Available online at www.sciencedirect.com
SCIENCE DIRECT®
Plant Physiology and Biochemistry 43 (2005) 363–373
Plant Physiology and Biochemistry
www.elsevier.com/locate/plaphy

Sucrose-induced lupine defense against *Fusarium oxysporum* Sucrose-stimulated accumulation of isoflavonoids as a defense response of lupine to *Fusarium oxysporum*

Iwona Morkunas [a,*], Łukasz Marczak [b], Jerzy Stachowiak [c], Maciej Stobiecki [b]

[a] *Department of Plant Physiology, August Cieszkowski Agricultural University, Wołyńska 35, 60-637 Poznań, Poland*
[b] *Institute of Bioorganic Chemistry, Polish Academy of Sciences, Noskowskiego 12/14, 61-704 Poznań, Poland*
[c] *Department of Chemistry, August Cieszkowski Agricultural University, Wojska Polskiego 75, 60-625 Poznań, Poland*

Received 20 August 2004; accepted 24 February 2005

Available online 17 March 2005

Abstract

Defense responses to inoculation with *Fusarium oxysporum* SCHLECHT f. sp. *lupini* were studied in embryo axes of *Lupinus luteus* L. cv. *Polo* cultured on a medium with sucrose (60 mM) or without it. Exogenous sucrose caused a marked endogenous increase in concentrations of sucrose, glucose and fructose in embryo axes. In axes cultured with sucrose, high performance liquid chromatography (HPLC) revealed generally higher levels of isoflavone glycosides (particularly until 48 h of culture) and free aglycones (genistein, wighteone, luteone). Inoculation resulted in a considerable decline in soluble carbohydrates between 24 and 72 h of culture. Simultaneously, the infection stimulated an increase in the level of free isoflavone aglycones in inoculated embryo axes, as compared to non-inoculated ones. Concentrations of free aglycones (i.e. genistein, wighteone and luteone) after infection were particularly high in inoculated embryo axes fed with sucrose. Genistein was a better inhibitor to *F. oxysporum* growth than genistein 7-O-glucoside tested. Exogenous sucrose also stimulated the activity of phenylalanine ammonialyase (PAL, EC 4.3.1.5)—an important enzyme initiating phenylpropanoid metabolism. After infection of tissues, a strong increase was observed in the activity of PAL and β-glucosidase (EC 3.2.1.21)—an enzyme hydrolyzing isoflavone glycosides. Furthermore, the growth of inoculated embryo axes cultured with sucrose was less inhibited as a result of infection than inoculated axes cultured under carbohydrate deficiency conditions. Additionally, it had been reported previously that disease symptoms of embryo axes growing in the presence of sucrose were less intensive [30]. These results suggest that soluble sugars are involved in the mechanism of resistance, as they can stimulate phenylpropanoid metabolism and contribute to the increase in concentration of isoflavonoids, which are important elements of the defense system of legumes.

Keywords: Defense response; *Fusarium oxysporum*; β-glucosidase; Isoflavonoids; *Lupinus luteus*; Phenylalanine ammonialyase; Sucrose

1. Introduction

In plants, sugars are essential as substrates in the carbon and energy metabolism and in polymer biosynthesis. In addition, sugars have important hormone-like functions as primary messengers in signal transduction, regulating cell metabolism. The latest progress in research on molecular mechanisms of sugar sensing and signaling in plants showed that signal molecules include not only glucose but also sucrose and trehalose [36]. Sucrose plays a particularly important role, as it is the major form of translocated sugars in plants and is the most frequently used sugar in studies of plant sugar responses in gene regulation and development [21,23]. However, in many cases the effects of sucrose could be completely substituted by a hexose, such as glucose or fructose [20].

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 the resultant metabolic and developmental reactions

Abbreviations: HPLC, high performance liquid chromatography; PAL, phenylalanine ammonialyase; *p*-NP, *p*-nitrophenol.

* Corresponding author. Fax: +48 61 848 7179.
E-mail address: morkunas@jay.au.poznan.pl (I. Morkunas).

0981-9428/$ - see front matter © 2005 Elsevier SAS. All rights reserved.
doi:10.1016/j.plaphy.2005.02.011

[23,39]. It has been revealed that sucrose level affects the intensity of biochemical processes, such as respiration, amino acid metabolism, and mobilization of storage compounds in germinating seeds [3,31]. Disturbances in carbohydrate distribution between the donor (cotyledons) and the acceptor (embryo axis) in germinating seeds are caused by both environmental factors and too deep sowing. . Perturbations in the transport of these metabolites may impair its defense capacity. In a previous paper it was reported that the decrease in sucrose level caused increases in the intensity of disease symptoms induced by *Fusarium oxysporum* [30]. In the published literature, only little information attests unambiguously to the participation of sugars in plant defense responses, which prevent or limit invasion of pathogens. The nature of sugar-based plant resistance to fungal pathogens is not clear. There is some direct and indirect evidence suggesting that soluble sugars-especially sucrose, which is an easily accessible source of carbon in host cells and takes part in many physiological reactions—participate in the induction of plant resistance to fungal pathogens. An important piece of direct evidence is that carbohydrates are osmolytes, while fungal pathogens usually require high values of water potential for optimum development, so a decrease in water potential of host cells may limit fungal growth [5,11]. A piece of indirect evidence is the fact that sucrose and hexoses induce the expression of many genes including also plant resistance genes that determine the production of peroxidase and pathogenesis-related (PR) proteins [18].

Ehness et al. [9] revealed that carbohydrate signal and stress-related pathogen infection independently activate different intracellular signaling pathways that ultimately are integrated to regulate coordinately source and sink metabolism and activate defense responses.

A spectrum of reactions that are sequentially activated in response to pathogens has been elucidated [25]. An important component of plant defense response is the transcriptional activation of phenylpropanoid metabolism, because the reactions are common to a range of pathways leading to functionally diverse defense-related products. In plants of the family Leguminosae these include isoflavonoids-derivatives varying in structure, which are synthesized and accumulated as phytoalexins in response to infection with pathogenic microbes [6]. A key enzyme of this pathway is phenylalanine ammonialyase (PAL), which catalyzes the deamination of L-phenylalanine. Moreover, sugar-induced gene expression has been confirmed for sink-specific enzymes involved in responses to pathogens or other stress factors. This applies to e.g. chalcone synthase [38], proteinase inhibitor II of potato [22] and PAL [9].

The experimental protocol used in this study is based on a model system: embryo axes from germinating yellow lupine seeds were cultured in vitro on a medium with or without sucrose after inoculation with a spore suspension of *F. oxysporum* f. sp. *lupini*. This pathogen causes *Fusarium* wilt and pre-emergent sprout root rot and post-emergent seedling rot. The participation of sucrose was analyzed in defense responses of germinating yellow lupine to inoculation with this fungus. More precisely, we investigated the relationship between sucrose level and accumulation of isoflavonoids, which are a varied group of polycyclic compounds that occur widely in legumes and are important elements of the defense system in interactions with pathogenic microorganisms. Groups of these secondary metabolites may occur either in a free state or conjugated as esters or glycosides [14,15]. Biological activity in interaction with pathogenic microorganisms is also shown by free isoflavone aglycones, released from glycosides with the use of glucosidases. For this reason, we also analyzed in this study changes in the activity of β-glucosidase (EC 3.2.1.21), which hydrolyses isoflavone glucosides, and PAL (EC 4.3.1.5), which plays a key role in linking primary metabolism to phenylpropanoid metabolism. At the same time the effect of vascular wilt pathogen *F. oxysporum* on the length and fresh weight (FW) of lupine embryo axes cultured on medium with sucrose and without sugar was analyzed.

2. Results

2.1. Changes in concentrations of sucrose and soluble monosaccharides

Levels of sucrose and soluble monosaccharides in non-inoculated and inoculated lupine embryo axes both for the time of 0 h and those cultured on the medium with and without sucrose are presented in Fig. 1. Already at 0 h, i.e. within 20 min after inoculation and before axes were transferred onto the medium, a slight decrease was observed in inoculated embryo axes in the levels of sucrose, glucose and fructose. After the addition of exogenous sucrose to the medium, its endogenous level in embryo axes (+ Sn axes) from 24 to 72 h of culture was twice as high as in tissues cultured without sucrose (–Sn axes). A slight reduction of sucrose level was observed after 48 h of culture in + Sn and –Sn embryo axes. It is worth noting that a strong decline in sucrose level occurred in tissues infected by *F. oxysporum* on the medium with sucrose (+ Si axes). After 72 h of culture, +Si axes contained only half as much sucrose as +Sn axes non-inoculated tissues.

Glucose content of +Sn embryo axes after 24–72 h of culture reached 32–38 mg•g^{-1} FW, while in –Sn embryo axes, it amounted to 5–15 mg•g^{-1} FW. Fructose content of + Sn embryo axes was 27 and 23 mg•g^{-1} FW after 24 and 48 h of culture, respectively, and later it increased to 38 mg•g^{-1} FW.

Special attention should be paid to the concentration of soluble monosaccharides in + Si embryo axes. In this tissue a decrease in glucose and fructose contents was recorded between 24 and 72 h of culture. It is also noteworthy that at 72 h after inoculation, concentrations of glucose and fructose in +Si tissues decrease by 50% and 60%, respectively, in comparison with non-inoculated (+ Sn) tissues.

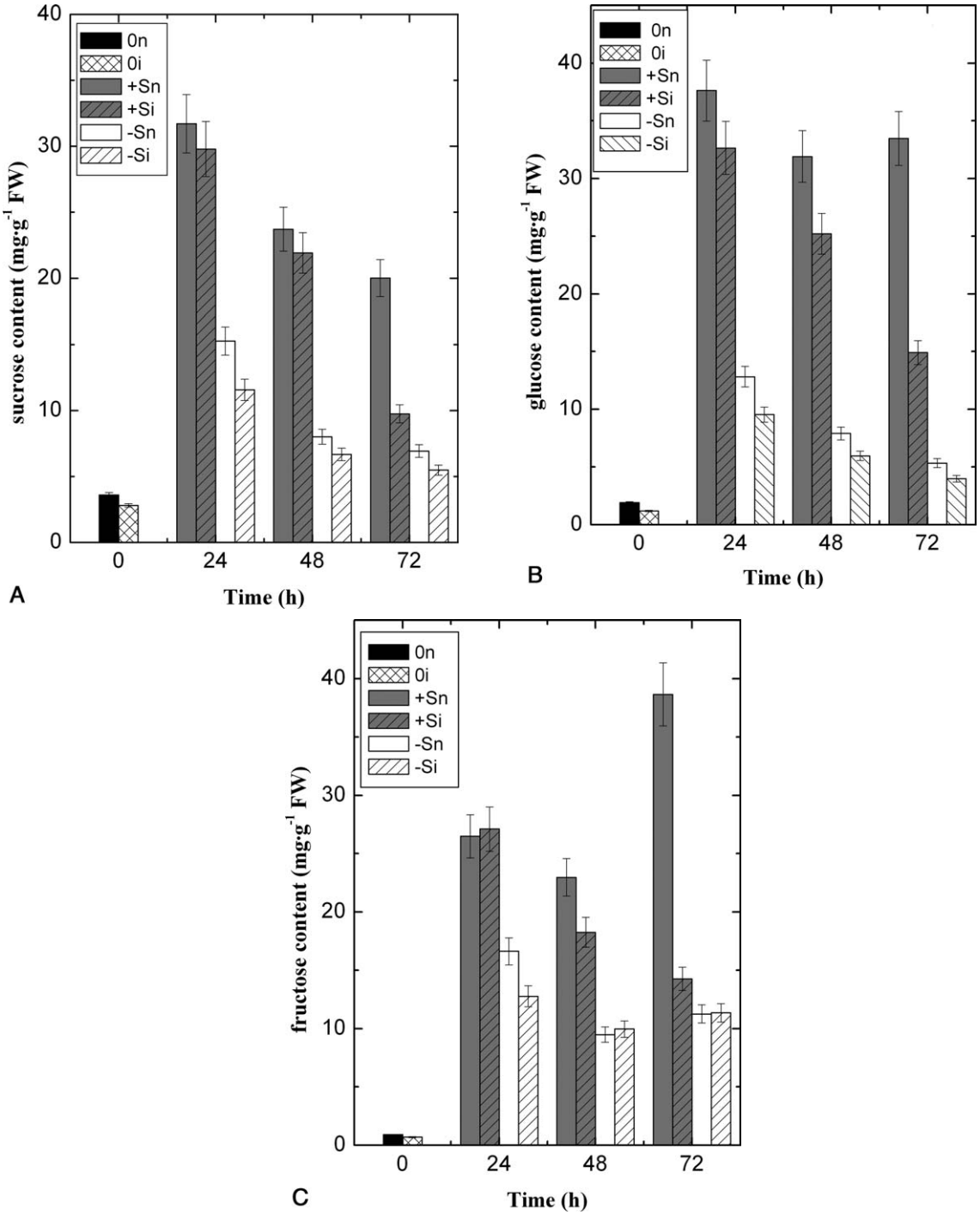

Fig. 1. Effects of *Fusarium oxysporum* SCHLECHT f. sp. *lupini* on concentrations of sucrose (**A**) and soluble monosaccharides (**B**, **C**) in yellow lupine embryo axes cultured in vitro on the medium with and without sucrose. 0n, uninoculated embryo axes, at 0 h immediately before transfer onto medium; 0i, inoculated embryo axes, at 0 h immediately before transfer onto medium; +Sn, uninoculated embryo axes cultured on Heller medium with 60 mM sucrose; +Si, inoculated embryo axes cultured on Heller medium with 60 mM sucrose; –Sn, uninoculated embryo axes cultured on Heller medium without sucrose; –Si, inoculated embryo axes cultured on Heller medium without sucrose.

2.2. Assay of phenolic compounds by liquid chromatography (LC/UV)

2.2.1. Concentrations of glycosides and free isoflavone aglycones

Isoflavonoids extracted from yellow lupine embryo axes were identified by analysis of UV chromatograms and comparison of retention times of phenolic compounds with corresponding data for standard substances (see Section 5). The isoflavonoid profile of yellow lupine embryo axes (Fig. 2) revealed the presence of isoflavone glycosides (genistein 4′,7-O-diglucoside and genistein 7-O-glucoside) and isoflavone aglycones (genistein, 2′-hydroxygenistein, wighteone, luteone). After 24 h since the transfer of incubation tissues onto

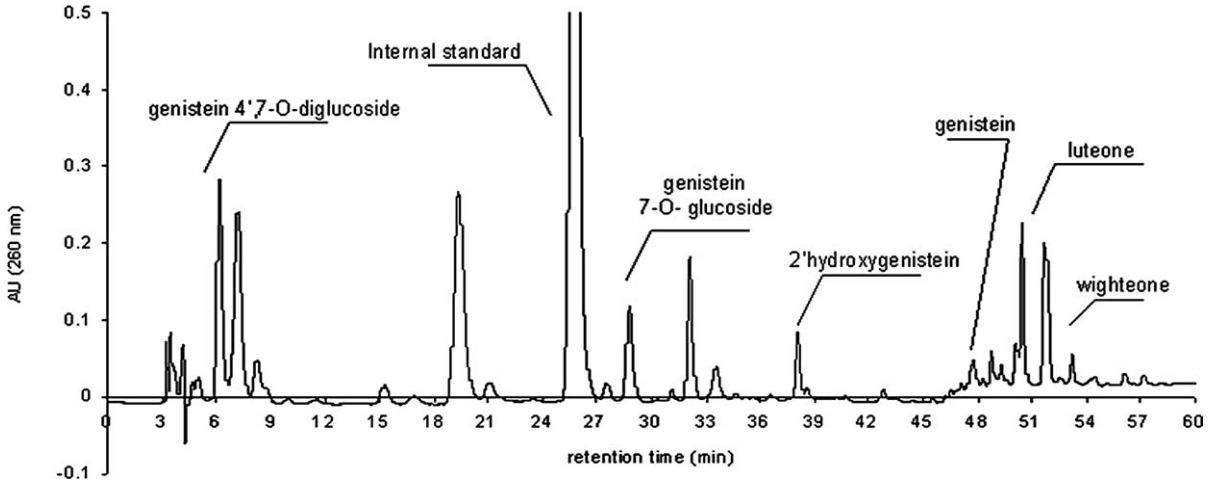

Fig. 2. LC chromatogram of phenolic compounds from yellow lupine embryo axes inoculated with *F. oxysporum* and cultured in vitro on the medium with and without sucrose.

the medium an increase was observed in the levels of glycosylated and deglycosylated isoflavonoids. Concentrations of genistein 4′,7-O-diglucoside and genistein 7-O-glucoside in +Sn embryo axes were higher than in –Sn embryo axes, both at 24 and 48 h of culture (Fig. 3A, B). The level of genistein 4′,7-O-diglucoside markedly declined in both + Sn and + Si embryo axes between 24 and 72 h of culture (Fig. 3A). A very strong decrease in the concentration of this compound was observed in inoculated and non-inoculated tissues at 48 h on the medium with and without sucrose. Genistein 7-O-glucoside level increased slightly in + Si embryo axes between 24 and 72 h of culture (Fig. 3B).

Concentrations of free isoflavone aglycones (genistein, 2′-hydroxygenistein, wighteone, luteone) in inoculated and non-inoculated embryo axes on the medium with and without sugar are shown in Fig. 4A–D. As a result of inoculation, concentrations of all the aglycones except 2′-hydroxygenistein were higher in + Si than in + Sn embro axes. After 48 h of culture, levels of the three compounds were about 2.5 times higher in + Si inoculated than in + Sn embryo axes. Their levels in inoculated tissues cultured on sucrose remained high also after 72 h of culture. By contrast, concentrations of 2′-hydroxygenistein in both + Si and + Sn embryo axes clearly declined between 24 and 72 h Generally, concentrations of isoflavone aglycones in + Si tissues were much higher than in –Si tissues.

2.3. Effect of isoflavonoids on F. oxysporum growth

To determine which of the two applied isoflavonoids, i.e. whether genistein 7-O-glucoside and isoflavone aglycone genistein have fungitoxic properties, *F. oxysporum* growth was measured on potato dextrose agar (PDA) medium containing these compounds at a concentration of 75 μM. It was found that genistein was the better inhibitor than genistein 7-O-glucoside, as its inhibition of *F. oxysporum* growth was 24% in comparison with control, while by genistein 7-O-glucoside only 10%, respectively.

2.4. Activity of β-glucosidase

Results of the assay of β-glucosidase activity are presented in Fig. 5. No inducing effect of sucrose was found on the activity of this enzyme. Thus, after 24 h culture β-glucosidase activity was by 14% lower in + Sn than in –Sn embryo axes, and at longer culture times differences in the enzyme activity in these tissues were negligible. Due to infection, its activity increased both immediately after inoculation in 0i embryo axes and between 24 and 72 h of culture in + Si and –Si embryo axes. After 72 h of culture, it was twice as high in + Si embryo axes than in + Sn ones. A very strong stimulating influence of infection on β-glucosidase activity was observed after 48 and 72 h of culture in –Si embryo axes, where it was 2–3.5 times higher than in –Sn tissues.

2.5. Variations in activity of phenylalanine ammonialyase (PAL)

The enzyme assays reveal than sucrose stimulated PAL activity in lupine embryo axes (Fig. 6). PAL activity in + Sn embryo axes was 2–3.5 times higher than in –Sn embryo axes between 24 and 72 h of culture. A particularly strong stimulation of PAL was noted after infection of embryo axes on the medium with sucrose. After 48 and 72 h of culture PAL activity in + Si tissues was over 2–2.5 times higher than in + Sn tissues. Moreover, as a result of infection an increase in PAL activity was also found in –Si embryo axes both after 24 and 48 h culture. It must be emphasized that PAL activity in –Si embryo axes after 24 and 48 h of culture was much lower (4.21–5.38 μmol cinnamic acid•mg^{-1} protein•h^{-1}) than in + Si embryo axes (9.47–15 μmol cinnamic acid•mg^{-1} protein•h^{-1}).

2.6. Growth of yellow lupine embryo axes

Changes in the length and FW of lupine embryo axes of non-inoculated and inoculated *F. oxysporum* cultured on the medium with and without sucrose are shown in Fig. 7. The

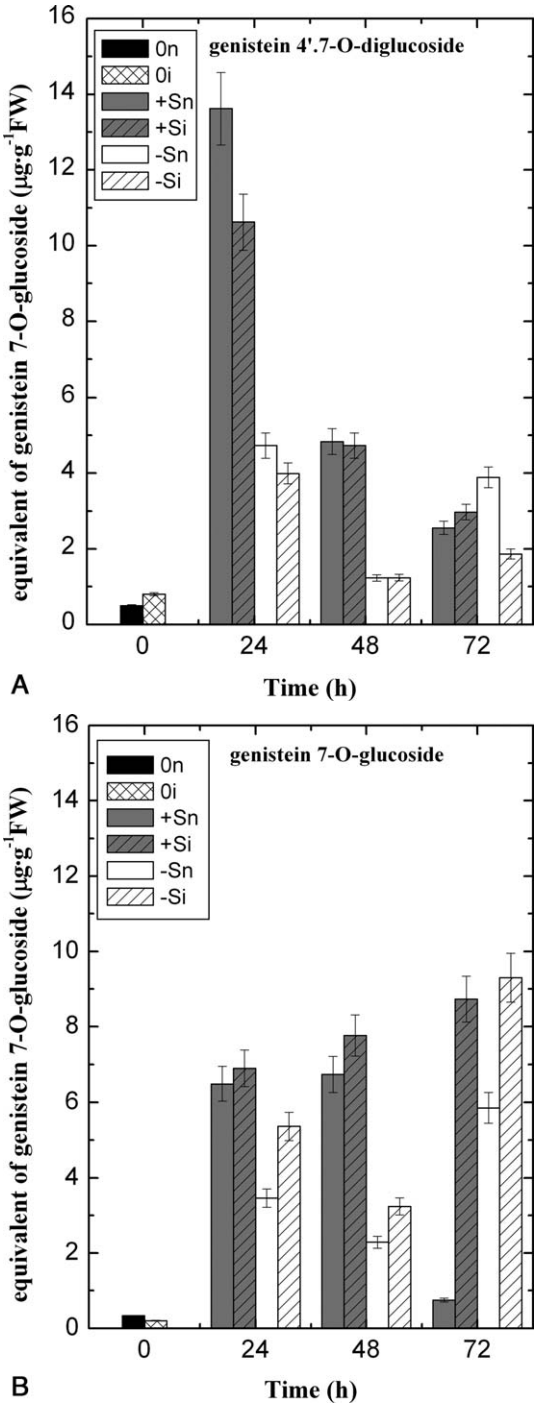

Fig. 3. Concentrations of isoflavone glycosides in yellow lupine embryo axes inoculated with *F. oxysporum* and cultured in vitro on the medium with and without sucrose: (**A**) genistein 4′,7-O-diglucoside, (**B**) genistein 7-O-glucoside. Amounts of metabolites are expressed as equivalents of genistein 7-O-glucoside (µg g^{-1} FW).

growth of embryo axes was retarded as a consequence of inoculation. However, significant differences in growth between non-inoculated and inoculated embryo axes were observed primarily starting from 48 h after inoculation. It needs to be emphasized that infection inhibited more significantly the growth of –Si embryo axes rather than + Si axes. Thus, at the time of the most intensive infection, i.e. 72 h after inoculation, the length of + Si embryo axes was 12 mm and their weight was 0.016 g lower than those of + Sn embryo axes, whereas the length of –Si embryo axes was 14 mm and their weight by 0.025 g lower than those of –Sn. Additionally, apart from a considerable decrease in FW, indicative of a developing disease in –Si embryo axes, it was also found that they were flabby and covered with large amounts of mucilage, which was not observed in + Si embryo axes.

3. Discussion

3.1. Concentration of soluble carbohydrates after inoculation of embryo axes

At the early stages of growth and development, soluble sugars (sucrose in particular) can modify the rate of important processes, such as seed germination and seedling development, and can affect their defense mechanisms triggered in response to infection by pathogens [27]. Engström and Strömberg [10] showed that the total amount of sugars dramatically decreased (65%) 1 day after experimental inoculation of potato plants with *Phytophthora infestans*, but recovered to normal levels by day 9. The presented results were interpreted by those authors as symptoms of defense response to the attack of the pathogen, although they did not exclude the possibility that fungi might metabolize sugars.

With the use of high performance liquid chromatography (HPLC), we revealed in this study that inoculation of yellow lupine tissues with *F. oxysporum* caused a marked decrease in levels of soluble carbohydrates in + Si embryo axes between 24 and 72 h of culture (Fig. 1A–C). There may be two independent reasons for this. Firstly, the low levels of soluble sugars in inoculated axes may be due to their partial metabolization by fungi, as they are easily accessible sources of carbon in host cells. Secondly, it could be a symptom of initiation of a defense response by the plant. If the level of sugars in tissues was high, the infection lowered it, but if the level was already initially low, no substantial lowering was observed after infection. This suggests that the low level was necessary for the maintenance of primary metabolism. Thus, embryo axes fed with sugar may initiate the defense mechanism, as they have an excess of sugars, while embryo axes cultured on the medium without sucrose may not, as they have too little sugars.

3.2. Levels of isoflavone glycosides and aglycones after inoculation of embryo axes cultured on the medium with and without sucrose

HPLC showed that as a result of infection, concentrations of free isoflavone aglycones in + Si embryo axes were generally higher than in + Sn ones (Fig. 4A–D). After 48 and 72 h of culture, concentrations of genistein, wighteone and luteone in inoculated tissues were about two to three times

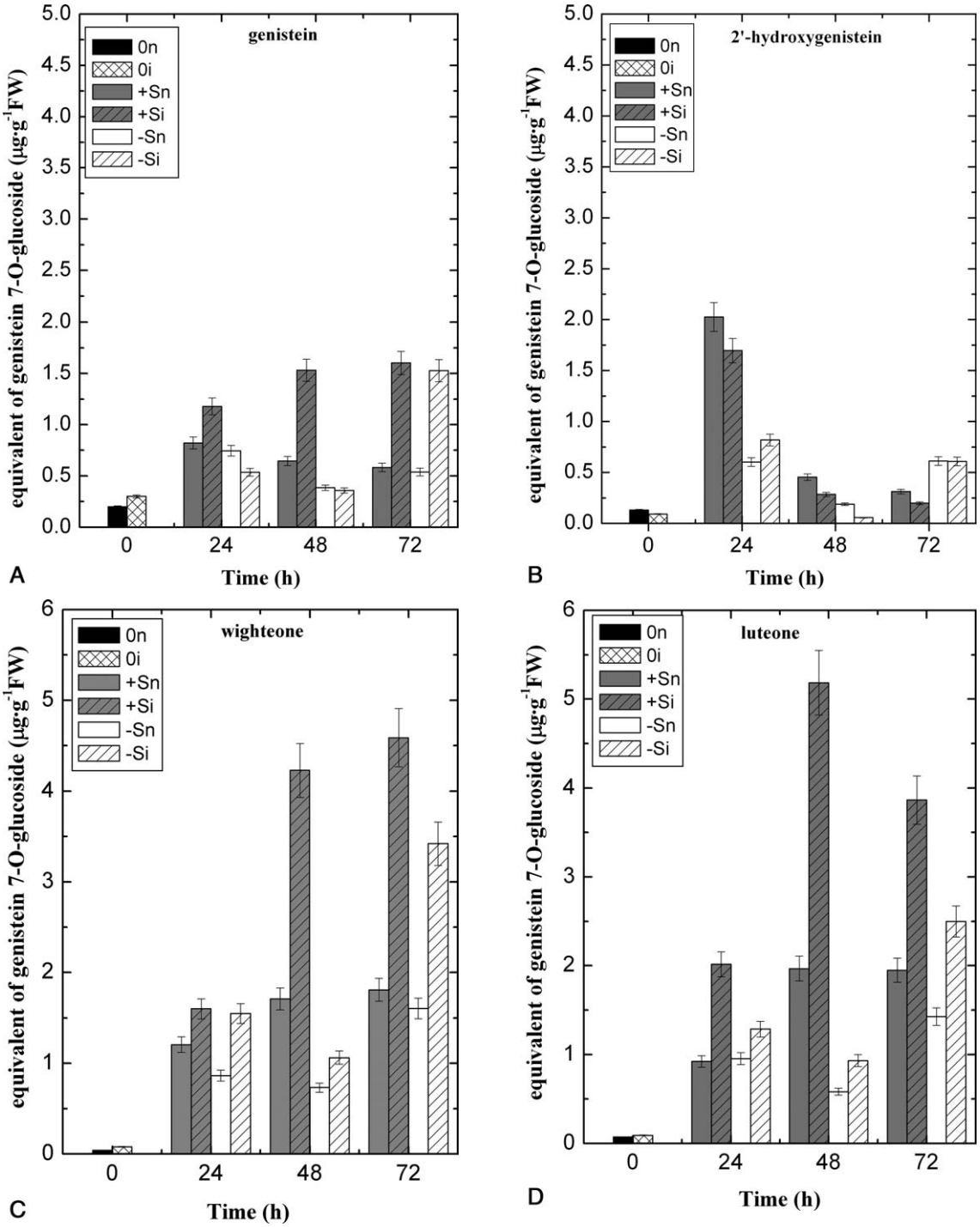

Fig. 4. Concentrations of isoflavone aglycones in yellow lupine embryo axes inoculated with *F. oxysporum* and cultured in vitro on the medium with and without sucrose: (**A**) genistein, (**B**) 2′-hydroxygenistein, (**C**) wighteone, (**D**) luteone. Amounts of metabolites are expressed as equivalents of genistein 7-O-glucoside (μg g^{-1} FW).

higher than in non-inoculated tissues. The increase in luteone level was accompanied, especially until 48 h of culture, by a decrease in 2′-hydroxygenistein (precursor of luteone) in + Si embryo axes (Fig. 4B, D). Moreover, concentrations of isoflavone aglycones (genistein, wighteone and luteone) and glycosides (genistein 4′,7-O-diglucoside and genistein 7-O-glucoside) (Fig. 2) in +Si embryo axes on the medium with sucrose were generally higher than in those –Si embryo axes (Fig. 3A, B and Fig. 4A, C, D). We suppose that sucrose and hexoses, as sources of carbon skeletons, may be directed to secondary metabolism and consequently contribute to the increased level of isoflavonoids, which are important elements of plant defense against microbial pathogens. However, it needs to be emphasized that the amount of sugars consumed is three orders of magnitudes (i.e. 1000-fold) higher than the amount of isoflavones produced.

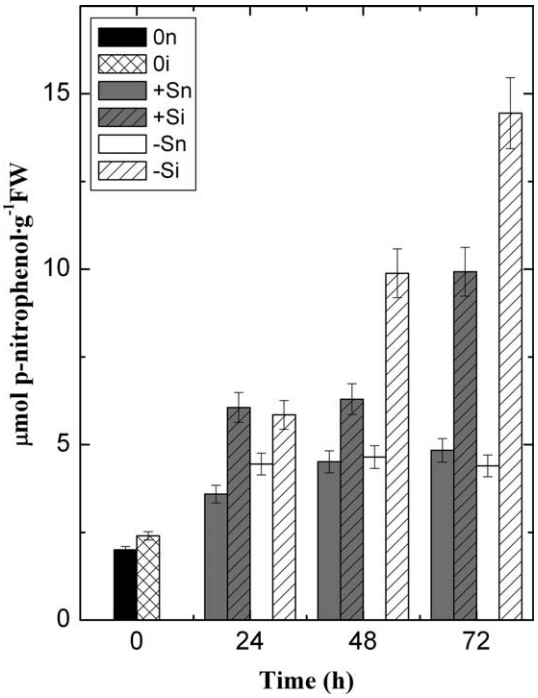

Fig. 5. Changes in β-glucosidase activity in yellow lupine embryo axes inoculated with *F. oxysporum* and cultured in vitro on the medium with and without sucrose.

Ingham et al. [19] also showed that infection of lupine by pathogenic fungi stimulates in lupine cells de novo synthesis and accumulation of isoflavone glycosides and mainly free aglycones, which thanks to their high fungitoxicity are effective elements of the defense response of the plant host to fungal pathogens.

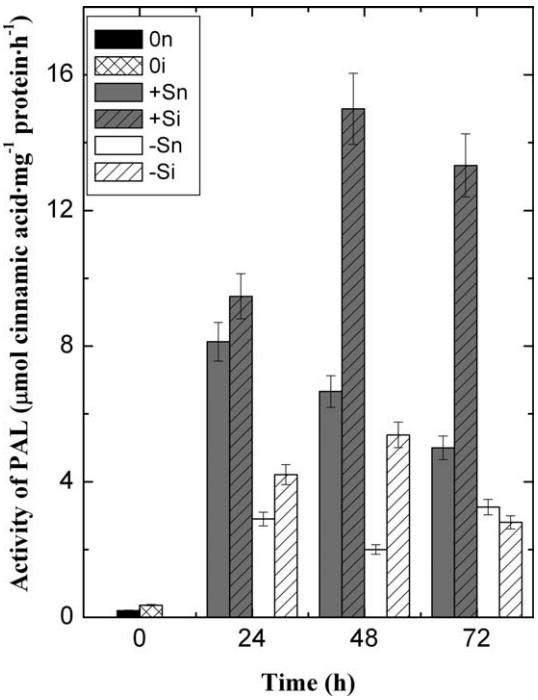

Fig. 6. Changes in PAL activity in yellow lupine embryo axes inoculated with *F. oxysporum* and cultured in vitro on the medium with and without sucrose.

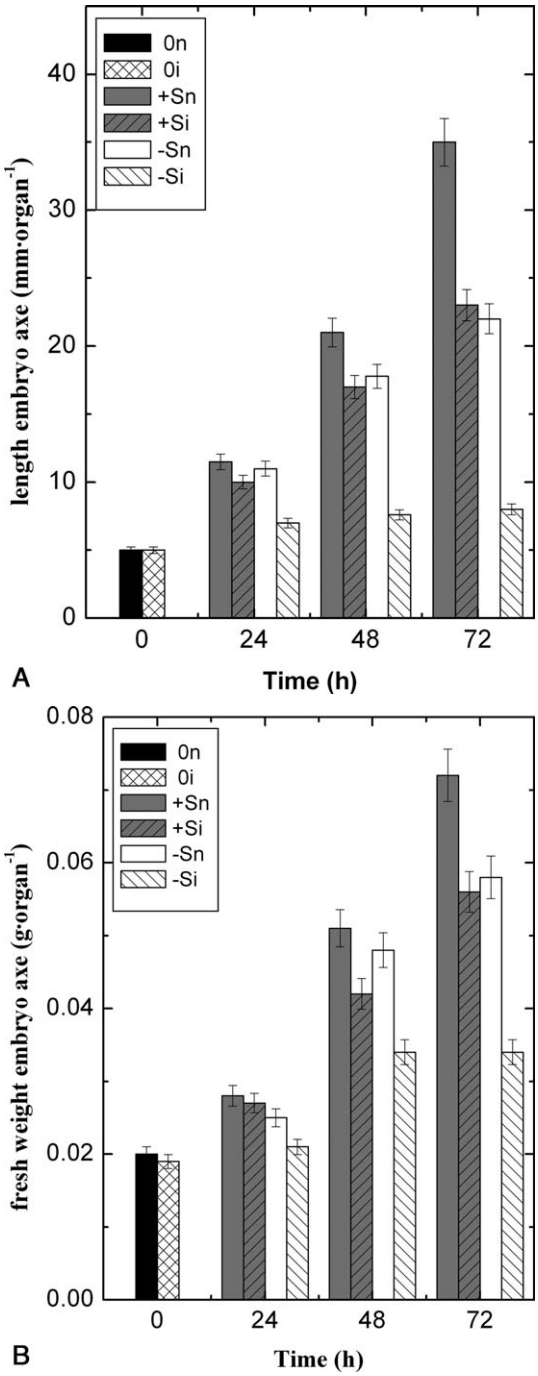

Fig. 7. Effects of *F. oxysporum* on the length (A) and FW (B) of yellow lupine embryo axes cultured in vitro on the medium with and without sucrose.

Our results are similar to those reported by Bednarek et al. [1], who studied responses of *Lupinus album* L. to an elicitor from yeast cell walls. As a result of the application of this elicitor, concentrations of free aglycones (genistein, 2′-hydroxygenistein, and their prenylated derivatives: wighteone and luteone) increased two to four times in lupine leaves. Graham et al. [13] showed that injection of an elicitor from *Phytophthora sojae* into soybean tissues caused an increase in the concentration of another phytoalexin, glyceollin, especially at the site of injection. High glyceollin content was also observed by Lozovaya et al. [28] in inoculated *Fusarium*

solani hairy roots of soybean. At the same time it was found that glyceollin was the most inhibitory to fungi growth among isoflavonoids tested, i.e. daidzin, daidzein, genistin, genistein, glycitein, glycitin and coumestrol. In these studies HPLC analyses showed that 20 h after inoculation in the resistant soybean cultivar total isoflavone concentrations increased, whereas after 72 h they decreased, both in the susceptible and resistant cultivar. Moreover, in contrast to our results, genistein contents in hairy roots of the resistant soybean cultivar 24 h after inoculation were lower than in controls.

It is an interesting issue whether the glycoside or its aglycone have a stronger antimicrobial action, if any. One of the glycosides and one of the free aglycones investigated in this study, i.e. genistein 7-O-glucoside and genistein, were also studied by Lozovaya et al. [28]. It was found in their study that the glucoside exhibits a stronger inhibitory action than the aglycone towards mycelium *F. solani* f. sp. *glycines*. In case of *F. oxysporum* f. sp. *lupini* investigated in our study an opposite reaction was observed.

In our study, beside the high level of free aglycones, also a slight increase in genistein glucoside was observed in + Si tissues until 72 h of culture (Fig. 3B). The increase in genistein glucoside level probably results from the fact that intercellular transport of isoflavonoids is possible mainly thanks to glycosylated forms. An inverse trend was recorded in this material for genistein 4′,7-O-diglucoside, which concentration declined between 24 and 72 h after inoculation.

Bednarek et al. [1] observed a slight increase in concentrations of conjugates of 2′-hydroxygenistein, namely 2′-hydroxygenistein 7-O-glucoside and 2′-hydroxygenistein 7-O-(6′-O-malonyl)-glucoside, 24 and 48 h after infiltration of the elicitor. By contrast, a decrease was recorded by those authors after elicitation in the levels of genistein glucosides, i.e. genistein 7-O-glucoside and genistein 7-O-(6′-O-malonyl)-glucoside.

There also exists a relationship between the level of phenylpropanoid compounds and resistance to diseases. For example, an increased production of isoflavonoid phytoalexins may enhance the resistance of *Medicago sativa* to diseases [16] as well as limit fungal growth and decrease pathogenicity, as reported for infected pea [29]. Because of their antifungal properties, many secondary metabolites–including isoflavonoids—are regarded as natural fungicides [35].

3.3. Activity of β-glucosidase

Measurements of the activity of β-glucosidase (an enzyme hydrolyzing isoflavone glycosides) showed that infection generally stimulated an increase in enzymatic activity, which was not found both in the presence and absence of sucrose. Sucrose alone had no effect action (Fig. 5). However, it appeared to delay the increase in + Si tissue in the 24–48 h time interval when compared to –Si tissues, in which an approximately twofold increase was observed, comparable to the increase at 72 h in + Si tissue. We suppose that the observed increase in its activity in inoculated embryo axes is due to the progress of infection, as in embryo axes cultured on the medium without sugar it was more advanced than in those cultured on the medium with sucrose.

A post-infection increase in β-glucosidase activity was observed in many plant species because this enzyme plays an important role in the plant–environment interaction, as it releases biologically active aglycones from glycosides [24,32]. However, some researchers report that under conditions of biotic stress, the activity of this enzyme may be reduced if the infecting factors are viruses or bacteria [8].

3.4. Activation of phenylalanine ammonialyase (PAL) by sucrose

PAL is a key enzyme initiating the transition from primary metabolism to phenylpropanoid metabolism and simultaneously controlling the biosynthesis of isoflavonoids and other phenylpropanoid compounds. Douglas [7] reported that the biosynthesis of phenylpropanoids requires an efficient inflow of carbon for the synthesis of phenylalanine (a substrate for the reaction catalyzed by PAL) through shikimate and pathways of aromatic amino acids.

The enzyme assays carried out in this study indicate that the addition of exogenous sucrose to the medium stimulated PAL activity in lupine embryo axes even without infection, but infection markedly enhanced the stimulation (Fig. 6).

The stimulating effect of sugars on the activity of phenylpropanoid pathway enzymes was already reported by Ehness et al. [9] and previously demonstrated by Tsukaya et al. [38]. Moreover, our results are consistent with published reports suggesting that infection by pathogens induces a strong activation of PAL [26,34].

The strong activation of PAL and the high concentrations of isoflavonoids recorded in this study in + Si embryo axes, as compared with those of –Si embryo axes, probably increase the resistance of lupine tissues to infection and development of *F. oxysporum*.

4. Conclusions

The measurements of concentrations of isoflavonoids and enzyme assays performed in this study indicate that soluble carbohydrates may be involved in the mechanism of resistance of yellow lupine embryo axes to infection and development of *F. oxysporum*, because the carbohydrates can be used as carbon skeletons for synthesis of isoflavonoids, which are important elements of the defense system of legumes.

5. Methods

5.1. Plant material

Yellow lupine (*Lupinus luteus* L. cv. *Polo*) seeds of S-elite class were used in the experiments. The seeds were surface-

sterilized, immersed in sterile water and left in a thermostat (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 imbibition. After subsequent 18 h, seed coats were removed from the imbibed seeds and cotyledons were cut off, to isolate embryo axes. Embryo axes were either inoculated (see below), or not, and immediately within the next 20 min, after being cut off the cotyledons, i.e. at 0 h, non-inoculated (0n) and inoculated with a *F. oxysporum* spore suspension (0i), were placed in groups of 4, on Whatman filter paper and subsequently transferred to sterile glass test tubes (diameter of 3 cm, height 13.5 cm) containing 14 ml mineral Heller's medium [17], where they were suspended so that one end of the axis was immersed in the medium and empty space was left below the paper to allow better aeration. After removal of cotyledons, embryo axes were dependent on the carbon source provided by the medium. Four culture variants were applied: on Heller's medium supplemented with 60 mM sucrose, non-infected (+Sn) (control) and infected (+Si) tissues were grown, but also on Heller's medium without addition of sucrose, non-infected (–Sn) (control) and infected (–Si) tissues were cultured. The embryo axes were incubated in darkness at 25 °C. Samples were collected for analyses at 0 h and after 24, 48, and 72 h of culture, when they were either killed by freezing in liquid nitrogen for the purpose of the analysis of isoflavonoids, assay of PAL and β-glucosidase activity or were thrown into a boiling ethanol solution for sugar analysis. Results of the measurements of length and FW were means for 20 embryo axes.

5.2. Preparation of spore suspension and inoculation

F. oxysporum f. sp. *lupini* strain K-1018 (further named as *F. oxysporum*) was obtained from the collection of Plant Pathogenic Fungi, Institute of Plant Protection, Poznań. The pathogen was incubated at 25 °C in Petri dishes (9 cm diameter) on PDA medium (Difco, pH 5.5) in the dark. After 3 weeks of growth, *F. oxysporum* spore suspension was prepared. The spore suspension was obtained by washing the mycelium with sterile water and shaking with glass pears. Then the number of spores was determined by means of the Bürker hemocytometer chamber. The embryo axes were inoculated with the spore suspension at a concentration of 5×10^6 of 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.

To assess fungicidal properties of isoflavones, mycelial plugs were cut off from 3-week *F. oxysporum* cultures, which were placed in the center of a Petri dish with PDA medium (3.9% w/v) with no isoflavones added (control) and containing isoflavones, i.e. genistein 7-O-glucoside or its free aglycone genistein. Compounds were added to the medium before solidification in 100% ethanol to give a final concentration of 75 μM. The concentration of ethanol in each medium was 1% (v/v) including the control. Colony diameters were measured on the 7th day of growth at 25 °C in the dark.

5.3. Carbohydrate analysis

5.3.1. Extraction

Samples of approximately 0.5 g of embryo axes each were subjected to extraction for 30 min with 10 ml of ethanol/water (80:20, v/v) as an extraction solvent at 80 °C [33]. The solution was filtered and evaporated to dryness under nitrogen. Finally the aliquot of the filtrate was redissolved in 0.5 ml of 0.1 mM ethylenediaminetetraacetic acid calcium disodium salt (CaEDTA) solution and filtered through a 0.45 μm Milipore filter.

5.3.2. HPLC analyses

Soluble sugars were analyzed by HPLC (Waters Alliance 2695) using a Sugar-Pak I column (Waters). The mobile phase (filtered water with 0.1 mM CaEDTA) was pumped through the column at a flow rate of 0.4 ml min^{-1}. The temperature of the oven was adjusted to 70 °C, while that of the cuvette, to 40 °C. An RI monitor (Waters 2414) was used. Sucrose, glucose and fructose were identified by their retention times and were quantified according to standards.

5.4. Analysis of isoflavonoids

5.4.1. Isolation of phenolic compounds

Prior to LC profiling of isoflavone glucosides, frozen plant tissue was homogenized in 80% methanol (20 ml g^{-1} FW) at 4 °C and sonicated for 3 min with a VirTis Model VirSonic 60 sonicator. The suspension was filtered through a Büchner funnel and concentrated under vacuum at 40 °C. Samples of plant extracts for LC analyses were prepared from 0.5 g FW of lupine tissue. They were purified and concentrated by solid phase extraction (SPE) on cartridges containing a cation exchanger and RP C-18 silica gel (Alltech, Carnforth, UK) combined in tandem, according to Stobiecki et al. [37].

5.4.2. 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 Superspher 100 RP-18 column (250 mm × 2 mm; Merck). To each analyzed sample, 125 μM *p*-hydroxybenzoic acid was added as an internal standard (LC retention time and UV spectral data did not interfere with those of studied compounds). Quantification of total isoflavones was achieved by integration of UV chromatograms at 259 nm normalization to the peak of a standard and comparison with a respective calibration curve for genistein 7-O-β-D-glucoside prepared in the range 10–500 μM. The concentrations of lupine isoflavone glucosides and free aglycones were expressed as equivalents of genistein 7-O-glucoside (μg g^{-1} FW). 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, 48–60 min isocratic at 100% of A. Free isoflavone 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 in the same chromatographic condition. The above mentioned standards were obtained and characterized during earlier studies in our laboratory [12].

5.5. Extraction and assay of β-glucosidase activity

Frozen embryo axes (200 mg) were homogenized at 4 °C with a mortar and pestle in 2 ml of 0.1 M phosphate buffer (pH 7.0) containing 0.5% polyethylene glycol (PEG 6.000). Polyclar AT (10 mg per 100 mg tissue) was added during extraction. Supernatants obtained after centrifugation (at 10,000 × *g* for 15 min) were used to determine β-glucosidase (EC 3.2.1.21) activity. The activity was determined by the method of Nichols et al. [32]. The mixture containing 0.2 ml of extract and 0.2 ml of 4-nitrophenyl-β-D-glucopyranoside as substrate (2 mg ml^{-1}) was incubated for 1 h at 35 °C. After that time, 0.6 ml of 0.2 N Na_2CO_3 was added. The formation of *p*-nitrophenol (*p*-NP) was measured at 400 nm (the Perkin–Elmer Lambda 11 spectrophotometer).

5.6. Extraction and assay of phenylalanine ammonialyase (PAL) activity

PAL (EC 4.3.1.5) was extracted at 4 °C by using 0.1 M Tris–HCl buffer at pH 8.9 (4 ml per 300 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 × *g* for 30 min. PAL activity was determined with a modified method of Cahill and McComb [4]. The incubation mixture contained 80 mM of borate buffer (pH 8.9), 30 mM of 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 2 N 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 Bradford [2].

5.7. Statistical analysis

All determinations were performed in at least three replicates in three independent experiments. Statistical analysis was based on Student's *t*-test, with the level of significance $\alpha = 0.05$; standard deviation was calculated and its range is shown in the figures.

Acknowledgements

The authors would like to thank Professor L. Ratajczak, the Adam Mickiewicz University, Poznań, for fruitful discussions, and Dr. Dorota Remlein-Starosta, Institute of Plant Protection, Poznań, for cultures of *F. oxysporum* SCHLECHT f. sp. *lupini*. This work was supported by the Polish Committee for Scientific Research (KBN, grant no. 3P06R 052 24)

References

[1] P. Bednarek, R. Frański, L. Kerhoas, J. Einhorn, P. Wojtaszek, M. Stobiecki, Profiling changes in metabolism of isoflavonoids and their conjugates in *Lupinus albus* treated with biotic elicitor, Phytochemistry 56 (2001) 77–85.

[2] M. Bradford, A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.

[3] R. Brouquisse, F. James, P. Raymond, A. Pradet, Study of glucose starvation in excised maize root tips, Plant Physiol. 96 (1991) 619–626.

[4] D.M. Cahill, J.A. McComb, A comparison of changes in phenylalanine ammonia-lyase activity, lignin and phenolic synthesis in the roots of *Eucalyptus calophylla* (field resistant) and *E. marginata* (susceptible) when infected with *Phytophtora cinnamoni*, Physiol. Mol. Plant Pathol. 40 (1992) 315–332.

[5] R.J. Cook, R.I. Papendick, Role of water potential in microbial growth and development of plant disease with special reference to postharvest pathology, Hortsciences 13 (1978) 559–564.

[6] F.D. Dakora, D.A. Phillips, Diverse functions of isoflavonoids in legumes transcend anti-microbilal definitions of phytoalexins, Physiol. Mol. Plant Pathol. 49 (1996) 1–20.

[7] C.J. Douglas, Phenylpropanoid metabolism and lignin biosynthesis: from weeds to trees, Trends Plant Sci. 1 (6) (1996) 171–178.

[8] A. Edreva, V. Sotirova, I.D. Georgieva, E. Stoimenova, R. Rodeva, N. Bogatzevska, Differential expression of α-glucosidase in tomato-stress stimuli systems, Acta Physiol. Plant. 22 (3) (2000) 274–277.

[9] R. Ehness, M. Ecker, D.E. Godt, T. Roitsch, Glucose and stress independently regulate source and sink metabolism and defense mechanisms via signal transduction pathways involving protein phosphorylation, Plant Cell 9 (1997) 1825–1841.

[10] K. Engström, A. Strömberg, Changes in sugar content during induction of systemic acquired resistance to late blight caused by *Phytophthora infestans* (Mont.) de Bary in potato, J. Phytopathol. 144 (1996) 33–36.

[11] J.F. Farrar, Fluxes and turnover of sucrose and fructans in healthy and diseased plants, J. Plant Physiol. 134 (1989) 137–140.

[12] R. Frański, P. Bednarek, P. Wojtaszek, M. Stobiecki, Identification of flavonoid diglycosides in yellow lupin (*Lupinus luteus* L.) with Mass Spectrometric techniques, J. Mass Spectrom. 34 (1999) 486–495.

[13] M.Y. Graham, T.L. Graham, Wound-associated competency factors are required for the proximal cell responses of soybean to the *Phytophthora sojae* wall glucan elicitor, Plant Physiol. 105 (1994) 571–578.

[14] J.B. Harborne, Phenolic glycosides and their natural distribution, in: J.B. Harborne (Ed.), Biochemistry of Phenolic Compounds, Academic Press, New York, 1964, pp. 129–169.

[15] J.B. Harborne, S.W. Simmonds, The natural distribution of the phenolic aglycones, in: J.B. Harborne (Ed.), Biochemistry of Phenolic Compounds, Academic Press, New York, 1964, pp. 77–127.

[16] X.Z. He, R.A. Dixon, Genetic manipulation of isoflavone 7-O-methyltransferase enhances biosynthesis of 4′-O-methylated isoflavonoid phytoalexins and disease resistance in alfalfa, Plant Cell 12 (2000) 1689–1702.

[17] R. Heller, Recherches sur la nutrition minerale des tissues vegetaux cultives in vitro, Ann. Sci. Nat. Bot. Biol. Veg. 14 (1954) 1–223.

[18] K. Herbers, P. Meuwly, W.B. Frommer, J.P. Metraux, U. Sonnewald, Systemic acquired resistance mediated by the ectopic expression of invertase: possible hexose sensing in the secretory pathway, Plant Cell 8 (1996) 793–803.

[19] J.L. Ingham, S. Tahara, J.B. Harborne, Fungitoxic isoflavones from *Lupinus albus* and other *Lupinus* species, Z. Naturforschung 38c (1983) 194–200.

[20] J.C. Jang, P. Leon, L. Zhou, J. Sheen, Hexokinase as a sugar sensor in higher plants, Plant Cell 9 (1997) 5–19.

[21] J.C. Jang, J. Sheen, Sugar sensing in higher plants, Trends Plant Sci. 2 (1997) 208–214.

[22] R. Johnson, C.A. Ryan, Wound-inducible potato inhibitor II genes: enhancement of expression by sucrose, Plant Mol. Biol. 14 (1990) 527–536.

[23] K.E. Koch, Carbohydrate modulated gene expression in plants, Annu. Rev. Plant Physiol. Plant Mol. Biol. 47 (1996) 509–540.

[24] M. Kozłowska, Activity of β-D-glucosidase and syringaldazine oxidase and susceptibility of red raspberry canes to *Didymella applanata* (Niessl) Sacc, Phytopathol. Polonica 6 (1993) 7–12.

[25] C. Lamb, R.A. Dixon, The oxidative burst in plant disease resistance, Annu. Rev. Plant Physiol. Plant Mol. Biol. 48 (1997) 251–275.

[26] M.A. Lawton, R.A. Dixon, K. Hahlbrock, C.J. Lamb, Elicitor induction of mRNA activity. Rapid effects of elicitor on phenylalanine ammonia-lyase and chalcone synthase mRNA activities in bean cells, Eur. J. Biochem. 130 (1983) 131–139.

[27] P. Leon, J. Sheen, Sugar and hormone connections, Trends Plant Sci. 8 (3) (2003) 110–116.

[28] V.V. Lozovaya, A.V. Lygin, O.V. Zernova, S. Li, G.L. Hartman, J.M. Widholm, Isoflavonoid accumulation in soybean hairy roots upon treatment with *Fusarium solani*, Plant Physiol. Biochem. 42 (2004) 671–679.

[29] S.F. Mackintosh, D.E. Matthews, H.D. VanEtten, Two additional genes for pisatin demethylation and their relationship to the pathogenicity of *Nectria haematococca* on pea, Mol. Plant Microbe Interact. 2 (1998) 354–362.

[30] I. Morkunas, M. Kozłowska, W. Ratajczak, The role of carbohydrates in early metabolic responses of germinating lupine seeds to *Fusarium oxysporum* f. sp. *lupini*, Acta Agrobotanica 55 (1) (2002) 247–254.

[31] I. Morkunas, M. Garnczarska, W. Bednarski, W. Ratajczak, S. Waplak, Metabolic and ultrastructural responses of lupine embryo axes to sugar starvation, J. Plant Physiol. 160 (2003) 311–319.

[32] E.J. Nichols, J.M. Beckman, L.A. Hadwiger, Glycosidic enzyme activity in pea tissue and pea-*Fusarium solani* interaction, Plant Physiol. 66 (1980) 199–204.

[33] J.H. Nygarad, G. Vibe, K.K.E. Bach, Influence of extraction solvent and temperature on the qualitative determination of oligosaccharides from plant materials by high-performance liquid chromatography, J. Agric. Food Chem. 44 (1996) 1470–1474.

[34] L. Pellegrini, O. Rohfritsch, B. Fritig, M. Legrand, Phenylalanine ammonia-lyase in tobacco. Molecular cloning and gene expression during the hypersensitive reaction to tobacco mosaic virus and the response to a fungal elicitor, Plant Physiol. 106 (1994) 877–886.

[35] A.K. Picman, E.F. Schneider, J. Picman, Effect of flavonoids on mycelial growth of *Verticillium albo-atrum*, Biochem. Syst. Ecol. 23 (1995) 683–693.

[36] F. Rolland, B. Moore, J. Sheen, Sugar sensing and signaling in plants, Plant Cell 14 (2002) 185–205.

[37] M. Stobiecki, P. Wojtaszek, K. Gulewicz, Application of solid phase extraction for profiling of quinolizidine alkaloids and phenolic compounds in lupin tissue, Phytochem. Anal. 8 (1997) 153–158.

[38] H. Tsukaya, T. Ohshima, S. Naito, M. Chino, Y. Komeda, Sugar-dependent expression of the CHS-A gene for chalcone synthase from petunia in transgenic *Arabidopsis*, Plant Physiol. 97 (1991) 1414–1421.

[39] S.M. Yu, Cellular and genetic responses of plants to sugar starvation, Plant Physiol. 121 (1999) 687–693.