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The influence of *Fusarium oxysporum* infection and low temperatures on the activity of soybean esterase and PR proteins

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SUMMARY

Some aspects of the interaction within the soybean/*Fusarium oxysporum* Snyd. et Hans. pathosystem have been elucidated. The reaction of soybean varieties in relation to their resistance has been evaluated against an artificial background infection, prepared from the *Fusarium* fungi characterised by an increased pathogenicity, using conventional phytopathological techniques. Esterase and PR-2 proteins (1,3-β-glucanases) were extracted from plantlet rootlets of the soybean varieties, treated and not treated with the *Fusarium oxysporum* culture filtrate, and cultivated under optimal temperature conditions (24°C) and subnormal temperatures (8°C). The electrophoretical analysis of esterase activity has shown the expression of different isozymes of this enzyme in soybean under the action of the *Fusarium* fungi and subnormal temperatures. The enzymes 1,3-β-glucanases activity was higher in the resistant varieties which suggests the role of these enzymes in the development of resistance to the *Fusarium* infection. The subnormal temperatures enhanced their activity. Thus, it is possible that there is a linkage between the genes responsible for the soybean resistance to the *Fusarium* infection and subnormal temperatures.

Key words: 1,3-β-glucanases, *Fusarium*, *Glycine max* (L.), low temperature, PR proteins, soybean.

YFIRLIT

Áhrif smitunar með Fusarium oxysporum *og lághita á virkni esterasa og PR-próteina í sojabaunum*

Skýrð er nokkur samspilsatriði í sjúkdómsferli *Fusarium oxysporum* Snyd. Et Hans á sojabaunum. Með hefðbundnum smitaðferðum voru viðbrögð sojabaunastofna og þol þeirra metin eftir *Fusarium* smit með *Fusarium* sveppum sem einkennast af auknum sýkingarmætti. Esterasi og PR-2 prótein (1,3-β-glucanasar) voru einangruð frá plönturótum sojabaunastofna sem voru smitaðir eða ósmitaðir með *Fusarium oxysporum* vökva og ræktaðar við kjörhita (24°C) eða undir kjörhita (8°C). Rafdráttargreining á virkni esterasa sýnir mismunandi „isozyma“ af þessu ensími þar sem *Fusarium* er virkur undir kjörhita. Virkni ensímsins 1,3-β-glukanasa var hærri í þolnum stofnum, sem bendir til að þessi ensím gegni hlutverki í þróun *Fusarium* þols. Hiti undir kjörhita eykur virkni þeirra og bendir það til sambands á milli erfðavísa sem stýra *Fusarium* þoli og lághita.

INTRODUCTION

Successful infection of plants by pathogenic fungi involves many different phases. The primary phase involves contact between plant and pathogen comprising adhesion and surface recognition. This phase can be followed by penetration, which may require hydrolytic degradation of host cell walls. Establishment and maintenance within the host species are further requirements for successful infection. These latter phases may depend on different

pathogenically determinants that may differ between pathogens. These involve suppressing and inactivating host defense responses by the fungus. In addition, a nutritional relation between host plant and fungus should be established for some time. In resistant plants, specific recognition of the invading fungus may occur, leading to activation of defense responses, preventing further growth of the pathogen in the plant.

In recent years, many new techniques have been developed facilitating the study of infection processes at the molecular level. The interaction between different pathogens and plants has been described in greatest detail, with emphasis on putative pathogenically factors, race-specific elicitors, and resistance gene-dependent defense responses (Wubben, 1994; Kuc, 1997).

Host-specificity of fungal pathogens may be determined by host-selective toxins (HST) (Walton and Panaccione, 1993). HST are known only among fungal pathogens, especially in the genera of *Alternaria* and *Cochliobolus*. The majority of known HSTs are low molecular weight secondary metabolites. They are involved in aggressiveness and pathogenicity: a fungus that produces a HST causes more disease on its host compared to non-producing strains of the same species. Insensitivity of a plant to HST confers increased resistance to the producing organism.

Several low molecular weight antimicrobial compounds have been regarded as possible means by which plants defend themselves against fungal pathogens. These compounds may either be synthesized constitutive or in response to microbial infection. For successful infection, detoxification of plant antimicrobial compounds may be a necessity for the fungus (VanEtten *et al.*, 1993; Bowyer *et al.*, 1994).

Recently, new molecular strategies have been employed to isolate factors possibly involved in pathogenicity of plant pathogenic fungi (Talbot *et al.*, 1993). It was shown that a number of fungal genes, possibly involved in the infection process, was induced specifically during plant infection. Several strategies have been designed to dissect the infection process at the molecular level, based on induced expression of genes involved in the infection process, as has been shown before (Joosten *et al.*, 1994; Van den Ackerveken *et al.*, 1993). Examples of genes identified by the differential screening approach was described (Pieterse *et al.*, 1993; Talbot *et al.*, 1993).

Several plant proteins have been found to accumulate 2–4 days earlier in incompatible interaction between *Cladosporum fulvum* and tomato than in compatible interaction (De Wit *et al.*, 1986). These proteins were identified as pathogenesis-related (PR) proteins (Joosten *et al.*, 1990). PR protein accumulation has been observed in many plant species, induced upon infection by pathogenic organisms such as viruses, bacteria and fungi (Stintzi *et al.*, 1993). Since PR proteins accumulate in plants upon pathogen infection and are usually associated with acquired resistance (Pan *et al.*, 1992), it has been suggested that they are involved in defense of plants against pathogens.

PR-2 proteins (1,3-β-glucanase) have been shown to catalyze the hydrolysis of 1,3-β-glucan polymers. This indicates the possibility that PR-2 proteins play a role in plant defense, targeted against fungal pathogens with 1,3-β-glucan-containing cell walls. Basic, vacuolar isoforms of 1,3-β-glucanase had, in general, higher specific activity than acidic, extracelular isoforms. In addition to the potential antifungal activity, a role for these enzymes in development of healthy plants has been suggested, in view of the expression of PR-2 and PR-2-like genes in flowers and roots (Henning *et al.*, 1993; Memelink *et al.*, 1990; Ori *et al.*, 1990). Recently, it has been reported that purified 1,3-β-glucanases from different plant species were able to inhibit growth of several fungi *in vitro* (Mauch *et al.*, 1988; Sela-Buurlage *et al.*, 1993) indicating a possible involvement in active defense of plants against pathogenic fungi. A role for 1,3-β-glucanases in active

defense of soybean against *Fusarium* infection remains to be elucidated.

Due to a great economical importance of soybean, the number of research on the genetical structure of soybean varieties has increased significantly during the recent years (Morgante *et al.*, 1994; Sivolap *et al.*, 1998; Cheng and Chandlee, 1999). The data have been accumulated until present, regarding the employment of isoenzymes to identified genes of the resistance to diseases. Isoenzymes of peroxidases can serve as indicators of specific metabolic reaction of soybean under biotic and abiotic stresses (Glazko and Sozinov, 1993; Koretsky and Cotofana, 1997).

The aim of the work was to elucidate some aspects of the interaction of the *Fusarium*-soybean pathosystem in resistant and sensitive varieties. The studies on the effect of the *Fusarium* infection and subnormal temperatures on the esterase isozyme polymorphism and PR-2 protein (1,3-β-glucanase) activity present a special interest.

MATERIALS AND METHODS

Studies on the reaction of soybean varieties to the Fusarium *infection under field conditions against an artificial infections background*

The soybean varieties, Bucuria and KOO3, were sown against an artificial infection background, formed from the inoculum of the *Fusarium* fungi characterized by enhanced pathogenicity. To produce the inoculum, the *Fusarium* fungi were isolated from the infected soybean plants, their morphology studied, and followed by the assessment of the pathogenicity using a method of direct infection of soybean rootlets *in vitro* and screening of the fungi with increased pathogenicity. The soybean seeds (150 of each genotype) were sown in rows (50 seeds per row), the distance between the rows being 50 cm. Replicates were three. The degree of the attack of the *Fusarium* root and cotyledon rot was estimated at the plantlet stage.

The variety resistance was evaluated according to a 5 ranking scale: 0, 1, 2, 3, 4, 5.

In vitro *cultivation of plantlets and* Fusarium *fungi*

The seeds were washed with sterile distilled water and imbedded into the filtrate of the *Fusarium oxysporum* fungus with an increased pathogenicity for 24 h. To produce the filtrate, the fungi were cultivated on a solid medium for 14 days, then on a Czapec liquid medium for 14–21 days. The filtrate produced was used to treat the soybean seeds. After 24 hours the seeds were removed from the filtrate, rinsed with distilled water and placed on Petri dishes covered with sterile filtrate paper, humified with sterile distilled water. The Petri dishes containing the seeds of both varieties were placed under optimal temperature conditions (24°C) for 24 h to initiate the growth, and then transferred to low temperature (8°C) to intensify the *Fusarium* fungus attack. The soybean seeds of the water treated genotypes served as a control. The esterases extraction from the plantlet rootlets was carried out at the plantlet stage (by the 7th day of incubation in the thermostat), followed by electrophoresis.

Esterase extraction

After 7 days incubation in the thermostat, esterases were extracted from the rootlets of the plantlets. Preparation of the material for the esterase extraction were as follow: the rootlets were ground in the extraction buffer, the extract poured into Epindorf tubes and centrifuged for 5–7 min at 5–7000 rpm in a microcentrifuge. Then, 5–10 µl of the extract were loaded into gel for electrophoresis.

The solution for the visualisation and evaluation of the distribution and intensity of the esterase fraction extracted from the different genotypes was prepared in the following way: 40 mg of naphthyl acetate and α-naphthyl acetate were dissolved in 2 ml of acetone, and then diluted with 100 ml of 0.1 M phosphate buffer, pH=6.0; with 100 mg of methylene blue. The gel was incubated in this mixture prior to electrophoresis. After electrophoresis the gel was incubated in dark

Table 1. The degree of the *Fusarium* infection (*Fusarium* root rot and *Fusarium* cotyledon rot) of soybean different genotypes under field conditions.
1. tafla. Fusarium *smitstig (*Fusarium *rótarrot og* Fusarium *rótarhálsrot) mismunandi sojabaunastofna í akurtilraun*.

No.	Genotype	Year	Infection degree, $\overline{X}\pm m_{\bar{x}}$	
			Fusarium root rot	*Fusarium* cotyledon rot
1	Bucuria	1997	3.14±0.33	2.90±0.34
2	Bucuria	1998	4.15±0.36	3.36±0.28
3	Bucuria	1999	3.53±0.28	3.40±0.4
4	KOO3	1997	1.89±0.24	1.33±0.2
5	KOO3	1998	2.0 ±0.30	2.20±0.40
6	KOO3	1999	1.75±0.85	2.0 ±0.91

for 4–8 hours, followed by visualization of the molecular fraction spectrum of the esterases. The photographs of the electrophoregrams were taken using a Micrat-300 photo film.

Electrophoresis

Composition of gel, electrophoresis buffers, and histochemical reactions were similar to those of Bousquet *et al.* (1987).

The quantitative analyses of 1,3-β-glucanase

The activity of 1,3-β-glucanase was estimated by a calorimetric method which measures the enzyme's capacity to split KMC to a reducted substances (Radionova *et al.*, 1966). In this case, 1 ml of the extract and 9 ml of NaKMC (pH=6.0) were incubated at 37°C for 1 h, then boiled in water bath (15 min) to inactivate the enzyme, followed by the addition of 1 ml of the Somogi's reagent and 1 ml of the Nelson's reagent. The amount of enzyme forming 1 mg of glucose in the inactivation mixture were taken as one unit of activity. The optical density was measured. For control sample 1 ml of water was added instead of the extract to the 9 ml of reaction mixture. Protein extraction from the soybean seedlings (850 mg) was through their rubbing with 4.5 ml of 0.5 M Na-acetate.

RESULTS AND DISCUSSION

The findings of the phytopathological assessment of the different soybean varieties following *Fusarium* infection under field conditions against an artificial infections background are presented in Table 1.

The data show that the degree of infection of the Bucuria and KOO3 varieties is different, the Bucuria variety being less resistant to *Fusarium* infection. Also, the infection with *Fusarium* root rot was 3.14–4.15 according to a 5-ranking scale, while the *Fusarium* cotyledon rot was 2.90–3.40. The KOO3 variety proved to be more resistant to the *Fusarium* fungi, the degree of the *Fusarium* root rot and the *Fusarium* cotyledon rot was 1.75–2.0 and 1.33–2.20, respectively.

The mechanism of soybean resistance to *Fusarium oxysporum* and low temperatures and the cause of variety differences in resistance are not known. Since enzymes are principal regulatory components of the cell, the changes in the enzymatic system induced by stress factors seem to play an important part in the adaptation processes occurring in plants under stress conditions.

The activity of two resistance-related enzymes was also investigated in KOO3 and Bucuria during infections with *Fusarium oxysporum* at optimal (24°C) and low temperatures (8°C). Rootlets infected with *Fusarium oxysporum* culture medium and untreated root-

Table 2. The activity of 1,3-β-glucanase in soybean rootlets under the action of *Fusarium oxysporum* at subnormal temperatures.
2. tafla. Virkni 1,3-β-glukanasa í sojabaunarótum eftir smit með Fusarium oxysporum *undir kjörhita*.

No.	Treatment	Variety resistance[a)]	Temperature °C	Glucanases activity
1	Bucuria	S	24	3.19
2	Bucuria + *Fusarium oxysporum*	S	24	2.31
3	Bucuria + *Fusarium oxysporum*	S	7	4.95
4	Bucuria	S	7	5.25
5	KOO3	R	24	3.08
6	KOO3 + *Fusarium oxysporum*	R	24	3.08
7	KOO3 + *Fusarium oxysporum*	R	7	6.60
8	KOO3	R	7	8.80

a) S: Sensitive; R: Resistant.

lets (as control) were used and the activity of esterases and 1,3-β-glucanases in each variety. Polyacrylamide gel electrophoresis for analysis of esterase spectrum was performed in vertical plates on two layers.

We detected that *Fusarium oxysporum* induced the expression of esterase in both varieties, particularly when incubated at low temperatures, but the response was stronger and more rapid in the more resistant variety KOO3 (Figure 1). A specific reaction of the esterase at the level of the variety resistance, to the thermostresogene factor and biotic stress was revealed. The reaction of the isoenzymes with Rf= 0.35–0.40, 0.50–0.60, 0.80–0.90 was more specific for the action of *Fusarium oxysporum* under low temperature conditions. Hence, these isoforms might serve as a molecular-biochemical marker in identufying the soybean varieties more resistant to *Fusarium oxysporum* and low temperatures.

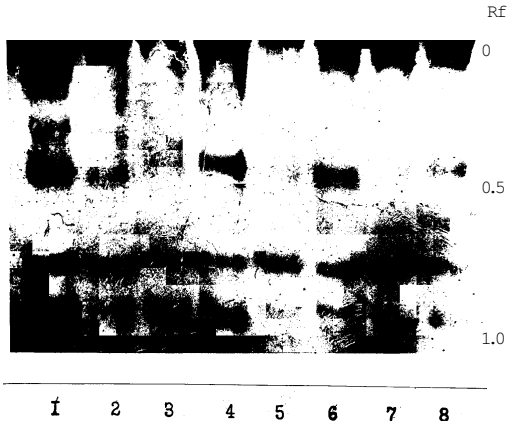

Figure 1. The influence of *Fusarium oxysporum* and low temperatures on the polymorphism of soybean esterase. 1–4: sensitive cultivator (Bucuria); 5–8: resistant cultivator (KOO3); 1, 5: *Fusarium oxysporum*; 2, 6: control; 3, 7: subnormal temperature (8°C); 4, 8: *Fusariun oxysporum* + subnormal temperatures.
1. mynd. Áhrif Fusarium oxysporum *við lághita á fjölbreytni sojabaunaesterasa. 1–4: næmur stofn (Bucuria); 5–8: þolinn stofn (K003); 1, 5:* Fusarium oxysporum*; 2, 6: viðmið; 3, 7: undir kjörhita (8°C); 4, 8:* Fusarium oxysporum + *undir kjörhita*.

Quantitative analysis of the activity of 1,3-β-glucanase showed higher expression in the more resistant variety, both under the effects of *Fusarium oxysporum* and low temperatures (Table 2).

CONCLUSIONS

The fact that the more resistant variety has higher 1,3-β-glucanase expression than the less resistant one indicate that the genes encoding these enzymes play an important role in developing the resistance against *Fusarium oxysporum*. The effect at low temperatures on PR-2 expression underlines the complex mechanism of soybean plants resistance to *Fusarium oxysporum* which was demonstrated on the resistance testing backround.

Consequently, low temperatures induce resistance to *Fusarium oxysporum* which has the ability of maximum plant infection under low temperatures. It is quite possible that a certain linkage exist between genes that contribute to resistance of cultivars to *Fusarium oxysporum* and low temperatures.

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