CHAPTER SIX

CONTROL OF *PENICILLIUM DIGITATUM* GROWTH ON CITRUS FRUIT USING TWO PLANT EXTRACTS AND THEIR MODE OF ACTION

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Abstract

Extracts from two plant species *Withania somnifera* and *Acacia seyal* selected from Ethiopia were used in this study to evaluate their potential as a natural biopesticide and to study their mode of action. Ethanolic extracts of these plants were tested *in vivo* on citrus fruits for their efficacy to control *Penicillium digitatum* when applied as a spray and wound application. Up to 70% of wound inoculated fruits did not develop decay symptoms for up to 21 days of storage at 25 ºC and >85%RH. Soluble phenolic concentrations, which inversely correlated with an increase of cell wall bound phenolics was found in treated fruit. Scanning electron microscopy revealed deposition of crystal plant material sticking to the pathogen and around the wound site. The application of plant extracts increased the epiphytic background total microbial population but decreased diversity.

***Key words*:** Soluble phenolics; Insoluble phenolics; Postharvest disease; Host resistance; Ferulic acid

6.1 INTRODUCTION

Pre- and postharvest pathogens negatively affect the quality of citrus fruits (Eckert and Brown, 1986). Mould decay caused by *Penicillium digitatum* Sacc is the main postharvest disease affecting fruit quality and is initiated through injuries before or during harvesting, packing and processing (Eckert and Brown, 1986). 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 (Tripathi and Dubey, 2004). Chemical control with imazalil, quazatine and thiabindazole remains the main options to reduce postharvest diseases (Poppe *et al*., 2003). The commercial use of postharvest fungicides has become restricted because of public health concerns (Unnikrishnan and Nath, 2002), development of pathogen resistance (Fogliata *et al*., 2001; Dianz *et al*., 2002) and environmental issues (Janisiewicz and Korsten, 2002). This effect instigated the search for natural control options using plant extracts and/or microbial antagonists.

The potential of plant extracts for control of plant diseases have long been recognized (Ark and Thompson, 1959). There are about 250 000 species of higher plants of which only 5-15% have been studied for their therapeutic value (Rojas *et al*., 2003). The use of plants for human disease control attracts more attention compared to its use in plant and animal disease control (Hernandez *et al*., 2003; Newton *et al*., 2002; Cano and Volpato, 2004). In crop protection studies, various natural plant products have been identified and used to control postharvest diseases of fruits 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 alternate* (Fr.) Keissler on eggplant and pepper fruits (Fallik and Grinberg, 1992), strawberry volatiles against postharvest fungal pathogens (Vaugh *et al*., 1993; Moline *et al*., 1997; Droby *et al*., 1999); glucosinolates from mustard and horseradish against microbial pathogens (Ishiki *et al*., 1992), citral against *P. digitatum*, *Penicillium italicum* Wehmer and *Geotrichum candidum* Link ex Pers (Klieber *et al*., 2002) and garlic against citrus green and blue moulds (Obagwu and Korsten, 2003)] are indications of the potential use 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 (Capdeville *et al*., 2002; Porat *et al*., 2002). The

mechanism involved in the former direct host reaction however is less understood (Porat *et al*., 2002). Host resistance induction on the other hand may involve several complex mechanisms including hypersensitive responses, build up of cell wall barriers, increase production of phytoalexins, accumulation of pathogenesis-related (PR) proteins, and fungal cell wall hydrolases (El-Ghaouth *et al*., 2002; Porat *et al*., 2002).

In this particular study, the antifungal activity and mode of action of two selected plant extracts *Withania somnifera* L. Dunal (code H_2), *Acacia seyal* Del var. Seyal (code I_1) were studied for preventive application against *P. digitatum* decay on citrus. Information of these plants for their use in plant disease control in general and postharvest use in particular is lacking. Limitations in the natural distribution of plants and/or the youngness of the field towards making use of 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 mould. The non-target effect of the plant extracts on the citrus micro-biota was also evaluated.

6.2. MATERIALS AND METHODS

6.2.1. Fruits

Untreated freshly harvested Valencia fruits were collected from Rustenburg citrus packhouse, Northwest Province, South Africa. Fruits were surface sterilized with sodium hypochlorate (1%) for two minutes and air-dried before use.

6.2.2. The pathogen

The pathogen, *P. digitatum* was obtained from the culture collection of Plant Pathology Laboratories, University of Pretoria, South Africa and its pathogenicity was confirmed. The pathogen was grown on Potato Dextrose agar (PDA) (Biolab, Merk, Johannesburg, South Africa) at 25°C. Ten to twenty milliliters of sterilized distilled water was added to the surface of a 14 day old culture, surface rubbed with a glass rod and the collected spore concentration was determined using a haemacytometer. A conidial suspension (10^5 conidia ml^{-1}) was prepared (Janisiewicz *et al*., 2000) and used immediately and/ or stored in the fridge (0-4°C) until further use.

6.2.3. Plant materials

Two species of plant samples *A. seyal* and *W. somnifera* were collected from Ethiopia, prepared, imported (Permit No. P0017192) and processed as described in chapter 5.

6.2.4. Plant extraction

A methanol/ acetone/ water (7:7:1, v:v) solvent was used as extraction system (Regnier and Macheix, 1996). Three successive extractions were conducted from the dried plant powder (1:20 w/v). The 1st and 2nd extraction suspension were mixed with a vortex (VM-300) and placed on a rotary shaker for one hour at 170 rpm. Samples were cold centrifuged in a micro-centrifuge (Denver instrumental Company, USA) at 3913 x g for 10 minutes. The 3rd extraction was placed over night on the rotary shaker and centrifuged as described above. The combined supernatants were concentrated to 1 ml under vacuum and freeze-dried for 48h. Tubes were refilled uniformly with sterilized distilled water to a volume of 10 ml and re-sterilized using a hypodermic syringe driven filter paper (0.22 µm pore size). Samples were either immediately used or kept in the refrigerator at 4 ºC ± 1°C until further use.

6.2.5. *In vivo* antifungal assay

In vivo preventive antifungal activities of plant extracts were tested using the method described by (Poppe *et al*., 2003), with some modifications. Wound (3 x 3 mm) and/ or spray applications of extracts were applied to the fruit 12 h prior to challenging inoculation with the pathogen. Each fruit was wounded on the opposing sides of the fruit on the middle between the stem and styler end of the fruit. 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}. For the fruit wound (FW) experiment the following treatments were included: FW only, wounding followed by application of *P. digitatum* (10^5 spore ml^{-1}) (30µl) only, wounding followed by *W. somnifera* extract (30µl) only, wounding followed by *A. seyal* extract (30µl) only, wounding followed by *W. somnifera* challenged with *P. digitatum* after 12 h of application of the extract and wounding followed by ethanolic extract of *A. seyal* challenged with *P. digitatum* after 12 h of application of the extract. Wounding followed by the application of commercial chemicals [decodone (Greifswald, Germany) and thiabendazole (Tecto 90, Johannesburg) 1000ppm for 30 sec] challenged with *P. digitatum* was included for comparison.

For spray experiment the following treatments were included: fruit surface spraying with *P.*

digitatum (10^5 spore ml^{-1}) only, surface spraying with *W. somnifera* extract only, surface spraying with *A. seyal* extract only, surface spraying with *W. somnifera* followed by drying and spraying with *P. digitatum* (10^5 spore ml^{-1}) after 12 h of application of the extract and surface spraying with *A. seyal* and challenged with *P. digitatum* after 12 h of application of the extract. Spray application of commercial chemicals [decodone and thiabendazole, 1000ppm for 30 sec] followed by the application of *P. digitatum* was included for comparison.

For each of the wound and spray treatment, twenty fruits were used and the experiment was done in triplicate and repeated once. Treated fruits were packed in boxes and incubated at 25 ºC with >85% RH for 21 days. Evaluation was done every two days and data was recorded as number of lesions developing. Efficacy of treatment application was determined according to Vero *et al*. (2002).

6.2.6. Non-target effect of plant extracts on orange surface microbial flora

The non-target effect of the plant extracts on the natural fruit micro-flora was evaluated by determining the total microbial count and the population of bacteria, yeast and mycelial fungi. The natural microflora background was determined on freshly harvested Valencia fruits and on fruits spray treated with extracts as described in section 2.5 and stored for 21 days at 25 ºC. Nine fruits were randomly selected from three boxes per treatment before and after 21 days of storage. Each fruit was placed in 500 ml Ringer's (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 and serially diluted. A 100µl of each diluted sample was spread plated on three different media [PDA, Standard 1 Nutrient agar (STD-1 NA) and Malt Extract Agar (MEA), each of which were amended with 0.002 g L^{-1} of rifampicin, cyclohexamide to discriminate growth of bacteria and fungi respectively]. Dilution plates were done in triplicate and plates were incubated at 25 ºC for two weeks. Total colony counts (cfu ml^{-1}) were computed using the following formula and log transformed for analysis (Zhang *et al*., 2005).

$$N = \frac{\sum C}{(n_1 + 0.1 * n_2)d}$$

Where, $\sum C$, is the some 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

6.2.7. Induce resistance study:

6.2.7.1. 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 untreated controlled side (cs) of the fruit. Samples were freeze-dried for 48 h, reduced to powder, sieved with a strainer (0.05 µm pore size) and kept in sterilized Scott bottles for subsequent use.

6.2.7.2. 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 Kim *et al*. (1991) with slight modifications. One milliliter of dichloromethane was poured in 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 (Centronix, 1236) for 10 minutes at 3913 x g. The supernatant was transferred to a new Eppendorf tube and the extraction repeated once. One milliliter of petroleum ether was added to the remaining peel residue, mixed and centrifuged as described. The extraction procedure was repeated once. The supernatant was dried under vacuum and 500µl methanol was added to stock the final volume. The residual extract was either stored at 4 ºC or used immediately for subsequent extraction of cell-bound phenolics.

6.2.7.3. Extraction of wall-bound phenolic compounds

Residual peel powders obtained from section 2.7.2 were used for extraction of non-soluble phenolic compounds using blowing Pasteur pipettes. A Pasteur pipette was modified to a blowing apparatus by gentle flame heating the tip and simultaneous mouth air blowing into it. The tip was sealed and cooled in air. One millilitre of 0.05 N NaOH transferred into a blowing Pasteur Pipette was mixed with 0.01g of peel powder and the pipette was sealed before transfer into a water bath (95 ºC) for one hour. Pipettes were removed from the water bath, kept on ice for 10 minutes before the tips were opened and the contents transferred into an Eppendorf tube. Sixty millilitres of concentrated HCL (10M) was added to reduce the pH to ± 5. Samples were centrifuged in a micro centrifuge at 3913 x g (Denver Instrumental Company, USA) for two minutes and the supernatant was transferred into a new Eppendorf tube. One millilitre of diethyl ether was added to the remaining residue, vortexed and centrifuged for two minutes. 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 into dryness under vacuum and 250 µl methanol was added to stock the final volume for subsequent use.

6.2.7.4. Quantification of total phenolics

The concentration of total soluble and/ or wall-bound phenolics was determined using the Folin-Ciocalteu method as described in chapter 5, section 5.2.6.1.

6.2.8. Plant extracts activity against *Penicillium digitatum* on citrus peel

Surface attachment and colonization of the pathogen were determined according to Chan and Tian (2005). 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 scanning electron microscope (SEM) preparation. Wound lesions were cut transversely into four slices (4 x 4 mm) after 0, 12, 24, and 48 h of treatment application on fruit wounds. The cut peel tissue was fixed, mounted and viewed as described in chapter 4, section 4.2.11.

6.2.9. Statistical analyses

Data was analyzed using the SAS computer program (version 8.1, 2002). 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. 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 (Zhang *et al*., 2005).

6.3 RESULTS

6.3.1. *In vivo* antifungal activity of plant extracts

Wound application of *W. somnifera* and *A. seyal* extracts against the pathogen showed significant reduction of disease incidence by 70-75 %, respectively (Table 6.1). Spray application of plant extracts on the other hand exhibited 100 % protection against the postharvest pathogen *P. digitatum* (Table 6. 2).

Table 6.1 *In vivo* antifungal activity of plant extracts with wound treatment

Treatments*	(%) Disease incidence	(%) Intact fruit**
Fruit wound only	10	90[a]
Wound application of extract *W. somnifera* only	0	100[a]
Wound application of extract *A. seyal* only	0	100[a]
Wound inoculation of *P. digitatum* only	100	0[d]
Wound inoculation of extract *W. somnifera* followed by *P. digitatum*	30	70[b]
Wound inoculation of extract *A. seyal* followed by *P. digitatum*	25	75[b]
Wound application of decodone followed by *P. digitatum*	30	55[c]
Wound application of thiabendazole followed by *P. digitatum*	25	70[b]

Legend: * = Mean of sample size for each treatment done in triplicate.

** =Values in the same column followed by different superscripts are significantly different (P <0.05).

Table 6.2 *In vivo* antifungal activity of plant extracts on citrus with spray treatment

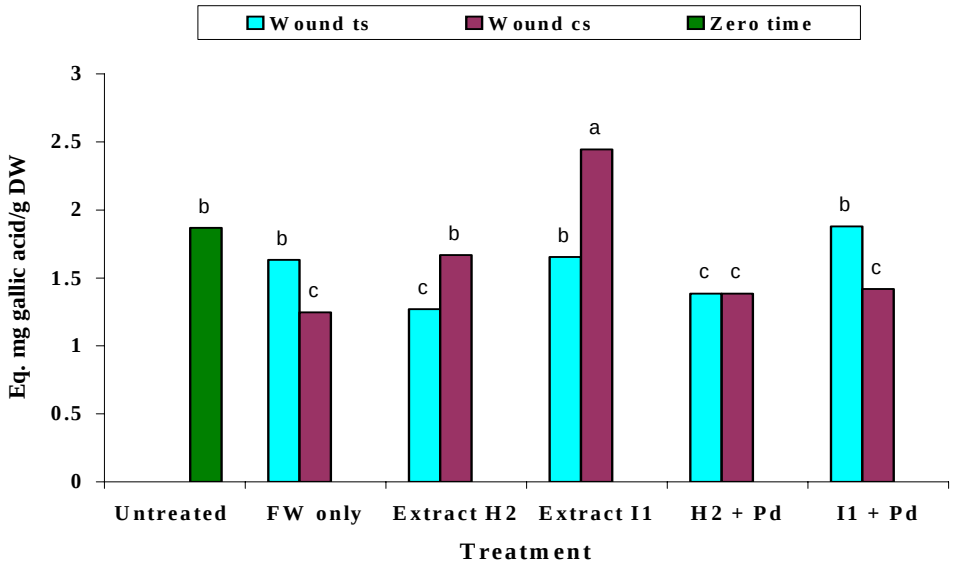
Treatments*	Disease incidence (%)	Intact fruit** (%)
Spray application of *P. digitatum* only	60	40[b]
Spray application of extract *W. somnifera* only	0	100[a]
Spray application of extract *A. seyal* only	0	100[a]
Spray application of extract *W. somnifera* followed by *P. digitatum*	0	100[a]
Spray application of extract *A. seyal* with *P. digitatum*	0	100[a]
Spray application of decodone followed by *P. digitatum*	0	100[a]
Spray application of thiabendazole followed by *P. digitatum*	0	100[a]

Legend: * = Mean of sample size for each treatment done in triplicate.

** = Values in the same column followed by different superscripts are significantly different (P <0.05).

6.3.2 Quantification of total soluble phenolics

Wound treated oranges with extract *A. seyal* showed significant increase in the concentration of total soluble phenolics around the control side (cs) of the rind. In other wound treatments [(cs) of fruit wound (FW) alone, treated side (ts) of extract *W. somnifera* alone, (ts) and (cs) of extract *W. somnifera* + *P. digitatum* (Pd) treated fruits showed significant decrease in their total soluble phenolics concentration (Fig. 6.1)].



Legend: Bars represent ± SE of the means. Bars with the same letter are not significantly (P <0.05) different according to Fisher's protected LSD and t-grouping. Abbreviations/ words used for the various treatments are indicated as follows:

□ (ts) = treated side of a fruit

□ (cs) = Control, untreated side of a fruit

FW only = fruit wound only

Extract H_2 = *Withania somnifera* L. Dunal extract treatment

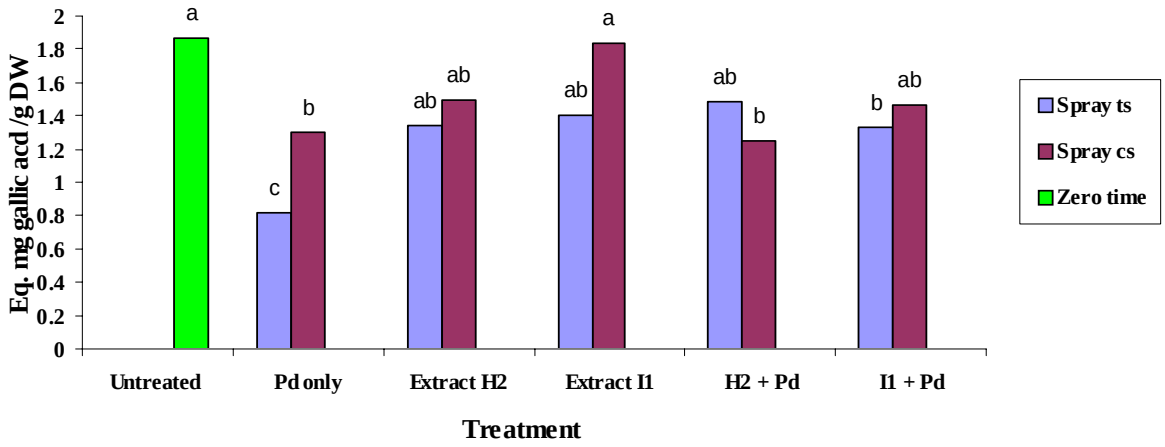
Extract I_1= *Acacia seyal* Del. var. Seyal extract treatment

H_2 + Pd = *Withania somnifera* L. Dunal extract followed by *Penicillium digitatum*.

I_1 + Pd = *Acacia seyal* Del. var. Seyal extract followed by inoculation with *P. digitatum*.

Fig. 6.1. Soluble phenolic concentrations in wound treated orange peels using plant extracts.

Spray treated fruits exhibited no significant increase in their total soluble phenolics concentration. Treated (ts) and control sides (cs) of Pd, (cs) of extract H_2 + Pd and (cs) of extract I_1 + Pd treated fruit rinds showed significant (P <0.05) decrease in the amount of total soluble phenolics (Fig. 6.2).

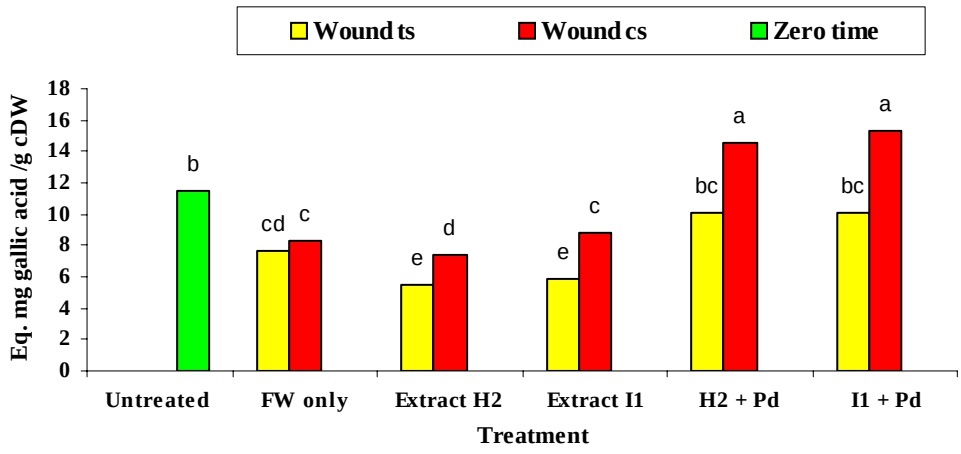


Legends: Bars represent ± SE of the means. Bars with the same letter are not significantly (P <0.05) different according to Fisher's protected LSD and t- grouping. For designated codes given to treatments refer to figure 6.1.

Fig. 6.2. Soluble phenolic concentrations in spray treated orange peels using plant extracts.

6.3.3. Quantification of total cell wall-bound phenolics

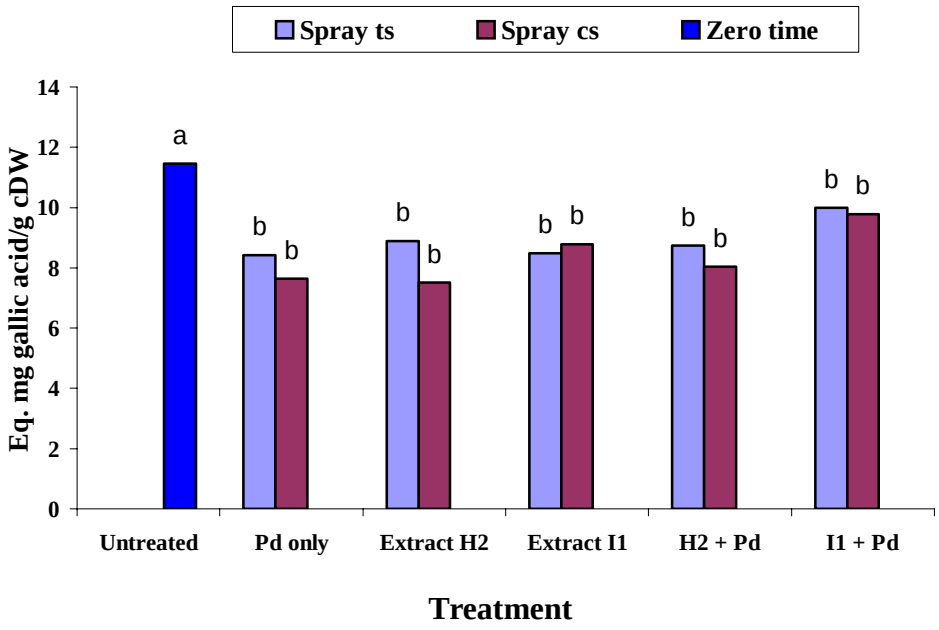
Wound and spray applications of treatments showed significant difference in cell wall-bound phenolics concentration of treated fruits (Fig. 6. 3 and 6. 4). Wound treated oranges with extract H_2 + Pd and extract I_1 + Pd showed significant increase (P <0.05) in their total insoluble phenolic concentrations at the control side (cs) of the orange rind. The concentrations decreased significantly (P <0.05) in the treated (t) and control (c) side of (FW), *W. somnifera* and *A. seyal* extracts alone treated fruits (Fig. 6. 3).



Legends: Bars represent ± SE of the means. Bars with the same letter are not significantly (P <0.05) different according to Fisher's protected LSD and t- grouping. For designated codes given to treatments refer to figure 6.1.

Fig. 6.3. Insoluble (cell wall-bound) phenolic concentrations in wound treated orange peels using plant extracts.

Spray applications of Pd alone, *W. somnifera* extract alone, *A. seyal* extract alone and *W. somnifera* extract followed by challenge treatment with Pd showed significant decrease in the total insoluble phenolic concentrations at both sides of the fruit treated (ts and cs). Spray application of *A. seyal* extract challenged with Pd did not exhibit any significant increase in the total insoluble phenolic concentration as compared to the control (Fig. 6.4).



Legend: Bars represent ± SE of the means. Bars with the same letter are not significantly (P <0.05) different according to Fisher's protected LSD and t-grouping. For designated codes given to treatments refer to figure 6.1.

Fig. 6. 4. Insoluble (cell wall-bound) phenolics concentration in spray treated orange peels using plant extracts.

6.3.4 Non-target effect of plant extracts on orange micro-flora

The post-treatment effect of plant extracts on the total microbial flora is depicted in (Table 6. 3-4). Wound applications of *W. somnifera* alone, and wound and spray applications of extract I_1 alone showed a positive impact in augmenting the growth of yeasts (Table 6. 4). The percentage growth of moulds increased significantly with wound applications of the pathogen (Pd) (Table 6. 3). Spray applications of the pathogen (Pd) showed a positive effect on increasing the total bacteria and mould counts (Table 6. 4). Preventive wound applications of *W. somnifera* and preventive wound and spray applications of *A. seyal* extracts against the pathogen, *P. digitatum* exhibited an increase in total bacteria count (Table 6. 3-4). These population shifts were significant for treatments to be further evaluated in integrated trials.

6.3.5 Effect of plant extracts against *Penicillium digitatum* on citrus

Scanning electron microscope (SEM) examination of wound treated orange peels with preventive application *A. seyal* and *W. somnifera* depicts a complex set of mode of actions against *P. digitatum* (Fig.6. 5A-P). The mechanism involved showed direct reaction of the plant extract with the pathogen by sticking and/ or deposition of crystal like substances around the wound site (Fig. 6. 5I-P). Control experiments showed fungal mass deposition around the wound site of infected fruit (Fig. 6. 5E-H).

Table 6. 3 The non-target effect assessment of the two plant extracts (*Acacia seyal* Del. var. Seyal (I_1) and *Withania somnifera* Dunal (H_2) on orange wound treated fruit surface microflora

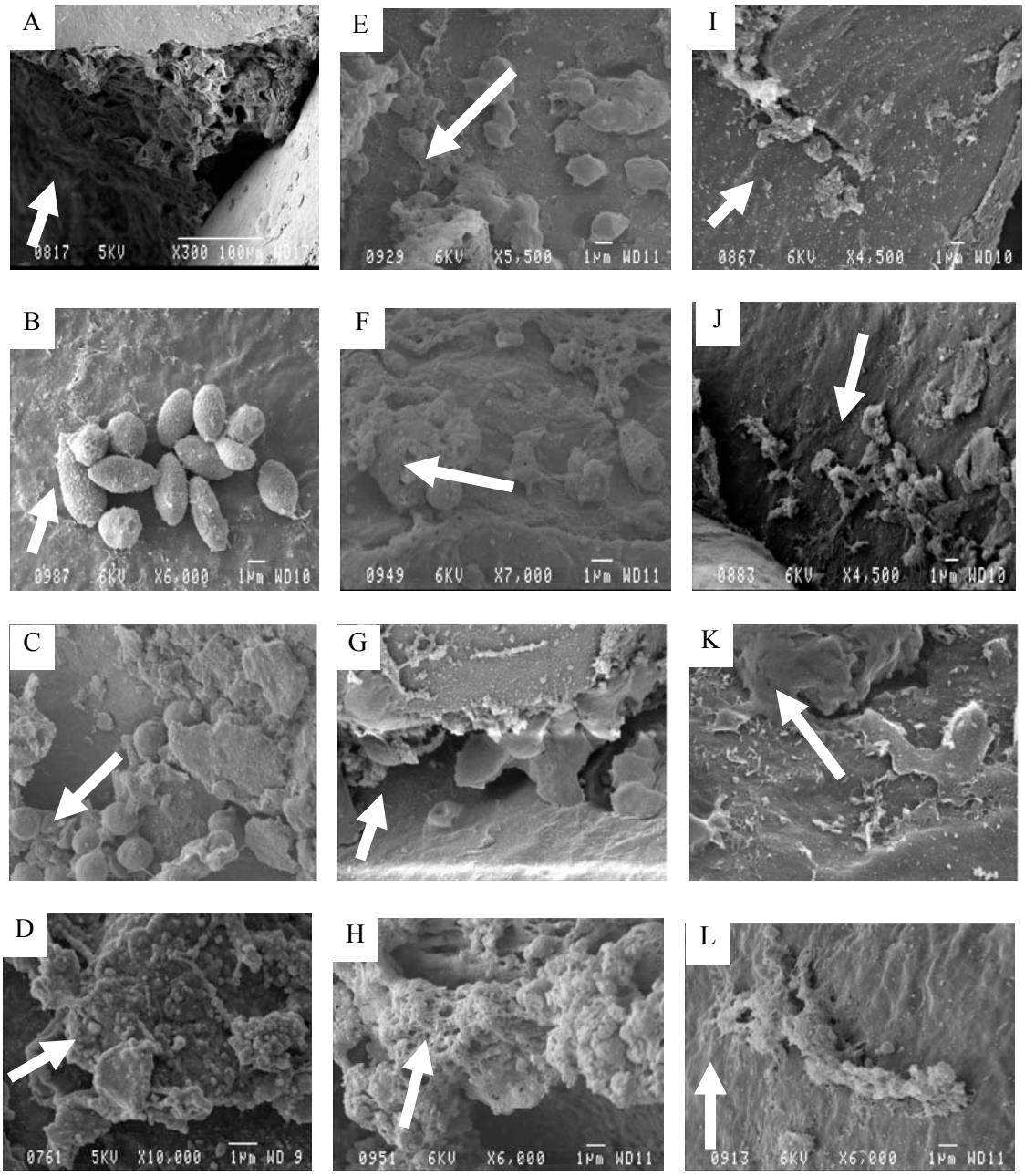
Treatments	Treatment application					
	Total microbial count (Log $_{10}$ cfu ml $^{-1}$)					
	STD-1NA		PDA		MEA	
	Bacteria	Mould	Yeast	Mould	Yeast	Mould
Untreated control	$5.02^{a} + 0.04$	$3.87^{b} + 0.10$	$3.07^{b} + 0.04$	$3.86^{b} + 0.11$	$3.90^{b} + 0.11$	$4.25^{a} + 0.03$
Extract H_2 alone	$3.14^{c}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

Legend: [a] = Untreated zero time fruit wash regarded as a control. Treated fruits incubated at 25^{o}C for 3 weeks. Relative humidity (RH) maintained between 80-90%. H_2 = *Withania somnifera* L. Dunal; I_1 = *Acacia seyal* Del. var. Seyal, Pd = *Penicillium digitatum.* Means in each column with the same letter are not significantly different by Fisher's protected LSD and t- grouping (P<0.05).

Table 6. 4 The non-target effect of the two plant extracts *Acacia seyal* Del. var. Seyal (I_1) and *Withania somnifera* Dunal (H_2) on spray treated orange fruit surface microflora.

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

Legend: For designated abbreviations refer the legend description in table 6. 3.



Legend: Preventive application of plant extracts. Images from **A-D** showed fruit wound lesion with or without *Penicillium digitatum* application: **A**= just after wounding, **B**= just after *P. digitatum* application, B= 6 h later, **C**= 12 h later, **D**= 24 h later; **E-H** showed wound + *A. seyal* extract + *P. digitatum* application: **E**= just after application, **F**= 6 h later, **G**= 12 h later, and **H**= 24 h later against the pathogen. Spore growth inactivation by sticking together and flooding the surface seems the major mode of action of *A. seyal* extract. Images **I-L** showed wound + *W. somnifera* extract + *P. digitatum* reaction: **I**= just after application, **J**= 6 h later, **K**= 12 h later, and **L**= 24 h later against the pathogen. Spore growth inactivation by deposition of substances around glandular openings seems the major mode of action of *W. somnifera* extract.

Fig. 6.5. Mode of action study of plant extracts on Valencia oranges.

6.4 DISCUSSION

This study reports on two plant extracts for their postharvest disease control efficacy and the mechanism involved in host resistance induction. Leaf extracts from *W. somnifera* and *A. seyal* exhibited 70-75% *in vivo* inhibitory efficacy against the postharvest fruit pathogen, *P. digitatum*. These plant species were initially selected for their broad spectrum antimicrobial activity against human and plant pathogens. Comparative *in vitro* studies with these plant extracts showed better performance as compared to commercial chemicals.

In this study, all fruit spray treated with plant extracts and wound application of the two extracts on their own showed 100% protection of the orange fruit against *P. digitatum*. This report indicates similar results as described by Porat *et al*. (2003) with the application of elicitors. Reports on the traditional use of *W. somnifera* for control of human ailments in Ethiopia (Demissew, 1989; Bekele, 1993; Desissa and Binggeli, 2002), India (Bhatia *et al*., 1987) and *A. seyal* in East Africa (Duke, 1983) are indicators for safe and potential use of these plant extracts for postharvest disease control.

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 to the control. A decrease or an increase in the total soluble phenolics concentration of a plant tissue indicates host defence reaction system involving certain mode of action against the pathogen. According to Robards and Antolovich (1997), any environmental stimuli applied on the host tissue may increase the total soluble phenolics concentration through phenylpropanoid pathway. Treatment side (ts) and control sides (cs) of wound inoculated fruits with plant extracts exhibited significant change in the total soluble phenolics concentration. Wound application of extract (I_1) alone showed significant increase in the total soluble phenolics concentration in the (cs) of an orange rind. According to Cheng and Breen (1991), this reaction could show the high potential of the plant material in induction of the key enzyme phenyl alanine lyase (PAL) activity towards the synthesis of soluble phenolics. On the other hand, in treatment side (ts) of a fruit with extract H_2 alone, (ts) and (cs) of extract H_2 + Pd treated fruits, the concentration of soluble phenolics were found decreased. In this interaction, the host defence mechanisms against the pathogen involved another mechanism other than oxidation of soluble phenolics (Harborne, 1964). As reported by Cruickshank and Perris (1964), phenolic compounds at low concentrations do not have any inhibitory effect on plant pathogens instead they have a stimulatory effect on host

defence 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 involve induction of a key enzyme (PAL) in the phenylpropanoid pathway to synthesis ferulic acid, a lignin monomer that conjugated with glucose to form a cell wall bound phenolics, lignin (Cruickshank and Perris, 1964). Lignin, as a major cell wall component of a plant tissue builds up cell wall barriers and increase host resistance. Induced defence reactions of a fruit can be restricted to tissues close to the wound site of the stimulus or can be spread or expressed throughout the neighbouring tissues (El-Ghaouth *et al*., 2002). Significant increase in the total cell wall bound phenolics concentration was exhibited on the control side (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 actions that could be involved in the defence mechanism of the host. Deposition of crystal-like substances on the wound side and direct interaction of the extract with the pathogen by sticking the spores together were identified as possible mechanisms observed in the healing process of an infected fruit. The mode of action shown by accumulation of crystals around the wound site is a similar mechanism as described by Porat *et al*. (2002). The other mechanism involved with direct reaction to the pathogen by sticking indicates their putative involvement in the physical and biochemical defence responses against the pathogen. The latter mechanism however is the first to be reported.

The non-target effect of the plant extracts on the orange fruit micro-flora showed a general trend of decrease in microbial diversity while favouring 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. Reports by Leben *et al*. (1965) showed similar results of plant extracts effect in enhancing growth of epiphytic yeasts and bacterial strains. The abundance of epiphytic micro flora on the peel of citrus fruits confirms the importance of natural protection against microbiological alterations by natural antagonists, which are capable of competing for nutrients and space (Arras, 1996; Janisiewicz *et al*., 2000). The mode of actions 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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