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BIORATIONALS FROM *Nicotiana* PROTECT CUCUMBERS AGAINST *Colletotrichum lagenarium* (Pass.) Ell. & Halst DISEASE DEVELOPMENT

B.S. KENNEDY,[1] M.T. NIELSEN,[1,*] and R.F. SEVERSON[2]

[1]*Department of Agronomy*
University of Kentucky
Lexington, Kentucky 40546

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Abstract—Trichome exudate compounds isolated from leaves of *Nicotiana tabacum, N. glutinosa* (accessions 24 and 24a), and 21 other *Nicotiana* species were evaluated for biorational activity against *C. lagenarium*, the anthracnose pathogen of cucumber. Exudate compounds tested were the cembrane diterpenes α- and β-4,8,13-duvatriene-1,3-diols (DVT diols); the labdane diterpenes (13-*E*)-labda-13-ene-8α,15-diol (labdenediol) and (13-*R*)-labda-14-ene-8α,13-diol (sclareol); extracts from *N. gossei*; and various sugar ester isolates. In dose–response experiments test compounds were applied to a water agar surface that was then inoculated with a conidial suspension. Low levels of most test compounds reduced or completely inhibited germination of *C. lagenarium* conidia. IC_{50} values, concentrations (micrograms per square centimeter) at which conidium germination was reduced 50%, were 6.3 for DVT diols, 19.3 for sclareol, 1.0 for labdenediol, 2.8 for a mixture of sclareol and labdenediol, 1.2 for an *N. gossei* sucrose ester and 4.1 for the *N. gossei* crude extract. Higher levels of DVT diols and the sclareol–labdenediol mixture were required to reduce lesion size and number on inoculated cucumber leaves. At the highest concentration tested, 100 $\mu g/cm^2$, the DVT diols and sclareol–labdenediol mixtures protected cucumbers against lesion development by 93 and 98%, respectively. Sugar ester mixtures from 20 *Nicotiana* species and three different sugar ester fractions from *N. bideglovii* had in vitro antifungal activity at a concentration of 48 $\mu g/cm^2$. Sugar esters from nine of the species including *N. acuminata, N. attenuata, N. clevelandii, N. maritima, N. miersii, N. noctiflora, N. occidentalis, N. rustica*, and fractions 10, 12, and 13 from *N. bideglovii* completely inhibited *C. lagenarium* conidium from germinating. Sugar ester mixtures from only four species, *N. plumbaginifolia, N. bonar-*

*To whom correspondence should be addressed.
[2]Deceased, formerly ARS-USDA, Richard B. Russell Agricultural Research Center, Athens, Georgia 30613.

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iensis, *N. simulans*, and *N. palmerii*, had no significant effect on *C. lagenarium* conidium germination. These results suggest the potential of *Nicotiana* exudates as biorationals in reducing disease development.

Key Words—*Nicotiana*, *Colletotrichum lagenarium*, biorationals, trichome exudates, leaf surface chemistry, diterpenes, sugar esters.

INTRODUCTION

Secondary plant products derived from glandular trichome exudates have been implicated in host-plant resistance in certain genera of the Solanaceaous family including *Lycopersicon* and *Solanum* (Gentile and Stoner, 1968; Gibson, 1971). Trichome exudate compounds or crude leaf surface extracts from different *Nicotiana* species have also been shown to have activity against certain phytophagous insects and plant pathogens (Severson et al., 1985, 1991). Earlier, we reported that sporangium germination of *Peronospora tabacina* Adam, the blue mold pathogen of tobacco, was greatly inhibited following exposure to diterpenes and sucrose esters derived from the leaf surface of several *Nicotiana* species (Kennedy et al., 1992). The importance of these compounds as biorationals, natural products with pesticide activity, could increase greatly if they are found to be active against disease and insect pests of other plant species. Recently, foliar applications of sucrose esters, derived from the leaf surface of *N. gossei*, on field grown cotton showed similar or greater control of certain whitefly species than approved pesticides (R.F. Severson, unpublished observations, 1993). The objective of our research was to determine if compounds derived from *Nicotiana* have biorational activity against a fungal pathogen of an unrelated plant species. The following trichome exudate compounds were evaluated for activity against the cucumber pathogen *Colletotrichum lagenarium* (Pass) Ell & Halst.: a mixture of the cembrane diterpenes α- and β-duvatrienediols; two labdane diterpenes, sclareol and labdenediol; extracts from *N. gossei*; and sugar ester mixtures from 20 different *Nicotiana* species.

METHODS AND MATERIALS

Isolation and Purification of Leaf Surface Compounds. Cuticular components were extracted from *N. tabacum* cultivars NC 2326 and NFT, *N. glutinosa* (accessions 24 and 24a), and 24 other species grown in field plots at the Georgia Coastal Plain Experiment Station, Tifton, Georgia, and the Crops Research Laboratory, Oxford, North Carolina. Six to eight weeks after transplanting, tops cut from plants were extracted with methylene chloride (Baker Resianalyzed reagent grade) as previously described (Jackson et al., 1986; Severson et al., 1988). The α- and β-4,8,13,-duvatriene-1,3-diols (from NC 2326) were isolated

in 98 +% purity as described by Severson et al. (1988). *Nicotiana glutinosa* was the source of labdenediol, (13-*E*)-labda-13-ene-8α,15-diol and the sclareols, a mixture of (13-*R*)- and (13-*S*)-labda-14-ene-8α,13-diol (sclareol and episclareol). Labdenediol and sclareols were obtained in 98+% purity as described by Jackson et al. (1986).

Cuticular extracts from the *Nicotiana* species in Table 1 were taken to dryness on a rotoevaporator, and the residue was partitioned between hexane and 80% methanol–water (Severson et al., 1988). The methanol–water-soluble fraction (100–200 μg) containing the sugar esters was dissolved in 0.5 ml of 90% methanol–water, loaded into an injection loop, and chromatographed on a Bio-Sil C-18 (HL Silica, fully encapped; 40–63 μm; Bio-Rad Laboratories) preparative reverse-phase column (1.25 cm ID × 15 cm). The column was eluted with 225 ml 60% methanol–water, 90 ml 90% methanol–water, and 125 ml 100% methanol, and 5-ml fractions were collected. The solvent was removed on a Savant Speed Vac, and fractions with observable residue were derivatized and analyzed by capillary gas chromatography (Severson et al., 1984; Jackson et al., 1986). Fractions containing glucose and/or sucrose esters were combined to yield the sugar ester tested. The preparative chromatography cleanly separated the glucose and sucrose ester fractions in the *N. attenuata* sample, and they were combined separately.

Inoculum Preparation. Colletotrichum lagenarium (Pass) Ell. & Halst. race 1 was obtained from J. Kuć, Department of Plant Pathology, University of Kentucky. It was maintained on green bean agar at ±20–24°C in the dark (Goode, 1958). Conidial suspensions were prepared in sterile distilled H_2O from 7-day-old cultures and conidium concentrations were determined with a hemacytometer.

In vitro Bioassay. Efficacy of the test compounds and extracts was determined by assaying germination of *C. lagenarium* conidia on water agar covered uniformly with the test compounds. Sugar ester mixtures from 20 different *Nicotiana* species were tested at a concentration of 48 $\mu g/cm^2$. In separate experiments, DVTs, sclareol, labdenediol, a mixture of sclareol and labdenediol, a sucrose ester mixture from *N. gossei*, and a crude exudate mixture from *N. gossei* were tested at six concentrations ranging from 0.75 to 48 $\mu g/cm^2$. Desired concentrations were prepared by diluting portions of the stock solution with 100% acetone. Treatments were applied in 50-μl aliquots to the surface of 1.5% water agar in 35- × 10-mm Petri plates. A bent Pasteur glass pipet was used to evenly distribute the compound over the agar surface. Plates were set aside for the carrier solvent to evaporate before a 200-μl conidial suspension of *C. lagenarium* (1×10^5 sp/ml) was added to each plate. Plates were incubated overnight at 24°C in the dark. All treatments were replicated four times and contained two control treatments: (1) no compound or acetone was applied prior to the conidial suspension, and (2) application of 100% acetone, the carrier

solvent. Conidium germination was determined microscopically, and activity of each test compound was expressed as percent inhibition: [(% germination of control − % germination for each treatment)/% germination of control] × 100. Where appropriate, the log dose (concentration)–response relationship was linearized using probits, and inhibitory concentrations (IC_{50}) were estimated (SAS Institute Inc., 1985).

Plant Assay. Seeds of the anthracnose-susceptible cucumber cultivar SMR-58 were sown in 3 1/2-in. plastic pots containing a commercial medium. They were watered daily over the top until emergence of the first cotyledon and then were watered from the bottom of the pot for the duration of each experiment. A water soluble fertilizer, Peters 20:10:20 (N/P/K) was applied weekly. Plants were maintained in a greenhouse at 24–30°C under high-pressure sodium lights for a 14-hr photoperiod. Exudate compounds, α- + β-DVT-diols and mixture of sclareol and labdenediol, were evaluated in the greenhouse for controlling anthracnose disease development on the leaf surface of cucumbers. The DVT diols and the sclareol–labdenediol mixture were assayed in separate experiments. The first true leaves of 13-day-old cucumber plants were uniformly sprayed with different concentrations of the test compounds contained in 35% acetone (v/v) using an airbrush sprayer (Paashe Airbrush Co., Harwood Heights, Illinois) at 50 psi. Plants were returned to the greenhouse bench to dry overnight in absence of artificial light. Approximately 18 hr later, treated plants were inoculated following a procedure adapted by Kuć et al. (1975). Each treated leaf was inoculated with 30 10-μl drops of a conidial suspension of *C. lagenarium* (1×10^5 sp/ml). Thirty 10-μl drops of sterile water were placed on control plants. Plants were incubated in closed moist chambers for 24 hr in the greenhouse and then partially opened within the next 12 hr to establish air temperature equilibrium. Plants were returned to the greenhouse bench under high-pressure sodium lights and rated for symptom development one week after inoculation. Lesion number and necrotic area were determined for each leaf treatment. Treatments were replicated four times in each experiment and both experiments were done twice. Percent protection was calculated in a similar manner for each *in vivo* experiment: % protection = $[(C - T)/C] \times 100$ where C is the percent necrotic area for the acetone control treatments and T is the percent necrotic area for the various treatments.

RESULTS

Conidia germination rates for the control treatments were between 80 and 100% in all *in vitro* experiments (Table 1 and Figure 1). Acetone applied to the agar had no significant effect on conidium germination compared to the untreated agar plates. The acetone apparently evaporated completely and had no residual

TABLE 1. EFFECT OF SUCROSE AND/OR GLUCOSE ESTERS ISOLATED FROM *Nicotiana* SPECIES ON GERMINATION OF *C. lagenarium* CONIDIA IN AN *In Vitro* EXPERIMENT[a]

Treatment (48 $\mu g/cm^2$)	Inhibition (%)
N. plumbaginifolia (43a)	1.1
N. bonariensis (11a)	9.4
N. simulans (66)	13.6
N. palmerii (39)	14.2
N. nudicaulis (36)	26.5[b]
N. debneyi (17)	36.9[b]
N. langsdorffii (28b)	61.8[b]
N. velutina (62)	77.5[b]
N. cavicola (68)	78.6[b]
N. pauciflora (41)	94.9[b]
N. hesperis (67)	98.4[b]
N. acuminata (2)	100.0[b]
N. attenuata (7a)	100.0[b]
N. bigelovii (10)	100.0[b]
N. bigelovii (12)	100.0[b]
N. bigelovii (13)	100.0[b]
N. clevelandii (14)	100.0[b]
N. martima (31)	100.0[b]
N. miersii (33)	100.0[b]
N. noctiflora (35)	100.0[b]
N. occidentalis (37b)	100.0[b]
N. rustica (48)	100.0[b]

[a]The germination rates for the conidia in the control treatments of water and acetone (the carrier solvent) were 94.8% and 93.5%, respectively.
[b]Denotes significant differences ($P < 0.05$) from the acetone control treatment according to a paired *t* test.

effect on the conidia. Thus, activities of the various chemicals were based on comparisons to the acetone control.

Sugar ester mixtures from 20 *Nicotiana* species and three different sugar ester fractions from *N. bideglovii* had *in vitro* antifungal activity at a concentration of 48 $\mu g/cm^2$. *C. lagenarium* conidia were completely inhibited from germinating when exposed to sugar esters from nine of the species, *N. acuminata*, *N. attenuata*, *N. clevelandii*, *N. maritima*, *N. miersii*, *N. noctiflora*, *N. occidentalis*, *N. rustica*, and fractions 10, 12, and 13 from *N. bideglovii*, when compared to the control treatments (Table 1). Conidia germination was reduced more than 25% when exposed to sugar esters derived from *N. nudicaulis*, *N. debneyi*, *N. langsdorffii*, *N. velutina*, *N. cavicola*, *N. pauciflora*, and *N. hesperis*. Sugar ester mixtures from only four species, *N. plumbaginifolia*, *N. bon-*

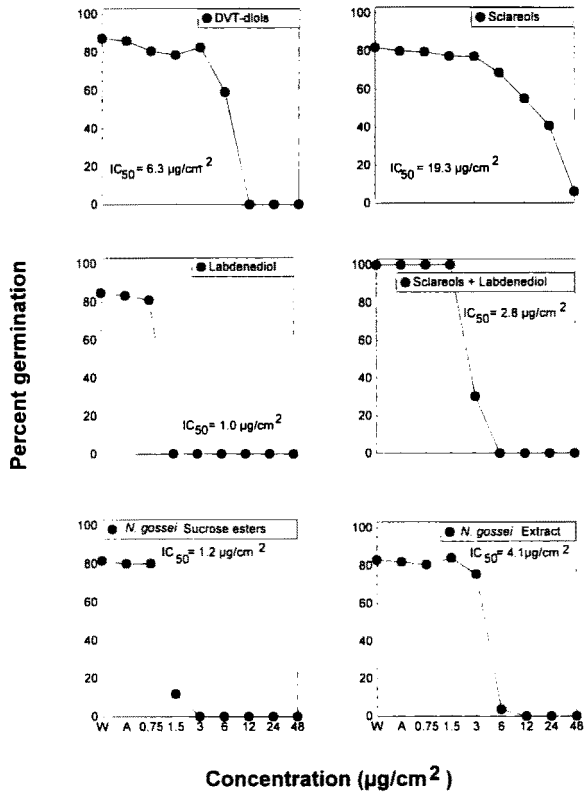

FIG. 1. Percent germination of *C. lagenarium* conidia in vitro after exposure to trichome exudates. Control treatments are represented by: W, no chemical or acetone; and A, acetone only.

ariensis, *N. simulans*, and *N. palmerii*, had no significant effect on *C. lagenarium* conidium germination.

Microscopic examination of conidia in all of the *in vitro* experiments revealed that for those conidia that did germinate, germ tube length and morphology was similar to that observed for conidia in the control treatments.

Inhibition of conidium germination was nearly complete when exposed to the highest concentrations of the six different test compounds for which dose responses were assessed (Figure 1). IC_{50} values, concentrations at which conidium germination was reduced 50%, were well under 5 $\mu g/cm^2$ for labdenediol, the sclareol–labdenediol mixture, *N. gossei* sucrose esters, and the *N. gossei* extract. IC_{50} values were 6.3 $\mu g/cm^2$ for the α- and β-DVT-diol mixture. The

most active compound tested was labdenediol with an IC_{50} value of 1 $\mu g/cm^2$ and the least active was sclareol with an IC_{50} value of 19.3 $\mu g/cm^2$. Figure 1 also reveals that for most of the tested compounds, germination inhibition was not linearly related to concentration. In fact, for labdenediol, DVTs, and the *N. gossei* compounds, a critical response to concentration was observed. This is best illustrated by the experiments with labdenediol in which conidium germination was little affected at 0.75 $\mu g/cm^2$ of labdenediol, but was greatly inhibited at the next higher chemical concentration of 1.5 $\mu g/cm^2$.

Mixtures of: (1) α- and β-DVT-diols and (2) sclareol and labdenediol were evaluated at different concentrations for control of anthracnose disease development on cucumber leaves. These two mixtures were chosen for the disease control experiments because both mixtures are abundant on *Nicotiana* leaf surfaces, DVT diols on most *N. tabacum* genotypes and on *N. glutinosa* accession 24a. Furthermore, abundant quantities of these mixtures were available for testing. Because cucumber leaves are very sensitive to chemical applications, we were initially concerned about using acetone as a carrier solvent for our exudates. However, when the concentration of acetone was reduced to 35%, no phytotoxicity was observed among the sprayed leaves in any of the treatments including the controls.

Preliminary experiments indicated that low concentrations of both mixtures were less effective in controlling anthracnose than they were in preventing *C. lagenarium* conidium germination in vitro. Therefore, higher concentration ranges were chosen for the disease control experiments.

There was significantly less disease as measured by lesion number and total necrotic area at the lowest concentration (40 $\mu g/cm^2$) of DVT diols. At a treatment concentration of 15 $\mu g/cm^2$, the sclareol–labdenediol mixture had significantly lower disease scores than the acetone control. At a concentration of 100 $\mu g/cm^2$, DVT diols and the sclareol–labdenediol mixture protected cucumbers against anthracnose disease development by 93 and 98%, respectively (Tables 2 and 3).

DISCUSSION

Controlling plant diseases in agricultural crops has traditionally been accomplished by using multidisciplinary approaches consisting of host-plant resistance, use of appropriate cultural practices including crop rotation and good sanitation, and the application of pesticides. However, some producer's options for disease control are becoming increasingly limited. This is due primarily to the lack of land available for crop rotation and the growing concern over the use of synthetic chemicals to control plant disease pests. The use of natural plant products, acting as applied pesticides or as factors in host-plant resistance,

TABLE 2. PERCENT PROTECTION OF SMR58 CUCUMBER LEAVES AGAINST DISEASE DEVELOPMENT OF *C. lagenarium* BY α- + β-DVT DIOL FOLIAR SPRAYS IN GREENHOUSE EXPERIMENTS.

Spray treatments	Average No. of lesions per leaf[a]	Average lesion size (mm^2)	Total necrotic area (mm^2)[b]	Protection (%)[c]
Untreated	29.9 a[d]	9.3	277.5 a	
35% acetone	29.3 a	10.5	308.9 a	
40 $\mu g/cm^2$ DVT diols	18.1 b	5.7	107.3 b	65
50	15.1 bc	6.0	78.6 bc	75
60	13.0 bcd	8.6	109.1 b	65
70	9.8 cde	5.4	52.7 bc	83
80	8.6 de	4.9	36.8 bc	88
90	6.5 e	4.5	32.4 bc	90
100	5.0 e	5.5	22.5 c	93
Untreated/water	0.0 e		0.0 c	
35% acetone/water	0.0 e		0.0 c	

[a] Average number of lesions resulting from 30 inoculations per leaf.
[b] Average total necrotic area from each leaf.
[c] Percent protection was calculated for each treatment by using the following formula: % protection = [(35% acetone X TNA–treatment X TNA)/35% acetone X TNA] × 100, where TNA is the total necrotic area.
[d] Means followed by the same letter are not significantly different at $P < 0.05$ according to the least significant difference.

is becoming increasingly desirable. One approach is to identify biorationals—naturally occurring products that have pesticide activity. Our hypothesis was that plant compounds that have already been classified as determinants of resistance in one host plant–pathogen relationship could possibly serve as biorationals against pathogens of other plant species.

Earlier investigations by our group and others (Kennedy et al., 1992, Menetrez et al., 1990) reported that trichome exudates produced on the leaf surface of some *N. tabacum* and *Nicotiana* species significantly reduced germination of *Peronospora tabacina* Adam, the obligate fungal pathogen responsible for the blue mold disease in tobacco. In our present study, we determined that many of these same compounds could be classified as biorationals effective against disease development of anthracnose in cucumbers.

The compounds were selected for our biological assays primarily because of their relative abundance on the leaf surface and their ease in isolation. The duvatriene diols (DVT) were relatively effective in inhibiting germination of *C. lagenarium* conidia in vitro and somewhat effective at higher concentrations in preventing disease development on the inoculated cucumber leaves. These compounds are of particular interest because they are present at high concentrations in the trichome exudate of many *Nicotiana* genotypes. Indeed, we estimate that

TABLE 3. EFFECT OF SCLAREOL AND LABDENEDIOL FOLIAR SPRAYS ON DISEASE DEVELOPMENT OF SMR58 CUCUMBERS BY *C. lagenarium* IN GREENHOUSE EXPERIMENTS

Spray treatments	Average no. of lesions per leaf[a]	Average lesion size (mm^2)	Total necrotic area (mm^2)[b]	Protection (%)[c]
Untreated	27.3 a[d]	5.8	159.5 ab	
35% acetone	29.5 a	6.4	192.8 a	
5 $\mu g/cm^2$ DVT diols	26.8 a	5.1	139.3 ab	23
10	26.0 a	5.5	149.3 ab	28
15	21.3 b	5.3	126.0 b	35
20	13.8 c	3.8	54.8 c	72
40	3.9 d	2.8	12.1 c	94
80	2.5 d	2.4	6.1 c	97
100	1.1 d	3.5	3.1 c	98
Untreated/water	0.0 d		0.0 c	
35% acetone/water	0.0 d		0.0 c	

[a] Average number of lesions resulting from 30 inoculations per leaf.
[b] Average total necrotic area from each leaf.
[c] Percent protection was calculated for each treatment by using the following formula: % protection = [(35% acetone X TNA—treatment X TNA)/35% acetone X TNA] × 100, where the TNA is the total necrotic area.
[d] Means followed by the same letter are not significantly different from $P < 0.05$ according to the least significant difference.

in certain genotypes, the DVTs could represent as much as 10% of the total leaf dry weight.

Sclareol and labdenediol are labdene diterpenes found primarily on the leaf surface of the *Nicotiana* species *glutinosa*. In *in vitro* experiments, we found sclareol to be the least active (IC_{50} value 19.3 $\mu g/cm^2$) and labdenediol to be the most active compound (IC_{50} value 1.0 $\mu g/cm^2$). The two compounds were mixed and concentrated as they naturally occur on the leaf surface in a 65:35 (sclareol–labdenediol) ratio. *C. lagenarium* conidium germination was completely inhibited when exposed to 6 $\mu g/cm^2$ of the mixture in vitro. Foliar applications of this mixture at 40 $\mu g/cm^2$ or greater protected the leaves of cucumbers from disease development by over 90% when compared to the control treatments.

Conidium germination was completely inhibited when exposed to concentrations of 3 $\mu g/cm^2$ and 12 $\mu g/cm^2$ of the *N. gossei* sucrose ester and extract, respectively. These compounds were included in our screen because they have been implicated as biorationals for control of some insects including whiteflies.

Only four of the sugar ester mixtures from the 20 different *Nicotiana* species did not significantly effect germination of *C. lagenarium* conidia. Sugar esters from nine of the species inhibited conidium germination completely. These sugar

esters vary with the type of acid substitution on the glucose or sucrose moieties (Severson et al., 1991) and are considered to be a very diverse class of compounds, having high potential for genetic manipulation. We screened a large number of sugar esters for biorational activity in hopes of correlating this activity with a particular acid substitution. Although we were unable to establish any relationship between biorational activity and any particular type of sugar ester, the positive results from the assays warrant further investigation.

Results of this study further demonstrate the potential of *Nicotiana* exudates as biorationals in reducing disease development. The effectiveness of these exudates in preventing or retarding disease progress under natural disease pressure needs to be investigated. Additional research is also necessary to estimate the overall value of these compounds as antifungal agents at the whole-plant level, including efforts to characterize these exudates for their biorational activity against a wide range of different plant pathogens and to determine appropriate application methodology. *Nicotiana* exudates are quantitatively and qualitatively different among genotypes and can be manipulated through breeding by the type and density of the trichome and by selection for specific compounds (Nielsen and Severson, 1990). New research should be initiated so that biorationals can efficiently and effectively be utilized as sources for developing additional alternatives to pest control that broaden opportunities for reducing fungicide use.

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