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Compatibility of systemic acquired resistance and microbial biocontrol for suppression of plant disease in a laboratory assay

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Abstract

Systemic acquired resistance (SAR) and microbial biocontrol each hold promise as alternatives to pesticides for control of plant diseases. SAR and *Bacillus cereus* UW85, a microbial biocontrol agent, separately suppress seedling damping-off diseases caused by oomycete pathogens. The purposes of this study were to investigate how expression of SAR affected the efficacy of biocontrol by UW85 and if UW85 treatment of plants induced SAR. We devised a laboratory assay in which seedling damping-off disease, induction of SAR, and growth of UW85 could be quantified. Seedlings of *Nicotiana tabacum* Xanthi nc were germinated on moist filter paper and transferred after 7 days to water agar plates (40 seedlings per plate). Zoospores of oomycete pathogens (*Pythium torulosum, Pythium aphanidermatum,* or *Phytophthora parasitica*) were applied at concentrations that caused ≈ 80% seedling mortality within 10 days. Seedling mortality was dependent on zoospore inoculum concentration. The level of disease suppression caused by treatment with UW85 depended on the UW85 dose applied. SAR was induced with 0.5-mM salicylic acid or 0.1-mM 2,6-dichloroisonicotinic acid. Expression of an SAR-related gene was confirmed by northern analysis with a probe prepared from a tobacco PR-1a cDNA. Induction of SAR suppressed disease caused by each of the oomycete pathogens, but did not alter the growth of UW85 on roots. Treatment of seedlings with UW85 did not induce the expression of PR-1a. The combination of induction of SAR and treatment with UW85 resulted in additive suppression of disease as measured by seedling survival.

Keywords: *Bacillus cereus*, biological control, pathogenesis related protein, *Phytophthora parasitica*, *Pythium aphanidermatum*, *Pythium torulosum*, systemic acquired resistance

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Introduction

Systemic acquired resistance (SAR) is a widespread response of plants to infection by pathogens. SAR occurs in many plant species, is effective against a broad range of pathogens, and it can last for several weeks to months after its induction. (Chester 1933; Ross 1961a,b; Kuc 1982; Sequeira 1983). SAR is associated with the coordinate expression of a suite of genes (Ward *et al.* 1991), some of which confer resistance to specific pathogens when the genes are individually and constitutively expressed in transgenic plants (Alexander *et al.* 1993a,b; Liu *et al.* 1994). A wide array of pathogens and certain chemicals (White 1979; Kuc 1990; Metraux *et al.* 1991; Kessmann *et al.* 1994) induce SAR.

Use of SAR for crop protection, either through construction of transgenic plants or through the use of SAR-inducing chemicals, seems increasingly practical (Metraux *et al.* 1991; Ryals *et al.* 1992; Kessmann *et al.* 1994), but has also been the subject of some controversy. One concern is that treatment of crops to induce SAR or modification of the expression of SAR-related genes by genetic engineering may alter associations with beneficial plant-associated microorganisms. Miller (1993), for example, raised the possibility that over-expression of SAR transgenes might compromise the beneficial effects of mycorrhizal associations with crops.

Another concern about the use of SAR as a crop protection method is the possibility of nontarget effects on the

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ecology of rhizosphere bacteria, either indigenous or applied, that may be effective as biological control agents to suppress plant disease. SAR and microbial biological control are examples of emerging alternatives for disease management that are of interest because of the current focus on making farming systems more ecologically based. Such alternatives can be expected to be used in combinations, and as a result may interact in unexpected or unpredicted ways. Therefore, ecological research is needed to understand whether and how disease management methods interact or interfere. Such research also offers new opportunities to increase our understanding of plant and microbial ecology at the molecular level.

We report here the results of the first study to evaluate the interactions between chemically induced SAR and a microbial biological control agent. The microbial biological control agent used, *Bacillus cereus* strain UW85, suppresses a range of plant diseases in laboratory tests (Handelsman *et al.* 1990, 1991) and field trials (Halverson & Handelsman 1991; Osburn *et al.* 1995). UW85 will soon be available commercially for control of root rot disease in soybeans. The mechanisms of disease suppression by UW85 are not fully understood, but production of antibiotics is a major factor in disease control in the laboratory (Silo-Suh *et al.* 1994), and UW85 influences microbial communities associated with plant roots in the field (Gilbert *et al.* 1993).

We developed an *in vitro* seedling assay system designed to enable us concurrently to induce SAR with chemical inducers, to measure the expression of SAR-related plant genes, to treat the plants with UW85 and measure its root-dependent growth, and to inoculate with zoospores and score disease incidence and severity caused by oomycete pathogens. Here we describe the details of this assay system and the results of molecular and microbiological studies of the interaction between SAR and UW85. Specifically, we measured the influence of SAR induction on UW85 growth on roots, determined whether UW85 induces expression of PR-1a, an SAR-related gene, and evaluated the independent and concerted use of UW85 and SAR for disease suppression.

Materials and methods

Seedling bioassay system

Seeds of *Nicotiana tabacum* Xanthi nc were surface-disinfested (Handelsman *et al.* 1991) and germinated in Petri dishes under Cool White fluorescent lights at 24 °C on Whatman #1 filter paper moistened with 0.25-strength Hoagland solution. Seven days after germination, seedlings were transplanted into Petri dishes (100 mm × 15 mm) containing 1.5% water agar (40 seedlings per plate). Petri dishes were wrapped with Parafilm and placed in a growth chamber at 24 °C with 12 h of light (244 μEinsteins/m^2/s) provided from Cool White fluorescent bulbs.

Oomycete pathogens

The *P. aphanidermatum* isolate we used was isolated from turfgrass (K. P. Smith, personal communication). The *P. torulosum* isolate used was isolated from alfalfa roots grown in Hancock, Wisconsin (D. W. Johnson, personal communication). Taxonomic determinations for the *Pythium* isolates used in this study were provided by Dr Donald Barr, Agriculture Canada, Ottawa. Zoospore inocula for both *Pythium* spp. were produced in culture on V8 agar according to a method previously described (Smith *et al.* 1993). *P. aphanidermatum* usually produced 20 000 zoospores/mL. Zoospore concentrations produced by *P. torulosum* were typically between 100 000 and 500 000/mL.

Phytophthora parasitica was provided by William C. Nesmith, University of Kentucky. *P. parasitica* was grown on oatmeal agar for 5–7 days. Zoospores were produced in sterile distilled water by the method described previously (Handelsman *et al.* 1991). Zoospore concentrations produced in *P. parasitica* cultures were typically 20 000/mL.

All three pathogens were maintained in culture (24 °C) and subcultured twice each month. Seedlings were inoculated with a suspension containing the appropriate number of zoospores 2 days after the seedlings were transplanted to the water-agar plates. To maintain pathogenicity, which can be lost in culture, every three months the pathogens were inoculated to seedlings growing on water agar plates and after several days reisolated from surface disinfested diseased tissues. Reisolation was on water agar (*Pythium* spp.) or V8 agar (*Phytophthora*).

Induction of SAR

One day before transplanting, seedlings were flooded with 15 mL per Petri dish of 0.5-mM Na-salicylate (SA; Sigma) or 0.1-mM 2,6-dichloroisonicotinic acid (INA; Ciba-Geigy). Control seedlings received the same volume of sterile distilled water (as control for SA) or the wettable powder formulation without INA (Ciba-Geigy; as control for INA). After incubation at room temperature overnight (12 h), seedlings were washed three times with sterile distilled water and transplanted onto water-agar plates as described above.

UW85 culture and growth measurements

Fully sporulated cultures of UW85 were obtained by growing the bacteria in well-aerated 0.5 strength tryptic soy broth at 28 °C for 4 days. The concentration of cells in the culture was determined by serial dilution and plating on 0.5 strength tryptic soy agar plates. For inoculation of

UW85 on water agar plates, UW85 cultures were diluted in sterile distilled water to the desired concentration (c.f.u./mL), and 1 mL of the diluted culture was distributed evenly over the surface of the agar plate.

Growth of UW85 in the seedling bioassay system was determined by removing 8-mm-diameter samples of water agar surrounding and containing a segment of seedling root (with-root sample), and from a similar area between roots (without-root sample). Three with-root and three without-root samples were taken at random from each plate immediately after addition of UW85 and at two-day intervals thereafter for 10 days. Each sample was placed individually in 1 mL of sterile water in a 1.5-mL Eppendorf tube and sonicated for 30 s in a Branson 2000 waterbath sonicator. UW85 was enumerated with a Spiral Biotech Model 3000 spiral plater.

Experimental design and analysis

The experimental design was a randomized complete block design with three replications. Means and standard errors were calculated for each treatment at each sampling time. Analysis of variance for treatments at each sampling time was conducted using the GLM procedure (SAS 1990). All experiments were repeated at least twice. The data reported are from single experiments that are representative.

RNA extraction and northern blotting

Seedling samples were taken for RNA extraction immediately before SAR induction and every 2 days thereafter for 14 days. Total RNA was isolated from tobacco seedlings using a method modified from that described previously (Verwoerd *et al.* 1989). The modification was in the use of an equal volume of 7.5-M ammonium acetate rather than 4-M LiCl for RNA precipitation. Isolated total RNA was fractionated by formaldehyde-agarose gel electrophoresis at 20 μg of total RNA per lane. RNA was transferred to a nylon membrane (Nytran; Schleicher and Schuell) and cross-linked with UV light (UV StrataLinker 2400; Stratagene). RNA blots were hybridized with ^{32}P-labelled PR-1a cDNA probe. The blots were then stripped and rehybridized with a labelled pCNT6 probe to confirm equal loading of RNA. pCNT6 is a cDNA clone of a constitutively expressed gene in tobacco plants (Memelink *et al.* 1987). Probes were prepared using random primers (Ambion) according to the manufacturer's directions.

Results

Pathogen infection of seedlings

The uninoculated seedlings were healthy and their mass increased linearly from 0.5 mg (fresh weight) at transplanting to 1.7 mg 12 days after transplanting. All three oomycete pathogens infected the tobacco seedlings, as evidenced by reisolation from 100% of the seedlings inoculated (data not shown), and the oomycetes caused seedling damping-off as evidenced by typical disease symptoms. The symptoms observed on diseased seedlings were specific to each pathogen. Death of seedlings inoculated with zoospores of *P. torulosum* was slow and was preceded by browning and water-soaking, whereas death of seedlings inoculated with *P. aphanidermatum* and *P. parasitica* was rapid, characterized by desiccation rather than water-soaking. Disease incidence was inoculum-density dependent across a wide range of zoospore concentrations

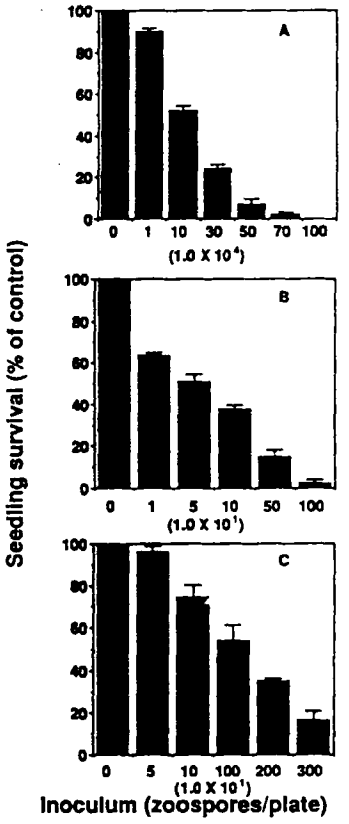

Fig. 1 Survival of tobacco seedlings ($n = 120$) 10 days after inoculation with various zoospore concentrations of (A) *P. torulosum* (B) *P. aphanidermatum*, and (C) *P. parasitica*. Seeds were surface-disinfested and germinated in Petri dishes at 24 °C on Whatman #1 filter paper moistened with 0.25-strength Hoagland solution. Seven days after germination, 40 seedlings were transplanted onto 1.5% water agar in 100-mm × 15-mm Petri dishes, which were then sealed with Parafilm and placed in a growth chamber at 24 °C. Seedlings were inoculated with a suspension containing the appropriate number of zoospores 2 days after the seedlings were transplanted to the water-agar plates. All steps in this procedure were in a chamber supplied with light (12 h per 24-hour day, 244 μEinsteins/m²/s) provided from Cool White fluorescent bulbs.

(Fig. 1) for each of the pathogens, although the range of inoculum density required to cause disease symptoms differed for each pathogen.

Suppression of diseases by UW85

Fully sporulated, undiluted cultures of UW85 containing 10^8 c.f.u./mL provided almost complete suppression of symptoms caused by infection with *P. torulosum*, *P. aphanidermatum*, or *P. parasitica* at the zoospore inoculum concentrations tested. Symptom suppression was correlated with seedling survival and both were dependent on the cell concentration of UW85 used (Fig. 2). UW85 at 10^6 c.f.u./mL provided ≈ 50% suppression against all three pathogens at the zoospore inoculum concentrations used in these tests (see legend to Fig. 2). UW85 was applied at 10^6 c.f.u./mL to test for the interaction between UW85 and induced SAR in suppression of diseases.

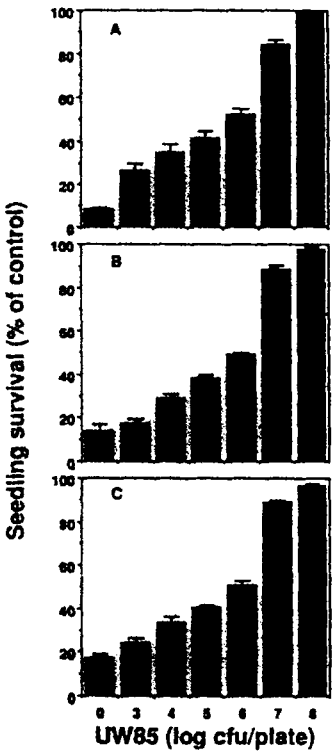

Fig. 2 Survival of tobacco seedlings ($n = 120$) grown on water-agar plates 10 days after treatment with various concentrations of UW85 and inoculation with (A) 5×10^5 zoospores of *P. torulosum* (B) 500 zoospores of *P. aphanidermatum*, and (C) 2500 zoospores of *P. parasitica*. Seedling germination, transplanting, and inoculation with zoospores were as described in legend to Fig. 1. Fully sporulated cultures of UW85 grown in well-aerated 0.5 strength tryptic soy broth at 28 °C for 4 days were diluted with sterile distilled water to the cell concentrations shown (c.f.u./mL). One millilitre of the diluted culture was distributed evenly over the surface of the agar plate.

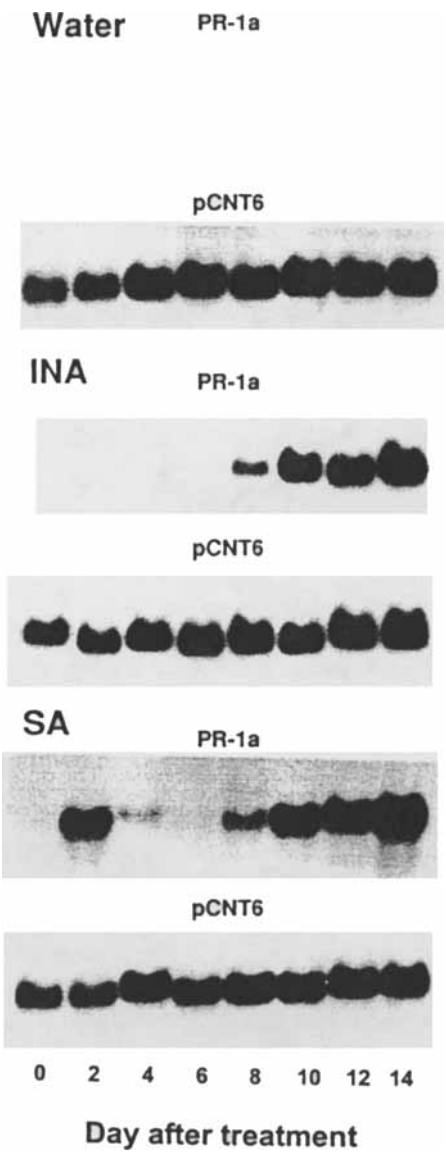

Fig. 3 Time-course (days) of PR-1a induction in tobacco seedlings following treatment with sterile distilled water, 0.1 mM of INA, or 0.5-mM SA. One day before transplanting (see Fig. 1 legend), germinated seedlings on Whatman filter paper were flooded with 15 mL per Petri dish of sterile distilled water, 0.5-mM Na-salicylate or 0.1-mM 2,6-dichloroisonicotinic acid. After incubation at room temperature overnight (12 h), seedlings were washed three times with sterile distilled water and transplanted onto water-agar plates as described in legend to Fig. 1. Seedling samples were taken for RNA extraction immediately before SAR induction and every 2 days thereafter for 14 days. Total RNA was isolated from tobacco seedlings, fractionated by formaldehyde-agarose gel electrophoresis, transferred to a nylon membrane and cross-linked with UV light. RNA blots were hybridized with ^{32}P-labelled PR-1a cDNA probe prepared using random primers. The blots were then stripped and rehybridized with a labelled pCNT6 probe to confirm equal loading of RNA. pCNT6 is a cDNA probe complementary to a transcript expressed constitutively in tobacco (Memelink *et al.* 1987).

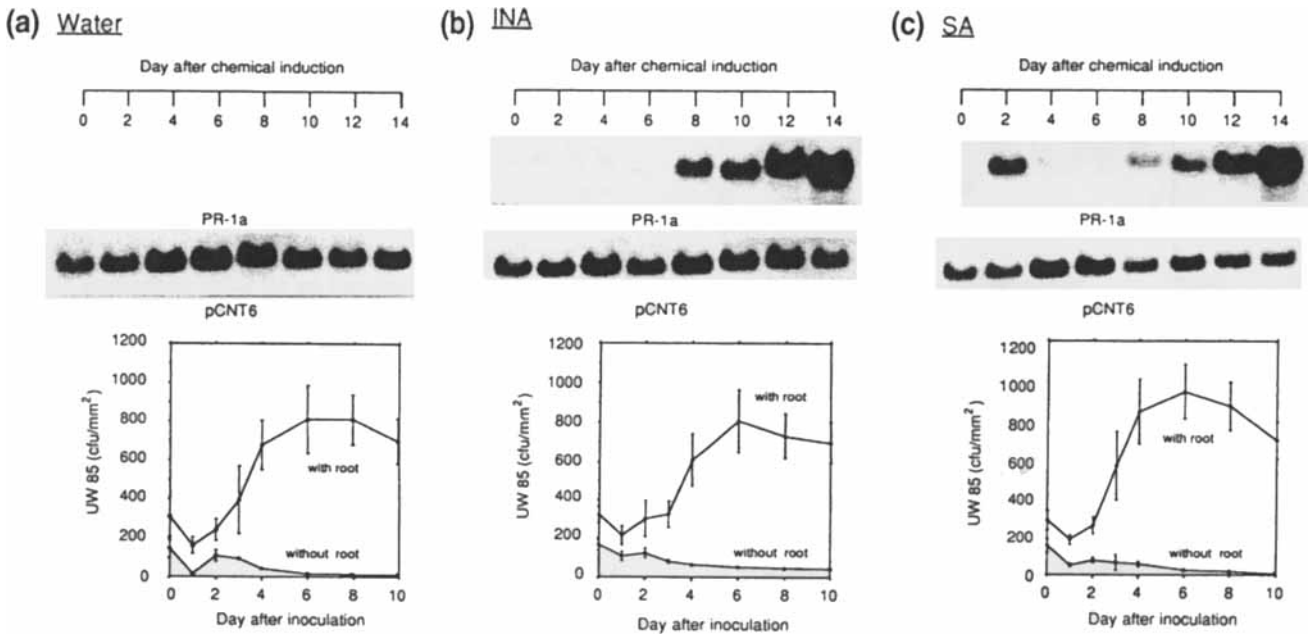

Fig. 4 Time-course of PR-1a expression in tobacco seedlings treated (a) with sterile distilled water, (b) with 0.1 mM INA and (c) with 0.5 mM SA and of UW85 growth on roots of the seedlings. Seedlings were germinated, transplanted, and treated with UW85 (at 10^6 c.f.u./plate of 40 seedlings) as described in legends to Figs 1 and 2. Northern analysis was as described in the legend to Fig. 3. Bars represent standard error.

Induction of SAR in tobacco seedlings

SAR was induced in seedlings either by treatment with 0.1-mM INA or 0.5-mM SA, and induction was monitored by measuring the expression of a pathogenesis-related (PR) gene, which has been used widely as a marker for induction of SAR (Ryals *et al.* 1994). SAR induction was determined by northern analysis with a labelled probe prepared from a full-length cDNA clone of tobacco PR-1a gene (Fig. 3). The pattern of induction in seedlings treated by INA showed an increase in PR-1a mRNA accumulation from 2 to 14 days after induction. Following SA induction, PR-1a mRNA levels were high on day 2, followed by lower levels between days 4 and 6, followed by an increase again from day 8 to day 14 (Fig. 3). The pattern of PR-1a mRNA levels following SA induction was consistent from experiment to experiment. Rates of seedling mortality during this period, however, did not differ between SA and INA treatments (data not shown).

PR-1a expression was also induced by two (*P. parasitica* or *P. torulosum*) of the three pathogens, but at very much lower levels than when treatment was with SA or INA (see Fig. 5). PR-1a expression was not induced in the control seedlings.

Growth of UW85 on roots of SAR-induced seedlings

The population of UW85 increased on water agar in the presence of seedling roots and decreased in their absence (Fig. 4). Thus, UW85 growth was dependent on the presence of plants. Analysis of variance at each sampling time (data not shown) indicated that induction of SAR in seedlings did not significantly alter the growth of UW85 on roots (Fig. 4b,c) in comparison with non-induced controls (Fig. 4a). The presence or growth of UW85 on seedlings did not induce the expression of SAR as determined by northern analysis using the PR-1a as a probe (Fig. 4a).

Concerted use of SAR induction and UW85 to suppress disease

SAR induction and UW85 treatment used in concert resulted in greater disease suppression than did either alone, as measured by seedling survival in the water-agar system (Fig. 5). Survival of control seedlings (not SAR induced or UW85 treated) was adjusted to 10–20% by adjusting the zoospore inoculum (5×10^5 zoospores per plate for *P. torulosum*, 500 zoospores for *P. aphanidermatum*, and 2500 zoospores for *P. parasitica*). Application of UW85 (10^6 c.f.u./mL) to oomycete-inoculated seedlings resulted in 40% seedling survival. The survival of SAR-induced seedlings inoculated with zoospores but not with UW85 was about 60%, whether INA or SA was used for induction. Concerted use of SAR induction and UW85 treatment in these experiments resulted in more than 80% seedling survival.

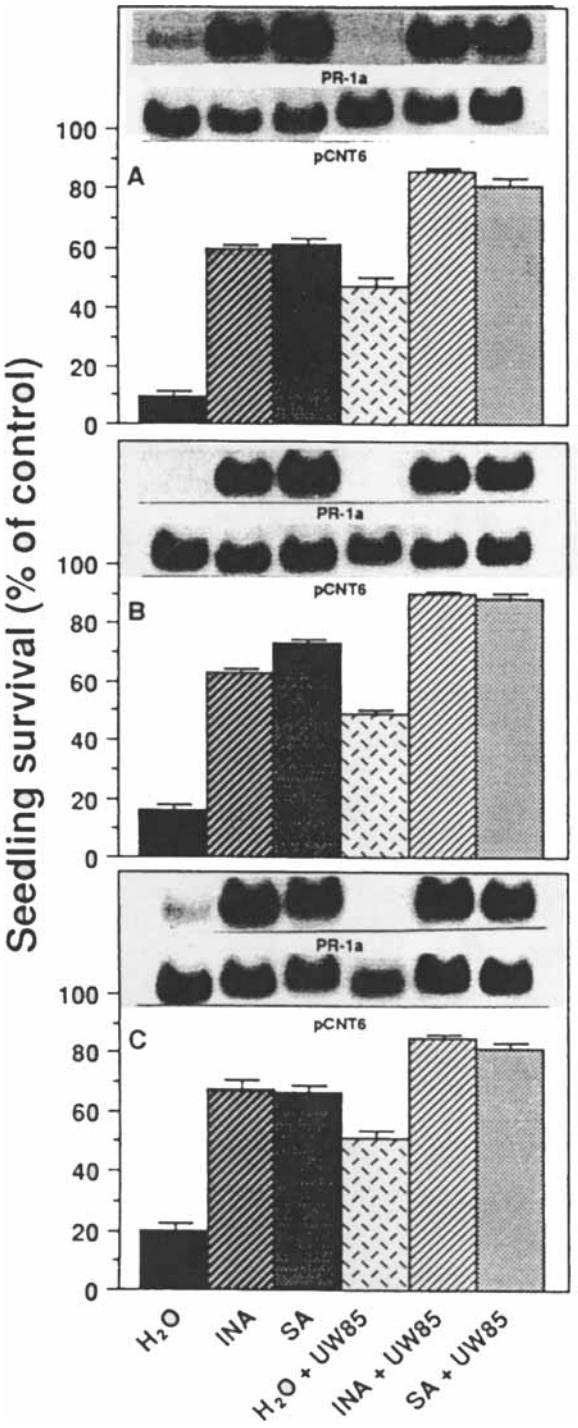

Fig. 5 Survival of tobacco seedlings ($n = 120$) treated as indicated and grown on water-agar plates for 10 days after inoculations with (A) 5×10^5 zoospores of *P. torulosum* (B) 500 zoospores of *P. aphanidermatum*, or (C) 2500 zoospores of *P. parasitica*. Seedlings were germinated, transplanted, and treated with water, SA or INA and UW85 (at 10^6 c.f.u./plate of 40 seedlings) as described in legends to Figs 1–3. Northern analysis was as described in the legend to Fig. 3. Bars represent standard error. LSDs ($P < 0.05$) were 6.9 (A), 4.3 (B), and 7.6 (C).

Discussion

We have characterized the interactions between SAR and UW85 in an experimental system allowing measurements of disease and biocontrol as well as SAR gene expression and UW85 population growth. The results indicate that both INA and SA induce tobacco SAR gene expression at the seedling stage, and that SAR provides significant suppression of disease caused by all three pathogens studied. *B. cereus* UW85 suppresses disease caused by the same pathogens. Induction of SAR does not alter the growth of UW85 on roots, and UW85 does not appear to induce SAR. SAR and UW85 act additively to suppress disease. Thus, at the level of this model system, there are no evident barriers to the use of SAR and UW85 together for disease suppression and the approaches appear to be complementary. This is a limited first step in the assessment of whether and how expression of SAR in plants may have ecological impacts on other beneficial microbes in addition to their desired effects toward pathogens. Further work in an experimental setting that more closely resembles the field situation will be necessary to examine whether concerted use of SAR and biocontrol will be effective in the field.

Seedling germination and emergence are critical in establishing plant populations. In agriculture, seedling damping-off diseases, many of which are caused by the oomycetes, are a significant factor and the justification for use of substantial amounts of pesticides. Laboratory and greenhouse studies have shown that SAR or the expression of SAR-related transgenes may offer promising alternatives or adjuncts to pesticide use for control of these pathogens (Alexander *et al.* 1993a,b; Liu *et al.* 1994). Likewise, microbial biocontrol with UW85 shows promise, in both laboratory (Handelsman *et al.* 1990, 1991) and field (Osburn *et al.* 1995) studies, for control of seedling damping-off and root rot diseases caused by the oomycetes. In both cases, however, the results thus far reported suggest that, as is sometimes the case with pesticides, control is variable. Work in our laboratories and others is directed toward understanding the reasons underlying this variability. Based on the results we have reported here, concerted use of SAR and biocontrol is one avenue for further investigation toward understanding and possibly controlling variability.

There are aspects of the interaction among seedlings, pathogens, and the microbial biological control agent that call for further study. The *P. parasitica* and *P. torulosum* isolates used in this study were found to induce low levels of PR-1a in this system. We do not yet know if these oomycetes induce SAR, but induction of PR-1a is consistent with previous evidence in tobacco that PR proteins are induced by *P. parasitica* (Bonnet *et al.* 1986) and that resistance (Kamoun *et al.* 1993) and SAR gene expression are induced by the *Phytophthora* elicitin parasiticein (S.

Kamoun and B. M. Tyler, personal communication; M. Moraes, personal communication). Previous work showed PR-protein accumulation in spruce roots induced by *Pythium* (Sharma *et al.* 1993), and our results extend this to tobacco and to the messenger RNA level.

At the doses tested (up to 10^8c.f.u./mL) and under these experimental conditions, UW85 did not induce PR-1a gene expression (Fig. 4a and other data not shown). That PR-1a gene expression was not induced by UW85 treatment suggests that SAR may not be involved in the mechanisms of biocontrol by UW85. We will test this hypothesis further by analysing the expression of other SAR-related genes following UW85 treatment. We also plan to assess whether UW85 treatment induces other gene expression in treated plants. Preliminary results indicate that UW85 treatment in this system may induce expression of certain genes associated with SAR (data not shown). These experiments are ongoing, as are attempts to determine whether resistance or tolerance to pathogens, separate from the direct effect of UW85 or its antibiotics on pathogens, can be attributed to such gene expression. Other rhizosphere bacteria, notably certain isolates of *Pseudomonas* spp., the plant growth-promoting rhizobacteria (PGPR), are reported to induce an SAR-like response (Alstrom 1991; van Peer *et al.* 1991; Wei *et al.* 1991; Zhou & Paulitz 1994). Little is yet known from experiments with PGPR about the nature of the genes induced, if any, or their relationship to mechanisms of resistance.

A full accounting of the ecology underlying microbial biological control will likely reveal a complex picture. Interactions among the host plant, the biocontrol agent, and the pathogen seem to be significant in the biocontrol activity of UW85. We have previously found that UW85 cultures, culture supernatants, and a UW85-produced antibiotic, zwittermicin A (He *et al.* 1994), delay germination of *Pythium* spp. zoospore cysts and the growth rate of germ tubes on roots (H. Shang *et al.*, in preparation). Other effects of UW85, culture supernatants, and UW85-produced antibiotics previously reported include reduced germ tube elongation in culture (Silo-Suh *et al.* 1994) and the lysis (Gilbert *et al.* 1990) of *Phytophthora* spp. zoospores. Moreover, we previously showed that under some conditions UW85 alters microbial communities associated with plant roots (Gilbert *et al.* 1993). The potential for SAR to influence microbial communities and the possibility that changes in microbial community composition and dynamics mediated by UW85 and SAR may contribute to disease suppression are the focus of continuing work on the molecular ecology of these interactions.

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