Oxidative Burst and Cell Death Induced by β-Quercinin and Zoospores of *Phytophthora quercina* in Tobacco Cell Cultures

J. KOEHL[1], E. F. ELSTNER[1], W. OßWALD[2] and I. HEISER[1]*

[1]Department of Phytopathology, Center of Life Sciences, TU-München, 85350 Freising, Germany; [2]Department of Forest Phytopathology, TU-München, 85350 Freising, Germany

[*]E-mail: j.koehl@lrz.tum.de

Abstract

Mode of action of β-quercinin, a novel elicitin on tobacco cell suspension cultures (cvs. Bel B and Bel W3) was investigated by measuring the oxidative burst and cell death in these cell cultures. β-quercinin induced an oxidative burst comparable to that excited by zoospores from *P. quercina*. Adding superoxidedismutase, catalase and diphenyleneiodonium to elicited cell cultures, it could be demonstrated, that the induction of cell death in tobacco cell cultures is not correlated to the oxidative burst.

Keywords: *P. quercina*; elicitins; tobacco; oxidative burst; cell death

INTRODUCTION

P. quercina is a newly described *Phytophthora* species (JUNG *et al.* 1999) highly pathogenic on oak trees. *P. quercina* is very likely involved in phenomena called oak decline or oak disease. This study was done to investigate the interaction of elicitors from *P. quercina* such as zoospores and β-quercinin, released by the pathogen. β-quercinin is a novel basic protein which belongs to the elicitin family. These proteins comprising 98 amino acids and a molecular weight of about 10 kDa are exclusively released by *Phytophthora* and some *Pythium* species into their culture medium (RICCI *et al.* 1989). All elicitins screened so far show the common feature of inducing a hypersensitive reaction in tobacco plants and cell cultures (RICCI *et al.* 1989; BONNET *et al.* 1996). Our study includes two tobacco cultivars (Bel B and Bel W3) that are highly related but reveal different responses concerning ozone sensitivity. The experiments were performed to examine the action of zoospores and a new elicitin of *P. quercina* on two different tobacco cell culture cultivars.

MATERIAL AND METHODS

Elicitin. Isolation of β-quercinin was performed according to KOEHL *et al.* (2002, submitted for publication).

Production of zoospores. *P. quercina* was grown in V8 liquid medium on a horizontal shaker at 18°C in the dark. After 7 days the mycelium was removed from the liquid and transferred into root exsudate from *Quercus robur*, that enhances the generation of zoosporangia. After a period of 12 days zoosporangia were produced. To induce release of zoospores the mycelium was transferred to 4°C for 30 min. Zoospore suspension was diluted to a concentration of 0.5×10^3 per ml. 1 ml was added to the cell cultures.

Tobacco cell suspension cultures. Suspension cultures of tobacco cvs. Bel B and Bel W3 were cultivated in NTD medium at 27°C in the dark. Tobacco cells were transferred to fresh medium every 7 days. They were subcultured in Fe-free NTD medium for 2 days after transfer and then used for experiments.

Oxidative burst. Generation of H_2O_2 was detected using the dye Amplex Red (10-acetyl-3,7, dihydroxyphenoxazin), a Resorufin derivate. For the Amplex Red stock solution (2mM) 1 mg Amplex Red was dissolved in 200 µl DMSO and diluted up to 1.8 ml with H_2O_{bd}. The assay was performed with a 100 µM Amplex Red solution. 320 µl 0.2M phosphate buffer pH 7.5, 80 µl horse radish peroxidase (1 U/ml), 400 µl culture medium and 200 µl of Amplex Red. The solution was measured photometrically at 571 nm. Total amount of generated H_2O_2 was calculated by a H_2O_2 calibration curve. Catalase was added in a final concentration of

500 U/ml to the cell cultures, shortly after elicitiation. 1 ml of SOD stock solution (1900 U/ml) was added to aim a final concentration of 100 U/ml. DPI (diphenyleniodonium) was dissolved in 100% DMSO for a 40 mM stock solution. DPI solution was diluted with water to a final concentration of 10 μM DPI and 0.025% DMSO.

Cell death. Estimation of cell death was performed by using a 0.05% Evans Blue dye. 1 g cells per sample were incubated in the dye for 20 min. Cells were then washed with water until washing solution was clear. 0.1 g of cells were extracted with 1% SDS and centrifuged at 8800 g for 3 min. Supernatant was then measured photometrically at 600 nm.

RESULTS

Zoospores of *P. quercina* as well as its basic elicitin β-quercinin induce an oxidative burst in tobacco cell cultures which can be detected by measuring a transient H_2O_2 production. As shown in Figure 1 ozone sensitive cultivar Bel W3 (Figure 1B) also reacts more sensitive than Bel B (Figure 1A) concerning the elicitin concentration. 0.2nM β-quercinin are sufficient to induce an oxidative burst in Bel W3 (Figure 1B) while a higher concentration is needed in Bel B (Figure 1A). On the other hand, a β-quercinin concentration of 20 nM inhibits an oxidative burst in cultivar Bel W3 completely (Figure 1B) while this concentration induces an accumulation of H_2O_2 in Bel B (Figure 1A). Contrary to β-quercinin the elicitation of the two cultivars with zoospores from *P. quercina* did not reveal great differences in induction of an oxidative burst (Figures 1A and 1B).

Figure 2 shows induction of cell death in cvs. Bel B and Bel W3 after treatment with β-quercinin and zoospores of *P. quercina*. In Bel B only 20nM β-quercinin induces an increase of cell death compared to the corresponding control. Neither 0.2nM β-quercinin nor zoospores were sufficient to induce cell death in Bel B, but do so in cv. Bel W3 (Figure 2).

The interaction of oxidative burst and cell death in tobacco cells cv. Bel W3 was examined using catalase, superoxidedismutase and dpi. Figure 3 clearly shows no reduction of cell death in tobacco cell culture Bel W3 after adding any of these agents, whereas the oxidative burst was inhibited by catalase and DPI.

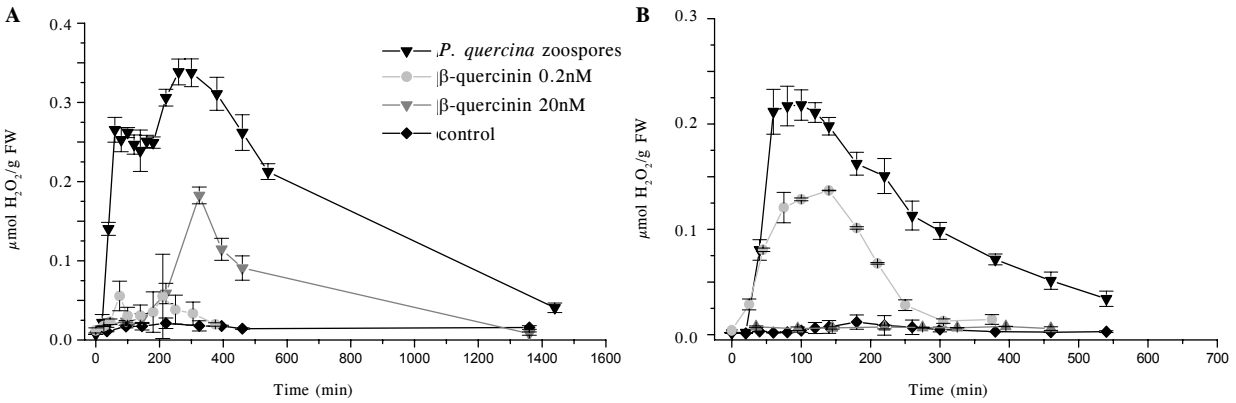

Figure 1. Oxidative burst in tobacco cell cultures Bel B (A) and Bel W3 (B), elicited with β-quercinin and zoospores from *P. quercina*

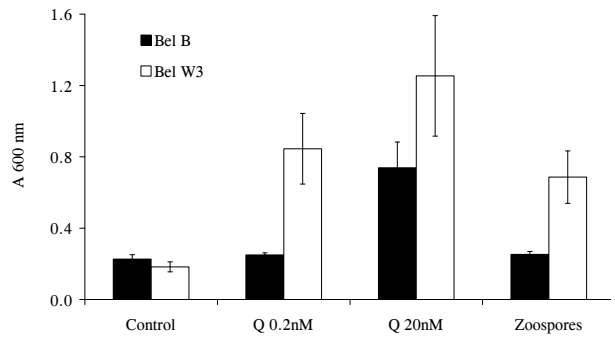

Figure 2. Cell death in tobacco cell cultures Bel B and Bel W3 24 h after elicitation

DISCUSSION

Elicitation of tobacco cell cultures with zoospores of the incompatible pathogen *P. quercina* reveals a monophasic oxidative burst in both cultivars (Figure 1). ABLE *et al*. (2000) who treated tobacco cells with zoospores of an incompatible strain of *P. parasitica* var. *nicotianae* obtained a biphasic production of H_2O_2. Kinetic of the oxidative burst produced in cv. Bel B indicates a possible second phase which could not be measured in cv. Bel W3 (Figures 1A and 1B). The production of β-quercinin induced H_2O_2 accumulation is dose dependent up to a certain quercinin concentration. Ozone sensitive cv. Bel W3 does not

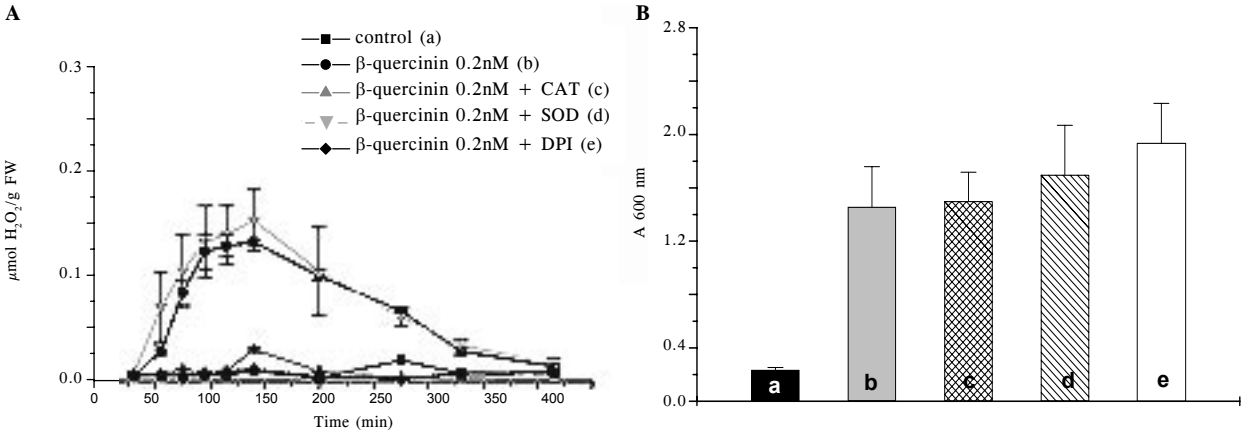

Figure 3. Oxidative burst (A) and cell death (B) in tobacco cell culture Bel W3 after elicitation with β-quercinin

reveal an oxidative burst at high concentrations (20nM) probably due to early cell death that is supported by a strong cell death development after 24 h (Figure 2). On the other hand a very low quercinin concentration (0.2nM) is sufficient to induce an oxidative burst in this cultivar (Figure 1B), whereas none H_2O_2 induction could be measured in Bel B at this concentration (Figure 1A). Other authors measured generation of H_2O_2 after elicitin treatment in tobacco cell cultures that also developed dose dependent features, but did not put it down to an early cell death induction inhibiting the generation of H_2O_2 in these cells (RUSTERUCCI *et al*. 1996; BOURQUE *et al*. 1998).

Independence of cell death and oxidative burst could be demonstrated by adding the H_2O_2 degrading enzyme catalase and NADPH-oxidase inhibiting DPI to elicited cell cultures. No oxidative burst was detected but cell death remained undiminished (Figure 3). These results agree with a variety of others where no correlation between cell death and reactive oxygen species could be found. YANO *et al*. (1999) and JABS *et al*. (1997) both showed an induction of H_2O_2 generation in tobacco cells elicited with an elicitor form *P. infestans* and parsley cells incubated with *Pseudomonas syringae* but could not measure cell death in these cell cultures. Addition of catalase, dpi and nac which all inhibit oxidative burst but not cell death strongly support this result (YANO *et al*. 1999; SASABE *et al*. 2000).

References

ABLE A., GUEST D., SUTHERLAND M. (2000): Hydrogen peroxide yields during the incompatible interaction of tobacco suspension cells inoculated with *Phytophthora nicotianae*. Plant Physiol., **124**: 899–910.

BONNET P., BOURDON E., PONCHET M., BLEIN J.-P., RICCI P. (1996): Aquired resistance triggered by elicitins in tobacco and other plants. Eur. J. Plant Pathol., **102**: 181–192.

BOURQUE S., PONCHET M., BINET M.-N., RICCI P., PUGIN A., LEBRUN-GARCIA A. (1998): Comparison of binding properties and early biological effects of elicitins in tobacco cells. Plant Physiol., **118**: 1317–1326.

JABS T., TSCHÖPE M., COLLING C., HAHLBROCK K., SCHEEL D. (1997): Elicitor-stimulated ion fluxes and O2- from the oxidative burst are essential components in triggering defense gene activation and phytoalexin synthesis in parsley. P. Natl. Acad. Sci., **94**: 4800–4805.

JUNG T., COOKE D., BLASCHKE H., DUNCAN J., OßWALD W. (1999): *Phytophthora quercina* sp. nov., causing root rot of European oaks. Mycol. Res., **103**: 785–798.

RICCI P., BONNET P., HUET J., SALLANTIN M., BEAVAIS-CANTE F., BRUNTEAU M., BILLARD V., MICHEL G., PERNOLLET J. (1989): Structure and activity of proteins from pathogenic fungi *Phytophthora* eliciting necrosis and acquired resistance in tobacco. Eur. J. Biochem., **183**: 555–563.

RUSTERUCCI C., STALLAERT V., MILAT M., PUGIN A., RICCI P., BLEIN J. (1996): Relationship between active oxygen species, lipid peroxidation, necrosis and phytoalexin production induced by elicitins in *Nicotiana*. Plant Physiol., **111**: 885–891.

SASABE M., TAKEUCHI K., KAMOUN S., ICHINOSE Y., GOVERS F., TOYODA K., SHIRAIHI T., YAMADA T. (2000): Independent pathways leading to apoptotic cell death, oxidative burst and defense gene expression in response to elicitin in tobacco cell suspension culture. Eur. J. Biochem., **267**: 5005–5013.

YANO A., SUZUKI K., SHINSHI H. (1999): A signaling pathway, independent of the oxidative burst, that leads to hypersensitive cell death in cultured tobacco cells includes a serine protease. Plant J., **18**: 105–109.