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Effects of plant defence activators on anthracnose disease of cashew

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Abstract

The plant defence activators acibenzolar-S-methyl (Benzo[1,2,3]thiadiazole-7-carbothioic acid-S-methyl ester, ASM), 2,6-dichloro-isonicotinic acid (DCINA), salicylic acid (SA), and dibasic potassium phosphate (K_2HPO_4) were tested for their ability to protect cashew (*Anacardium occidentale*) seeds and leaves from anthracnose disease caused by *Colletotrichum gloeosporioides*. No inhibition of the early stages of pathogen development was caused by concentrations equal to or lower than 1.1 mM a.i. ASM, 1.2 mM a.i. DCINA, 5 mM SA and 50 mM K_2HPO_4. Maximum reduction of the disease in detached leaves, without phytotoxic effects, was obtained with 0.07 mM a.i. ASM and DCINA, 5 mM SA, and 50 mM K_2HPO_4, with a time interval of at least 72 h between application of the activator and inoculation with the pathogen. On attached leaves, foliar sprays were slightly more efficient than soil drench treatments, with 5 mM SA being the most effective treatment, while 50 mM SA as well as 0.3 mM a.i. ASM and DCINA caused phytotoxic effects. In field-grown plants, protection was conferred by a soil drench of concentrations as low as 12.6 μM a.i. ASM and DCINA and 2.6 mM SA. These concentrations were not phytotoxic suggesting that plant defence activators have potential for control of anthracnose disease in the field.

Introduction

Cashew (*Anacardium occidentale* L.) is a perennial evergreen tree, native to the lower Amazon and north-east coast of Brazil (Mitchell and Mori, 1987). The cashew crop is of major importance in Brazil, generating around 20,000 jobs in industries and 280,000 in farms, with a production of 182,000 metric tons of nuts from 680,000 ha of crop in 1997. The majority (c. 90%) of the Brazilian cashewnut and cashew-nut shell liquid (CSNL) production is exported, earning around 150 million dollars annually (F.A.O., 1998; Leite, 1994). The major constraint to cashew production in the region is inflorescence blight, fruit rot and anthracnose of leaves caused by the fungus *Colletotrichum gloeosporioides* Penz. All the commercially available clones are susceptible to the pathogen, although disease incidence varies between clones (Cardoso et al., 1999). Prospects for conventional chemical control are limited, as fungicides are difficult to apply, given the stature of the plants, and treatment increases production costs and might have environmental effects (Freire and Cardoso, 1995).

Induced resistance may provide an alternative approach to plant protection especially for problems not satisfactorily controlled by conventional methods (Schoenbeck, 1996). Local and systemic induced resistance can be activated in plants by low level persistent stress, including infection by pathogens, and also by chemical activators such as salicylic acid (SA) and analogues, or sodium and potassium salts (Cohen, 1994; Reuveni et al., 1994a,b, 2000; Reuveni and Reuveni, 1998; Reuveni et al., 1998). Plants such as tobacco, cucurbits, cocoa, nectarines, grapevines, and mango

trees treated with these compounds develop resistance against a broad spectrum of diseases (Mucharromah and Kuć, 1991; Reuveni and Reuveni, 1995; Fought and Kuć, 1996; Okey and Sreenivasan, 1996). Induced resistance most likely includes more than one phenomenon (van Loon et al., 1998; Hammerschmidt, 1999), of which the best characterised is systemic acquired resistance (SAR). Many studies have indicated an essential role for SA in the pathway leading to SAR (Delaney et al., 1994, Dempsey et al., 1999). 2,6-dichloro-isonicotinic acid (DCINA) and benzo[1,2,3]thiadiazole-7-carboxylic acid derivatives are functional analogues of SA and induce SAR in several plant species (Kessman et al., 1994; Kunz et al., 1997). Despite extensive experimental analysis of SAR in model species such as tobacco and *Arabidopsis* (Delaney, 1997; Sticher et al., 1997), relatively little information is available on induced resistance in perennial woody crops. Recently, Ishii et al. (1999) and Brisset et al. (2000) induced resistance respectively in Japanese pears and apples using acibenzolar-S-methyl (Benzo[1,2,3]thiadiazole-7-carbothioic acid-S-methyl ester, ASM, Bion®-Novartis). The local and systemic protection of Golden Delicious apple seedlings against *Erwinia amylovora* was associated with the activation of peroxidases and β-1,3-glucanases, and was sustained for at least 17 days. These results suggested that ASM promoted systemic resistance in apple by increasing defence-related enzymes. However, nothing is known regarding this phenomenon in cashew.

The aim of the present study was to evaluate the effects of the defence activators ASM, DCINA, and SA, and K_2HPO_4 on the disease reaction type of cashew leaves to infection by *C. gloeosporioides*. Experiments were done in controlled environment and glasshouse conditions, as well as in field plots in North-east Brazil, to assess whether this approach might have potential application for the control of anthracnose disease in the cashew crop.

Materials and methods

Cashew clones and fungal isolates

Two Precocious-Dwarf cashew (*A. occidentale*) clones were selected for this study, on the basis of their differential responses to two isolates of *C. gloeosporioides*, LARS 905, from leaves of *Rubus brasiliensis*, and LARS 910, from *A. occidentale* (both from Maceio, Alagoas, Brazil). Clone CCP-76 was susceptible to both isolates, while clone CCP-1001 was resistant to isolate LARS 905 and susceptible to isolate LARS 910 in inoculation tests in controlled environments (Lopez and Lucas, 1998). Seeds of both clones were supplied by the Brazilian National Centre of Research in Tropical Agroindustry (CNPAT/EMBRAPA, Fortaleza, Brazil), and stored at 10 °C. Seeds were sown in sterilized compost (soil-sand-peat, 1 : 1 : 1, enriched with 2.5 kg m^{-3} of triple superphosphate and 1 kgm^{-3} of potassium chloride, pH 5.5) in 21 plastic pots and maintained in a controlled environment chamber (16 h day, 280 µmol PAR m^{-2} s^{-1}, 80% RH, 35 °C). The pots were watered daily and after 10–15 days, when the seedlings emerged, the temperature of the chamber was adjusted to 25 °C. Forty-five-day old seedlings were maintained in the same environmental cabinet, or transferred to glasshouse conditions (25 °C, 12 h day, 500 µmol PAR m^{-2} s^{-1}) for further assays.

Inoculum of isolates LARS 905 and LARS 910 was prepared as conidial suspensions by flooding 7-day-old cultures grown on CM (*Colletotrichum* medium; Mathur et al., 1950) with sterilised distilled water (SDW), and adjusting the concentration of spores to c. 10^6 ml^{-1}.

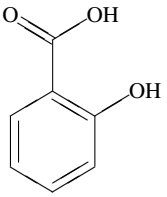
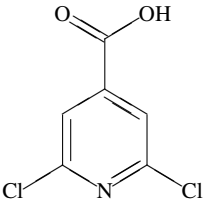
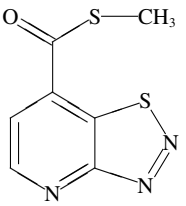
Chemical activators

Table 1 lists the compounds tested and their sources. Fresh suspensions (ASM, DCINA and SA) or solutions (K_2HPO_4) were prepared by dissolving the products in deionised water to obtain different concentrations, and maintained at room temperature until use. Prior to application, the pH of the ASM, DCINA, and SA suspensions was checked and adjusted to 7.0 with 0.1 M NaOH or HCl as necessary.

Effects of activators on mycelial growth, spore germination and appressorium formation in vitro

The compounds (Table 1) were first dissolved in ethanol and then separately added (1%) to molten CM-agar after autoclaving, to give the following concentrations: DCINA (0, 0.5, 5.0, 50, 500, and 1225 µM a.i.), ASM (0, 0.44, 4.4, 44, 440, and 1096 µM a.i.), SA (0, 0.5, 5, and 50 mM) and K_2HPO_4 (0, 0.05, 0.5, and 50 mM). The amended media were dispensed in 9 cm dia. Petri dishes. After solidification of the media, each dish was inoculated centrally with an agar disc (c. 4 mm dia.) cut from a 7-day-old mycelial culture of

Table 1. Compounds assayed for their ability to induce systemic resistance against *C. gloeosporioides* in cashew (*A. occidentale*)

Class and compound	Common name or industrial code	Source	Formula
Hydroxybenzoic acids			
2-hydroxybenzoic acid	Salicylic acid (SA) (salt)	Sigma	
Phosphates			
Potassium phosphate dibasic	Potassium phosphate (salt)	Merck	K_2HPO_4
Nicotinic acids			
2,6-dichoro-isonicotinic acid	DCINA (CGA-41396) (Wettable powder, 25% a.i.)	Syngenta	
Benzothiadiazoles			
Benzo(1,2,3)thiadiazole-7-carbothioic acid-S-methyl ester	Acibenzolar-S-methyl (ASM) Bion® (CGA-245704) (Granule, 50% a.i.)	Syngenta	

either isolate LARS 905 or LARS 910. The dishes were incubated in the dark at 25 °C for 5 days, and the diameter of each colony was measured. There were 5 replicate dishes for each isolate × compound-concentration (treatments), and the dishes were arranged in a complete randomised design for each compound used. Data were transformed to $\sqrt{(x + 0.5)}$ for analysis of variance and comparison between the means by the least significant difference (LSD) test.

To determine effects on spore germination and appressorium formation, 200 µl of an aqueous suspension of each concentration of ASM (see above) was mixed with 200 µl of conidial suspension (c. 10^6 ml^{-1}) of each isolate and 12 µl of the mixture dispensed in wells on glass slides on metal supports inside plastic boxes lined with moist filter paper. There were 25 replicate wells per treatment. The slides were incubated in the dark at 25 °C and percentages of spores germinated and appressoria formed were assessed after 15 and 30 h, respectively.

Effect of different concentrations of chemical activators on disease reaction type of detached cashew leaves

First or second fully-expanded leaves were excised, the petioles sealed with warm molten paraffin wax, and placed adaxial surface down on square polypropylene sheets inside transparent plastic boxes (24 × 24 × 12 cm^3) lined with moist tissue paper. They were treated by spraying their abaxial surface until just prior to run-off (c. 1.0–1.5 ml per leaf) with a range of concentrations of the chemical activators, or SDW, using a Humbrol (Hull, UK) power pack atomiser. The treatments were aqueous suspensions or solutions of DCINA (0.07, 0.1, and 0.3 mM), ASM (0.03, 0.06, 0.09, 0.13, 0.3, 0.33, 0.44, 0.66, 0.88, 6.6, and 13.2 mM), SA (0.1, 0.5, 1.0, 2.5, 5, 10, 25, and 50 mM) and K_2HPO_4 (1, 5, 10, 25, and 50 mM). The treated leaves of each clone (10 per treatment per box) were maintained for 72 h in a controlled environment chamber (25 °C, 100% RH,

16 h day, 280 μmol PAR m^{-2} s^{-1} light intensity). Each leaf was inoculated with 10–16 drops (according to the leaf size) of a conidial suspension (10 μl of c. 10^6 spores ml^{-1}) of either isolate of *C. gloeosporioides* and incubated under the same conditions for 96 h. Disease severity was rated using a visual scale as follows: 0 = no symptoms; 1 = some small flecks c. 1 mm or 5% necrotic tissue; 2 = flecks 2–3 mm/limited lesions or 6–20% necrotic tissue; 3 = many coalescent flecks, lesions > 3 mm, 21–35% necrotic tissue; 4 = water soaked lesions >5 mm, 36–60% necrotic tissue; 5 = spreading coalescent lesions with sporulation or >60% necrotic tissue. Scores 0–2 were classed as resistant, 3 as intermediate, and 4–5 as susceptible. There were 10 leaves for each treatment, and for each clone-isolate combination, the boxes were arranged in a completely randomised plot design. These experiments were repeated to complete three replicates in total. The data were transformed to $\sqrt{(x + 0.5)}$ for analysis of variance, and comparison between means by the LSD test.

An alternative treatment was carried out by applying the activator suspensions to the under surface of one half of each leaf, using 10 μl droplets. After 72 h the other half of each leaf was inoculated by drops, incubated for 96 h, and scored for disease symptoms as above.

Effect of time between treatment and challenge inoculation on disease reaction type of detached leaves

Detached leaves were sprayed with an aqueous suspension of ASM (0.09 mM) 12, 24, 48, 72, 96, and 120 h prior to inoculation with 10–16 droplets of conidial suspension (10 μl, c. 10^6 spores ml^{-1}). For each one of the time intervals tested, there were 10 leaves inoculated with either isolate of *C. gloeosporioides*. For each clone-isolate combination, the boxes were arranged in a completely randomised plot design. These experiments were repeated to complete a total of three replicates. Symptoms were scored at 96 h after inoculation. Alternatively a half-leaf treatment was included as before.

In vivo *germination and appressorium formation of* C. gloeosporioides *on ASM-treated leaves*

The percentages of spore germination and appressorium formation by isolates LARS 905 and LARS 910 were assessed 15 and 36 h after inoculation of detached leaves (3 per box) treated 72 h previously with different concentrations of ASM (0.4, 4.4, 44, 440, and 1100 μM) or SDW. Four leaf discs (c. 4 mm dia.) from three leaves of each clone-isolate combination × treatment were cut from beneath inoculum drops, cleared in 0.15% (w/v) trichloroacetic acid in ethanol : chloroform (3 : 1 v/v), and stained with 0.025% (w/v) aniline blue. The percentages of germinated conidia and appressoria formed were recorded for at least 100 conidia for each leaf disc.

Seed treatment with ASM

Three batches of 12 seeds of each clone (CCP-76 and 1001) were used. Seeds in the first and second batch were imbibed in an aqueous suspension of ASM (0.44 mM), while the third control batch was imbibed in SDW for 24 h. The seeds were air dried on filter paper for 2 h at room temperature, and transferred into separate plastic boxes lined with wet paper towels. Seeds of the second (ASM treated) and third (SDW treated) batches were inoculated with four 10 μl drops of a conidial suspension of isolate LARS 905 (c. 10^6 conidia ml^{-1}) while seeds of the first batch were treated in the same way with SDW. After incubation for 96 h in an environmental cabinet, the percentages of sporulating and restricted lesions were assessed. The seeds were sown in compost and maintained in the same environmental cabinet. The percentages of emerging seedlings without anthracnose symptoms were recorded after 35 days.

Systemic protection in seedlings

Two groups of 27 plants of each clone were treated with the chemicals (Table 1) using two different application methods. In the first method, the 3rd, 4th and 5th leaves (numbered from apex to base) of the plants were sprayed until just prior to run-off (c. 1.0–1.5 ml per leaf) with one of the following aqueous suspensions or solutions: DCINA (98 and 300 μM), ASM (88 and 300 μM), K_2HPO_4 (5 and 50 mM), SA (5 and 50 mM) and SDW (control). The second group of plants received a soil drench with 50 ml of the same suspensions, solutions or SDW (control). There were three plants per treatment. The plants were maintained under glasshouse conditions (see above) for 96 h. All leaves were inoculated on both surfaces by spraying until run-off (c. 1.0–1.5 ml per leaf) with a conidial suspension

of isolate LARS 905 (c. 10^6 conidia ml^{-1}), and incubated in moist polyethylene bags in the environmental cabinet (see above) for 48 h. The bags were removed. Disease reaction type of the first and second leaves (numbered from apex to base) was assessed using the 0–5 severity scale 5 days after inoculation. The experiments were numbered in a completely randomised design for each clone-isolate combination, and replicated three times in total. The data were transformed to $\sqrt{(x + 0.5)}$ for analysis of variance and comparison between means by the LSD test.

Field experiment

The experiment was carried out during the cashew blooming season, in late July and August 1997, at the CNPAT/EMBRAPA field station in Pacajus, 55 km south–east of Fortaleza, Ceara, North-east Brasil, (4°10′S and 38°27′W at 60 m above sea level, climate dry/subhumid with an average annual precipitation of c. 1300 mm over the previous 4 years). The field used for the trial was originally set up to supply uniform vegetative material to propagate 8 clones (CCP-06, CCP-09, CCP-76, CCP-1001, CAP-06, CAP-12, CAP-14, and CAP-18). The trees were raised from seedling progeny in 1993, in a 4 × 3 spacing covering 7020 m^2 (15 columns (3 m) and 39 rows (4 m)). The first and last columns and rows consisted of border plants (CCP-76), and 45 plots (7 plants per plot, 5 plots per column, 7 plots of CCP-76, 7 plots of CCP-1001, 6 plots of CCP-14, and 5 plots of the other clones) arranged in a complete randomised design. Each group of three rows of plants was separated by a column without plants, to facilitate husbandry. The soil at the site is a uniform sandy-clay (70% sand, 20% clay, pH 6.4), nutrient poor, with high hydraulic activity (56 cm h^{-1}) (Oliveira and Ramos, 1995).

Given the layout of the clones in the field it was possible to arrange a factorial experiment (7 × 3) in a strip-plot design, with three clones (CCP-76, CCP-1001, and CAP-14) as horizontal treatments, and 7 plots (A = distilled water; B = 12.6 μM ASM; C = 126 μM ASM; D = 12.6 μM DCINA; E = 126 μM DCINA; F = 26 mM SA; G = 87 mM SA) as the vertical treatments, with five replicates. The plants received no pesticides for at least 3 months prior to the experiment. As part of normal husbandry, pruning of the plants was conducted one week prior to the experiment. As rainfall was low (171 mm precipitation during May–August, which included the experimental period) the trees received irrigation (20 l water per plant) twice weekly, to increase humidity. The chemicals were applied by soil drench. Stock suspensions of ASM (5.0 mM), DCINA (5.0 mM) and SA (35 mM) were first prepared by diluting the products in 10 l of distilled water with the pH adjusted to 7.0. In the field, these stock suspensions were diluted with tap water to give the appropriate concentration for application. To reduce loss of the chemical by drainage or evaporation, and to improve uptake, each plant was watered with 20 l of tap water 2 h prior to treatment, and drenched with 20 l of the treatment suspension applied over a 1.5 m radius. As environmental conditions during the trial period were unfavourable for natural infection by anthracnose, the plants were artificially inoculated at sunset 10 days after activator treatment by spraying until run-off with a conidial suspension of isolate LARS 910 (c. 10^6 conidia ml^{-1}, 1 l per plant) using a 20 l spray canister with pressure chamber. The experiment was evaluated 12 and 15 days after inoculation by scoring the young leaves of 4 cardinal branches of each plant using the disease scale described previously and calculating the average disease severity. The data were transformed to $\sqrt{(x + 0.5)}$ for analysis of variance and comparison between means by the LSD test.

Results

Effect of activators on mycelial growth, germination, and appressorium formation in vitro

No significant effects on mycelial growth (Table 2), spore germination or appressorium formation (data not shown) were observed for any of the compounds at most of the concentrations used, with the exception of DCINA at 1.225 mM and SA at concentrations higher than 5 mM .

Effect of different concentrations of chemical activators on disease reaction type of detached cashew leaves

Treatment with aqueous suspensions or solution of ASM, DCINA, SA, and K_2HPO_4 reduced disease severity in detached leaves. Tables 3 and 4 show transformed data respectively for clone CCP-76 (susceptible to both isolates used) and for CCP-1001 (resistant to isolate LARS-905, susceptible to isolate LARS-910). The greatest reduction in disease was seen

Table 2. Effect of ASM, DCINA, SA, and K_2HPO_4 on mycelial growth of *C. gloeosporioides* (isolates LARS 905 and LARS 910)

Chemicals and quantity	Mycelial growth[1] (cm)	
	LARS 905	LARS 910
ASM (μM)		
0.0	2.61 ± 0.02	2.29 ± 0.12
0.4	2.58 ± 0.06	2.20 ± 0.17
4.4	2.57 ± 0.05	2.31 ± 0.03
44.0	2.54 ± 0.04	2.34 ± 0.03
440.0	2.55 ± 0.03	2.26 ± 0.05
1100.0	2.54 ± 0.04	2.24 ± 0.07
LSD 1%[2]	0.07	0.16
DCINA (μM)		
0.0	2.50 ± 0.01	2.23 ± 0.04
0.5	2.49 ± 0.01	2.20 ± 0.06
5.0	2.47 ± 0.01	2.20 ± 0.05
50.0	2.50 ± 0.03	2.21 ± 0.04
500.0	2.47 ± 0.03	2.18 ± 0.04
1225.0	2.27 ± 0.01**	2.19 ± 0.05
LSD 1%[2]	0.04	0.08
SA (mM)		
0.0	2.50 ± 0.01	2.23 ± 0.04
0.5	2.51 ± 0.04	2.20 ± 0.03
5.0	2.17 ± 0.07**	1.94 ± 0.05**
50.0	0.71 ± 0**	0.71 ± 0**
LSD 1%[2]	0.06	0.05
K_2HPO_4 (mM)		
0.0	2.50 ± 0.01	2.23 ± 0.04
0.05	2.54 ± 0.04	2.21 ± 0.04
0.5	2.51 ± 0.03	2.20 ± 0.05
5.0	2.54 ± 0.04	2.20 ± 0.17
50.0	2.51 ± 0.03	2.20 ± 0.06
LSD 1%(2)	0.06	0.14

[1]Means ± standard errors of the transformed data ($\sqrt{x+0.5}$) of five replications per treatment.
[2]LSD test: means are significantly different from the control at 1% level (** $P \leq 1\%$).

at concentrations of ASM and DCINA equal or lower than 0.3 and 0.44 mM, respectively, and of SA equal or lower than 5 mM. At higher concentrations, chlorotic flecking developed within 48 h (SA) or after 72 h (ASM and DCINA) and disease severity was eventually similar to the control treatment. With K_2HPO_4, the highest concentrations provided the greatest protection. With clone CCP-1001 (Table 4), chemical treatment did not significantly alter the level of resistance to isolate LARS 905, while resistance to isolate LARS 910 was increased. In the latter clone × isolate combination, the disease reaction type was effectively shifted from susceptibility to resistance. When droplets of the activators were placed on the abaxial surface of half-leaves 72 h prior to inoculation of the opposite half-leaf, chlorotic spots were again observed beneath the droplets. Resistance to the fungus was increased at concentrations equal to or lower than 0.44 mM ASM or 5 mM SA (data not shown). Doses higher than these caused necrosis.

Table 3. Effect of chemical activators on disease reaction of detached cashew leaves (clone CCP-76) to *C. gloeosporioides*

Chemical treatment (mM)		Average reaction type[1]	
		LARS 905	LARS 910
Control (SDW)		2.29 ± 0.15	2.30 ± 0.09
DCINA	0.07	1.18 ± 0.36**	1.44 ± 0.18**
DCINA	0.10	1.33 ± 0.17**	1.56 ± 0.26**
DCINA	0.30	1.33 ± 0.17**	1.56 ± 0.26**
ASM	0.03	1.33 ± 0.17**	1.43 ± 0.28**
ASM	0.07	1.18 ± 0.36**	1.26 ± 0.33**
ASM	0.13	1.26 ± 0.33**	1.35 ± 0.27**
ASM	0.30	1.26 ± 0.33**	1.44 ± 0.18**
ASM	0.44	1.44 ± 0.18**	1.57 ± 0.22**
ASM	0.70	1.86 ± 0.25**	1.81 ± 0.22**
ASM	0.90	1.86 ± 0.25**	1.91 ± 0.21**
ASM	6.60	1.91 ± 0.21**	1.96 ± 0.23**
ASM	13.20	2.01 ± 0.09**	2.12 ± 0.00**
SA	0.10	1.64 ± 0.13**	1.75 ± 0.15**
SA	0.50	1.57 ± 0.22**	1.70 ± 0.15**
SA	1.00	1.50 ± 0.26**	1.70 ± 0.15**
SA	2.50	1.48 ± 0.33**	1.56 ± 0.26**
SA	5.00	1.43 ± 0.28**	1.56 ± 0.26**
SA	10.00	1.75 ± 0.15**	1.81 ± 0.22**
SA	25.00	1.86 ± 0.22**	1.91 ± 0.21**
SA	50.00	1.96 ± 0.23**	2.01 ± 0.09**
K_2HPO_4	1.00	1.70 ± 0.15**	1.81 ± 0.22**
K_2HPO_4	5.00	1.48 ± 0.33**	1.64 ± 0.13**
K_2HPO_4	10.00	1.44 ± 0.18**	1.57 ± 0.22**
K_2HPO_4	25.00	1.37 ± 0.18**	1.43 ± 0.28**
K_2HPO_4	50.00	1.19 ± 0.29**	1.33 ± 0.17**
LSD 5%[2]		0.22	0.18

[1]Means of transformed data ($\sqrt{x+0.5}$) ± standard errors of 10 leaves per treatment.
[2]LSD test: means are significantly different from the control at 5% level (** $P \leq 5\%$).

Effect of time between treatment and challenge inoculation on disease reaction type of detached leaves

With clone CCP-76, ASM reduced the severity of anthracnose infection on detached leaves when sprayed at least 48 h prior to inoculation (Table 5). The response of leaves maintained for shorter periods (12 or 24 h) was not significantly different to untreated controls. The same reaction was observed when the clone CCP-1001

Table 4. Effect of chemical activators on disease reaction of detached cashew leaves (clone CCP-1001) to *C. gloeosporioides* (isolates LARS 905 and LARS 910)

Chemical treatment (mM)		Average reaction type[1]	
		LARS 905	LARS 910
Control (SDW)		1.35 ± 0.27	2.32 ± 0.19
DCINA	0.07	1.19 ± 0.45	$1.56 \pm 0.26^{**}$
DCINA	0.10	1.18 ± 0.36	$1.56 \pm 0.26^{**}$
DCINA	0.30	1.26 ± 0.33	$1.44 \pm 0.18^{**}$
ASM	0.03	1.26 ± 0.33	$1.32 \pm 0.41^{**}$
ASM	0.07	1.10 ± 0.30	$1.16 \pm 0.41^{**}$
ASM	0.13	1.19 ± 0.45	$1.33 \pm 0.17^{**}$
ASM	0.30	1.22 ± 0.49	$1.44 \pm 0.18^{**}$
ASM	0.44	1.51 ± 0.50	$1.70 \pm 0.15^{**}$
ASM	0.70	1.56 ± 0.26	$1.86 \pm 0.25^{**}$
ASM	0.90	$1.81 \pm 0.22^{**}$	$1.86 \pm 0.25^{**}$
ASM	6.60	$1.86 \pm 0.25^{**}$	$1.91 \pm 0.21^{**}$
ASM	13.20	$1.86 \pm 0.22^{**}$	$1.96 \pm 0.23^{**}$
SA	0.10	1.33 ± 0.17	$1.75 \pm 0.15^{**}$
SA	0.50	1.26 ± 0.33	$1.70 \pm 0.15^{**}$
SA	1.00	1.29 ± 0.51	$1.64 \pm 0.13^{**}$
SA	2.50	1.23 ± 0.32	$1.56 \pm 0.26^{**}$
SA	5.00	1.15 ± 0.46	$1.57 \pm 0.22^{**}$
SA	10.00	1.50 ± 0.26	$1.81 \pm 0.22^{**}$
SA	25.00	$1.75 \pm 0.15^{**}$	$1.86 \pm 0.22^{**}$
SA	50.00	$1.91 \pm 0.21^{**}$	$2.02 \pm 0.13^{**}$
K_2HPO_4	1.00	1.32 ± 0.41	$1.70 \pm 0.15^{**}$
K_2HPO_4	5.00	1.26 ± 0.33	$1.57 \pm 0.22^{**}$
K_2HPO_4	10.00	1.16 ± 0.41	$1.48 \pm 0.33^{**}$
K_2HPO_4	25.00	1.19 ± 0.29	$1.43 \pm 0.28^{**}$
K_2HPO_4	50.00	1.16 ± 0.41	$1.37 \pm 0.18^{**}$
LSD 5%[2]		0.31	0.20

[1]Means of transformed data ($\sqrt{x + 0.5}$) standard errors of 10 leaves per treatment.
[2]LSD test: means are significantly different from the control at 5% level ($^{**}P \leq 5\%$).

Table 5. Effect of time interval between the application of ASM (0.07 mM) and inoculations with *C. gloeosporioides* on reaction type of detached cashew leaves

Clone	Time interval (h)	Average reaction type[1]	
		Isolate LARS 905	Isolate LARS 910
CCP-76	12	2.07 ± 0.11	2.12 ± 0
	24	1.86 ± 0.25	1.91 ± 0.21
	48	$1.19 \pm 0.45^{**}$	$1.44 \pm 0.18^{**}$
	72	$1.04 \pm 0.37^{**}$	$1.33 \pm 0.17^{**}$
	96	$1.18 \pm 0.36^{**}$	$1.19 \pm 0.45^{**}$
	Control	2.29 ± 0.15	2.32 ± 0.19
LSD 0.5%[2]		0.48	0.37
CCP-1001	12	1.23 ± 0.32	2.02 ± 0.13
	24	1.19 ± 0.45	1.91 ± 0.21
	48	1.16 ± 0.26	$1.56 \pm 0.26^{**}$
	72	1.10 ± 0.30	$1.44 \pm 0.18^{**}$
	96	1.00 ± 0.33	$1.18 \pm 0.36^{**}$
	Control	1.26 ± 0.33	2.28 ± 0.11
LSD 0.5%[2]		0.52	0.35

[1]Means of transformed data ($\sqrt{x + 0.5}$) $\pm$ standard errors of 10 leaves per treatment.
[2]LSD test: means are significantly different from the control at 0.5% level ($^{**}P \leq 0.5\%$).

was treated with ASM and infected by the isolate LARS 910 of *C. gloeosporioides* (susceptible interaction). Hence cashew leaves treated with ASM appear to require a threshold period for acquired resistance to develop.

In vivo *germination and appressorium formation* C. gloeosporioides *on ASM-treated leaves*

Microscopic studies showed that, with the exception of the highest concentration used (1.1 mM), ASM applied 96 h prior to inoculation did not affect spore germination or appressorium formation by *C. gloeosporioides* on treated leaves (Table 6). Since 1.1 mM ASM had no effect on fungal development (mycelial growth and/or conidial germination) *in vitro* it is possible that the compound at this concentration induces some host response on the leaf surface.

Seed treatment with ASM

Despite the well-known antibiotic properties of the cashew nut testa and shell (Nayudama and Rao, 1967; Kubo et al., 1993), a wide spectrum of fungi can survive saprophytically on cashew seeds. Following seed germination, young tissues may be susceptible to some of these fungi. Imbibing seeds of clone CCP-76 in an aqueous suspension of ASM (0.44 mM) for 24 h prior to inoculation significantly reduced (60%) the lesion development on seeds by isolate LARS-905, compared with seeds imbibed in water (data not shown). Most of the treated seeds exhibited a dark flecking reaction at the inoculation site, while on water-treated seeds larger sporulating lesions were formed. In addition, seedlings emerging from treated and inoculated seeds showed no symptoms of anthracnose disease, while 33% of seedlings emerging from non-treated seeds had necrotic lesions on the cotyledons, hypocotyl, or epicotyl.

Table 6. Effect of ASM on the pre-infectional behaviour of *C. gloeosporioides* (isolates LARS 905 and LARS 910) on detached cashew leaves

Clones	ASM (μM)	Germination (%)[1]		Appressoria formed (%)[1]	
		LARS 905	LARS 910	LARS 905	LARS 910
CCP-76	SDW	67.1 ± 1.8	66.8 ± 2.8	63.9 ± 3.2	63.7 ± 3.2
	0.4	65.9 ± 1.6	65.5 ± 2.4	62.4 ± 2.0	63.9 ± 3.2
	4.0	65.6 ± 2.0	65.4 ± 3.6	62.1 ± 1.9	64.5 ± 2.6
	44.0	65.6 ± 1.5	65.7 ± 1.5	62.8 ± 2.7	63.3 ± 2.2
	440.0	65.8 ± 1.5	64.5 ± 3.8	63.3 ± 3.9	62.1 ± 1.9
	1100.0	54.6 ± 1.3**	49.3 ± 1.1**	52.3 ± 1.5**	53.4 ± 1.7**
LSD 0.5%[2]		2.3	3.8	3.8	3.6
CCP-1001	SDW	66.0 ± 3.1	66.7 ± 3.3	64.5 ± 3.8	65.0 ± 2.7
	0.4	65.3 ± 2.7	65.6 ± 2.3	63.3 ± 2.2	62.6 ± 2.8
	4.0	65.4 ± 3.6	65.0 ± 2.1	63.9 ± 3.2	63.0 ± 2.5
	44.0	65.6 ± 2.3	65.6 ± 2.3	63.1 ± 2.1	61.4 ± 2.1
	440.0	64.5 ± 3.8	63.7 ± 3.2	63.4 ± 2.4	61.2 ± 1.6
	1100.0	52.5 ± 1.4**	52.2 ± 1.5**	52.6 ± 2.0**	49.3 ± 1.1**
LSD 0.5% [2]		4.2	3.5	3.8	3.1

[1]Means ± standard errors of the transformed data (arcsin $\sqrt{x\%}$) from 12 leaf-discs per treatment. Data recorded 15 and 36 h after inoculation (carried out 96 h after the treatments).
[2]LSD test: means are significantly different from the control at 0.5% level (** $P \leq 0.5\%$).

Systemic protection in seedlings

In susceptible interactions, almost all treatments (chemicals and concentrations used), whether applied to leaves 3–5 (Table 7), or as a soil drench (data not shown), increased the resistance of leaves 1 and 2 to infection by *C. gloeosporioides*. The lower concentration of K_2HPO_4 (5 mM) had a lower protective effect in the majority of cases. The highest concentrations of SA (50 mM) as well as ASM and DCINA (0.3 mM) caused some phytotoxic symptoms on sprayed leaves. In plants treated with a soil drench at the highest concentration of these activators, some older leaves abscissed; this was not seen with K_2HPO_4. In the more resistant clone × isolate combination (CCP-1001 × LARS 905) none of the treatments appeared to enhance protection (Table 7). This suggests that, while the defence activators may shift a susceptible disease reaction type towards resistance, they do not boost the reaction of clones already expressing a degree of resistance to the anthracnose pathogen.

Field experiment

Table 8 shows the results of a field experiment using three cashew clones, based on averages of 5 replicates. Table 9 shows the corresponding result of ANOVA tests. All the chemical treatments had less anthracnose symptoms than the water control, and differed significantly from the control at the 1% level. There were no significant differences, however, in the responses of the three clones, or in the interaction between clones and treatments. The most effective treatments were ASM or DCINA at 130 μM, or both concentrations of SA. These treatments did not differ significantly in their ability to reduce anthracnose symptoms in the field. No phytotoxic effects were observed with drench treatments of any of the chemicals under field conditions.

Discussion

The introduction of the commercial plant defence activator, acibenzolar-S-methyl (ASM, Bion®; Kunz et al., 1997) provided a new option for chemical management of plant diseases based on the induction of host resistance. Application of nutrients, especially phosphate salts (Reuveni and Reuveni, 1998), and vitamins (Dong and Beer, 2000) can have systemic effects on plant resistance to pathogens. The mechanisms of induction, signal pathways, and expression of induced resistance are being intensively studied in annual crops and genetic models such as *Arabidopsis* (Delaney, 1997; Hammerschmidt, 1999). Much less is known, however, about the nature and expression of such systemic effects

Table 7. Effect of previous treatment of 3rd, 4th, and 5th leaves of cashew with chemicals on the resistance of the 1st and 2nd leaves against infection by *C. gloeosporioides* (isolates LARS 905 and LARS 910)

Clone	Spray treatment of the 3rd, 4th, and 5th leaves (mM)		Average severity[1]			
			LARS 905		LARS 910	
			1st	2nd	1st	2nd
CCP-76	SDW		2.27 ± 0.1	2.12 ± 0.0	2.34 ± 0.0	2.15 ± 0.14
	DCINA	0.1	$1.44 \pm 0.4^{**}$	$1.34 \pm 0.2^{**}$	$1.53 \pm 0.1^{**}$	$1.58 \pm 0.0^{**}$
	DCINA	0.3	$1.58 \pm 0.0^{**}$	$1.34 \pm 0.2^{**}$	$1.68 \pm 0.2^{**}$	$1.46 \pm 0.2^{**}$
	ASM	0.1	$1.46 \pm 0.2^{**}$	$1.58 \pm 0.0^{**}$	$1.44 \pm 0.4^{**}$	$1.34 \pm 0.2^{**}$
	ASM	0.3	$1.58 \pm 0.0^{**}$	$1.46 \pm 0.2^{**}$	$1.58 \pm 0.0^{**}$	$1.46 \pm 0.2^{**}$
	K_2HPO_4	5.0	$1.77 \pm 0.2^{**}$	$1.58 \pm 0.0^{**}$	$1.87 \pm 0.0^{**}$	$1.77 \pm 0.2^{**}$
	K_2HPO_4	50.0	$1.58 \pm 0.0^{**}$	$1.58 \pm 0.0^{**}$	$1.68 \pm 0.2^{**}$	$1.58 \pm 0.0^{**}$
	SA	5.0	$1.22 \pm 0.0^{**}$	$1.22 \pm 0.0^{**}$	$1.46 \pm 0.2^{**}$	$1.22 \pm 0.0^{**}$
	SA	50.0	$1.34 \pm 0.2^{**}$	$1.22 \pm 0.0^{**}$	$1.58 \pm 0.0^{**}$	$1.22 \pm 0.0^{**}$
LSD 5% [2]			0.31	0.17	0.29	0.25
CCP-1001	SDW		1.58 ± 0.0	1.46 ± 0.2	2.27 ± 0.1	2.20 ± 0.1
	DCINA	0.1	1.17 ± 0.4	1.00 ± 0.5	$1.68 \pm 0.2^{**}$	$1.58 \pm 0.0^{**}$
	DCINA	0.3	1.34 ± 0.2	1.05 ± 0.3	$1.58 \pm 0.0^{**}$	$1.34 \pm 0.2^{**}$
	ASM	0.1	1.22 ± 0.0	1.05 ± 0.3	$1.68 \pm 0.2^{**}$	$1.58 \pm 0.0^{**}$
	ASM	0.3	1.17 ± 0.4	1.05 ± 0.3	$1.68 \pm 0.2^{**}$	$1.46 \pm 0.2^{**}$
	K_2HPO_4	5.0	1.46 ± 0.2	1.17 ± 0.4	$1.77 \pm 0.2^{**}$	$1.77 \pm 0.1^{**}$
	K_2HPO_4	50.0	1.00 ± 0.5	1.17 ± 0.4	$1.58 \pm 0.0^{**}$	$1.46 \pm 0.2^{**}$
	SA	5.0	1.05 ± 0.3	0.87 ± 0.3	$1.22 \pm 0.0^{**}$	$1.22 \pm 0.0^{**}$
	SA	50.0	1.22 ± 0.0	1.05 ± 0.3	$1.05 \pm 0.3^{**}$	$1.22 \pm 0.0^{**}$
LSD 5% [2]			0.53	0.62	0.27	0.25

[1]Means of transformed data ($\sqrt{x + 0.5}$) $\pm$ standard errors of three plants (first and second leaves from the top to the bottom) per treatment.
[2]LSD test: means are significantly different from the control at 5% level ($^{**} P \leq 5\%$).

Table 8. Disease reaction of three cashew clones (CCP-76, CCP-1001, and CAP-14) to infection by *C. gloeosporioides* (isolate 910) in the field, following soil drench with water or chemical activators (in different concentrations) in a strip-plot design

Treatment	Mean disease score[1] of clones		
	CCP-76	CCP-1001	CAP-14
Control	3.00 (1.76)	3.00 (1.76)	2.16 (1.63)
ASM 13 µM	1.58 (1.44)**	1.58 (1.44)**	1.38 (1.37)**
ASM 130 µM	0.33 (0.91)**	0.33 (0.91)**	0.16 (0.81)**
DCINA 13 µM	1.19 (1.30)**	1.38 (1.37)**	1.19 (1.30)**
DCINA 130 µM	0.16 (0.81)**	0.33 (0.91)**	0.16 (0.81)**
SA 2600 µM	0.16 (0.81)**	0.16 (0.81)**	0.33 (0.91)**
SA 8700 µM	0.00 (0.71)**	0.00 (0.71)**	0.00 (0.71)**

[1]The back-transformed means of anthracnose score of five replicate trees, and the average of transformed data ($\sqrt{x + 0.5}$) in brackets. LSD values for comparing the two chemical treatments over the three clones are 0.24097 at 5% level of significance and 0.32656 at 1% level.

in woody perennial plants. In the case of cashew trees, nothing is known about induced systemic resistance.

The results of the present study revealed that different concentrations of ASM, DCINA, SA, and K_2HPO_4 reduced the severity of anthracnose disease caused by *C. gloeosporioides* in cashew leaves. The maximum reduction of the disease in detached leaves, without significant phytotoxic effects, was obtained with 0.07 mM ASM and DCINA, 5 mM SA and 50 mM K_2HPO_4, with an interval of at least 48 h between the spray application and inoculation. According to Siegrist et al. (1997), a minimum period of 96 h was necessary for ASM or DCINA (0.1 mM) to induce resistance on bean leaves against *Uromyces appendiculatus*, *Rhizoctonia solani, Colletotrichum lindemuthianum* and *Xanthomonas campestris* pv. *phaseoli*. Ishii et al. (1999) reported that ASM induced resistance in cucumber against anthracnose and scab as quickly as 3 h prior to inoculation with the pathogens. Aguilar et al. (1998) showed that when 0.22 mM ASM was applied to cocoa, the best protection against *Crinipellis perniciosa* was obtained with an interval of 48 h between treatment and inoculation.

Tests *in vitro*, along with microscopic examination of treated, inoculated leaves showed that concentrations lower than 1.1 mM ASM, 1.2 mM DCINA, 5 mM SA, and 50 mM K_2HPO_4 were not inhibitory to the first steps of pathogen development. This is in

Table 9. Analysis of variance of data from the field trial (7 × 3 factorial experiment in a strip-plot design)

Source of variation[1]	Degrees of freedom	Sum of squares	Mean square	Computed F[2]	Tabular F	
					5%	1%
Replication	$(r-1)=4$	0.416	0.104			
Horizontal factor						
(Chemicals) (A)	$(a-1)=6$	12.817	2.136	20.94**	2.51	3.67
Error (a)	$(r-1)(a-1)=24$	2.453	0.102			
Vertical factor						
(Clones) (B)	$(b-1)=2$	0.052	0.025	1.72	4.46	8.65
Error (b)	$(r-1)(b-1)=8$	0.120	0.015			
A × B	$(a-1)(b-1)=12$	0.249	0.0207	1.74	1.96	2.58
Error (c)	$(r-1)(a-1)(b-1)=48$	0.573	0.0119			
Total	$(rab-1)=104$	16.666				

[1] cv(a) = 10.98%; cv(b) = 28.98%, and cv(c) = 9.87%.
[2] ** = significant at 1% level.

agreement with data from previous studies on other pathogens (Doubrava et al., 1988; Kessmann et al., 1994; Freidrich et al., 1996). The only exceptions were higher concentrations of DCINA and especially SA. Direct inhibitory effects of SA have been reported for both bacterial (Lyon et al., 1995) and fungal pathogens (Fought and Kuć, 1996). The reduction in disease severity on leaves treated with lower concentrations of compound is most likely due to mechanisms affecting pathogen infection stages after appressorium formation. Görlach et al. (1996) showed that ASM applied to wheat plants at 0.3 mM did not affect appressorium formation by the powdery mildew fungus *Blumeria* (*Erysiphe*) *graminis* f.sp. *tritici*, but reduced the formation of haustoria.

Different application methods have been used for the chemical induction of systemic resistance in plants. Pennazio et al. (1985) reported that a soil drench is an effective method for salicylate application on tobacco, while Walters and Murray (1992) found spraying to be more effective. Okey and Sreenivasan (1996) showed that salicylic acid applied either as a seed treatment, spray or soil drench induced systemic resistance in cacao against *Phytophthora palmivora.* Seed treatment with defence activators also enhanced resistance to soilborne and foliar pathogens in bean (Siegrist et al., 1997) and brassicas (Jensen et al., 1998). In the present study, on attached leaves, foliar sprays with the chemicals were slightly more effective than soil drench treatments, with 5 mM SA being more effective than the other treatments, while 50 mM SA as well as 0.3 mM ASM or DCINA caused significant phytotoxic effects. Only a narrow safety margin separates the rates at which exogenous SA is effective and the rate at which it is strongly phytotoxic (Kessmann et al., 1994). Imbibing seeds of the susceptible clone CCP-76 with 0.4 mM ASM protected seedlings emerging from such seeds inoculated with the anthracnose pathogen, while one third of untreated seedlings developed disease lesions. In field-grown plants, protection was conferred by a soil drench of concentrations as low as 0.01 mM ASM or DCINA, and 2.6 mM SA. These concentrations were not phytotoxic. It should be noted that natural disease pressure in the field was low at the time of the experiment, due to the predominantly dry conditions. Any natural inoculum present was therefore augmented by artificial inoculation of clones to ensure infection. This may have influenced results and the observed effects need to be confirmed in subsequent trials, ideally under natural epidemic conditions.

In experiments with seedlings under glasshouse conditions, significant systemic protection against anthracnose was seen using phosphate treatments. Other studies with woody hosts, e.g. mangos (Reuveni et al., 1998), have shown that foliar sprays with phosphate salts can significantly reduce infection by fungi such as powdery mildew. It is not yet clear whether such protection is due to induced resistance, or to some tonic effect. Recently, Orober et al. (1998) showed that K_2HPO_4 induces the same molecular markers of systemic acquired resistance as the biotic agent tobacco necrosis virus (TNV) in cucumber plants, although different to those induced by ASM. According to Reuveni and Reuveni (1995), the rapid absorption of phosphates by plants and their extreme mobility within tissues, as well as their low cost, low animal toxicity, comparative environmental safety and nutrient value, make them ideal foliar

fertilisers which, in addition, may contribute to disease control.

The current results suggest that defence activators and phosphate salts have the potential for control of anthracnose disease in cashew plantations, and should prove useful in integrated disease control, especially when combined with more resistant host genotypes (Cardoso et al., 1999).

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