Mycorrhiza (2001) 11:101–106

ORIGINAL PAPER

Stefanie Schröder · Ulrich Hildebrandt
Hermann Bothe · Karsten Niehaus

Suppression of an elicitor-induced oxidative burst reaction in *Nicotiana tabacum* and *Medicago sativa* cell cultures by corticrocin but not by mycorradicin

Accepted: 20 March 2001

Abstract The biological activities of mycorradicin, the major component of the yellow pigment formed in maize and other grasses upon colonization by arbuscular mycorrhizal fungi, and corticrocin from the ectomycorrhizal fungus *Piloderma croceum* were analysed in cell cultures of tobacco (*Nicotiana tabacum* L.) and alfalfa (*Medicago sativa* L.). Tobacco and alfalfa suspension cell cultures react to elicitor treatment by alkalinization of the culture medium and generation of activated oxygen species, the so-called oxidative burst. In the present study, the addition of corticrocin suppressed the elicitor-induced oxidative burst reaction but not the alkalinization. The suppression of the oxidative burst by corticrocin was dose dependent. Mycorradicin in either its methylated or free form had no effect on the oxidative burst or the alkalinization.

Keywords Corticrocin · Mycorrhiza · Mycorradicin · Oxidative burst · Suppression

Introduction

In their natural habitats, plants are confronted with many different organisms, such as mycoplasms, bacteria, fungi, nematodes, insects and other herbivorous animals. The interaction between plants and other species may reduce the fitness of the host plant in the case of a pathogenic relationship or can be beneficial to both partners in a symbiosis. More than 80% of terrestrial plants form symbioses with mycorrhizal fungi, particularly under stress conditions (Bonfante and Perotto 1995; Perotto and Bonfante 1997). During the last decade, evidence has accumulated that not only pathogenic but also symbiotic microbes are subjected to the defence systems of their host plants. Mutants of the symbiotic soil bacterium *Sinorhizobium meliloti* that fail to produce extracellular polysaccharides induce the plant defence system and cannot infect the host plant alfalfa (Niehaus et al. 1993). Levels of phenylalanine ammonia-lyase and chalcone-synthase transcripts increased during the colonization of *Medicago truncatula* by the arbuscular mycorrhizal fungus *Glomus versiforme* (Harrison and Dixon 1994), consistent with increased flavonoid biosynthesis. The level of the phytoalexin medicarpin increased only transiently during the early stages of colonization. Interestingly, isoflavone transcripts encoding an enzyme specific for medicarpin biosynthesis decreased below the control levels in roots during later stages of the interaction (Harrison and Dixon 1994). Similar findings in the *Medicago sativa–Glomus intraradices* symbiosis indicate that plant defence reactions are induced during the early colonization of plant roots and are subsequently suppressed during the established symbiosis (Volpin et al. 1995). However, phenolic and flavonoid compounds may also stimulate the growth of some fungal species or induce specific developmental steps such as branching of the invading hyphae (Tsai and Phillips 1991; Kape et al. 1993; Giovannetti et al. 1996). In the case of ectomycorrhiza, chitin oligosaccharides arising from the fungal cell wall can stimulate defence reactions, while this defence-stimulating signal is inactivated by chitinases during the establishment of the symbiosis (Salzer et al. 1996, 1997). Pathogenesis-related (PR) proteins, widely used as markers for the onset of defence reactions, are only weakly induced in mycorrhizal symbioses. At the stage of highest fungal activity, no dramatic changes were observed in the expression of the *PR1* gene in the parsley–*Glomus* interaction (Franken and Gnädinger 1994). In tobacco, the *PRb1* gene was found to be weakly activated in mycorrhizal roots and the protein was located in the interfacial matrix of the arbuscules (Gianinazzi-Pearson et al. 1992). Direct histochemical evidence for a local defence reaction against the invading fungi was

S. Schröder · K. Niehaus (✉)
University of Bielefeld, Faculty of Biology, Genetics,
P. O. Box 100131, 33501 Bielefeld, Germany
e-mail: kniehaus@genetik.uni-bielefeld.de
Fax: +49-521-1065626

U. Hildebrandt · H. Bothe
University of Cologne, Institute of Botany, Gyrhofstrasse 15,
50923 Cologne, Germany

found in the form of activated oxygen species (AOS). Salzer et al. (1999) showed a local oxidative burst in areas of direct contact between the fungal hyphae and the plant cell wall. Accumulation of hydrogen peroxide was pronounced in cells containing clumped and less-branched arbuscules and around hyphal tips attempting to penetrate a host cell. In contrast, no hydrogen peroxide accumulation was observed in hyphal tips growing along the middle lamella or in appressoria or vesicles. Based on these observations, the authors suggest that a locally restricted oxidative burst is involved in the temporal and spatial control of the intracellular colonization of *Medicago truncatula* cells by *Glomus intraradices*.

Mycorrhizal roots of many plants show a characteristic yellow colour (Jones 1924) due to the carotenoid mycorradicin, which consists of 14 carbon atoms including two terminal carboxylic groups (Klingner et al. 1995a). The ectomycorrhizal fungus *Piloderma croceum* forms corticrocin, which is also a 14-carbon polyene with two terminal carboxyl groups but no side-chain methyl groups. This pigment was purified and identified as all-E-tetradeca-2,4,6,8,10,12,-hexaene-1,14-dioic acid (Erdtman 1948; Schreiner et al. 1998). The highly unsaturated nature of these compounds makes them candidates for scavengers of AOS, probably protecting the invading microbe from oxidative stress. To test whether these yellow pigments fulfil such a function, elicitor-treated tobacco cell cultures were used as a test system in the present investigation.

Materials and methods

Mycorradicin and corticrocin

Mycorradicin, mycorradicin dimethylester and corticrocin were isolated or synthesized as described (Schreiner et al. 1998) and the identity of all compounds was verified by HPLC and NMR.

Preparation of the yeast elicitor

The yeast elicitor (YE) was prepared as described by Baier et al. (1999). Briefly, baker's yeast was stirred for 30 min in 1.5 l citrate buffer (20 mM, pH 7.5). After autoclaving (121°C, 1.2 bar), the preparation was centrifuged (20 min, 7,000 *g*). The supernatant was filtered and 1 volume EtOH (96% v/v) was added. After stirring overnight at 4°C, the precipitate was collected by centrifugation. The pellet was dissolved in water for approximately 12 h, centrifuged (30 min), and the supernatant freeze-dried. Approximately 10 g of crude YE was obtained from 1 kg of baker's yeast. The YE fraction was dialysed (Spectra-Pore MWCO 1000, Serva) prior to use.

Growth conditions for tobacco and alfalfa cell cultures

The cell suspension cultures used were from the tobacco variety Petit Havanna SR1 (Maliga et al. 1973) and the alfalfa variety Du Puits (Baier et al. 1999). Cells were cultured in MS medium (Murashige and Skoog 1962) supplemented with 2,4-dichlorophenoxyacetic acid (1 mg l^{-1}) and kinetin (0.1 mg l^{-1}) at 24°C on a rotary shaker (Certomat R, B. Braun, 115 rpm) in the dark. Suspension cultures were subcultured at intervals of 10 days into 15 ml fresh medium in 100-ml Erlenmeyer flasks with aluminium caps. In alkalinization experiments, the pH of the medium was continuously monitored by a glass pH electrode (Baier et al. 1999).

Detection of hydrogen peroxide in plant cell cultures

Hydrogen peroxide was determined by peroxide-dependent chemoluminescence of luminol (Warm and Laties 1982). Three to 5 days after transfer to fresh medium, cell suspension cultures of tobacco were diluted in preincubation medium (3% sucrose in 0.4× MS medium) to a final concentration of 250 mg cell mass per ml medium and incubated on an orbital shaker for 3–5 h. An aliquot of this suspension (200 µl) was mixed with 700 µl of 50 mM potassium phosphate buffer (pH 7.9) in a chemoluminescence reaction vial. After placing the vial in the sample chamber of the luminometer (1250; BioOrbit, Turku, Finland), 100 µl of 1.2 mM luminol in potassium phosphate buffer (pH 7.9) was added and the reaction started by adding 100 µl of 10 mM potassium ferricyanide.

Results

An assay system for the determination of AOS in tobacco cell suspension cultures was used to test whether mycorradicin or corticrocin affect plant defence reactions. AOS production following treatment with YE was first determined to ensure the elicitor-responsiveness of the cell cultures (Fig. 1). A transient increase in hydrogen peroxide was observed after the addition of 200 µg ml^{-1} YE to the cells. This oxidative burst was measurable 5 min after addition of the elicitor, reached its maximum after about 20 min and decayed to near basal level within 45 min (Fig. 1). In order to analyse the mode of AOS generation during the oxidative burst, alfalfa and tobacco cell cultures were treated with diphenylene iodonium (DPI) to block the AOS-producing enzyme NAD(P)H oxidase. The oxidative burst induced by YE was inhibited when the cell cultures were incubated with 7.5 µM DPI for 30 min prior to the addition of YE (data not shown).

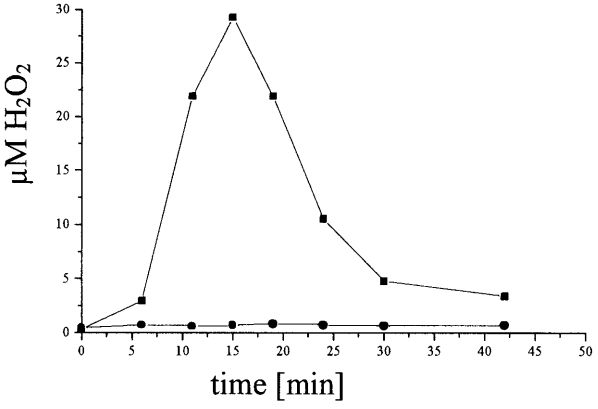

Fig. 1 Release of activated oxygen species into the medium of tobacco cell suspension cultures induced by a yeast elicitor (YE) fraction. The activated oxygen species were monitored in the supernatant by luminol-induced luminescence and are expressed as equivalents of hydrogen peroxide (● control; ■ 200 µg/ml YE)

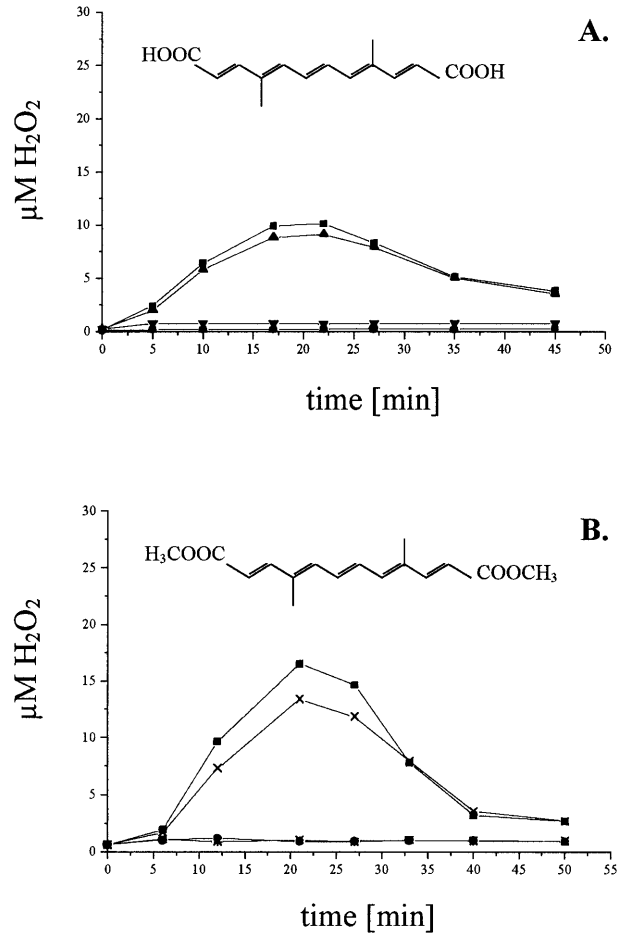

Fig. 2 Induction of the oxidative burst in tobacco cell cultures by YE in the presence of mycorradicin in its free (**A**) or methylated (**B**) form as measured by the generation of hydrogen peroxide after application of YE, or mycorradicin alone or in combination. The structures of free and methylated mycorradicin are shown [■ 200 µg ml^{-1} YE, ● 30 µg ml^{-1} mycorradicin (*A*) or mycorradicin methyl ester (*B*), ▲ 30 µg ml^{-1} mycorradicin together with 200 µg ml^{-1} YE, ✕ 30 µg ml^{-1} mycorradicin methyl ester together with 200 µg ml^{-1} YE, ▼ control]

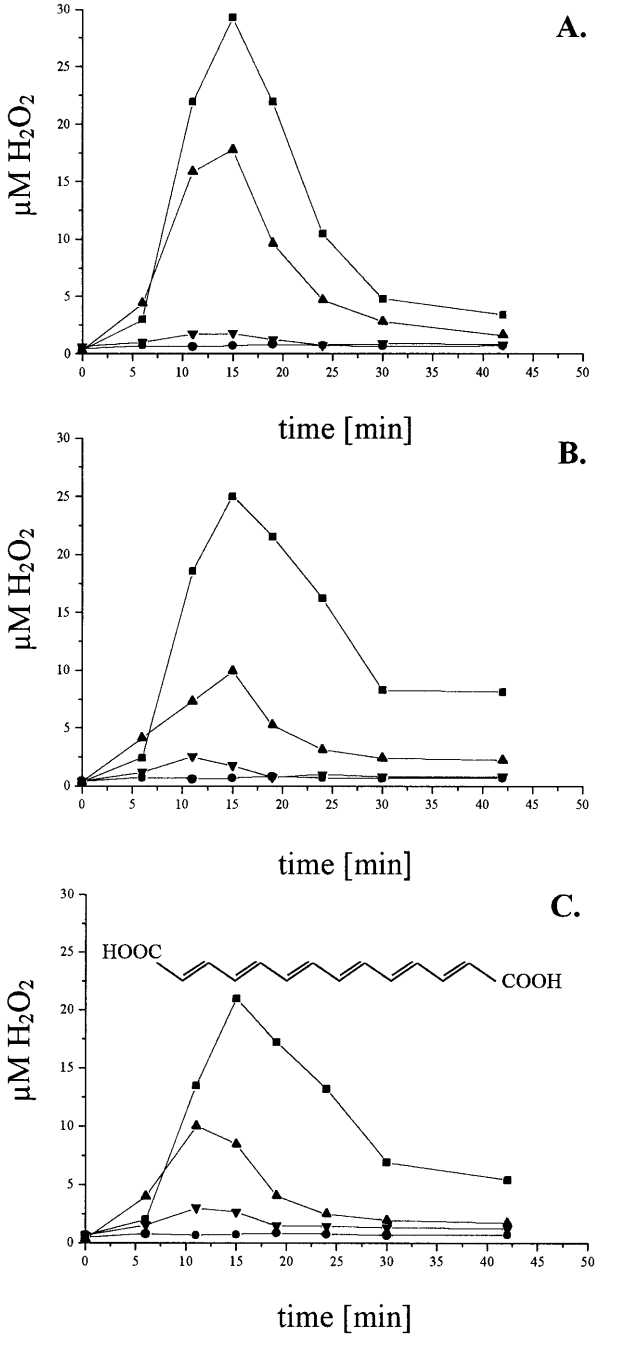

Fig. 3A–C Suppression of the elicitor-induced oxidative burst reaction in tobacco cell suspension cultures by corticrocin. The generation of peroxide was analysed after application of YE or corticrocin alone or in combination (■ 200 µg ml^{-1} YE alone, ▼ 30 µg ml^{-1} corticrocin alone, ▲ corticrocin at 10 µg ml^{-1} (**A**), 20 µg ml^{-1} (**B**), or 30 µg ml^{-1} (**C**) added together with with 200 µg ml^{-1} YE, ● cell cultures supplemented with 100 µl growth medium as a control)

The tobacco cell cultures were treated with YE plus either mycorradicin (Fig. 2A), its methyl ester (Fig. 2B) or corticrocin (Fig. 3C). All compounds were dissolved in dimethyl sulfoxide (DMSO) because of their hydrophobic nature. Control treatments showed that up to 0.5% DMSO was tolerated by the cell cultures without severe effects on the elicitor-induced oxidative burst. Mycorradicin (30 µg ml^{-1}) or its methyl ester, dissolved in 30 µl DMSO, together with 200 µg ml^{-1} YE did not alter the oxidative burst generated by 200 µg ml^{-1} YE alone (Fig. 2). However, the addition of corticrocin simultaneously with 200 µg ml^{-1} YE resulted in a significant suppression of the elicitor-induced oxidative burst (Fig. 3). The tobacco cell suspension cultures responded to corticrocin in a dose-dependent manner. Corticrocin reduced the elicitor-induced oxidative burst at a concentration as low as 10 µg ml^{-1} (approximately 40 µM). The corticrocin effect was concentration dependent up to 30 µg ml^{-1}. While the course of the hydrogen peroxide outburst curve was dependent on the amount of corticrocin added to the cell cultures, the onset and termination of peroxide formation was concentration independent (Fig. 3). Mycorradicin, its methyl ester or corticrocin alone had no effect on AOS production by tobacco

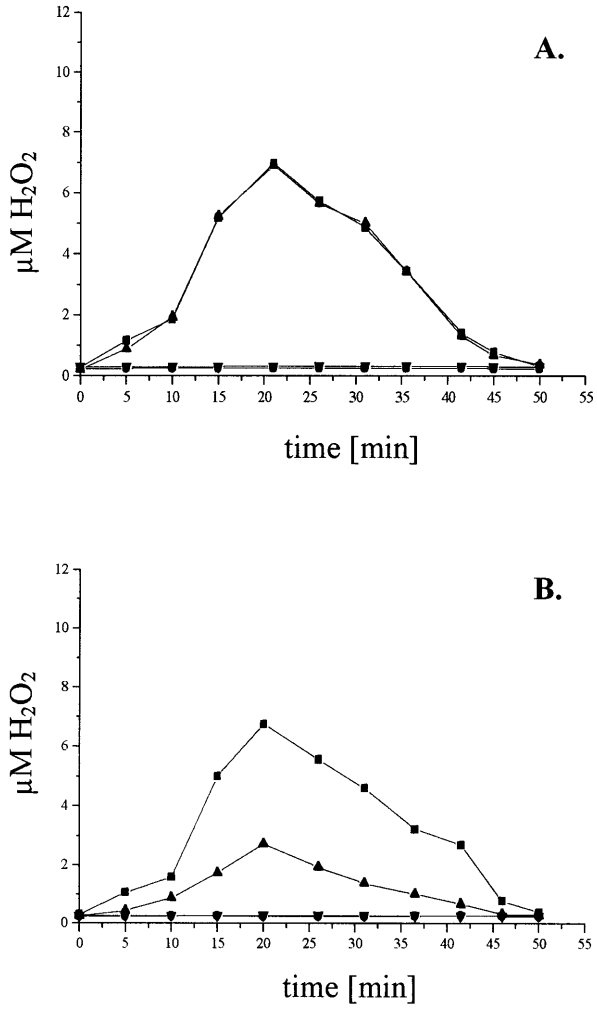

Fig. 4 Induction of the oxidative burst in alfalfa cell cultures by YE in the presence of mycorradicin (**A**) or corticrocin (**B**). Generation of hydrogen peroxide in alfalfa cell cultures was analysed after application of mycorradicin or corticrocin alone or each in combination with YE [■ 200 µg ml^{-1} YE, ● 30 µg ml^{-1} mycorradicin (A) or corticrocin (B), ▲ 30 µg ml^{-1} mycorradicin (A) or corticrocin (B) in combination with 200 µg ml^{-1} YE, ▼ 10 µL DMSO as a control]

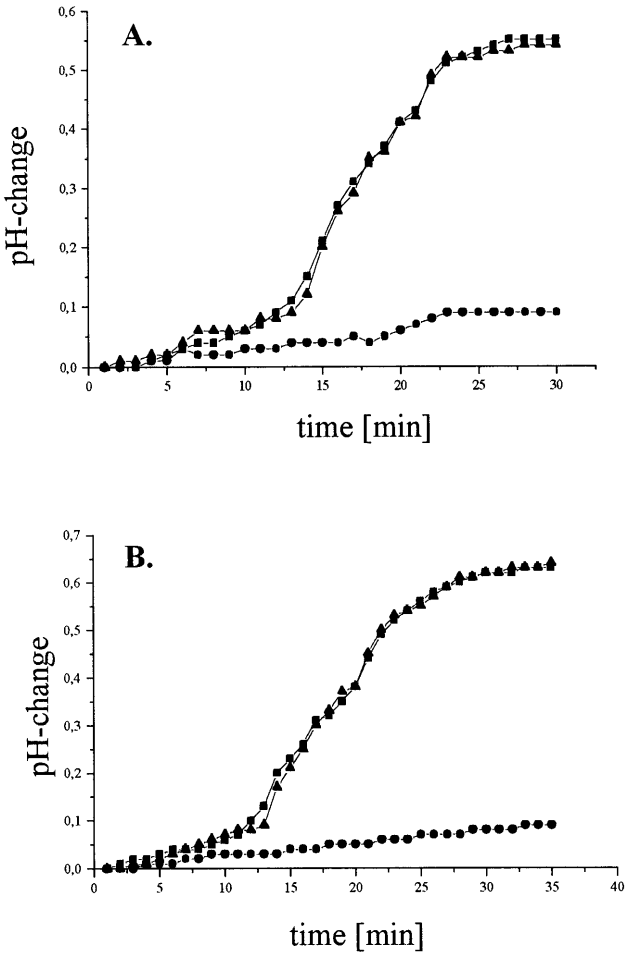

Fig. 5 Elicitor-induced alkalinization reaction in tobacco (**A**) and alfalfa (**B**) cell cultures in the presence of corticrocin (■ 400 µg ml^{-1} YE, ● 30 µg ml^{-1} corticrocin, ▲ both substances together)

cell cultures (Figs. 2, 3). In summary, only corticrocin suppressed the elicitor-induced oxidative burst in tobacco cell cultures.

To determine whether suppression of the elicitor-induced oxidative burst by corticrocin is specific to tobacco cell cultures, the experiments repeated using alfalfa cell cultures. As in tobacco, AOS production by alfalfa cells upon treatment with YE was first determined to ensure elicitor responsiveness (Fig. 4A). A transient increase in peroxide was observed after the addition of 200 µg ml^{-1} YE to alfalfa cell cultures. In contrast to tobacco, maximal hydrogen peroxide production by alfalfa was only 7 µM, although the kinetics of the reaction were comparable. Treatment of alfalfa cell cultures with 30 µg ml^{-1} mycorradicin did not alter the oxidative burst generated by 200 µg ml^{-1} YE alone (Fig. 4A). In contrast, the addition of 30 µg ml^{-1} corticrocin simultaneously with 200 µg ml^{-1} YE led to a significant suppression of the induced oxidative burst (Fig. 4B). Preincubation (30 min) of the cell cultures with 30 µg ml^{-1} corticrocin resulted in the same degree of suppression (data not shown). Thus, suppression of the elicitor induced oxidative burst by corticrocin is not cell culture or plant species specific.

The suppression of elicitor-induced AOS production in tobacco and alfalfa cells by corticrocin may involve changes in signal transduction in the plant cells and/or corticrocin may act as a scavenger of AOS. To distinguish between these possibilities, the elicitor-induced alkalinization of the cell culture medium (Felix et al. 1999) was used as an early indicator of signal transduction events. YE at low concentrations induced a significant alkalinization of the medium of tobacco and alfalfa cell suspension cultures. Normally, these cell suspension cultures showed a pH range of 5.3–5.8, depending on growth and age after subculture (Fig. 5). The addition of 400 µg ml^{-1} YE caused an alkalinization of the medium with a maximal change in pH (ΔpH_{max}) of 0.57 after 24 min in tobacco (Fig. 5A) and 0.62 after 30 min in

alfalfa (Fig. 5B). The elicitor-induced alkalinization in either cell culture was not affected by the addition of up to 30 µg ml^{-1} corticrocin (Fig. 5) or by mycorradicin or its methyl ester (data not shown).

Discussion

It is somewhat surprising that corticrocin but not mycorradicin (either in its free or methylated form) suppressed the elicitor-induced oxidative burst in cell cultures as these two pigments appear to be similar at first glance. Both are polyenes with 14 C-atoms and with carboxylic acids at both ends. However, they are otherwise very dissimilar. Mycorradicin is most likely a degradation product of 40-C carotenoids synthesized via the recently discovered methylerythritol phosphate pathway of isoprenoids (Walter et al. 2000). The two rings of the carotenoids (xanthophylls) are split to two C_{13} intermediates which are glycosylated to form blumenin and related compounds (Maier et al. 1997); the remaining 14 C-atoms constitute mycorradicin (Klingner et al. 1995a; Walter et al. 2000). As side-chain methyl groups cannot (easily) be split off from a polyene, corticrocin may be synthesized as acetogenin. In line with this, the formation of corticrocin in *P. croceum* has been shown to be inhibited by light (Schreiner et al. 1998), in contrast to carotenoid biosynthesis. Mycorradicin is deposited into the plant vacuole (Klingner et al. 1995b) and is thus spatially separated from the fungal partner. In contrast, corticrocin is synthesized by *P. croceum* independent of the host and is deposited outside the fungal cytoplasm (Schreiner et al. 1998). Microscopically, corticrocin granules on the hyphal cell surface form plaques covering distinct patches of the fungal cell. Their different cytological localization is consistent with their biological activities. Only corticrocin on the surface of the fungus but not mycorradicin inside the plant cell can directly affect the peroxide outburst of the plant cells. As shown by Salzer et al. (1999), localized oxidative-burst reactions are observed during infection of the host plant by mycorrhizal fungi. Corticrocin probably serves the fungal partner as a defence against oxidative stress.

The specificity of the suppression of the elicitor-induced AOS production is striking. A direct effect of corticrocin on NADPH oxidase, an enzyme involved in hydrogen peroxide generation, or peroxidase is possible. In comparison, direct scavenging of radicals by corticrocin is unlikely, since mycorradicin with almost the same content of p-bonds would also be active. More likely, a component of the signal transduction chain in tobacco and alfalfa cells responsible for the initiation of AOS production recognizes corticrocin, but mycorradicin with its two side-chain methyl groups and subsequently shorter chain length is not recognized. Elicitor-induced alkalinization of cell cultures is considered to be an indicator of elicitor perception and early signal transduction events (Felix et al. 1999). Inhibition of ion fluxes leading to the alkalinization reaction also resulted in inhibition of the oxidative burst (Woitaszek 1997), indicating that alkalinization precedes the oxidative burst. Neither corticrocin nor mycorradicin had an effect on elicitor-induced alkalinization. The results thus suggest that corticrocin interferes with a compound of the elicitor-induced signal transduction cascade which is specific for the onset of the oxidative burst.

Acknowledgements The authors are indebted to Professor F-J. Marner, Institute of Biochemistry, The University of Cologne, for supplying chemically synthesized mycorradicin and corticrocin. This work was kindly supported by grants to both K. N. and H. B. from the Deutsche Forschungsgemeinschaft within the priority program “Molekulare Grundlagen der Mykorrhiza-Symbiosen”.

References

Baier R, Schiene K, Kohring B, Flaschel E, Niehaus K (1999) Alfalfa and tobacco cells react differently to chitin oligosaccharides and *Sinorhizobium meliloti* nodulation factors. Planta 210:157–164

Bonfante P, Perotto S (1995) Strategies of arbuscular mycorrhizal fungi when infecting host plants. Tansley Review No. 82. New Phytol 130:3–21

Erdtman H (1948) Corticrocin, a pigment from the mycelium of a mycorrhiza fungus II. Act Chem Scand 2:209–219

Felix G, Duran JD, Volko S, Boller T (1999) Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. Plant J 18:265–276

Franken P, Gnädinger F (1994) Analysis of parsley arbuscular endomycorrhiza: infection development and mRNA levels of defence-related genes. Mol Plant Microbe Interact 7:612–620

Gianinazzi-Pearson V, Tahiri-Alaoui A, Antoniw JF, Gianinazzi S, Dumas E (1992) Weak expression of the pathogenesis related PR-b_1 gene and localization of related protein during symbiotic endomycorrhizal interactions in tobacco roots. Endocytobiosis Cell Res 8:177–185

Giovannetti M, Sbrana C, Citernesi AS, Avio L (1996) Analysis of factors involved in fungal recognition responses to host-derived signals by arbuscular mycorrhizal fungi. New Phytol 133:65–71

Harrison MJ, Dixon RA (1994) Spatial patterns of expression of flavonoid/isoflavonoid pathway genes during interactions between roots of *Medicago truncatula* and mycorrhizal fungus *Glomus versiforme*. Plant J 6:9–20

Jones FR (1924) A mycorrhizal fungus in the roots of legumes and some other plants. J Agric Res 29:459–470

Kape R, Wex K, Parniske M, Goerge E, Wetzel A, Werner D (1993) Legume root metabolites and VA-mycorrhiza development. J Plant Physiol 141:54–60

Klingner A, Bothe H, Wray V, Marner FJ (1995a) Identification of a yellow pigment formed in maize roots upon mycorrhizal colonization. Phytochemistry 38:53–55

Klingner A, Hundeshagen B, Kernebeck H, Bothe H (1995b) Localization of the yellow pigment formed in roots of gramineous plants colonized by arbuscular fungi. Protoplasma 185:50–57

Maier W, Hammer K, Dammann U, Schulz B, Strack D (1997) Accumulation of sesquiterpenoid cyclohexenone derivatives induced by an arbuscular mycorrhizal fungus in members of the Poaceae. Planta 202:36–42

Maliga P, Breznowitz A, Marton L (1973) Streptomycin-resistant plants from callus cultures of haploid tobacco. Nature New Biol 244:29–30

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Niehaus K, Kapp D, Pühler A (1993) Plant defence and delayed infection of alfalfa pseudonodules induced by an exopolysaccharide (EPS I) deficient *Rhizobium meliloti* mutant. Planta 190:415–425

Perotto S, Bonfante P (1997) Bacterial associations with mycorrhizal fungi: close and distant friends in the rhizosphere. Trends Microbiol 5:496–501

Salzer P, Hebe G, Reith A, Zitterell-Haid B, Stransky H, Gaschler K, Hager A (1996) Rapid reactions of spruce cells to elicitors released from the ectomycorrhizal fungus *Hebeloma crustuliniforme* and inactivation of these elicitors by extracellular spruce cell enzymes. Planta 198:118–126

Salzer P, Hebe G, Hager A (1997) Cleavage of chitinous elicitors from the ectomycorrhizal fungus *Hebeloma crustuliniforme* by host chitinases prevents induction of K^+ and Cl^- release, extracellular alkalinization and H_2O_2 synthesis in *Picea abies* cells. Planta 203:470–479

Salzer P, Corbière H, Boller T (1999) Hydrogen peroxide accumulation in *Medicago truncatula* roots colonized by the arbuscular mycorrhiza-forming fungus *Glomus intraradices*. Planta 208:319–325

Schreiner T, Marner FJ, Hildebrandt U, Bothe H (1998) Chemical and biological characterization of corticrocin, a yellow pigment formed by the ectomycorrhizal fungus *Piloderma croceum*. Z Naturforsch 53:4–8

Tsai SM, Phillips DA (1991) Flavonoids released naturally from alfalfa promote development of symbiotic *Glomus* spores in vitro. Appl Environ Microbiol 57:1485–1488

Volpin H, Phillips DA, Okon Y, Kapulnik Y (1995) Suppression of an isoflavonoid phytoalexin defence response in mycorrhizal alfalfa roots. Plant Physiol 108:1449–1454

Walter MH, Fester T, Strack D (2000) Arbuscular mycorrhizal fungi induce the non-mevalonate methylerythritol phosphate pathway of isoprenoid biosynthesis correlated with accumulation of the "yellow pigment" and other apocarotenoids. Plant J 21:571–578

Warm E, Laties GG (1982) Quantification of hydrogen peroxide in plant extracts by the chemoluminescence reaction with luminol. Phytochemistry 21:827–831

Woitaszek P (1997) Mechanisms for the generation of reactive oxygen species in plant defence responses. Acta Physiol Plant 19:581–589