Control of *Penicillium digitatum* on Citrus Fruit Using Two Plant Extracts and Study of Their Mode of Action

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Extracts from two plants from Ethiopia, *Withania somnifera* and *Acacia seyal*, were evaluated for their potential as natural biopesticides and to study their mode of action. Methanolic extracts of these plants were tested *in vivo* on citrus fruit for their efficacy to control *Penicillium digitatum* when applied on wounded or unwounded fruit surfaces. Relative to the control, 70% and 75% of wound – inoculated fruit did not develop decay symptoms for up to 21 days of storage at 25°C and >85% r.h. An increase in cell wall-bound phenolics was evident in wounded fruit treated with plant extracts and inoculated with a spore suspension of *P. digitatum*. Scanning electron microscopy revealed deposition of crystalline plant material sticking to the pathogen and around the wound site. The application of the plant extracts increased the epiphytic background total microbial population but decreased diversity.
KEY WORDS: Citrus fruit; postharvest diseases; natural compounds; plant phenolics; host resistance.

INTRODUCTION

Pre- and postharvest pathogens greatly affect the quality of citrus fruit (13). Decay caused by *Penicillium digitatum* Sacc. is an aspect of the main postharvest disease initiated through injuries before or during harvesting, packing and processing (13). The importance and impact of wound pathogens may differ from country to country. In countries where protection and proper handling of fresh fruit is inadequate, losses during transit and storage may be as high as 50% of the harvested crop (29). Chemical control with imazalil, guazatine and thiabendazole is an option used to reduce postharvest diseases (25). The commercial use of postharvest fungicides has become restricted because of public health concerns (30), development of pathogen resistance (11), and environmental factors (16). These issues led us to the search for natural control options using plant extracts and/or microbial antagonists.

The potential of plant extracts to control plant diseases has long been recognized (1). There are approximately 250,000 species of higher plants, of which only 5%–15% have been studied for their therapeutic value (28). The use of plants for human disease control attracts more attention, compared with its use in plant and animal disease control (6,22). In crop protection studies, various natural plant products have been identified and employed to control postharvest diseases of fruit and vegetables.

The use of volatile compounds – *Hinokitiol* (*β-thujaplicin*) from the roots of *Hiba arboruitae* (Japanese cypress) against *Botrytis cinerea* Pers. ex Fr. and *Alternaria alternata*

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(Fr.) Keissler on eggplant and pepper fruit (14); strawberry volatiles against postharvest fungal pathogens (21); citral against *P. digitatum*, *P. italicum* Wehmer and *Geotrichum candidum* Link ex Pers. (19); and garlic against citrus green and blue filamentous fungi (23) – are indications of the potential applications of plant extracts for plant disease control.

The activity of natural plant products on the host tissue may involve direct interaction with the pathogen or induction of host resistance; the mechanism involved in the former, however, is the less understood one (25). Host resistance induction, on the other hand, may involve several complex mechanisms including hypersensitive responses, buildup of cell wall barriers, increased production of phytoalexins, accumulation of pathogenesis-related (PR) proteins, and fungal cell wall hydrolases (14,25).

In the present work the antifungal activity of two plant extracts, from *Withania somnifera* (code H_2) and *Acacia seyal* (code I_1), were studied for protective application against *P. digitatum* decay on citrus. These plants were selected for their wide use by traditional healers to control human ailments and their high *in vitro* antimicrobial activity screened against human and plant pathogens. Information about these plants for plant disease control in general and postharvest use in particular is lacking. Limitations in the natural distribution of plants and/or lack of a track record to employ these plants for postharvest disease control may hinder their use.

The aim of this study was to evaluate the efficacy of the two selected plant extracts by wound and spray treatment applications and to investigate the mode of action involved in the healing mechanisms of the fruit wound against green mold by scanning electron microscopy. The non-target effect of the plant extracts on the microflora of citrus fruit surfaces and wounds was also evaluated.

MATERIALS AND METHODS

Fruit Untreated freshly harvested orange fruits, cv. 'Valencia', were collected from the Rustenburg citrus packinghouse, Northwest Province, South Africa. Fruits were surface sterilized with sodium hypochlorite (1% chlorine) for 2 min and air-dried before use.

The pathogen The isolate *P. digitatum* used in the experiments was obtained from the culture collection of Plant Pathology Laboratories, University of Pretoria, South Africa, and pathogenicity was confirmed. The pathogen was grown on potato dextrose agar (PDA, Biolab, Johannesburg) at 25°C. A conidial suspension (10^5 ml^{-l}) was prepared as described by Janisiewicz *et al.* (17). Ten to 20 ml of sterile distilled water was added to the surface of a 14-day-old culture, surface-rubbed with a glass rod, and the collected spore concentration determined with a hemacytometer.

Plant material Two species of plants, *Withania somnifera* L. Dunal and *Acacia seyal* Del. var. 'Seyal', were collected from Awash Valley and Hursso, respectively, east of Addis Ababa, Ethiopia. They were selected based on their broad-spectrum activity and efficacy against postharvest pathogens (data not shown). Fresh leaf samples, collected in brown paper bags, were transported to the Plant Pathology Laboratory (Dept. of Plant Science, Alemaya University, Ethiopia), placed in the shade until dry, reduced to powder in a blender (Russell Hobbs, Germany) and stored in plastic bags at ambient temperature until needed. Samples were transferred to the Plant Pathology Laboratories of the University of Pretoria (South Africa) for biocontrol and phytochemical studies. Strict quarantine handling, processing and plant destruction protocols were followed during and after processing of

samples according to the standard operational procedures of the laboratories.

Plant extraction A methanol/acetone/water (7:7:1, v:v) solvent was used as the extraction system (28). Three successive extractions were conducted from the dried plant powder (1:20, w/v). The first and second extraction suspensions were mixed with a vortex (VM-300) and placed on a rotary shaker for 1 h at 170 rpm. Samples were centrifuged at 4°C in a micro-centrifuge (Denver Instrument Co., Arvada, CO, USA) at 7200 *g* for 10 min. The third extraction was placed overnight on a rotary shaker and centrifuged as described above. The combined supernatants were concentrated to 1 ml under vacuum and freeze-dried for 48 h. Tubes were refilled uniformly with sterile distilled water to a volume of 10 ml and the suspension was re-sterilized using a hypodermic syringe-driven filter paper (0.22 μm pore size). Samples were either used immediately or kept in the refrigerator at $4^\circ \pm 1^\circ$C until further use.

***In vivo* antifungal assay** Antifungal activities of plant extracts were tested *in vivo* using the method described by Poppe *et al.* (24), with some modifications. Wounded (3×3 mm) and unwounded fruits were used throughout the experiment. Plant extracts were applied to wounded and unwounded fruit 12 h prior to the challenge inoculation with the pathogen. Ten percent of the original concentration of the plant extracts was used indiscriminately in all trials. The pathogen concentration was standardized at 10^5 conidia ml^{-1}. The fruit wound (FW) experiment included the following treatments: FW only; FW followed by application of 30 μl *P. digitatum* (10^5 spore ml^{-1}); FW followed by methanolic extract of *W. somnifera* (30 μl); FW followed by methanolic extract of *A. seyal* (30 μl); FW followed by methanolic extract of *W. somnifera* challenged with *P. digitatum;* and FW followed by methanolic extract of *A. seyal* challenged with *P. digitatum*. Wounding followed by the application of commercial chemicals [alkyl-dimethyl benzyl ammonium chloride (UTopharm Co. Ltd., Shanghai, China) 800 mg l^{-1}; and thiabendazole (Tecto 90, Johannesburg, So. Afr.) 1000 mg l^{-1}] challenged with *P. digitatum* was included for comparison purposes.

For spray experiments the following treatments were included: fruit surface spraying with *P. digitatum* (10^5 spore ml^{-1}); surface spraying with methanolic extract of *W. somnifera;* surface spraying with methanolic extract of *A. seyal*; surface spraying with methanolic extract of *W. somnifera* followed by drying and spraying with *P. digitatum* (10^5 spore ml^{-1}) 12 h after application of the extract; and surface spraying with methanolic extract of *A. seyal* and challenged with *P. digitatum* 12 h after application of the extract. Spray application of commercial chemicals [alkyl-dimethyl benzyl ammonium chloride (UTopharm) 800 mg l^{-1}; and thiabendazole (Tecto 90) 1000 mg l^{-1}] followed by the application of *P. digitatum* was included for comparison purposes.

For each wound or spray treatment, 20 fruits were used; the experiment was done in triplicate and performed twice. Treated fruits were packed in boxes and incubated at 25°C and >85% r.h. for 21 days. Evaluation was done every 2 days and data were recorded as number of lesions developing. Efficacy of treatment application was determined according to Vero *et al.* (31).

Non-target effect on microbial population of fruit The non-target effect of the plant extracts on the natural fruit microflora was evaluated by determining the total microbial count and the population of bacteria, yeasts and mycelial fungi in particular. The natural microflora population was determined on freshly harvested orange fruit and on fruit spray-

treated with extracts and stored for 21 days at 25°C and >85 r.h. Three fruits were randomly picked for evaluation. Each fruit was placed in 500 ml Ringer's solution (Merck, South Africa) and sonicated for 30 sec. The wash water was filter sterilized with a membrane (0.45 μm pore size) under vacuum. A filter membrane was placed in 10 ml Ringer's solution and serially diluted. One hundred μl of each diluted sample was spread-plated on three different media [PDA (Biolab), standard-1 nutrient agar (STD-1NA, Biolab) and malt extract agar (MEA, Biolab), each of which was amended with 2 mg l^{-1} each of rifampicin and cyclohexamide to discriminate growth of bacteria and fungi, respectively]. Dilution plates were prepared in triplicate and plates were incubated at 25°C for 2 weeks. Total colony counts (cfu ml^{-1}) were computed using the following formula and log-transformed for analysis (31).

$$N = \sum C$$

$$(n_1 + 0.1 * n_2)d$$

where $\sum$C is the sum of colonies counted on all plates retained
n_1 is the number of plates retained in the first dilution
n_2 is the number of plates retained in the second dilution
d is the dilution factor corresponding to the first dilution

Induced resistance study

Orange peel powder preparation Two fruit samples were randomly picked from each treatment before and after treatments and used for orange peel preparation. Forty-six samples were used from the treatment side (ts) and the untreated controlled side (cs) of a fruit. Orange peel samples taken from the ts and 10 cm distant from the cs were regarded as a control. Samples were freeze-dried for 48 h, reduced to powder, sieved with a strainer (0.05 μm pore size) and kept in white sterilized Scott bottles for subsequent use.

Extraction of soluble phenolic compounds Two successive citrus peel soluble phenolic tests were conducted before and after treatment application, using dichloromethane and petroleum ether as extraction solvents according to the method described by Kim *et al.* (18), with slight modifications. One ml of dichloromethane was poured into an Eppendorf tube containing 0.05 g of orange peel collected from the previously described treatments. The sample was mixed with a vortex for 1 min and centrifuged in a Centronix 1236 (National Labnet Co., Woodbridge, NJ, USA) for 10 min at 5000 *g*. The supernatant was transferred to a fresh Eppendorf tube and the extraction was repeated once. One milliliter of petroleum ether was added to the remaining peel residue, mixed and centrifuged as described above. The extraction procedure was repeated once. The supernatant was dried under vacuum and 500 μl methanol was added to stock the final volume. The residual extract was either stored at 4°C or used immediately for subsequent extraction of cell-bound phenolics.

Extraction of wall-bound phenolic compounds Residual peel powders obtained from extraction of soluble phenolic compounds were used for extraction of non-soluble phenolic compounds using Pasteur pipettes. The pipette was modified into a blowing apparatus by gentle flame-heating of the tip while simultaneously blowing air into it. The tip was sealed and cooled in air. One ml of 0.05 N NaOH was transferred into a blowing Pasteur pipette and mixed with 0.01 g of peel powder; the pipette was sealed before transfer into a water bath (95°C) for 1 h. Pipettes were removed from the water bath and kept on ice for

10 min before the tips were opened and the contents transferred into an Eppendorf tube. Sixty ml of concentrated HCL (10 M) was added to reduce the pH to ∼5. Samples were centrifuged in a microcentrifuge at 5000 *g* (Denver Instrument Co., Denver, CO, USA) for 2 min and the supernatant was transferred into a new Eppendorf tube. One ml of diethyl ether was added to the remaining residue, vortexed and centrifuged for 2 min. The supernatant was transferred into the tubes containing the concentrated suspension and extraction with diethyl ether was repeated four times. The combined supernatants were reduced to dryness under vacuum and 250 ml methanol was added to stock the final volume for subsequent use.

Quantification of orange peel total phenolics The concentration of total soluble and/or wall-bound phenolics was determined using the Folin-Ciocalteu reagent as described by Bray and Thorpe (5).

SEM study Surface attachment and colonization of the pathogen were determined according to Chan and Tian (7). Treatment combinations included in this experiment were: fruit wound only; wounding followed by *P. digitatum* only; wounding followed by *A. seyal* extract and *P. digitatum;* and wounding followed by *W. somnifera* and *P. digitatum*. Control experiments included plant extracts applied to the fruit wound without the pathogen and the pathogen on its own. For each treatment, six fruits were used and four fruits were used at random for SEM preparation. Wound lesions were cut transversely into four slices (4× mm) 0, 12, 24 and 48 h after treatment application on fruit wounds. The cut peel tissue was fixed by immersion in 2.5% glutaraldehyde in 0.075 M phosphate buffer at pH 7.0 for 24 h at room temperature. Samples were rinsed for 1 h (four or five changes) with 0.075 M sodium phosphate buffer (pH 7.2) and dehydrated in a series of ethanol concentrations before critical point drying. Dried tissues were mounted on aluminum stubs, coated with gold-palladium, and observed at 6 kv with a scanning electron microscope (Joel JSM 840, Tokyo, Japan).

Statistical analyses Data were analyzed using the SAS computer program (version 8.1, 2002). After ANOVA had been applied, differences between means were tested using least significant differences and treatment means were compared with Fisher's protected LSD test ($P<0.05$) and t-grouping. Typically, if percentage values exceed a range of 40%, homogeneity of variance tests, such as Leven's, fail and an arcsin transform is needed before ANOVA can be applied. To determine the microflora population on treated and untreated fruit surfaces, the cfu ml^{-1} of fruit wash data were transformed to logarithms to improve the homogeneity of variances.

RESULTS

***In vivo* antifungal activity of plant extracts** Wound application of extracts H_2 (*W. somnifera*) and I_1 (*A. seyal*) against the pathogen showed significant reduction of disease incidence, by 70% and 75%, respectively (Table 1). On the other hand, spray application of plant extracts provided 100% protection against the postharvest pathogen *P. digitatum*.

Quantification of total soluble phenolics Wounds treated with extract I_1 (*A. seyal*) showed a significant increase in the concentration of total soluble phenolics around the control side of the rind. In other wound treatments [cs of FW alone; ts of extract H_2 alone; ts and cs of extract H_2 + *Pd*-treated fruit showed a significant decrease in their total soluble phenolics concentration (Fig.1)].

TABLE 1. *In vivo* antifungal activity of plant extracts[z] applied to artificial wounds on orange fruit (test pathogen = *Penicillium digitatum* [*Pd*], applied 12 h after plant extracts)

Treatment	Disease incidence (%)
Fruit wound alone	10c
Extract H_2 alone	0d
Extract I_1 alone	0d
Inoculation with *Pd* alone	100a
Extract H_2 + challenge inoculation with *Pd*	30b
Extract I_1 + challenge inoculation with *Pd*	25b
Alkyl-dimethyl benzyl ammonium chloride + challenge inoculation with *Pd*	30b
Thiabendazole + challenge inoculation with *Pd*	25b

[z] H_2 – *Withania somnifera*; I_1 – *Acacia seyal*.

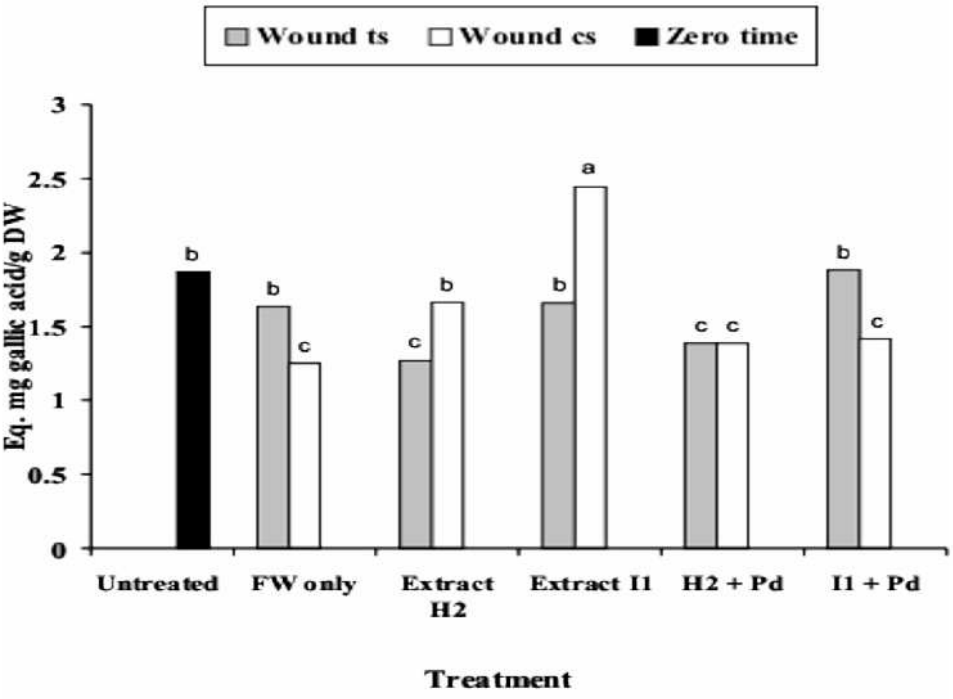

Fig. 1. Soluble phenolic concentrations in artificial wounds of orange fruit 7 days after treatment with plant extracts. Columns with the same letter do not differ significantly (means $\pm$S.E.; $P<0.05$) according to Fisher's protected LSD and t-grouping. ts = treated side of a fruit; cs = control (untreated) side of a fruit; FW only = fruit wound only; Extract H_2 = *Withania somnifera* extract treatment; Extract I_1= *Acacia seyal* extract treatment; H_2 + *Pd* = *W. somnifera* extract + challenge inoculation with *Penicillium digitatum*; I_1 + *Pd* = *A. seyal* extract + challenge inoculation with *P. digitatum*.

Spray-treated fruits exhibited no significant increase in their total soluble phenolics concentration. Treated and control sides of *Pd*, cs of extract H_2 + *Pd*-treated and cs of extract I_1 + *Pd*-treated fruit rinds showed a significant ($P<0.05$) decrease in the amount of total soluble phenolics (Fig. 2).

TABLE 2. The non-target effect of *Withania somnifera* (H_2) and *Acacia seyal* (I_1) on the microflora of artificially wounded orange fruit (test pathogen = *Penicillium digitatum* [*Pd*], applied 12 h after plant extracts

Treatment	Total microbial count ($\log_{10}$ cfu ml^{-1})					
	STD-1NA		PDA		MEA	
	Bacteria	Filamentous fungi	Yeasts	Filamentous fungi	Yeasts	Filamentous fungi
Untreated control[z]	5.02a ± 0.04[y]	3.87b ± 0.10	3.07b ± 0.04	3.86b ± 0.11	3.90b ± 0.11	4.25a ± 0.03
Extract H_2 alone	3.14c ± 0.07	2.51cd ± 0.07	3.31a ± 0.02	2.56c ± 0.13	4.05a ± 0.06	2.61bc ± 0.03
Extract I_1 alone	3.21c ± 0.11	2.40d ± 0.09	3.36a ± 0.07	2.83c ± 0.15	4.15a ± 0.06	2.71b ± 0.05
Pd alone	2.47d ± 0.12	4.24a ± 0.83	2.70c ± 0.07	4.67a ± 0.13	2.33c ± 0.03	4.28a ± 0.02
Extract H_2 ± *Pd*	4.53b ± 0.21	2.22e ± 0.09	3.38a ± 0.07	2.76c ± 0.12	4.09a ± 0.03	2.55c ± 0.04
Extract I_1 ± *Pd*	4.44b ± 0.22	2.64c ± 0.04	3.40a ± 0.08	2.70c ± 0.16	4.08a ± 0.03	2.69b ± 0.10

[z]Zero-time fruit wash.
[y]Within columns, means ± SD followed by a common letter do not differ significantly by Fisher's protected LSD and t-grouping ($P<0.05$).

TABLE 3. The non-target effect of *Withania somnifera* (H_2) and *Acacia seyal* (I_1) on the microflora of spray-treated orange fruit (test pathogen = *Penicillium digitatum* [*Pd*], applied 12 h after plant extracts)

Treatment	Total microbial count ($\log_{10}$ cfu ml^{-1})					
	STD-1NA		PDA		MEA	
	Bacteria	Filamentous fungi	Yeasts	Filamentous fungi	Yeasts	Filamentous fungi
Untreated control[z]	5.13a ± 0.07[y]	3.89a ± 0.10	3.08c ± 0.03	3.19b ± 0.09	3.90b ± 0.11	4.22a ± 0.09
Extract H_2 alone	3.39d ± 0.12	2.41c ± 0.07	3.54b ± 0.05	2.60de ± 0.07	4.11a ± 0.02	2.89d ± 0.06
Extract I_1 alone	3.70c ± 0.06	2.37c ± 0.12	3.51b ± 0.02	2.71cd ± 0.11	4.17a ± 0.05	2.90d ± 0.04
Pd alone	4.21b ± 0.06	2.17d ± 0.05	2.87d ± 0.09	4.72a ± 0.06	3.17c ± 0.08	3.26b ± 0.02
Extract H_2 ± *Pd*	3.44e ± 0.04	2.60b ± 0.03	3.59ab ± 0.06	2.81c ± 0.07	4.20a ± 0.07	2.19e ± 0.06
Extract I_1 ± *Pd*	4.29b ± 0.03	2.58b ± 0.04	3.69a ± 0.13	2.55e ± 0.04	4.19a ± 0.03	3.04c ± 0.03

[z]Zero-time fruit wash.
[y]Within columns, means ± SD followed by a common letter do not differ significantly by Fisher's protected LSD and t-grouping ($P<0.05$).

Quantification of total cell wall-bound phenolics Wound and spray applications showed a significant difference in cell wall-bound phenolics concentration of treated fruit (Figs. 3, 4). Wounded oranges treated with extract H_2 + *Pd* and extract I_1 + *Pd* showed a significant increase ($P<0.05$) in their total insoluble phenolic concentrations at the cs of the orange rind. The concentrations decreased significantly ($P<0.05$) in the ts and cs of FW, extract H_2-alone and extract I_1-alone treated fruit (Fig. 3).

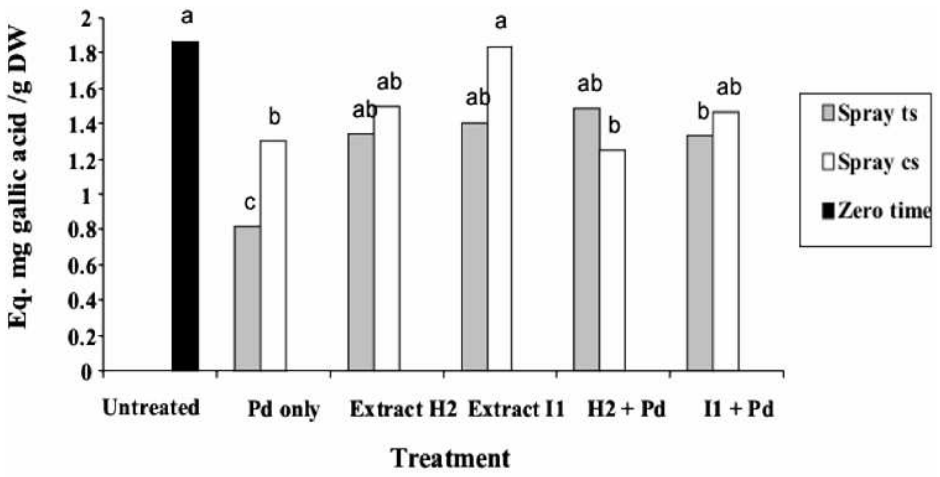

Fig. 2. Soluble phenolic concentrations in spray-treated orange fruit 7 days after treatment with plant extracts. Columns with a common letter do not differ significantly (means ±S.E.; $P<0.05$) according to Fisher's protected LSD and t-grouping. ts = treated side of a fruit; cs = control (untreated) side of a fruit; *Pd* only = *Penicillium digitatum* only; Extract H_2 = *Withania somnifera* extract treatment; Extract I_1 = *Acacia seyal* extract treatment; H_2 + *Pd* = *W. somnifera* extract + challenge inoculation with *Pd*; I_1 + *Pd* = *A. seyal* extract + challenge inoculation with *P. digitatum*.

Spray applications of *Pd* alone, extract H_2 alone, extract I_1 alone, and extract H_2 followed by challenge treatment with *Pd*, showed a significant decrease in the total insoluble phenolic concentrations both at the ts and cs of treated oranges. Spray application of extract I_1 challenged with *Pd* did not exhibit any significant increase in the total insoluble phenolic concentration as compared with the control (Fig. 4).

Non-target effect of plant extracts on orange microflora The post-treatment effect of plant extracts on the total microbial flora is recorded in Tables 2 and 3. Wound applications of extract H_2 alone, and wound and spray applications of extract I_1 alone, had a positive impact in augmenting the growth of yeasts (Table 3). The percentage growth of filamentous fungi increased significantly with wound applications of *Pd* (Table 2). Spray applications of *Pd* increased the total bacteria and mold counts (Table 3). Preventive wound applications of extract H_2 (*W. somnifera*) and preventive wound and spray applications of extract I_1 (*A. seyal*) against *P. digitatum* caused an increase of total bacteria and yeast count (Tables 2 and 3).

SEM study Scanning electron microscope examination of wounded orange peels treated with preventive application of *A. seyal* or *W. somnifera* shows a complex set of antagonistic

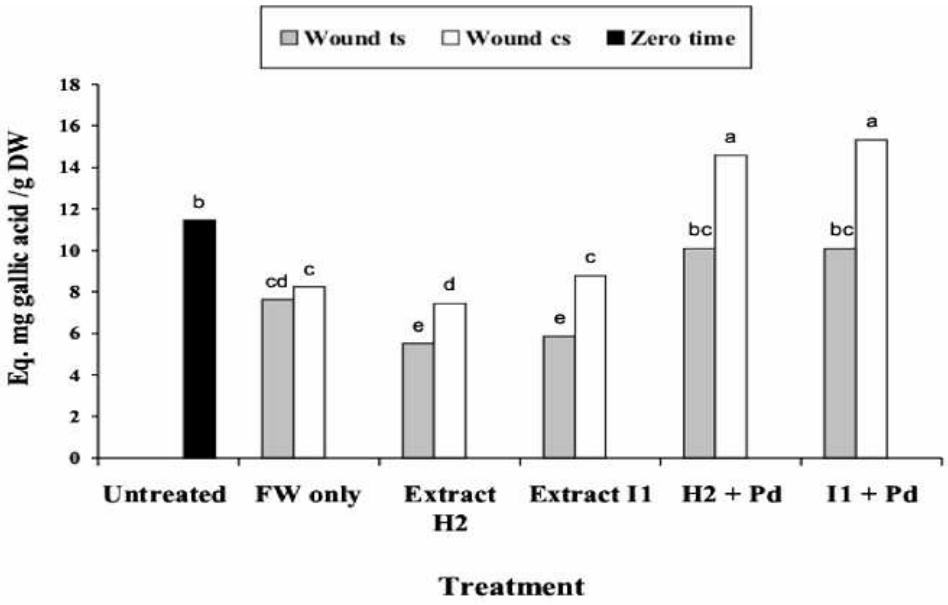

Fig. 3. Insoluble (cell wall-bound) phenolic concentrations in artificial wounds of orange fruit 7 days after treatment with plant extracts. Columns with a common letter do not differ significantly (means ±S.E.; $P<0.05$) according to Fisher's protected LSD and t-grouping. ts = treated side of a fruit; cs = control (untreated) side of a fruit; FW only = fruit wound only; Extract H_2 = *Withania somnifera* extract treatment; Extract I_1= *Acacia seyal* extract treatment; H_2 + *Pd* = *W. somnifera* extract + challenge inoculation with *Penicillium digitatum*; I_1 + *Pd* = *A. seyal* extract + challenge inoculation with *P. digitatum*.

reactions against *P. digitatum* (Fig. 5A-L). The mechanism involved exhibited a direct reaction of the plant extract with the pathogen by adhesion and/or deposition of crystal-like substances around the wound site (Fig. 5E-L). Control experiments showed fungal mass deposition around the wound site of infected fruit (Fig. 5A-D).

DISCUSSION

The efficacy of two plant extracts and their control mechanism – which involved host resistance induction against postharvest diseases – are reported. Leaf extracts from *W. somnifera* and *A. seyal* exhibited respectively 70% and 75% *in vivo* inhibitory efficacy against the postharvest fruit pathogen *P. digitatum*. These plant species were initially selected for their broad spectrum activity against human and plant pathogens. Comparative *in vitro* studies with these plant extracts showed better performance than commercial chemicals.

All orange fruit treated by spraying with plant extracts and wound application of the two extracts on their own showed 100% protection against *P. digitatum*. Our results are similar to those described by Porat *et al.* (25) with the application of elicitors. Reports on the traditional use of *W. somnifera* for control of human ailments in Ethiopia (3,10) and

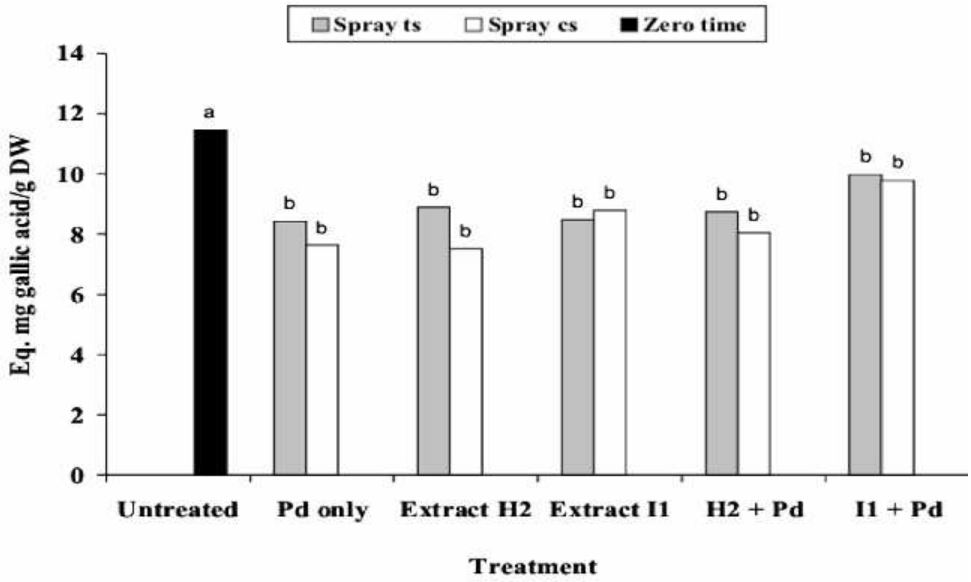

Fig. 4. Insoluble (cell wall-bound) phenolics concentration in spray-treated orange fruit 7 days after treatment with plant extracts. Columns with the same letter do not differ significantly (means ±S.E.; $P<0.05$) according to Fisher's protected LSD and t-grouping. ts = treated side of a fruit; cs = control (untreated) side of a fruit; FW only = fruit wound only; Extract H_2 = *Withania somnifera* extract treatment; Extract I_1= *Acacia seyal* extract treatment; H_2 + *Pd* = *W. somnifera* extract + challenge inoculation with *Penicillium digitatum*; I_1 + *Pd* = *A. seyal* extract + challenge inoculation with *P. digitatum*.

India (4) and of *A. seyal* in East Africa (12) have not shown that they are injurious to human health.

Wound and/or spray application of a plant extract alone and/or preventive application against the pathogen *P. digitatum* showed a change in the total phenolics concentration of orange peels as compared with the control. A decrease or increase in the total soluble phenolics concentration of a plant tissue indicates a host defense reaction system involving a certain mode of action against the pathogen. According to Robards and Antolovich (27), any environmental stimulus applied to the host tissue may increase the total soluble phenolics concentration through the phenylpropanoid pathway. The treatment and control sides of wounded fruit inoculated with plant extracts exhibited a significant change in the total soluble phenolics concentration. Wound application of extract I_1 alone showed a significant increase in the total soluble phenolics concentration in the cs of an orange rind. According to Cheng and Breen (8), this reaction could indicate a high potential of the plant material in induction of the key enzyme phenylalanine lyase (PAL) activity towards the synthesis of soluble phenolics. On the other hand, in the ts of a fruit treated with extract H_2 alone, and the ts and cs of extract H_2 + *Pd*-treated fruit, the concentration of soluble phenolics was found to be decreased. In this interaction, the host defense against

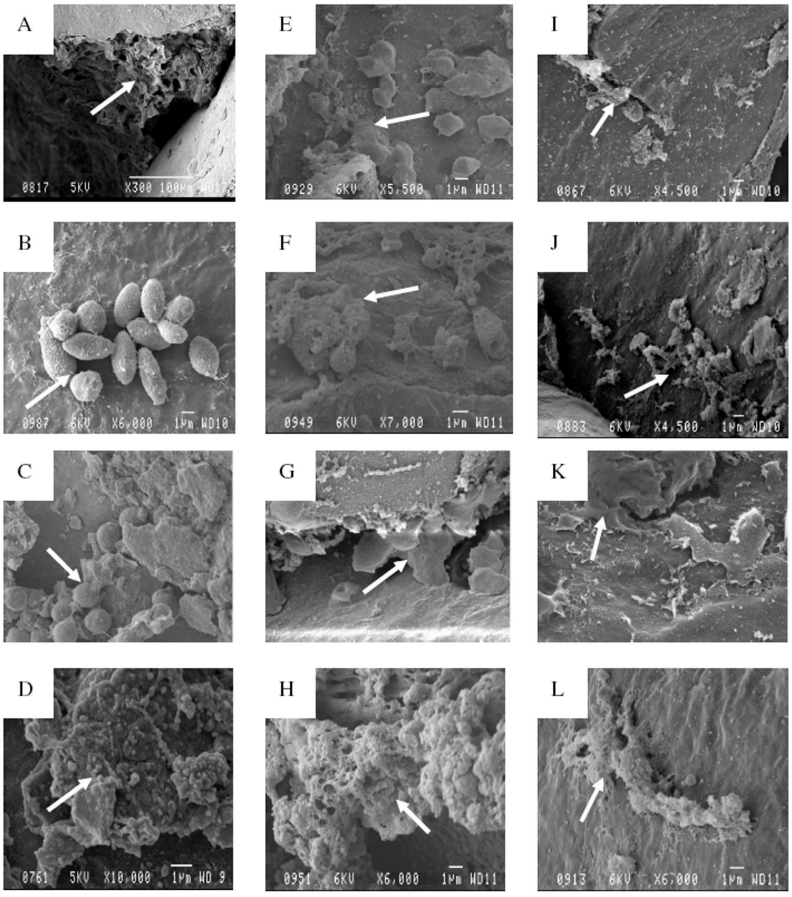

Fig. 5. Appearance of pathogen reaction to plant extracts (I_1 = *Acacia seyal*; H_2 = *Withania somnifera*) on wounded Valencia oranges. **A-D,** fruit wound lesions: **A** = just after wounding; **B,C,D,** with *Penicillium digitatum* application: **B** = 6 h after inoculation, **C** = 12 h after, **D** = 24 h after; **E-H,** wound + *A. seyal* extract + *P. digitatum* application: **E** = just after application, **F** = 6 h after, **G** = 12 h after, **H** = 24 h after. Adhesion of spores together and inactivation of pathogen growth was characteristic of the *A. seyal* extract. **I-L,** wound + *W. somnifera* extract + *P. digitatum* application: **I** = just after application, **J** = 6 h after, **K** = 12 h after, **L** = 24 h after. Pathogen growth inactivation by adhesion of spores and deposition of substances around glandular openings was characteristic of the *W. somnifera* extract.

the pathogen involved a mechanism other than oxidation of soluble phenolics (15). As reported by Cruickshank and Perrin (9), phenolic compounds at low concentrations do not have any inhibitory effect on plant pathogens; instead, they have a stimulatory effect on the host defense mechanism to build up the lignified tissues of the wall. A decrease in the total soluble phenolics concentration of an orange peel and healing of the wound surface involved a synthesis of cell wall-bound phenolics that could serve as a physical and biological barrier to invading pathogens. The stimulatory reaction involved induction of a key enzyme (PAL) in the phenylpropanoid pathway to synthesize ferulic acid, a lignin

monomer that conjugated with glucose to form a cell wall-bound phenolic, lignin (9). Lignin, as a major cell wall component of a plant tissue, builds up cell wall barriers and increases host resistance. Induced defense reactions of a fruit can be restricted to tissues close to the wound site of the stimulus or can be spread over or expressed throughout the neighboring tissues (13). A significant increase in the total cell wall-bound phenolics concentration was exhibited on the cs of an orange rind with preventive application of extract H_2 + *Pd* and I_1 + *Pd*. In other wound – treatment combinations, the total insoluble phenolics content was significantly decreased.

Images viewed through SEM showed two possible modes of action that could be involved in the defense mechanism of the host. Deposition of crystal-like substances on the wound side and direct interaction of the extract with the pathogen by adhesion of the spores together were identified as possible mechanisms observed in the healing process of an infected fruit. The antagonistic reaction depicted by accumulation of crystals around the wound site is similar to the mechanism described by Porat *et al.* (26). The other mechanism involved with direct reaction to the pathogen by adhesion indicates their putative involvement in the physical and biochemical defense responses against the pathogen. The adhesion mechanism, however, is first reported in this study.

The non-target effect of the plant extracts on the orange fruit microflora showed a general trend of decrease in microbial diversity while favoring surface colonization by yeasts and bacteria. Wound and/or spray application of extracts H_2 and I_1 in combination with *P. digitatum* showed establishment of yeast and bacterial population on the surface of the fruit. Leben *et al.* (20) reported a similar effect of the plant extracts in enhancing growth of epiphytic yeasts and bacteria. The abundance of epiphytic microflora on the peel of citrus fruit confirms the importance of natural protection against microbiological alterations by natural antagonists, which are capable of competing for nutrients and space (2,18). The appearance of antagonistic reactions exhibited by these plant extracts is desirable for postharvest application. Further semi-commercial studies are recommended for verification of the product for commercial use.

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