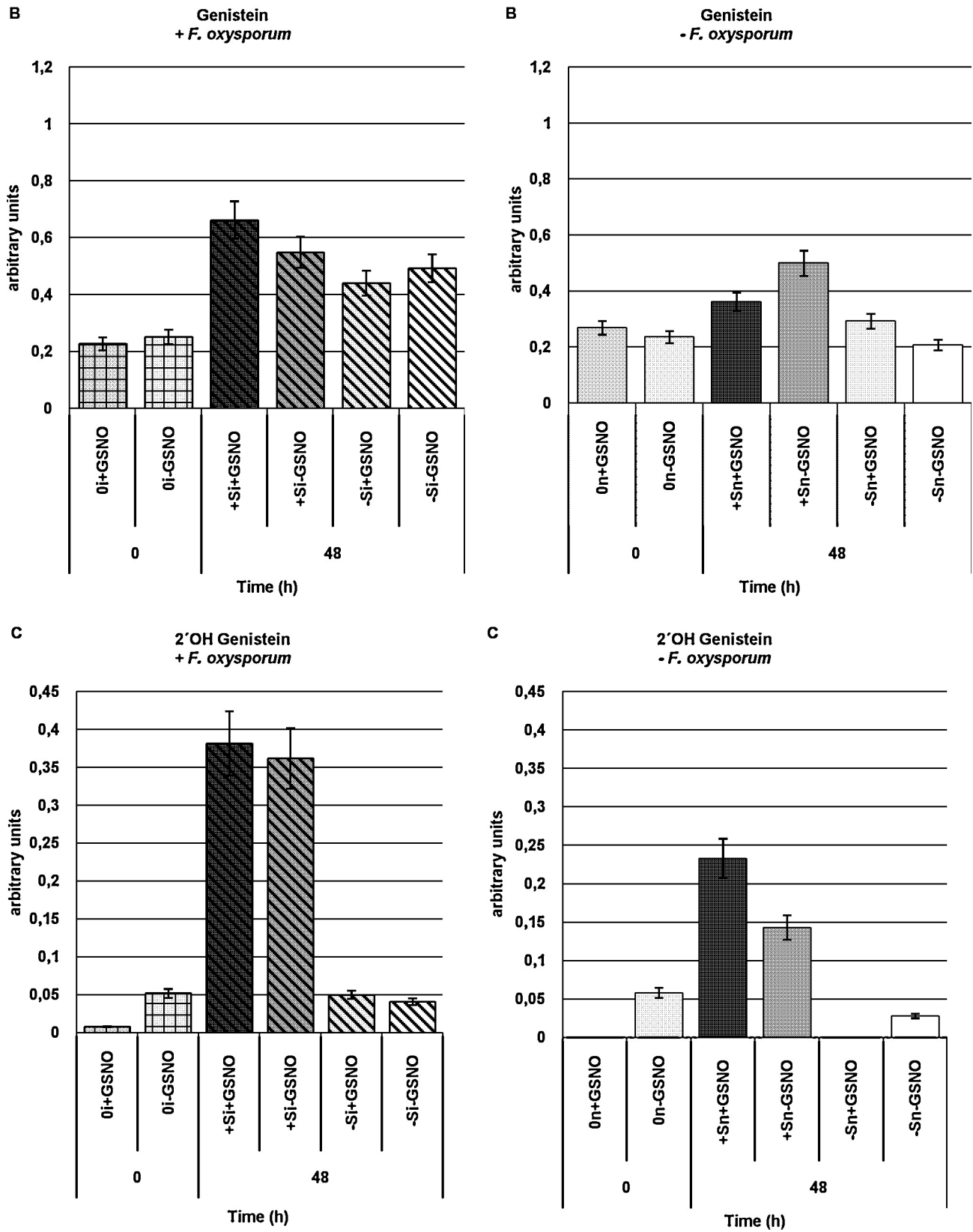
**B** Genistein + *F. oxysporum*



**B** Genistein - *F. oxysporum*



**C** 2′OH Genistein + *F. oxysporum*



**C** 2′OH Genistein - *F. oxysporum*

**Fig. 2.** (*Continued*)

effect on post-infection reduction of growth inhibition. Moreover, SNP pretreatment of embryo axes inoculated with *F. oxysporum* caused tissue browning, although the strongest effect was observed on +Si+SNP axes. Disease symptoms in pretreated SNP and non-pretreated SNP embryo axes of *L. luteus* L. cv. Juno infected with *F. oxysporum* f. sp. *lupini* and cultured *in vitro* on Heller medium with and without sucrose are shown in supplementary data (Table S1, supplementary data).
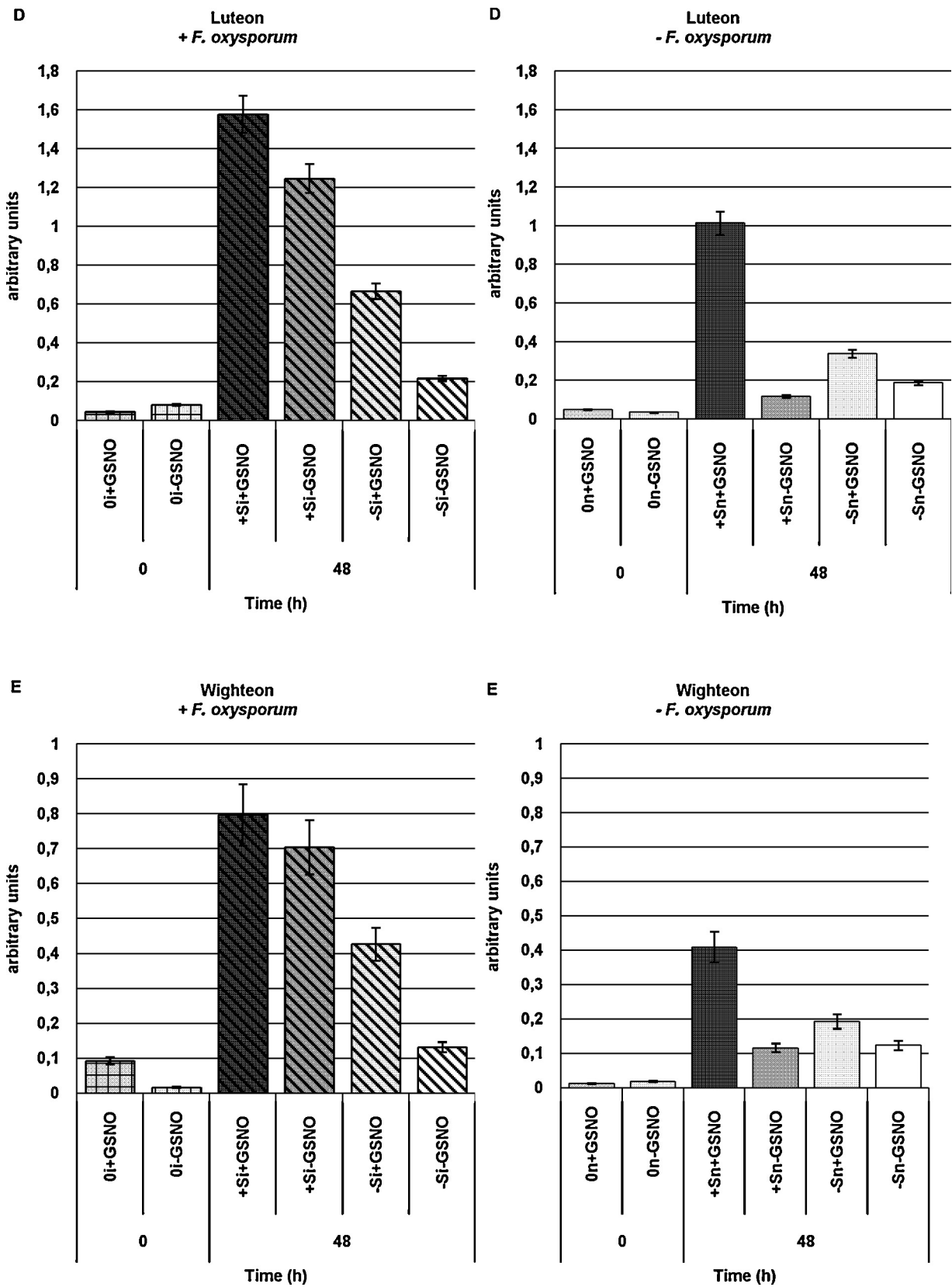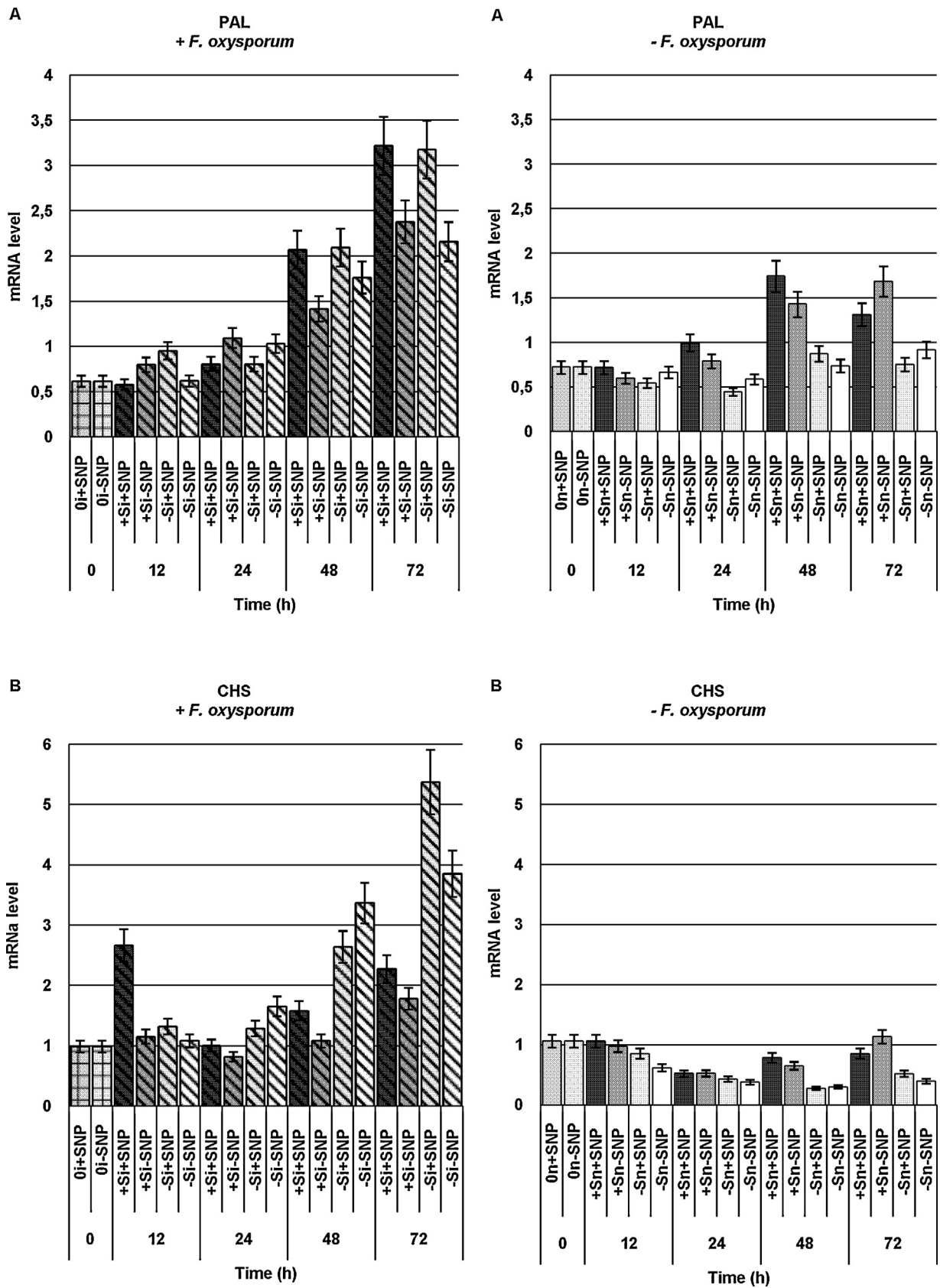
Fig. 2. (Continued)

**Fig. 3.** The effect of interactions between SNP and sucrose on expression levels of the isoflavonoid biosynthetic pathway genes, i.e. phenylalanine ammonia-lyase (PAL) (A), chalcone synthase (CHS) (B), chalcone isomerase (CHI) (C) and isoflavone synthase (IFS) (D) in *in vitro* cultured embryo axes of *Lupinus luteus* L. cv. Juno infected with *F. oxysporum*.
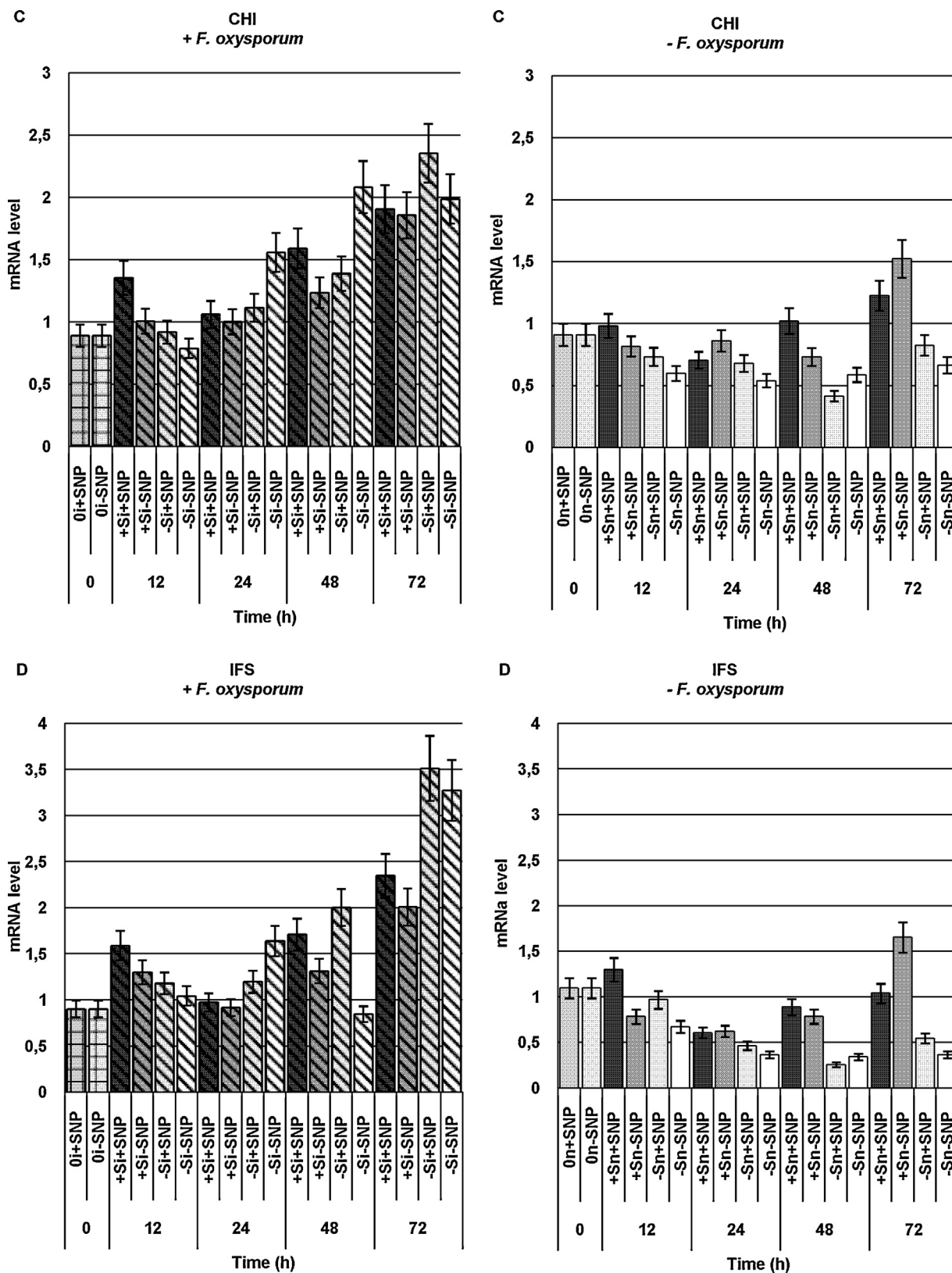
**Fig. 3.** (*Continued*)

## 4. Discussion

The results presented in this study are of paramount importance for present-day plant biology, since there is a lack of information on the effect of cross-talk between nitric oxide (NO) and sucrose signaling pathways and their effect on the mechanism of isoflavonoid synthesis, i.e. molecules potentially involved in the defense response of plants to fungal pathogens. This study strongly argues that sucrose and the $NO^+$ donor, SNP, when applied exogenously, enhanced the synthesis of isoflavonoids in yellow lupine in

**Fig. 4.** The effect of SNP and sucrose on phenylalanine ammonia-lyase (PAL) activity in *in vitro* cultured embryo axes of *Lupinus luteus* L. cv. Juno infected with *F. oxysporum*.

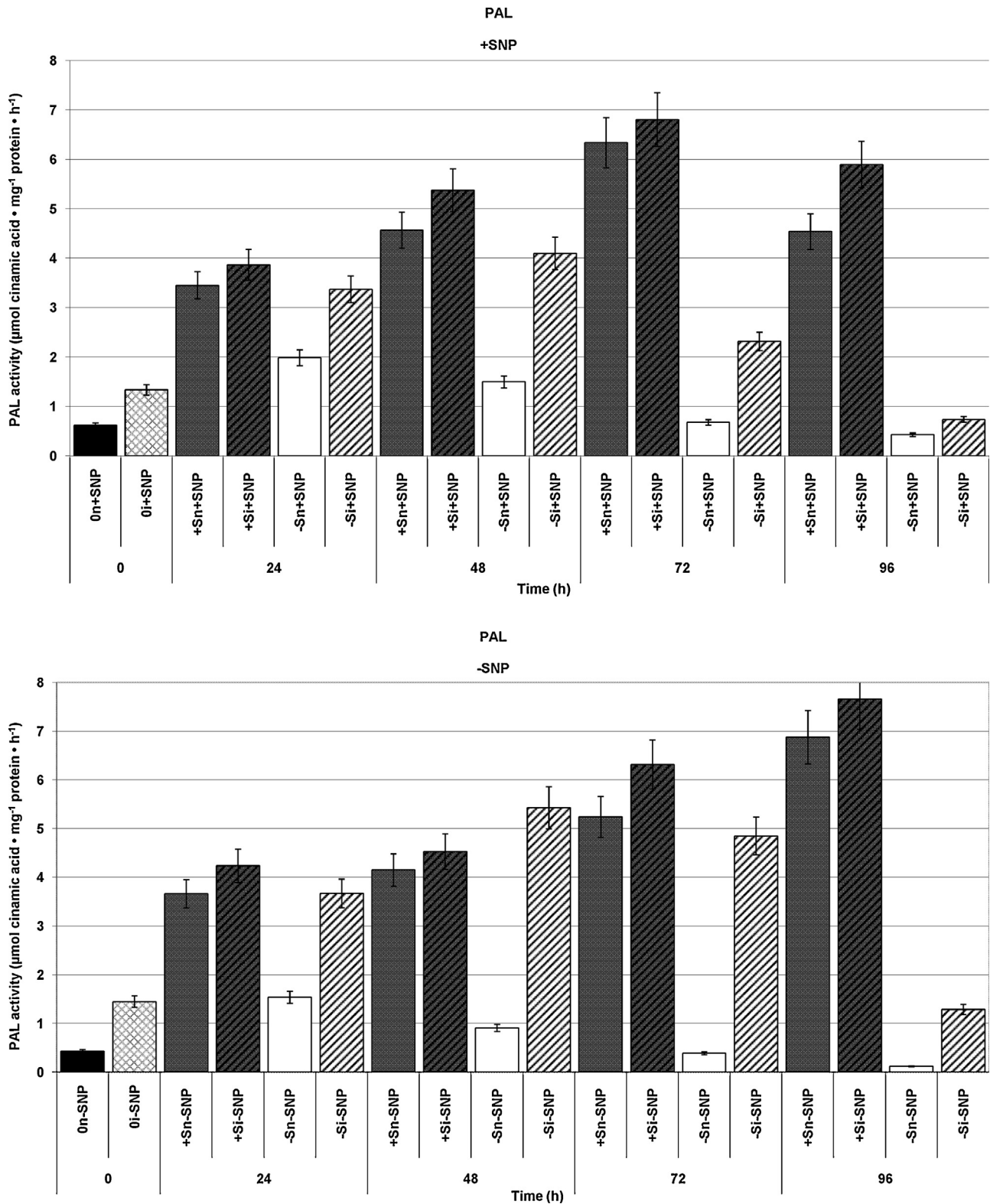response to infection with the hemibiotrophic fungus *F. oxysporum*. In order to strengthen our hypothesis on NO-dependent effects, we also performed additional experiments using another NO donor(s), S-nitrosoglutathione (GSNO).

The strong accumulation of free isoflavone aglycones, such as genistein, 2′hydroxygenistein, wighteone and luteone, recorded in our study in inoculated *F. oxysporum* embryo axes of yellow lupine

pretreated with SNP and cultured on the medium with sucrose (+Si+SNP) is the result of amplification of the signal coming from sucrose, the NO$^+$ donor and the pathogenic fungus (Fig. 1B–E). A similar effect to the above was obtained by using GSNO. Therefore, infection with *F. oxysporum* strongly enhanced the level of isoflavone aglycones at 48 h in embryo axes pretreated with GSNO with a high sucrose level (+Si+GSNO) (Fig. 2B–E). Thus integration of
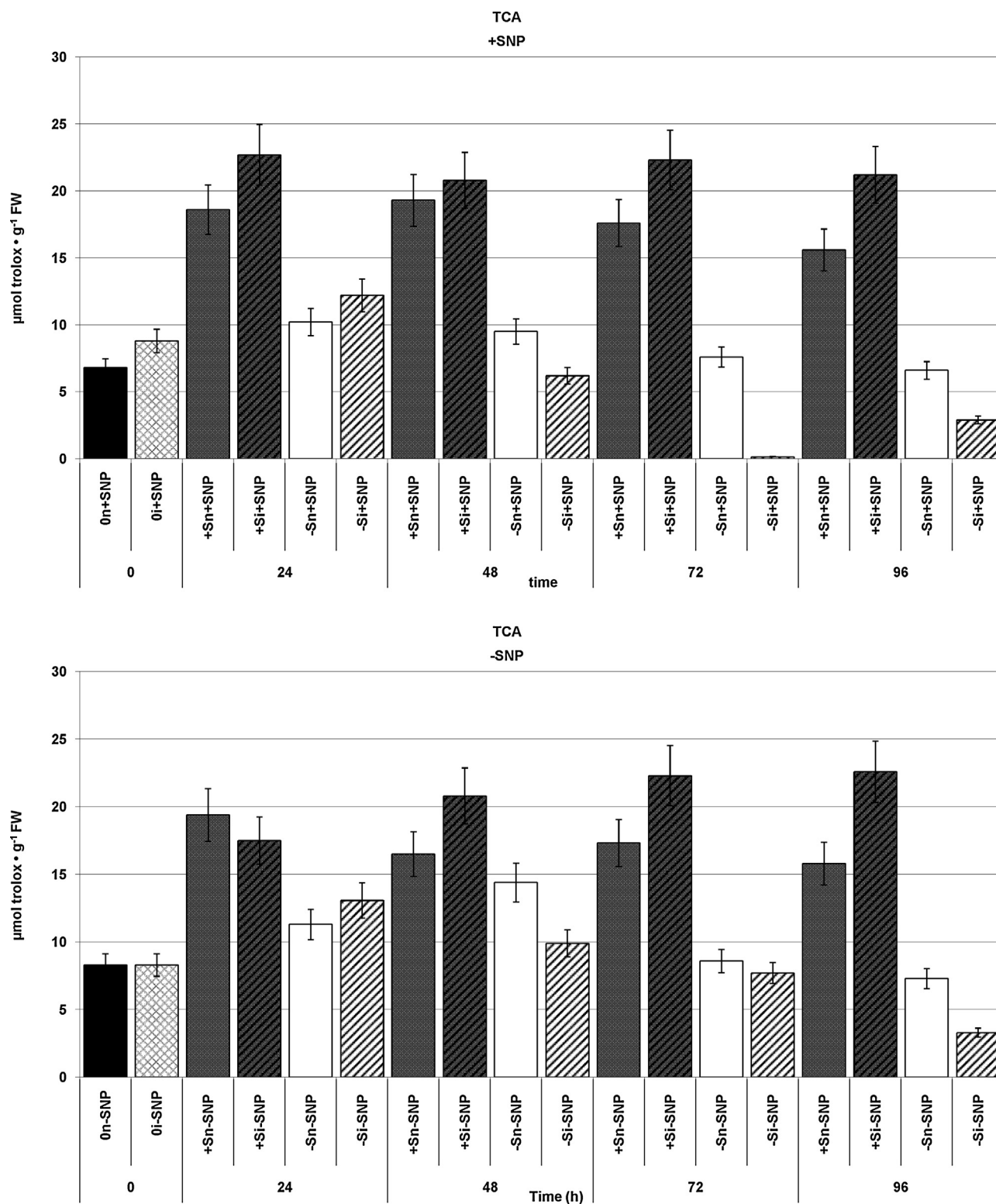
**Fig. 5.** The effect of SNP and sucrose on total antioxidant capacity (TAC) of embryo axes of *Lupinus luteus* L. cv. Juno infected with *F. oxysporum*.

these three signaling pathways in +Si+SNP/+Si+GSNO axes provides an enhanced defense response. Free isoflavone aglycones may be synthesized *de novo* or originate from hydrolysis of already existing isoflavonoid glucosides, which at pathogen attack are transported from the vacuoles to the cytoplasm [87,88]. Biological activity in interaction with pathogenic fungi was shown by free isoflavone aglycones, released from glycosides with the use of β-glucosidase

[58,59]. Our findings indicate that already at the early stage of infection in +Si+SNP/+Si+GSNO embryo axes strong accumulation of free isoflavone aglycones occurred. At the same time, already at 12 h after inoculation in +Si+SNP axes an increase was recorded in the level of expression in genes of the specific isoflavonoid biosynthesis pathway, i.e. genes encoding chalcone synthase (CHS), chalcone isomerase (CHI) and isoflavone synthase (IFS), in relation to the

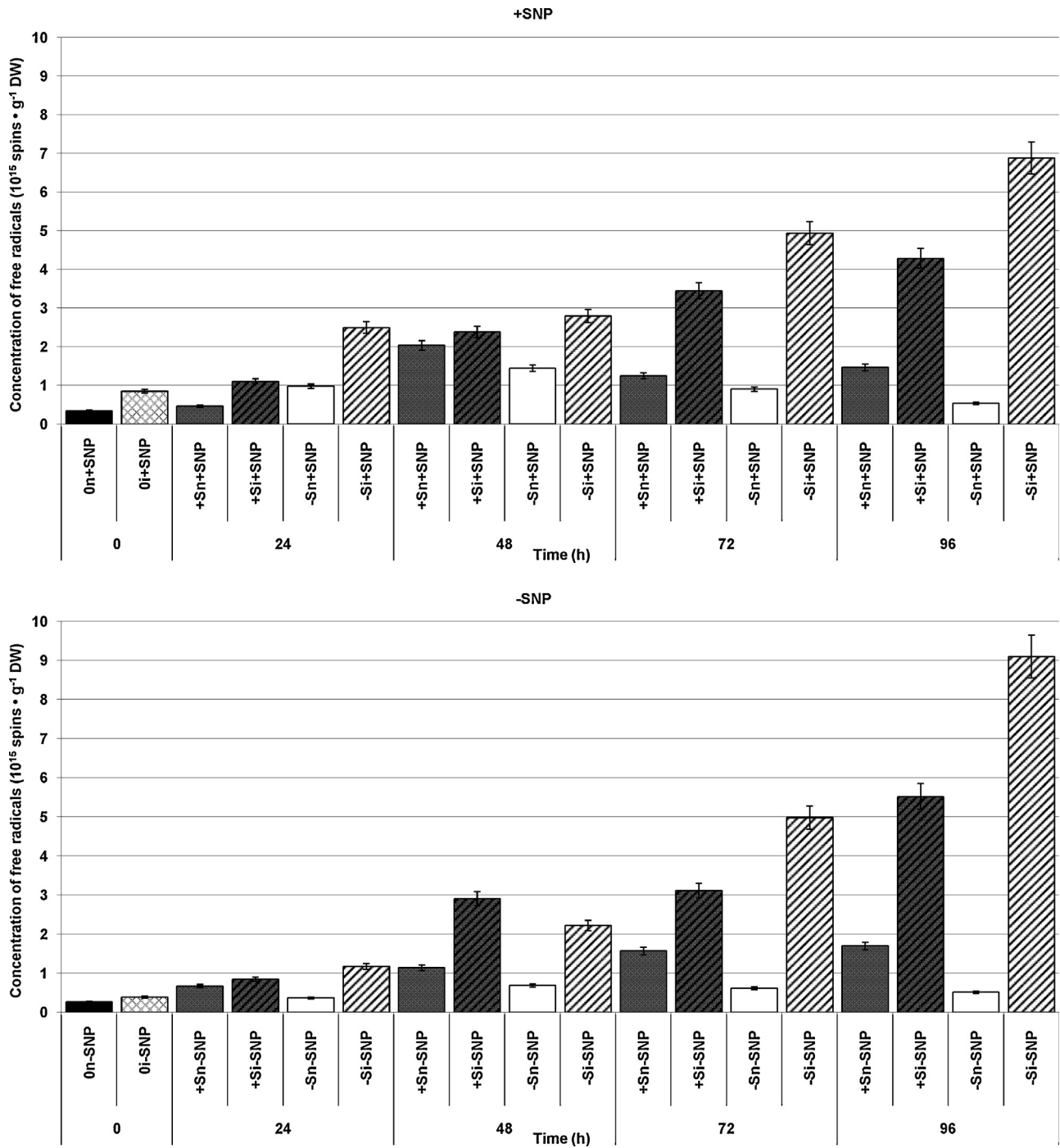**Fig. 6.** The effect of SNP and sucrose on semiquinone radical generation in response to infection with *F. oxysporum*.

other experimental variants (Fig. 3). Additionally, in +Si+SNP axes the activity of phenylalanine ammonia-lyase (PAL), an enzyme initiating phenylpropanoid metabolism, was highest up to 72 h among the tested experimental variants (Fig. 4). Furthermore, a reduction in the level of 2′OH genistein 7-O-glucoside in relation to +Sn+SNP axes was noted (Fig. 1A). This reduction in +Si+SNP axes was evident only at 72 and 96 h after inoculation, while in +Si−SNP axes this decrease occurred at all time points after inoculation. At 48 h in +Si+GSNO axes there was also observed a reduction in the level of 2′OH genistein 7-O-glucoside (Fig. 2A). Therefore, strong accumulation of free isoflavone aglycones with the properties of toxic fungi in +Si+SNP/+Si+GSNO embryo axes can indicate initiation of an active defense mechanism.

Moreover, Kretzschmar et al. [89] documented the prominent increase in some induced-defensive flavonoids (daidzein, coumestrol, genistein, luteolin, apigenin) in soybean cotyledons in response to an elevated $CO_2$ concentration and induction with NO and a fungal elicitor. An elevated $CO_2$ concentration resulted in a significant increase in the levels of total non-structural carbohydrate in cotyledons (TNC).

The results mentioned above indirectly support our research, where under elevated NO and sucrose conditions pathogen infection led to the accumulation of free isoflavone aglycones as the natural end products of the phenylpropanoid pathway.

It is known that in plants there are several induction pathways for the signal originating from NO, including cyclic nucleotides,

such as cGMP and cADPR [90]. Also, a study by [5] showed that the introduction of animal NOS to tobacco leaves or treatment of tobacco cell suspension with an NO donor (GSNO) induced a rapid increase of cGMP levels. Moreover, it was documented that defense-related genes encoding pathogenesis-related 1 protein and phenylalanine ammonia-lyase (PAL) can be activated by NO, cGMP and cADPR. Research results presented by [39] suggest that cGMP acts as a second messenger to activate the expression of genes for enzymes involved in the flavonoid biosynthetic pathway in soybean. It was documented that cyclic GMP (cGMP) and nitric oxide (NO) can regulate the expression of genes, such as cinnamate4-hydroxylase (C4H), anthocyanidin synthase (ANS), UDP-glucose: isoflavone7-O-glucosyltransferase (IFGT) and 2-hydroxy isoflavanone dehydratase (HIDH).

Thus the administration of an exogenous nitric oxide to lupine embryo axes could have influenced the synthesis of cGMP, on which the synthesis of isoflavonoids may be dependent, and additionally next to sucrose and infection it could enhance the accumulation of these free isoflavone aglycones.

It is known from the results reported by [38] that following the administration of a nitric oxide, i.e. 10 mM SNP, to soy seedlings accumulation of isoflavones of the phenylpropanoid pathway, i.e. genistein and daidzein was recorded. It needs to be stressed that the accumulation of these phytoalexins in soy leaves was much faster and higher after the administration of NO than after treatment of the elicitor fungus *Diaporthe phaseolorum* f. sp. *meridionalis* (Dpm). Moreover, the results of this study indicate the incorporation of an NOS-like enzyme serving a similar function as nitric oxide synthase (NOS) in animal cells, in the response of soy cotyledons to the Dpm elicitor. Production of phytoalexins induced by Dpm was inhibited when cotyledons were pretreated with NOS inhibitors. The above-mentioned effect was not observed at the joint treatment with the elicitor and SNP. Recorded results suggest that the Dpm fungal elicitor activated the phenyl propanoid pathway by NO produced by NO synthase (NOS-like). Thus after Dpm elicitation the accumulation of phytoalexins occurred only after NOS-like activation, while the administration of NO additionally stimulated the synthesis of secondary metabolites. Also, treatment of potato tuber tissues with the NO donor NOR-18 induced accumulation of the phytoalexin rishitin, an endogenous antibiotic compound [91]. Recently, some studies have shown that NO is involved in fungal elicitor-induced production of secondary metabolites, such as ginseng saponin [92], hypericin [93] and puerarin [94]. These observations suggest the existence of an NO-mediated signaling pathway in biosynthesis of induced secondary metabolites in plant cells. Furthermore, Zheng et al. [95] reported that the oligosaccharide-induced artemisinin production could be potentiated by the NO donor SNP. In turn, Floryszak-Wieczorek et al. [96] showed that nitric oxide plays an important role in the mobilization of the defense strategy in pelargonium leaves in relation to the necrotrophic fungus *Botrytis cinerea*. Irrespective of plant cells it was found that isoflavones, such as genistein and daidzein in cells collected from the human organism (RAW264.7) may activate nitric oxide synthase (iNOS), and thus induce NO production [97]. A study by [98] on *Rhodococcus* APG1 cells showed that the administration of sucrose stimulated NO production and NOS activity might be connected with the regulatory action of sucrose. Moreover, Van Ree et al. [99] identified a correlation between the reported ability of Nitric Oxide Associated 1 (NOA1) to accumulate nitric oxide with growth on sucrose-supplemented media. They reported that provision of exogenous sucrose enables NOA1 to accumulate NO. Furthermore, Mandal et al. [100] reported that NO production in plants can be regulated also via oleic acid (18:1).

Free isoflavone aglycones may constitute an important line of defense both in +Si+SNP and +Si−SNP axes in relation to the pathogenic fungus *F. oxysporum*. The results obtained for 48 h +Si+GSNO embryo axes support this conclusion (Fig. 2B–E). Analysis of disease symptoms showed that inoculated axes with high levels of sucrose and pretreated with SNP (+Si+SNP) showed a limitation of infection development and fusariosis, similarly as in +Si−SNP axes in relation to inoculated axes with carbohydrate deficit, i.e. −Si+SNP and −Si−SNP (Table S1). However, it needs to be stressed that SNP pretreatment of infected axes with high sucrose levels caused their strong browning, which was not observed in the case of +Si−SNP axes, and stronger than in −Si+SNP axes, which may indicate melanization of these tissues (Table S1). Pretreatment with SNP also caused a slight reduction of elongation in axes cultured on the medium with sucrose (Fig. S2, supplementary data).

In turn, strong accumulation of flavonoids as a result of amplification of two signals, i.e. sucrose and the pathogenic fungus *F. oxysporum*, in cells of yellow lupine L. cv. Polo was revealed with the use of confocal microscopy by [57]. It needs to be stressed that high levels of sucrose in embryo axes and infection with the pathogenic fungus *F. oxysporum* strongly enhanced expression level of the flavonoid biosynthetic genes. This effect was most apparent in the early phase of infection.

What is more, an interesting finding of this study is connected with the fact that sucrose alone without SNP stimulated the accumulation of 2′OH genistein 7-O-glucoside, stronger than it was with SNP. The level of this glucoside in non-inoculated and inoculated tissues with sucrose was higher than in tissues with carbohydrate deficit (−Si+SNP, −Si−SNP). Thus sucrose as a source of carbon may be involved directly in phenylpropanoid metabolism pathways, which in consequence leads to increased synthesis of isoflavonoids. However, it needs to be stressed that sucrose alone in non-inoculated axes generally did not cause accumulation of free isoflavone aglycones. In turn, differently to the SNP, as a result of amplification of the signal coming from GSNO and sucrose alone there was observed high accumulation of genistein glucoside, 2′hydroxygenistein, wighteone and luteone at 48 h in +Sn+GSNO axes, significantly higher than in +Sn−GSNO and −Sn−GSNO axes (Fig. 2C–E).

Moreover, Shin et al. [101] revealed that changes in levels of endogenous $Ca^{2+}$, a second messenger in sugar signaling in plants, can modulate sucrose-induced sugar uptake in *Arabidopsis*, which in turn regulated anthocyanin accumulation. It has also been revealed that pathways involving NO, Ca and sugar might be equally important in abiotic stress responses. For example, the results presented by [102] prove that SNP in association with $CaCl_2$ can play a role in enhancing the tolerance of plants to abiotic stress such as salt stress by improving the antioxidative defense system, osmolyte accumulation and ionic homeostasis.

A study by [103] showed that NO may influence the maintenance of homeostasis in cells under stress conditions, which means that in the presence of endogenous nitric oxide in wounded tissues an increase was observed in the total antioxidant pool in cells. A significant role of NO in maintaining the functional homeostasis in normal (unstressed) systems as well as in protection against biotic and abiotic stresses was reported by [104]. Khan et al. [102] reported that nitric oxide (NO) is not only a small, diffusible, ubiquitous bioactive molecule, acting as a pro-oxidant as well as an antioxidant, but also regulates a remarkable spectrum of plant cellular mechanisms.

In this study, in inoculated axes pretreated and not pretreated with SNP with a high level of sucrose (+Si+SNP, +Si−SNP) we need to focus on the very high total antioxidant capacity (TAC) dependent on the pool of fast antioxidants (ascorbic acid and glutathione) (Fig. 5). However, the highest TAC level among the tested experimental variants was recorded at 24 h in +Si+SNP axes, where its high level was also maintained at the subsequent time points. This result indicates that NO in tissues with high levels of sucrose (where the level of isoflavonoids, particularly free aglycones was very high),
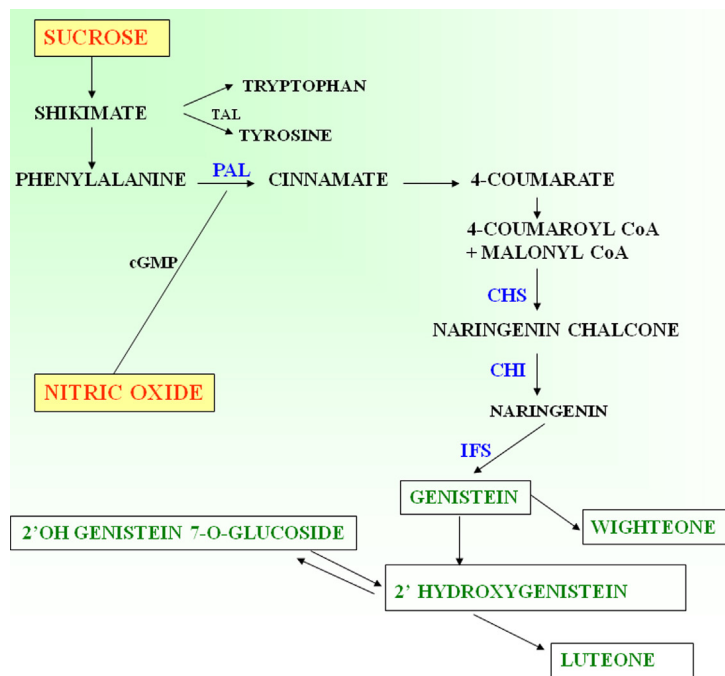
**Fig. 7.** Biosynthetic pathway enhanced in yellow lupine cultivated under elevated NO (SNP) and sucrose conditions by pathogenic fungus *F. oxysporum*.

may have an effect on the increase in TAC. This in turn may increase the maintenance of homeostasis in cells infected with *F. oxysporum* in +Si+SNP axes. Moreover, this result may suggest the interaction between antioxidants. Flavonoids are known to be stress protectors [105]. On the other hand, Bandy and Bechara [106] revealed that flavonoids found in such membranes as epicatechin and quercetin may regenerate ascorbate in the aqueous phase.

Moreover, in inoculated tissues with high levels of sucrose (+Si+SNP, +Si−SNP), where TAC dependent on the pool of fast antioxidants was high, much higher than in inoculated tissues with carbohydrate deficit, postinfection generation of semiquinone radicals was lower (Fig. 6). On the one hand, the high antioxidant pool could have limited generation of these radicals, while on the other hand these semiquinone radicals may be incorporated in such polymers as lignins by binding with reactive oxygen species (ROS) [107]. In addition, high levels of isoflavonoids in inoculated tissues with an enhanced sucrose level (+Si+SNP, +Si−SNP) could act as antioxidants that scavenge free radicals. Numerous studies have reported that sucrose may directly act as an antioxidant and play a role as ROS scavengers [108–116].

Moreover, sugar signals may also contribute to immune responses against pathogens and probably function as priming molecules leading to pathogen-associated molecular patterns (PAMP)-triggered immunity and effector-triggered immunity in plants [117], [118]. The process of priming involves prior exposure to an eliciting factor making plants more tolerant to future stress exposure [119]. As reported by Sonnewald et al. [120], activation of plant defense responses, such as reinforcement of the cell wall, production of reactive oxygen species or the accumulation of antimicrobial compounds, requires energy, reducing power and carbon skeletons that can be fueled by carbohydrates. The increased metabolic activity is probably fed by an enhanced flow of sucrose to the site of infection or reduced sucrose export.

In conclusion, the strong accumulation of isoflavonoids, particularly free aglycones, in *F. oxysporum*-inoculated embryo axes of yellow lupine pretreated with nitric oxide with a high level of sucrose is the result of amplification of the signal coming from sucrose, the nitric oxide donor and the pathogenic fungus. Sucrose

and nitric oxide act as signal molecules, which alter gene expression, leading to specific metabolic responses (Fig. 7).

### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2013.07.007.

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