European Journal of Plant Pathology **107:** 129–136, 2001.

Oxycom™ under field and laboratory conditions increases resistance responses in plants

Y.C. Kim**, K.A. Blee, J. Robins and A.J. Anderson*
*Department of Biology, Utah State University, Logan, Utah 84322-5305, USA; *Author for correspondence (E-mail: anderson@biology.usu.edu); **Current address: Applied Plant Science Division and Institute of Biotechnology, College of Agriculture, Chonnam National University, KwangJu 500-757, Korea*

Accepted 14 September 2000

Key words: active oxygen species, defense genes, induced systemic resistance

Abstract

Several different types of chemicals induce resistance in plants. Oxycom™ that contains active oxygen species has under commercial field conditions improved the performance of a diversity of plant species exposed to a range of naturally-occurring pathogens, including *Pythium*, downy mildew and powdery mildew. Oxycom™ had only weak antifungal activity when assayed in the laboratory at the concentrations showing efficacy in the field. Northern hybridization using probes of genes involved in defense was performed on bean, *Phaseolus vulgaris*, after sprays of Oxycom™ or water. The transcript abundance of genes encoding proteins concerned with phenolic metabolism and plant cell wall strengthening was increased by treatment with Oxycom™ compared with water treatment. Similar patterns of gene induction were observed when benzothiadiazone (BTH), a chemical known to trigger systemic resistance in plants, was used. These laboratory findings are consistent with the products in Oxycom™ being able to increase the resistance potential of the plant.

Introduction

Plants possess several strategies to resist pests that include preformed as well as induced resistance mechanisms. Both localized and systemic resistance mechanisms are induced against microbial pathogens. The localized response of hypersensitivity normally is triggered in the plant by recognition of an avirulent pathogen. The plant cell at the site of contact becomes necrotic but the further spread and growth of the pathogen is halted. A burst of hydrogen peroxide production is observed early in the response. Hypersensitive cell death also induces systemic resistance measures effective at a distance from the induction site (Alvarez et al., 1998). Systemic resistance may also be a consequence of the colonization of plants by non-pathogenic microbes or by treatment with chemical inducers, such as isonicotinic acid (INA), benzothiadiazone (BTH), probenazole and salicylic acid (SA) (De Meyer et al., 1999; Gorlach et al., 1996; Kessmann et al., 1994; Métraux et al., 1991; Pieterse et al., 1998; Sakamoto et al., 1999). Two different signal transduction pathways are recognized to be involved in induced systemic resistance. One mechanism is dependent on SA, whereas the second involves jasmonate (Reymond and Farmer, 1998; Thomma et al., 1998). These pathways involve the induction of different sets of defense-related genes.

Some of the induced genes encode proteins, such as phenylalanine ammonia lyase (PAL), chalcone synthase (CHS) and peroxidases, that function in phenolic metabolism. Other genes correspond to proteins, such as the hydroxyproline-rich glycoproteins (HYP) involved in the cell wall strengthening. Genes encoding enzymes that will hydrolyse fungal cell walls, glucanase and chitinase, or the antifungal defensins appear widespread in induced resistance in plants, yet other induced proteins such as the PR-l proteins have an undefined role (Reymond and Farmer, 1998).

Increasingly, there is demand for environmentally friendly chemicals to improve plant health in the field. The synthesized chemicals INA, BTH, and probenazole or the materials in Messenger™ (Eden Biosciences, WA, USA) fall into this category. In this paper, studies of another commercial product, Oxycom™ (Redox Chemicals, Idaho, USA), are reported. Data are provided from commercial fields, where it was compared to industry standards, to show that Oxycom™ had efficacy under conditions of disease pressure. The trials involved different crops and pests indicating that the improved performance is of a general nature.

The laboratory studies reported in this paper were performed to determine the mechanism by which Oxycom™ was active in limiting disease pressure in the field. Inhibition of mycelial growth of fungal pathogens by Oxycom™ was tested to investigate whether there was a direct effect on fungal growth. Whether Oxycom™ altered the expression of defense genes in the plant also was examined. Using *Phaseolus vulgaris* as a test plant, probes were used to detect enhanced expression of genes for peroxidase, PAL, CHS, and HYP. The responses to treatments with hydrogen peroxide, BTH and SA were compared. A role for SA in induced systemic resistance has been suggested for bean (De Meyer et al., 1999).

Materials and methods

Oxycom™

This product consists of two components. Component A is a 5% v/v stabilized solution of peracetic acid, containing 10–12% acetic acid and 20–22% hydrogen peroxide. Component B contains a mixture of plant nutrients. The two products are packaged separately and mixed at the time of use. The Oxycom™ mix is diluted to appropriate concentrations and applied using commercial pesticide application systems such as overhead booms, or in the irrigation watering system. The application rate is in the range of 1000–5000 ppm active ingredient. Application frequency is crop dependent, generally being between 5 and 20 days.

Field trials

Oxycom™ was tested for efficacy for the control of *Bremia lactucae*, downy mildew, on lettuce. Lettuce was grown in a loam soil. Six replications were employed with a replication size of 0.38 ha where there were 8820 plants. Oxycom™ was applied as a foliar spray using 508 ml/ha, starting 3 weeks after planting. The applications were continued every 14 days for a total of 5 applications. The commercial standard fungicides applied were Aliette 3 (0.68 kg/ha) and Rovral (1.4 kg/ha) with the same 14-day frequency as Oxycom™. The incidence of mildew was recorded based on observations of between 111 and 164 heads per replicate in May 1998.

Carrots were grown in a sandy loam-soil with known problems with nematodes and *Pythium*. Each replication consisted of a 0.49 ha plot with 387,100 plants per hectare. There were 4 replications of each treatment. Oxycom™ was applied using 16.3 l/ha at germination followed by 10.9 l/ha applications at days 10 and 20 after planting. Vapam (metam sodium) was applied through sprinklers 21 days prior to planting at a rate of 544 l/ha. Carrots were harvested onto processing trucks in the last week of January 1998 when the crop was 95 days old. Each sample assayed for data was of 22.7 kg containing 248–421 carrots.

Assessment of the effects of Oxycom™ on grapes with infections with powdery mildew (*Uncinula necator*) was determined with susceptible cultivar Carignane. There were 4 replications of treatments with 5 vines per plot in a randomized block design. Treatments were applied with Echo Air Blast Backpack Sprayers with a setting of 40–900 l/ha and a number 3 nozzle. The Oxycom™ product was applied at 2500 ppm together with a standard of Microthiol at 7.0 kg/ha at intervals of 7 days. Microthiol alone was used as the standard treatment at 7.0 kg/ha and at the 7 day interval. Mildew cluster evaluation was performed on July 8 1999 by rating 20 clusters from the middle three vines of each plot. Berry counts were made from 10 randomly selected clusters to get an average number of berries per cluster. Berry weight was determined from a 100-berry sample harvested on August 4 1999 from the same middle three vines of each plot previously assessed for mildew incidence.

Assessment of fungal growth inhibition by Oxycom™

Oxycom™ was added at 0, 2000, 5000 and 10,000 ppm to autoclave-sterilized potato dextrose agar (PDA) medium (Difco, Detroit, MI) cooled to 50 °C. The plates were inoculated in the center with a 0.5 by 0.5 cm plug of mycelium removed from the growing edge of

cultures previously grown for 5 days at 26 °C. The plates were sealed with Parafilm and incubated at 26 °C. At 3 days the diameters of the growing fungal colonies were measured.

Plant treatments

Bean seeds (*Phaseolus vulgaris* L. cv. Dark Red Kidney, Idaho Seed Bean Co., Twin Falls, ID) were surface sterilized with 10% sodium hypochlorite and washed extensively with sterile distilled water (Blee and Anderson, 1998). Each seed was planted into soil matrix in a plastic pot. The plants were maintained under a 14 h photoperiod at 22 ± 4 °C. Ten days after planting, the primary leaves were fully developed. The plants were sprayed with sterile water as control or exposed to different chemical treatments.

RNA isolation and northern analysis

At defined times, three leaves from three different plants were removed and ground to a fine powder in liquid nitrogen. Total RNA was isolated with Tri-Reagent following the manufacturer's protocol (Molecular Research Center Inc., Cincinnati, OH). Northern analysis was performed using the nonradioactive Genius system (Roche Biochem, Indianapolis, IN) with dig-labeled probes (Blee and Anderson, 1996; 1998). Total RNA was loaded (20 μg/lane), separated on 1.2% agarose gels containing formaldehyde, and then blotted onto positively-charged nylon membranes. The blots were prehybridized for 2 h and hybridized with antisense RNA probes. The probes were the transcripts from the cDNA clones from bean pPAL5, 1.75 kb, pCHS1, 1.4 kb; pCHT12.2, 0.65 kb; pHYP3.6, 1.3 kb (Blee and Anderson, 1996; 1998). Five dig-labeled peroxidase probes from bean were used (Blee et al., unpublished). These sequences are deposited in Genbank with accession numbers FBP1, AF149277; FBP3, AF149278; FBP4, AF149279; FBP5, AF149280 and FBP6, AF149281.

Results

Field studies

Results from three crops, lettuce and downy mildew, carrots with *Pythium* and nematode pressure and grapes with powdery mildew infection, grown under commercial field conditions are reported.

Lettuce and downy mildew

The efficacy of Oxycom™ quantified as percent of heads infected, was assessed using a generalized linear model fit with PROC GENMOD in SAS Release 7.0. Oxycom™ reduced the disease level compared with the applications of the standard fungicides from 12.2% to 6.0% (p =0.006).

Pythium and nematode damage on carrots

Using a generalized linear model computed with PROC GENMOD in SAS Release 7.0, the proportion of forked carrots and the proportion of the *Pythium*-infected carrots were statistically smaller in the Oxycom™ treatment compared with the Vapam treatment:

	Oxycom™	VAPAM	p value
% forked	2.8%	11.2%	0.005
% diseased	0.13%	3.0%	0.009

Grape and powdery mildew

The percentage of incidence of powdery mildew was rated as a 100% for both the control and the Microthiol treatments. The combined Oxycom™–Microthiol treatment was lower at 96% (SE 2.3). The percent severity values for control, Microthiol, and Oxycom™–Microthiol treatments were significant at $p < 0.001$ when assessed using analysis of variance of a one-way factorial. All treatments were different from one another when pairwise comparison among treatments were controlled for experimentwise Type 1 error using Tukey's method. The data were $\log_e$-transformed for this analysis. The percent severity was highest for the control treatment (90.5%), intermediate for Microthiol and lowest for the combined Oxycom™–Microthiol treatments (29.1%). There was no evidence of a treatment effect for grams/berry ($p = 0.757$) when the differences between the Microthiol and combined Oxycom™–Microthiol treatments were assessed using analysis of variance of a one-way factorial.

Fungicidal activity of Oxycom™

The addition of Oxycom™ to growth media impaired the hyphal growth of fungi in a dose dependent manner (Table 1). Fungi were differentially inhibited by the presence of Oxycom™ with *Sclerotinia minor* being more sensitive than others assayed (Table 1). At the median concentration of Oxycom™ used in the field,

Table 1. Effect of Oxycom™ on the mycelial growth of plant pathogens

Isolate	Relative growth* on medium amended with Oxycom™ at ppm			
	10,000	5000	2000	0
Rhizoctonia solani	0.28 (±0.02)	0.84 (±0.05)	1.00 (±0.05)	1.00 (±0.00)
Fusarium proliferatum	0.28 (±0.05)	0.81 (±0.02)	0.90 (±0.04)	1.00 (±0.05)
Phytophthora infestans	0.48 (±0.02)	0.69 (±0.03)	0.77 (±0.04)	1.00 (±0.00)
Sclerotinia minor	0.16 (±0.06)	0.50 (±0.08)	0.83 (±0.08)	1.00 (±0.02)

*Growth was assessed on PDA plates after 3 days of incubation at 26 °C. Mycelial plugs were transferred into the center of the PDA plate and radial growth was measured. The results shown are from one study where each treatment was replicated three times. The study is one of four trials. The values are relative to the growth on the control plate and are the means with the standard deviations.

2500 ppm, there was generally less than 50% inhibition of the fungal growth on the plate medium.

Increased accumulation of transcripts of defense related genes in plants treated with Oxycom™

Bean leaves 24 h after spraying with Oxycom™ had increased accumulations of mRNA corresponding to several defense genes compared with the levels from control plants that were treated with water (Figure 1). The experiment was repeated at least three times with the same results: data from one of the studies are shown. Analysis of hybridization with six different clones for peroxidases from bean showed that two probes, FBP 3 and FBP 6, had increased accumulation of mRNA when the plants were treated with Oxycom™. Transcripts for genes involved in the isopropanoid pathway, PAL and CHS, were also increased in accumulation. A probe of the gene family for the cell wall structural protein hydroxyproline-rich glycoprotein, HYP, showed elevated transcript levels in the Oxycom™ treatment.

Both of the components of Oxycom™, the active oxygen species in product A, and the components in product B, stimulated expression of these five defense-related genes (Figure 1). Synergism was noted for the combined applications for the FBP 6 probe. Genes encoding chitinase and three other bean peroxidases, were not changed in expression by the Oxycom™ treatment in leaves sprayed and harvested 24 h later (data not shown). Treatments with Oxycom™ at 50, 500 and 2500 ppm had similar effects on the accumulations of CHS, HYP, PAL and FBP 3 (Figure 2) in the 24 h period.

After treatment of the leaves with BTH at 0.005% and 0.5% for 24 h the accumulation of mRNA for the defense genes PAL, CHS, HYP, and FBP3 increased (Figure 3). Hydrogen peroxide and SA also caused increases in a dose dependent manner but their effects were less strong than BTH (Figure 3).

Effect of time and distance on the response to Oxycom™

Assay of the leaves that were sprayed with Oxycom™ showed that accumulation of mRNA for the certain of the defense genes occurred by 6 h after treatment and was elevated at 12 and at 24 h. By 48 h the accumulations had decreased (Figure 4).

To investigate systemic effects of the induction of defense-related genes, northern analysis was performed on extracts from treated or non-treated leaves from same plant. Accumulations of mRNA for CHS, PAL, HYP and FBP 3 were to the same extent in the nontreated leaves, of plants with other leaves previously treated with Oxycom™, as those that were sprayed directly with Oxycom™ at 24 h after treatment (Figure 5).

Discussion

Field studies performed under commercial growing conditions showed that applications of Oxycom™ were as effective or gave better protection than industry standards in limiting pathogen-caused problems. Compatibitility of Oxycom™ with micronized sulfur, a contact chemical, was observed in the grape-powdery mildew trial. Thus, as has been proposed for the commercial use of BTH, the possibility of teaming systemic resistance inducers with other control measures acting directly on the pathogen appears feasible. The crop and the potential pathogens in these studies were varied indicating

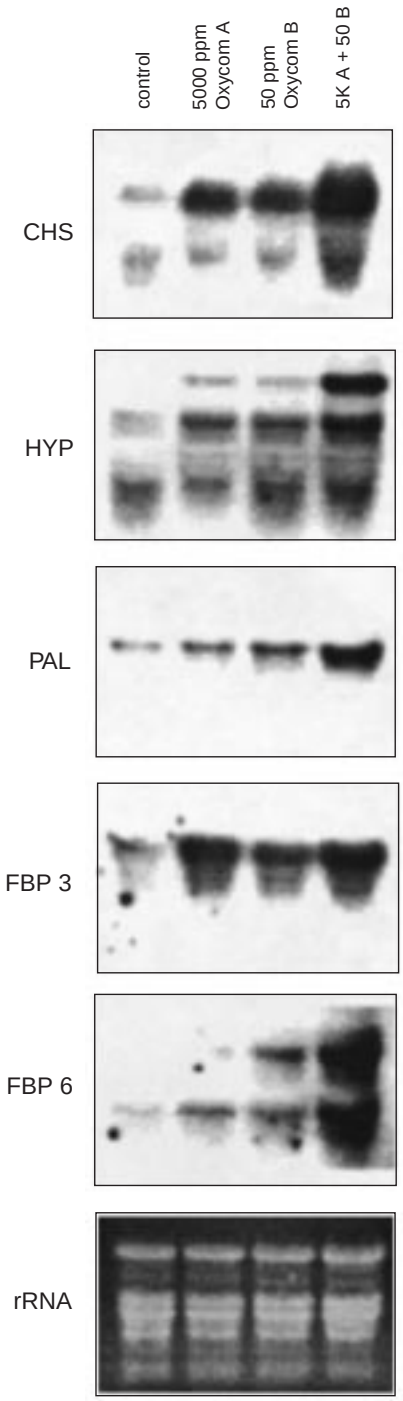

Figure 1. Increased accumulation of transcripts for defense-related genes after 24 h of treatment of bean leaves by Oxycom™. Bean leaves were treated with water or Oxycom™ A and/or B at the dilutions shown for 24 h prior to extraction of mRNA and hybridization analysis as described in Materials and methods. Hybridization to transcripts for CHS, HYP, PAL, FBP 3 and 6 are shown. The pattern of rRNA bands is provided to show equal lane loading.

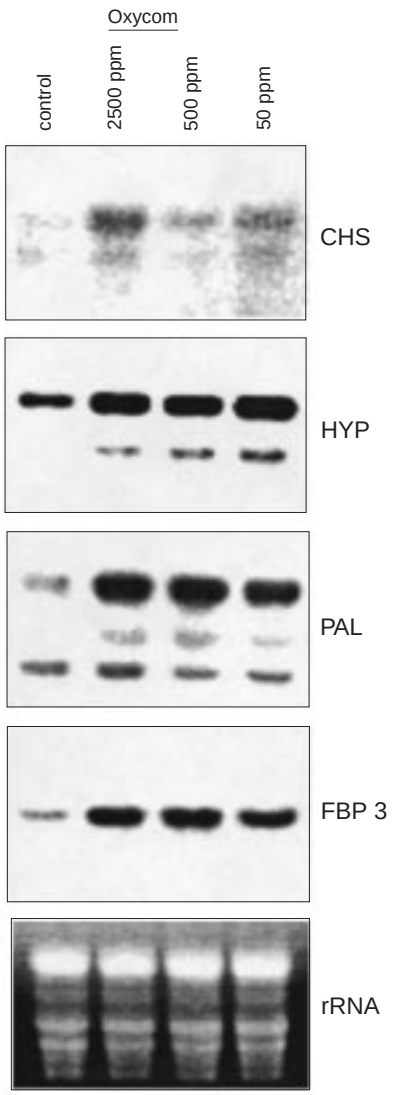

Figure 2. Effect of dosage on the accumulation of defense gene transcripts after 24 h treatment with Oxycom™. Bean leaves were treated with water or Oxycom™ for 24 h prior to extraction of mRNA and hybridization analysis as described in Materials and methods. Data are for one of three studies that show the same trend. The conditions for treatment were control: water: 2500 ppm of A and B; 500 ppm of A and B; and 50 ppm of A and B. Hybridization to transcripts for CHS, HYP, PAL and FBP 3 is shown. The pattern of rRNA bands is provided to show equal lane loading.

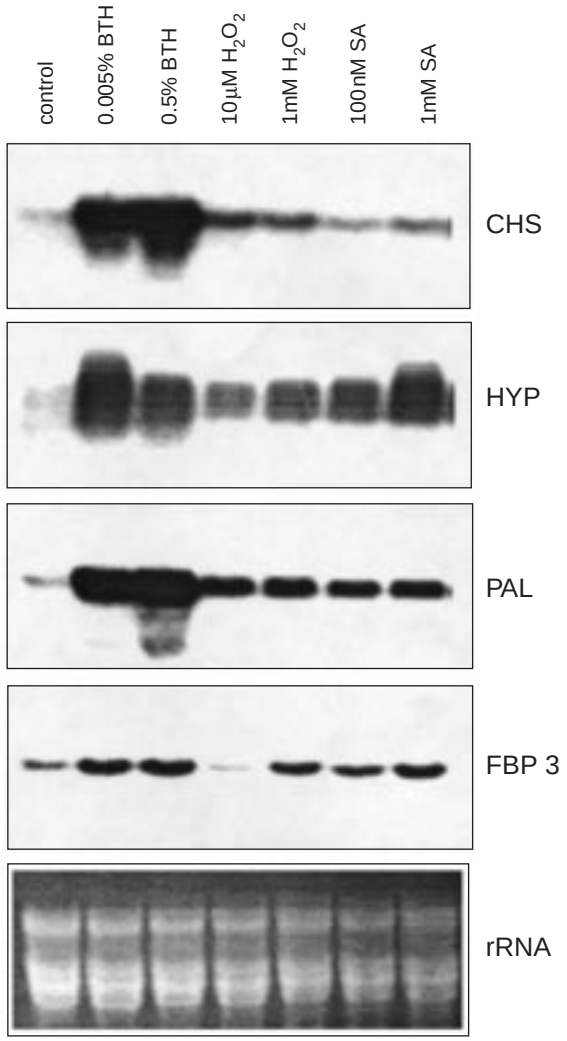

Figure 3. Effect of treatment with inducers of defense responses in bean, BTH, hydrogen peroxide and SA. Bean leaves were treated with water or BTH (0.005 and 0.5%), hydrogen peroxide (10 µM or 1 mM) or SA (100 nM or 1 mM) 24 h prior to extraction of mRNA and hybridization analysis as described in Materials and methods. Data are for one of three studies that show the same trend. Hybridization to transcripts for CHS, HYP, PAL and FBP 3 is shown. The pattern of rRNA bands is provided to show equal lane loading.

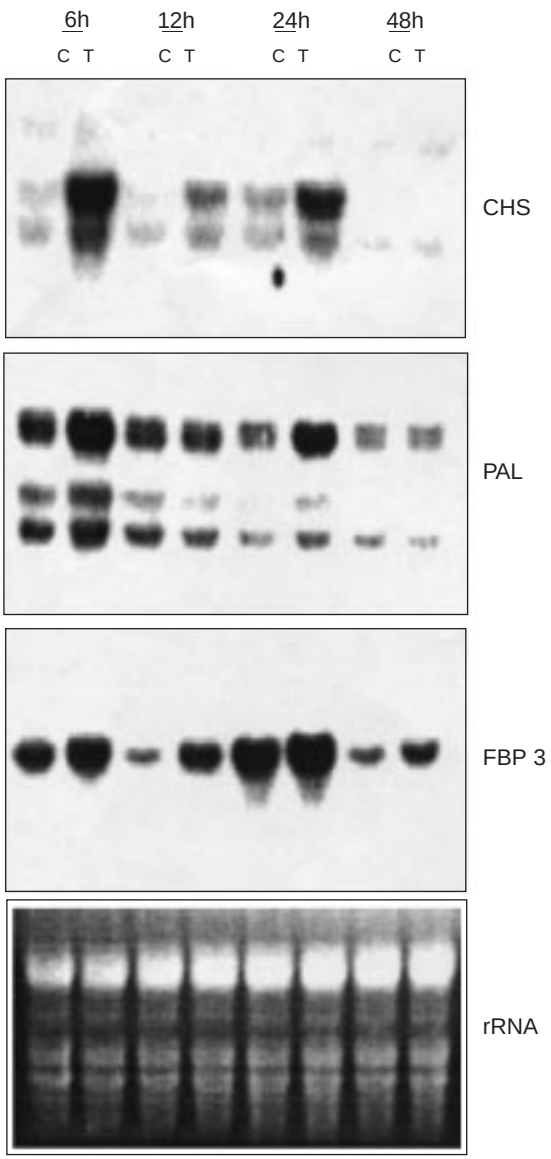

Figure 4. Effect of time on the accumulation of defense-related transcripts after treatment with Oxycom™. Leaves were treated with water (C) or Oxycom™ (5000 ppm A and 50 ppm B) and harvested at 0, 6, 12, 24 and 48 h prior to mRNA hybridization analysis as described in Materials and methods. Hybridization to transcripts for CHS, PAL and FBP 3 is shown. The pattern of rRNA bands is provided to show equal lane loading.

that the Oxycom™ effects were general rather than having plant or microbial specificity.

Our laboratory studies demonstrate that Oxycom™ at sufficiently high concentrations limited mycelial growth of pathogenic fungi. Other studies revealed that both acidity and the active oxygen species present in Oxycom™ will inhibit the growth of the plant-associated bacterium *Pseudomonas putida* in a dose-dependent manner (Miller and Anderson, unpublished data). The active oxygen species in Oxycom™

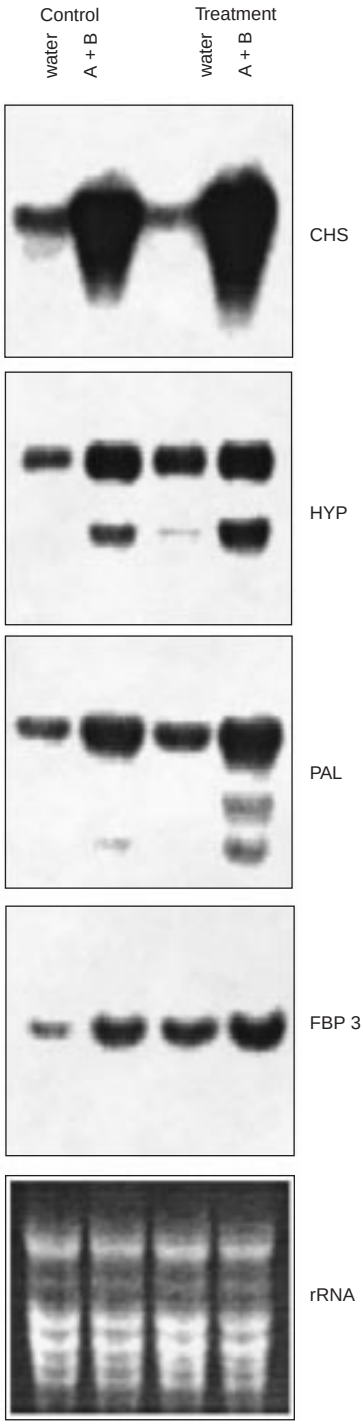

Figure 5. Systemic effect of application of Oxycom™ on the elevation of mRNA transcripts for defense genes. One leaf on control plants was sprayed with water and one leaf on test plants with Oxycom™ (5000 ppm A and 50 ppm B). At 24 h the sprayed leaves from both the control and Oxycom™-treated plants were harvested. Leaves adjacent to the sprayed leaves that had no prior treatment were also harvested from the water or the Oxycom™-treated plants. Hybridization analysis on extracted mRNA was performed as described in Materials and methods. Hybridization to transcripts for CHS, HYP, PAL and FBP 3 is shown. The pattern of rRNA bands is provided to show equal lane loading.

product A are at high enough concentration toxic to all organisms through inactivation of metabolic enzymes or by causing lipid peroxidation, disruption of structural proteins and DNA damage (Freeman and Crapo, 1982; Fridovich, 1986; Halliwell and Gutteridge, 1984). However, the effectiveness Oxycom™ in the field is more likely due to its ability to stimulate defense responses in the plant. At field levels Oxycom™ had limited effect on fungal pathogen growth, whereas, when applied foliarly, plant mRNAs encoding defense-related products rapidly accumulated. The potential for resistance based upon phenolic production and cell wall strengthening was increased because transcripts from PAL, CHS, FBP and HYP genes accumulated. This ability of Oxycom™ to stimulate a defense response confirms prior findings that hydrogen peroxide and acidification of plant cells act as triggers for altered transcription of defense genes (Chamnongpol et al., 1998).

A systemic action of Oxycom™ was observed in bean because transcripts of the defense genes accumulated in the nontreated leaves of plants bearing other leaves that were exposed. The effects of Oxycom™ were time related with mRNA accumulations being noted as soon as 6 h but declining by 48 h. These findings suggest that Oxycom™ may rapidly help protect the plant but concur with the use of repetitive applications of Oxycom™ in the field. The systemic nature of the response to Oxycom™ resembles the effect of other chemicals studied in other plants. Indeed, similar patterns of induction for the defense genes CHS, PAL, FBP and HYP in bean were found for both Oxycom™ and BTH. The classic demonstration of induced systemic resistance by BTH treatment was with wheat where induction of lipoxygenase and cysteine-rich proteins was documented (Gorlach et al., 1996). In contrast, probenazole protected against rice blast without PAL induction, although peroxidase, lipoxygenase, and chitinase were induced (Sakamoto et al., 1999). In bean, SA treatments resulted in higher levels of PAL activity in a dose-dependent and time-transient manner and correlated with induced systemic resistance to

Botrytis cinerea (De Meyer et al., 1999). The present studies extend the information for bean to the mRNA level where SA was found to increase accumulation of the PAL transcript.

In the studies with the bean peroxidase probes, the Oxycom™ treatment caused differential expression of the peroxidase gene family members. The pattern of expression was distinct from bean treated with an elicitor preparation from *Colletotrichum lindemuthianum* where the hypersensitive response is mimicked. Increased transcripts for FBP 3 and FBP 6 were only observed with the Oxycom™ treatment, whereas transcripts for FBP 4 and FBP I accumulated by 1.5 h in the elicited cells (Blee et al., unpublished). It is believed that the FBP 1 gene peroxidase is the peroxidase that accounts for the hydrogen peroxide burst in the hypersensitive response. Thus, our studies suggest that different pathways are involved in elicitor-induced resistance and treatment with Oxycom™. The interconnections between the various signal transduction pathways involved in systemic resistance measures are being intensively studied. Probenazole, SA, and BTH had a common effect on rice, each causing induction of the protein RPRI (Sakamoto et al., 1999). Both the SA pathway and the bacterial-induced jasmonic acid pathway in *Arabidopsis* share the transcriptional factor, NPRl (Pieterse et al., 1998). Further studies are needed to resolve the signaling pathways activated by the Oxycom™ products and to determine interactive effects of this product with other inducers of plant defense.

Acknowledgements

The authors thank Drs. C. Miller and P. Timmer for comments on the manuscript and Moon Enterprises for support. We are grateful for the statistical assistance provided by Susan Durham, MS. Utah Agricultural Experiment Station Paper Number 5900.

References

Alvarez ME, Pennell RI, Meijer P-J, Ishikawa A, Dixon RA and Lamb CJ (1998) Reactive oxygen intermediates mediate a systemic network in the establishment of plant immunity. Cell 92: 773–774

Blee KA and Anderson AJ (1996) Defense-related transcript accumulation in *Phaseolus vulgaris* L. colonized by the arbuscular mycorrhizal fungus *Glomus interadices* Schenck and Smith in mycorrhizal bean roots. Plant Physio 110: 675–688

Blee KA and Anderson AJ (1998) Regulation of arbuscule formation by carbon in the plant. The Plant J 16: 523–530

Chamnongpol S, Willekens H, Moeder W, Langebartels C, Sandermann H Jr, von Montagu MC, Inke D and Van Camp W (1998) Defense activation and enhanced pathogen tolerance induced by H_2O_2 in transgenic tobacco. Proc Natl Acad Sci USA 95: 5818–5823

De Meyer G, Capieau K, Audenaert K, Buchala A, Metraux J-P and Hotfte M (1999) Nanogram amounts of salicylic acid produced by the rhizobacterium *Pseudomonas aeruginosa* 7NSK2 activate the systemic acquired resistance pathway in bean. MPMI: 12 450–458

Freeman BA and Crapo JD (1982) Biology of disease. Free radicals and tissue injury. Lab Invest 27: 412–426

Fridovich I (1986) Biological effects of the superoxide radical. Arch Biochem Biophys 247: 1–11

Gorlach J, Volrath S, Knauf-Beiter G, Hengy O, Beckhove U, Kogel K-H, Oostendorp M, Staub T, Ward E, Kessmann H and Ryals J(1996) Benzothiadiazone, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. Plant Cell 8: 629–643

Halliwell B and Gutterridge JMC (1984) Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem J 219: 1–14

Kessmann H, Staub T, Hofmann C, Maetzke T and Herzog J (1994) Induction of systemic acquired disease resistance in plants by chemicals. Annu Rev Phytopathol 32: 439–459

Métraux JP, Ahl-Goy P, Staub T, Speich J, Steinmann A, Ryals J and Ward E (1991) Induced resistance in cucumber in response to 2,6-dichloroisonicotinic acid and pathogens. In: Hennecke H and Verma DPS (eds.) Advances in Molecular Genetics of Plant–Microbe Interactions. Vol. 1 (pp 432–439) Dordrecht, The Netherlands: Kluwer Academic Publishers

Pieterse CM, Wees SC, van Pelt JA, Knoester M, Laan R, Gerrits H, Weisbeek PJ and van Loon LC (1998) A novel signaling pathway controlling induced systemic resistance in *Arabidopsis*. Plant Cell 10: 1571–1580

Reymond P and Farmer E (1998) Jasmonate and salicylate as global signals for defense gene expression. Cur Opin Plant Biol 1: 404–411

Sakamoto K, Tada Y, Yokozeki Y, Akari H, Hayashi N, Fujimura T, and Ichikawa N (1999) Chemical induction of disease resistance in rice is correlated with the expression of a gene encoding a nucleotide binding site and leucine-rich repeats. Plant Molec Biol 40: 847–855

Thomma BPHJ, Eggermont K, Penninckx IRMA, Mauch-Mani B, Volgelsang R, Cannue BPA and Broekkaert W (1998) Separate jasmonate-dependent and salicylate-dependent defense-response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens. Proc Natl Acad Sci USA 95: 15107–15111