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Commercialization of Plant Systemic Defense Activation: Theory, Problems and Successes

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17.1 Introduction

Crop protection can reduce losses by 10% to 60% depending on the disease, the locality and the crop (Crop Protection Compendium, 2002). An array of different strategies to reduce the consequences of pathogen pressure is available. Of these methods, the use of commercial products that stimulate defense reactions in the plant host to reduce plant pathogen success is in its infancy. Although the activation of systemic resistance has been demonstrated reproducibly in the laboratory for many plant species, utilizing a wide range of activating materials, it is not yet a proven technology widely accepted in commerce. A general view is that field results are too variable, and therefore risky, for many farmers when the alternative strategies for protection are perceived as more reliable. Systemic defense activation, however, offers attractive features:

- Ecological compatibility, with some products fulfilling the requirements for the "organic" farming label.
- Protection for the whole plant, with effects extending post harvest.
- Protection against pathogens that are not controlled by available methods, which is especially valuable for those pathogens with resistance to a chemical pesticide.
- Function through plant-based mechanisms rather than a direct attack on the pathogenic organism, thus, avoiding direct but undesired effects on non-pathogenic organisms.
- Provision of protection to a broad range of challenges including microbes, insects and nematodes.
- Compatibility with short time reentry and short time preharvest applications.
- Applications may be teamed with other differently based strategies to provide better protective coverage.
- The array of genes activated in systemic resistance may be beneficial in thwarting other stresses in the field, such as heat, cold, drought, and damage from the blue to UV irradiances of sunlight.

Detriments to commercial use include:

- The protection requires time to become effective in the plant, especially when the stimulating treatment is not applied to the whole plant.
- Variability in performance, especially in instances where biologicals are used to activate the defenses.
- Activation of defense against one pathogen may promote greater susceptibility to other pathogens using different strategies to attack the plant.
- Fine-tuning of the activation mixture, the method of application and the timing between applications for maximum effectiveness.
- Overexpression of defenses may lead to stunting and reduced productivity.

In this review, we introduce the mechanisms leading to induced plant defenses and illustrate some peculiarities of systemic resistance compared with the hypersensitive response (HR). We discuss how molecular and biochemical knowledge has participated in the development and understanding of the mode of action of commercial products that stimulate plant systemic defense in the field. We describe the nature of products that are commercially available with their division into chemical and microbial categories. We close with summaries and speculations.

17.1.1 Molecular Understanding of the Pathways for Systemic Resistance

Two pathways for systemic resistance that have drawn the main attention of researchers involve salicylic acid (SA) or jasmonic acid (JA)/ethylene as key signaling compounds (Dong, 1998; Reymond and Farmer, 1998). As discussed in detail by Nawrath et al. and Pieterse et al. (Chapters 7 and 8, this volume) these pathways result in the accumulation of the products of different defense genes. Examples of these differences are illustrated in Figure 17.1.

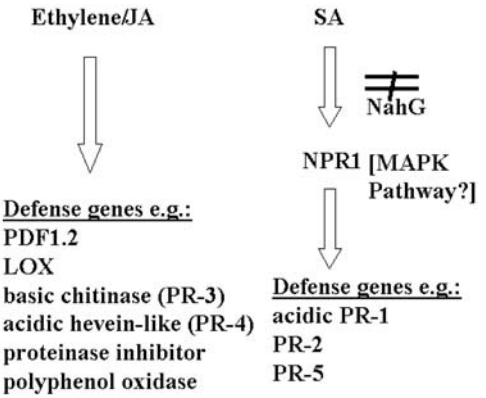

FIGURE 17.1. Differential defense gene activation by pathways involving ethylene/JA or SA.

The defense participants include the pathogenesis related (PR) proteins, discussed by Tuzun et al. (Chapter 6, this volume). The functions of this group are diverse and some are not as yet fully resolved, e.g., some members of the PR-1 group are antifungal by unknown mechanisms (Alexander et al., 1993). Other PR proteins have enzymatic activities that will degrade components in fungal cell walls (glucanases and chitinases) or help to generate phenolic radicals (the peroxidases) to produce barriers, such as cell wall lignification, or other antifungal materials in the plant. The marker protein most commonly ascribed to the SA pathway is the acidic PR-1, whereas PDF1.2 and the protease inhibitor genes are correlated with the JA/ethylene pathway. Expression of genes encoding the basic PR proteins, generally ascribed to a vacuolar location, is attributed more to the JA/ethylene-regulated defense pathway (van Loon, 1997). In contrast, the acidic PR proteins are generally thought to be apoplastic and associated with the SA-regulated pathway. However, global gene expression analysis reveals that several defense and metabolic genes are coregulated by both SA and JA/ethylene (e.g., Schenk et al., 2000). Crosstalk between metabolic pathways that involve genes encoding defense proteins controlled by such different plant growth regulators as JA, ethylene, SA, and abscisic acid is observed (e.g., Audenaert et al., 2002a; Gazzarrini and McCourt, 2003; Kunkel and Brooks, 2002). Thus, although these pathways can be viewed academically as being distinct, it is likely that effective resistance in the field will arise as a result of crosstalk between several pathways controlling defense gene expression.

The effectors that activate the SA and JA/ethylene regulated defense pathways may differ, as illustrated in Figure 17.2. Activation of the SA-regulated pathway is associated with events that cause necrosis. Thus, the pathway is aligned with the hypersensitive response (HR) where programmed plant cell death is part of the mechanism by which a pathogen is constrained to the initial invasion site. Cell death by HR initiates resistant events, termed local resistance, in the cells surrounding the containment site (Dangl et al., 1996).

With time, expression of defense genes occurs at greater distance to result in a systemic effect (Epple et al., 2003). Pathogens that cause necrosis as part of their symptomology also elicit the SA-regulated pathway (Ward et al., 1991). The classic findings of the significance of the SA pathway stemmed in part from studies with the lesion-causing virus, tobacco mosaic virus (Ross, 1961). Other pioneering work from Kuć (1982) showed that necrotizing bacterial and fungal pathogens would confer induced systemic resistance. An increase in the level of SA is associated with the induction of the systemic resistance phenomenon (Ward et al., 1991). Thus, this effect is not apparent in plants that are transformed to express the *nahG* gene encoding a bacterial salicylic hydrolase (Delaney et al., 1994). Metabolism of SA to catechol by the hydrolase in these plants is presumed to limit the accumulation of SA and prevent the expression of the SA-regulated genes (Figure 17.1; Neuenschwander et al., 1995).

In contrast, the JA/ethylene-regulated pathway of defense is associated with chewing insects where both wounding and specific components from insects participate in the stimulation (Figure 17.2; Kessler and Baldwin, 2002; Korth and

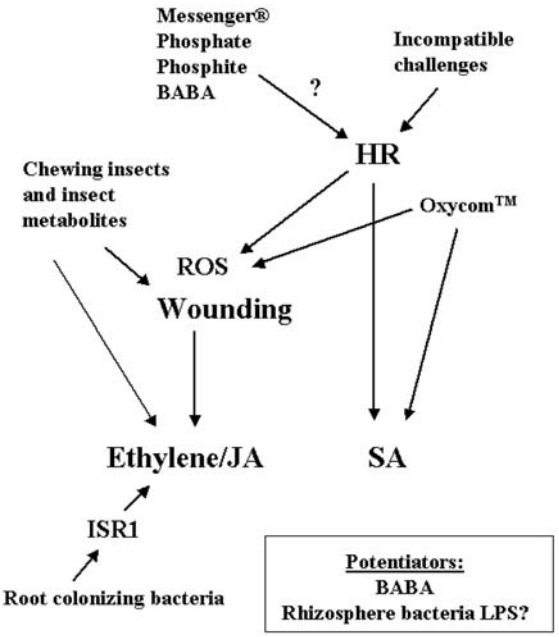

FIGURE 17.2. Differential effectors for pathways regulated by ethylene/JA or SA.

Thompson, Chapter 11 of this volume). Bacterial lipopolysaccharides also activate genes in this pathway (Dow et al., 2000). Elucidation of the JA/ethylene-regulated defense pathways was founded with the observation of systemic induction of proteinase inhibitors in the plants as a response to chewing (Ryan and Pearce, 1998; Kessler and Baldwin, 2002). Impaired insect digestion is correlated to the induced accumulation of proteinase inhibitors as well as to the effects of induced polyphenol oxidases in the plant tissues (Kessler and Baldwin, 2002). Ryan's studies in solanaceous plants revealed the crucial role of the synthesis of a novel peptide systemin in the signaling pathway which leads to oxylipin production and to altered gene expression. Although systemin appears to be restricted to certain solanaceous plants, the oxylipin pathway has been demonstrated for many plants (Turner et al., 2002). Interestingly, some of the volatile oxylipins are associated indirectly with plant defense because they act as attractants for predators of the insect pests (Kessler and Baldwin, 2002; van Poecke and Dicke, 2002). Although the JA/ethylene-regulated pathway is involved in insect resistance, other studies now reveal that it also is a major player in resistance to certain microbial pathogens (see Table 17.1). Likewise, the SA-regulated pathway is associated with resistance to an insect, the gall midge (Ollerstam and Larsson 2003).

For both the JA/ethylene- and SA-regulated pathways, signaling events include activation of members of the MAPK-cascade of protein kinases. Phosphorylation of the tobacco signal transduction MAPK member, salicylic acid-induced protein

TABLE 17.1. Spectrum of pest suppression associated with the salicylic acid (SA) and jasmonic acid (JA)/ethylene-regulated pathways.

	Pest	Reference
SA-regulated pathway	Downy mildew	Thomma et al. (2001); Ton et al. (2002)
	Powdery mildew	Thomma et al. (2001)
	Tobacco mosaic virus	Delaney et al. (1994)
	Turnip crinkle virus	Ton et al. (2002)
	Gall midge	Ollerstam and Larsson (2003)
JA/ethylene-regulated pathway	*Alternaria brassicicola*	Kunkel and Brooks (2002); Thomma et al. (2001); Ton et al. (2002)
	Botrytis cinerea	Diaz et al. (2002); Kunkel and Brooks (2002); Thomma et al. (2001)
	Erwinia carotovora	Kunkel and Brooks (2002); Thomma et al. (2001)
	Fusarium oxysporum	Garaats et al. (2002)
	Pythium spp.	Garaats et al. (2002); Kunkel and Brooks (2002); Thomma et al. (2001)
	Rhizopus stolonifer	Garaats et al. (2002)
	Thielaviopsis basicola	Garaats et al. (2002)
	Beet armyworm	Kessler and Baldwin (2002)
	Colorado potato beetle	Kessler and Baldwin (2002)
	Egyptian cotton worm	Stotz et al. (2002)
	Manduca sexta	Kessler and Baldwin (2002)
	Noctuid moth	Kessler and Baldwin (2002); Stout et al. (1999)
SA- and JA/ethylene-regulated pathways	Powdery mildew	Ellis et al. (2002)
	Pseudomonas syringae	Ellis et al. (2002)
	Xanthomonas campestris	Ton et al. (2002)
	Green peach aphid	Ellis et al., (2002); Kessler and Baldwin (2002)

kinase (SIPK), is rapid after SA treatment (Zhang and Klessig, 1997) and activation of wound-induced protein kinase (WIPK) initiates JA synthesis (Turner et al., 2002). Both SIPK and WIPK activation occurs as a result of the recognition event between the products of the *Cladosporium fulvum* avirulence gene, *avr9,* and its cognate resistance gene, *cf9*, responsible for HR (Romeis et al., 1999). The

on–off-switch protein, CTR1, in ethylene signaling is believed to be a MAPKKK (Wang et al., 2002). In *Arabidopsis* a MAPK, MAPK4, acts as a repressor for the SA-regulated pathway, thus, promoting JA/ethylene effects (Turner et al., 2002; Wang et al., 2002). A plethora of transcriptional activators are implicated in altering defense gene expression (Eulgem et al., 1999; Chen and Chen, 2002; Turner et al., 2002; Wang et al., 2002). This complex situation means that defense genes are expressed and proteins are produced at different times in the response, e.g., phenylalanine ammonia-lyase (PAL) versus PR-1 [Guo et al., 2000]).

Other factors such as plant age also influence when defense genes are expressed. Certain defense genes are increased in expression by elevated sugar levels *in planta* (Ehness et al., 1997; Herbers et al., 1996). Studies by several groups find increased expression of certain defense genes in senescent tissues (e.g., Hanfrey et al., 1996; Quirino et al., 1999; Zhu et al., 2001). A recent paper (Yoshida et al., 2002) indicates that the *cpr5* gene, which causes constitutive expression of defense genes, is allelic with *hys1* that regulates senescent-induced defense gene expression. Further exploration is needed to clarify how the SA-independent expression of defense genes in these aging tissues relates to sugar sensing (Rolland et al., 2002). Another speculation is that gene regulation by the plant growth regulators ABA and ethylene may explain the sugar-linked expression of the defense genes (Gazzarrini and McCourt, 2003). Likewise, how plant aging affects systemic expression of the defense genes also has been little studied, although this factor is of vital importance for field efficacy.

17.1.2 Induced Plant Defense Responses and Field Protection

Induction of systemic resistance in crops is an attractive protective strategy because it can activate defenses throughout the plant. It complements existing plant-based strategies of preformed defenses and the localized induced response of HR. Cell death in HR is localized to the challenged cell and is initiated by recognition between the host and pathogen factors conditioned by resistance genes and avirulence genes, respectively. Because the response is dependent upon single genes for recognition, breeding for plant genes to confer HR has been a primary strategy to provide high-level protection against specific pests. However, frequently the pathogen population change to lose the effective avirulence gene. Thus, control based only on HR-based resistance may have limited time efficacy in the field. In contrast to the hypersensitive response, the plant cells expressing systemic resistance do not undergo programmed cell death *en masse*. Consequently, the systemically resistant plant maintains growth and production while offering pest protection. Because so many different types of stimuli may be involved in induction of the process, and its implementation may involve crosstalk between several defense pathways, pathogen resistance to plant systemic mechanisms may be less likely to develop.

Plants utilize some of the same chemical and physical ploys of the hypersensitive response to limit pathogen ingress in systemic resistance. Early inhibition of ingress and growth is a typical response observed upon challenge of a systemically

protected plant (Hammerschmidt, 1999a). For systemic resistance to be effective, activation before the pathogen pressure reaches a crisis point is essential. Depending on the trigger used, such as the biologicals where signals have to be transduced from the root to leaf tissues, considerable pretreatment time is required for commercial applications to be successful.

Altered transcription and protein synthesis associated with defense gene activation may bring about a cost to the plant (Heil, 2002; Heil and Baldwin, 2002; Heil and Bostock, 2002). Choices must be made by the plant in how to allocate energy and metabolic resources. The view of Heil and Baldwin (2002) is that overexpression of defense traits in either of the pathways will result in poor growth and impaired reproduction. They cite the occurrence of stunted growth for 11 plant lines that were transformed to have increased expression of defense-related genes. However, they make the case for the need of more studies on the trade-off of protection versus metabolic cost under natural environmental conditions. Chemical overstimulation of defense also may result in poor plant performance. Although resistance to bacterial spot in bell pepper was induced by BTH, acibenzolar-S-methyl, weekly applications during the entire crop season reduced yield (Romero et al., 2001).

Responses in addition to protection against pathogen challenge may result from activation of the SA- and JA/ethylene-regulated pathways for gene expression. For example, tomatoes have an enhanced resistance to low temperatures when these pathways are stimulated (Ding et al., 2002). Protection against heat-induced oxidative damage in *Arabidopsis* involves responses orchestrated by ethylene, ABA, and SA (Larkindale and Knight, 2002). As revealed by gene microarray analyses (e.g., Chen and Chen, 2002; Cheong et al., 2002), these same growth regulators are key players in governing expression of defense genes.

17.1.3 Consequences of Multiple Defense Pathways: The Good and The Bad

The genes encoding defense functions associated with the SA- or the JA/ethylene-regulated pathways are differentially effective against different pests (Thomma et al., 2001; Ellis et al., 2002; Garaats et al., 2002; Kunkel and Brooks, 2002; Table 17.1). Which defense gene products are key in limiting each pathogen have not been resolved. For instance, although *Pseudomonas syringae* pv. *tabaci* incites the production of PR-1 in tobacco, neither this protein nor the PR proteins 3 and 5 appear to account for inhibition of growth of this pathogen (Thomma et al., 2001). The fact that different defense ploys are effective against different pathogens means that activation of only one pathway, (e.g., the SA-regulated pathway), may leave plants protected against some but not against all pests (i.e., chewing insects).

In the field there will be multiple interactive effectors and pathogenic challenges (Cui et al., 2002) and these may affect the responses of the plant. Systemic resistance effective against the cabbage looper in *Arabidopsis* was induced only by a pathogen-induced hypersensitive response and not by mutations that result in increased levels of SA, although this is one of the consequences of HR believed

to be involved in establishing systemic resistance (Cui et al. 2002). These findings illustrate that there are complexities in these pathways that currently we do not understand. New studies continue to bring more questions of the accepted pathways, for example the evidence for an SA defense response independent of the transcription regulator, NPR1 (Figure 17.1; Wang et al., 2002). Such branching and crosstalk between pathways (Feys and Parker, 2000; Gazzarrini and McCourt, 2003) is of significance when considering protection against an array of pathogens.

Protection from one set of pathogens over another may also result from negative interactions between the two pathways. SA applications strongly impair the functioning of the JA-regulated pathway, in part by inhibiting key enzymes in oxylipin synthesis (Thaler et al., 2002). JA appears to be inhibitory, but to a lesser extent, to the SA-regulated pathway (e.g., Seo et al., 1997; Ellis et al., 2002). Abscisic acid (ABA) antagonizes SA-regulated responses (Audenaert et al., 2002a). Again the commercial impact of such antagonism would be that, although protected against one set of pathogens, the plants might be more susceptible to others. For SA-treated plants, an increase in feeding by insects that normally would be repressed by the JA/ethylene-regulated defense genes has been reported (Felton et al., 1999; Preston et al., 1999; Stout et al., 1999; Thaler et al., 1999).

Not all interactions between pathways are negative. Studies of the expression of distinct defense genes reveal synergism in effectors. Ethylene and SA act synergistically on the expression of several defense genes including *PR-2c*, *PR-3a*, *PR-3b*, *PR-4* and *PR-5* (van Loon, 1997). Such crosstalk between pathways may depend to some extent on potentiation. Certain activators of systemic resistance when present with very low levels of SA result in very effective expression of such genes as *PR-1* (Conrath et al., 2002). Because microbial challenge of plants can act to potentiate effects, perhaps through modification of SA or ethylene levels, the presence or absence of microbial challenges under field conditions within a time frame of application of a systemic resistance inducer may have dramatic effects.

Reactive oxygen species (ROS) are another important class of chemicals with field significance for defense. These arise during the early events in hypersensitivity, or they are produced naturally through plant metabolism or as a result of plant irradiation by the UVA/B spectrum of sunlight (Mittler, 2002; Neill et al., 2002). Recent gene-chip array studies to detect hydrogen peroxide-responsive plant genes confirm induction of a subset of defense-associated genes (Desikan et al., 2001). ROS signaling includes certain members of the MAPK families and transcriptional activators that are also involved in the SA pathway, so some crossover in defense products exists (Kovtun et al., 2000; Mittler, 2002). For instance, interaction between SA and hydrogen peroxide was suggested from studies of tobacco with a catalase deficit that under oxidative stress, imposed by high light, responded with elevated expression of *PR-1* (Chamnongpol et al., 1996, 1998). Although SA applications triggered localized increases in *PR-1*, the systemic response in the transformed plants was observed only under high light, suggesting that ROS was involved for long distance signaling. Also the increased expression of *PR-1* occurred without plant cell death, possibly because the ROS caused ethylene to be produced which enhanced the effect of SA on gene expression (Chamnongpol

et al., 1998). However, negative as well as positive interactions between ROS and ethylene have been noted for other systems (Wang et al., 2002). These findings have relevance to certain of the commercial products discussed in the next section.

Another finding in research on systemic resistance is that in some cases tolerance rather than resistance is induced (Kloek et al., 2001). Although the treatment leads to loss in symptom formation, assessment of pathogen numbers reveals that colonization has not been impeded. Thus, the induced defense responses may act to reduce symptom formation rather than limiting pathogen growth. In the field, this could be a problem in that the method would not reduce inoculum input for another growing cycle.

At present the extent to which the SA- and JA/ethylene-regulated pathways are represented in each plant genus and the level to which there is cultivar specificity is unknown. Indeed, resistance in bean to the necrotrophic fungus *Botryis cinerea* requires the SA pathway whereas for *Arabidopsis*, the JA/ethylene pathway is more important (Díaz et al., 2002). The SA-regulated pathway is also required in tomato for defense against *B. cinerea* (Audenaert et al., 2002a). Moreover, it appears that the JA and ethylene pathways in tomato act independently whereas they are intertwined in *Arabidopsis* (Diaz et al., 2002). Another example of plant variability is that the application of the systemic inducer β-aminobutyric acid (BABA) is more effective against late blight in tomato than potato (Cohen, 2002). Crop variability in induced protection by BTH is also documented (Oostendorp et al., 2001). Thus, our knowledge is far from complete in understanding how a treatment inducing a systemic response in one plant under laboratory conditions will have an impact on the wide spectrum of crops in agriculture, horticulture, and forestry. Ease of genetic transformation and the information from genomic sequencing projects has favored acquiring knowledge in *Arabidopsis* and tobacco with other plants being less studied, especially the monocots. As we identify key genes involved in the pathways in these model plants, the variability of responses in other crop plants will be more easily predicted. The current 2002/3 NSF Initiative to understand the functioning of all of the *Arabidopsis* genes will spearhead this effort. Similarly, the completion of genomic sequencing for other plants (corn, tomato, rice) is hastening our ability to harness the power of the plant in defense strategies.

17.2 Current Commercial Products

Products in commerce that induce systemic resistance include chemicals and biologicals. Those products registered by EPA in the USA as biopesticides are listed at http://www.epa.gov/pesticides/biopesticides. This site covers all products with biocontrol activity irrespective of mechanism, including those that induce systemic resistance. A reoccurring statement for most of these products is their relative safety to the environment and to human health. A listing with references of chemicals inducing systemic resistance, updated to May 2003, is provided by the Scottish Research Institute in Dundee, Scotland (http://www.scri.sari.ac.uk/). Microbes and

their metabolites that have biocontrol activity are also listed in the review article by McSpadden-Gardener and Fravel (2002). A similar list is compiled and updated, currently to April 2003, by the American Phytopathological Society Committee on Biological Control (available at http://www.oardc.ohio-state.edu/apsbcc). In these lists only four microbial products (three bacilli and a *Streptomyces* species) are cited with plant defense activation being a proven mechanism. However, as discussed by McSpadden-Gardener and Fravel (2002), not all products with plant defense-inducing potential are registered currently as a pesticide but rather, perhaps because of the expenses associated with registering a product as a pesticide, they only have the classification of fertilizers or plant growth promoters. As illustrated by the list of about 40 companies achieving EPA biopesticide registration between 1995 and 2000, most of these companies are relatively small with niche markets in comparison to the larger companies associated with production of the synthetic, chemically based, direct-impact pesticides. The politics of registration is posing problems. For instance, in California there has been a legal issue on whether a substance that is only registered as a fertilizer, phosphite, but which has proven resistance potential against the oomycete pathogens, can be used in attempts to control sudden oak death caused by a *Phytophthora*-like fungus.

17.2.1 The Chemical Inducers

Our review of the chemicals that induce resistance extends the review of Oostendorp et al. (2001). The chemical products with the potential to induced resistance fall under three classifications: inorganic, synthesized, and natural products.

Inorganic

Phosphates and Phosphites. Both phosphite and phosphate salts are demonstrated to induce systemic resistance. When applied as a foliar spray phosphate salts induce resistance under field conditions (Reuveni and Reuveni, 1998). Di- and tri-basic sodium and potassium salts at alkaline pH were proven effective (Gottstein and Kuć, 1989) as part of the pioneering studies of induced resistance from Kuć group. Systemic protection against fungi, bacteria, and viruses is reported (Mucharromah and Kuć, 1991).

Interpretation of the findings with phosphites is more complex because of debates on their mode of action. Salts are termed phosphites when in dry powder form. In water they are converted to phosphonates. Phosphonates are taken up and redistributed in the plant through the xylem and then the phloem (Rickard, 2000). They are used commercially as alternative phosphate ("P") fertilizers, and increase plant growth. Oxidation to phosphates is a presumed mechanism. A direct fungicidal effect of phosphonates is observed, especially for the fungal-like pathogens, *Pythium, Phytophthora* and downy mildews. This knowledge has, in part, stemmed from studies with the commercial registered fungicide, Aliette, that produces aluminum tris-ethyl phosphonate. However, the same antifungal potential

is displayed by inorganic phosphonates. The phosphonates are believed to exert their effect by limiting polyphosphate formation in the fungi (Niere et al., 1994). Activation of plant defenses is another proposed mode of action of the phosphonates (Smillie et al., 1989). Product information from Bayer for the commercial fungicide phosphonate marketed as Chipco indicates that enhanced plant defenses including the production of antimicrobial phytoalexins are part of the modes of action of this chemical. Products formulated to produce inorganic phosphonates include Nutri-Phite® (Biagro Western, USA), Ele-Max® (Helena Chemical Co, USA), and Phytogard® (CATE, France).

Both commercial and technical grade phosphites were effective in controlling the root and crown rot caused by *Phytophthora capsici* (Förster et al., 1998). Studies in lettuce (Pajor et al., 2001) showed that Phytogard® protected against downy mildew in a dose and systemic manner. Current work with Nutri-Phite® on citrus by the team of Graham and McLean (personal communication) reveals increased resistance in fruit as it develops on the tree against *Phytophthora palmivora*, between 30 and 60 days after application.

How the phosphates and phosphites are perceived by the plant, or which pathways are involved in the induced resistance phenomena, are little resolved. In cucumber, dipotassium hydrogen phosphate treatments were associated with localized cell death at the sites of application (Orober et al., 2002). This treatment caused systemic protection against cucumber anthracnose in cucumber. The chemical applications mimicked the hypersensitive response further because both superoxide anion and hydrogen peroxide were detected. The response was likened to HR induced by the tobacco necrosis virus (Orober et al., 2002). However, in lettuce, treatment with Phytogard® did not increase the PR-1 protein anticipated from activation of a SA pathway (Bécot et al., 2000). None of the PR proteins (PR-1, PR-5 and PR-9) examined were elevated in level. Studies in our laboratory confirm activation of defense or growth related genes. Rapid, strong, and lasting increased expression of transcripts for genes encoding phenylalanine ammonia lyase, peroxidase, chalcone synthase, and the cell wall protein hydroxyproline rich glycoprotein were stimulated in bean (Kim et al., unpublished data) after sprays with Nutri-Phite®. Small lesions were seen on the bean foliage within two days following application.

Oxycom™. Oxycom™ is produced by Redox Chemicals, USA and currently it is not registered as a biopesticide, although laboratory and field tests have demonstrated promotion of plant health and productivity of several crops under conditions of pathogen pressure (Kim et al., 2001; Yang et al., 2002). The active product is a mixture of reactive oxygen species, salicylic acid, and compounds with fertilizer activity. Application is by spray and by drench with repeat applications as needed for each crop. Oxycom™ protects tobacco against infection by *Pseudomonas syringae* pv. *tabaci* (Yang et al., 2002). Abuse of the application system by repeated root saturation results in stunting of tomatoes in a greenhouse trial (Anwar et al., 2003). In contrast, a single application prior to inoculation of root knot nematodes conferred a tolerance response. Although nematode populations were not reduced

there was no deleterious effect on foliar growth (Anwar and McKenry, 2002). Our studies with Oxycom™ further illustrate how application method may be important. We found that spraying on leaves induced confluent activation from the PR-1 promoter whereas application to roots induced a veinal pattern of activation of this promoter in the leaves (Blee et al., 2004). Thus, targeting application to the feeding strategies of the pathogen may be important for field control.

Systemic induction of defense genes associated with the SA- and the ethylene-regulated pathways has been observed in bean and tobacco after Oxycom™ applications (Kim et al., 2001; Yang et al., 2002). Gene chip array data analysis of the response of *Arabidopsis* to Oxycom™ treatments supports the concept of activation of the SA- and JA/ethylene-regulated pathways. We speculate that, like the findings of Chamnongpol et al. (1996, 1998), it is the simultaneous presence of ROS with SA that, in part, determines the defense activation potential of the product.

Synthesized Organic Chemicals

BABA β-aminobutyric acid. Induced resistance by the nonprotein amino acid (BABA) was reviewed recently by Cohen (2002). Registration is being pursued currently. BABA treatment results in different plant responses (induced physical barriers such as lignification, phytoalexin, and PR production) for each pathosystem studied (Cohen, 2002). Effective resistance is generated for a wide range of plant–pathogen systems (e.g., Shailasree et al., 2001). Curative effects of BABA treatment, a feature not observed with other chemical systemic resistance activators, are observed in some pathosystems. In tobacco, cell death accompanied by the generation of superoxide and hydrogen peroxide was induced by BABA treatment (Siegrist et al., 2000). Thus, association with the SA-regulated pathway would be expected. However, in cauliflower (Silue et al., 2002) induction of the typical barrage of PR proteins expected from this pathway (PR-1, PR-2, PR-5) were not detected. Rather only PR-2 accumulated significantly after challenge with downy mildew for which protection was apparent. In common with other activators, the involvement of the SA pathway in BABA-stimulated resistance is variable with the pathogen studied. It is possible that some of the variability in response relates to effective dose. As discussed by Conrath et al. (2002), activators of systemic resistance responses may cause plant cell death at high concentrations yet act at lower doses to potentiate the defense response in conjunction with other effectors. Studies with BABA in *Arabidopsis* suggest that potentiation of defense gene expression in response to another agonist is a likely mode of action (Zimmerli et al., 2000). Such potentiation was demonstrated with the observed resistance to the necrotrophic fungus *Botrytis cinerea* in BABA-treated plants (Zimmerli et al., 2001).

BTH Benzo(1,2,3)thiadiazole-7-carboxylic acid derivatives. One of the most academically studied chemicals with systemic inducing activity is BTH, marketed by Syngenta (www.syngenta.cropprotection-us.com) under the name of Bion® in Europe or Actigard® in the USA. The compound is formulated as a

water-dispersible granule to be applied as a drench. It was approved in 2002 for use on tobacco, tomato, lettuce, and spinach in the USA. The longevity of the protection afforded by BTH is variable, with a longer efficacy in monocots than in dicots (Staub, 2001). Data compiled by Tally et al. (1999) illustrate that BTH has crop specificity. Although resistance is induced in tomato against late blight there was no activation of defenses for potato late blight. Thus, the spectrum for effectiveness must be determined for each plant–pathogen system (Tally et al., 1999).

Effectiveness on 12 crops with activity against bacteria, viruses, fungi, insects, and nematodes was summarized by Oostendorp et al. (2001). Efficacy of Bion® against rhizoctonia leaf spot and wild fire in tobacco has been reported in other field studies (Cole, 1999). Suggested use of BTH is not as a "stand-alone" product but in conjunction with other protection methods. For example, a mixture of Bion® and copper hydroxide was more effective than single treatments in controlling bacterial spot of pepper (Buonaurio et al., 2002).

The BTH compounds are believed to stimulate the SA pathway downstream of SA before the transcriptional activator NPR1 (Figure 17.1). Early laboratory studies showed applications of BTH to barley promoted a rapid and effective HR-like response in the treated plant when challenged by powdery mildew (Gorlach et al., 1996) although other defense mechanisms were also stimulated. As listed in the report of SAR activators from the Scottish Research Institute, BTH is associated with increased accumulations of acidic PR-1, PR-2, and PR-5, each of which is an accepted marker for the SA-regulated pathway. BTH has potentiator activity enhancing the production of PR-1 and PAL with treatments by SA (Conrath et al., 2002). Potentiation after infection with a pathogen also has been noted (e.g., Benhamou and Belanger, 1998; Latunde-Dada and Lucas, 2001), a response requiring the *NPR1* gene (Kohler et al., 2002). Such potentiation means that under field conditions where the SA pathway may already be activated by challenge with a necrotizing microbe, BTH may enhance the activation of defense pathways.

Probenazole (3-allyloxy-1,2-benzisothiazole-1,1-dioxide). Probenazole, formulated as Oryzemate, is used in rice to provide protection against the rice blast fungus *Magnaporthe grisea* and bacterial blight, *Xanthomonas oryzae* (Watanabe et al., 1977, 1979). Spray or paddy applications result in uptake and metabolism into benzoate and saccharin-based products. A leucine-rich repeat (LRR) nuclear binding protein, RPR1, changes in level upon application of probenazole to rice, suggesting the potential for an interaction that resembles the recognition between microbial avirulence effectors and the resistance gene products that trigger the hypersensitive response (Sakamoto et al., 1999). Additional studies show that RPR1 belongs to the Pib family of genes associated with rice blast resistance genes (Wang et al., 2001; Chauhan et al., 2002) and resides on the chromosome in regions that show extensive cultivar variability. This situation resembles the clustering of genes encoding LRR proteins genes that are part of the signaling pathways determining resistance against *Pseudomonas syringae* pv. *tomato* (Salmeron et al., 1996). Gene expression of the *Pib* family is regulated by several environmental factors,

including SA. Indeed, Probenazole causes SA to accumulate in Arabidopsis and requires NPR1-dependent defense gene activation (Yoshioka et al., 2001). Failure of *NahG* plants to show defense indicates that the chemical acts apparently upstream of SA. These findings illustrate that, although debated, a defense pathway involving SA regulation is likely to operate in rice and that this pathway can be successfully activated under commercial conditions to boost plant productivity.

Natural Products

Chitin and Chitosan. A chitosan product called Elexa® is sold by SafeScience, USA, and is EPA approved for use on cucumber, vines, potatoes, strawberry, and tomato as "an alternative for traditional fungicides" and a "plant defense booster". Although direct effects of growth inhibition of fungal pathogens are reported as a mode of action, chitosan also activates plant defenses (Hadwiger et al., 1994; Chang et al., 1995). The activity of chitosan in stimulating plant defenses was established when researchers were screening fungal cells wall components as elicitors of HR. Chitosan treatments of pea caused an array of defense genes to be expressed and phytoalexins to accumulate (Hadwiger et al., 1994). Responses in other plants include elicitation of both PAL and peroxidase activities in wheat leaves (Vander et al., 1998). Treatment of tomato with chitosan enhanced resistance to the crown and root rot pathogen *Fusarium oxysporum* f. sp. *radicis-lycopersici* and stimulated defense responses, such as reinforcement of the plant cell wall and the alteration of the plasma membrane (Benhamou and Theriault, 1992). Synergism between chitosan and a root-colonizing protectant *Bacillus* isolate against Fusarium wilt infection has been observed (Benhamou et al., 1998). Rapid formation of plant cell wall modifications (chitin-enriched and callose deposits) was cited as limiting penetration of the fungus into the bacterized root.

Perception of chitosan may be initiated by electrostatic disruptions in the plant plasmalemma (Benhamou and Theriault, 1992). The signaling pathway for chitosan involves rapid induction of a 48 kDa MAPK activity in tomato that is independent of JA signaling (Stratmann and Ryan, 1997) and hydrogen peroxide production through the oxylipin pathway (Orozco-Cardena and Ryan, 1999). A burst of oxylipin synthesis was detected after rice was treated with chitosan (Rakwal et al., 2002). Involvement of ROS and MAPK activation (the ROS responsive-AtMAPK3 in *Arabidopsis*) after chitin treatments was demonstrated (Link et al., 2002; Zhang et al., 2002). However, the activation of two chitin-stimulated genes in *Arabidopsis* was independent of functional ethylene, JA and SA pathways (Zhang et al., 2002), although another required JA or SA regulation. The ethylene/JA-regulated pathways also were implicated in the defense response induced by chitin in pepper, where a specific chitin-binding protein was detected (e.g., Lee et al., 2001).

Messenger®. Messenger® is marketed by Eden Biosciences, USA, and is a preparation of a secreted peptide from the bacterium *Erwinia amylovora*. This peptide, termed a "harpin", triggers changes in plant tissues typical of HR (Yang

et al., 1993; Desikan et al., 1998). Consistent with these findings is the observation that harpin elicits disease resistance in Arabidopsis in a SA-dependent manner (Dong et al., 1999). The water-soluble powdered product is applied as a foliar spray and is stated to exert the required changes in the plant within three to five days. Eden Biosciences indicates efficacy on 40 crops, including specialty crops of strawberries, citrus, and ornamentals. Data sheets for applications for several crops are available from their website http://www.edenbio.com. Studies described in a patent for Messenger® indicate that there is also a strong plant growth-promoting activity associated with the product.

Strobilurins. Several products from wood-associated fungi are marketed as strobilurins, which have both indirect and direct effects on fungal pathogens (Ypema and Gold, 1999). Formulations include: Quadris and Abound, containing azoxystrobin, Trifloxystrobin, formulated as Flint, Stratego, and Compass; and pyraclostrobin, formulated as Cabrio EG and Headline, Amistar, Bankit, Priori, Ortiva, and Heritage. New products are being commercialized (e.g., Acanto, a picoxystrobin from Syngenta targeted toward emergent wheat). They are approved for 85 crops ranging from cereals including rice, to vines, fruits, vegetables, turf, and ornamentals. Strobilurins are designated as "reduced risk" products by the EPA. The products have direct fungicidal activity, by inhibiting mitochondrial respiration in the fungus at the site of complex III, the ubiquinine oxidation center. For some of the products their mobility in the plant is a benefit. However, they also activate plant defenses. The formulation Pyraclostrobin F 500 from BASF Inc., demonstrated NahG-independent protection against *Pseudomonas syringae* pv. *tabaci* (Herms et al., 2002). Although the strobilurin did not cause PR-1 accumulation itself, it primed tobacco for greater production when subsequently challenged with the wild-fire pathogen. Resistance to TMV generated by the strobilurin treatment was variable and cultivar dependent.

Although the rapid development of resistance in pathogens to strobilurins appears to be a problem, causing restricted and intregrated use with chemicals of different modes of action, studies as yet do not reveal whether strobilurins' ability to induce resistance will still have commercial importance.

Summary for Chemical Activators

The chemicals that stimulate systemic resistance display a wide range of structures and activate a diversity of plant defense genes. At present there are no commercial products based on the stimulating components that are naturally produced by insects (Kessler and Baldwin, 2002). A common thread for many of the activators is that under some conditions they mimic events occurring in HR (Messenger®, BABA, phosphite, Oxycom™). The commercial use of SAR inducers that function through causing "local lesions" was questioned by Oostendorp et al. (2001). However, field studies with these compounds demonstrate that any induced phytotoxicity is not adverse because beneficial effects against pathogen pressure have been shown. Several of the products also have potentiation activity. Although

at low concentration these seem to be only weak activators of defense, in combination with other factors they promote more rapid and greater activation of resistance.

Enhanced plant growth is another observation associated with use of chemicals that induce systemic resistance (e.g., phosphate, BABA, Messenger®, Oxycom™). Improved growth under field conditions is also a common effect of colonization of plant roots with beneficial microbes, hence the descriptive term "plant growth-promoting-bacteria". Reasons for improved growth are not resolved. One debated theory is that the growth of minor pathogens is reduced and, thus, the plants have more energy to divert to plant growth. Additionally, the metabolites, such as SA, involved in plant defense may also participate in regulating cell size. Expression of an effector gene, AvrBs3, results in enlarged mesophyll cells and increased transcripts of auxin-related genes and expansin, genes associated with cell expansion (Marois et al., 2002). Plant cells surrounding isolated dead cells, generated by changes in SA accumulation or by infection with a necrotic pathogen, were observed to grow abnormally large (Vanacker et al., 2001). Thus, roles for SA and the regulatory protein, NPR1, in controlling the balance between plant cell death and cell growth are suggested (Vanacker et al., 2001). Understanding the value of this growth effect of chemicals associated with plant defense toward their field efficacy will be most interesting.

Probably the most neglected factor involved in the significance to field protection is the role of nutrition to the plant. Nutrition may not be a notable factor in controlled greenhouse/laboratory studies where long-term plant growth is not the norm. In the field, the plants must have adequate nutrition to permit the required changes in gene expression to be accomplished. Whether plants purposely treated with effectors of systemic resistance under commercial conditions require specialized nutrition awaits rigorous examination.

17.2.2 Microbial Stimulants of Plant Defense

The EPA-registered Biopesticides with stated ability to induce resistance include bacilli. YieldShield from Gustafson, Inc. (www.gustafson.com) is a powdered formulation of *Bacillus pumilus* GB34 and is used as a seed treatment to confer protection on soybean for root pathogens. The APS listing indicates that YieldShield is currently under registration as a biopesticide. Serenade from AgraQuest, Inc. (www.agraquest.com) is based on *Bacillus subtilis* QST716. The preparation is reported to control a variety of pathogens (powdery mildew, downy mildew, Cercospora leaf spot, early blight, late blight, brown rot, and the bacteria, *Erwinia amylovora*) on a range of crops (vegetables, cucurbits, grapes, hops, peanuts, pome fruits, stone fruits). The product description indicates that the mode of action of the bacterium includes activation of host defenses but no further information is available.

The marketing of organisms as biocontrol agents that stimulate plant defenses, as opposed or in addition to a direct effect on the pathogen, is strongly supported by laboratory studies. Indeed the YieldShield *Bacillus* species were initially discovered in screens of bacteria for plant growth promoting and protection activities

(Raupach and Kloepper, 1998). Colonization of the plant by these biological control agents activates genes associated with both the SA- and the ethylene/JA-regulated pathways. For instance, certain fluorescent pseudomonads and *Bacillus* isolates stimulate expression from the *PR-1* gene in colonized tobacco (Park and Kloepper, 2000). Accumulation of protein regulated by the *PR-1* gene promoter is time dependent, requiring about 10 days for sizable activation (Park and Kloepper, 2000). This finding stresses the need for treatments with microbial inducers well before the disease pressure exists so that the plant is preconditioned for resistance. By comparison, defense genes associated with systemic resistance pathways are activated generally less than 24 hours after chemical application.

SA-independent activation of systemic resistance is reported after colonization of plant roots with the fluorescent pseudomonad WCS417r (Pieterse et al., 1996). The term induced systemic resistance, ISR, has been used to determine such microbially induced resistance [the term "ISR" has also been used to indicate resistance that is induced and systemic, regardless of the eliciting agent]. Resistance was induced toward the fungal root rot pathogen, *Fusarium oxysporum* f. sp.*raphani*, and the leaf pathogens blue mold (*Peronospora tabacina*), *Xanthomonas campestris* and *Pseudomonas syringae* pv. *tomato*. The lack of induced resistance in the JA-response mutant, *jar1*, and the ethylene-response mutant, *etr1*, is consistent with involvement of the JA/ethylene-regulated pathway. However, although sensitivity to the JA/ethylene pathway is essential, activation of ACC synthase or defense gene expression associated with these pathways was not observed (Knoester et al., 1999). Rather a rapid increase in a specific JA-regulated gene was observed only after pathogen challenge was detected (van Wees et al., 1999), suggesting that potentiation is occurring. A locus conditioning this sensitivity, ISR1, has been identified in *Arabidopsis* (Ton et al., 2001). Cultivars that fail to develop induced resistance are altered in this locus and lack ethylene sensitivity in their roots (Ton et al., 2001). Similar genetic differences in commercial crops could result in differential effectiveness of the microbials in inducing ISR.

How do the bacteria induce the response? The activity of some bacteria may correspond to their production of SA (Mercado-Blanco et al., 2001). Other activators for systemic resistance are extracellular bacterial surface structures, flagellin and its major structural protein, lipopolysaccharides (LPS) or the secreted siderophores. In 1999, a conserved domain from the N-terminus of flagellins was shown to stimulate alkalization of the medium of cultured plant cells, K^+ efflux and elicit ROS production, thus mimicking HR (Felix et al., 1999; Gómez-Gómez et al., 1999). A flagellin from *Pseudomonas (Acidivorax) avenae* incompatible on rice was also shown to cause ROS production and HR in rice (Che et al., 2000; Tanaka et al., 2003). In contrast, flagellins from compatible isolates were inactive (Che et al., 2000).

Using a synthetic peptide corresponding to a conserved 15-amino acid sequence from the N terminus of flagellin, a receptor was identified in *Arabidopsis* as a leucine-rich repeat kinase encoded by a single locus (Gómez-Gómez et al., 2001). The signal transfer chain involved in flagellin perception in *Arabidopsis* was further probed and was shown to include specific members of the MAPK pathway

(Asai et al., 2002). These MAPKs (AtMAPK3/6) are also known participants in oxidative-stress signaling (Kovtun et al., 2000) and again these findings are consistent with flagellin stimulating a HR-like response. Commercialization of chemical inducers based on flagellin structure seems unlikely at present because their use stunts plant growth.

Early work on plant recognition of LPS structures demonstrated that infusions of LPS from a range of enteric bacteria created a localized effect that nullified growth of both incompatible and compatible challenges from *Ralstonia solanacearum* on a temporary basis. This work, from Sequiera's lab, is placed into context with current findings in the review of Dow et al. (2000). Whereas the core of the enteric LPS was needed for a localized protective response, in other systems the core, its conserved sugar residues or the variable O-antigen side chains was involved. Although LPS from xanthomonads alone has weak elicitor activity, exposure to the LPS potentiated defense processes upon subsequent microbial challenge (Newman et al., 2002). This finding shows that the LPS effects are similar to the chemical inducers, BABA and BTH or the strobilurins, for which potentiation has been demonstrated. Speculation is raised that the *hrp* system required for microbial pathogenesis suppresses the potential for the LPS to otherwise induce resistance Dow et al. (2000).

LPS from saprophytic root-colonizing pseudomonads also induces systemic resistance responses. LPS from *Pseudomonas fluorescens* accounted for the systemic resistance against Fusarium wilt induced in radish when roots were colonized by this bacterium (Leeman et al., 1995). LPS from *P. fluorescens* strain WCS417r also was an inducing factor in certain *Arabidopsis* ecotypes (van Wees et al., 1997).

Bacterial siderophores, iron-binding compounds that are secreted when iron is limited, are demonstrated to cause ISR. The activity has been demonstrated with siderophores from *P. putida* (Leeman et al., 1995); P. *fluorescens* (Mauhofer et al., 1994); *P. aeruginosa* (Audenaert et al., 2002b), and a *Serratia marcescens* strain (Press et al., 2001). An interaction between the antifungal phenazine, pyocyanin, and the siderophore, pyochelin, both produced by *P. aeruginosa* 7NSK2, is proposed to account for the ability of this strain to cause ISR (Audenaert et al., 2002b). SA-regulated genes are demonstrated to be important in this system (Audenaert et al., 2002b). Because of the dependence on iron availability to induce siderophore production, the use of such ISR-inducing bacteria as an inoculant to induce resistance may be effective only in iron-deficient soils, such as those that a have a basic pH.

These findings raise the possibility of whether synthetic chemicals based on LPS or siderophore structures could be commercially marketed. Such products could be used for specialty high-profit crops, such as ornamentals.

Summary and Comments on Microbials as Inducers of Systemic Resistance

The potential for commercialization of microbes with defense stimulating properties seems endless. Surveys of published findings suggest that many microbial

isolates have the potential to activate defenses. For instance, although the hydrogen peroxide that is produced by *Talaromyces* species is assumed biocidal in its biological control potential (Stosz et al., 1996), this ROS could play a role in stimulating plant defense. The elicitor activity of the xylanase secreted from *Trichoderma viride* (Yano et al., 1998) suggests that induced resistance may also account for biocontrol activity of such *Trichoderma* isolates. Additionally, the chitosan and/or glucan oligomers released from fungal walls being degraded by *Trichoderma* could have elicitor activity. Such factors could explain why root colonization by a *Trichoderma* isolate was suggested to induce a systemic resistance response (Yedidia et al., 1999).

The limitation of commercial development of the microbials themselves, rather than the products they produce (e.g., harpin, chitosan), is in our weak ability to manipulate the field environment to provide the beneficial organisms at the right time, at the right place, and with expression of the needed set of genes. Basic studies on genes involved in colonization and survival may provide the understanding to better implement microbials in the field. For instance, identification of genes that underlie effective root colonization by pseudomonads may provide tools for better screening for isolates excelling in the field (Lugtenberg et al., 2001).

Work on formulations of the organism so that field applications have maximal effect is needed. Here the understanding of how microbials overcome adverse environmental conditions (e.g., Beattie and Lindow, 1995; Lindow and Leveau, 2002) will be useful. Generally microbials are raised under conditions where cells are produced at maximum growth rates to highest density. However, such rich-medium growth conditions may not generate cells that are optimum in expressing traits required for field survival. Expression of the genes for resistance to heat, dessication, and UV light may be stimulated by modified culture conditions and result in microbials that survive better when applied in the field. Genetic engineering of plants to excel as hosts for beneficial microbes may come into play. Recent findings (e.g., Fray et al., 1999) with plants engineered to produce the acyl homoserine lactones that are signals for altered bacterial expression of genes involved in quorum sensing, survival, and competition illustrate how we can manipulate the behavior of associated microbials to minimize pathogen and maximize biocontrol effects.

17.3 Summary

The recent years of laboratory studies are starting to explain at the molecular levels the complexities of pathogen-resistance mechanisms. More studies under commercial field condition are required to test the robustness of stimulation of the systemic defenses that lab studies demonstrate plants possess. With all of the natural modes of stimulation from microbial contacts, is it feasible to use these induced mechanisms in the field? In answering this question, Heil and Baldwin (2002) indicate that the mechanisms associated with chewing insect defense in field plants can still be elevated. Enhanced levels of control over what is available

from nature may result from genetic modification of genes in the defense pathways or genes controlling the effector structures in the microbes. Teaming the inducers of systemic resistance with traditional methods may be beneficial (e.g., Friedrich et al., 2001).

We need to maximize protection against crop losses in yield and quality through studies of dose, application frequency, and application techniques to understand the load in altered metabolism that plants can endure under field conditions. These studies of the profitable side of pest control must be balanced with long-term studies to deduce possible environmental consequences on biodiversity incurred by purposeful manipulation of the plants systemic defense responses. Many questions arise in this area. How will insect visitation be altered if activation of the SA pathway changes the emission of volatiles that other insect use as cues for predation or for finding food? Will some pathogens evolve into superpathogens as they mutate to avoid the systemic resistance measures? Will changes in PR proteins, such as the proteinase inhibitors or polyphenol oxidases, alter the digestibility of the foods for desirable consumers (animals and humans)? Will the fact that several PR proteins are allergens in humans (Salcedo et al., 1999; Ebner et al., 2001) have an effect on workers when the plant materials are processed or the products are ingested? These questions illustrate the increased need for interaction between researchers with expertise in such different areas as plant pathology, entomology, microbiology, and immune responses to work together to help formulate successful products to stimulate systemic resistance in the field with optimal effectiveness.

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