ANTIFUNGAL PROPERTIES OF NORWAY SPRUCE (*PICEA ABIES* (L.) KARST.) SEEDLING HOMOGENATES

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(Received: November 5, 1998. Accepted: June 20, 1999)

ABSTRACT

The presence of antimicrobials in root, hypocotyl and cotyledon homogenates of Norway spruce was studied using *in vitro* assays with soil-borne pathogens. For the studies presented here Norway spruce (*Picea abies* (L.) Karst.) as a typical host and forest tree and *Pythium* as a typical soil-borne pathogen were used. The highly virulent species *Pythium ultimum* and the less virulent species *Pythium irregulare* were chosen for all experiments. They are both the causal agents of damping-off disease, which can affect plants at a very early stage.

The strongest antimicrobial effect was observed using medium prepared from older seedlings and containing extracts from cotyledons. The influence of various treatments on antimicrobials accumulation in spruce extracts was also tested.

Seed coat shedding was observed to affect mortality of *Picea abies* seedlings infected by *Pythium*. Seedlings which had shed their seed coats were more resistant to *Pythium* attack. This phenomenon could be correlated with antimicrobial production in well developed cotyledons.

KEY WORDS: soil borne pathogens, *Pythium ultimum*, *P. irregulare*, *Picea abies*.

INTRODUCTION

Damping-off caused by various fungi is one of the diseases that can affect plants at a very early stage. Many biotic and abiotic factors play a role in the severity of the damping-off. The constitutive inhibitory substances could be a very important factor in host-pathogen interactions between young seedling and soil fungi. Antimicrobial compounds could be divided into two groups: constitutive inhibitory compounds (*phytoanticipins*) and induced antimicrobial compounds (*phytoalexins*) (Pearce 1996; Daniel and Purkhayastra 1995; Gorham 1989; Hoque 1982). They are part of the complex defence mechanisms against pathogenic organisms. Although the antimicrobial compounds of woody species have been studied since many decades (reviewed by Pearce 1996; Hoque 1982; Popoff et al. 1975) and are well described in many publications concerning general plant-pathogen interactions (Schweizer and Métraux 1996; Schneider et al. 1996) – the role of these compounds in the defence of trees remains little studied and poorly understood (Pearce 1996). The biochemical defence could be important since it acts almost immediately after pathogen attack, before any morphological symptoms can be detected (Hoque 1982). The most represented biochemical classes are proteins (reviewed by Bowles 1990, and by Stinzi et al. 1993) terpenoids, phenolics and fatty

acids (Pearce 1996). Among proteins the pathogenesis-related proteins (PR's) play an important role in resistance against many pathogens (Stinzi et al. 1993; Kozłowski and Métraux 1998). Some of them have been also found in *Picea abies* tissue and have been postulated to be important antifungal and stress proteins in this species (Sharma et al. 1993, Asiegbu et al. 1994; Kärenlampi et al. 1994; Sauter and Hager 1989). Important secondary metabolites are phenols, stilbenes, lignins, phenylpropanoids, flavonoids, terpenoids and alkaloids (Pearce 1996). In spruce, phenolics are one of the major class of secondary metabolites (Kraus and Spiteller 1997). Especially needles, but also roots of *Picea abies* contain many various phenolics from which some are thought to be antifungal (Strack et al. 1989; Klick and Hermann 1988). Stilbenes are very often regarded as the most important conifer antimicrobial phenolic group (Schweckendiek 1992, see also review by Gorham 1989). They were shown to be active against root- and butt-rotting fungi of spruce (Toscano and Pearce 1992) and they accumulate after treatment with fungal elicitors (Lange et al. 1994).

In the present study, experiments are described concerning constitutive and induced antimicrobials in roots, hypocotyls and cotyledons of young spruce seedlings. For the study presented here Norway spruce as a typical host and *Pythium* as atypical soil-borne pathogen were used. The effect of spruce extracts was studied on growth of two *Pythium* species (*Oomycetes*): *P. ultimum* and *P. irregulare*. Various compounds known as inducers of resistance or inducers of stress were also tested, in order to study their influence on the production of antimicrobials in spruce seedlings. The influence

Abbreviations:

SA – salicylic acid, SH-PCA – potato carrot agar medium containing spruce homogenates

of seed coat shedding as well as the developmental stage of cotyledons on mortality of spruce seedlings have been also determined.

MATERIALS AND METHODS

Biological material

Seeds of Norway spruce (*Picea abies* (L.) Karst.) were obtained from the Swiss Federal Institute for Forest, Snow and Landscape Research, Birmensdorf, Switzerland. They were collected from the experimental spruce stand in Tägerwil, Switzerland. *Pythium* sp. isolates were obtained from culture collections of CIBA, Basle, Switzerland.

Culture conditions

Seeds were surface-sterilized by dipping for 10 min in 30% hydrogen peroxide (v/v) followed by washing in 4 changes of sterile distilled water. Seeds were germinated on wet filter papers in darkness at room temperature for 8-10 days, they were then transferred into pots (10 cm x 25 cm, 1000 ml) containing a sand-vermiculite mixture (1:1, v/v). Plants were grown in a growth chamber (22°C day / 18°C night temperature 80% rel. humidity, 14 hours light). For preparation of spruce homogenate, 2 to 9 weeks old plant material was taken.

All *Pythium* species were maintained in petri-dishes on potato carrot agar (PCA) (2%, w/v, using a 1:1 potato and carrot homogenate and 1.8% (w/v) bacteriological agar, Unipath Ltd., Hampshire, England), in darkness at room temperature.

Salicylic acid and NaCl treatment

Ten days old spruce seedlings (cultivated as above), were treated with salicylic acid (2 mM) and salt solution (300 mM) by soil drench application, during 4 days before homogenate preparation.

Preparation of SH-PCA medium for antimicrobials tests

Roots, hypocotyls and cotyledons of *Picea abies* seedlings were homogenized in water and homogenates were added to potato carrot agar (PCA) medium (0.05-0.5 g fresh weight of spruce per petri dish). The petri dishes containing spruce homogenate medium (SH-PCA medium) were inoculated with sporangia of *Pythium irregulare* and *Pythium ultimum*. After 5 days, the mycelium was scraped off and its wet weight was measured.

Seed coat tests

Spruce seedlings (cultivated as above) were separated into two developmentally different groups, into separate pots. The first group consisted only of seedlings with seed coats still covering their cotyledons. The second group consisted of seedlings which had already shed the seed coats, and with well developed cotyledons. Both groups were then inoculated with *Pythium* species and the differences in mortality was estimated after two weeks.

RESULTS

Roots, hypocotyls and cotyledons of spruce seedlings were homogenized and added in different concentrations to potato carrot agar (PCA) medium. Using this method, an effect of spruce seedling extracts on *Pythium* sp. growth and development was observed. Figure 1 shows differences in weight of

Fig. 1. In vitro growth of *Pythium ultimum* (A) and *Pythium irregulare* (B) on different spruce homogenates – potato carrot agar (SH-PCA) media. Wet weight of mycelia 5 days after inoculation, means of 6 independent experiments, $\pm$ SD. The numbers correspond to the following ages and seedling organs used for SH-PCA preparation: 1-10 days old roots and hypocotyls; 2-30 days old roots and hypocotyls; 3-30 days old cotyledons; 4-60 days old roots and hypocotyls; 5-60 days old cotyledons; 6-60 days old roots and hypocotyls; 7-60 days old cotyledons; C – control PCA medium without spruce homogenate. Concentration: 0.1 g of fresh weight (FW) per petri dish.

virulent (*Pythium ultimum*, Fig. 1A) and less virulent (*Pythium irregulare*, Fig. 1B) species which were cultivated on medium containing spruce extracts from roots, hypocotyls and cotyledons of different ages. *Pythium ultimum* was the most affected species. Figure 1A shows that this species produces much less mycelium in comparison with the same fungus growing on control PCA medium. The decrease in mycelium weight was independent from age and organ from which the homogenate was prepared. Figure 1B shows the growth of the less virulent species on SH-PCA medium. For this species, the decrease in mycelium weight could be detected only on medium containing homogenates of older seedlings. The tendency of homogenates of older seedlings to be more inhibitory was also seen in Figure 1A. A stronger decrease of mycelium growth was also observed using homogenates from cotyledons in comparison with media containing homogenate from roots and hypocotyls (Fig. 1A and 1B) indicating different biochemical composition of these plant parts.

The next experiments show an influence of different treatments (salicylic acid SA and NaCl) on changes in antimicrobials content in spruce tissue. Figure 2A shows that *Pythium ultimum* growth was affected mostly by SH-PCA prepared with extracts from seedlings treated with SA. Treatment with

Fig. 2. In vitro growth of *Pythium ultimum* (A) and *Pythium irregulare* (B) on different spruce homogenate – potato carrot agar (SH-PCA) media. Wet weight of *Pythium* mycelia 5 days after inoculation. Means of 3 independent experiments $\pm$ SD. The media were prepared from 2 weeks old spruce seedlings treated with different biochemicals as described in Materials and Methods. The numbers correspond to the following fresh weight (FW) concentrations (per petri dish) and treatments: 1-0.1 g FW without treatment; 2-0.1 g FW with NaCl treatment; 3-0.1 g FW with salicylic acid treatment; 4-0.5 g FW with salicylic acid treatment; C – control PCA medium without spruce homogenate.

NaCl made the extracts less antimicrobial. Figure 2B shows the effect of the same extracts on *Pythium irregulare* growth. The most antimicrobial extract was SH-PCA media prepared from seedlings treated with SA. The decrease in *Pythium* growth was again closely dependent on concentration of spruce extract used for SH-PCA preparation: the more extract used the more decrease in fungal mycelium production.

Since the cotyledons used for SH-PCA preparations showed the strongest anti-*Pythium* activity, influence of the developmental stage of the cotyledons on resistance of the whole plant against *Pythium* was investigated. An effect of seed coat shedding could be observed on the tolerance of *Picea abies* seedlings to *P. ultimum* (Fig. 3). The seedlings with well developed cotyledons were less susceptible to *Pythium ultimum* compared to seedlings which had still the cotyledons enclosed in seed coats (Fig. 3).

Figure 4 shows that seedlings which have already shed seed coats, and which have well developed cotyledons – are more resistant to *Pythium irregulare* than to *Pythium ultimum*, indicating differences between virulent and less virulent species. The less virulent *Pythium irregulare* caused only 25% damp-

Fig. 3. Influence of seed coat shedding on mortality of *Picea abies* seedlings inoculated with *Pythium ultimum*. Means of 3 independent experiments, each consisting of 80 spruce seedlings, $\pm$ SD.

Fig. 4. Mortality of *Picea abies* seedlings which have shed the seed coats after inoculation with *Pythium ultimum* and *Pythium irregulare*. Means of 6 independent experiments, each consisting of 80 spruce seedlings, $\pm$ SD.

ing off, whereas *Pythium ultimum* caused up to 50% mortality of the seedlings.

DISCUSSION

Many biologically active substances, either preformed in the host or upon pathogen contact, may take part in the host-pathogen interaction. Some of these compounds belong to the general class of secondary metabolites and have either *in vivo* or *in vitro* fungitoxic properties. The experiments presented here were designed to test if homogenates of spruce contain fungitoxic substances active against fungal growth of *Pythium*.

Experiments presented in figure 1 and 2 show the presence of antimicrobials existing in *Picea homogenates*. The strongest anti-*Pythium* activity were observed in extracts from cotyledons and young needles (Fig. 1). Reports in the literature indicate that this part of the seedling is rich in phenolics (phenylpropanoids, hydroxybenzoic acids, lignans, neolignans, hydroxycinnamic acids, coumarins, stilbenes and flavonoids

(Strack et al. 1989; Klick and Hermann 1988; Kraus and Spitteller 1997). Our experiments do not give any indication of the biochemical nature of the antifungal substance involved. The fact that an antifungal activity was observed against *P. ultimum* would warrant further studies to identify the compound(s) involved and to elucidate its chemical structure. This in turn could possibly help to determine if enough of the compound is indeed present in the correct active chemical form in cotyledons to explain the antifungal properties of cotyledon homogenate.

The presence of the strongest antimicrobial activity in cotyledons was also apparent in experiments where whole seedlings were placed on a petri dish that was subsequently inoculated with *Pythium*. Colonization of the seedling tissue took place with the exception of the cotyledons (data not presented). Antifungals in the cotyledons could be one possible explanation why those seedlings which have shed the seed coats and have well developed cotyledons are much more resistant to *Pythium* colonization in comparison with very young seedlings still without cotyledons (Fig. 3 and 4). However, cotyledons having shed their seed coats might also be developmentally different in other aspects than the content in secondary metabolites only.

Increased levels of phenolic compounds have been reported in trees of many species (Shain 1967; Popoff et al. 1975; Yamada 1992; Kozłowski and Métraux 1998). Often these compounds are absent in healthy tissue, but induced at the site of infection (e.g. in *Pinus taeda* [Shain 1967]). Wood extracts of trees (containing gallic acid and lignans) have been shown to be inhibitory in vitro to wood-colonizing fungi, however the concentrations required for activity might be greater than those found in healthy trees (Pearce 1996). Stilbenes pinosylvin and pinosylvin monomethyl ether in sapwood of *Pinus densiflora* were reported to inhibit completely growth of *Heterobasidion annosum* (Dumas et al. 1983). These compounds are constitutively present in tissues of *Pinus* spp. but accumulate after pathogen attack in reaction zones and heartwood, thus they exhibit many features in common with induced antimicrobial compounds (phytoalexins) (Pearce 1996).

Another aspect of the studies on antimicrobials was to test the effect of chemical treatments on the production of antimicrobials in spruce seedlings. Figure 2 show clearly that spruce reacts differently to such treatments. Although any dramatic increase of antifungal properties of these extracts on *Pythium* have been observed, the growth of *Pythium irregulare* and *Pythium ultimum* was clearly affected by extracts prepared from seedlings treated with salicylic acid (SA). This suggests a stimulating role of SA on the production of antifungal compounds. However, direct effect of SA was also observed against *Pythium* in vitro, making it difficult to precisely ascertain the mode of action in of SA.

ACKNOWLEDGEMENTS

Funds for this research were provided by COST 813, grant No. OFES C92.0023. Additional support was obtained from a grant from the SN5F (No. 31-37098.92).

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PRZECIWGRZYBOWE DZIAŁANIE WODNYCH HOMOGENIZATÓW
SPORZĄDZANYCH Z SIEWEK ŚWIERKA (*PICEA ABIES* (L.) KARST.)

STRESZCZENIE

Badano obecność substancji przeciwgrzybowych w wodnych homogenizatach korzeni, hypokotyli i liści siewka. Używano metod *in vitro*, a otrzymane homogenizaty testowano przeciw patogenom glebowym. Do badań użyto siewek świerka (*Picea abies* (L.) Karst.) – typowego gospodarza zgorzeli siewek i pospolite drzewo leśne; oraz *Pythium* – typowy grzyb glebowy. Do eksperymentów wybrano bardzo wirulentny gatunek *Pythium ultimum* i słabo wirulentny *Pythium irregulare*. Oba gatunki powodują zgorzel siewek, chorobę atakującą rośliny w bardzo wczesnym stadium rozwojowym.

Najsilniejszy efekt przeciwgrzybowy zaobserwowano, stosując pożywkę sporządzaną z homogenizatów ze starszych siewek i zawierającą ekstrakty z liści. Testowano również wpływ różnych zabiegów na akumulację substancji przeciwgrzybowych w ekstraktach świerkowych.

Badano także wpływ zrzucania łusek nasiennych na śmiertelność siewek świerka, zaatakowanych przez *Pythium*. Siewki, które zrzuciły łuskę nasienną były bardziej odporne na zgorzel siewek powodowaną przez *Pythium*. Zjawisko to może być związane z produkcją substancji przeciwgrzybowych w prawidłowo rozwiniętych liściach.

SŁOWA KLUCZOWE: patogeny glebowe, homogenizaty wodne, *Pythium ultimum*, *P. irregulare*, *Picea abies*.