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INDUCTION OF SYSTEMIC RESISTANCE IN BROAD BEAN TO RUST USING SALICYLIC ACID AND TWO DERIVATIVES

by

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A thesis submitted for the degree of

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ABBREVIATIONS USED IN THE THESIS

AcSa	: Acetyl-Salicylic acid
EDTA	: Ethylene-diaminetetraacetate dihydrate
Ina	: Isonicotinic acid
IR	: Induced resistance
HR	: Hypersensitive response
MW	: Molecular weight
NaSa	: Sodium Salicylate
ND	: Not detached
Pa	: Polyacrylic acid
PAL	: Phenylalanine ammonia-lyase
PR-	: Pathogenesis-related (protein)
p.v.	: Pathovar
RH	: Relative humidity
Sa	: Salicylic acid
sem	: Standard error of the mean
SR	: Systemic resistance
SIR	: Systemic induced resistance
TMV	: Tobacco mosaic virus
TNV	: Tobacco necrosis virus

SUMMARY

Investigations were made on the induction of systemic resistance (SR) to rust (*Uromyces viciae-fabae*) infection in broad bean (*Vicia faba*), using exogenous inducers. *In vivo* and *in vitro* experiments were carried out in order to study various aspects of the systemic induced resistance (SIR). In addition, studies on the possible involvement of endogenous salicylic acid (Sa) in SIR were initiated. Thus, in broad bean, SR to rust infection could be induced using Sa, acetylsalicylic acid (AcSa), and sodium salicylate (NaSa). SIR was expressed as a reduction in the number of pustules cm^{-2} of the challenge-inoculated leaf. Increasing the interval between treatment of the first leaves with Sa, NaSa, or AcSa and inoculation of the third leaves had little effect on SIR to rust infection in broad bean.

Sa, or a signal(s) produced after treatment with salicylates moved both acropetally and basipetally in broad bean plants, although the protection was greater in leaves above the induced leaves than below the induced leaves. A lag-period of at least 28 h after induction with Sa, NaSa, or AcSa was required before SR was expressed in broad bean. It is clear that the treated leaves were essential for SR to be induced, in the upper or lower leaves. Sa did not have a direct antifungal effect on broad bean rust, although NaSa and AcSa appeared to affect the rust directly. High concentrations of Sa, NaSa, and AcSa caused damage to the sprayed leaves, although the toxicity to plant tissue is unlikely to have caused the observed decrease in rust infection by Sa-, NaSa-, or AcSa treatments in experiments examining SIR. The effect of the salicylates on *in vitro* fungal growth was dependent on the particular fungus used (*Botrytis cinerea* or *Pyrenophora avenae*) and also on the pH of the medium. Growth reductions caused be the salicylates in these fungi

appeared the be the result of the low pH of the medium. However, it seems unlikely that the reduction in rust infection in upper or lower leaves, caused by salicylate-treatment of lower or upper leaves respectively, was due to a direct effect of these compounds on the fungus.

The variability between experiments observed in this work may reflect differences in environmental conditions, natural variability in broad bean (which is notoriously variable) or metabolism and/or conjugation of the salicylates.

Salicylic acid in plant tissue could not be determined using TLC followed by spectrophotometry or spectrofluorometry. Using these procedures it seems likely that Sa-conjugates and/or other phenolic compounds were also being detected.

INTRODUCTION

In practical agriculture, major efforts are made by the farmer to minimize losses in crop yields and include the application of crop protection compounds. However, substantial crop losses still occur every year due to plant diseases. Not all the pathogens which thrive on crops can be controlled by chemical means (Beicht, 1984). Because many pesticides are pathogen-specific, the pathogens fight back by overcoming the toxic effects of the compounds, thus becoming more resistant and more difficult to control. Intensive crop production increases disease pressure, resulting in greater use of chemicals, thus increasing resistance to chemicals. Enhanced awareness and care for the environment led to realisation that pesticides should be handled with more care. Application of pesticides is expensive and the development of new, less toxic compounds is even more expensive.

It is not surprising therefore that alternatives to conventional disease control are much sought-after. One such alternative is biological control. This can involve a direct control of the pathogen, using antagonist microorganisms e.g. the control of *Botrytis fabae* on broad bean using antagonistic bacterial and fungal isolates (Jackson *et al.*, 1991).

In addition to this direct control, there is also interest in more indirect approaches to biological control. Factors such as the environment cannot be controlled easily, but much research has concentrated on a better understanding of the physiological and pathological processes during disease resistance expression in plants. It has been suggested that the difference between resistant and susceptible plants is not that susceptible plants lack genes for resistance, but that these genes are not activated in susceptible plants (Kuc, 1987a). Much research effort is now being directed at understanding and ultimately manipulating the triggering of resistance genes in susceptible plants. This could lead to a novel and exciting addition to existing crop protection methods.

1 LITERATURE REVIEW

1.1 Resistance

Plants respond with postinfectional biochemical reactions after exposure to pathogens, in order to resist infection. These multiple biochemical responses result in resistance in the host or virulence in the pathogen. Disease resistance can be described as the capability to prevent, restrict, or delay the development of a disease, while virulence originates from the infectious agent and is the ability to overcome resistance (Bell, 1981). Both processes are found in high, moderate, or low levels, which results in different reactions in the various host/pathogen interactions. The differences derive from genetic differences both in the plant and in the pathogens (Dean and Kuc, 1985). Resistance levels are also strongly influenced by environmental conditions such as temperature and to a lesser extent also by light, moisture and nutrient levels. Plants react differently to invaders while they are in different developmental stages and this resistance varies depending upon the plant or tissue (Bell, 1981). Ultimately, disease resistance is likely to be a coordinate expression of many different resistance mechanisms which are not totally effective by themselves, but in concert they can restrict pathogen development (Pan *et al.*, 1992), and are the likely result of multiple genes for resistance mechanisms, or defense genes.

Resistance is but one of three protection mechanisms available to the plant (Kleinjan, 1990). The other two are tolerance, allowing the pathogen to develop within the plant without causing serious damage to the plant, and avoidance, in which the plant avoids contact with the potential pathogen.

The defence mechanisms of the plant consist of passive resistance, which is always present and includes mechanisms such as hairs or other physical barriers on the leaf surface, as well as the presence of permanent chemical protectants (Ryder *et al.*, 1986; Kleinjan, 1990). In addition to this passive or preexisting protection, plants have active systems of defence (Ryder *et al.*, 1986; Enyedi *et al.*, 1992). An intimate contact between the host and the pathogen is essential to activate this protection. Active resistance includes a number of resistance mechanisms, including lignification, the production of hydrolytic enzymes, the accumulation of phytoalexins and the hypersensitive response.

1.2 Systemic induced resistance

1.2.1 General

The phenomenon of systemic induced resistance (SIR) was already observed some centuries ago, although the first documentations are to be found in the 19th century (Chester, 1933). SIR derived from the concept that resistance or susceptibility are not absolute features in the plant. In most cases resistance or susceptibility are partial and gradual, and the magnitude of the disease suppression is strongly related to the particular host-pathogen interaction. This is due to the race-specific and qualitative character of the vertical (absolute) resistance, which is under the control of a single or a few genes, while in contrast, horizontal resistance is race-nonspecific and quantitative, controlled by multiple sets of genes (Ouchi, 1983). Thus, a lack of resistance in susceptible plants is likely not a result of the lack of genes for resistance, but rather that the genes in susceptible plants are not activated.

Systemic resistance (SR) can therefore be induced in susceptible plants by human interference, by activating and regulating the expression of the genetic potential for resistance mechanisms available in the plant. This should result in the maintenance of the activity of gene products for resistance and lead to the expression of resistance (Madamanchi and Kuc, 1991; Pan *et al.*, 1992). Thus, in susceptible plants a compatible interaction may be inhibited, preventing the spread of the pathogen by induction of SR. The induction is based upon the activation of plant defence mechanisms, which sensitizes plant tissue enabling the plant to respond rapidly to subsequent pathogen infections. An interaction which would be compatible under non-induced circumstances can therefore become an incompatible interaction (Madamanchi and Kuc, 1991).

Both in induced resistance in susceptible plants and in noninduced resistance, the same chemical processes seem to be responsible for protection, though there is a difference in rapidity and magnitude of gene expression coordinating the speed of recognition of plant pathogens. Pan *et al.* (1992) describes and summarises reports which found that the general metabolic responses associated with systemic induced resistance are similar, if not identical, to those associated with natural resistance of noninduced resistant plants.

The induced SR is effective against a wide range of pathogens, including fungal, bacterial, and viral pathogens. In addition, SIR possibly gives an enhanced protection against chemical and abiotic influences (Beicht, 1984). Unlike the usual specificity of plant-pathogen interactions, induction of SR is effective against representatives of all classes of pathogens. This can be achieved by inoculation of a single pathogen or treatment with a nonspecific chemical (see paragraph 1.2.2). In SIR often a cross protection against unrelated pathogens is established, e.g. fungi against viruses, or viruses against fungi, but it is always essential to inoculate with necrotrophic (or in some cases biotrophic) pathogens

in order to induce the systemic resistance (Métraux *et al.*, 1990). In tobacco for example, infection with *Colletotrichum lagenarium* or tobacco necrosis virus (TNV) was effective against obligate and facultative parasitic fungi, wilt fungi and bacteria, as well as viruses causing local and systemic lesions and those causing restricted and non-restricted lesions (Dean and Kuc, 1985).

SIR can last for several weeks e.g. broad bean (Murray, 1990), or for the life of annual plants, but is not seed-transmitted, with rare exceptions e.g. tobacco (Madamanchi and Kuc, 1991; Wieringa-Brant and Dekker, 1987). However, SIR can be transmitted to regenerants via tissue culture (Kuc, 1987b), and also young developing leaves are protected.

The protection which is obtained by SIR is rarely absolute. Instead, it is commonly expressed as fewer and/or smaller lesions. Thus, by inducing SR disease development is suppressed rather than completely eliminated. Induced resistance to tobacco mosaic virus (TMV) in tobacco expresses itself as a reduction of lesion size, rather than in differences in the number of hypersensitive response (HR) lesions on the leaf (Enyedi *et al.*, 1992) Most work on SIR is performed in the greenhouse or under laboratory conditions, but the induction can also be highly effective under field conditions, as demonstrated in tobacco against metalaxyl-tolerant strains of *Peronospora tabacina* in Mexico (Tuzun *et al.*, 1992). Growth and yield of induced plants are not reduced or are enhanced in the absence of the pathogen. However, Tuzun *et al.* (1986) used techniques in the field such as stem injection and high N fertilization to overcome stunting of systemically protected plants. Indeed, induced resistance (IR) has not been subjected to field tests intensively in order to determine its stability and persistence under high natural pathogen pressure. Although

some objections may be raised about experimental conditions, SIR can be considered as stable since it involves a variety of defence mechanisms in the plant (Strömberg, 1989). Supplemental N is required during the induction period in the plant to maintain yield at normal levels, and this demonstrates that SIR is an energy-demanding process (Sequeira, 1983). Indeed, recent work showed that in the upper leaves of broad bean, where systemic resistance had been induced by the prior inoculation of the lower leaves with rust, rates of net photosynthesis were increased (Murray and Walters, 1992). Moreover, if this increase in photosynthesis was reduced by shading, systemic resistance was also reduced, although not in proportion to the reduction in photosynthesis. Nevertheless, this suggests that the increase in photosynthesis in systemic-protected broad bean leaves was important in providing energy for the expression of resistance (Murray and Walters, 1992).

Systemic resistance could be compared to the protection mechanisms in humans and animals (described by Beicht, 1984). Because plant defences are mainly latent, they must be activated, as with immunization in humans and animals. It is difficult to compare SIR and immunization because of the fundamental differences in the protection against diseases between in animals and plants (Beicht, 1984). Nevertheless, there are similarities between them, although the differences seem to reveal themselves in some important factors which exist in animals and humans, but are lacking in plants. Humans and animals have a strictly separated blood circulation throughout the body and a local transport of fluid in intercellular spaces. Though plants do not have such a comparable circulation system, by using the xylem, phloem, and apoplast, transport of compounds is possible throughout the plant. However, plants seem to lack antigens which would confer protection against specific diseases. The presence of interferon in plants is debatable, but perhaps there are

substances which form part of the plant defence mechanisms which have comparable activities to interferon in humans/animals.

Since (S)IR is expressed in many plant/pathogen interactions and could be activated using various elicitors, disease resistance can be activated without the necessity of using toxic substances to control diseases, or changing the genetic information of the plant (Kuc, 1987a). However, practical application of SIR will depend on the longevity and range of protection of the (S)IR, and the development of reliable means of induced resistance (Beicht, 1984).

1.2.2 Plant defences induced during SR

Molecules that signal plants to start processes of biosynthesis are called elicitors (Darvill and Albersheim, 1984). SIR has a broad effectiveness, which strongly presumes that the SIR is a result not just of a single elicitor, but rather of the involvement of a multicomponent elicitor. The recognition by the plant of signals from elicitors results in a subsequent activation of one or more biochemical pathways and various other metabolic changes involving hormones, second messengers and enzymatic alterations, leading to formation of resistance related compounds (Malamy *et al.*, 1990). The elicitors stimulate the expression of those genes responsible for encoding the enzymes required for synthesis of resistance related compounds.

The difference between resistant plants which can inhibit pathogen development in plant tissue and susceptible plants, which cannot prevent the replication and spread of the pathogen, may be found in the ability of resistant plants to activate biosynthetic pathways leading to resistance, while susceptible plants fail to trigger such resistance responses upon

infection (Malamy *et al.*, 1990). Little is known about the effect of elicitors on gene expression, and to what extent elicitors are race/cultivar specific.

Whether or not a plant/pathogen relationship will lead to resistance responses should not be ascribed solely to the capability of the defence responses of the plant. Information provided by the pathogen during the interaction may also trigger plant defence mechanisms. When the wild-type strain of *Pseudomonas syringae* p.v. *syringae* was inoculated onto cucumber leaves, there was the systemic appearance of SR-related compounds, while, in contrast, the Tn5 mutant of that strain was unable to induce systemic resistance or peroxidase activity (Rasmussen *et al.*, 1991).

Systemic resistance can be induced using fungi, bacteria and viruses, when applied to plants using different inoculation techniques. Tobacco plants could be systemically protected against blue mold by inoculation with tobacco mosaic virus (TMV) or stem injection with sporangiospores of *Peronospora tabacina* (Pan *et al.*, 1992). The maximum expression of SIR was accompanied by activities of acidic chitinases C5 and C6 in systemically-protected leaves. Pan *et al.* (1992) described eight chitinase isozymes, including c1-c4, c7 and c8 which were always present in plant tissue, and c5 and c6 which increased significantly in systemically-protected plants. This provides further evidence that the induction of chitinases may be one of the general metabolic responses which are involved in resistance to many pathogens. A systemic increase in chitinase was also demonstrated in tobacco necrosis virus (TNV-) inoculated cucumber leaves, and was associated with reduced lesion formation and chitin deacetylase production by *Colletotrichum lagenarium* (Siegrist and Kauss, 1990).

Tobacco "N"-cultivars were persistently systemically protected against the virus TMV, the fungi *Peronospora tabacina* and *Phytophthora parasitica* var. *nicotianae*, and the

bacterium *Pseudomonas tabaci*, when prior controlled inoculation with TMV took place (Ajlan and Potter, 1992). It was possible to induce accumulation of PR-1 proteins in tobacco "nc"-cultivars when interstitial fluid extracts from an interspecific Nicotiana hybrid infected with TNV were injected into leaves of Xanthi-nc tobacco (Gordon-Weeks *et al.*, 1991). The accumulation was both at the site of the infection and further up the plant. Cucumber could be protected for several weeks against numerous pathogens when a leaf was prior-inoculated with the fungus *C. lagenarium*, the bacterium *Pseudomonas lachrymans*, or TNV (Taylor, 1987). An increase in the spore density used to induce SR increased the extent and the duration of the protection, which could be prolonged into the fruiting period by a second booster inoculation (Kuc and Richmond, 1977). In cucumber, systemically-protected by prior inoculation with *C. lagenarium*, a systemic increase in peroxidase activity was also observed (Madamanchi and Kuc, 1991), although increased levels of peroxidase activity in tobacco did not seem to be involved in disease defense (Nadolny and Sequeira, 1980). Uncertainties relating to the involvement in SIR of hydroxyproline-rich glycoproteins such as extensin have also not yet been elucidated (Madamanchi and Kuc, 1991). In immunized cucumber, penetration by appressoria of *C. lagenarium* is reduced and the development of *C. lagenarium* and *Cladosporium cucumerinum* was restricted by rapid lignification after challenge (Kuc, 1987a).

Some biotic elicitors of SIR are carbohydrates from fungal and plant cell walls, and microbial enzymes. Sequeira (1983) did not review reports of SR induced using surface polysaccharides or other cell-wall components, but suggested that SIR results from the release of endogenous compounds. However, their elicitation is still required. Schönbeck *et al.* (1980) report that SR was induced using fungal and bacterial culture filtrates, but it is suggested that the protectants were possibly neither lipid nor carbohydrate.

However, since chitin and glucanpolysaccharides such as ß-1,3-glucan are major cell wall components of many pathogenic fungi (Pan *et al.*, 1992; Darvill and Albersheim, 1984), it has been suggested that the enzymes involved in their breakdown, chitinase and ß-1,3-glucanase respectively, play a role in defense against pathogen attacks. Indeed, their enzymes are closely associated with the expression of induced resistance (Rasmussen *et al.*, 1991).

Indeed, the accumulation of ß-1,3-glucanase was preceded by activation of the corresponding gene near infection sites, which spread rapidly throughout potato leaves infected with *Phytophthora infestans*, as well as in uninfected leaves (Schröder *et al.*, 1992). These two enzymes degrade fungal cell walls partially (Scholtens-Toma *et al.*, 1991; Pan *et al.*, 1992) and their formation is induced by ethylene in response to fungal infection of cells (Salisbury and Ross, 1992; Schlumbaum *et al.*, 1986). Pan *et al.* (1992) also describes an increase in chitinase and glucanase activated in plant tissue as a result of ethylene, but also after infection and elicitor treatment. Given that these two enzymes together inhibit growth of most fungi tested, and that a parallel increase in activities may be essential for their function, it seems that chitinase and ß-1,3-glucanase play a role in host defense by inhibiting fungal growth (Schlumbaum *et al.*, 1986).

Olio- and polysaccharides such as ß-glucans and polysaccharides, containing high amounts of glucosyl, have also been shown to be elicitors of phytoalexins (Darvill and Albersheim, 1984). These saccharides were isolated from mycelial walls of various fungi. Also, glycoproteins of various compositions, identified as peptidogalactoglucomannans, were able to induce phytoalexin accumulation.

Chitosan, a polyamine isolated from some fungal cell walls, can also elicit phytoalexin accumulation, but may do that in a similar way to abiotic elicitors which damage cell walls

(Darvill and Albersheim, 1984). However, although the production of phytoalexins in cell suspension cultures of parsley was accompanied by rapid and dramatic increases in relative amounts of mRNA coding for PR-proteins, commonly associated with SIR (Somssich *et al.*, 1986), it is not fully established whether phytoalexins are involved in SIR.

In response to insect and pathogen attack, the synthesis of proteinase I and II are induced, which is a response to wound damage to leaves (Farmer and Ryan, 1992). The accumulation of proteinase I and II is observed both at the wound sites and in distal leaves, and could be induced by oligouronides from plant cell walls, the growth regulators abscisic acid and auxin, a polypeptide called systemin, methyl jasmonate, jasmonic acid (mentioned in Farmer and Ryan, 1992), as well as octadecanoid precursors of jasmonic acid (Farmer and Ryan, 1992). These authors suggest that octadecanoid precursors may participate in a lipid-based signalling system that activates proteinase inhibitor synthesis in response to insect and pathogen attack (Farmer and Ryan, 1992). However, it is not known whether jasmonic acid is involved in SIR.

Exogenous elicitors induce a response in the plant by mimicking particular aspects of pathogen attack, which are essential for the induction of resistance (Uknes *et al.*, 1992). A range of abiotic agents are known which can induce the molecular and biochemical systems in the plant, which express themselves as SIR. These chemicals have been shown to have no direct antibiotic effect, but act as elicitors of the latent immune-system of the plant (Kuc, 1987a; Uknes *et al.*, 1992). These elicitors includes naturally-occurring compounds and systemic chemicals (White, 1979 ; Métraux *et al.*, 1991). Abiotic elicitors may function in the activation of inactive biotic elicitors, and may therefore to interfere positively in compatible host-pathogen interactions, by inducing localized and/ or systemic resistance.

In 1959 it was observed that the resistance of apple to *Venturia inaequalis* could be increased using D- and DL-isomers of phenylalanine, but not with the L-isomers (Kuc, 1987a). Protection of pear seedlings against fire blight could be increased by injection of cell-free sonicates of *Erwinia amylovora* or *Erwinia herbicula* (Kuc, 1987a). In addition to induction with *Colletotrichum lindemuthianum*, SR could be induced in *Phaseolus vulgaris* to *C. lagenarium* using injection with conidia, culture filtrates of the pathogen or a dialysis retentate of these filtrates (Cloud and Deverall, 1987). Although the protection in the case of the pear/fire blight may not have been systemic, these reports may nevertheless indicate that endogenous compounds are required for the induction of SIR. However, work in *Arabidopsis thaliana* revealed alterations in the mycelium of *Peronospora parasitica* caused by the non-endogenous compound isonicotinic acid (Ina) which were similar to changes in mycelia of other fungi treated with ß-1,3-glucanase and chitinase or zeamatin (Mauch *et al.*, 1988; Roberts and Selitrennikoff, 1988, 1990; in Uknes *et al.*, 1992). Abiotic elicitors are apparently able to induce the same expression of PR-related proteins as pathogen infection (Uknes *et al.*, 1992) However, Métraux *et al.* (1991) noted a faster chitinase accumulation when systemic resistance in cucumber was induced with an isonicotinic acid (Ina) then after TNV-treatment. This may indicate that Ina influences resistance in a different way than pathogen infection.

A range of phosphate salts (K_3PO_4, K_2HPO_4, Na_3PO_4, and Na_2HPO_4) were able to induce SR to *C. lagenarium* in the third and fourth leaves, when the first and second leaves of cucumber were sprayed (Gottstein and Kuc, 1989). The SIR was not merely a result of an alkaline pH, chlorotic stippling or rapid necrotic cell death. Exogenously applied K_2HPO_4 induced a two-fold or higher increase in peroxidase and chitinase in untreated leaves, and induced SR to *C. lagenarium* in cucumber (Irving and Kuc, 1990).

K_3PO_4 also elicited increased resistance to rust infection in upper, untreated leaves, when the lower leaves were given an earlier treatment with the compound (Walters and Murray, 1992). These authors also observed that treatment of the first leaves of broad bean with EDTA induced SR, while a subsequent treatment with calcium nitrate abolished the effects of K_3PO_4 and EDTA. These authors and Gottstein and Kuc (1989) suggest that the phosphates sequester intracellular calcium, thereby damaging cell walls and releasing fragments of cell walls. These cell wall fragments, in turn, elicit resistance responses and/or lead to the production of systemic signals which induce resistance elsewhere in the plant. It is suggested that oxalates, extracted from spinach and rhubarb leaves, have similar effects to phosphates in the induction of SR in cucumber to *C. lagenarium* (Doubrava *et al.*, 1988).

1.2.3 Involvement of a signal in SIR

Since SR can be induced in parts of the plant distal from the site of infection or treatment, a signal is essential to sensitise those sites following a primary induction. In tobacco, induction of SR in distal parts of the plant could be inhibited by girdling of the stem above the site of inoculation, but below the site of infection SR was still observed (Tuzun and Kuc, 1985). These authors suggested the presence of a protection factor which was produced either by the plant or by the fungus growing in the plant tissue. This factor may be an antifungal compound, or may condition cells to become resistant. Tuzun and Kuc (1985) suggested that it is a signal for the induction of SR. The signal (or signals) are unlikely to be of pathogen origin entirely, since many forms of induction can be used, including biotic elicitors of pathogen and plant origin, as well as chemicals occurring naturally in the plant, and exogenous and synthetic compounds. Work by Dean and Kuc

(1986) suggests that the infected leaf is the source of the signal (or signals) and that the signal(s) is (are) not remobilized from, or produced in, systemically-protected leaves. It seems therefore that a persistent stress in the inducer leaf is required to produce a continuous supply of the signal (Kuc, 1983; Dean and Kuc, 1985; Doubrava *et al.*, 1988). The signal appears to be translocated in the outer phloem, in which a bi-directional movement of compounds occurs. Therefore, the signal may be translocated through the plant both acropetally as well as basipetally, possibly protecting all plant tissues. The observed lag-period between the induction and the expression of SR also supports the production and movement of a signal. Indeed, in recent work (Métraux *et al.*, 1990) where SR was induced by prior induction of the lower leaves of cucumber with *C. lagenarium*, changes in the content of the phloem sap revealed an increase of a fluorescent metabolite after infection, which reached a peak before SIR was observed. The compound was identified as salicylic acid, a known exogenous inducer of resistance (Van Loon, 1985). It was observed that in parallel with the increase in salicylic acid levels, there was also an increase in pathogenesis-related (PR-) proteins (Yalpani *et al.*, 1991). These results suggest that salicylic acid is an endogenous signal involved in the induction of SR, including local and systemic induction of PR-1 proteins and possibly other compounds related to SIR (Métraux *et al.*, 1990; Malamy *et al.*, 1990; Yalpani *et al.*, 1991).

1.3 Salicylic acid in plants

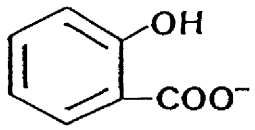
1.3.1 History of salicylates

Salicylic acid (Sa) belongs to a special group of plant phenolics, the salicylates. A brief historic background of salicylates has been given by Raskin (1992a). Parts of plants were used to cure aches and fevers or to relieve pains long before scientists discovered the therapeutic effects of salicylates. In the 19th century, salicylates were isolated from a variety of plants such as spirea, wintergreen, and willow. The isolated salicylates were mainly methylesters and glucosides, which are easily converted to Sa. The name salicylic acid originates from the Latin *Salix*, a willow tree, and was given to the compound by Raffaele Piria in 1838. Synthetic Sa was marketed commercially in 1874, but was replaced by acetylsalicylic acid (AcSa, trade name Aspirin) in 1898. Aspirin was not as irritating to the digestive system, but was as effective as Sa and is now one of the best-selling drugs in the world. Despite the common use, the medical mode of action of salicylates is still not clearly understood.

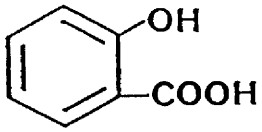
1.3.2 Some properties of Sa and derivatives

Salicylates are an exceptionally varied group of plant phenolics, and are defined as substances that possess an aromatic ring, bearing a hydroxyl group or its functional derivative such as non-aromatic ring structures. The molecular structure of the basic form of a salicylate, and of compounds used in this research are (see following page):

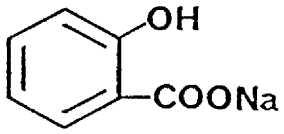
Salicylate; molecular weight (MW) = 137.1;



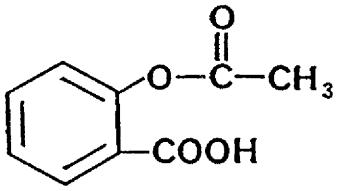
Salicylic acid (Sa); 2-hydroxybenzoic acid; MW = 138.1;



Sodium salicylate (NaSa); MW = 160.1;



Acetylsalicylic acid (AcSa); MW = 180.2;



Free Sa is a crystalline powder, with a melting point of 157-159 °C. Its solubility in water is moderate, but very good in polar organic solvents. The pH of a saturated aqueous solution of Sa is 2.4. Sa fluoresces at 412 nm when excited at 301 nm. Yalpani *et al.*(1991) mention a recently developed mathematical model, which indicates that the physical properties of Sa are almost ideal for long-distance transport in the phloem.

Sa seems to be ubiquitous in the plant kingdom, since the presence of Sa was confirmed in leaves and reproductive structures of 34 species of agronomical importance (Raskin *et al.*, 1990). The Sa levels in rice, crabgrass, green foxtail, barley, and soybean even exceeded 1 μg g^{-1} fresh weight. The highest levels of Sa were observed in the inflorescence of thermogenic plants and in plants infected with necrotizing pathogens. Salisbury and Ross (1992) and Raskin (1992b) suggest that endogenous Sa is a plant hormone, since it clearly meets the necessary criteria.

When aspirin is dissolved in aqueous solutions, AcSa will be hydrolysed spontaneously to Sa. Research in medical circles has shown that the hydrolysis of aspirin in blood plasma is catalyzed by nonspecific arylesterases. Since these arylesterases occur in most living tissues, it is assumed that in plants, exogenously applied aspirin is quickly converted to Sa (Raskin, 1992a). Because the results obtained using aspirin were similar to those using Sa, many scientists use both AcSa and Sa, although AcSa does not occur naturally in plant tissues. In animals, aspirin can block prostaglandin biosynthesis by donating an acetyl-group, but this important difference between aspirin and Sa in not yet known to be relevant in plants, because prostaglandins have not been identified in plants.

1.3.3 Effects of Sa on flowering and heat production

Sa is an important endogenous messenger in thermogenic plants. A well-known physiological phenomenon is heat production and production of aromas (due to amines and indoles) in the appendix of the inflorescence of *Arum* lilies (Salisbury and Ross, 1992; Raskin, 1992ab). Recent research has shown that Sa, produced in staminate flower primordia, is translocated to the appendix where it induces processes leading to heat production and volatilization of the closely linked scent production in thermogenic plants (Meeuse and Raskin, 1988). In addition to the activation of the alternative oxidase, the cyanide-resistant non-phosphorylating electron transport pathway in plant mitochondria, thermogenicity involves activation of the glycolytic and Krebs cycle enzymes.

Cleland describes Sa as "a naturally occurring regulator", with a main effect on flower initiation, and a slight stimulating effect on flower development. Changes in the critical daylength required for flower induction could be caused by using concentrations of Sa as low as 3.2 μM (Cleland and Tanaka, 1979). Sa inhibits ethylene biosynthesis in pear cell

suspension cultures and thus Sa also seems to be involved in the mechanism that increases flower longevity (Raskin, 1992ab), although no effects on ethylene production were observed in soybean cuttings. However, flowering induction can be induced by the exogenous application of many monohydroxybenzoic acids and other molecules. Although there is a well-established role for Sa in thermogenicity, the precise role of Sa in flower induction is still unclear.

1.3.4 Effects of Sa on disease resistance

Another possible role of Sa in plants is in the promotion of resistance to certain plant pathogens. Treatment with Sa or AcSa can induce at least some resistance to pathogens, and stimulates the production of some pathogenesis-related (PR-) proteins in most systems studied, except of Soybean (Salisbury and Ross, 1992; Raskin, 1992a). Xanthi-nc tobacco, containing the "N" gene for hypersensitive (HR) responses to TMV, was used in experiments in 1979 to reveal the protective function of salicylates (White, 1979). Application of Sa and AcSa in various ways (injection, spraying, or watering via the soil) prior to TMV inoculation resulted in reductions in lesion number and lesion size, the magnitude of which was dependent on the mode of application. Also, when cucumber plants were inoculated with *Colletotrichum lagenarium* after treatment with exogenous Sa, subsequent resistance was observed as reductions of total necrotic area (lesion size) and decreased appressorium penetrations. No visible direct effects of Sa on spore germination or appressorium formation were observed, while toxicity of Sa to cucumber tissue was observed when concentrations of 10 mM or higher were used (Rasmussen *et al.*, 1991). Differences in IR were observed between young cotyledons and older cotyledons (Rasmussen *et al.*, 1991; Mills and Wood, 1984).

In response to TMV inoculation or Sa application, an increase of mRNAs coding for basic and acidic isozymes of ß-1,3-glucanase, a member of the PR-2 group, was observed (Linthorst *et al.*, 1990). Aspirin also induced synthesis of PR-1 proteins at the transcription level, while Sa stimulated the accumulation of mRNA coding for extracelluar endochitinase belonging to the PR-3 group of proteins. This endochitinase could also be induced systemically using viral, bacterial, or fungal infections (Métraux *et al.*, 1988).

Apart from Sa and aspirin, only a related compound, 2,6-dihydroxybenzoic acid could induce PR-proteins and resistance to viruses, without increasing ethylene biosynthesis (Van Loon, 1983). However, it is not clear to what extent the resistance induced by Sa is caused by an stimulation of PR-protein production. It has been suggested that Sa-signalling may be essential for some resistance responses e.g. PR-gene and SIR expression, but is not necessarily involved in the establishment of localized induced resistance expressed as necrosis formation and HR (Malamy *et al.*, 1992).

There are two reports of Sa suppressing resistance mechanisms (Raskin, 1992a). Thus, Sa pretreatment of tomato plants inhibited the systemic accumulation of proteinase inhibitor, which may provide some protection against insect predation (Doherty *et al.*, 1988). In addition, when Sa was added to a fungal extract containing elicitors of resistance in carnation cuttings, no phytoalexin synthesis took place and the cuttings remained susceptible to subsequent infection of *Phytophthora parasitica* (Ponchet, 1983).

Isonicotinic acid (Ina), although not phenolic, is also an immunomodulator of induced resistance in plants (Métraux *et al.*, 1991; Uknes *et al.*, 1992). In may be analogous to the proposed endogenous signal (Ward *et al.*, 1991), but can only act as an exogenous elicitor since Ina does not occur naturally in plants.

1.3.5 Sa as a signal in SIR

The involvement of a signal in systemic induced resistance has been hypothesized and is the subject of intensive studies in the tobacco-TMV interaction, where resistance is single-gene inherited and therefore very useful in these studies (Raskin, 1992a). The physical properties of Sa are nearly ideal for long-distance transport in the phloem and Sa could therefore be translocated rapidly from the application site or point of synthesis to other parts of the plant (Yalpani *et al.*, 1991). It has been suggested that although Sa is rapidly conjugated in tobacco, it is the free form of Sa that is phloem-mobile (Enyedi *et al.*, 1992). In a study of SIR in tobacco, Sa levels in TMV-resistant tobacco increased almost 50-fold in TMV-inoculated leaves, but also increased 10-fold in uninfected leaves, in parallel with the induction of PR-1 genes. Sa was also transported from the infection site to uninfected parts of the plant (Yalpani *et al*, 1991), although the highest concentrations of Sa in its free form were measured in and around hypersensitive lesions on the inoculated leaf (Malamy *et al.*, 1990).

Research in cucumber inoculated with tobacco necrosis virus (TNV) or the fungal pathogen *Colletotrichum lagenarium*, detected transient increases in a fluorescent metabolite, identified as Sa, in the phloem sap after induction (Métraux *et al*, 1990). These authors concluded that the appearance of Sa was dependent on the timing of disease development of the induced leaf, since a more rapid accumulation of Sa resulted from TNV inoculation compared to *C. lagenarium* infection. Symptom development following the *C. lagenarium* infection took 2 days longer than following the TNV infection (Métraux *et al.*, 1990).

Typically, the amount of exogenously applied Sa required to induce SIR is much higher than the concentrations of Sa measured in the phloem after induction with TNV or *C.*

lagenarium. Experiments with ^{14}C-labelled Sa showed a rapid translocation of the organic acid, but a major proportion of the applied Sa was either sequestered or metabolized. It is concluded that a rapid turn-over of exogenously applied Sa takes place (Métraux *et al.*, 1990). Recent work by Rasmussen *et al.* (1991) showed that SR could be induced in the upper leaves of cucumber just 4 h after the leaves were inoculated with *Pseudomonas syringae*. However, increased Sa concentrations in the phloem sap moving out of the induced leaf were not detected until 8 h after inoculation. Those authors suggested therefore that although Sa was involved in SIR in cucumber, it was not the primary signal. However, it has been suggested that the relatively insensitive analytical method employed in the study of Rasmussen and co-workers does not preclude the possibility that there was a sufficient amount of Sa present in the phloem moving out of the inoculated leaves within the first 4 h to induce SR, thus still functioning as a primary signal (Raskin, 1992a). However, the role of Sa as the primary signal in SIR could not be confirmed by Enyedi *et al.* (1992), working on tobacco/TMV since they could not determine whether the systemic increases in Sa were due to the phloem transport of Sa, a conjugate, or another mobile signal molecule produced after infection.

1.3.6 Sa conjugates

Concomitant with the increases in free Sa following inoculation of tobacco with TMV, levels of Sa conjugates increased substantially above those of free Sa, whereas in uninfected or mock-inoculated plants only very low concentrations of Sa conjugates were present (Malamy *et al.*, 1992). These authors were about to show that Sa conjugates, mainly glucosides, were newly synthesized, like free Sa, after infection. Free phenolic acids like Sa are likely to be converted to sugar conjugates in the plant, since the acids in

their free form may be toxic to the plant (Harborne, 1980) Conjugation of *de novo* synthesized Sa in large quantities may therefore be a common self protection process for detoxifying free phenolic acids after pathogen infection (Malamy *et al.*, 1992). It appears likely that exogenously applied Sa will be converted to the glucoside, which is the same conjugate as observed in infected tissue.

1.3.7 Biosynthesis of Sa

Research should be intensified on the link between pathogen-induced necrosis and the induction of SIR, since the activity of phenylalanine ammonia-lyase (PAL) is considerably increased by necrosis induced by TMV in tobacco and other plant-pathogen interactions (Ward *et al.*, 1991). PAL is necessary for the conversion of phenylalanine to *trans*-cinnamic acid, which in turn can be transformed to Sa. It is not known whether, in conjunction with the activation of PAL, enzymes of Sa biosynthesis are also activated during necrosis. Although the Sa that accumulates during SIR may come from the pathogen, it is more likely that the accumulation is a result of the plant/pathogen interaction. Not known is the intracellular site of Sa synthesis, although increased levels are observed in leaf tissue surrounding lesions of HR in TMV-infected tobacco (Enyedi *et al.*, 1992).

The pathway involved in the biosynthesis of Sa is likely to be the shikimic acid pathway, because the formation of benzoic acids in plants is accomplished mainly by the side-chain degradation of cinnamic acids, which are important intermediates in this pathway (Gross, 1981). It is likely that Sa can be formed in plants from cinnamic acid from either benzoic acid or *o*-couramic acid (Chadha and Brown, 1974). In infected young tomato plants, the *ortho*-hydroxylation of cinnamic acid to *o*-couramic acid, followed by

its ß-oxidation to Sa, was increased, while in non-infected plants the pathway via benzoic acid was most active (Raskin, 1992a).

Trans-cinnamic acid, when hydroxylated by *trans*-cinnamate-4-hydroxylase, eventually forms lignin, flavonoids, and ubiquinone. The enzymes which convert *trans*-cinnamic acid into *o*-couramic acid might be cytochrome P-450 monoxygenases, some of which catalyze gibberellin biosynthesis and herbicide metabolism (West, 1980). However, little is known about the enzymes involved in the biosynthesis of Sa from cinnamic acid.

1.4 Environmental conditions for SIR

The induction of SR to TMV infection in tobacco is temperature dependent (Malamy *et al.*, 1992). At 32°C no PR-1 gene induction or necrotic lesion formation took place and no rise in Sa levels in response to TMV inoculation was observed. However, when TMV inoculated tobacco plants were shifted to lower temperatures, necrotic lesions and PR-1 mRNAs appeared, and prior to those changes the block of Sa production was eliminated, leading to a dramatic increase in Sa. These results suggest a link between changes in Sa levels and induction of PR-genes, and the hypersensitive response. The factor which is responsible for the temperature sensitivity of SIR in tobacco/TMV has been shown to precede the production of endogenous Sa (Malamy *et al.*, 1992). Light sensitivity of SIR in tobacco was observed since Sa accumulation, Sa exudation and the number of lesions were approximately twice as great under light conditions than in the dark (Yalpani *et al.*, 1991). This last observation is interesting, since the induction of systemic resistance against obligately biotrophic fungi needs special conditioning in the plant if there is to be

successful and efficient (Falkhof *et al.*, 1988). It is well-established that changes in light, temperature, water potential and nutritional status of the plant can affect plant disease resistance (Bell, 1981). This indicates that results obtained under isolated and artificial standard conditions in the laboratory may have to be interpreted with a certain amount of reserve when they are applied to more natural circumstances or to plants in their natural environment (Falkhof *et al.*, 1988).

1.5 Description of host and pathogen used in this study

1.5.1 Host

Broad bean (*Vicia faba* L.) belongs to the order *Leguminasae*, which produce the so called nitrogen root nodules via the symbiotic interaction of the plant with root infectious bacteria of the genus *Rhizobium*. The bean is a papilionaceous flowering plant of the genera *Vicia* and the species *faba*. In the faba group 3 subspecies occur: *V. faba major* L., originating from the Mediterranean area and which was used in this project; *V. faba equina* L. and *V. faba minor* L., originating from West-Asiatic areas. Unripe seeds, cooked or raw, are used for human consumption and is a good source of protein, vitamins, and a moderate source of minerals. The typical taste originates from tannin, and as disadvantageous compounds are seen glucosides, hemagglutins and trypsine-inhibitors. Major production areas in Europe are Great-Britain, Germany, Italy, France, Spain and the Netherlands. The faba bean is also grown in the Middle East, North Africa, Australia and Western Canada (Neuvel, 1991).

1.5.2 Pathogen

The rust used in this study was *Uromyces viciae-fabae* (Pers.) Schroet and belongs to the family of *Pucciniaceae* of the order *Uredinales* (Drok, 1988). It belongs together with the *Ustilaginaes* to the *Teliomycetidae*, a subclass of the *Basidiomycetes*, and are separated on the basis of the different appearance of the basidium on the teliospore. Rust is an obligately biotrophic parasite and a common disease of faba bean, obtaining its nutrients entirely from the host. Yield losses of 5-20 % in Egypt and up to 45 % in Australia have been reported (in Rashid and Bernier, 1991). Crop protection could increase the grain yield and thousand-kernel weight of plots in England by 17 % and 16 %. In Canada 11 races of *U. viciae-fabae* infecting the genera of *Vicia*, *Lathyrus*, and *Pisum* have been identified, with substantial variability in pathogenicity (Rashid and Bernier, 1991). Differences in varieties of *Vicia faba* show that there are slow-rusting varieties, which are more tolerant to rust than other populations, indicating different resistance responses of different populations (Rashid and Bernier, 1991).

1.6 Aims of the project

The main aim of the project was to investigate the induction of systemic resistance to rust in broad bean using salicylic acid and two derivatives. Various aspects of the use of those compounds as inducers of systemic resistance were studied.

A secondary aim of the project was to initiate studies on the detection of salicylic acid in plant tissues as a foundation for future work in this laboratory.

2 MATERIALS AND METHODS

2.1 Induction of systemic resistance in broad bean to rust using salicylic acid, sodium salicylate, and acetylsalicylic acid

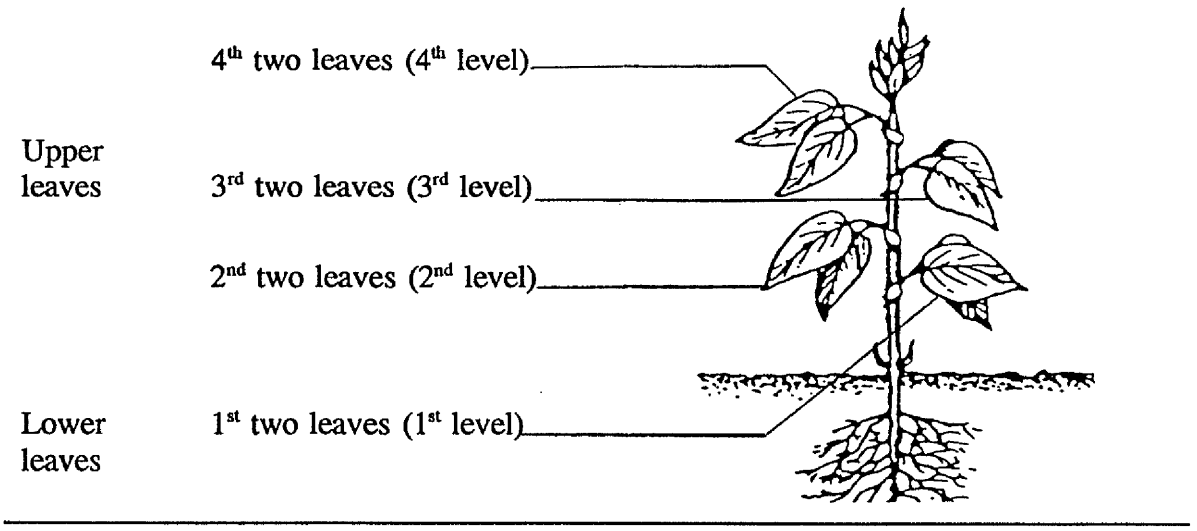
There are only three reports in the literature that systemic resistance (SR) can be induced in broad bean (*Vicia faba*) (Murray, 1990; Murray and Walters, 1992; Walters and Murray, 1992). In this experiment the ability of salicylic acid (Sa), sodium salicylate (NaSa), and acetylsalicylic acid (AcSa) to induce SR to rust infection in broad bean was examined.

2.1.1 Plant material

Seeds of broad bean (*Vicia faba* L. cv Bunyard Exhibition) were germinated and grown in Fisons Levington M3 compost in a heated greenhouse, using three seeds per pot. The average day temperature during the course of the experiment was 20°C, with mean maximums of 22-25°C during the day and mean minimums of 14-17°C at night. In addition to natural daylight plants were grown under 400 W mercury vapour lamps to provide a 16 h photoperiod during October-March. Mean irradiation in the greenhouse at midday was 600 μmol m^{-2} s^{-1} photosynthetically-active radiation (PAR). Plants were used for experiments when most of them had six fully-developed leaves (3 pairs) and two young developing leaves (i.e. when the plants were $\pm$ 22 days old; Fig 1).

Bean plants were supported with wooden sticks during most of the experiment. During the course of the experiments plants were watered only, and were not provided with nutrients.

Figure 1. Diagram to indicate the leaves used in the experiments.



2.1.2 Maintenance of the pathogen

The fungus *Uromyces viciae-fabae* (Pers.) Schroet was maintained on broad bean plants, which were kept in the greenhouse. Beans were grown as described in paragraph 2.1.1 and when the plants had three fully-developed levels of leaves, all leaves were sequentially inoculated with rust as described in paragraph 2.1.3, using spores which had developed on pustules ± 2½ weeks after inoculation.

2.1.3 Induction of systemic resistance

Systemic resistance was induced by spraying leaves to run-off with solutions of Sa, NaSa, and AcSa in 0.01 % (v/v) Tween 80 (BDH chemicals; to act as a surfactant) and 10 % (v/v) acetone (to dissolve Sa and AcSa). In this experiment the upper surfaces of the first level of two leaves were treated in this way. In these initial experiments, three concentrations (5 mM, 10 mM, and 20 mM) of the compounds were used. In some later

treatments, the concentration which gave the best results in the initial experiments was used, in order to reduce the number of assessments. Two controls were used in this experiment: one control received no treatment but was challenge-inoculated, while the other control was treated with the spray solution (0.01 % (v/v) Tween 80 and 10 % (v/v) acetone) only and was challenge-inoculated. The pH of all spray solutions was adjusted to pH 7, using NaOH and HCl. Plants were treated in a special pesticide application room and after induction, were left for 2 h to let the treated leaves dry. They were then returned to the greenhouse.

2.1.4 Challenge-inoculation of systemic induced plants

Treated plants were inoculated with the rust *U. viciae-fabae* one day after induction, by painting a spore suspension in 0.01% (v/v) Tween 80 onto the upper surfaces of third level leaves. Control plants were challenge-inoculated like the other treatments. Inoculated plants were covered with clear plastic bags for 2 days in order to provide the high relative humidity necessary for infection and development of the fungus. In most experiments an average spore solution concentration of 5×10^4 spores ml^{-1} was used, unless otherwise stated. Spores were collected from stock plants $\pm$ 2½ weeks after inoculation.

Rust infection was assessed by counting the number of rust pustules on the leaves, 14 days after inoculation. This was then calculated as the number of pustules per cm^2 of leaf. In all experiments, the plants were placed at random on the benches in the greenhouse.

2.2 Effect of varying the interval between treatment and challenge-inoculation, on the development of systemic induced resistance

In some cases, induction of SR has been reported to protect plants from pathogen attacks throughout the life of the plant (Strömberg, 1989; Kuc, 1987a; Tuzun and Kuc, 1987). In this experiment an examination was made of the longevity of systemic induced resistance (SIR) in broad bean to rust infection.

2.2.1 Plant material and maintenance of the pathogen

Broad bean plants were grown as described in paragraph 2.1.1 and the rust fungus was maintained as described in paragraph 2.1.2. The plants were grown under conditions that were approximately 3°C warmer than described in par. 2.1.1.

2.2.2 Induction of systemic resistance

Plants were induced by spraying leaves on the first level as described in paragraph 2.1.3. To make this experiment more manageable, only one concentration of each chemical was used: Sa, 10 mM; NaSa, 5 mM; and AcSa, 10 mM. These concentrations were chosen, based on the results obtained in the previous experiment.

2.2.3 Challenge-inoculation of systemic induced plants

Plants were inoculated on the third leaf level as described in paragraph 2.1.4. The interval between induction and challenge-inoculation was varied from one to seven days.

The spore concentrations used to inoculate the plants in the experiment were as follows:

1 day : 3.8×10^4 (± 0.30)
2 days: 7.1×10^4 (± 0.60)
3 days: 4.0×10^4 (± 0.43)
5 days: 3.5×10^4 (± 0.41)
7 days: 4.3×10^4 (± 0.46)

Numbers are mean concentrations of spores ml^{-1} ($\pm$ sem) from 54 readings.

2.3 Effect of varying the distance between the inducer leaves and the challenge-inoculated leaves, on the induction of systemic resistance

In this experiment a study was made of the magnitude of SIR in relation to the distance between the induced leaf and the challenged leaf within the plant. Although it is likely that the endogenous signal involved in SIR moves mainly in an acropetal direction (Murray, 1990), it is possible that the signal might also move in the opposite direction. It is possible therefore, that the Sa, NaSa, and AcSa used in this experiment might be transported both up and down the plant. Therefore, an additional part of this experiment involved an investigation of the treatment of the upper leaves on induction of SIR in the lower leaves.

2.3.1 Plant material and maintenance of the pathogen

Broad bean plants were grown as described in paragraph 2.1.1 and the rust fungus was maintained as described in paragraph 2.1.2.

The plants were only used for the experiment when they had four fully-developed leaf levels.

2.3.2 Induction of systemic resistance

Plants were induced by spraying leaves on the first level or on the fourth level as described in paragraph 2.1.3.

2.3.3 Challenge-inoculation of systemic induced plants

Plants were inoculated on the first to the fourth leaf level as described in paragraph 2.1.4, using a spore concentration of 4.5×10^4 spores ml^{-1}.

2.3.4 Experiment plan

The experiment was set up as follows:

A: Signal moving upwards in the plant.

Leaves on the first level were induced with 10 mM Sa, 5 mM NaSa or 10 mM AcSa. Subsequently, the first, second, third, or fourth leaf levels were challenge-inoculated. One pot containing three plants was used per treatment. On each plant one leaf level was induced and one leaf level was challenged.

B: Signal moving downwards in the plant.

In contrast with treatment A, in this part of the experiment the fourth leaf level was induced with the inducer compounds. The remainder of the experiment was identical to that of treatment A.

Plan of experiment described in paragraph 2.3

Inducer compound	Experiment A		Experiment B	
	Induced leaves	Challenged leaf levels	Induced leaves	Challenged leaf levels
Control	1	1,2,3, or 4	4	1,2,3, or 4
Sa	1	,,	4	,,
NaSa	1	,,	4	,,
AcSa	1	,,	4	,,

In addition to counting of the number of pustules per leaf, the percentage leaf area covered with rust pustules was estimated, using a standard area diagram of a chocolate spot key (Key no 2, draft 2, Plant Pathology Laboratory, 41168).

2.4 Effect of detaching the inducer leaves at different times after induction, on the development of systemic induced resistance

It is not known whether the signal for SIR is produced entirely at the infection site on the inducer leaf, or whether other parts of the plant are also stimulated to produce the signal (Dean and Kuc, 1986). By detaching the induced leaf level after the induction of SIR, the importance of the inducer leaf can be determined. This was examined in the following experiment.

2.4.1 Plant material and maintenance of the pathogen

Broad bean plants were grown as described in paragraph 2.1.1 and the rust fungus was maintained as described in paragraph 2.1.2.

2.4.2 Induction of systemic resistance

Plants were induced by spraying leaves on the first level as described in paragraph 2.1.3. The inducer (sprayed) leaves were detached at different times after induction, by cutting them off at the base of the petiole.

2.4.3 Challenge-inoculation of systemic induced plants

Plants were inoculated on the third leaf level as described in paragraph 2.1.4, using a spore concentration of 4.2×10^4 spores ml^{-1}.

2.4.4 Experiment plan

The experiment was set up as follows:

Time between induction and detaching (h)	Inducer compound	Induced and detached leaves	Inoculated leaves
4	Control	First	Third
12	Sa	,,	,,
24	NaSa	,,	,,
28	or AcSa	,,	,,
36	,,	,,	,,
not detached	,,	,,	,,

2.5 Direct effects of Sa, NaSa and AcSa on rust infection

The previous experiments showed that Sa, NaSa, and AcSa can induce SIR in broad bean. However, it is possible that, instead of being an elicitor of plant defence mechanisms, the chemicals were taken up from the leaf by the rust, thus exerting a direct antifungal effect on the fungus. This has been examined in the following experiment.

2.5.1 Plant material and maintenance of the pathogen

Broad bean plants were grown as described in paragraph 2.1.1 and the rust fungus was maintained as described in paragraph 2.1.2.

2.5.2 Inoculation and spraying

The plants were inoculated with rust on the first, second, and third leaf levels as described in paragraph 2.1.4.

Two days after inoculation, when the clear plastic bags were removed, the three leaf levels were sprayed with 5 mM, 10 mM, or 20 mM of Sa, NaSa, or AcSa as described in paragraph 2.1.3.

Rust infection was observed 14 days after inoculation as described previously.

2.6 Direct effect of Sa, NaSa, and AcSa on *Botrytis cinerea* and *Pyrenophora avenae* in vitro

From experiment 2.5 is still not clear whether the chemicals have a direct antifungal effect on the rust or whether the result is due to an indirect effect on the rust via the plant. To determine more precisely the effects of the compounds on the fungus, *U. viciae-fabae* should be grown *in vitro*. However, as rusts are obligate biotrophs, they cannot be grown *in vitro* easily. Therefore *Botrytis cinerea* and *Pyrenophora avenae* were used in this experiment, to examine the direct effects of the chemicals on fungal growth.

2.6.1 Maintenance of the pathogens

Stock cultures of *B. cinerea* and *P. avenae* were grown on Czapek Dox medium (modified; Oxoid), using 45.5 g l^{-1} of distilled water. The media were autoclaved at 120°C for 15 minutes and the cultures were kept in an incubator at a temperature of 20°C. Fresh cultures were initiated on a 2 to 3 week rota.

2.6.2 Growth of the fungi on media amended with Sa, NaSa, or AcSa

B.cinerea and *P.avenae* were grown on Czapek Dox medium containing Sa, NaSa, or AcSa at 5 or 20 mM. The medium was made up, autoclaved and allowed to cool down to 40-50°C. The chemicals were dissolved in 10 or 20 ml solution containing 6-10% acetone and were filter-sterilized using Ministart blue (0.2 μm) filters (Sartorius, Surrey, England) before being added to the cooled medium. Control plates consisted of Czapek Dox medium

only. Cylindrical plugs of mycelium were transferred from stock cultures to the amended media and placed in the centre of the petri-dishes.

2.6.3 Measurements of fungal growth

Fungal growth on amended media was determined by measuring surface expansion of the mycelial plug, i.e. by measuring radial growth of new mycelium from the edge of the original mycelial plug. Three to five replicates per treatment were used and 3 measurements were taken per petri-dish. The experiment was performed twice; in one experiment the pH of the medium was adjusted to 7.0, while in the other experiment the pH was not adjusted.

2.7 Analysis of salicylic acid in phloem exudates

Various workers have suggested that Sa is the endogenous signal involved in SIR (e.g. Métraux et al., 1990; Enyedi et al., 1992; Rasmussen et al., 1991). The experiments described so far in this thesis have examined the use of Sa and Sa-derivatives on the induction of SR in broad bean. To investigate the possible involvement of endogenous Sa in SIR in broad bean it was essential to determine any changes in Sa-levels in broad bean tissue after induction of SR. This section describes some initial trials which were carried out to collect phloem exudates (paragraph 2.7) from broad bean. A start was also made on the determination of Sa in plant tissues (paragraph 2.8).

2.7.1 Plant material and inoculation of the pathogen

Broad bean plants were grown as described in paragraph 2.1.1 and leaves of the first level were inoculated with the rust as described in paragraph 2.1.4.

2.7.2 Collection of phloem exudates

Plants were used when they had three fully-developed leaf levels. Plants were transferred from the greenhouse to an incubator, in which the humidity was kept at 80 or 90 %, and were watered thoroughly before experiments began. Phloem exudates were collected using a method described previously (Ahmad et al., 1982; King and Zeevaart, 1974). Leaves were excised under a solution of 5 or 20 mM disodium ethylene-diaminetetraacetate dihydrate (EDTA; pH 7.0) at the base of the petiole and immediately transferred into 1.5 ml microtubes containing fresh EDTA solution. To collect phloem exudates from petioles still connected to the rest of the plant, the petiole was cut under EDTA close to the leaf end of the petiole, after which a microtube containing 1.5 ml fresh EDTA solution was attached to the petiole using parafilm. Plants were placed at an angle in order to keep the EDTA solution in the microtubes. The plants and the excised leaves were placed under a clear plastic hood or in a clear plastic bag, and kept in an incubator under normal light conditions. The EDTA solutions were changed at 2 h intervals for 6 h. In order to increase the rate of exudation the following changes in the procedure were adopted: plants and excised leaves were kept uncovered or covered; humidity in the incubator was kept at 80% or 90%; temperature was kept at 17°C or 20°C; leaves were sprayed with distilled water during the 6 h period at 1 h intervals or not.

However, numerous problems were encountered with attempting to obtain exudates from broad bean (*Vicia faba*) plants, including severe dehydration of leaves. Attempts to obtain reliable, consistent exudation were finally abandoned.

2.8 Analysis of salicylic acid in plant tissue

2.8.1 Plant material and inoculation

Broad bean plants were grown as described in paragraph 2.1.1.

Plants were inoculated on leaves of the first level as described in paragraph 2.1.4.

2.8.2 Collection of plant tissue

At various times after inoculation (i.e after 0, 4, 12, 24, 48, and 72 h) the plants were cut into the following sections:

1: stem under first leaf level
2: first, induced leaf level
3: stem between first and second leaf level
4: second leaf level
5: stem between second and third leaf level
6: third leaf level

The different tissues were placed in sealed, clear plastic bags and were frozen within 1 h of excision.

2.8.3 Analysis of Sa

Plant tissues were stored at - 20 °C for 2 months before they were used for analysis of Sa. Sa was determined using a modification of the methods described by Rasmussen *et al.* (1991) and Harborne (1984). Defrosted tissue was weighed and then macerated with 99.7 % ethanol (2.5 ml g^{-1}). After maceration, the extracts were decanted into 15 ml centrifuge tubes and centrifuged for 10 min. at 4500 rev min^{-1}. Fifty ml of the ethanol extracts were spotted onto activated (1 h. at 85°C) silica gel 60 A K6F chromatography plates (Whatman) and developed in toluene:1,4-dioxane:acetic acid (90:25:4, v/v) for 1 h. Spots were visualized on the plate by viewing under short wave U.V.light. The bands corresponding to Sa were scraped off the plate and eluted in 5 ml of 99.7 % ethanol, and then centrifuged at 4500 rev min^{-1} for 10 min. The decanted extract was used for quantitative analysis. UV absorption was measured using a Pye Unicam SP8-400 UV/VIS Spectrophotometer, while absorption spectra were produced using a Pye Unicam SP1800 U.V. Spectrophotometer coupled to a Pye Unicam AR25 Linear Recorder. Fluorescence was measured using a SP5 spectrofluorimeter (Perkin Elmer; excitation wavelength = 310 nm, emission wavelength = 400 nm).

2.9 Statistical analysis

Data was analyzed using the statistical software Minitab, Release 7.2 Standard Version (Minitab, Inc. 1989) to calculate means, standard error of the means, using one way-analysis of variance. Confidence intervals were calculated using the Students t-test. Statistical results, including means, se means, t-values and significant differences are shown in appendix I, and not on the histograms. In the graphs of the results, significant differences are shown as P = 0.10 s, P = 0.05 *, P = 0.01 **, P = 0.001 ***. All experiments described in this thesis were repeated, with similar results.

3 RESULTS

3.1 Induction of systemic resistance in broad bean to rust using Sa, NaSa, and AcSa

Because not all the treatments using the different compounds were carried out at the same time, the results will be given separately, with the different results for the controls related their respective treatments.

During this research, considerable variability was observed in the induction of SR using Sa, NaSa and AcSa. Possible reasons for this variability are discussed later (see Discussion).

In these experiments, spray damage was observed when the higher concentrations of Sa, NaSa, and AcSa were used. When concentrations of Sa lower than 5 mM were used, no damage to the sprayed leaves was observed.

There were no significant differences between the control which was not treated and the control which was mock-induced (see Materials and Methods, paragraph 2.1.3). Statistical calculations, i.e. means, se means, t-values, and P-values, are shown in appendix I.

Sa treatments

SR to rust infection in broad bean could be induced using 5, 10, and 20 mM Sa (Fig. 2). The greatest reduction in rust infection on the leaves of the third level was observed when the first leaf level was sprayed with 5 mM Sa (Fig. 2). When Sa was applied at concentrations lower than 5 mM, SR was still induced, although only the reductions in rust infection observed after treatment with 1 and 5 mM were significantly different from the control (Fig. 3). The 3 mM and 4 mM treatments did not differ from the control.

NaSa treatments

When NaSa was used to induce SR, treatment of the first leaves with 5, 10, or 20 mM NaSa significantly reduced rust infection on the third leaves (Fig. 4), although greatest reduction was observed when 5 mM was applied.

AcSa treatments

Great variability was observed using AcSa to induce SR. Thus, 5 mM AcSa had little effect on SR, while 20 mM AcSa did induce SR in the third leaves, although the reduction in rust infection observed after treatment with 20 mM was not significantly different from the control (Fig. 5). Treatment of the first leaves with 10 mM AcSa actually increased rust infection on the third leaves (Fig. 5).

Figure 2. Induction of systemic resistance to rust infection using different concentrations of Salicylic acid. Sa was applied to the first leaves and the third leaves were challenge-inoculated. Values are the mean of 6 replicates, while the control treatment is the mean of 4 replicates. Significant differences are shown as P = 0.10 s, P = 0.05 *. See table 1 in appendix I.

Figure 3. Induction of systemic resistance to rust infection using different concentrations of Salicylic acid. Sa was applied to the first leaves and the third leaves were challenge-inoculated. Values for 1, 2, and 4 mM are the means of 8 replicates, while values for 3, and 5 mM are the means of 6 replicates and the control is the mean of 10 replicates. Significant differences are shown as P = 0.05 *. See table 2 in appendix I.

Figure 4. Induction of systemic resistance to rust infection using different concentrations of Sodium salicylate. NaSa was applied to the first leaves and the third leaves were challenge-inoculated. Values are the means of 6 replicates, while the control is the mean of 4 replicates. Significant differences are shown as P = 0.10 s, P = 0.05 *. See table 3 in appendix I.

Figure 5. Induction of systemic resistance to rust infection using different concentrations of Acetylsalicylic acid. AcSa was applied to the first leaves and the third leaves were challenge-inoculated. Values are the means of 10 replicates, while the control is the mean of 6 replicates. significant differences are shown as P = 0.05 *. See table 4 in appendix I.

Figure 2

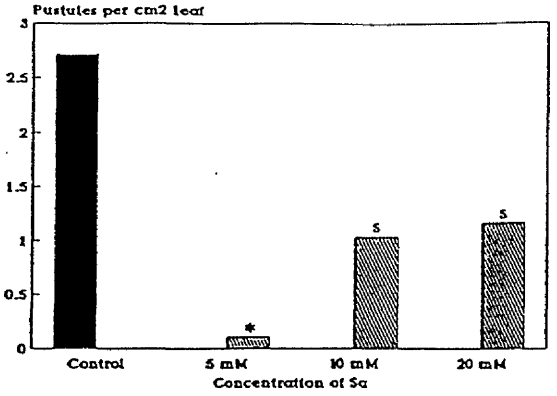


Figure 3

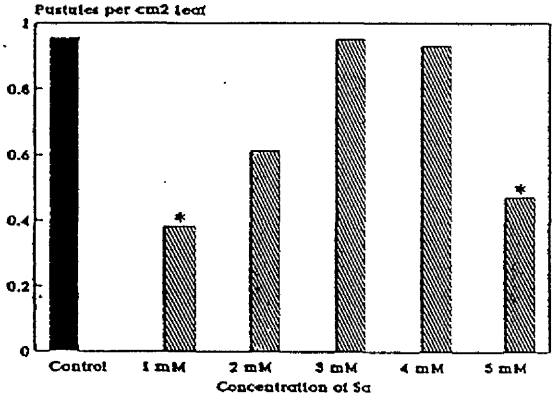


Figure 4

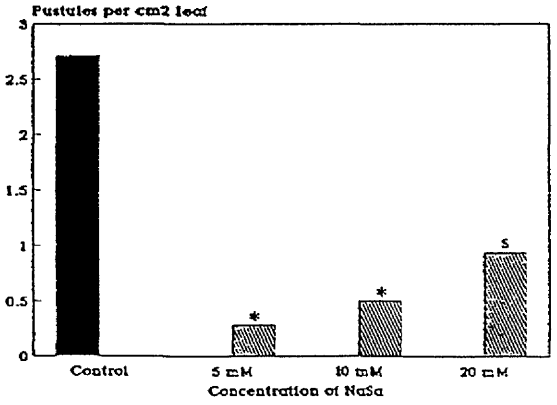
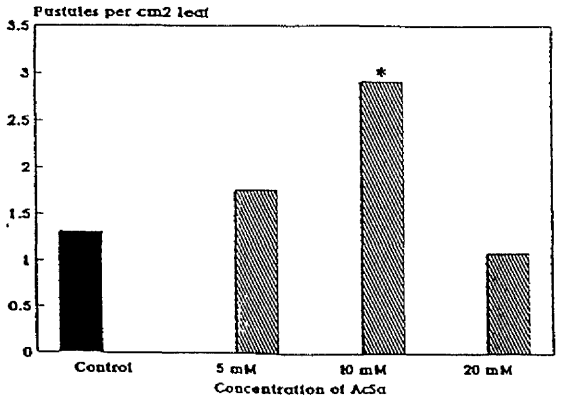


Figure 5



3.2 Effect of varying the interval between treatment and challenge-inoculation, on the development of SIR

Sa treatments

Treatment of the first leaves with 10 mM Sa 1, 2, 5, or 7 days prior to challenge inoculation of the third leaves, led to reductions in rust infection (Fig. 6), although only when the first leaves were treated 1 or 7 days before inoculation, were these decreases significantly different from the control. SR was not induced in the third leaves when the first leaves were treated 3 days prior to challenge-inoculation (Fig. 6).

NaSa treatments

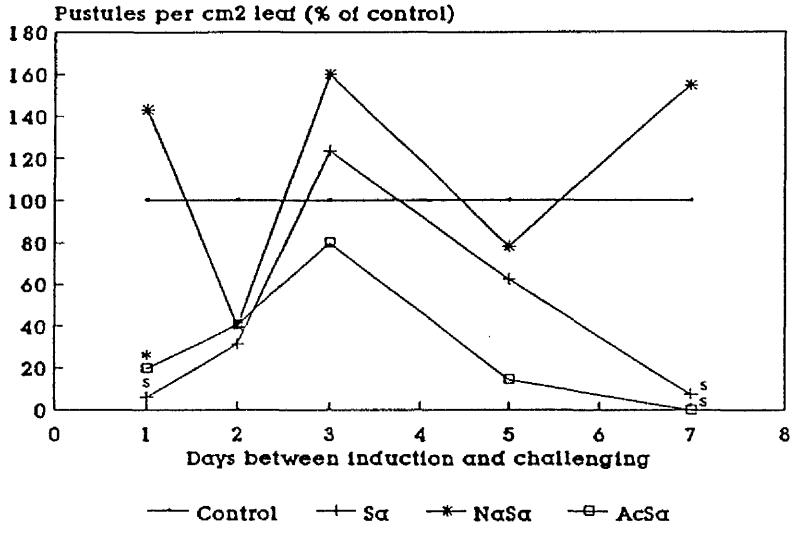
Great variability was observed when the interval between treatment of the first leaves with NaSa and challenging of the third leaves was varied. Rust infection was increased compared with the control when the interval between induction and challenge-inoculation was 1, 3, and 7 days, (Fig. 6). However, SR was induced in the third leaves when the first leaves had been induced with NaSa 2 days earlier, while there was no significant effect on SR when the interval between induction and challenge-inoculation was 5 days (Fig. 6).

AcSa treatments

Substantial reductions in rust infection on the third leaves were found when the first leaves were treated with AcSa (Fig. 6). Thus, considerable reductions in rust infection were observed when the first leaves had been treated 1, 2, 5 or 7 days earlier. However, SR was not induced in the third leaves when the interval between induction and challenge inoculation was 3 days (Fig. 6).

Figure 6. Effect of varying the interval between treatment and challenge-inoculation, on the development of systemic induced resistance to rust infection. The third leaves were challenge-inoculated at different times after Sa, NaSa, or AcSa were applied to the first leaves. Values are the means of 6 replicates. Significant differences are shown as P = 0.10 s, P = 0.05 *. See table 5 in appendix I.

Figure 6


Pustules per cm2 leaf (% of control)
180
160
140
120
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
Days between induction and challenging
Control
Sα
NaSα
AcSα

3.3 Effect of varying the distance between the inducer leaves and the challenged-inoculated leaves, on the induction of SR

3.3.1 Signal moving upwards in the plant

Sa treatments

SR was observed in leaves of the second and third levels when the first level leaves were induced using Sa, while in leaves of the fourth level an increase in rust infection was observed, when compared to the control (Fig. 7). The decrease in rust infection in the third leaves was significant (Fig. 7).

NaSa treatments

NaSa treatment of the first leaves led to a significant increase in rust infection on the second and fourth leaves, and a reduction in rust infection on the third leaves (Fig. 8).

AcSa treatments

SR to rust infection was induced in the second leaves, while rust infection was increased on the fourth leaves, when the first leaves had been previously treated with AcSa (Fig. 9). There was a small increase in rust infection on the third leaves of induced plants, compared to controls (Fig. 9).

Figure 7. Effect of varying the distance between the inducer leaves and the challenge-inoculated leaves, on the induction of systemic resistance to rust infection, using Salicylic acid. Sa was applied to the first leaves, and the first, second, third, or fourth leaves were challenge-inoculated. Values are the means of 6 replicates, while the control for the fourth leaves is the mean of 8 replicates, and Sa treatment of the third leaves is the mean of 4 replicates. Significant differences are shown as P = 0.05*. See tables 6 and 8 in appendix I.

Figure 8. Effect of varying the distance between the inducer leaf and the challenge-inoculated leaf, on the induction of systemic resistance to rust infection, using Sodium salicylate. NaSa was applied to the first leaves, and the first, second, third, or fourth leaves were challenge-inoculated. Values are the means of 6 replicates, and the control for the fourth leaves is the mean of 8 replicates. Significant differences are shown as P = 0.10 s. See tables 6 and 8 in appendix I.

Figure 9. Effect of varying the distance between the inducer leaf and the challenge-inoculated leaf, on the induction of systemic resistance to rust infection, using Acetylsalicylic acid. AcSa was applied to the first leaves, and the first, second, third, or fourth leaves were challenge-inoculated. Values are the means of 6 replicates, and the control for the fourth leaves is the mean of 8 replicates. No significant differences were observed. See tables 6 and 8 in appendix I.

Figure 7

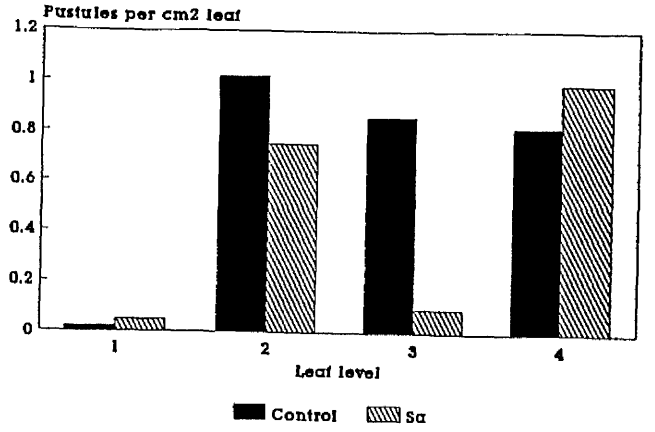


Figure 8

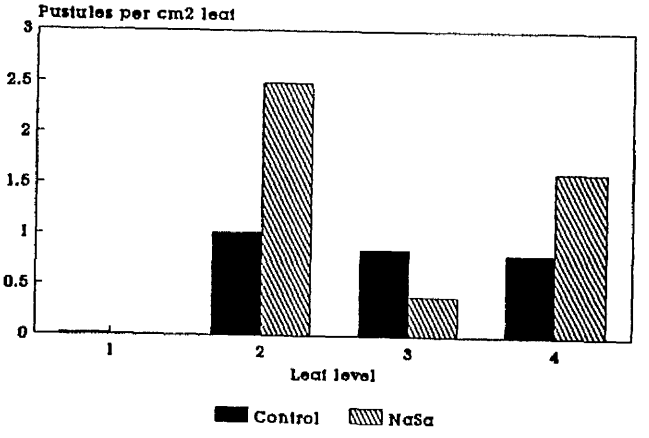
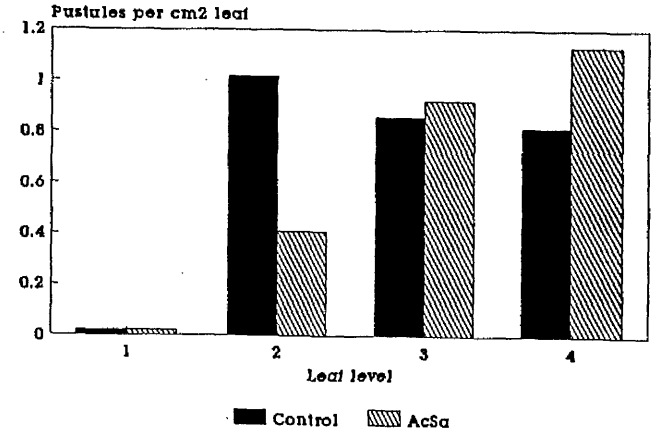


Figure 9



3.3.2 Signal moving downwards in the plant

In these experiments the fourth leaves were induced prior to challenge-inoculation of the first, second, third, and fourth leaves with rust.

Sa treatments

Sa treatment of the fourth leaves induced SR to rust infection in the first and second leaves (Fig. 10), although the decreases in rust infection did not differ significantly from the controls. A significant increase of rust infection was observed in the third leaves (Fig. 10). In addition, rust infection on the treated fourth leaves was significantly reduced compared to the control (Fig. 10).

NaSa treatments

When fourth leaves were treated with NaSa, reductions in rust infection were observed in the first and second leaves, while an increase in rust infection on the third leaves was observed (Fig. 11). Rust infection on the treated leaves was reduced compared to the control, although none of the changes were significantly different from the control values (Fig. 11).

AcSa treatments

When AcSa was used to treat the fourth leaves, there was an increase in rust infection on the first and third leaves, while infection on the second leaves was not significantly altered, compared to the control (Fig. 12). However, there was a significant reduction in rust infection on the treated, fourth leaves (Fig. 12).

Figure 10. Effect of varying the distance between the inducer leaf and the challenge-inoculated leaf, on the induction of systemic resistance to rust infection, using Salicylic acid. Sa was applied to the fourth leaves, and the first, second, third, and fourth leaves were challenge-inoculated. Values are the means of 6 replicates, while Sa treatment of the first and second leaves are the means of 4 replicates. Significant differences are shown as P = 0.10 s. See tables 7 and 8 in appendix I.

Figure 11. Effect of varying the distance between the inducer leaf and the challenge-inoculated leaf, on the induction of systemic resistance to rust infection, using Sodium salicylate. NaSa was applied to the fourth leaves, and the first, second, third, and fourth leaves were challenge-inoculated. Values are the means of 6 replicates. No significant differences were observed. See tables 7 and 8 in appendix I.

Figure 12. Effect of varying the distance between the inducer leaf and the challenge-inoculated leaf, on the induction of systemic resistance to rust infection, using Acetylsalicylic acid. AcSa was applied to the fourth leaves, and the first, second, third, and fourth leaves were challenge-inoculated. Values are means of 6 replicates. Significant differences are shown as P = 0.10 s, P = 0.05 *. See tables 7 and 8 in appendix I.

Figure 10

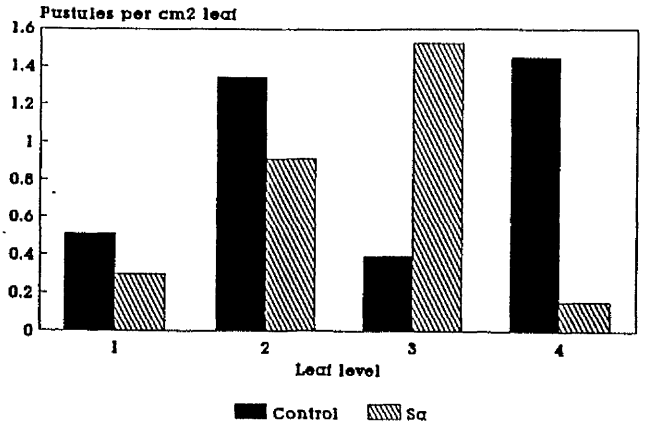


Figure 11

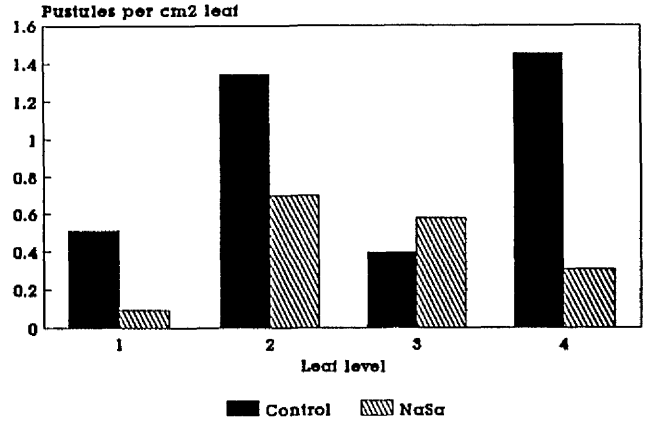
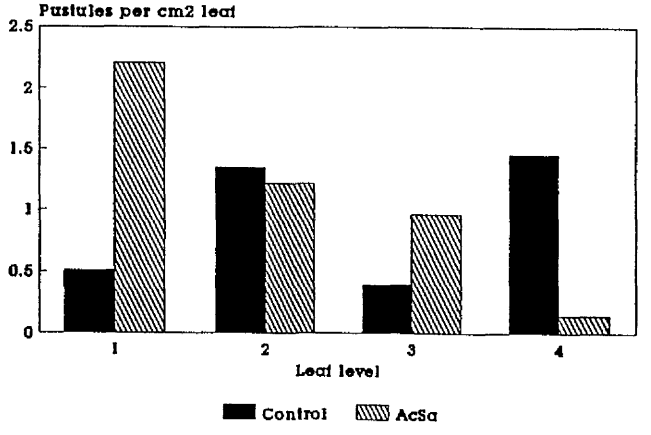


Figure 12



3.4 Effect of detaching the inducer leaves at different times after induction, on the development of SIR

In this experiment the effect of detaching the first leaves, at various times after treatment with Sa, NaSa, and AcSa, on rust infection of the third leaves was examined. To minimize any effects due to leaf detachment only, leaves of control plants were also detached at the varying times after induction of the lower leaves. Although large differences in the control treatment were observed, no general trend could be determined.

Sa treatments

Rust infection on the third leaves was reduced when the Sa-treated first leaves were detached 4 h, 12 h, or 36 h after induction (Fig. 13), but was increased if leaves were detached 24 h and 28 h after induction, or when the leaves were not detached (Fig. 13). The differences observed when the inducer leaves were detached 24 h and 36 h after induction were significantly different from the control (Fig 13).

NaSa treatments

Detaching NaSa treated inducer leaves 4 h, 12 h, or 28 h after induction, resulted in an increase of rust infection on the third leaves (Fig. 14), while SR was induced when the inducer leaves were detached 24 h or 36 h after induction, or were not detached (Fig. 14). The changes observed when the leaves were detached after 36 h or when the leaves were not detached were significantly different from the control (Fig. 14).

AcSa treatments

AcSa induced SR in the third leaves, when the inducer leaves were detached 24 h or 36 h after induction or when the inducer leaves were not detached (Fig. 15), but AcSa increased rust infection when the inducer leaves were detached 4 h, 12 h, or 28 h after induction (Fig. 15). None of these changes were significantly different from the control.

Figure 13. Effect of detaching the inducer leaves at different times after induction, on the development of systemic induced resistance to rust infection, using Salicylic acid. Sa was applied to the first leaves, which were detached at different times after induction. The third leaves were challenge-inoculated. Values are the means of 6 replicates. The value for the Sa-treated leaves detached after 24 h is the mean of 4 replicates. ND = inducer leaves not detached. Significant differences are shown as P = 0.10 s, P = 0.05 *. See tables 9 and 10 in appendix I.

Figure 14. Effect of detaching the inducer leaves at different times after induction, on the development of systemic induced resistance to rust infection, using Sodium salicylate. NaSa was applied to the first leaves, which were detached at different times after induction. The third leaves were challenge-inoculated. Values are the means of 6 replicates. The values for the NaSa-treated leaves detached after 12 h and 24 h are the means of 4 replicates. ND = inducer leaves not detached. Significant differences are shown as P = 0.10 s, P = 0.05 *. See tables 9 and 10 in appendix I.

Figure 15. Effect of detaching the inducer leaves at different times after induction, on the development of systemic induced resistance to rust infection, using Acetylsalicylic acid. AcSa was applied to the first leaves, which were detached at different times after induction. The third leaves were challenge-inoculated. Values are the means of 6 replicates. The value for the AcSa-treated leaves detached after 12 h is mean of 4 replicates. ND = inducer leaves not detached. No significant differences were observed. See tables 9 and 10 in appendix I.

Figure 13

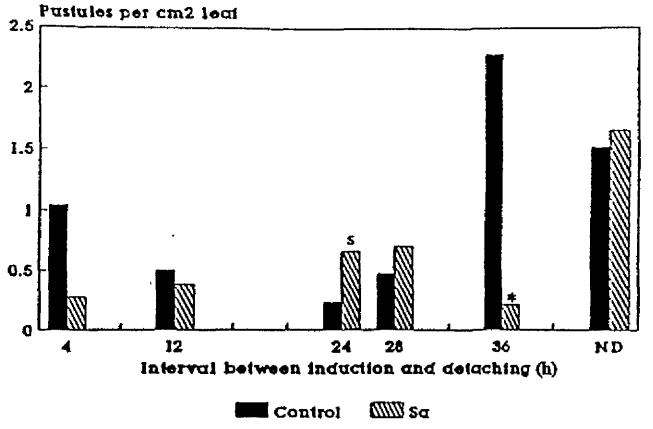


Figure 14

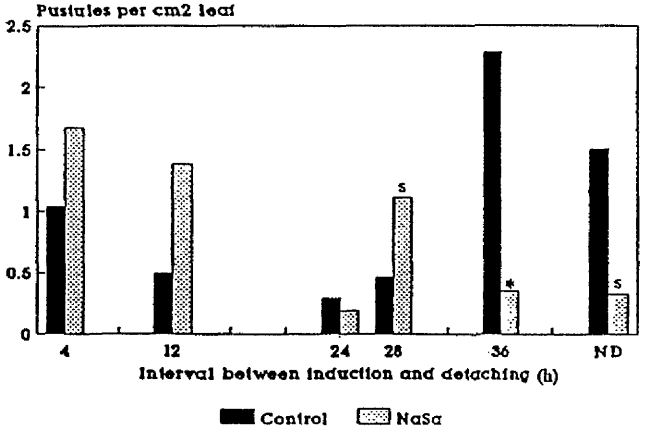
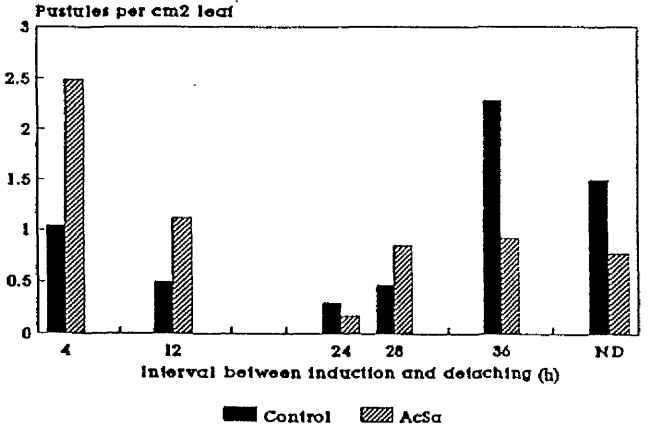


Figure 15



3.5 Direct effects of Sa, NaSa, and AcSa on rust infection

Sa treatments

Increases in rust infection were observed when 5 mM or 20 mM Sa was sprayed onto the inoculated leaves, and a reduction in infection was observed when 10 mM was used (Fig. 16), although the differences were not significant. Damage to the inoculated leaves was observed especially when 20 mM Sa was used (Fig 19). This damage had increased when plants were observed again 6 and 12 days after spraying (Figs 20, and 21), while leaf damage observed when 10 mM Sa was applied did not change with time (Figs 19, 20, and 21). When the size of the pustules was observed 14 days after inoculation, significantly smaller pustules were observed following 5 and 10 mM Sa treatment, while larger pustules were observed when 20 mM Sa was used (Fig. 22).

NaSa treatments

Substantial reductions in rust infection were observed when 5 and 20 mM NaSa were applied to inoculated leaves, while no significant reduction was observed when 10 mM NaSa was sprayed onto the leaves (Fig. 17). Less then 5 % of the leaf surface was damaged when the leaves were treated with 5 or 10 mM NaSa, while nearly 48 % of the leaf area was damaged when 20 mM was applied (Figs. 19, 20, and 21). No significant differences in pustule sizes were observed between the NaSa treatment and the control treatment (Fig. 22).

AcSa treatments

AcSa treatment led to significant decrease in the number of pustules, when 5 or 20 mM concentrations were used, but no significant difference was observed when 10 mM AcSa was applied to the leaves (Fig. 18). Treatment with 20 mM AcSa caused considerable damage to leaves (Figs. 19, 20, and 21). The size of the pustules was smaller in the AcSa treatments than in the controls, although only the decrease followed 5 mM AcSa treatment was significantly different from the control (Fig. 22).

Figure 16. Direct effects on rust infection, using different concentrations of Salicylic acid. Sa was applied to three leaf levels 2 days after the leaves were inoculated. Values are the means of 18 replicates, the control is the mean of 60 replicates and the treatment with 20 mM Sa is the mean of 16 replicates. No significant differences were observed. See table 11 in appendix I.

Figure 17. Direct effects on rust infection, using different concentrations of Sodium salicylate. NaSa was applied to three leaf levels 2 days after the leaves were inoculated. Values are the means of 18 replicates, the control is the mean of 60 replicates. Significant differences are shown as P = 0.001 ***. See table 11 in appendix I.

Figure 18. Direct effects on rust infection, using different concentrations of Acetylsalicylic acid. AcSa was applied to three leaf levels 2 days after the leaves were inoculated. Values are the means of 18 replicates, the control is the mean of 60 replicates. Significant differences are shown as P = 0.001 ***. See table 11 in appendix I.

Figure 16

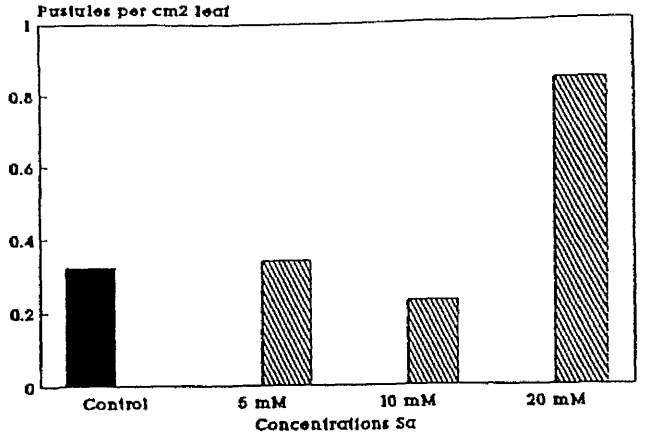


Figure 17

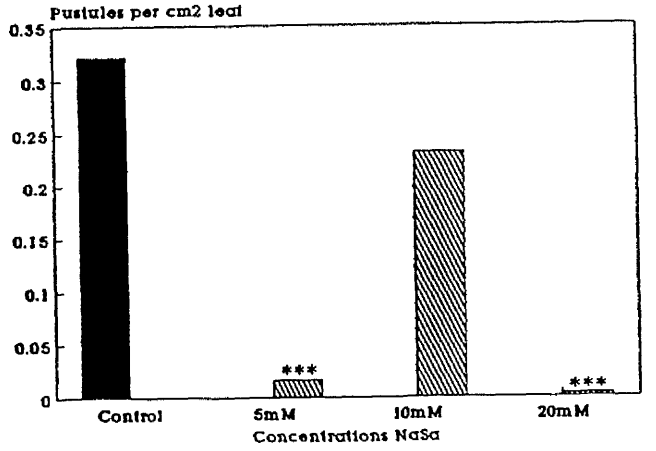


Figure 18

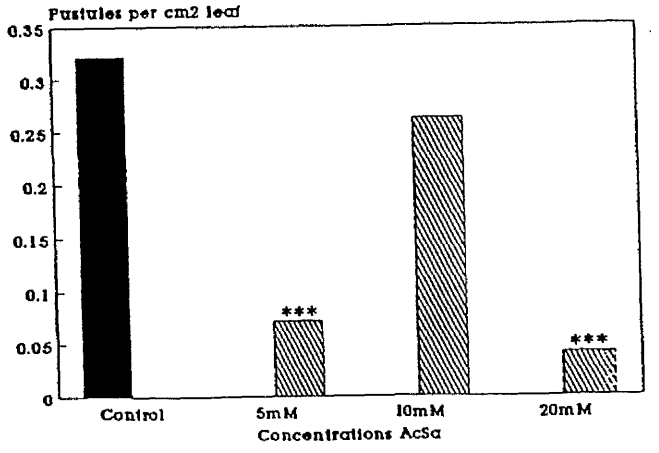


Figure 19. Direct effect of different concentrations of Salicylic acid, Sodium salicylate, or Acetylsalicylic acid on Broad Bean leaves. Three leaves were treated per plant, and damage was observed 3 days after treatment (5 days after inoculation of those leaves). Values are the mean of 18 replicates. The value for treatment with 20 mM Sa is the mean of 16 replicates. No damage was observed in the control plants. See table 12 in appendix I.

Figure 20. Direct effect of different concentrations of Salicylic acid, Sodium salicylate, or Acetylsalicylic acid on Broad Bean leaves. Three leaves were treated per plant, and damage was observed 6 days after treatment (8 days after inoculation of those leaves). Values are the means of 18 replicates. The value for treatment with 20 mM Sa is the mean of 16 replicates. No damage was observed in the control plants. See table 12 in appendix I.

Figure 21. Direct effect of different concentrations of Salicylic acid, Sodium salicylate, or Acetylsalicylic acid on Broad Bean leaves. Three leaves were treated per plant, and damage was observed 12 days after treatment (14 days after inoculation of those leaves). Values are the means of 18 replicates. The value for treatment with 20 mM Sa is the mean of 16 replicates. No damage was observed in the control plants. See table 12 in appendix I.

Figure 22. Direct effect of different concentrations of Salicylic acid, Sodium salicylate, or Acetylsalicylic acid on the size of the rust pustules. Rust was inoculated on three leaf levels two days before 5, 10, or 20 mM of Sa, NaSa, or AcSa was applied. Pustule sizes were observed 14 days after inoculation. Values are the means of 1-44 replicates. Significant differences are given as $P = 0.10$ s, $P = 0.001$ ***. See table 13 in appendix I.

Figure 19

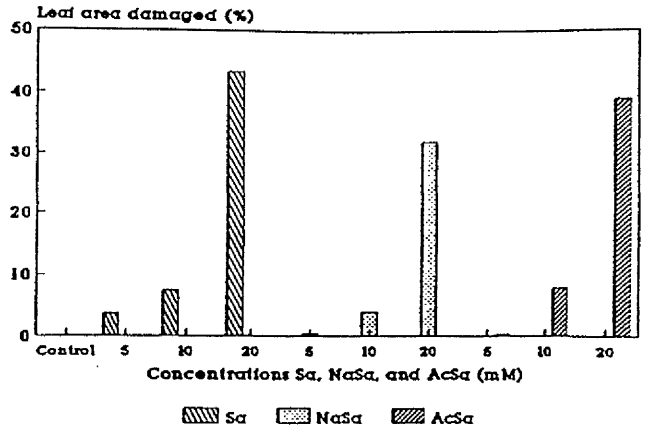


Figure 20

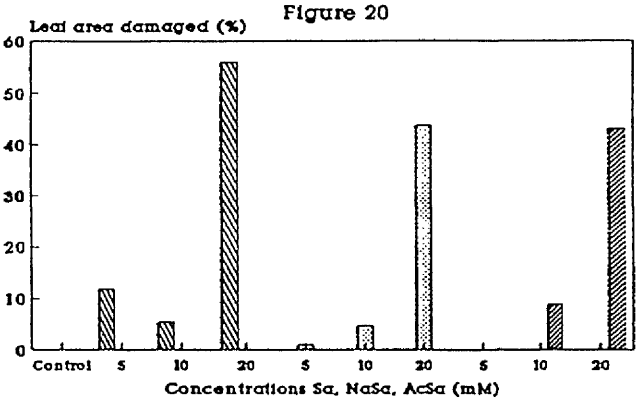


Figure 21

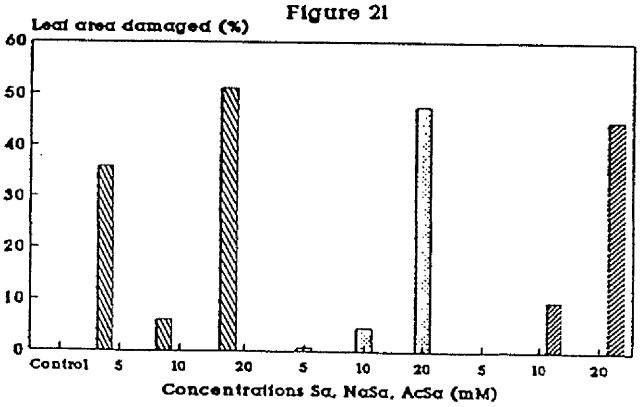
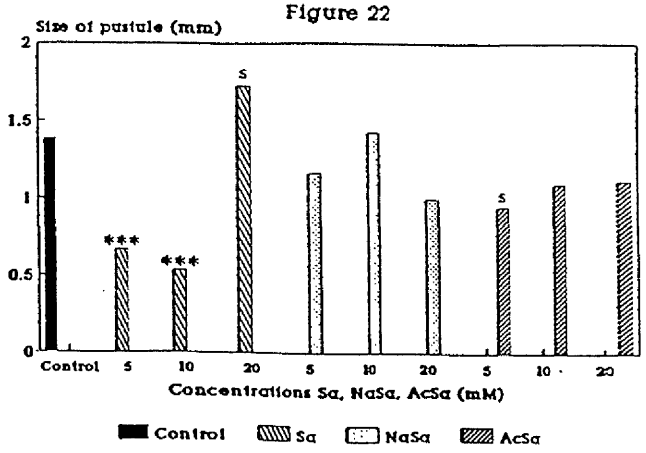


Figure 22



3.6 Direct effect of Sa, NaSa, and AcSa on *Botrytis cinerea* and *Pyrenophora avenae* in vitro

This work was performed twice. In one experiment the pH of the medium was not adjusted, while in the other experiment the pH was adjusted to 6.7 (for Sa and NaSa). The adjusted pH of AcSa-amended media was 5.1 in the inoculations with *B. cinerea*, and 4.4 and 3.5 in the inoculations with *P. avenae*. The pH values of the unadjusted media were as follows: control, 6.8; 5 mM Sa, 2.8; 20 mM Sa, 2.4; 5 mM NaSa, 7.2; 20 mM NaSa, 7.5; 5 mM AcSa, 4.6; 20 mM AcSa, 3.8. There was no visible change in the appearance of *B. cinerea* or *P. avenae* mycelium (i.e. colour, surface texture or sporulation) in these experiments.

Sa treatments

B. cinerea, pH of medium not adjusted

Growth of *B. cinerea* was significantly reduced when the fungus was grown on medium amended with 5 mM Sa (Fig 23). No fungal growth was observed when 20 mM Sa was added to the medium.

B. cinerea, pH of medium adjusted

In contrast, an increase in surface expansion of *B. cinerea* mycelium was observed when the fungus was grown on medium amended with 5 mM Sa (Fig. 24). The 20 mM treatment started off slower than the control, but no significant differences were observed 6 days or later after inoculation.

P. avenae, pH of medium not adjusted

When Sa was added to the medium, no mycelial growth of was observed (Fig. 25).

P. avenae, pH of medium adjusted

Growth of *P. avenae* was significantly reduced compared to the control , when grown on media with the pH adjusted to 6.7 (Fig. 26).

NaSa treatments

B. cinerea, pH of medium not adjusted

When 5 mM NaSa was added to the medium, growth of *B. cinerea* was reduced (Fig. 27), although by 6 days after inoculation, mycelial growth was actually greater than that observed in the control inoculation. An increased mycelial growth compared to the control was observed when *B. cinerea* was grown on medium amended with 20 mM NaSa (Fig. 27).

B. cinerea, pH of medium adjusted

No significant reductions in mycelial growth of *B. cinerea* could be observed when grown on 5 mM NaSa (Fig. 28), while 20 mM NaSa significantly slowed down surface expansion of the mycelium (Fig. 28).

P. avenae, pH of medium not adjusted

Treatment with 5 mM NaSa did not lead to differences in mycelial growth of *P. avenae*, while 20 mM NaSa caused some reduction in growth, although these differences were not significant compared to the control (Fig. 29).

P. avenae, pH of medium adjusted

When *P. avenae* was grown on NaSa-amended medium, reductions in mycelial growth were observed at 5 mM (Fig. 30), although significant reductions were only observed 6 and 8 days after inoculation. Amending the medium with 20 mM NaSa led to an increase in mycelial growth of *P. avenae* (Fig. 30).

AcSa treatments

B. cinerea, pH of medium not adjusted

Amendment of the medium with AcSa led to significant reductions of mycelial growth of *B. cinerea*, (Fig. 31).

B. cinerea, pH of medium adjusted

When *B. cinerea* was grown on media, with the pH adjusted, no difference compared to the control was observed when the medium was amended with 5 mM AcSa, while 20 mM AcSa caused a significant delay in growth of *B. cinerea* (Fig. 32).

P. avenae, pH of medium not adjusted

P. avenae, when inoculated onto medium amended with AcSa, did not grow (Fig. 33).

P. avenae, pH of medium adjusted

No difference in surface expansion of the mycelium compared to the control, was observed when 5 mM AcSa was added to the medium, while 20 mM AcSa inhibited mycelial growth of *P. avenae* (Fig. 34).

Figure 23. Direct effect of different concentrations of Salicylic acid on the surface expansion of *Botrytis cinerea* mycelium. The pH of the medium was not adjusted (pH for 5 mM was 2.8 and for 20 mM was 2.4). Values are the means of 5 replicates. Significant differences are shown in table 14 in appendix I.

Figure 24. Direct effect of different concentrations of Salicylic acid on the surface expansion of *Botrytis cinerea* mycelium. The pH of the medium was adjusted to pH 6.7. Values are the means of 5 replicates. Significant differences are shown in table 14 in appendix I.

Figure 25. Direct effect of different concentrations of Salicylic acid on the surface expansion of *Pyrenophora avenae* mycelium. The pH of the medium was not adjusted (pH for 5 mM was 2.8 and for 20 mM was 2.4). Values are the means of 5 replicates. Significant differences are shown in table 17 in appendix I.

Figure 26. Direct effect of different concentrations of Salicylic acid on the surface expansion of *Pyrenophora avenae* mycelium. The pH of the medium was adjusted to pH 6.7. Values are the means of 3 replicates. Values for the control, observed at day 6 are the mean of 6 observations, and at day 8 of 1 observation. Significant differences are shown in table 17 in appendix I.

Figure 23

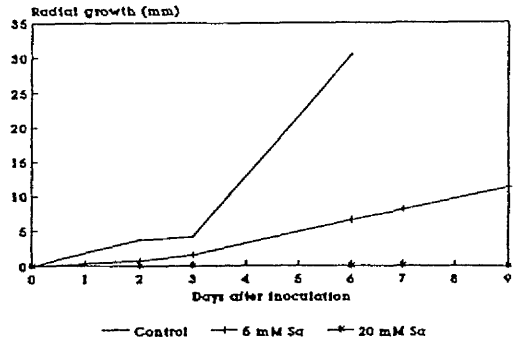


Figure 24

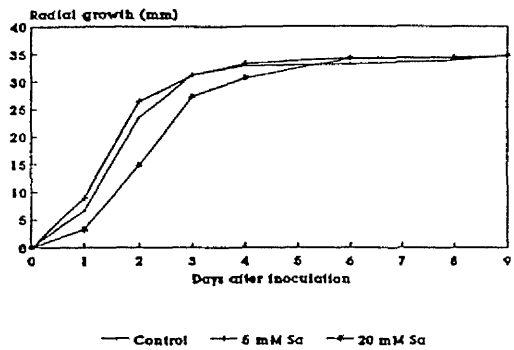


Figure 25

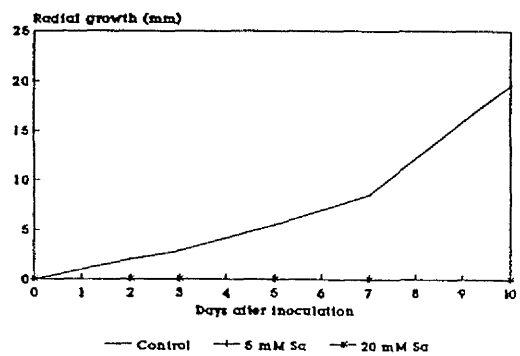


Figure 26

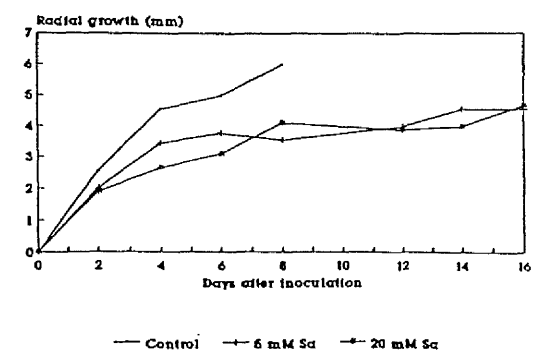


Figure 27. Direct effect of different concentrations of Sodium salicylate on the surface expansion of *Botrytis cinerea* mycelium. The pH of the medium was not adjusted (pH for 5 mM was 7.2 and for 20 mM was 7.5). Values are the means of 5 replicates. Significant differences are shown in table 15 in appendix I.

Figure 28. Direct effect of different concentrations of Sodium salicylate on the surface expansion of *Botrytis cinerea* mycelium. The pH of the medium was adjusted to pH 6.7. Values are the means of 5 replicates. Significant differences are shown in table 15 in appendix I.

Figure 29. Direct effect of different concentrations of Sodium salicylate on the surface expansion of *Pyrenophora avenae* mycelium. The pH of the medium was not adjusted (pH for 5 mM was 7.2 and for 20 mM was 7.5). Values are the means of 5 replicates. Values of the observations at day 10 are means of 8 observations for 5 mM, and 2 observations for 20 mM. Significant differences are shown in table 18 in appendix I.

Figure 30. Direct effect of different concentrations of Sodium salicylate on the surface expansion of *Pyrenophora avenae* mycelium. The pH of the medium was adjusted to pH 6.7. Values are the means of 5 or 3 replicates. Values for the control observed at day 6 are of 6 observations, and at day 8 of 1 observation. Significant differences are shown in table 18 in appendix I.

Figure 27

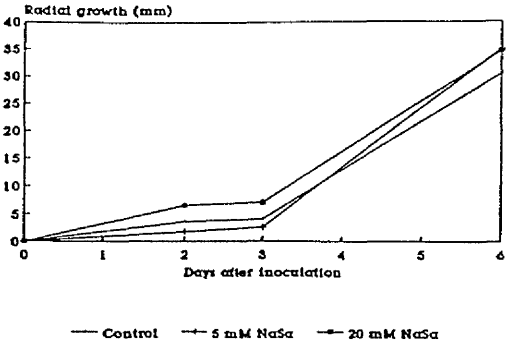


Figure 28

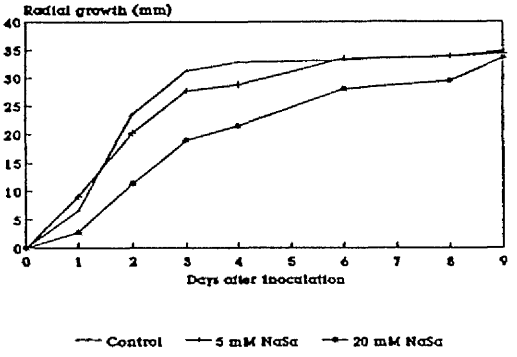


Figure 29

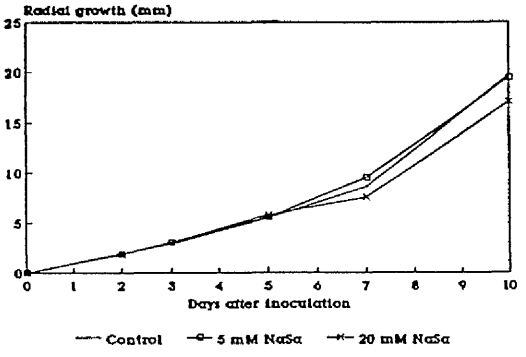


Figure 30

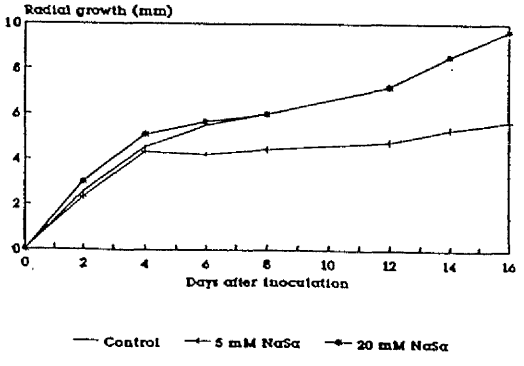


Figure 31. Direct effect of different concentrations of Acetylsalicylic acid on the surface expansion of *Botrytis cinerea* mycelium. The pH of the medium was not adjusted (pH for 5 mM was 4.6 and for 20 mM was 3.8). Values are the means of 5 replicates. Values for 20 mM AcSa observed at days 6, 7, and 9 are the means of 3 replicates. Significant differences are shown in table 16 in appendix I.

Figure 32. Direct effect of different concentrations of Acetylsalicylic acid on the surface expansion of *Botrytis cinerea* mycelium. The pH of the medium was adjusted to pH 5.1. Values are the means of 5 replicates. Significant differences are shown in table 16 in appendix I.

Figure 33. Direct effect of different concentrations of Acetylsalicylic acid on the surface expansion of *Pyrenophora avenae* mycelium. The pH of the medium was not adjusted (pH for 5 mM was 4.6 and for 20 mM was 3.8). Values are the means of 5 replicates. Significant differences are shown in table 19 in appendix I.

Figure 34. Direct effect of different concentrations of Acetylsalicylic acid on the surface expansion of *Pyrenophora avenae* mycelium. The pH of the medium was for 5 mM adjusted to 4.4 and 3.5 for 20 mM. Values are the means of 3 replicates. The control observed at day 6 is the mean of 6 readings, and at day 8 of 1 observation. Significant differences are shown in table 19 in appendix I.

Figure 31

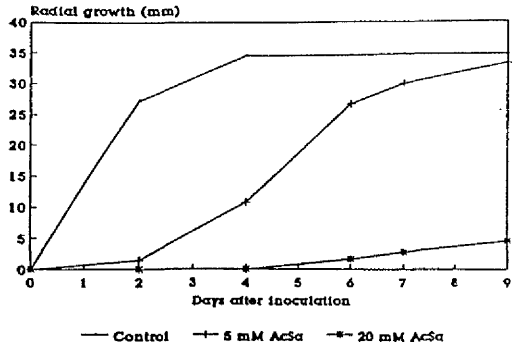


Figure 32

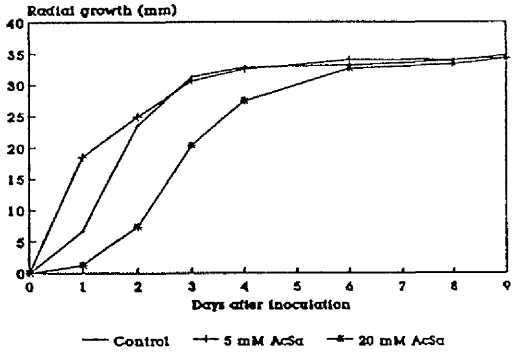


Figure 33

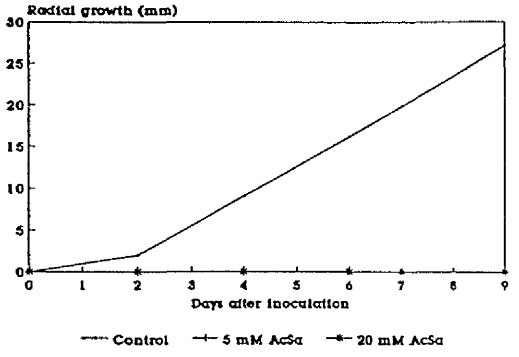
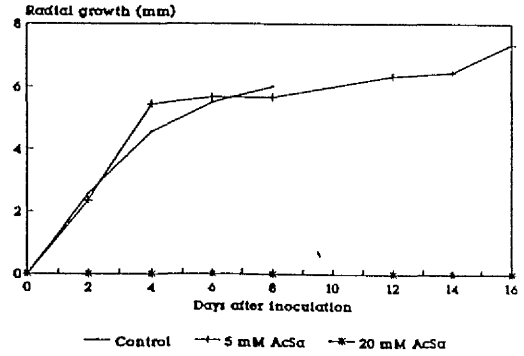


Figure 34



3.7 Analysis of salicylic acid in plant tissue

The absorption spectrum of Sa (50 μg in 5 ml of 99.5% ethanol) had maximum absorption at 212 nm, a smaller peak in absorption at 232 nm, and another, still smaller peak in absorption at 297 nm (Fig. 35). Even when the concentration of the standard solution was reduced, a similar absorption spectrum was obtained. Solution concentrations lower than 5 μg lacked the absorption peak at 235 nm, but absorption at 295 nm was visible when the concentration was as low as 0.5 μg in 5 ml. The peak in absorption at 212 nm moved towards 207 nm when the amount of Sa in 5 ml ethanol became 10 μg or lower.

However, although substantial absorption could be measured in extracts of control and inoculated samples, no absorption peak was observed at 295 nm (Figs. 36 and 37). Almost no absorption could be observed at $\pm$ 235 nm, and the highest absorptions was observed at 208 nm (Figs. 36 and 37).

Increased levels of absorbing compounds were observed when inoculated tissues were compared with the control (Fig 38). Fluorescence of the tissue extracts was measured, but much lower levels of absorbing compounds were observed (Fig. 39).

Figure 35. Analysis of Salicylic acid in plant tissue. Absorption spectrum of Sa (50 μg (A) or 2.5 μg (B) Sa diluted in 5 ml 95 % ethanol). See table 20 in appendix I.

Figure 36. Analysis of Salicylic acid in plant tissue. Absorption spectrum of the TLC spot corresponding to the Sa marker. Sample is an extract of the first, uninoculated leaf. See table 20 in appendix I.

Figure 37. Analysis of Salicylic acid in plant tissue. Absorption spectrum of the TLC spot corresponding to the Sa marker. Sample is an extract of the first leaf, inoculated with rust. See table 22 in appendix I.

Figure 35

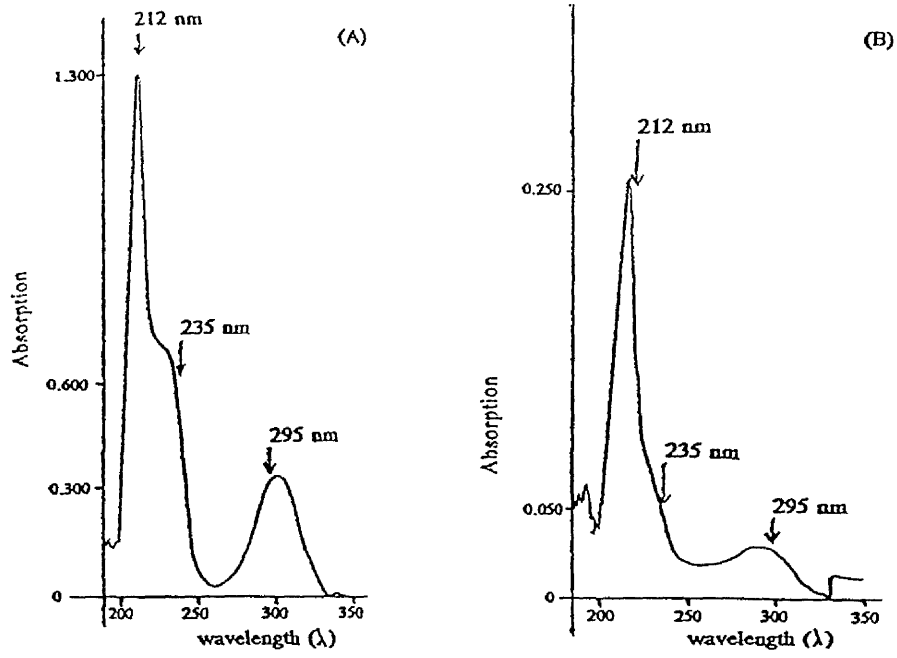


Figure 36

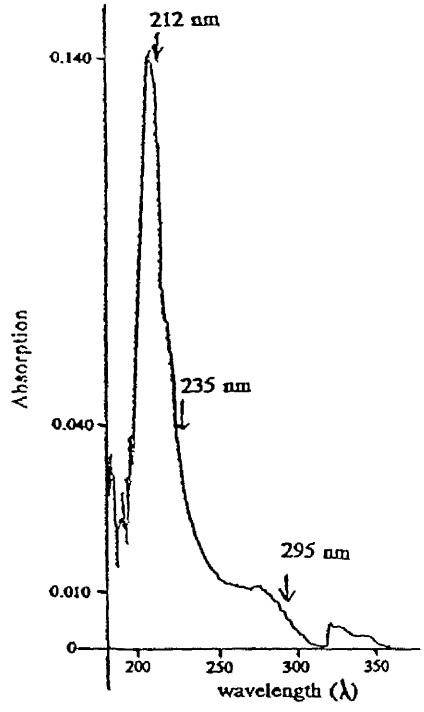


Figure 37

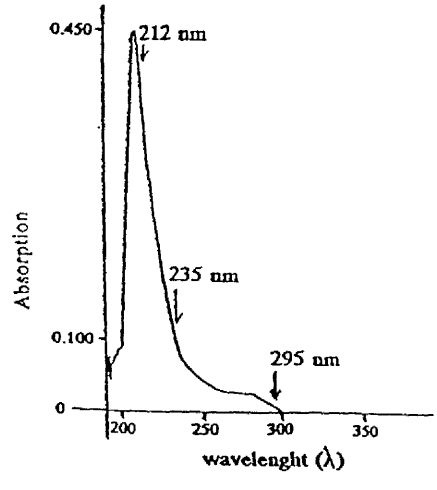


Figure 38. Analysis of Salicylic acid in plant tissue. Amount of absorbing compounds present in uninoculated first leaves and inoculated first leaves. Values are the means of absorption at three wavelengths (212 nm, 235 nm, 295 nm). See table 21 in appendix I.

Figure 39. Analysis of Salicylic acid in plant tissue. Amount of fluorescing compounds present in inoculated first leaves. See table 22 in appendix I.

Figure 38

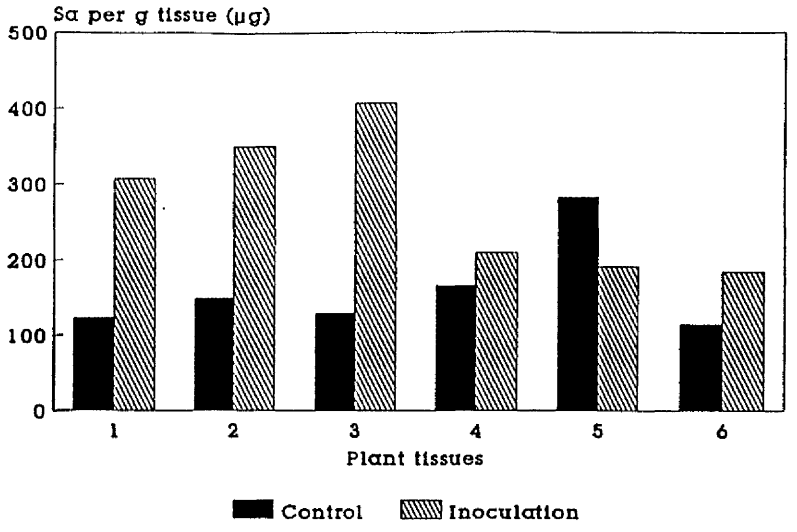
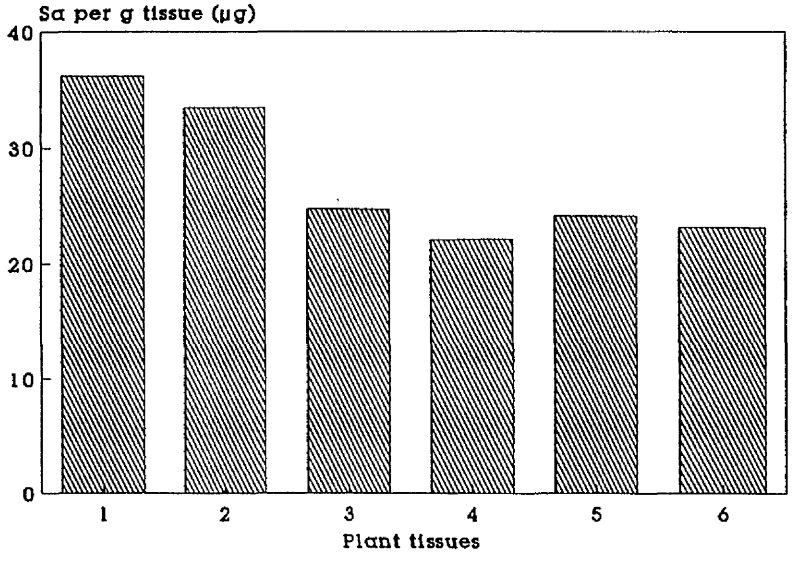


Figure 39



4 DISCUSSION

The crop protection problems, which growers encounter year after year, are reflected in the problems that have to be dealt with by both extension workers and researchers. However, it is important to remember that such research should not be an aim in itself, but rather should affect the way in which agriculturists deal with crop protection problems to minimize disease development and to increase yield. This is important for broad bean (*Vicia faba*), a crop of moderate importance in Western Europe. Broad bean is susceptible to a range of diseases, including chocolate spot, grey mould, leaf spot, sclerotinia and downy mildew, which all flourish well under moist conditions (Neuvel, 1991). Mildew and rust appear in the crop late in the growth season, but high disease pressure can result in substantial yield losses (Rashid and Bernier, 1991). Therefore, research is necessary in order to increase resistance to those pathogens.

Plants possess a wide array of effective mechanisms for disease resistance. In susceptible plants, colonization of the host by the pathogen is not due to a lack of resistance genes, but rather that activation of the genes is not rapid enough and subsequent resistance responses occur too late to prevent the development of the pathogen (Kuc, 1987a). Research has revealed that these latent resistance genes can be activated exogenously using elicitors of pathogen-, plant- or abiotic origin. The induction of resistance to rust fungi has been demonstrated in cereal crops and sunflower (Buchenauer, 1982), and recently Murray and Walters (1992) demonstrated the induction of SR to rust in the upper (second) uninfected leaves of broad bean by prior inoculation of the lower (first) leaves with rust.

There is now much emphasis on the signals involved in SIR. Work is presently concentrated around salicylic acid (Sa), since Malamy *et al.* (1990) postulated that Sa acts as an endogenous signal in the resistance response of tobacco to viruses, and Métraux *et al.* (1990) suggested that Sa is involved in the signalling of SIR in cucumber inoculated with TNV or *Colletotrichum lagenarium.*

In broad bean, SR to rust infection could be induced using Sa, Sodium salicylate (NaSa), and Acetylsalicylic acid (AcSa) (see experiment 1 and figures 2-5). SR was induced at all the concentrations of Sa used, although application of 1, 2, or 5 mM Sa to the first leaves resulted in as much reduction of rust infection in the third leaves, as application of 10 mM or 20 mM Sa. The application of 3 or 4 mM Sa hardly reduced rust infection in the third leaves. NaSa, only once previously mentioned as a possible inducer of SR (Mitchell, 1992), also significantly reduced rust infection, while AcSa had little effect on SR in broad bean. Curiously, in the second experiment, NaSa occasionally increased rust infection (Figure 6), while AcSa induced SR in the third leaves. This variability may be the result of differences in the rates of conversion of the compounds to Sa (particularly AcSa), which may be dependent on environmental conditions both on the leaf surface and in the plant tissue. Exogenously applied AcSa may rapidly be converted to Sa by spontaneous hydrolysis, catalyzed by arylesterases (Mitchell and Broadhead, 1967; Morgan and Truitt, 1965; Harthon and Hedstrom, 1971). It is reported that AcSa causes similar effects to those of Sa (Raskin, 1992a). However, although this was occasionally true in bean (see Figs. 6, 9, 12, 13, 15), it was not always the case. This contradicts observations on the interchangeable use of AcSa and Sa (Raskin, 1992a). However, such results should not be unexpected. For example, attempts to induce

resistance to plant viruses, or stimulation of PR-protein production, in soybean using exogenous salicylate were unsuccessful (Roggero and Pennazio, 1991).

Induction of SR using AcSa and Sa was observed in 1979, when tobacco plants were partially protected against inoculation with TMV (White, 1979), but the first report of SIR to a fungal pathogen using AcSa and Sa was some years later, when Mills and Wood (1984) demonstrated the induction of SR in cucumber to *C. lagenarium* using Polyacrylic acid (Pa), AcSa, and Sa.

Mills and Wood (1984) used similar concentrations of AcSa and Sa (0.02 %, which is 11.1 mM AcSa and 14.5 mM Sa) in their experiments to those used in the experiments reported in this thesis. Interestingly, when endogenous levels of Sa were analyzed recently, much lower concentrations of Sa were observed during SIR, than the concentrations of exogenous Sa required for the induction of SR (Ward *et al.*, 1991; Métraux *et al.*, 1990; Malamy *et al.*, 1992). Exogenous Sa is likely to be rapidly converted in the plant to a conjugated form (Malamy *et al.*, 1992). It is not surprising therefore that high exogenous concentrations are required to induce SR. Mills and Wood (1984) also used phosphate buffers to dissolve Pa, AcSa, and Sa, thus raising questions about the results they obtained, since later work showed that SR to anthracnose (caused by *C. lagenarium*) in cucumber could be induced using phosphates (Gottstein and Kuc, 1989).

The observation that Sa and its derivatives did not always yield similar results, is clear from the results obtained in experiment 2 (Fig. 6). While NaSa hardly induced SR, and actually increased rust infection, Sa and AcSa were very effective in inducing SR. It was expected that, as the interval between induction and challenge-inoculation was increased, SR in the third leaves would decrease. However, substantial SIR was observed when the

interval was 1 or 2 days, SIR almost disappeared on the third day, while on days 5 and 7 an increase in SIR was observed when the inducer leaves were treated with Sa and AcSa, but not with NaSa. This has been observed previously (Murray, 1990). The increase of rust infection on the third day might have been due to the use of a different rust spore concentration in that day, or to differences in light, temperature, RH, and aging of the challenged tissue (Murray and Walters, 1992). Nevertheless, although fluctuations were observed within the time-intervals, an increase of the interval between treatment of the first leaves and inoculation of the third leaves had little effect on the SIR to rust infection. Similar results were observed when broad bean plants were induced using rust, potassium phosphate or EDTA (Murray and Walters, 1992; Walters and Murray, 1992) or when SR was induced in tobacco (Tuzun and Kuc, 1985). A maximal level of SR in barley to infection by *Erysiphe graminis* f.sp. *hordei* was achieved sooner after induction with NaSa than using Sa or AcSa as inducers (Mitchell, 1992), but in broad bean such differences could not be observed.

However, when the distance between the inducer leaves and the challenge-inoculated leaves was increased, significant changes in rust infection were observed (Figs. 7-12). When Sa was applied to the first leaves, SR was induced in the second and third leaves, but no SR was observed in the fourth leaves (Fig. 7). A similar pattern was observed when NaSa and AcSa sprays were applied (Figs. 8 and 9), although SR was not observed in the second leaves when NaSa was applied. Hardly any rust infection was observed on the first treated leaves, which was not the result of the spray solution itself. Although this might have been due to local induced resistance, it may be that not enough light could penetrate through to those lower leaves, thus restricting growth of the pathogen, as shown in tobacco

exhibiting SR to TMV (Yalpani *et al.*, 1991). A different pattern of rust infection altogether was observed when the fourth leaves were treated prior to inoculation of the first to the fourth leaves. No decrease in rust infection was observed in the challenged third leaves immediately below the treated fourth leaves (Figs 10-12), but SR was observed in the first and second leaves, except the first leaves of the AcSa treated plants, which showed enhanced levels of rust infection. It can be suggested that the salicylates (and/or other signals produced after induction of SR) move in both acropetal and basipetal directions in broad bean plants. These results are in agreement with observations made in tobacco systemically-protected against blue mold (Tuzun and Kuc, 1985), and observations that protection is greater in leaves above than below the induced leaves (Dean and Kuc, 1985). Systemic accumulation of Sa was also observed both above, below, and at the site of infection in cucumber (Rasmussen *et al.*, 1991). It would appear that endogenous Sa, moving acropetally following exogenous salicylate application, is highly likely to be metabolized or sequestered (Métraux *et al.*, 1990). Thus, greatest protection will be observed in leaves close to the site of induction. Indeed, when the first leaves of broad bean were treated with salicylates, greatest reductions in rust infection were observed immediately above the inducer leaves, while no reductions were observed in the fourth leaves (Figs. 7-9). However, it is difficult to explain why greatest protection was observed in first and second leaves when the fourth leaves were induced (Figs 10-12).

Various workers have observed a lag-period after induction before the SR is expressed (Dean and Kuc, 1985, 1986; Rasmussen *et al.*, 1991). It was observed in broad bean that very little induced resistance could be observed when the inducer leaves, treated with Sa, NaSa, or AcSa, were removed from the plant 28 h or earlier after induction. However,

great reductions in rust infection were observed when the inducer leaves were detached after 36 h, or were not detached (Figs. 13-15). As observed for cucumber systemically-protected against *C. lagenarium*, these results provide more evidence that the treated or induced leaves are the source of the signal(s) for SIR and it further suggests that intact inducer leaves are necessary for the induction and maintenance of SIR (Dean and Kuc, 1986). The observation of a lag-period for SIR expression after induction was also documented by Rasmussen *et al.* (1991), who demonstrated that the inoculated cucumber leaf had to remain attached for at least 4 h after inoculation with *P.s.* p.v. *syringae*, in order to result in the systemic accumulation of Sa. Why the lag-period for SIR in cucumber is only 4 h, while it in broad bean is 28 h, is difficult to explain, although the following differences in experimental procedures and host plant material should be noted:

1. Cucumber exhibits a greater rate of phloem exudation than broad bean, which may indicate a more rapid transport of phloem mobile components. This could result in a more rapid sensitisation of other parts of the plant. Moreover, Rasmussen *et al.* (1991) examined phloem exudates of the induced first leaf and petiole exudates of the second leaf, while in broad bean the first leaves were treated and rust infection was observed in the third leaves.

2. *P.s.* p.v. *syringae*, which is a rapidly-growing bacterium, was used for inoculation and induction of SIR in the work of Rasmussen *et al* (1991), while *U. viciae-fabae*, a more slowly-developing rust fungus, was used in the work reported in this thesis. Observations using *C. lagenarium* (Métraux *et al.*, 1990) show more similar results to the SR observed in broad bean.

3. In the work of Métraux *et al.* (1990) the systemic accumulation of Sa occurred within 3-4 days of treatment, while expression of SIR was evident between 7-9 days after

treatment. In contrast, in the work in this thesis SIR to rust infection was expressed as reductions in pustule numbers 14 days after inoculation.

Although Sa had no direct antifungal effect on rust, NaSa and AcSa applied as 5 or 20 mM concentrations, both reduced rust infection (Figs. 16, 17, 18). Exactly why Sa and NaSa should have such different effects is not known, especially since NaSa is the Na^+ salt of Sa. It is unlikely that the difference in uptake was due to a pH effect, since the pH of the Sa and NaSa solutions were adjusted to near neutral. However, a difference in uptake may explain why AcSa reduced rust infection directly. It is well-known that addition of methyl or acetyl groups has been used to enhance uptake of drugs or antifungal compounds (Pera *et al.*, 1986).

Spray damage was observed when the higher concentrations of Sa, NaSa, or AcSa were used (Figs. 19-21). Concentrations of Sa lower than 5 mM did not damage the sprayed leaves, although substantial damage was observed using 5 mM Sa. Concentrations of 5 or 10 mM of NaSa or AcSa caused minimal damage to leaf tissue. The high percentage leaf area damaged when 20 mM NaSa or AcSa was used, might have been the cause of the reductions in rust infections in these cases (Figs. 16, 17, 18, and 13, 14, 15). The observed toxicity to plant tissue at higher levels of Sa, was also observed in cucumber (Rasmussen *et al.*, 1991). Interestingly, work on the coordinate expression of PR-genes in tobacco was carried out using exogenous applications of 50 mM NaSa, but no mention was made about toxic effects on plant tissue (Ward *et al.*, 1991). It might be that tobacco leaves are less sensitive to the salicylates or that damage to the plant tissue was not visible.

The size of rust pustules was reduced using Sa, but remained unchanged when NaSa or AcSa were applied to the inoculated leaves (Fig. 22). It is not known whether the

reduction is a result of a direct effect of Sa, or whether it also occurs when SR is induced. The latter was not examined in the present work.

The direct antifungal effects of 20 mM NaSa and AcSa are unlikely to interfere with the results of the experiments, since the compounds were applied to leaves other than the challenge-inoculated leaves. Treatment also took place prior to challenge-inoculation with the rust.

The damage to leaf tissue observed in this work is unlikely to be a cause of the decrease in rust infection in treated plants, since wounding was ineffective in raising the basal levels of Sa in leaf tissue and phloem of tobacco (Yalpani *et al.*, 1991). Also, severely abrading, slicing with a razor blade, or injury with dry ice chips did not increase the accumulation of endogenous Sa in tobacco, indicating that Sa induction and subsequent SIR are not a generalized response to wounding or tissue death (Malamy *et al.*, 1990).

The effects of the salicylates on fungal growth *in vitro* was dependent upon the particular fungus studied and the pH of the medium. Thus, growth of *Botrytis cinerea* was inhibited by Sa and AcSa, while NaSa did not have any effect on surface expansion of the mycelium (Figs. 23, 27, 31). However, when the pH of the Sa and AcSa-amended media was adjusted to pH 6.7, hardly any effect on mycelial growth could be observed (Figs. 24 and 32). This suggests that the growth reductions caused by amending the medium with Sa or AcSa were due to low pH rather than to the compounds themselves. This is supported by the finding that growth on medium amended with NaSa (near neutral pH) was not affected. Although similar results were obtained with *Pyrenophora avenae* (Figs. 25, 26, 29, 30, 33, and 34), this fungus was more sensitive to growth on amended media and even when the pH of the Sa and NaSa media were adjusted to pH 6.7, growth was still

reduced. This is very interesting in view of recent work (Mitchell, 1992) which showed that two isolates of *Pythium ultimum* responded very differently to growth on media amended with salicylates. Thus, isolate A was not affected by growth on Sa, NaSa, or AcSa, while growth of isolate B was greatly reduced in the presence of these compounds. It was found that isolate B had a substantially greater rate of uptake of ^{14}C-Sa (8-fold greater) than isolate B. Métraux *et al.* (1990) and Mills and Wood (1984) could find no effect of salicylates on growth of *Colletotrichum lagenarium in vitro*, although they did not examine the influence of the pH. To what extent the results obtained using *B. cinerea* or *P. avenae in vitro* can be applied to *in vivo* growth of rust is not known. The same fungus may respond differently to treatments when grown *in vivo* or *in vitro* and different fungi, as shown above, may respond differently. In any event, it is highly unlikely that salicylates applied to lower leaves will exert direct antifungal effects on upper leaves, since the Sa will be rapidly metabolized (conjugated) within the treated leave and the concentration accumulating in upper leaves will be very small. This was recently shown to be the case in barley seedlings, where more than 95 % of exogenously-applied Sa remained in the treated leaf (Hampson and Walters, unpublished results).

Initial studies were made to determine the involvement of endogenous Sa in the onset of and/or signalling of SIR in broad bean. Attempts to obtain reliable, consistent phloem exudation were abandoned, since problems of severe dehydration of leaves could not be prevented using the methods described in the literature. Such difficulties were not mentioned when phloem exudates were collected from *Perilla cripsa*, *Chenopodium rubrum*, and *Pharbitis nil*, although detached leaves were kept in darkness (King and Zeevaart, 1974) and in barley, in which the detached leaves were kept in a water vapour-

saturated air stream (Ahmad *et al.*, 1982; Tully and Hanson, 1979). To collect phloem exudates from cucurbits may require less advanced approaches, as described by Richardson *et al.* (1982) and Rasmussen *et al.* (1991).

As demonstrated by the absorption spectra (Figs 35-37), it is very unlikely that the absorptions detected in tissue extracts reflect the presence of Sa in leaf tissues. Although absorption spectra of the tissues, between wavelengths of 200-250 nm were similar to those of pure Sa, the difference appeared between 250-330 nm: where pure Sa showed a peak at $\pm$ 295 nm, this increase in absorption was lacking in tissue extracts. The enhanced levels detected in tissues of rust-inoculated plants may therefore not reflect increases in levels of endogenous Sa, but probably reflect accumulation of other phenolic compounds (Fig. 38). Fluorescence measurements of the tissue extracts yielded much lower levels of Sa-like compounds than the absorption spectra (Fig. 39). Since with fluorescence measurements smaller amounts can be detected compared with using absorption measurements, it may be that with fluorescence, Sa was measured, while by measuring absorption spectra, Sa-conjugates or other phenolic compounds were detected. Thus, the absorption spectra may represent salicylate-derivatives or conjugates, where no increase in absorption at 295 nm could be observed, perhaps because levels of Sa in the tissue extracts were too low to yield the characteristic absorption spectrum for Sa. Still, levels of Sa detected in broad bean tissues using fluorescence, greatly exceeded levels of Sa detected in systemically-protected tobacco or cucumber (Enyedi *et al.*, 1992; Malamy *et al.*, 1990, 1992; Métraux *et al.*, 1990; Yalpani *et al.*, 1991). Sa levels in the leaves of non-thermogenic angiosperms only exceeded 5 μg g^{-1} fresh weight in crabgrass (5.05 ± 1.45) and rice (37.19 ± 4.39) (Raskin *et al.*, 1990). Analysis of Sa in the work of

Rasmussen *et al.* (1991) was performed using HPLC and spectrofluorescence. This suggests that the fluorescence measurements made with broad bean tissue (Fig. 39) may still represent the presence of Sa and Sa-conjugates in the extracts. Further work is required if accurate and reliable determinations of free Sa in broad bean tissue is to be achieved.

5 CONCLUSIONS

Given the ever-present problems posed by attempts to keep crops healthy, it is not surprising that interest in alternative crop protection strategies has increased enormously in the past couple of decades. Early observations indicated that prior-inoculation of plants with pathogens could protect them against further infection (Chester, 1933), but only in the 1970's was induced resistance acknowledged as an important and interesting phenomenon. However, it took considerable research and another decade before the phenomenon of induced resistance became established as a concept. In 1983, Kuc was still puzzled about the few attempts to use systemic induced resistance (SIR) in practical agriculture and that not yet more research was being done to reveal the microbiological and biochemical features of SIR. Reports of SIR in the field are scarce, but molecular approaches to understand SIR have intensified since that time. Research is even more feverish now that Salicylic acid (Sa) is thought to be part of the signalling pathway or the signal itself, in SIR.

Exogenous Sa is able to induce the accumulation of compounds which are related to SIR, or to induce the SIR itself. Moreover, when SR is induced by pathogen infection, for example, endogenous Sa accumulates, concomitant with the appearance of enhanced activities of hydrolytic enzymes and other defense-related compounds.

Research presented in this thesis further supports the involvement of Sa in SIR. However, more research is required to examine the extent to which induction of SR using Sa or its derivatives is both biologically and biochemically similar to induction using pathogens or other methods, e.g. cell wall fragments, or phosphates.

Research on the involvement of Sa in SIR has been performed mainly in tobacco and cucumber, and little is known about Sa and SIR in other systems. Detailed work in other systems is necessary if this research is to benefit agriculture. It will be important to provide more data on the uptake of Sa or its derivatives into plants and into pathogens in order to provide evidence of the proposed non-toxicity of Sa and its derivatives to pathogens. It will also be necessary to know whether the salicylates are taken up into the plant, translocated, and accumulated at sites of SIR. Detailed work is also required on the resistance mechanisms involved in SIR induced by Sa and by other means.

Although it is known that Sa and AcSa can induce SIR in plants, it would be very useful to determine whether other Sa-like compounds can also induce SR. Application of effective Sa mimics might be an important and valuable addition to existing crop protection measures. More practical field work is required to provide information about longevity, range of protection, modes of application of methods for induction, and economic competitiveness with present crop protection methods, in order to provide a picture of the practical applicability of SIR to modern agriculture.

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Appendix I. Statistical calculations of observations

In this section the results of the experiments are shown, together with the s.e. of the mean, t-values, and P-values.

Table 1. Induction of systemic resistance, using Sa

	Rust infection[a]				Significance	
Treatment	Pustules per cm[b]		(%)		T-value	P-value
Control 1	2.723*	±0.59	100.0	±22.00	-	-
Control 2	0.244	±0.08	8.9	± 2.79	-	-
5 mM Sa	0.107	±0.06	3.9	± 2.26	4.35	0.022 *
10 mM Sa	1.026	±0.36	37.7	±13.30	2.43	0.060 s
20 mM Sa	1.162	±0.37	42.7	±13.60	2.22	0.077 s
P	0					

a = mean of 6 replicates ± sem
b = mean of 4 replicates
(%) = Control 1 (not induced) is used to express results as percentage of the control (mean % ±sem)
P = total significance
Significant differences: P = 0.10 s, P = 0.05 *
See figure 2.

Table 2. Induction of SR, using Sa at low concentrations

Treatment	Rust infection[a] (pustules/cm^2)		(%)[d]		Significance[e] T-value	P-value	Leaf area infection[f] (%)		T-value	P-value
Control	0.957[b]	±0.20	100.0	±21.3	-	-	1.435	±0.35	-	-
1 mM	0.383	±0.15	39.9	±16.0	2.25	0.040 *	0.458	±0.21	2.40	0.031 *
2 mM	0.613	±0.16	64.1	±16.5	1.33	0.20	0.712	±0.27	1.65	0.12
3 mM	0.952[c]	±0.26	99.5	±27.0	0.02	0.99	4.542	±3.50	-0.88	0.42
4 mM	0.930	±0.22	97.2	±23.2	0.09	0.93	1.406	±0.16	0.07	0.94
5 mM	0.472[c]	±0.08	49.3	± 8.0	2.23	0.048 *	0.917	±0.11	1.42	0.19
P	0.13		0.13				0.22			

a = mean of 8 replicates ± sem
b = mean of 10 replicates
c = means of 6 replicates
d = Control (mock-induction) is used to express results as percentage of the control (mean % ± sem)
e = Significances of pustule observations
f = equivalent percentages (mean ±se m) of leaf surface covered with rust infection
g = Significances of leaf area covered with rust infection
P = total significance
Significant differences : P = 0.05 *

See figure 3.

Table 3. Induction of SR, using NaSa

	Rust infection[a]				Significances	
Treatment	(pustules per cm^2)		(%)[c]		T-value	P-value
Control 1	2.723[b]	±0.59	100.0	±22.00	-	-
Control 2	0.107	±0.06	3.9	± 2.26	-	-
5 mM	0.283	±0.09	10.4	± 2.13	4.04	0.027 *
10 mm	0.501	±0.15	18.4	± 5.51	3.60	0.037 *
20 mM	0.938	±0.25	34.5	± 9.26	2.75	0.052 s
P	0					

a = mean of 6 replicates ± sem
b = mean of 4 replicates ± sem
c = Control 1 (not induced) is used to express results as percentage of the control (mean % ± sem)
P = total significance
Significant differences : P=0.1 s, P=0.05 *
See figure 4.

Table 4. Induction of SR, using AcSa

	Rust infection[a]				Significance	
Treatment	(pustules per cm^2)		(%)[c]		T-value	P-value
Control	1.320[b]	0.32	100.0	23.8	-	-
5 mM	1.766	0.56	133.8	42.30	-0.70	0.50
10 mM	2.918	0.49	221.1	37.24	-2.74	0.016 *
20 mM	1.103	0.41	83.6	31.02	0.42	0.68
P	0.04					

a = mean of 10 replicates ± sem
b = mean of 16 replicates ± sem
c = Control (mock-induced) is used to express results as percentage of the control (mean % ± sem)
P = total significance
Significant difference : P=0.05 *.
See figure 5.

Table 5. Effect of varying the interval between induction and inoculation on the development of SIR.

Treatment	Rust infection a (pustules per cm²)		(%) c		Significances T-value	P-value
1 days						
Control 1	3.55	±2.46	112.1	±77.7	-0.15	0.89
Control 2	3.17	±1.00	100.0	±31.6	-	-
Sa	0.19	±0.17	6.07	± 5.5	2.92	0.033 *
NaSa	4.53	±0.35	142.9	±11.0	-1.28	0.25
AcSa	0.62	±0.27	19.70	± 8.6	2.45	0.058 s
P	0.070					
2 days						
Control 1	4.48	±1.01	95.93	±21.6	0.11	0.92
Control 2	4.67	±1.47	100.0	±31.4	-	-
Sa	1.46	±1.35	31.20	±28.8	1.62	0.14
NaSa	1.85	±0.53	39.65	±11.4	1.81	0.12
AcSa	1.89	±0.32	40.47	± 6.74	1.86	0.12
P	0.087					
3 days						
Control 1	1.29	±0.57	32.88	±14.5	1.79	0.12
Control 2	3.92	±1.36	100.0	±34.7	-	-
Sa	4.83b	±2.59	123.3	±27.1	-0.48	>0.1
NaSa	6.27	±1.11	159.9	±28.2	-1.34	0.21
AcSa	0.14	±0.50	79.98	±12.8	0.54	0.61
P	0.012					
5 days						
Control 1	2.99	±0.85	50.40	±14.4	1.08	0.32
Control 2	5.94	±2.59	100.0	±43.5	-	-
Sa	3.72	±0.71	62.55	±11.9	0.83	0.45
NaSa	4.64	±0.84	78.09	±14.2	0.48	0.65
AcSa	0.85	±0.38	14.33	± 6.39	1.95	0.11
P	0.116					
7 days						
Control 1	3.09	±1.76	116.9	±66.7	-0.21	0.84
Control 2	2.64	±1.10	100.0	±41.6	-	-
Sa	0.19	±0.11	7.4	± 4.31	2.22	0.077 s
NaSa	4.08	±0.94	154.5	±35.5	-0.99	0.35
AcSa	0.0	± 0.0	0.0	± 0.0	1.833	0.10 s
P	0.031					

a = mean of 6 replicates ± sem
b = mean of 2 replicates ± sem
c = Control 2 (mock-induced) is used to express results as percentage of the contol (± sem)
P = total significance
Significant differences : P = 0.10 s, P = 0.05 * See figure 6.

Table 6. Effect of varying the distance between the inducer first leaves and the challeng-inoculated first to fourth leaves, on the induction of SR.

	Inducer compounds							
	Control		Sa		NaSa		AcSa	
Leaf level	Rust infection (pustules per cm^2)[a]							
1	0.019	±0.01	0.047	±0.03	0.004	±0.00	0.019	±0.02
2	1.018	±0.35	0.749	±0.31	2.487	±0.69	0.409	±0.28
3	0.856	±0.29	0.093[c]	±0.04	0.397	±0.13	0.921	±0.38
4	0.814[b]	±0.38	0.988	±0.27	1.620	±0.20	1.132	±0.08
P	0.16		0.02		0.0		0.02	

a = means of 6 replicates ± sem
b = mean of 8 replicates
c = mean of 4 replicates
P = Total significance.
See figures 7, 8, and 9.

Table 7. Effect of varying the distance between the inducer fourth leaves and the challeng-inoculated first to fourth leaves, on the induction of SR.

	Inducer compounds							
	Control		Sa		NaSa		AcSa	
Leaf level	Rust infection (pustules per cm^2)[a]							
1	0.510	±0.23	0.295[b]	±0.17	0.092	±0.05	2.206	±0.74
2	1.342	±0.46	0.914[b]	±0.20	0.701	±0.40	1.215	±1.01
3	0.393	±0.18	1.524	±0.48	0.578	±0.12	0.965	±0.16
4	1.451	±0.63	0.154	±0.06	0.304	±0.18	0.144	±0.06
P	0.19		0.02		0.26		0.18	

a = means of 6 replicates ± sem
b = mean of 4 replicates
P = Total significance.
See figures 10, 11, and 12.

Table 8. Effect of varying the distance between the inducer leaves and the challeng-inoculated leaves, on the induction of SR, significant differences

	Sa				NaSa				AcSa			
	signal moving :											
	acropetal		basipetal		acropetal		basipetal		acropetal		basipetal	
Level	T	P-value	T	P-value	T	P-value	T	P-value	T	P-value	T	P-value
1	-0.8[a]	0.44	0.74	0.48	1.03	0.35	1.75	0.14	0.02	0.98	-2.19	0.080 s
2	0.57	0.58	0.86	0.42	-1.9	0.10 s	1.06	0.32	1.35	0.21	0.11	0.91
3	2.59	0.05 *	-2.22	0.068 s	1.45	0.20	-0.85	0.42	-0.1	0.89	-2.37	0.042 *
4	-0.4	0.71	2.05	0.096 s	-1.9	0.09 s	1.75	0.14	-0.8	0.44	2.06	0.094 s

a = Each treatment is compared with its control treatment within the same challenge-inoculated level of leaves.
Significant differences : P = 0.10 s, P = 0.05 *
See tables 6 and 7, and figures 7 to 12.

Table 9. Effect of detaching the inducer leaves at different times after induction, on the development of SIR

	Rust infection (pustules per cm ^{2}) using Inducer compound :[a]							
Interval (h)	Control		Sa		NaSa		AcSa	
4	1.042	±0.49	0.275	±0.11	1.676	±0.83	2.488	±1.04
12	0.498	±0.24	0.380	±0.17	1.383[b]	±0.42	1.118[b]	±0.31
24	0.229	±0.07	0.654[b]	±0.15	0.192[b]	±0.07	0.172	±0.13
28	0.466	±0.10	0.693	±0.14	1.113	±0.27	0.857	±0.22
36	2.284	±0.70	0.211	±0.07	0.353	±0.15	0.927	±0.29
ND	1.508	±0.50	1.648	±0.31	0.328	±0.21	0.782	±0.26
P	0.01		0.00		0.11		0.60	

a = mean of 6 replicates ± sem
b = means of 4 replicates
ND = Inducer leaves not detached
P = total significance.
See table 10 and figures 13, 14, and 15.

Table 10. Effect of detaching the inducer leaves at different times after induction, on the development of SIR, significant differences

	Significant differences					
	Control compared with :					
	Sa		NaSa		AcSa	
Interval (h)	T-value	P-value	T-value	P-value	T-value	P-value
4	1.51	0.19	-0.66	0.53	-1.25	0.25
12	0.40	0.70	-1.83	0.14	-1.57	0.17
24	-2.59	0.061 s	0.36	0.73	0.39	0.71
28	-1.29	0.23	-2.27	0.064 s	-1.60	0.15
36	2.95	0.032 *	2.71	0.042 *	1.80	0.12
ND	-0.24	0.882	2.18	0.072 s	1.30	0.24

ND = inducer leaves not detached
Significant differences : P=0.10 s, P=0.05 *.
See table 9 and figures 13, 14, and 15.

Table 11. Direct effects of Sa, NaSa, and AcSa on rust infection

Compound	Rust infection[ab]		Significances T-values	P-values
Control	0.322[c]	±0.06	-	-
Sa				
5 mM	0.337	±0.12	-0.11	0.91
10 mM	0.229	±0.06	1.18	0.24
20 mM	0.840	±0.41	-1.24	0.23
NaSa				
5 mM	0.016	±0.01	5.30	0.0 ***
10 mM	0.232	±0.08	0.94	0.35
20 mM	0.003	±0.003	5.62	0.0 ***
AcSa				
5 mM	0.072	±0.03	3.85	0.0002 ***
10 mM	0.263	±0.13	0.41	0.69
20 mM	0.041	±0.02	4.55	0.0 ***

a = Mean number of pustules per cm2 ± sem
b = means of 18 readings from 3 plants
c = control is mean of 60 observations
Significant differences : P = 0.001 ***
See figures 16, 17, and 18.

Table 12. Damage on leaves, when treated with Sa, NaSa, or AcSa

Compound	Days post treatment with compound (a) 3		6		12	
Control	0.0[b]	±0.0	0.0	±0.0	0.0	±0.0
Sa						
5 mM	3.6[c]	±1.1	11.8	±3.9	35.9	±11.0
10 mM	7.5	±0.6	5.4	±0.7	6.1	±1.3
20 mM	43.3	±2.3	58.7	±6.7	51.1	±8.2
NaSa						
5 mM	0.4	±0.1	1.0	±0.4	0.8	±0.4
10 mM	3.9	±1.1	4.7	±1.0	4.7	±1.0
20 mM	31.7	±5.2	43.7	±6.3	47.5	±7.7
AcSa						
5 mm	0.3	±0.1	0.1	±0.1	0.0	±0.0
10 mM	7.9	±1.0	9.0	±1.5	9.8	±4.5
20 mM	38.9	±4.9	43.1	±6.5	44.9	±8.3

a = mean damage to treated leaves ± sem
b = means of 18 observations from 3 plants
c = means of 60 observations
No significant differences could be calculated.
See figures 19, 20, and 21.

Table 13. Size of rust pustules after treatment with Sa, NaSa, or AcSa

Compound	N	Size (mm)		Significance T-value	P-value
Control	44	1.4	±0.08	-	
Sa					
5 mM	9	0.7	±0.08	6.07	0 ***
10 mM	14	0.5	±0.04	9.30	0 ***
20 mM	11	1.7	±0.14	-2.08	0.053 s
NaSa					
5 mM	3	1.2	±0.44	0.49	0.67
10 mM	14	1.4	±0.14	-0.26	0.80
20 mM	1	1.0	-	-	-
AcSa					
5 mm	10	1.0	±0.19	2.11	0.057 s
10 mm	10	1.1	±0.21	1.28	0.23
20 mM	4	1.1	±0.31	0.80	0.48

a = Number of observations
b = Mean pustule sizes ± sem
Significant differences : P = 0.10 s, P = 0.001 ***
See figure 22.

Table 14. Direct effects of Sa on *Botrytis cinerea*

	Radial growth of mycelium plug on amended medium (mm)a			
Treatment	pH not adjusted		pH adjusted	
day 1				
Control	NM		6.60	±1.08
5 mM	NM		8.93	±3.20
20 mM	NM		3.20	±0.79 *
day 2				
Control	3.67	±0.86	23.53	±1.97
5 mM	0.73	±0.12 **	26.47	±2.58
20 mM	0.00	±0.00 ***	14.80	±2.80 *
day 3				
Control	4.07	±1.02	31.27	±0.76
5 mM	1.53	±0.24 *	31.20	±1.16
20 mM	0.00	±0.00 ***	27.40	±1.68 *
day 4				
Control	NM		32.87	±0.42
5 mM	NM		33.37	±0.49
20 mM	NM		30.67	±1.15 s
day 6				
Control	30.40	±1.73	33.13	±0.42
5 mM	6.53	±0.26 ***	34.27	±0.37 s
20 mM	0.00	±0.00 ***	34.13	±0.49
day 7				
5 mM	8.00	±0.26 ***	NM	
day 8				
Control	NM		33.87	±0.40
5 mM	NM		34.47	±0.38
20 mM	NM		34.33	±0.49
day 9				
Control	NM		34.73	±0.41
5 mM	11.27	±0.49 ***	34.67	±0.45
20 mM	NM		34.67	±0.45

a = mean of 5 replicates ± sem
NM = not measured
Significant differences : P = 0.10 s, P = 0.05 *, P = 0.01 **, P = 0.001 ***
See figures 23 and 24.

Table 15. Direct effects of NaSa on *Botrytis cinerea*

Treatment	Radial growth of mycelium plug on amended medium (mm)a			
	pH not adjusted		pH adjusted	
day 1				
Control	NM		6.60	±1.08
5 mM	NM		9.07	±2.45
20 mM	NM		2.73	±0.81 **
day 2				
Control	3.67	±0.86	23.53	±1.97
5 mM	1.80	±0.28 s	20.20	±2.97
20 mM	6.47	±1.46	11.33	±2.91 **
day 3				
Control	4.07	±1.02	31.27	±0.76
5 mM	2.60	±0.32	27.67	±2.59
20 mM	7.00	±1.39 s	18.93	±2.73 ***
day 4				
Control	NM		32.87	±0.42
5 mM	NM		28.80	±2.39
20 mM	NM		21.40	±2.69 ***
day 6				
Control	30.40	±1.73	33.13	±0.42
5 mM	34.87	±0.62 *	33.40	±0.64
20 mM	34.53	±1.15 s	28.00	±2.08 *
day 8				
Control	NM		33.87	±0.40
5 mM	NM		33.80	±0.69
20 mM	NM		29.47	±1.89 *
day 9				
Control	NM		34.73	±0.41
5 mM	NM		34.33	±0.58
20 mM	NM		33.53	±1.17

a = mean of 5 replicates ± sem
NM = not measured
Significant differences : P = 0.10 s, P = 0.05 *, P = 0.01 **, P = 0.001 ***
See figures 27 and 28.

Table 16. Direct effects of AcSa on *Botrytis cinerea*

	Radial growth of mycelium plug on amended medium (mm)a			
Treatment	pH not adjusted		pH adjusted	
day 1				
Control	NM		6.60	±1.08
5 mM	NM		18.47	±3.58 **
20 mM	NM		1.27	±0.38 ***
day 2				
Control	27.13	±2.37	23.53	±1.97
5 mM	1.47	±0.38 ***	24.93	±2.85
20 mM	0.0	±0.00	7.33	±1.74 ***
day 3				
Control	NM		31.27	±0.76
5 mM	NM		30.60	±1.89
20 mM	NM		20.33	±2.09 ***
day 4				
Control	34.47	±1.02	32.87	±0.42
5 mM	10.73	±1.64 ***	32.53	±1.02
20 mM	0.0	±0.00	27.40	±1.43 **
day 6				
Control	34.47	±1.02	33.13	±0.42
5 mM	26.60	±2.17 **	33.93	±0.62
20 mM	1.67b	±0.24 ***	32.60	±0.65
day 7				
Control	34.67	±0.98	NM	
5 mM	29.93	±1.73 *	NM	
20 mM	2.78b	±0.22 ***	NM	
day 8				
Control	NM		33.87	±0.40
5 mM	NM		34.00	±0.55
20 mM	NM		33.33	±0.65
day 9				
Control	34.67	±0.98	34.73	±0.41
5 mM	33.27	±0.84	34.27	±0.49
20 mM	4.56b	±0.18 ***	33.13	±0.61

a = mean of 5 replicates ± sem
b = mean of 3 replicates ± sem
NM = not measured
Significant differences : P = 0.05 *, P = 0.01 **, P = 0.001 ***
See figures 31 and 32.

Table 17. Direct effects of Sa on *Pyrenophora avenae*

	Radial growth of mycelium plug on amended medium (mm)[a]			
Treatment	pH not adjusted		pH adjusted	
Day 2				
Control	2.00[b]	±0.00	2.56	±0.18
5 mM	0.00	±0.00	2.00	±0.00
20 mM	0.00	±0.00	1.89	±0.31 s
Day 3				
Control	2.87	±0.09	NM	
5 mM	0.00	±0.00	NM	
20 mM	0.00	±0.00	NM	
Day 4				
Control	NM		4.56	±0.34
5 mM	NM		3.44	±0.18 *
20 mM	NM		2.67	±0.58 *
Day 5				
Control	5.47	±0.13	NM	
5 mM	0.00	±0.00	NM	
20 mM	0.00	±0.00	NM	
Day 6				
Control	NM		5.50	±0.34
5 mM	NM		3.78	±0.28 **
20 mM	NM		3.11	±0.72 *
Day 7				
Control	8.53	±0.48	NM	
5 mM	0.00	±0.00	NM	
20 mM	0.00	±0.00	NM	
Day 8				
Control	NM		6.00	±0.00
5 mM	NM		3.56	±0.96 *
20 mM	NM		4.11	±0.32 ***

a = mean of 5 replicates ± sem
NM = not measured
Significant differences : P = 0.10 s, P = 0.05 *, P = 0.01 **, P = 0.001 ***
See figures 25 and 26.

Table 17. Continued

Treatment	Radial growth of mycelium plug on amended medium (mm)[a]			
	pH not adjusted		pH adjusted	
Day 10				
Control	19.60	±0.59	NM	
5 mM	0.00	±0.00	NM	
20 mM	0.00	±0.00	NM	
Day 12				
Control	NM		-	
5 mM	NM		4.00	±0.37 s
20 mM	NM		3.89	±1.16 s
Day 14				
Control	NM		-	-
5 mM	NM		4.56	±0.34
20 mM	NM		4.00	±1.29
Day 16				
Control	NM		-	-
5 mM	NM		4.56	±0.34
20 mM	NM		4.67	±1.45

a = mean of 5 replicates ± sem
NM = not measured
Significant differences : P = 0.10 s
See figures 25 and 26.

Table 18. Direct effects of NaSa on *Pyrenophora avenae*

Treatment	Radial growth of mycelium plug on amended medium (mm)[a]			
	pH not adjusted		pH adjusted	
Day 2				
Control	2.00[b]	±0.00	2.56	±0.18
5 mM	1.87	±0.13	2.33	±0.17
20 mM	1.80	±0.11 s	3.00	±0.00 s
Day 3				
Control	2.87	±0.09	NM	
5 mM	3.00	±0.14	NM	
20 mM	2.80	±0.11	NM	
Day 4				
Control	NM		4.56	±0.34
5 mM	NM		4.33	±0.60
20 mM	NM		5.11	±0.46
Day 5				
Control	5.47	±0.13	NM	
5 mM	5.53	±0.17	NM	
20 mM	5.80	±0.21	NM	
Day 6				
Control	NM		5.50	±0.34
5 mM	NM		4.22	±0.60 s
20 mM	NM		5.67	±0.29
Day 7				
Control	8.53	±0.48	NM	
5 mM	9.47	±0.44	NM	
20 mM	7.53	±0.36	NM	
Day 8				
Control	NM		6.00	±0.00
5 mM	NM		4.44	±0.65 *
20 mM	NM		6.00	±0.44

a = mean of 5 replicates ± sem
NM = not measured
Significant differences : P = 0.10 s, P = 0.05 *
See figures 30 and 31.

Table 18. Continued

Treatment	Radial growth of mycelium plug on amended medium (mm)[a]	
	pH not adjusted	pH adjusted
Day 10		
Control	19.60 ±0.59	NM
5 mM	19.38 ±0.89	NM
20 mM	17.00 ±1.00	NM
Day 12		
Control	NM	- -
5 mM	NM	4.78 ±1.00
20 mM	NM	7.22 ±0.64 **
Day 14		
Control	NM	- -
5 mM	NM	5.33 ±1.19
20 mM	NM	8.56 ±0.71
Day 16		
Control	NM	- -
5 mM	NM	5.67 ±1.28
20 mM	NM	9.67 ±0.78

a = mean of 5 replicates ± sem
NM = not measured
Significant differences : P = 0.01 **
See figures 30 and 31.

Table 19. Direct effects of AcSa on *Pyrenophora avenae*

	Radial growth of mycelium plug on amended medium (mm)[a]			
Treatment	pH not adjusted		pH adjusted	
Day 2				
Control	2.00[b]	±0.00	2.56	±0.18
5 mM	0.00	±0.00	2.33	±0.17
20 mM	0.00	±0.00	0.00	±0.00
Day 4				
Control	9.00	±0.41	4.56	±0.34
5 mM	0.00	±0.00	5.44	±0.38 s
20 mM	0.00	±0.00	0.00	±0.00
Day 6				
Control	16.07	±0.71	5.50	±0.34
5 mM	0.00	±0.00	5.67	±0.41
20 mM	0.00	±0.00	0.00	±0.00
Day 7				
Control	19.80	±0.81	NM	
5 mM	0.00	±0.00	NM	
20 mM	0.00	±0.00	NM	
Day 8				
Control	NM		6.00	±0.00
5 mM	NM		5.67	±0.37
20 mM	NM		0.00	±0.00
Day 9				
Control	27.07	±0.95	NM	
5 mM	0.00	±0.00	NM	
20 mM	0.00	±0.00	NM	

a = mean of 5 replicates ± sem
NM = not measured
Significant differences : P = 0.10 s
See figures 30 and 31.

Table 19. Continued

Treatment	Radial growth of mycelium plug on amended medium (mm)[a]	
	pH not adjusted	pH adjusted
Day 12		
Control	NM	- -
5 mM	NM	6.33 ±0.27 *
20 mM	NM	0.00 ±0.00
Day 14		
Control	NM	- -
5 mM	NM	6.44 ±0.44
20 mM	NM	0.00 ±0.00
Day 16		
Control	NM	- -
5 mM	NM	7.33 ±0.58
20 mM	NM	0.00 ±0.00

a = mean of 5 replicates ± sem
NM = not measured
Significant differences : P = 0.05 *
See figures 33 and 34.

Table 20. Analysis of salicylic acid in 50 μl extracts of uninoculated tissue, collected after 48 h.

	Tissue[c]	Ethanol[d]	Absorption[e]				Sa in 50 μl (μg)				Multiple	
	weight (g)	added (ml)	wavelengths				wavelength				wavelengths	
I.D.[a]			212 nm	235 nm	295 nm	305 nm	212 nm	235 nm	295 nm	305 nm	3[f]	4[g]
1	1.761	4.420	0.165	0.023	0.004	0.004	5.821	2.134	0.159	0.862	2.454	0.838
2	1.284	3.210	0.189	0.028	0.005	0.005	6.910	2.791	0.336	1.033	2.978	1.072
3	1.261	3.153	0.139	0.026	0.004	0.006	4.640	2.600	0.159	1.204	2.571	1.154
4	1.611	4.027	0.189	0.032	0.005	0.005	6.910	3.172	0.336	1.033	3.298	1.252
5	0.458	1.145	0.223	0.059	0.007	0.006	8.454	5.748	0.690	1.204	5.639	2.496
6	1.291	3.240	0.145	0.022	0.004	0.004	4.613	2.218	0.159	0.862	2.280	0.873
Sa[b]	*	*	1.386	0.489	0.319	0.275	61.25	46.77	55.91	14.20	49.27	49.16
Sa	*	*	0.845	0.266	0.173	0.149	36.69	25.50	30.07	25.65	27.13	26.34
Sa	*	*	1.297	0.445	0.297	0.258	57.21	42.57	52.02	44.30	45.07	45.37

a = identity (see Materials and Methods)
b = 50 μg marker
c = g leaf or stem tissue
d = amount of ethanol (ml) added
e = 1 reading per wavelength, sample read at 4 wavelengths
f = μg in 50 μl calculated using the absorptions at 212nm, 235nm, and 295 nm
g = μg in 50 μl calculated using the absorptions of the four wavelengths
For calculation equations see Appendix 2
See figures 35, and 36

Table 21. Analysis of salicylic acid per g uninoculated tissue, collected after 48 h.

	Sa per g tissue (μg)		Sa per g tissue (μg)			
	multiple		wavelength[b]			
Identity[a]	3[c]	4[d]	212 nm	235 nm	295 nm	305 nm
1	123.2	42.1	292.2	116.2	8.0	43.3
2	148.9	53.7	345.5	139.6	16.8	51.7
3	128.6	57.7	232.1	130.0	8.0	60.2
4	164.9	62.6	345.6	158.7	16.8	51.7
5	282.0	124.8	442.7	287.4	34.5	60.2
6	114.4	43.8	246.6	111.4	8.0	43.3

a = identity (see Materials and Methods)
b = 1 reading per wavelength, sample read at 4 wavelengths
c = μg Sa per g tissue using the absorptions at 212nm, 235nm, and 295 nm
d = μg Sa per g tissue using the absorptions of the four wavelengths
For calculation equations see Appendix 2
See figures 35, 36 and 38

Table 22. Analysis of salicylic acid in 50 μg extracts of inoculated plants, collected 48 h after inoculation.

	Tissue	Ethanol	Absorption[d]			Sa in 50 μl[e]					
	weight (g)	added (ml)	wavelenghts			wavelengths			multiple	Fluorescence	(μg)
I.D.[a]			212 nm	235 nm	295 nm	212 nm	235 nm	295 nm	3		
1	1.210	3.02	0.250	0.063	0.012	9.680	6.130	1.575	6.147	114.8	0.724
2	1.137	2.84	0.280	0.071	0.018	11.042	6.893	2.637	7.001	105.2	0.670
3	1.372	3.42	0.288	0.085	0.017	11.405	8.229	2.460	8.145	70.0	0.497
4	1.211	3.04	0.135	0.045	0.011	4.459	4.413	1.398	4.156	56.7	0.441
5	1.033	2.58	0.126	0.040	0.020	4.050	3.936	2.991	3.824	67.1	0.484
6	1.297	3.24	0.133	0.039	0.012	4.368	3.841	1.575	3.680	62.5	0.464
50[b]	*	*	1.054	0.359	0.215	46.182	34.369	37.506	36.053	564.8	6.346
50[b]	*	*	1.350	0.495	0.314	59.620	47.343	55.029	49.518	583.0	6.701
100[c]	*	*	1.648	0.656	0.406	73.149	62.702	71.313	64.905	668.2	8.496

a = identity (see Materials and Methods)
b = 50 μg Sa marker
c = 100 μg Sa marker
d = 1 reading per wavelength, sample read at 3 wavelengths
e = μg Sa in 50 μl extract using the absorptions at 212nm, 235nm, and 295 nm
For calculation equations see Appendix 2
See figure 37

Table 23. Analysis of salicylic acid per g tissue of inoculated plants, collected 48 h after inoculation.

	Sa per g tissue (μg)[b]				
	wavelength			Multiple[c]	Fluorescence
Identity[a]	212 nm	235 nm	295 nm	3	(μg)
1	483.20	306.00	78.62	306.85	36.13
2	551.61	344.37	131.73	349.72	33.47
3	568.60	410.25	122.64	406.04	24.75
4	223.87	221.56	70.19	208.67	22.12
5	202.32	196.61	149.41	191.03	24.17
6	218.24	191.88	78.69	183.84	23.20

a = identity (see Materials and Methods)
b = 1 reading per wavelength, sample read at 3 wavelengths
c = μg Sa per g tissue using the absorptions at 212nm, 235nm, and 295 nm
For calculation equations see Appendix 2.
See figures 38 and 39

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Appendix II. Regression equations for calculation of μg Sa, and marking of plant tissue

Amount of Sa in 50 μl, spotted on the plate (μg)		
Absorption		
Wavelength	Regression equation:	R-sq (adj.)
212 nm	-1.670 + 45.4 x (212 nm)[ab]	97.8 %
235 nm	0.120 + 95.4 x (235 nm)	99.5 %
295 nm	-0.549 + 177.0 x (295 nm)	99.1 %
	Multiple regression equation:	
	-0.208 + 4.7 x (212 nm) + 79.9 x (235 nm) + 12.2 x (295 nm)	99.9 %
Fluorescence		
Equation:	0.262 + 0.023 x (fluorescence) + 0.000015 x (fluorescence)	96.3 %
Final amount of Sa per g tissue (μg)		
	(μg Sa in 50 μl) x ((ml ethanol added)/0.05) / weight of tissue	

a = Equations are calculated from Sa standard series ranging from 0.005 to 200 μg Sa per 5 ml 99.5 % ethanol.
b = Absorption at 212 nm etc.

Tissues were marked as follows:

1: stem under first leaf level;
2: first, induced leaf level;
3: stem between first and second leaf level;
4: second leaf level;
5: stem between second and third leaf level;
6: third leaf level.

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