Final report

The aim of the project was to get a deeper insight into the role of plant hormones in various plant-pathogen interactions. It has been known that the plant hormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) play a key role in plant immunity. However, recently many data support the important role of auxins, cytokinins, gibberellins, abscisic acid and brassinosteroids as well in the plant immune responses (Denance et al. 2013; Alazem and Lin 2015; Opara and Amarachi 2015). It is generally accepted that SA signaling induces defense against biotrophic, whereas JA/ET signaling against necrotrophic pathogens (Glazebrook 2005).

Brassinosteroids (BRs) are known not only influence many physiological processes (Xia et al. 2010) and stress tolerance (Janeczko et al 2005; Janeczko et al. 2007, Bajguz and Hayat, 2009; Hassan et al., 2008; Hayat et al., 2010), but BRs have been shown to play substantial roles also in disease resistance (Opara and Amarachi, 2015; Alazem and Lin, 2015).

We wanted to determine the protective effect of brassinosteroid (BR27) on oilseed rape cotyledons against infection by an incompatible wild type, of a hypersensitive response mutant and of saprophytic Pseudomonas bacteria. In this paper, membrane permeability, PSII efficiency and metabolic activity were analyzed. The following strains of *Pseudomonas* were used: *P. syringae* pv. *syringae* (Ps), *P. syringae* pv. *syringae* hrcC mutant (Pm) and P. fluorescence (Pf). The study was carried out using two cultivars of spring oilseed rape (*Brassica napus* L.): ‘Licosmos’ and ‘Huzar’. Pre-treatment of cotyledons with BR27 caused about 50–70% increase in ion leakage for both cultivars. However, BR27 pre-treatment significantly decreased ion leakage from cotyledons inoculated with Ps in both cultivars. Infection with Ps and Pf caused disturbances of energy flow in PSII by lowering its efficiency in rape cotyledons. We noted insignificant impact of 24-epibrassinolide on PSII efficiency if compared to absolute control, but generally it had a positive effect on plants infected with bacteria. The values of heat flow in all treatments, except for cotyledons infected with Ps, decreased during 20 h after inoculation. However, the curves of heat flow for Ps-infected cotyledons showed a completely different pattern with at least two peaks. BR27 pre-treated cotyledons infected with Ps had higher heat flow in comparison to Ps infected ones. BR27 treatment did not change specific enthalpy of cotyledon growth (Dgh) for both cultivars if compared with absolute control. However, infection with Ps markedly increased Dgh values by about 200% for both cultivars. We suggested protective action of BR27 in oilseed rape cotyledons after bacterial infection with Pseudomonas (Skoczowski et al. 2011).

Although brassinosteroids are known to protect plants against various abiotic and biotic stresses, however, very limited information is available about the role of progesterone. Therefore the effects of *Pseudomonas syringae* pv. s*yringae* (P.s.) wild type strain 61, its hrcC mutant, and the saprophytic *P. fluorescens* (P.f.) strain 55 were investigated in wild type *Arabidopsis thaliana* cv. Columbia and its rbohF knock-out mutant with and without progesterone pre-treatment. The reactions of wild type and rbohF mutant *Arabidopsis* to bacterial inoculations were similar although 2 h after injection of P.s. a larger increase of electrolyte leakage was measured in wild type than in rbohF knockout mutant leaves. The hrcC mutant caused weak necrotic symptoms and increased leakage in both types of Arabidopsis although to a much lesser extent than P.s. The P.f. did not induce any visible symptom but slightly increased the electrolyte leakage in both types of *Arabidopsis*. Inoculation by all *Pseudomonas* bacteria led to significant alterations in photosystem II efficiency as compared to control plants. Pre-treatment of leaves with progesterone diminished the necrotic symptoms, the electrolyte leakage and improve the efficiency of photosystem II caused by Pseudomonas bacteria (Janeczko et al. 2013).

The effect of light on ethylene and ethane production in damaged leaf tissues was also investigated. When whole leaves of tobacco cv. Samsun NN were damaged with liquid nitrogen the ethylene formation was the highest, if 100 % of leaves were injured and were kept in the light, the lowest when leaves after 100 % injury were kept in darkness. Ethane production (lipid peroxidation) could be detected only in damaged, but not in control leaves, and was much higher in light than in darkness. In addition, there was a strong degradation of chlorophyll of damaged leaves kept in light. In light aminoethoxy-vinylglycine (AVG) inhibited ethylene formation in control, non-damaged whole leaves effectively, but in leaves with 100% damage the inhibitory effect was much weaker and similar to the effect of propyl gallate (PG), a free radical scavenger. Both AVG and PG treatments decreased ethylene formation by control leaf discs and discs with 100 % damage. Ethane production was significantly inhibited by PG and slightly by AVG in the case of 100 % damage. In accordance, tobacco mosaic virus (TMV) infection on the necrotic host resulted in much larger amount of ethylene and ethane formation in light than in darkness (Barna et al. 2012b).

Plant pathogens can be divided into biotrophs and necrotrophs according to their different life styles; biotrophs prefer living, while necrotrophs prefer dead cells for nutritional purposes. Therefore tissue necrosis caused by reactive oxygen species (ROS) during pathogen infection increases host susceptibility to necrotrophic, but resistance to biotrophic pathogen. Consequently, elevation of antioxidant capacity of plants enhances their tolerance to development of necroses caused by necrotrophic pathogens. Plant hormones can strongly influence induction of ROS and antioxidants, thereby influencing susceptibility or resistance of plants to pathogens. Pathogen-induced ROS themselves are considered as signaling molecules. Generally, salicylic acid (SA) signaling induces defense against biotrophic pathogens, whereas jasmonic acid (JA) against necrotrophic pathogens. Furthermore pathogens can modify plant's defense signaling network for their own benefit by changing phytohormone homeostasis. On the other hand, ROS are harmful also to the pathogens; consequently they try to defend themselves by elevating antioxidant activity and secreting ROS scavengers in the infected tissue. The Janus face nature of ROS and plant cell death on biotrophic and on necrotrophic pathogens is also supported by the experiments with BAX inhibitor-1 and the mlo mutation of Mlo gene in barley. It was found that ROS and elevated plant antioxidant activity play an important role in systemic acquired resistance (SAR) and induced systemic resistance (ISR), as well as in mycorrhiza induced abiotic and biotic stress tolerance of plants. Upon request we wrote a review article on the role of reactive oxygen species (ROS) and plant hormones in plant resistance against necrotrophic and biotrophic pathogens (Barna et al. 2012a).

Fusarium culmorum causes root rot in barley (*Hordeum vulgare*), resulting in severely reduced plant growth and yield. Pretreatment of roots with chlamydospores of the mutualistic root-colonizing basidiomycete *Piriformospora indica* prevented necrotization of root tissues and plant growth retardation commonly associated with *Fusarium* root rot. Quantification of *Fusarium* infections with a real-time PCR assay revealed a correlation between root rot symptoms and the relative amount of fungal DNA. Fusarium infected roots showed reduced levels of ascorbate and glutathione (GSH), along with reduced activities of antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), dehydroascorbate reductase (DHAR), and monodehydroascorbate reductase (MDHAR). Consistent with this, *Fusarium*-infected roots showed elevated levels of lipid hydroperoxides and decreased ratios of reduced to oxidized forms of ascorbate and glutathione. In clear contrast, roots treated with *P. indica* prior to inoculation with *F. culmorum* showed levels of ascorbate and GSH that were similar to controls. Likewise, lipid peroxidation and the overall reduction in antioxidant enzyme activities were largely attenuated by *P. indica* in roots challenged by *F. culmorum*. These results suggest that *P. indica* protects roots from necrotrophic pathogens at least partly, through activating the plant's antioxidant capacity (Harrach et al. 2013).

Next, ion leakage, chlorophyll fluorescence and content, as well as expressions of some related genes in pepper leaves infected with a compatible or an incompatible tobamovirus were studied. Leaves of a pepper cultivar harboring the L3 resistance gene were inoculated with *Obuda pepper virus* (ObPV), which led to the appearance of hypersensitive necrotic lesions approx. 72 h post-inoculation (hpi) (incompatible interaction), or with *Pepper mild mottle virus* (PMMoV) that caused no visible symptoms on the inoculated leaves (compatible interaction). ObPV inoculation of leaves resulted in ion leakage already 18 hpi, up-regulation of a pepper carotenoid cleavage dioxygenase (CCD) gene from 24 hpi, heat emission and declining chlorophyll a content from 48 hpi, and partial desiccation from 72 hpi. After the appearance of necrotic lesions a strong inhibition of photochemical energy conversion was observed, which led to photochemically inactive leaf areas 96 hpi. However, leaf tissues adjacent to these inactive areas showed elevated FPSII and Fv/Fm values proving the advantage of chlorophyll a imaging technique. PMMoV inoculations also led to a significant rise of ion leakage and heat emission, to the up-regulation of the pepper CCD gene as well as to decreased PSII efficiency, but these responses were much weaker than in the case of ObPV inoculation. Chlorophyll b and total carotenoid contents as measured by spectrophotometric methods were not significantly influenced by any virus inoculations when these pigment contents were calculated on leaf surface basis. On the other hand, near-infrared FTRaman spectroscopy showed an increase of carotenoid content in ObPV-inoculated leaves suggesting that the two techniques detect different sets of compounds (Rys et al. 2014).

In addition, the efficiency of PSII, gas exchange, spectral reflectance and sugar contents were also studied in pepper leaves infected with a compatible or an incompatible tobamovirus. Inoculation of pepper leaves with ObPV decelerated the electron transport in PSII and lowered the efficiency of O_2 evolving centers, and increased also more strongly the leaf reflectance of pepper leaves than PMMoV. ObPV decreased net photosynthesis, stomatal conductance and transpiration rate, while elevated the internal CO_2 concentration of leaves.

Sugars, as well as hormone contents were studied in pepper leaves infected with a compatible or an incompatible tobamovirus by UHPLC-MS, or HPLC methods, supplemented with BR pre-treatment. In ObPV infected leaves a strong accumulation of glucose and fructose was accompanied with decreased sucrose, maltoheptose, nystose and trehalose contents in the infected leaves, while that of glucose-6-phosphate increased. PMMoV infection increased the levels of glucose, maltose and raffinose in the inoculated leaves. Pre-treatment with 24-epi-brassinolide significantly alleviated the effects of ObPV on PSII, and increased nystose and maltoheptose, while decrease glucose contents in ObPV infected leaves (Janecko et al. 2016 under review).

Furthermore ObPV-inoculation markedly increased not only the levels of salicylic acid (SA) (73-fold) and jasmonic acid (8-fold) but also those of abscisic acid, indole-3-acetic acid, indole-3-butyric acid, *cis*-zeatin, *cis*-zeatin-9-riboside and *trans*-zeatin-9-riboside in the inoculated pepper leaves 3 days post inoculation. In sharp contrast to ObPV, PMMoV infection in the inoculated leaves increased only the contents of gibberellic acid and SA. Concentrations of both BRs and progesterone increased both in ObPV- and PMMoV inoculated leaves, and to our best knowledge this is the first report about the occurrence of these steroids in pepper. ObPV-inoculation markedly induced the expression of two phenylalanine ammonia-lyase (*PAL*) genes, while that of an isochorismate synthase (*ICS*) gene was not modified by ObPV. These findings suggest that the phenylpropanoid pathway of SA biosynthesis is predominant in ObPV-inoculated pepper leaves. The expression of *PAL* and *ICS* genes did not change during the compatible pepper-PMMoV interaction. The transcript abundance of an 1-aminocyclopropane-1-carboxylate oxidase gene was strongly increased by both ObPV- and PMMoV, but the effect was more rapid in the ObPV-inoculated leaves. Pre-treatment of pepper leaves with exogenous 24-epi-brassinolide (24-epi-BR) prior to ObPV-inoculation strongly mitigated the visible symptoms caused by ObPV. In addition, 24-epi-BR pre-treatment markedly altered the endogenous levels of several hormones in pepper leaves following ObPV-inoculation. All these data indicate that ObPV- and PMMoV-inoculations lead to specific, intricate, but well harmonized responses that are largely determined by the incompatible or compatible nature of plant-virus interactions (Dzurka et al. 2016).

In conclusion, we demonstrated that brassinosteroid (BR) and progesterone pre-treatments remarkably decreased *Pseudomonas* bacteria induced symptoms, ion leakage and changes of photosystem II efficiency, as well as of heat flow in rape or in wild type and rbohF knock-out mutant of *Arabidopsis thaliana*. We could also show that ethylene and ethane production of damaged plant tissues is strongly induced by light and ROS are involved in this induction, strengthening the idea on the strong relationship between plant hormones and ROS/antioxidant systems. We proved that pre-treatment with *Piriformospora indica* significantly decreased the damage caused by the pathogen *Fusarium culmorum* on barley, by activating the plant’s antioxidant capacity. In addition, in pepper leaves infected with a compatible or an incompatible tobamovirus we demonstrated, that not only the amount of typical stress hormones like SA, JA or ABA changed significantly, but also the amount of cytokinins, auxins and gibberellins. Concentrations of both BRs and progesterone increased both in ObPV- and PMMoV inoculated leaves, and to our best knowledge this is the first report about the occurrence of these steroids in pepper. Similarly, the sugar analysis showed remarkable changes after virus infections. All these results indicate that plant hormones form a complex network which has significant effect on pathogen induced changes of ROS/antioxidant systems and soluble sugars in plants.

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