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Defence responses of Norway spruce seedlings to elicitors of ectomycorrhizal fungus *Pisolithus tinctorius* and pathogen *Heterobasidion annosum* are affected by zeatin riboside

Vpliv zeatin ribozida na obrambni odgovor kalic smreke po tretiranju z elicitorji mikorizne glive *Pisolithus tinctorius* in patogena *Heterobasidion annosum*

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Abstract: Cytokinins are known to attenuate defence responses of plants after elicitor application or inoculation with fungi. To evaluate their role in the regulation of colonisation of Norway spruce (*Picea abies*) seedlings with mycorrhizal and pathogenic fungus, we analysed the effects of zeatin riboside (ZR) on: i) growth of ectomycorrhizal fungus *Pisolithus tinctorius* and pathogen *Heterobasidion annosum* in axenic cultures, ii) colonisation intensity of selected fungi on *P. abies* seedlings and iii) induction of defence reactions of spruce seedlings following elicitor treatment. Mycorrhizal fungus *P. tinctorius* showed increased growth at concentrations higher than 10^{-2} µM ZR that was accompanied by increased ergosterol concentrations. In contrast, decreased growth of pathogen *H. annosum* was observed at the highest ZR (10 µM) concentration. ZR treatment also increased colonisation of spruce seedlings with the mycorrhizal fungus. Application of cell wall preparations of both fungi increased peroxidase (POD) activity in the roots of treated spruce seedlings, whereas only elicitors of *H. annosum* increased also phenylalanine ammonia-lyase (PAL) activity, levels of soluble phenolics and salicylic acis (SA) concentrations. Application of ZR negated the increased activity of POD that was observed in elicitor treated seedlings, accompanied by increased levels of soluble phenolics in the roots of elicitated seedlings. In contrast, no effects of ZR treatment on PAL activity and SA accumulation could be observed. Our results suggest involvement of ZR in the regulation of cell wall modifications during the fungal colonisation with *P. tinctorius* and formation of ectomycorrhizae, by affecting the growth of fungal partner and non-specific defence reactions of the plant host.

Keywords: cytokinins, peroxidases, phenolics, phenylalanine ammonia lyase, *Picea abies*

Izvleček: Iz literature je znano, da lahko citokinini vplivajo na obrambne reakcije rastlin, ki se sprožijo po aplikaciji elicitorjev ali ob inokulaciji z živo glivo. Za ovrednotenje njihove vloge pri regulaciji kolonizacije kalic smreke (*Picea abies*) z mikoriznimi in patogenimi glivami, smo preverili vpliv zeatin ribozida (ZR): i) na rast ektomikorizne glive *Pisolithus tintorius* in patogena *Heterobasidion annosum*

v aksenični kulturi, ii) na stopnjo kolonizacije kalic smreke z obema glivama in iii) na aktivacijo obrambnih reakcij smreke po tretiranju z elicitorji. V aksenični kulturi smo pri 10^{-2} µM koncentraciji ZR opazili pospešeno rast mikorizne glive *P. tinctorius*, ki jo je spremljala povečana koncentracija ergosterola v miceliju. Nasprotno je bila rast patogene glive *H. annosum* pri najvišji koncentraciji ZR v gojišču (10 µM) zavrta. Podobno kot v aksenični kulturi je dodaten ZR pospešil kolonizacijo kalic smreke z ektomikorizno glivo, medtem ko na stopnjo kolonizacije s patogenom ni imel učinka. Tretiranje smreke z elicitorji obeh gliv je povečalo aktivnost peroksidaz (POD) v koreninah kalic, samo elicitorji patogene glive *H. annosum* pa so povečali tudi aktivnost fenilalanin amonijeve liaze (PAL) in koncentracijo topnih fenolov ter proste salicilne kisline (SA). Dodatek ZR je znižal peroksidazno aktivnost v kalicah tretiranih z elicitorji obeh gliv in povečal koncentracijo topnih fenolov. Nasprotno, ZR ni imel nobenega vpliva na aktivnost PAL in akumulacijo salicilne kisline v koreninah smreke. Na podlagi naših rezultatov predvidevamo, da je ZR vpleten v regulacijo modifikacij celične stene ob glivni kolonizaciji z ektomikorizno glivo *P. tinctorius* in vzpostavitev ektomikorize, preko delovanja na glivnega partnerja in nespecifične obrambne reakcije gostitelja.

Ključne besede: citokinini, peroksidaze, fenil alanin amonijeva liaza, *Picea abies*

Introduction

Root exudate is a very diverse group of compounds released from the roots to the rhizosphere and contains along with amino acids, organic acids, proteins and sugars also several plant growth regulators, including cytokinins (Neumann and Römhled 2000). Cytokinins play a crucial role in regulating proliferation and differentiation of plant cells, and also control various processes in plant growth and development (Sakakibara 2006; Kyozuka 2007). Furthermore, cytokinins are known to influence the growth of fungi (Barker and Tagu 2000, Nasim and Rehman 2006).

Colonisation of plants by different symbiotic and pathogenic fungi triggers the expression of defence related genes and induces local and systemic host responses (Ryals et al. 1996; Hammerschmidt 1999). An almost ubiquitous feature of plant responses to fungal colonisation or elicitors treatment is the activation of phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) and peroxidases (EC 1.11.1.7), which are associated with phenolic chemistry and cell wall modifications (Chitoor et al. 1997). It was demonstrated that cytokinins can regulate activity and expression of peroxidases (Kadioglu and Durmus 1997, Limam et al. 1998) and can therefore affect the reactions leading to cell-wall reinforcements. When tobacco plants were wounded in the presence of the synthetic cytokinin, benzylaminopurine, production of jasmonic acid (JA) was accompanied by salicylic acid (SA) accumulation (Sano et al. 1996), which is normally not accumulated upon wounding (Pieterse and Van Loon 1999). These results indicated that cytokinins may be involved in accumulation of JA and SA (Sano et al. 1996), which transmits the defence activation signal from the point of infection throughout the plant (Yalpani et al. 1993). Though majority of information concerning the role of PAL, peroxidases and SA in plant defence emanates from research on angiosperms, several studies suggest a similar role in host responses of gymnosperm Norway spruce (Asiegbu et al. 1994, Kozlowski and Metraux 1998, Nagy et al. 2000, Nagy et al. 2004; Likar and Regvar 2008).

In the present study we tested the effects of zeatin riboside (ZR) on the growth of the ectomycorrhizal fungus *Pisolithus tinctorius* and necrotrophic pathogen *Heterobasidion annosum*, as differential effects of cytokinin treatment on growth and colonisation of host roots with both fungal groups (ectomycorrhizal vs. necrotroph) was assumed. Ergosterol content in the fungal mycelia was tested in combination with the fungal growth studies, due to its importance in the formation of sterol rich domains (Xu et al. 2001), which are involved in several important processes

such as endocytosis and hyphal growth (Alvarez et al. 2007). Further, the effects of ZR on the colonisation intensity and induction of defence reactions of spruce seedlings following elicitor treatment were examined, as cytokinins were already proven to affect activity and expression of some enzymes involved in the defence reactions (Kadioglu and Durmus 1997, Limam et al. 1998). Several differences in the spruce seedling responses after colonisation with *P. tinctorius* and *H. annosum* were observed previously (Likar and Regvar 2008) and are assumed to be at least in part influenced by cytokinins.

Material and methods

Effects of zeatin riboside on fungal growth

Cultures of ectomycorrhizal fungus *Pisolithus tinctorius* (Mich.: Pers.) Coker and Couch (isolate DB49) andet al. necrotroph *Heterobasidion annosum* (Fr) Bref. (isolate DB75) are maintained in the fungal collection of Plant Physiology Lab (Biotechnical Faculty, University of Ljubljana). Sequences of ITS-rDNA region of both fungal species can be obtained from GenBank under accession numbers EU559631 and EU559632. Fungal cultures were grown on Melin-Norkrans-Marx (MNM) media (Marx 1969), supplemented with zeatin riboside (ZR) at final concentrations: 0, 10^{-6}, 10^{-4}, 10^{-2}, 1 and 10 µM. Due to different growth rates, growth of the individual fungus was monitored for two weeks in case of *H. annosum* and five weeks in case of *P. tinctorius*, after which mycelia were removed and frozen in liquid nitrogen, prior to freeze-drying (Christ, Alpha 2-4).

Ergosterol analysis

Twenty mg of dried fungal material were used for ergosterol extraction following modified protocol of Martin et al. (1990). For ergosterol quantification, 25 µl of ethanolic supernatant was injected into the high performance liquid chromatography (HPLC) system consisting of Waters 2960 separation module with Waters 996 PDA detector. UV absorbing compounds were separated by reverse-phase chromatography on a 5-µm Spherisorb C18, 250 mm x 4.6 mm column. The mobile phase was 100% methanol at 1 ml min^{-1}. Ergosterol peak was identified by comparison of the retention time (absorbance at 280 nm) and absorbance spectra with the purified ergosterol standard (Nylund et al. 1992).

Elicitation and inoculation experiments

Seeds of Norway spruce (*P. abies* (L.) Karst. prov. Čermošnjice) were surface sterilised in 30% H_2O_2 (105 min), washed and sown on sterile peat: vermiculite (1:3, V/V) mixture supplemented with Knop's medium (Booth 1971). Seedlings were grown in growth chamber (16h of light, 23 °C) for three months, and then transferred to Petri dishes for the elicitation and colonisation experiments.

For the elicitor treatment, fungal cell wall extracts of both fungal species were prepared as described by Salzer et al. (1996). For elicitation of spruce seedlings, elicitors were added to the MNM media at final concentration of 0.1 g l^{-1} (Likar and Regvar, 2008). To half of the seedlings ZR was added to MNM media at final concentration of 10^{-3} µM (determined as optimal for spruce seedling growth prior to experiments), while sterile double distilled water was used for the control. After ten days of growth the seedlings were collected and whole roots excised. Roots were freeze-dried (Christ, Alpha 2–4) and grinded to powder using liquid nitrogen prior to extraction of proteins and soluble phenolics.

For the inoculation experiments three months old spruce seedlings were transferred to petri dishes and covered with semi-circles of cloth without (control) or with the fungi, according to Chilvers et al. (1986). An additional semicircle of cotton soaked with half strength liquid MNM media without sugars was laid over the roots. Inoculated seedlings were grown for one month, after which colonisation of the spruce seedlings was estimated under a stereomicroscope (Leica, MZ8) by counting the short roots that had been entered by the hyphae.

PAL and peroxidase activity

Soluble proteins were extracted in 100mM phosphate buffer (pH 7.0, 1:10 = w/V) as described

by Albrech et al. (1994). Supernatant was used for total protein quantification, as well as PAL and POD assays. A modified Lowry assay (Sandermann and Strominger 1972) with bovine serum albumin as the standard was used for quantification of total soluble proteins prior to enzyme assays. Concentrations of soluble proteins ranged from 1 to 2 mg ml^{-1}.

Peroxidase activity was measured as described by Bashan et al. (1987), using guaiacol as substrate in an assay mixture containing: 25 µl of the protein extract, 0.5 ml 100 mM phosphate buffer, 0.2 ml 1% guaiacol and 0.2 ml 10 mM H_20_2. The enzyme activity was measured by monitoring the increase in absorbance at 470 nm (extinction coefficient of 26.6 mM^{-1} cm^{-1}) during the polymerization of guaiacol into tetraguaiacol (Chance and Maehly 1955).

PAL was assayed spectrophotometrically following a modified method of Khan and Vaidyanathan (1986). The PAL assay was performed at 37 °C for 1 h in an assay mixture containing: 100 µl of the protein extract 450 µl 50 mM TRIS-HCl (pH 8.8) and 25 µl 100 mM L-phenylalanine. The PAL activity was measured by monitoring the increase in absorbance at 290 nm (extinction coefficient of 16.6 mM^{-1} cm^{-1}) during the production of t-cinnamic acid (Gomez-Vasquez et al. 2004).

Total soluble phenolics

Soluble phenolics in the roots of spruce seedlings were extracted subsequently in 90% methanol and 100% methanol (1:25, w/V). Total phenols in combined methanolic extracts were measured according to Marigó (1973). For this, 1 ml of 2% Na_2CO_3 and 75 µl of Folin-Ciocalteau reagent (Kemika, Zagreb) were added to 100 µl of phenolic extract. After 15 minutes of incubation at 25 °C in the dark, the absorbance at 750 nm was measured (HP 8452A spectrophotometer). Catechin (Sigma) was used as a standard.

Analysis of salicylic acid (SA)

SA was extracted following the modified procedure of Raskin et al. (1989). The initial extraction procedure was the same as for the extraction of soluble phenolics. Pooled methanol extracts were dried and resuspended in 0.5 ml 5% (w/V) trichloroacetic acid. After 1 min of mixing by sonification (35kHz, Elma, Transonic 460/H), the extracts were centrifuged for 10 min at 3,000 x *g* (4 °C), and the supernatants were extracted twice with 0.5 ml of ethylacetate: cyclopentane (1:1, V/V). The organic phases were pooled and dried at 40 °C. The dried extracts were resuspended in 1.5 ml 100% methanol and filtered (0.22 µm) before injecting onto the HPLC.

For the analysis of SA, we used the same HPLC system configuration as above with additional flourimetric 474 detector. SA was identified and quantified by monitoring fluorescence at 407 nm (the excitation wavelength was 305 nm). The elution was carried out using: A – 20 mM sodium acetate buffer (pH 5.0) with 0.02% sodium azide (Sigma); and B – 100% methanol (Merck). The gradient used was: 0–4.5 min: 75% A; 4.5–11 min: 75%-30% A; 11-15 min: 30% A, at a flow rate of 1 ml min^{-1}, with 100 µl of sample injected per run.

Statistical analysis

Experiments were repeated three times. As similar trends were observed in all experiments represented data is from a single experiment.

Effects of elicitor and ZR treatment were determined by analysis of variance according to general linear model procedure. Differences among various treatment means were separated by Holm-Sidak post hoc test, while effects of ZR treatment on colonisation were evaluated using t-test. All analyses were performed at the 0.05 level of probability in SigmaStat (SPSS).

Results

Growth and ergosterol content of fungi in axenic cultures

ZR treatment increased colony diameter and ergosterol concentration of mycorrhizal fungus *P. tinctorius* at concentrations higher than 10^{-2} µM ZR (Fig 1). In contrast, pathogen *H. annosum* showed a decrease in colony diameter at the highest ZR (10 µM ZR) concentration. Ergosterol concentrations in the mycelia of *H. annosum* showed only

marginal effects of ZR, as we could observe an increase in the ergosterol concentration only at 10^{-2} µM concentration of ZR.

Fungal colonisation

One month after inoculation, colonisation of spruce seedlings with ectomycorrhizal fungus *P. tinctorius* reached 40.2% ±5.3%, while pathogen *H. annosum* colonised 58.9%±6.7% of the root tips. ZR treatment of spruce seedlings significantly increased (t-test, $p < 0.05$) the colonisation with mycorrhizal fungus *P. tinctorius* to 73.4% ±9.0% (a 82% increase in colonisation levels). No effects of ZR treatment on the colonisation of spruce seedlings with the pathogen *H. annosum* were observed (colonisation of ZR treated seedlings was 63.0%±6.9%).

Enzyme activity and phenolics

Only treatment with elicitors from *H. annosum* increased the PAL activity in the roots of the treated seedlings (Fig. 2a), whereas no effects of elicitors of *P. tinctorius* or ZR on the PAL activity could be observed.

Treatment with elicitors from both fungi induced POD activity in the roots of the treated seedlings (Fig. 2b). ZR treatment decreased POD activity in seedlings treated with elicitors from both fungi, whereas no effects of ZR on POD activity were observed in the control seedlings.

Treatment with elicitors of necrotroph *H. annosum* increased root levels of soluble phenolics (Fig. 2c), whereas no increase in accumulation could be observed in seedlings treated with elicitors of *P. tinctorius*. ZR increased the levels of soluble phenolics for 20% in the roots of the seedlings treated with elicitors of *H. annosum* and for 28% in the seedlings treated with elicitors of *P. tinctorius*, but had no effect on the levels of soluble phenolics in the control seedlings.

Treatment with elicitors of *H. annosum* increased also the root levels of free SA to 1.6 times the levels of the control (Fig. 2d), whereas elicitors of *P. tinctorius* had no effect on the root SA levels. Similarly to PAL activity, ZR treatment did not affect the SA concentrations in the elicitor treated nor control seedlings.

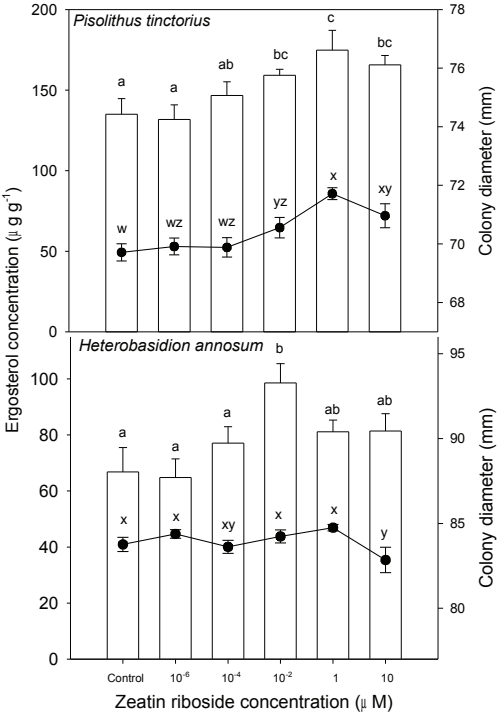

Figure 1: Ergosterol concentration (bars) and colony diameter (●) of *Pisolithus tinctorius* and *Heterobasidion annosum* treated with zeatin riboside at 10^{-6} to 10 µM concentration (Means±SE, n = 10 for colony diameter and n = 6 for ergosterol concentrations). Letters depict statistically significant difference of one-way ANOVA and Holm-Sidak post hoc test at $p < 0.05$.

Slika 1: Koncentracija ergosterola (stolpci) in premer kolonije (●) gliv *Pisolithus tinctorius* in *Heterobasidion annosum* rastočih na gojišču z 10^{-6} µM – 10 µM koncentracijo zeatin ribozida (SV±SN, n = 10 za premer kolonij in n = 6 za koncentracije ergosterola). Črke predstavljajo statistično značilno razliko testa enosmerne ANOVA in Holm-Sidak post hoc testa pri $p < 0,05$.

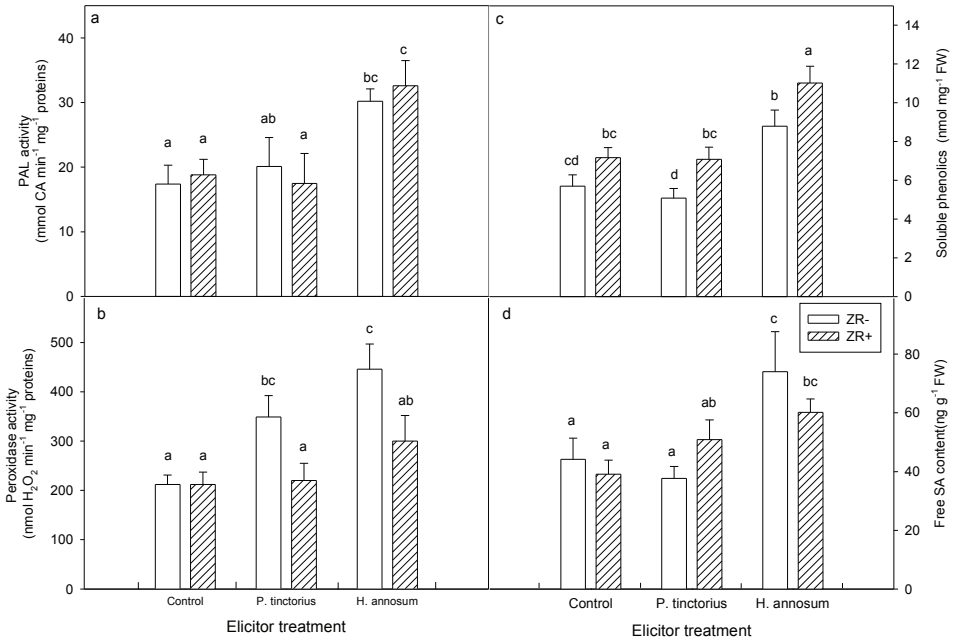

Figure 2: Impact of elicitors of *Pisolithus tinctorius* or *Heterobasidion annosum* and zeatin riboside treatment on: a) phenylalanine ammonia lyase activity, b) peroxidase activity, c) soluble phenolics and d) free salicylic acid concentrations in roots of the spruce seedlings (Mean±SE, n = 10). Letters depict statistically significant difference of one-way ANOVA and Holm-Sidak post hoc test at $p < 0.05$.

Slika 2: Vpliv tretiranja z elicitorji gliv *Pisolithus tinctorius* ali *Heterobasidion annosum* in zeatin ribozidom na: a) aktivnost fenilalanin amonijeve liaze, b) peroksidazna aktivnost, c) koncentracija topnih fenolov in d) koncentracija proste salicilne kisline v koreninah kalic smreke (SV±SN, n = 10). Črke predstavljajo statistično značilno razliko testa enosmerne ANOVA in Holm-Sidak post hoc testa pri $p < 0,05$.

Discussion

ZR treatment increased mycelial growth of ectomycorrhizal fungus *P. tinctorius* at concentrations, similar to the concentrations found in soil extracts (Van Staden and

Dimalla 1976) and *in vitro* cultures of mycorrhizal fungi (Wullschleger and

Reid 1990, Kovac and Žel 1995). Stimulating effects of cytokinins on growth of mycelia of ectomycorrhizal fungi were observed also by other authors (Gogala and Pohleven 1976, Pohleven 1988). Pohleven (1988) also observed increased fluidity of the plasma membranes of the hyphae of *Suillus variegatus* after cytokinin treatment, which is believed to contribute to increased growth of fungal mycelia. In our experiments ZR mediated increase in growth of *P. tinctorius* was accompanied by increased ergosterol concentrations. Ergosterol is an important constituent of the lipid rafts and sterol rich domains (SRD), which have been implicated in fungi in important processes such as endocytosis, virulence and hyphal growth (Alvarez et al. 2007; Steinberg et al. 2007). Due to the importance of ergosterol as a constituent of SRD and its high ability to promote the formation of SRDs (Xu et al. 2001), observed changes in ergosterol concentrations could be connected to morphological and physiological changes observed by Martin et al. (2001) and increased radial growth observed in our experiments. Positive effects on radial growth of ectomycorrhizal fungus *P. tinctorius* could support the importance of cytokinins in the regulation of the formation of the mycorrhiza, as was already pointed out by several authors (Gogala 1991; Barker and Tagu 2000;

Martin et al. 2001). Indeed in our experiments increased colonisation with mycorrhizal fungus was observed in ZR treated spruce seedlings. In experiments of Martin et al. (2001) treatment with zeatin changed the morphology of the hyphae of *P. tinctorius* and triggered enhanced accumulation of hyphaphorine, which plays an important role in the formation of the ectomycorrhizal root (Ditengou et al. 2000). Other studies have shown that cytokinins are able to supress host cell death in infected tissues, thereby allowing fungal development and growth within healthy tissue (Murphy et al. 1997). Furthermore it was confirmed that biotrophic and hemibiotrophic fungal pathogens are able to release the active forms of cytokinins from the pool of inactive *O*-glucosides (Cooper and Ashby 1998), thus influencing the host balance of growth regulators (Walters and McRoberts 2006). In contrast to biotrophic and hemibiotrophic fungal pathogens, no production of cytokinins or O-glucoside cleaving enzymes was observed in fungal necrotrophs (Cooper and Ashby 1998). In our experiments the colonisation levels of spruce seedlings with *H. annosum* were not affected by the applied ZR. Furthermore ZR treated *H. annosum* showed a decreased in colony growth at higher ZR concentrations, which seems to apply also to other necrotrophic fungi (Michniewicz et al. 1984).

Treatment with elicitors from both fungi increased content of soluble proteins and POD activity in the roots of spruce seedlings, whereas increased PAL activity, accompanied by accumulation of soluble phenolics and free SA, was observed only in seedlings treated with elicitors of *H. annosum*. Increased PAL and POD activity is often reported for Norway spruce treated with elicitors or after inoculation with ectomycorrhizal and pathogenic fungi (Asiegbu et al. 1994; Mensen et al. 1998; Nagy et al. 2004, Likar and Regvar, 2008) and is associated with accumulation of phenolics with function in wall strengthening (Hammerschmidt 1999). In Norway spruce seedlings the inoculation with *H. annosum* and *P. tinctorius* increased activity of guiaicol POD and ferulic acid POD and induced expression of new POD isoforms (Likar and Regvar 2008). Increased POD activity and induction of POD isoforms was observed also after treatment with the cell preparations of both fungi, suggesting increased cell-wall strengthening (Cahill and McComb 1992), and possibly formation of POD-generated fungitoxic compounds. Inhibitory effects of ZR on the POD activity observed in our present experiment, could thus severely affect the response of *P. abies* seedling to the colonisation by the tested fungi. Indeed, Beckman and Ingram (1994) showed that exogenous kinetin is able to inhibit hypersensitive response in potato inoculated with *Phytophtora infestans*, which could be due to inhibition of the apoplastic peroxidases (Bolwell et al. 2002). Cytokinins are known to regulate the expression of acid peroxidases (Limam et al. 1998), which play an important role in cell wall strengthening (Chitoor et al. 1997; Chitoor et al. 1999). Increased levels of soluble phenolics in ZR treated spruce seedlings in our experiments in combination with decreased POD activity could suggest accumulation of phenolic precursors destined for polymerisation in the cell wall, thus leading to improved conditions for fungal penetration and formation of ectomycorrhizae. Production of cytokinins in several ectomycorrhizal fungi (Kraigher et al. 1991) and improved mycorrhization of Norway spruce seedling after treatment wit ZR as observed by Gabrovšek and Gogala (1995) could support this hypothesis.

In contrast to POD activity, PAL activity and free SA accumulation, was increased only in seedling treated with *H. annosum* cell wall preparation. In addition to synthesis of precursors of several cell wall compounds, PAL can also play an important role in SA synthesis (Mauch-Mani and Slusarenko 1996) and as such in spreading the defence activation signal throughout the plant (Ryals et al. 1996). A positive correlation between SA and PAL activity, together with the accumulation of a SA precursors in the phenylpropanoid pathway was seen in *H. annosum*-inoculated spruce seedlings (Likar and Regvar 2008). As the synthesis of SA through the PAL pathway is linked to plant-cell death (Wildermuth et al. 2001), it was suggested that *H. annosum* exploits the plant-cell death for facilitation of its infection (Likar and Regvar 2008), as was observed also for *Botrytis cinerea-Arabidopsis thaliana* interactions (Govrin and Levine 2000). In our experiments, ZR treatment did not affect either PAL activity or free SA accumulation after the treatment with the elicitors and thus had no effect

on the induction of hypersensitive reaction. Based on our observations and the absence of cytokinin production in necrotrophic fungal pathogens, we assume that in contrast to biotrophic fungi, the cytokinins are not involved in the pathogenesis of the necrotrophs.

In conclusion, stimulating effects of ZR on *P. tinctorius* (growth, ergosterol concentrations) and improved colonisation of spruce seedlings, suggest that cytokinins can successfully alter the growth and colonisation success of mycorrhizal fungal symbiont. Effects of ZR on cell-wall strengthening responses after elicitation with elicitors of both fungi point to involvement of cytokinins in non-specific defence responses. In combination cytokinins could play an important role in regulation of cell wall modifications during the fungal colonisation and formation of ectomycorrhizae, by simultaneously affecting fungal growth and physiology, as well as the non-specific defence reactions of the plant host. In comparison with the ectomycorrhizal fungus *P. tinctorius*, elicitors of necrotroph *H. annosum* activated also PAL activity and the SA-dependant signal pathway, which showed no ZR-induced changes. Growth of the fungus in axenic culture and colonisation of spruce seedlings with the pathogen were not improved as in the case of the ectomycorrhizal fungus, suggesting a minor role of cytokinins in the pathogenesis of *H. annosum* on spruce.

Povzetek

Rastline s korenin sproščajo kompleksno mešanico spojin, ki tvorijo koreninski eksudat. Pole aminokislin, organskih kislin, proteinov in sladkorje, koreninski eksudat vsebuje tudi številne rastne regulatorje kot so npr. citokinini (Neumann in Römheld 2000). Citokinini igrajo pomembno vlogo pri regulaciji številnih procesov tekom rasti in razvoja rastlin (Sakakibara 2006, Kyozuka 2007), hkrati pa lahko vplivajo tudi na rast gliv (Barker in Tagu 2000, Nasim in Rehman 2006).

Iz literature je znano, da lahko citokinini vplivajo tudi na obrambne reakcije rastlin, ki se sprožijo po aplikaciji elicitorjev ali ob inokulaciji z živo glivo (Kadioglu in Durmus 1997, Limam et al. 1998, Sano et al. 1996). V pričujoči raziskavi smo, za ovrednotenje njihove vloge pri regulaciji kolonizacije kalic smreke (*Picea abies*) z mikoriznimi in patogenimi glivami, preverili vpliv zeatin ribozida (ZR): i) na rast ektomikorizne glive *Pisolithus tintorius* in patogena *Heterobasidion annosum* v aksenični kulturi, ii) na stopnjo kolonizacije kalic smreke z obema glivama in iii) na aktivacijo obrambnih reakcij smreke po tretiranju z elicitorji.

V aksenični kulturi smo pri 10^{-2} µM koncentraciji ZR opazili pospešeno rast mikorizne glive *P. tinctorius*, ki jo je spremljala povečana koncentracija ergosterola v miceliju. Nasprotno je bila rast patogene glive *H. annosum* pri najvišji koncentraciji ZR v gojišču (10 µM) zavrta. Podobno kot v aksenični kulturi je dodaten ZR pospešil kolonizacijo kalic smreke z ektomikorizno glivo, medtem ko na stopnjo kolonizacije s patogenom ni imel učinka. Tretiranje smreke z elicitorji obeh gliv je povečalo aktivnost peroksidaz (POD) v koreninah kalic, samo elicitorji patogene glive *H. annosum* pa so povečali tudi aktivnost fenilalanin amonijeve liaze (PAL) in koncentracijo topnih fenolov ter proste salicilne kisline (SA). Dodatek ZR je znižal peroksidazno aktivnost v kalicah tretiranih z elicitorji obeh gliv in povečal koncentracijo topnih fenolov.

Na podlagi naših rezultatov predvidevamo, da je ZR vpleten v regulacijo modifikacij celične stene ob glivni kolonizaciji z ektomikorizno glivo *P. tinctorius* in vzpostavitev ektomikorize, preko delovanja na glivnega partnerja in nespecifične obrambne reakcije gostitelja.

Povečana aktivnost PAL in aktivacija od SA odvisne signalne poti sta se izkazali za neobčutljive za dodatek ZR. Podobno dodan ZR ni imel pozitivnega vpliva na rast nekrotrofa *H. annosum* v aksenični kulturi ali na stopnjo kolonizacije kalic smreke, kar nakazuje, da imajo citokinini zanemarljivo vlogo pri patogenezi smreke s tem patogenom.

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