

# Comparative Studies on Inducible Antimicrobial Defense Reactions in Soybean Cultivars

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## ABSTRACT

Plants react to microbial attack with a number of defence mechanisms which include a rapid formation of reactive oxygen species (ROS), cross-linking of cell wall proteins and strengthening of the plant cell wall, induction of the phenylpropanoid pathway, synthesis of phenolic compounds, accumulation of antimicrobial compounds named phytoalexins, synthesis of fungal wall degrading enzymes (glucanases, chitinases) and a rapid localized cell death. These defence responses can also be induced by application of various biotic and abiotic substances (elicitors) to cell suspension cultures. Most analyses of defence reactions were performed on a few cultivars and elicitors. There is little knowledge about the reactivity of different cultivars against various stimuli. Therefore, suspension cultured soybean (*Glycine max* [L.] Merr.) cells of four cultivars (Willis, Lumut, Kalmit, Doko RC) were treated either with crude cell wall extracts of the fungal pathogens *Phytophthora sojae*, *Rhizoctonia solani*, *Athelia rolfsii* (Pmg-, Riso-, Aro-elicitor, respectively) or with two isolates of the bacterial pathogen *Pseudomonas syringae* pv. *glycinea* (Psg01/02). Cells of all four cultivars showed the same range of defence reactions but time courses and intensities differed significantly between the cultivars. Furthermore, the reactivity of the cultivars depended on the tested elicitor. A common response to elicitation is the rapid release of ROS called oxidative burst. Comparing the amount of H<sub>2</sub>O<sub>2</sub> induced by the fungal elicitors three cultivars produced significantly less H<sub>2</sub>O<sub>2</sub> after application of Pmg-elicitor than with Riso-elicitor, only Doko RC showed equal values for both elicitors. These differences in the expression of an oxidative burst corresponded with a rapid and transient alkalization of the cell culture medium after elicitation. Those elicitors that caused a strong oxidative burst were also capable of inducing a greater alkalization. Nevertheless there is no causal relationship between the two reactions as could be shown by specific inhibitors of the oxidative burst. Activation of the phenylpropanoid metabolism is an important step for the synthesis of various secondary compounds (i.e. [iso]-flavonoids, lignin-like substances, soluble phenolics). Phenylalanine ammonia-lyase (PAL), the key enzyme of phenylpropanoid metabolism, was activated after elicitor-treatment. The enzyme activity was stronger after addition of Riso-elicitor than after application of bacterial suspensions. The intensity of PAL-activity correlated with an increase of soluble phenolic compounds in the culture medium but not with an accumulation of the isoflavonoid phytoalexins. These results suggest that soybean cultivars recognise the same stimuli but differ in the intensity of elicitor-induced defence reactions. This is probably due to a different recognition of the elicitors at the plasma membrane.

**Key words:** Antimicrobial, *Glycine max*, fungal pathogen, elicitor

## INTRODUCTION

Plants are continually exposed to a vast array of different stress factors shown in Figure 1. On the one hand there are abiotic factors such as UV-light, heavy metal ions, detergents, xenobiochemicals, freezing or heating. On the other hand plants are challenged by a great number of biotic factors such as viruses, bacteria, fungi, nematodes, and insects (Barz, 1997).

Nevertheless, most plants exhibit natural resistance to microbial attack. This resistance is called non-host resistance or basic incompatibility. Non-host resistance is the consequence of either the inability of the parasite to recognise or infect a plant or the ability of a plant to rapidly and effectively activate its defence mechanism (Figure 2A) (Kombrink and Somssich, 1995). Higher plants express constitutively an array of defence mechanisms. They include mechanical barriers such as the outer layer of the epidermis or rhizodermis and its cutin or suberin layers as well as biochemical barriers such as preformed often toxic phenolic compounds-flavonoids, isoflavonoids, alkaloids, cyanogenic glycosides. Only in a few cases pathogens successfully parasitise a plant

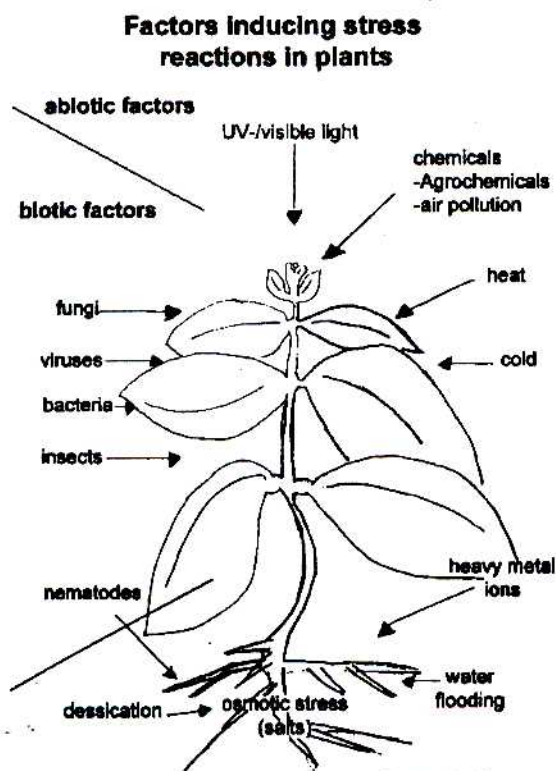
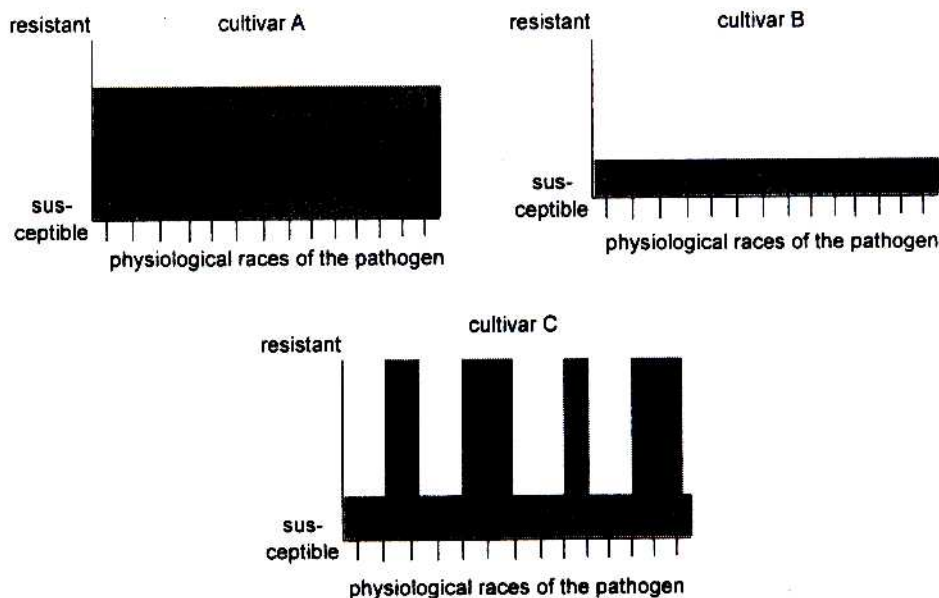


Figure 1. Schematic diagram showing factors that induce stress reactions in plants

host. In regard of a compatible interaction, the plant is susceptible and the pathogen virulent. The host range of a pathogen is usually very limited and often restricted to a single plant (Figure 2B) or even a single cultivar whereas other cultivars of the same plant are resistant (Figure 2C). This cultivar-resistance is supposed to be based on a gene-for-gene relationship of genetically defined resistance (*R*) genes of the host and corresponding avirulence (*Avr*) genes of the pathogen (Kombrink and Somssich, 1995). Most of high yielding crop varieties confer cultivar resistance against a specific race of a pathogen. A simple mutation can break this resistance so that the plant becomes susceptible and the pathogen virulent. Therefore the aim of breeding should be a plant with a broad spectrum disease resistance based on several genes.

Induction of active plant anti-microbial defence reactions results from the perception of signal molecules (elicitors) by the plant cells. Elicitors are molecules derived either from the pathogen (exogenous elicitors) or the host (endogenous elicitor). It is supposed that these elicitors mostly are recognised by receptors localised in the plasma membrane for a number of fungal elicitors or inside the plant cell for certain bacterial elicitors (Ebel and Mithöfer, 1998).

Many different molecules have been shown to behave as elicitors of defence responses when applied to plant tissue. Most of them elicit defence responses in a large variety of plant species and, within the same plant species, in a large variety of cultivars, so that they are called non-specific elicitors. In contrast, cultivar-specific elicitors are



Notes: A = non-specific, broad resistance, B = susceptibility, C = race-cultivar-specific resistance

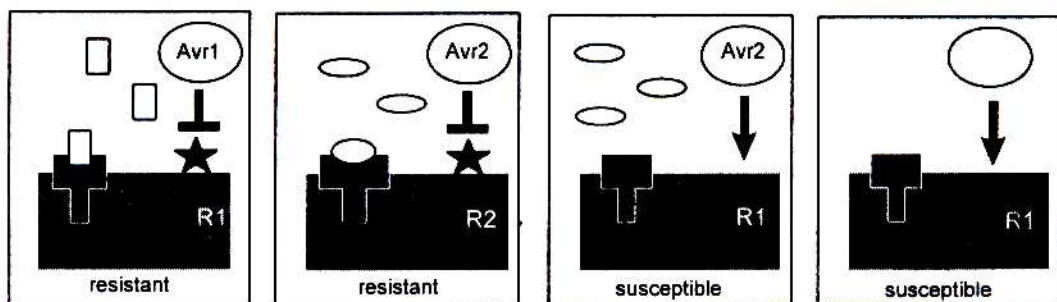
Figure 2. Schematic representation of race-cultivar specific and non-specific resistance (modified according to Heitefuss, 1987)

those molecules, direct or indirect products of *Avr*-genes, which are active only on the cultivars having the corresponding *R*-genes (Figure 3) (Cervone *et al.*, 1997).

Plants have developed active defence mechanisms against pathogens. In contrast to animal cells, each plant cell is capable of defending itself. After the perception of the pathogen a signal cascade is triggered. As a consequence, a number of defence reactions are induced, which include very rapid reactions independent of a *de novo* synthesis via gene activation as ion fluxes across the plasma membrane, accumulation of reactive oxygen species (ROS), cross-linking and strengthening of the plant cell wall. Slower reactions depend on transcriptional activation of specific genes and usually the following biochemical responses are observed: activation of enzymes of phenylpropanoid metabolism, synthesis and deposition of phenolic compounds and of hydroxyproline rich proteins, rapid localised cell collapse and death (hypersensitive response, HR), accumulation of antimicrobial phytoalexins, and synthesis of pathogenesis-related proteins (PR-proteins), including the two hydrolytic enzymes, chitinase and 1,3- $\beta$ -glucanase, which can attack the fungal cell wall (Figure 4) (Cervone *et al.*, 1997).

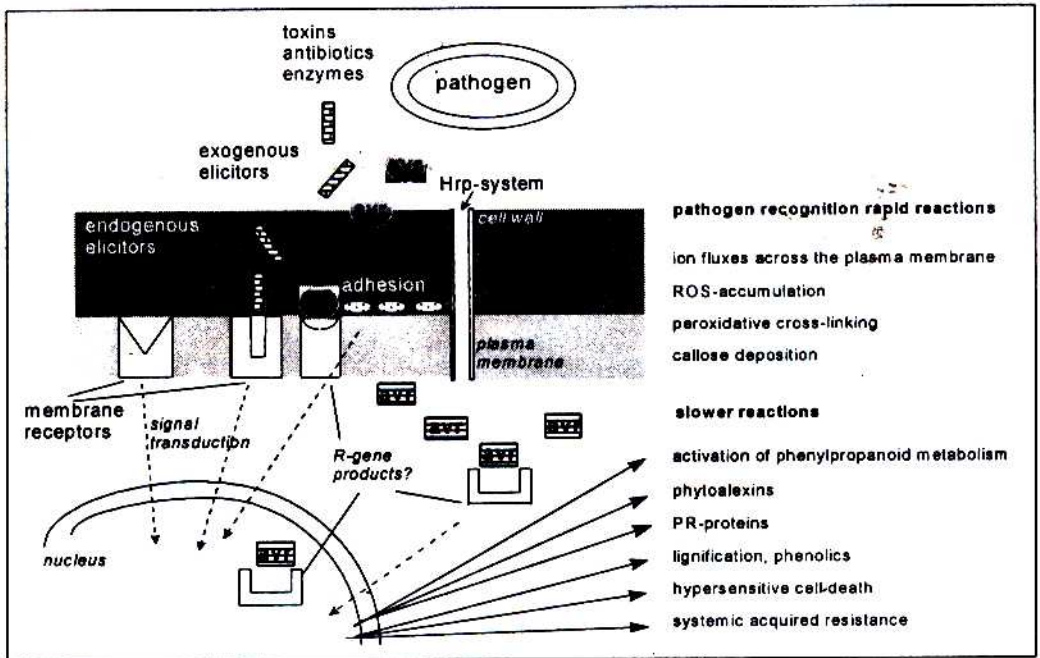
The defence mechanisms shown in Figure 4 are induced in plant-pathogen interactions as well as in a simplified model system using cell suspension cultures and elicitors. In contrast to the whole plant in the cell suspension culture defence reactions are not limited to a few cells around the infection site but occur in many cells simultaneously. The advantages are, that it is much easier to analyse early defence reactions, to apply specific inhibitors of signal transduction, to elucidate biochemical pathways and to get a large amount of secondary plant products.

This studies used cell suspension cultures of four soybean cultivars (Willis, Lumut, Kalmit, Doko RC) and various biotic elicitors. The elicitors tested were crude cell wall extracts of yeast and of the three fungal pathogens *Phytophthora sojae* (Pmg-elicitor), *Rhizoctonia solani* (Riso-elicitor), and *Athelia rolfsii* (Aro-elicitor) as well as two isolates



Notes: Different cultivars may have different resistance genes (*R*-genes), in contrast to different isolates of a pathogen which may have various avirulence genes (*avr*-genes). An incompatible interaction results if a specific *R*-gene, which encodes a receptor binds the complementary avirulence gene product of a pathogen

Figure 3. Model of the receptor ligand hypothesis of a gene-for-gene interaction (modified according to Dudler, 1997)



Notes: Neither all recognition mechanisms nor the whole spectrum of defence responses shown have to occur in every plant/pathogen interaction

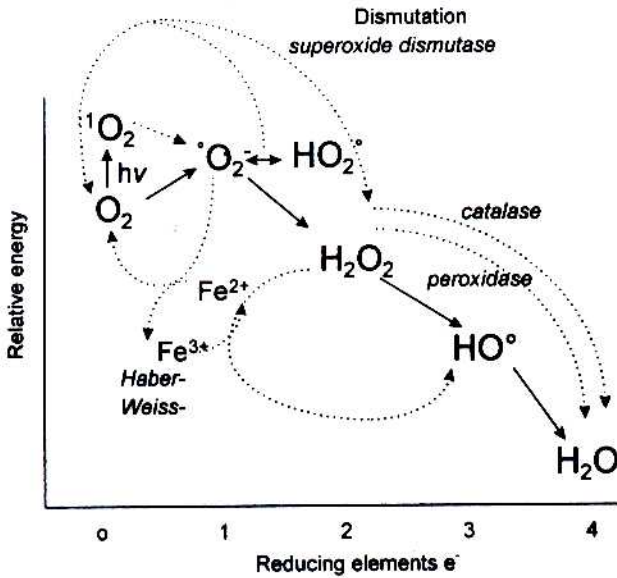
Figure 4. Schematic diagram of elicitor recognition and induction of plant defence responses

of the bacterial pathogen *Pseudomonas syringae* pv. *glycinea* (Psg01,02) collected from RIFCB Indonesia. These elicitors were non-specific because they induced defence reactions in cell suspension cultures of the four cultivars tested. Furthermore, Pmg-elicitor, and Riso-elicitor stimulate cell responses in other species (i.e. chickpea, parsley). The elicitor-active components are not known because purification of elicitor-active substances is very time-consuming and difficult. Besides it remained unclear if one or more components of the crude elicitor-preparations led to the defence responses.

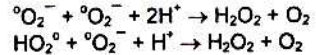
### Elicitor-Induced Oxidative Burst

As mentioned above one of the earliest defence reactions is a rapid and transient accumulation of ROS, the so called oxidative burst. The term ROS refers to species that result from the reduction of molecular oxygen: superoxid radical ( $^{\circ}\text{O}_2^-$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), and hydroxyl radical ( $^{\circ}\text{OH}$ ) (Figure 5).

Most cells possess the ability to produce and detoxify ROS. In some cases, however, especially under stress conditions these protective mechanisms are overridden by the rapid, transient production of huge amounts of ROS. It seems that this production serves not only as protectant against invading pathogens but could also be the signal



Dismutation:



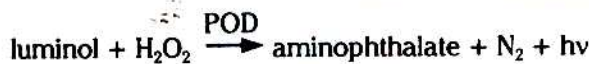
**Figure 5.** Active oxygen species derived from molecular oxygen and interconversion pathways that are supposed to exist in plants

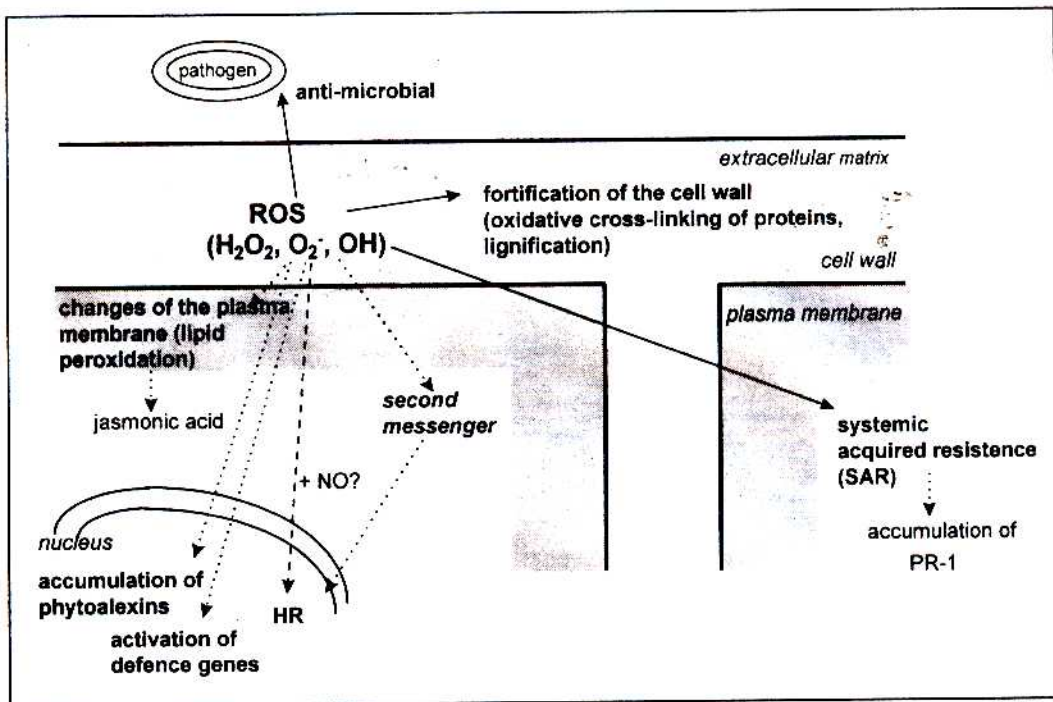
Source: Baker and Orlandi, 1995

activating further plant defence reactions (Wojtaszek, 1997). Figure 6 summarises reactions that are discussed to be related with the oxidative burst.

There is strong evidence that ROS are cytotoxic for invading pathogens (Peng and Kuc, 1992), that they are necessary for the peroxidative cross-linking of structural cell wall proteins (Otte and Barz, 1996), that they activate defence genes related to oxidative stress (Levine *et al.*, 1994) and that they are required but not sufficient for the induction of hypersensitive cell death (Glazener *et al.*, 1996). Eventually ROS act synergistically with nitric oxide to induce hypersensitive cell death (Delledonne *et al.*, 1998). Furthermore ROS seem to play a role in systemic acquired resistance which means that a signal transduction leads to immunity of plant tissue far away from the infection site (Alvarez *et al.*, 1998). In contrast, a general function as a second messenger and an influence on secondary metabolism is doubted. It can't be ruled out that different transduction mechanisms occur in different plant species.

Most of the data indicate that the major ROS building the oxidative burst is  $\text{H}_2\text{O}_2$  with possible participation of  ${}^{\circ}\text{O}_2^-$ . However the inherent interrelationship between  $\text{H}_2\text{O}_2$  and  ${}^{\circ}\text{O}_2^-$  makes it sometimes difficult to identify clearly the ROS behind the oxidative burst. A frequently used test is the oxidation of luminol by  $\text{H}_2\text{O}_2$  catalysed by peroxidase (POD) which can be measured as chemiluminescence.





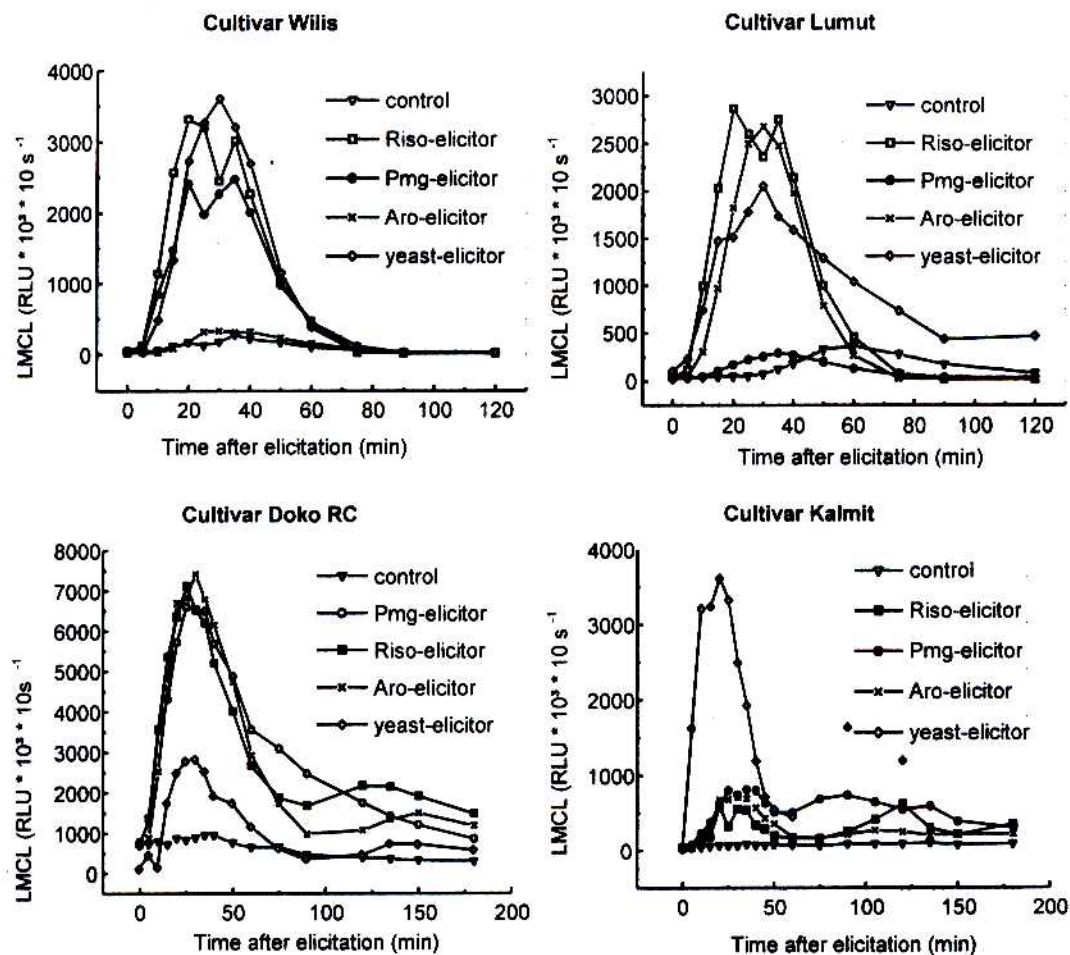
Notes: The dashed arrows indicate weak evidences for a role of reactive oxygen species. The responses shown have been demonstrated in different plant species and may not occur within a given species

**Figure 6.** Schematic diagram of defence responses that are supposed to be influenced by the elicitor-induced oxidative burst

Using this method cell suspension cultures of the four soybean cultivars showed a very rapid increase in H<sub>2</sub>O<sub>2</sub>, starting five minutes after addition of the fungal elicitors. After 20-40 minutes maximal values were reached and the reaction was completed around 70 minutes after elicitation. Nevertheless as shown in Figure 7 the time-course and intensity could vary depending on the cultivar and the challenging factor used. For example Pmg-elicitor induced a strong chemiluminescence in cell suspension cultures of cv. Willis, Doko RC, and Kalmit, but only a weak response in cv. Lumut. Furthermore, cell suspension cultures of cv. Doko RC and Kalmit showed a very weak second increase in H<sub>2</sub>O<sub>2</sub> after a strong first elicitor-stimulated oxidative burst.

A similar result was obtained using the bacterial elicitors. In suspension cultures of cv. Willis bacterial elicitor (Psg02) induced a sharp increase of ROS whereas cells of cv. Lumut showed a prolonged burst for nearly two hours. The experiments were performed with living as well as with heat-killed bacteria, but there was no clear result if living or dead cells were stronger inducers of the oxidative burst (Figure 8).

Using higher elicitor concentrations H<sub>2</sub>O<sub>2</sub> accumulation increased (Figure 9). Addition of Pmg-elicitor increased the H<sub>2</sub>O<sub>2</sub>-formation until an elicitor-concentration of

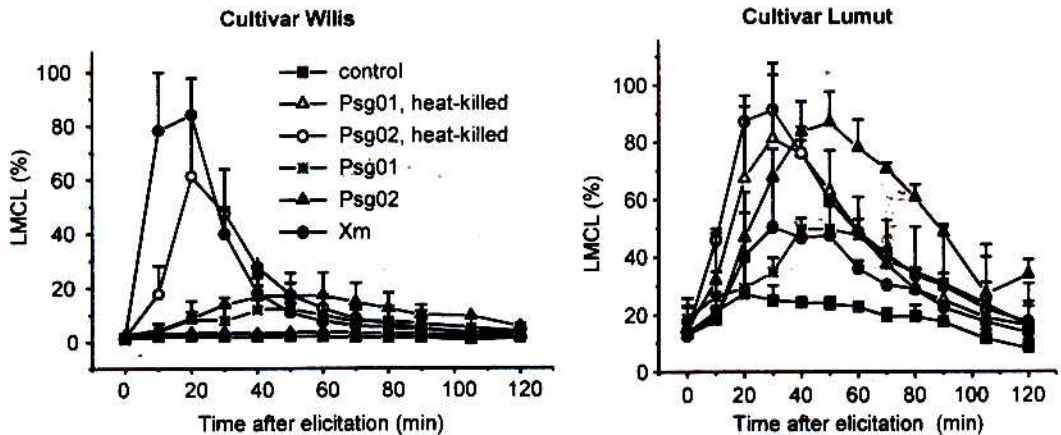


Notes: Five days after subculture cells of four cultivars were transferred to fresh medium, incubated for 2½ h and then elicited. The elicitors were crude cell wall preparations of *P. sojae* (Pmg-elicitor), *R. solani* (Riso-elicitor), *A. rolfii* (Aro-elicitor), and *S. cerevisiae* (yeast elicitor). As control an equal volume of *A. bidest* was added instead of elicitor. H<sub>2</sub>O<sub>2</sub> was measured as luminol-dependent chemiluminescence (LMCL)

Figure 7. Kinetics of H<sub>2</sub>O<sub>2</sub>-formation induced by various fungal elicitors in soybean cell suspension cultures

300 µg/ml medium was reached. Higher amounts of Pmg-elicitor only slightly further enhanced the oxidative burst. The highest intensity of the oxidative burst induced by Riso-elicitor in cell suspension cultures of cv. Lumut was obtained with a concentration of 100 µg/ml medium. In contrast, the reaction was not saturated in cv. Willis using 400 µg/ml. It seems that cells of cv. Lumut have a higher sensitivity for Riso-elicitor than cells of cv. Willis. Because of the saturation kinetics it is assumed that the two fungal elicitors are recognised by receptors.





Notes: Cells were prepared as described in Figure 8. As elicitors bacterial suspensions of two *P. syringae* pv. *glyciniae* isolates (Psg01, 02) were used. Bacterial cells lived (dark sign) or were heat-killed (20 min, 120°C). As control an equal volume of MES buffer was added in stead of elicitor. The results are means (+se) of two independent experiments. The maximal H<sub>2</sub>O<sub>2</sub>-accumulation of one charge was defined as 100%.

**Figure 8.** Kinetics of H<sub>2</sub>O<sub>2</sub>-formation induced by bacterial elicitors in soybean cell suspension cultures

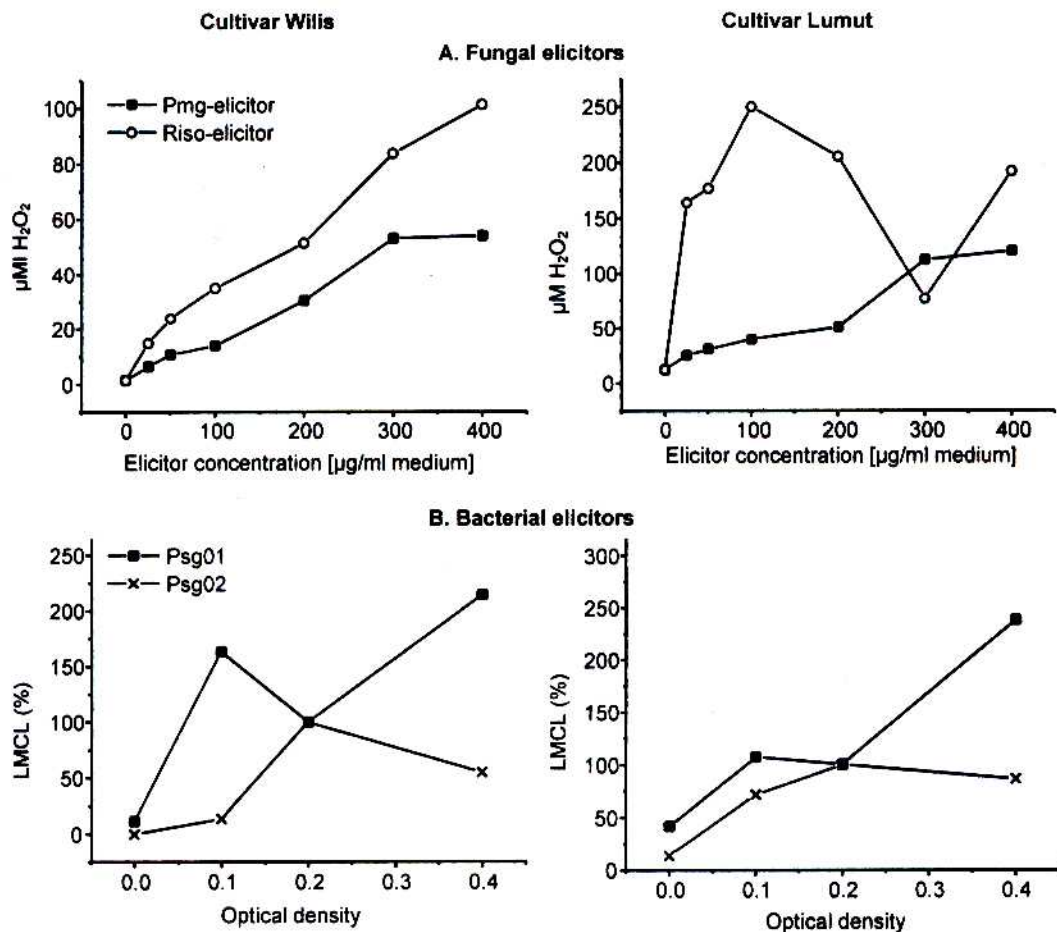
A comparison of the two elicitors shows that even great amounts of Pmg-elicitor resulted in a lower H<sub>2</sub>O<sub>2</sub>-accumulation than for Riso-elicitor. These results suggest that the two elicitor preparations contain different elicitor-active substances. The elicitor-active component(s) of Riso-elicitor seem to be more effective than the substance(s) of Pmg-elicitor.

Likewise, the intensity of the oxidative burst depended on the concentration (optical density) of the bacterial suspension. An optical density of 0.2 of Psg02 stimulated the highest chemiluminescence in both cultivars. In contrast, an optical density of 0.4 of Psg01 was not sufficient to saturate the reaction.

Comparing the maximal amounts of H<sub>2</sub>O<sub>2</sub> induced by the fungal elicitors cell suspension cultures of three cultivars produced significantly less H<sub>2</sub>O<sub>2</sub> after application of Pmg-elicitor than after addition of Riso-elicitor, only cells of cv. Doko RC showed equal values for both elicitors. This underlines the hypothesis that the elicitor-preparations contain different elicitor-active substances.

Furthermore the elicitor-induced H<sub>2</sub>O<sub>2</sub>-maximum for cell suspension cultures of cv. Willis was found to be three to four times lower compared with the three other cell lines. Using bacterial elicitors reactivity of cells from cv. Willis was equally strong for both isolates of *P. syringae*, unlike cells from cv. Lumut which produced much more H<sub>2</sub>O<sub>2</sub> after addition of isolate 02 than isolate 01 (Figure 10). These results indicate that the four cultivars have a differential sensitivity against different elicitor-preparations.

Summarising, these results indicate that the bacterial and fungal elicitors tested are recognised by the four soybean cultivars as all induced a transient increase in H<sub>2</sub>O<sub>2</sub>. It is

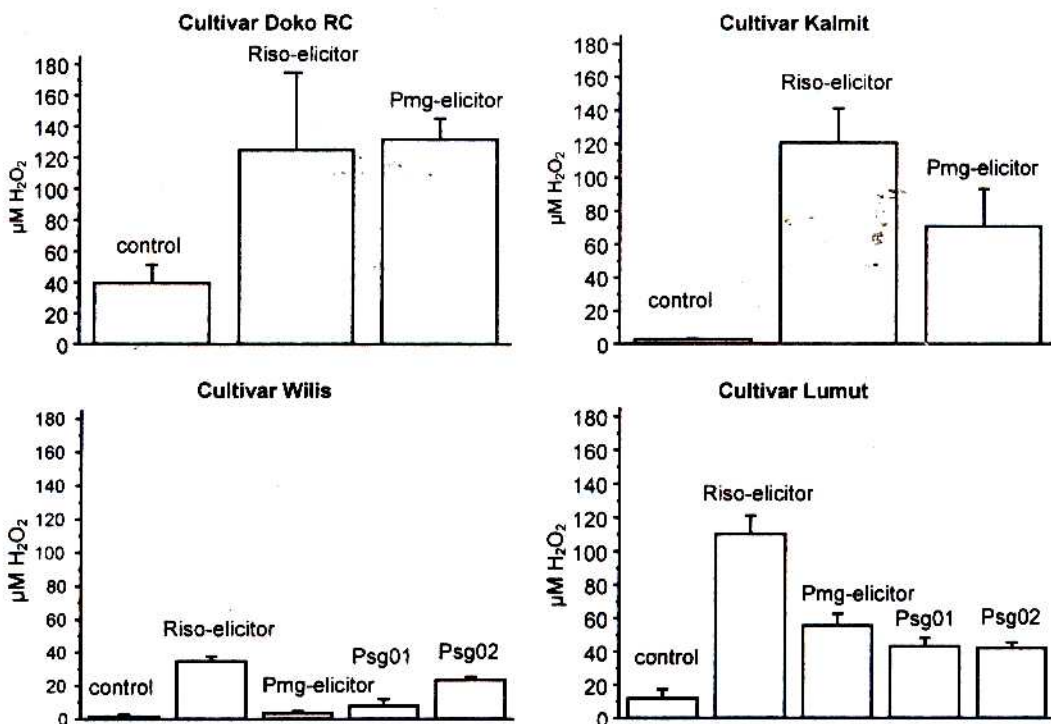


Notes: A. H<sub>2</sub>O<sub>2</sub>-formation was determined by luminol-dependent chemiluminescence (LMCL). The system was calibrated with a H<sub>2</sub>O<sub>2</sub> standard. For each elicitor-concentration the highest amount of H<sub>2</sub>O<sub>2</sub> after elicitation is shown. Cells were treated with crude cell wall extracts of *R. solani* (Riso-elicitor) or *P. sojae* (Pmg-elicitor)

B. H<sub>2</sub>O<sub>2</sub>-formation was determined by luminol-dependent chemiluminescence (LMCL). For each optical density (OD<sub>500</sub>) of the bacterial suspension the highest LMCL was determined. The highest LMCL of OD 0,2 was defined as 100%. Cells were treated with two isolates of *P. syringae* pv. *glycinea* (Psg01, 02)

Figure 9. Dose-dependency of the elicitor-induced oxidative burst in soybean cell suspension cultures of two cultivars

assumed that the elicitor preparations contain different elicitor-active components which may be perceived by specific receptors. Different cultivars seem to possess the same set of receptors, but probably they differ in sensitivity or the subsequent signal transductions are different.

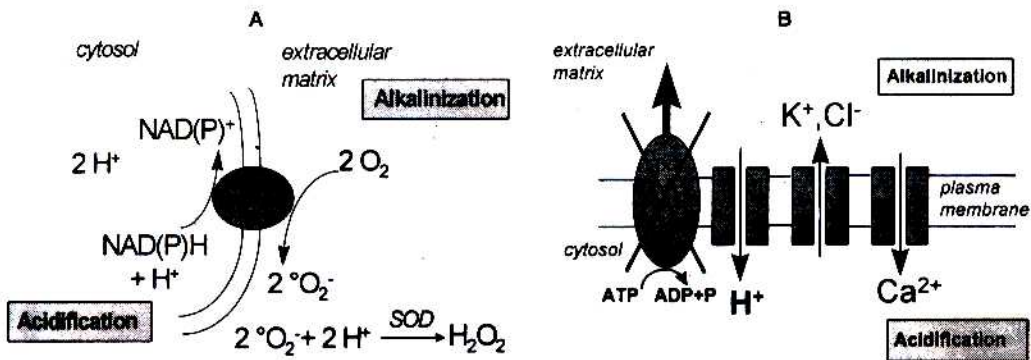


Notes: Cells were prepared as described in Figure 7. The elicitors used were crude cell wall extracts (100 µg/ml medium) of the fungal pathogens *R. solani* (Riso-elicitor) and *P. sojae* (Pmg-elicitor) as well as bacterial suspensions (optical density: 0.1; 50 µl/ml medium) of two isolates of *P. syringae* pv. *glycinea* (Psg01, 02). As control an equal volume of *A. bidez* or MES-buffer was added. H<sub>2</sub>O<sub>2</sub> was measured as luminol-dependent chemiluminescence. Results are means (+se) of three independent experiments. Variation among the two fungal elicitors and the cultivars except for cultivar Doko RC are significant at the 10 and 2.5% confidence level, respectively

Figure 10. Comparison of four soybean cultivars with regard to the maximal amounts of H<sub>2</sub>O<sub>2</sub> production induced after addition of different biotic elicitors

### Elicitor-Induced Changes of Extracellular Ph

Besides the oxidative burst another rapid response of plant cells to microbial signals is the alkalization of the cell culture medium (Tenhaken and Rübél, 1998). The biochemical function of the alkalization, as well as the detailed mechanism, are still unknown. Two hypotheses are discussed. On the one hand it is supposed that there is a causal relationship between the oxidative burst and the alkalization response. Most current models explaining the origin of the oxidative burst believe that a plasma membrane oxidase produces  $^{\circ}O_2^-$  through the reduction of external O<sub>2</sub>.  $^{\circ}O_2^-$  is then converted to H<sub>2</sub>O<sub>2</sub> consuming two protons from the apoplastic space (Figure 11A). On the other hand it is assumed that the two reactions are unrelated and the alkalization may be due to an inhibition of plasma membrane H<sup>+</sup>-ATPase and/or the opening of ion channels (Figure 11B).



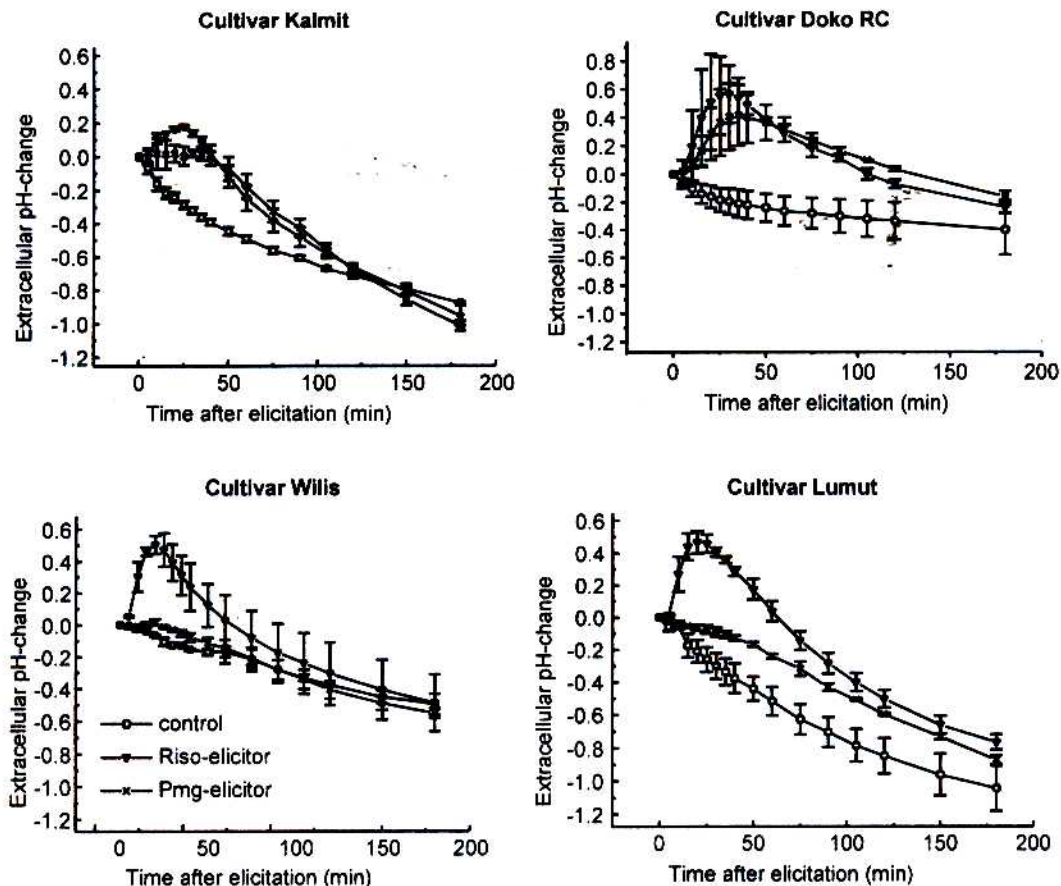
- Notes: A. It is supposed that H<sub>2</sub>O<sub>2</sub> is formed via the conversion of O<sub>2</sub><sup>-</sup> by superoxide dismutase, so that each molecule of H<sub>2</sub>O<sub>2</sub> needs two protons from the apoplast and thereby raises the pH
- B. Plasma membrane H<sup>+</sup>-ATPase is inhibited concomitant with an H<sup>+</sup>-influx and K<sup>+</sup>-efflux

**Figure 11.** Hypothetical models explaining the origin of the elicitor-induced alkalization of the culture medium

The fungal elicitors induced a transient pH-increase in the culture media of the four cultivars. pH-values started to rise 5 min after elicitation, reached a maximum after 20-40 min and around 70 min the initial pH-value was obtained again. This time-course and the intensities of the pH-response corresponded with the oxidative burst. Elicitors causing a strong oxidative burst were also capable of inducing a greater transient pH-increase (Figure 12). The maximal pH-increase stimulated by Riso-elicitor in cell suspension cultures of three cultivars was 0.5-0.6 pH-units. For cell suspension cultures of cv. Kalmit the increase was only 0.2 pH-units.

The strong correlation between the elicitor-stimulated oxidative burst and the extracellular pH-change indicated that there could be a causal relationship between the two reactions, but further analyses with an inhibitor of the oxidative burst (diphenyleneiodonium, DPI) showed that inhibition of the oxidative burst was stronger than suppression of the alkalization response (Figure 13). 25 μM DPI reduced the Riso-elicitor-induced oxidative burst by 65%, the extracellular alkalization decreased only by 25%.

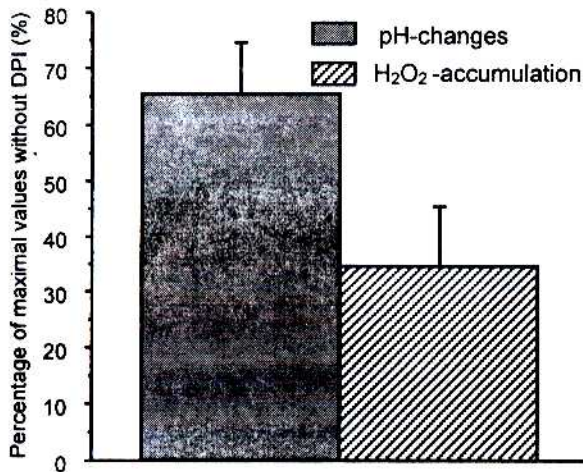
Therefore, proton consumption could not be the sole cause for alkalization. It is assumed that a transient inhibition of the plasma membrane-H<sup>+</sup>-ATPase together with an H<sup>+</sup>-influx and a K<sup>+</sup>-efflux led to the alkalization of the culture medium. In all cultivars the alkalization was followed by a strong acidification which is probably due to a reactivation of plasma membrane-H<sup>+</sup>-ATPase. The reversible activation of the plasma membrane-H<sup>+</sup>-ATPase seems to depend on phosphorylation/dephosphorylation by kinases and phosphatases (Felix *et al.*, 1991). Control cells as well showed an acidification of the culture medium. This is probably the consequence of the uptake of ions from the culture medium. Especially ammonium nutrition leads to an extracellular increase of protons (Kirkby and Mengel, 1962).



Notes: Five days after subculture cells were transferred to pH-medium (4% MS-salts, 3% sucrose), incubated for 2½ hours and then elicited. The elicitors were crude cell wall preparations (100 µg/ml medium) of *P. sojae* (Pmg-elicitor) and *R. solani* (Riso-elicitor). As control an equal volume of *A. bideist* was added in stead of elicitor. pH was measured continuously with a glass electrode. Results are means (±se) of three independent experiments

Figure 12. Comparison of four soybean cultivars with regard to extracellular pH-changes after addition of two fungal elicitors

In summary, the experiments indicate that the elicitor-induced alkalinization response and the production of H<sub>2</sub>O<sub>2</sub> are mostly independent. But because of the similarities in the intensity of the two reactions it is supposed that they are regulated by the same signal transduction pathway. Furthermore the elicitor-stimulated changes of extracellular pH underline the differential reactivity of various cultivars of one species.



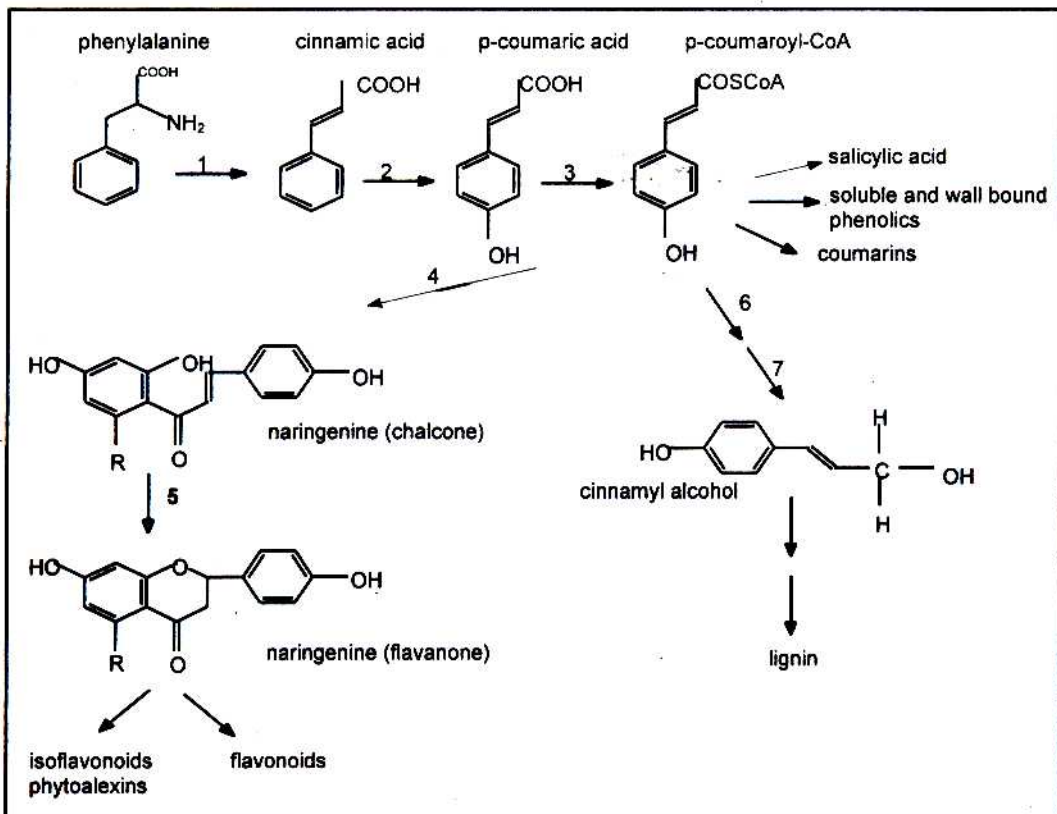
**Figure 13.** Influence of diphenyleneiodonium on the elicitor-induced oxidative burst and ion fluxes across the plasma membrane

### Induction of Phenylpropanoid Metabolism

Phenylpropanoid compounds encompass a wide range of structural classes and biological functions. All phenylpropanoids are derived from cinnamic acid, which is formed from phenylalanine by the action of phenylalanine ammonia-lyase (PAL). The phenylpropanoid metabolism is activated in response to a wide array of developmental and environmental cues, and, consequently, phenylpropanoids are synthesised during the normal process of development and under various stress conditions such as UV-irradiation, mechanical wounding, or pathogen attack (Dixon and Paiva, 1995). Fluctuation in PAL-activity has been shown to be a key step controlling the synthesis of phenylpropanoids, and in many cases, an increase in the amount of PAL-mRNA has been shown to underlie the increase in PAL-activity. The biosynthetic relationships among some of the classes of phenylpropanoids are outlined in Figure 14. Phenylpropanoid pathway supplies the precursors for flavonoid pigments, lignin, UV-protectants, salicylic acid, and isoflavonoid phytoalexins.

Phytoalexins have been defined as anti-microbial compounds of low molecular weight that both are synthesised by and accumulate in plants after exposure of the plant to micro-organisms. In the incompatible interaction between soybean and *P. sojae* accumulation of glyceollins was higher and faster than in the compatible interaction. It is assumed that the rapid production of phytoalexins is one factor that could limit the spread of the pathogens but is not the sole cause of resistance (Ebel 1986; Barz, 1997).

In soybean plants and cell suspension cultures the induction of PAL-activity correlated with an increase in enzymes of isoflavonoid metabolism and was followed by the accumulation of the soybean isoflavonoid phytoalexins glyceollin I-III (Habereder *et*

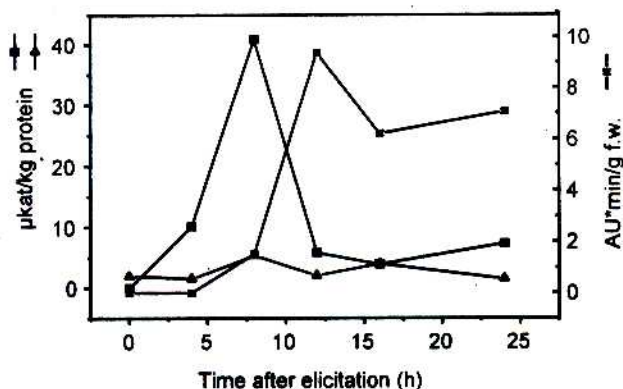


Notes: The enzymes marked by numbers are: 1 = -phenylalanine ammonia lyase, 2 = -cinnamate-4-hydroxylase, 3 = 4-coumarate:CoA ligase, 4 = -chalcone synthase, 5 = -chalcone isomerase, 6 = -cinnamoyl-CoA-oxidoreductase, 7 = -cinnamyl alcohol dehydrogenase

Figure 14. Schematic diagram illustrating the reactions of general phenylpropanoid metabolism and its branched specific pathways

*et al.*, 1989). Using yeast elicitor and cell suspension cultures of cv. Kalmit this result could be confirmed (Figure 15).

Comparative studies on the elicitor-induced activation of phenylpropanoid metabolism showed that in cell suspension cultures of the three soybean cultivars Wilis, Lumut and Kalmit PAL was stimulated by Riso-Elicitor and bacterial suspension. The enzyme activity of cells from cv. Wilis and Kalmit was much stronger after addition of Riso-elicitor than after application of bacterial suspension. Because of the high standard deviation there is no clear result for cells of cv. Lumut. Time courses and total activity varied between the cultivars and elicitors (Figure 16). The maximal enzyme activities in cell suspension cultures of cv. Lumut induced by Riso-elicitor was significantly higher (38  $\mu$ kat/kg protein) than for the two other cultivars (20  $\mu$ kat/kg protein).



Notes: Soybean cells were elicited with yeast elicitor (2 mg/ml medium) 5 days after subculturing. As control an equal volume of *A. bidesi* was added. After the indicated time cells were harvested and PAL-activity was determined. At the same time points the phytoalexin content of the medium was measured using HPLC. triangle-PAL-activity of control cells, square-PAL Elicitor-induced PAL-activity, asterix-Elicitor-induced glyceollin content

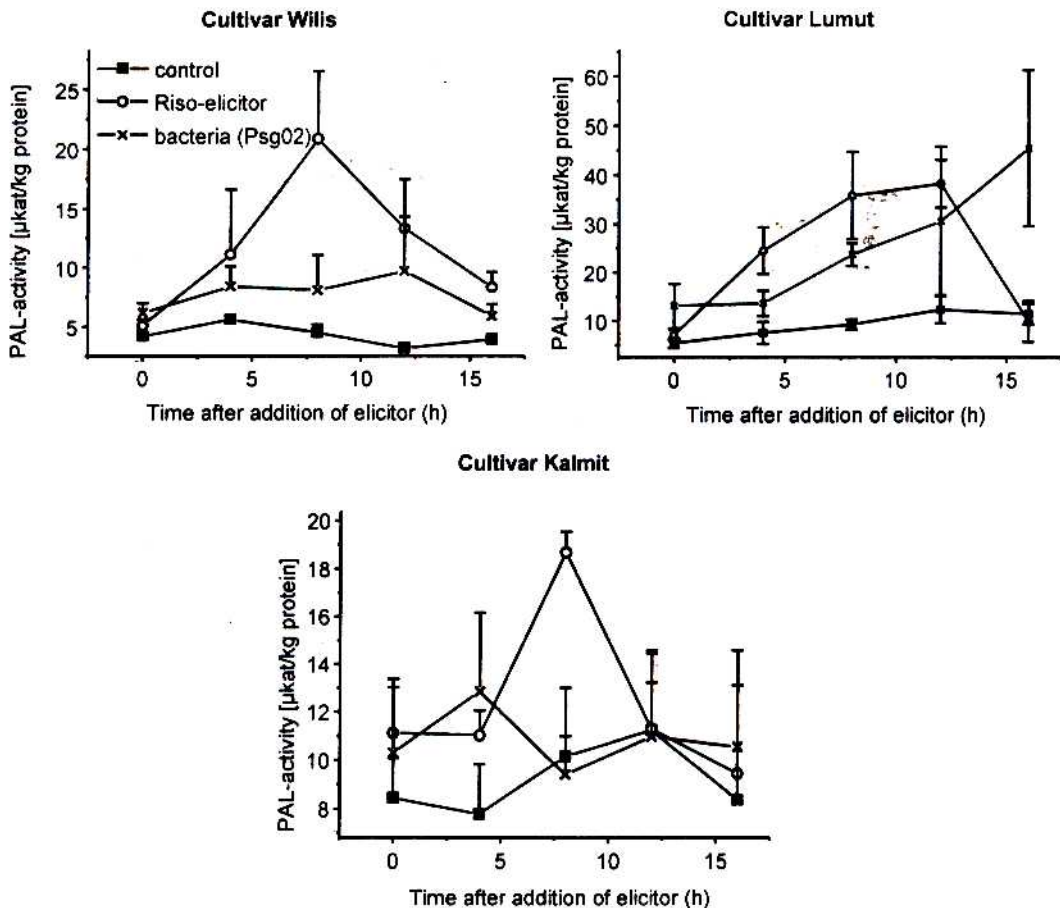
Figure 15. Elicitor-induced phenylalanine ammonia lyase (PAL) activity and phytoalexin content in the culture medium of soybean cell suspension culture

In contrast to the above cited literature and to the results obtained with yeast elicitor an accumulation of phytoalexins could not be observed in the three cultivars tested. Probably the whole isoflavonoid metabolism was not induced because there was no activation of its key enzyme chalcone synthase.

Other important elicitor-induced derivatives of phenylpropanoid metabolism are soluble and wall-bound phenolics. Biological functions of phenolics range from cell wall reinforcement, anti-microbial activity, plant hormones, or defence signalling compounds to scavengers of reactive oxygen species. Phenolics can be conjugated to various substances, for example carbohydrates, proteins or polyamines (Von Röpenack *et al.*, 1998). In cell suspension culture a visible sign for the secretion of phenolic compounds after elicitation is a browning reaction.

The fungal and bacterial elicitors tested induced an accumulation of soluble phenolics in the culture medium. There was a weakly stronger induction by the fungal elicitors than by the bacterial pathogens (Figure 17). Relative reactivity against the different elicitors was the same for the three cultivars but the total increase of soluble phenolics referring to control was higher in suspension cultures of cv. Lumut (160% after addition of Riso-elicitor) than of cv. Kalmit and Willis (120%). The amount of soluble phenolics correlated with the elicitor-stimulated increase in PAL-activity, so that it is suggested that derivatives of phenylpropanoid metabolism are used to produce soluble phenolics which are secreted in the culture medium.

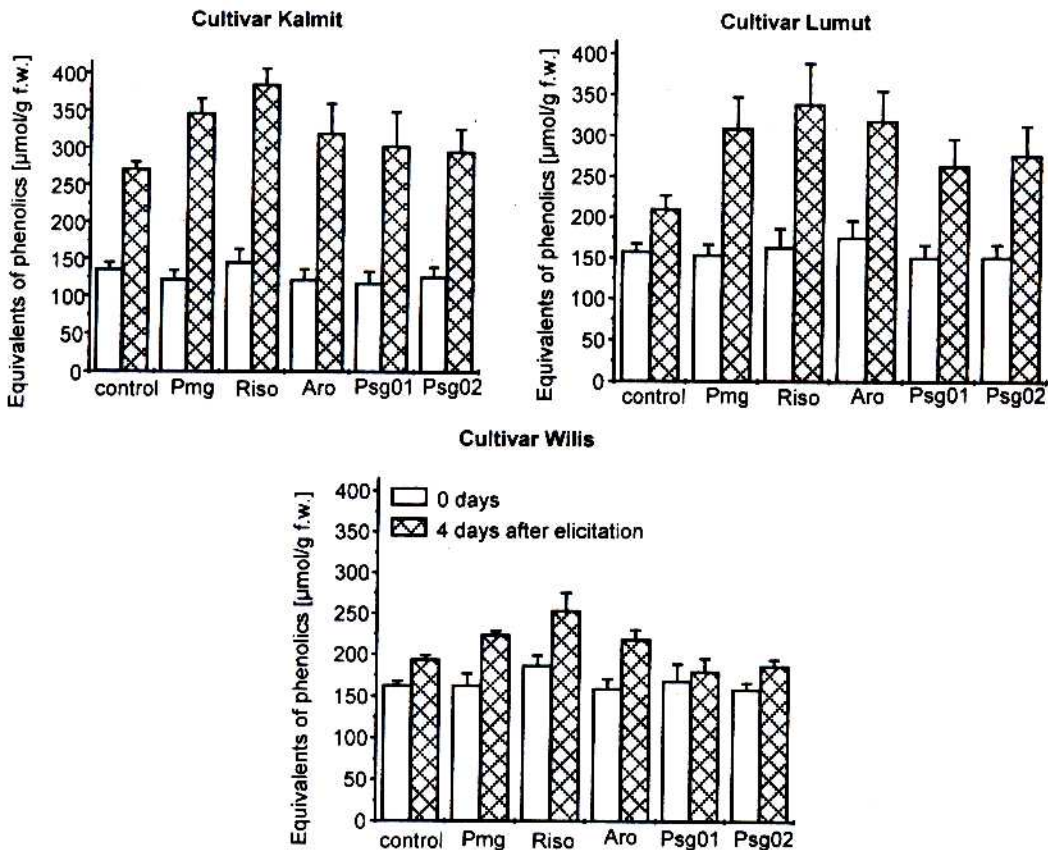




Notes: Five days old cell suspension cultures were elicited with a crude cell wall extract of *R. solani* (Riso-elicitor, 100 µg/ml medium) or with bacterial suspension (50 µl/ml medium, OD<sub>500</sub> = 0.2, Psg02). As control an equal volume of sterile MES-buffer (0.5 mM, pH 6.0) was added. Data are means (+se) of two independent experiments

Figure 16. Comparison of three soybean cultivars with regard to the elicitor-induced activity of phenylalanine ammonia lyase (PAL)

The data indicate that the late defence responses as demonstrated for the early defence mechanisms vary in intensity and duration depending on the cultivar and elicitor used. Besides the results show that the elicitor-stimulated activation of PAL probably provides the substances for the accumulation of soluble phenolics which are secreted in the culture medium.



Notes: Five days after subculture cells were transferred to fresh medium, incubated for 2½ h and then elicited. After 4 days the amount of phenolic compounds was determined in the culture medium using Folin Ciocalteus phenol reagent. Data are means (+se) of four independent experiments. Cells were treated with: *A. bidest* (control), crude cell wall extracts of *P. sojae* (Pmg), *R. solani* (Riso), *A. rolfsii* (Aro) or bacterial suspension of *P. syringae* pv. *glycinea* isolate 01 (Psg01), isolate 02 (Psg02)

Figure 17. Comparison of three soybean cultivars with regard to the elicitor-induced accumulation of soluble phenolics in the culture medium.

## CONCLUSION

The comparative studies of four soybean cultivars indicate that the reactivity of a cell suspension culture not only depends on concentration but as well on the elicitor tested. Furthermore cultivars differ in their reactivity. It is concluded that elicitors are recognised by receptors at the plasma membrane. For each elicitor there seems to be a receptor with a specific structure and sensibility. The different cultivars probably have the same set of receptors because they recognise the same stimuli but their sensitivity is supposed to be cultivar-specific.

With regard to selection of specific i.e. Al-resistant cultivars these results underline, that the intensity of one defence reaction is not sufficient as a marker for pathogen resistance. Using an *in vitro* selection system the observed great differences in the reactivity of the cultivars require the analyses of undisturbed defence reactions of each cultivar before and after the selection procedure.

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