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Involvement of antifungal compounds from rockmelon fruit rind (*Cucumis melo* L.) in resistance against the fruit rot pathogen *Fusarium oxysporum* f. sp. *melonis*.

Vinod Kumar · Robyn McConchie

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Abstract Fusarium rot caused by *Fusarium oxysporum* f. sp. *melonis*, causes significant postharvest losses in rockmelon crops. Although latent infection is often present in the field, symptoms of the disease may not appear until fruit maturity. The susceptibility of different-aged rockmelon fruit cv. "Colorado" was determined by inoculating fruit at different stages of development with a spore suspension of *F. oxysporum* f. sp. *melonis*. Disease symptoms appeared first and were more severe in older fruit compared to younger fruit. Disease symptoms on fruit 35 DAA (Days After Anthesis) and 42 DAA appeared within 3 days of inoculation and rapidly covered the fruit within 5 days. In contrast, disease symptoms on fruit 7 DAA appeared 6 days after inoculation and grew slowly. Extraction of antifungal compounds without involving acid hydrolysis from 7 DAA fruit rind did not show antifungal activity on TLC plates. However, hydrolysis of the ethyl acetate fraction resulted in a strong fungal inhibitory zone on agar plates against colonies of *F. oxysporum* f. sp. *melonis*. Separation of the hydrolysed crude extracts on TLC plates indicated the presence of two distinct antifungal zones with R_f 0.36 and 0.13 in young fruit 7, 14 and 21 DAA. The area of fungal inhibition of compound R_f 0.36 was greater than that of R_f 0.13 on the TLC plate. Extracts from mature fruit of 35 and 42 DAA did not have detectable levels of antifungal compounds. The decrease in the susceptibility of rockmelon fruit during maturity may be correlated to a decrease in the antifungal compounds in the fruit with maturity.

Keywords Induced resistance · Latent infection · Phytoalexin · Phytoanticipin · Systemic acquired resistance · Thin layer chromatography

V. Kumar (✉) · R. McConchie
Faculty of Agriculture Food and Natural Resources,
The University of Sydney,
Science Road,
Sydney, New South Wales 2006, Australia
e-mail: v.kumar@usyd.edu.au

R. McConchie
e-mail: r.mcconchie@usyd.edu.au

R. McConchie
Research Institute for Asia and the Pacific,
Old Teachers College, University of Sydney,
Manning Road,
Sydney, New South Wales 2006, Australia

Introduction

Fusarium rot caused by *F. oxysporum* f. sp. *melonis* is a major postharvest disease problem in rockmelon (*Cucumis melo* L) resulting in significant postharvest losses (Huang et al. 2000). The infection can enter the flower at anthesis and remain latent in the field throughout the growing period, with symptoms appearing as the fruit ripens or during postharvest storage (McConchie et al. 2007). Absence of infection during

the early stages of fruit development in rockmelon suggests that natural disease resistance mechanisms in young fruit may offer protection from pathogen attack.

Temporary halting of the pathogen infection process in immature fruit may result from several factors such as insufficient degradative enzyme production by the pathogen to invade the unripe fruit, insufficient nutrition for the pathogen, or presence of host preformed antifungal compounds (phytoanticipins) (Prusky 1996). Phytoanticipins are low molecular weight antimicrobial compounds that are present in the plant before challenge by microorganisms or are synthesized from pre-existing constituents in a host plant at the time of challenge (VanEttan et al. 1995). The role of antifungal compounds in natural disease resistance has been studied extensively in many horticultural crops (Prusky et al. 1990 and Terry et al. 2004) and often, quiescence of the pathogen is regarded as a mechanism to avoid the toxicity of these antimicrobial compounds (Prusky et al. 1983; Morrissey and Osbourn 1999).

Involvement of two classes of antifungal compounds, monoenes (1-acetoxy-2,4-dihydroxy-*n*-heptadeca-16-ene) and dienes (1-acetoxy-2-hydroxy-4-oxo-heneicosa-12,15-diene) have been identified in resistance against *Colletotrichum gloeosporioides* in avocado fruit (Prusky et al. 1990). Similarly, the peel of unripe mango fruit contains a mixture of the antifungal compounds 5-alkylated resorcinols, 5-12-*cis*-heptadecenyl resorcinol and 5-pentadecyl resorcinol (Droby and Prusky 1986). The concentration of antifungal compounds in both avocado and mango decreases with fruit maturity, resulting in the susceptibility of fruit to pathogen infection. In strawberry, a decrease in antifungal terpenes has been correlated with the decrease in natural disease resistance of fruit against *Botrytis cinerea* (Terry et al. 2004). In cucurbits, phenolic phytoalexins are reported to play an important role in resistance against foliar disease in cucumber (Daayf et al. 2000). These compounds are present in a glycosylated form in the fruit tissue and increase in concentration following the treatment with defence elicitor Milsana® (a plant extract from *Reynoutria sachalinensis*). However, the role of antifungal compounds in the natural defence mechanisms of rockmelon fruit has not been investigated.

Currently the melon industry in Australia relies on fungicidal dip treatments for the control of storage rots (Bokshi et al. 2007). Development of resistance against the conventional fungicides and growing concerns over food safety and effects on the environment, have focused interest on finding alternative means of disease control (Schirra et al. 2000). Inducing host resistance during the period of pathogen susceptibility (28 DAA–42 DAA) may be one strategy that provides an alternative to conventional methods of disease control. Enhanced natural disease resistance in horticultural crops may be achieved through use of elicitors to induce host defences, including increased levels of preformed antifungal compounds (Joyce and Johnson 1999). The present study was undertaken to evaluate the difference in the levels of resistance between unripe and mature rockmelon fruit against *F. oxysporum* f. sp. *melonis* and determine whether there is involvement of pre-formed antifungal compounds (phytoanticipins) in the resistance of unripe fruit against Fusarium rot.

Material and methods

Plant material

Seeds of rockmelon (*Cucumis melo* L) cv. Colorado (Syngenta Seeds Care, Australia) were germinated in trays using Premium Potting Mix (Debco Pty. Ltd., Australia) in a glasshouse in November 2005. After three weeks the seedlings were transplanted into raised beds (120 cm×7 m) covered with black plastic in a single row, spaced 60 cm apart. The plants were irrigated twice daily through drip irrigation for six minutes delivering 60 l water/day. The plants were fertilised by applying NPK (12:6:12) @1.2 t/ha as a basal dose and 1.8 kg of slow release Osmocote (Scotts® Australia, NSW, Australia) as band application (by cutting holes in the plastic) 2 weeks after transplanting. The plants were randomly divided into three blocks of 40 plants each.

At anthesis, female flowers were tagged to monitor the days after anthesis (DAA). At 7, 14, 21, 28, 35, and 42 DAA, three fruit from each block showing no sign of infection were randomly harvested. The fruit were washed with water, rinsed with 70% ethanol to remove any surface pathogens and were air dried.

Inoculum preparation

A fresh culture of the bioassay organism *C. cladosporioides* was obtained from the labeled collection at

Food Science Australia, North Ryde, NSW, Australia, and was maintained by subculturing every fortnight on V8 agar media (Hernandez-Perez and du Toit 2006). A culture of *F. oxysporum* f. sp. *melonis* was isolated from naturally infected melon fruits in the field, identified by the Fusarium laboratory at the University of Sydney and maintained on carnation leaf agar (Burgess et al. 1988). Koch's postulates were performed by inoculating mature melon fruit with the Fusarium strain as described in the following section. The pathogen was re-isolated from the infected fruit. Seven to ten day old cultures were used for all experiments.

Fruit inoculation and disease assessment

A total of nine fruit for each age group were analysed for disease assessment during the experiment. Fruit were wounded (3 mm long×2 mm wide×5 mm deep) with a metal scalpel and inoculated at eight sites along the shoulder with 20 µl of spore suspension of *F. oxysporum* f. sp. *melonis* (approx. 2×10^5 spores.ml^{-1} in Czapek Dox broth). Inoculated fruit were randomly placed in cardboard boxes, covered with plastic wrap and held in dark incubators at 23±2°C and 95–100% relative humidity (Terry et al. 2004). Fruit were assessed daily for 10 days for disease development and scored according to Huang et al. (2000) on the basis of number and area of lesions:

1- no symptoms; **2**-one lesion less than 1 cm in diameter (≤5% infection) ; **3**-one lesion between 1 and 3 cm or two lesions each with an area less than 2 cm (≥5% to ≤15% infection); **4**-one lesion larger than 3 cm but smaller than 5 cm or two lesions each of them larger than 2 but smaller than 3 cm (≥15% to ≤30% infection); **5-** one lesion larger than 5 cm or more than 3 lesions smaller than 4 cm (≥30% infection).

Extraction of antifungal compounds

Two methods were used for the extraction of antifungal compounds from the fruit rind. Freshly harvested uninoculated fruit were used in both methods.

Method 1: Extraction of non-conjugated (glycosylated free) antifungal compounds without acid hydrolysis.

Freshly harvested fruit tissue (20 g, 8–10 mm deep) were ground into fine powder in liquid nitrogen using a mortar and pestle and homogenised in 25 ml ethanol (95% v/v) and centrifuged at 1,500 g for 5 min. The homogenate was filtered and partitioned three times with petroleum ether. The petroleum ether fraction was named fraction 1 (F-1). The ethanolic fraction was concentrated to one third of its original volume and partitioned three times against equal volumes of ethyl acetate. Both ethanolic and ethyl acetate fractions were evaporated to complete dryness, re-suspended in 80% ethanol and named as fraction 2 (F-2) and fraction 3 (F-3) respectively. All the fractions were stored at −20°C for further use in bioassays.

Method 2: Extraction of glycosylated conjugated antifungal compounds with acid hydrolysis

Extraction of glycosylated compounds required a modified method as described by McNally et al. (2002). Fruit tissue (2 g, 10–12 mm deep) was ground in 80% acidified methanol (with 4 N HCl), pH-2 (10 ml methanol per gram leaf or fruit) and the homogenate was placed in a rotary shaker at 50 g for 24 h. The extract was vacuum filtered and the residue was washed with methanol repeatedly until the green colour was removed. The dark green solution was partitioned against an equal volume of petroleum ether three times to remove the pigments and lipophilic compounds. This fraction containing pigments was named fraction 1 (F-1). The methanolic fraction was roto-evaporated at 38°C to one third of its original volume. The concentrated aqueous fraction was then partitioned against an equal volume of ethyl acetate. The ethyl acetate fraction contained the non-glycosylated free phenolic compounds and named as fraction 4 (F-4). The methanolic fraction was acid hydrolysed with 4 N HCl at 100°C for 30, 60, 90 and 120 min. The hydrosylate was cooled and partitioned against an equal volume of ethyl acetate. Both methanolic and ethyl acetate fractions were evaporated to complete dryness, re-suspended in 80% ethanol and named as fraction 2 (F-2) and fraction 3 (F-3) respectively. All the fractions were stored at −20°C for further use in bioassays.

Fruit rind and flesh of unripe avocado was also extracted using the method of Prusky et al. (1982), and incorporated in experiments as a positive control.

A blank without the fruit tissue was used as a negative control in both methods.

Agar plate bioassay

The efficacy of antifungal compounds against *F. oxysporum* f. sp. *melonis* was tested using agar plate bioassay by scraping the antifungal compounds at R_f 0.36 and 0.13 from TLC plates and re-suspending them in 80% methanol. Twenty µl aliquots of active compounds were applied to 4 mm diameter filter paper discs and were placed in PDA petri-dishes freshly seeded with *F. oxysporum* f. sp. *melonis* (approx. 2×10^5 spores.ml^{-1}). The plates were covered and incubated at 25±2°C for 72 h, after which the area of fungal inhibition was recorded. Ten replicates were used for each agar plate bioassay.

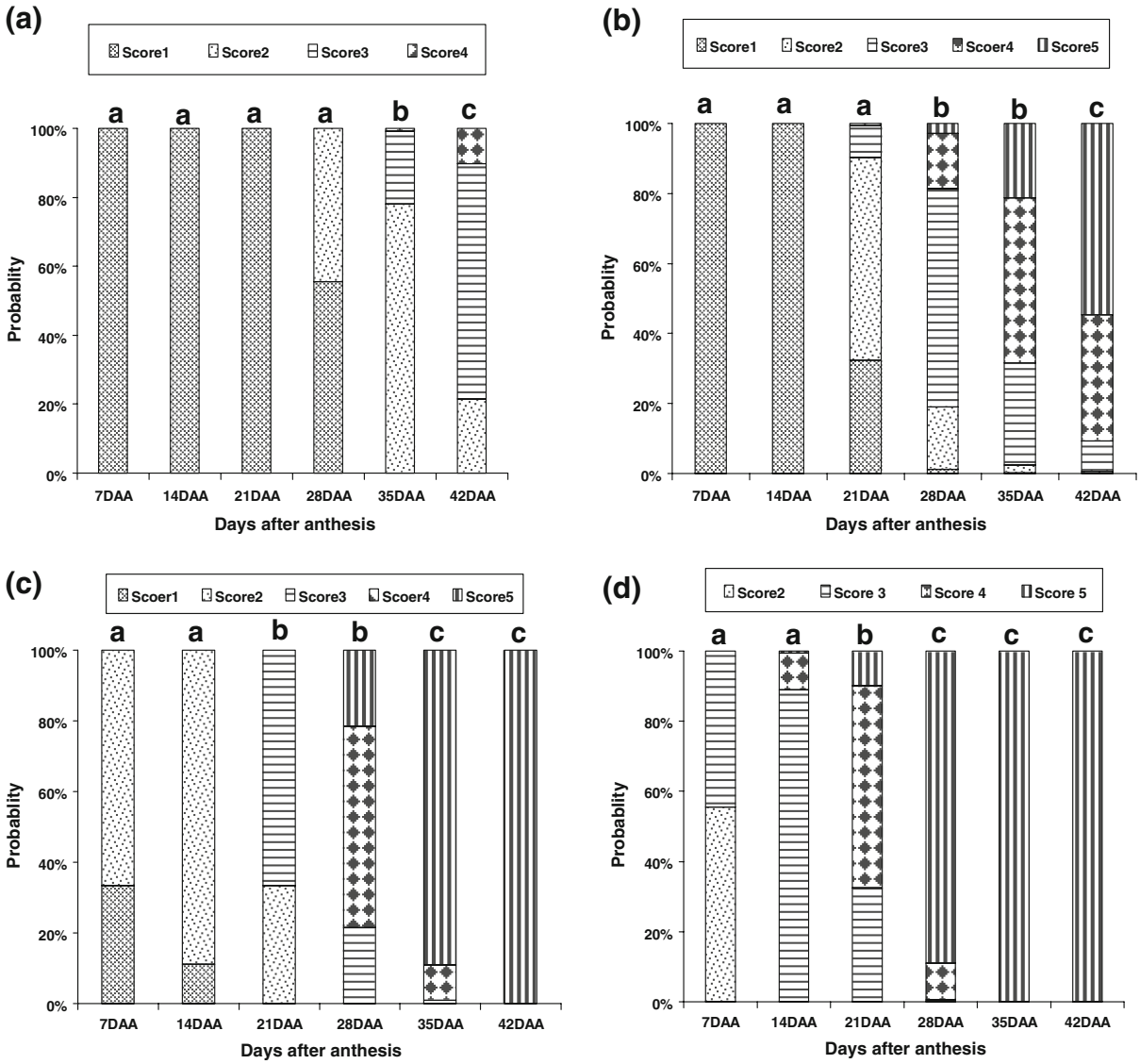

Fig. 1 Disease severity of rockmelon fruit **a** 4, **b** 5, **c** 6 and **d** 7 days after inoculation with *Fusarium oxysporium* fsp. *melonis*. Patterns in each column represent the percent probability of the score given for assessing the disease severity. ($P<0.001$) for all four days after inoculation. Letters on each bar represents the significant difference between the different fruit ages. Bars with the same letter are not significantly different from each other

TLC bioassay

TLC plates (20×20 cm) pre-coated with silica (POLYGRAM® SIL G/UV_{254}, MACHEREY-NAGEL GmbH & Co., Düren, Germany) were spotted using 60–180 µl of the crude extract from each fraction from both methods. Extracts of avocado, isolated as described earlier, were spotted on the plates as a positive control and a blank without the plant material was used as a negative control. Plates were developed in a TLC tank (30×20×10 cm) lined with filter paper to build a solvent saturated atmosphere using 100 ml of hexane: ethyl acetate: methanol (60:40:5 v/v/v) (Terry et al. 2003). Developed chromatograms were air dried and used for antifungal bioassays by spraying with a spore suspension of *C. cladosporioides* (approx. 2×10^7 spore ml^{-1}) in Czapek Dox nutrient solution. The *C. cladosporioides* was used because of its dark color mycelium which makes zones of fungal inhibition easily visible on TLC plates. Plates were incubated at 98% relative humidity and 25°C for 48 h. A zone of fungal inhibition, where mycelial growth was absent, indicated the presence of antifungal activity (Klarman and Stanford 1968). The retention factor (R_f) of the antifungal zone was recorded (Randerath 1966).

Statistical analysis

The disease severity data was analyzed by repeated measures ANOVA (Analysis of Variance) using STATISTICA (data analysis software system, StatSoft Inc. 2005), where significant differences among the various fruit ages were obtained, and means were separated by LSD. For disease assessment experiment, the disease score was converted into probability graphs using MINITAB 14. For agar plate bioassay, area of inhibition was compared by one-way ANOVA.

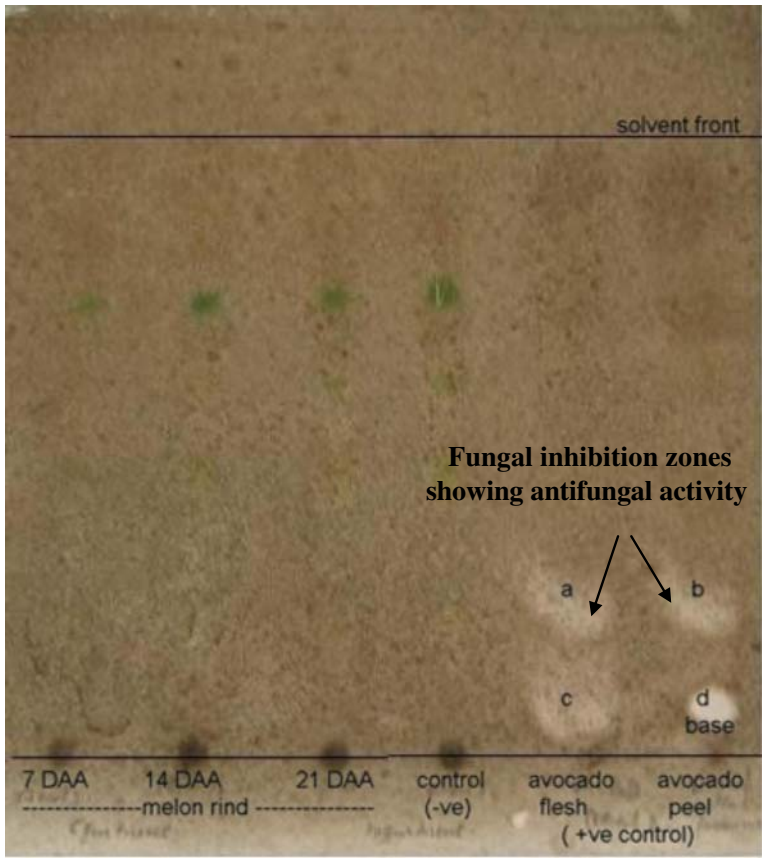

Fig. 2 TLC plate showing no sign of antifungal inhibition after spotting with crude extracts from rockmelon fruit (7, 14 and 21 DAA) without acid hydrolysis. A blank without the plant material and avocado extracts from flesh and peel were used as negative and positive controls respectively. a, b, c, d are the zones of fungal inhibition in flesh and peel of avocado fruit

Results

Susceptibility of rockmelon fruit against *F. oxysporum f. sp. melonis*

Disease symptoms appeared first and were more severe in older fruit compared to younger fruit. The overall probability of disease expression was significantly higher ($p<0.01$) in mature fruit (35 and 42 DAA) compared with other aged fruit (7, 14 21 and 28 DAA) (Fig. 1). Disease symptoms on the 35 and 42 DAA fruit first appeared three days after inoculation whereas no disease symptoms were observed on the 7 and 14 DAA fruit until six days after inoculation ($p<0.01$). Seven days after inoculation, (Fig. 1d) the probability of severe disease (greater than 30% of surface area) in the 35 and 42 DAA fruit was 100% ($p=0.01$). In contrast disease severity for fruit of 7 and 14 DAA was between 5 and 15% ($p=0.01$).

Separation and detection of preformed antifungal compounds in fruit rind

Without using acid hydrolysis for antifungal compound extraction (Method 1), no antifungal inhibition zone was apparent on the TLC Plate for 7, 14 and 21 DAA melon fruit extracts (Fig. 2). However two active compounds with R_f 0.07 (a and b) and 0.03 (c and d) showing antifungal activity were observed from both the peel and flesh of avocado (+ve control). A blank without the plant material used as a negative control also showed no antifungal zone.

Acid hydrolysed Fraction 3 (Method 2) from 7 DAA melon fruit indicated the presence of antifungal compounds on TLC plates with a white area deprived of germinated fungal spores (Fig. 3). Zones of fungal inhibition were detected at R_f 0.36 (compounds a, b and c) and 0.13 (compounds d, e and f), whereas no antifungal zone was present in the non-hydrolysed Fraction 2. The area of the antifungal zone expanded as

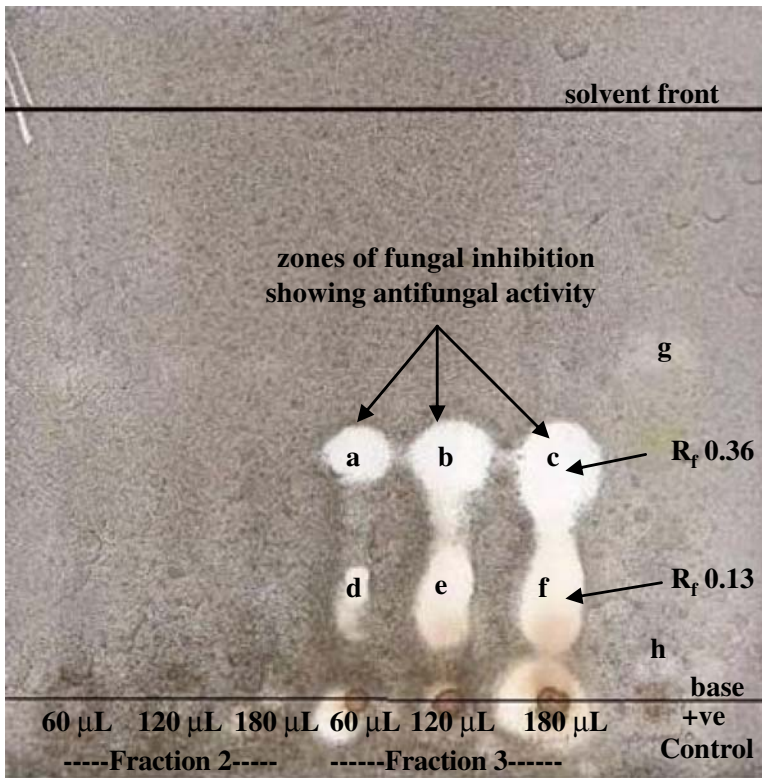

Fig. 3 TLC plate showing the difference in antifungal activity of Fraction 2 & 3 when spotted with 60, 120 and 180µl of crude extracts from 7 DAA melon fruit rind. Two antifungal zones with R_f 0.36 (a, b, and c) and R_f 0.13 (d, e, and f) were present in acid hydrolysed fraction 3. Two weak antifungal zone (g and h), which disappeared quickly were also present in the positive control

the volume of loaded sample increased from 60 µl to 180 µl in Fraction 3. Two weak antifungal zones appeared in the positive control using the crude extracts from peel of avocado fruits with R_f 0.45 (g) and 0.03 (h) respectively (Fig. 3) which disappeared within 3 days. Presence of antifungal zones was also found on the base line indicating that some of the compounds might not have moved along with solvent system.

Two different inhibitory zones at R_f 0.36 (compounds a, b, c, and d) and R_f 0.13 (compounds e, f, and g) were observed in samples taken 7, 14, 21 and 28 days after anthesis (Fig. 4), indicating the presence of antifungal compounds. The area of inhibition decreased with increasing fruit age, indicating that the antifungal activity of the crude extracts decreases with fruit maturity. The highest inhibition was observed in 7 and 14 DAA fruit whereas it decreased in 21 and 28 DAA fruit. No inhibitory zone was observed in mature fruit (35 and 42 DAA) and the control.

The inhibition zone of the compound at R_f 0.36 was larger compared with the compound at R_f 0.13 in younger fruit (7 and 14 DAA) (Fig. 4). The inhibitory zone of the compound at R_f 0.13 in both the TLC plates faded with time compared with the compound at R_f 0.36. Both compounds (R_f 0.36 and 0.13) were scraped from the TLC plates and tested against *F. oxysporum* f. sp. *melonis*. The compound at R_f 0.36 gave a larger inhibitory zone compared to the compound at R_f 0.13 (Fig. 5).

There was no difference in the antifungal activity at 30, 60, and 90 min hydrolysis but activity decreased when hydrolysed for 120 min (Fig. 6). An increase in antifungal activity was also observed when samples were applied with increasing volumes (20–120 µl).

Discussion

Results of the present study indicate that young rockmelon fruit have a natural defence mechanism which declines with fruit maturity. Development of disease symptoms in immature fruit was slow compared to mature fruit, indicating a higher degree of resistance in immature fruit. The absence of disease symptoms in young fruit (7 DAA), up to six days after inoculation, indicates a higher level of resistance to *F. oxysporum* f. sp. *melonis* compared to mature fruit (35–42 DAA) which showed symptoms covering between 5–30% of fruit surface area at the same time. Five days after

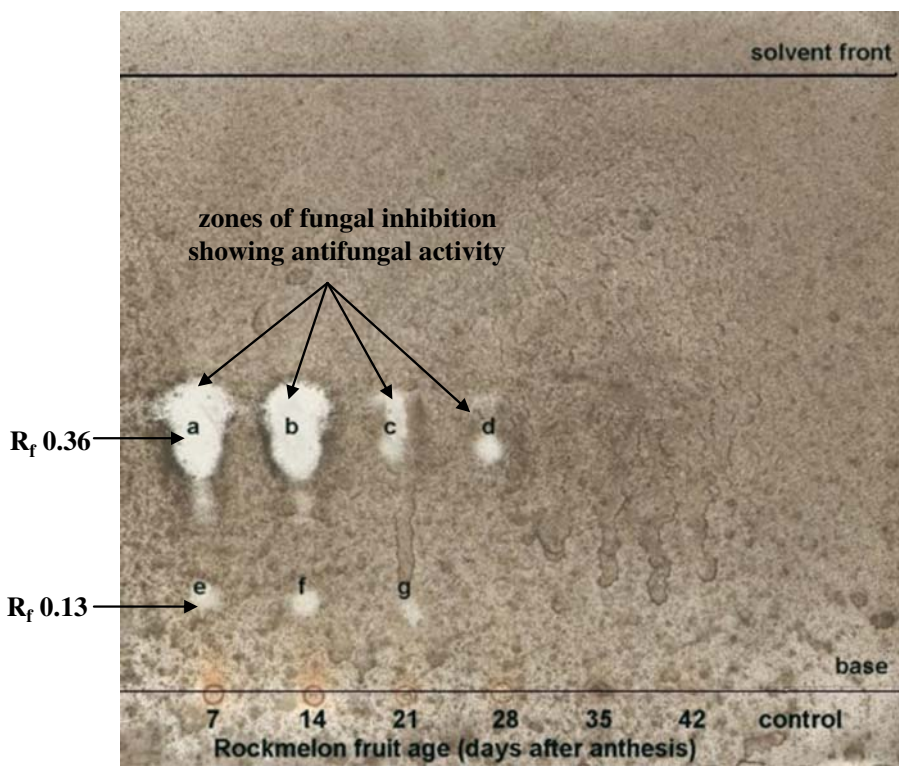

Fig. 4 TLC plate showing the zones of fungal inhibition in acid hydrolysed fraction 3 of crude extract (20 µl from rockmelon fruit rind). Two distinct zones with R_f 0.36 (a, b, c, and d) and R_f 0.13 (e, f, and g) appeared in fruit of 7, 14, 21, 28 and 7, 14 and 21 DAA respectively. No antifungal zone was observed in 35 and 42 DAA fruit and the control

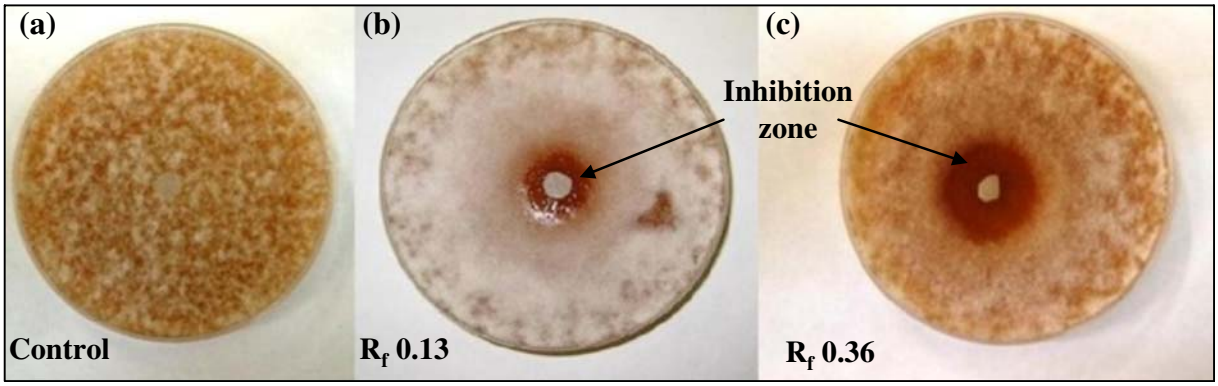

Fig. 5 Agar plate bioassay showing the antifungal activity of compounds with R_f 0.13 and 0.36 against the fruit rot causing organism *Fusarium oxysporium* f.sp. *melonis*

inoculation, the mature fruit were fully covered with mycelium, whereas young fruit were showing significantly less disease severity. In mature fruit, progression of disease was more rapid, suggesting a decline in resistance. These results are in agreement with Droby and Prusky (1986) who demonstrated that in mango fruit, *Alternaria alternata* remains quiescent until harvest, with disease symptoms only appearing in mature fruit. Similarly, in strawberry fruit, natural disease resistance against *Botrytis cinerea* and *C. cladosporioides* declines with fruit maturity (Terry et al. 2004).

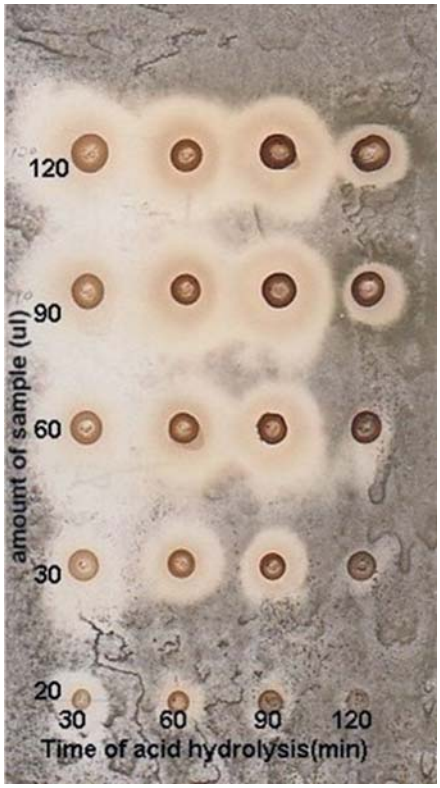

Fig. 6 Effect of hydrolysis time on the antifungal activity of crude extracts from rockmelon fruit rind (7 DAA) when applied to a TLC plate in different concentrations. Spotted TLC plates were sprayed with the bioassay organism *C. cladosporioides* without developing in any solvent system

The results presented in this study provide the first evidence that the rind of rockmelon fruit (*Cucumis melo* L) cv. 'Colorado' contains preformed antifungal compounds. Since no field infection was apparently present in the fruit, it is likely that the antifungal compounds found in the study were preformed (phytoanticipins). In other cucurbits, the presence of antifungal phytoalexins such as *p*-coumaric acid methyl ester (Daayf et al. 1997a) and rhamnetin, a flavonol aglycon, (Fawe et al. 1998) have been reported in cucumber leaves following treatment with Milsana® (an extract from the giant knotweed *Reynoutria sachalinensis*). A coniferyl aldehyde, *trans-p*-coumaric aldehyde has also been identified from green acorn squash (*Cucurbita maxima*) after elicitation with pectinase (Stange et al. 1999). However, this is the first report indicating the presence of antifungal compounds in rockmelon fruit of different maturities without involving a biological or chemical elicitor treatment.

Our results show that resistance in young rockmelon fruit at 7, 14 and 21 DAA is correlated with the presence of antifungal compounds. Two methods were used to extract the antifungal compounds from melon rind. The lack of antifungal activity in the non-hydrolysed samples suggested that the compounds

may be inactivated. Subsequent hydrolysis of the extracts led to the identification of two antifungal zones on the TLC plates indicating that the compounds may have been activated by deglycosylation. Glucose conjugated antifungal compounds have been reported in cucumber (Dercks and Buchenaure 1986; Daayf et al. 1997b, 2000; and Fawe et al. 1998). In their experiments, acid hydrolysis of the extracts was required to free the active compound from sugars to activate the antifungal nature of the compounds.

The timing of hydrolysis is critical to obtaining significant quantities of the aglycons without destroying antifungal activity (McNally et al. 2002). Some phenolic compounds such as flavonoid aglycons were easily degraded by acid hydrolysis (Merken et al. 2001), whereas other members of the same chemical family, such as C-flavonoids glycosides, are unaffected by acid hydrolysis and remain conjugated (Markham 1982). The present study found that maximal antifungal activity occurred after 30 min hydrolysis. No decrease in antifungal activity was observed when the extract was hydrolyzed for up to 90 min but activity did decline after 120 min hydrolysis. This result differs to the findings of McNally et al. (2002) for cucumber, where very low activity was found when the extract was hydrolysed for 30, 60 or 120 min and maximum antifungal activity was found when the extract was hydrolyzed for 90 min. The difference in the antifungal activity of the compounds may be due to species differences or because there are different compounds in melon rind and cucumber leaves and in their sensitivity to hydrolysis. None of the antifungal compounds relates to those present in cucumber and other cucurbitaceous crops. The decrease in the melon antifungal activity after 120 min of hydrolysis may also be due to the heat of the process denaturing of the antifungal compound, as reported by Merken et al. (2001).

The antifungal activity of the two compounds separated on the TLC plate differed when tested against the pathogen *F. oxysporum* f.sp. *melonis* on agar plates. There was a clear trend in declining resistance to *F. oxysporum* f.sp. *melonis* from the young melon fruit through to the mature fruit. Higher level disease resistance observed in the young melon fruit may be due to the presence of antifungal compounds in higher quantities, compared to mature fruit. In the present study, TLC assay shows that antifungal activity is higher in young melon fruit and decreases with fruit maturity. The decrease in the antifungal activity may have contributed towards susceptibility of the mature fruit. This evidence provides a strong argument that these preformed antifungal compounds play an active role in the resistance mechanism of the developing fruit. These results are in agreement with Prusky and Keen (1993) who stated that in avocado fruit antifungal diene decreases in fruit after harvest but increases in response to infection with *C. gloeosporioides*. Similar trends have also been reported in mango (Droby et al. 1986) and strawberry (Terry et al. 2004) where fruits have higher concentration of antifungal compounds when they are young but antifungal activity decreases with the fruit maturity.

However, there might be other factors which also contribute to quiescence of the pathogen in younger fruit in addition to antifungal compounds. Activity of peroxidases in muskmelon fruit correlates with the disease latency period and decreases with fruit maturity (Biles et al. 2000). They suggested that a decrease in peroxidase activity is associated with the susceptibility of the mature fruit. Apart from peroxidase activity, netting formation can also provide the pathogen the opportunity to start infection (Zhang and Bruton 1999). Development of netting normally starts 14 to 16 DAA and is completed around 28 to 30 DAA. Peroxidase activity is high during the netting formation and starts to decline after the netting formation is complete. This decline in peroxidase activity and completion of netting correlates with the start of susceptibility in the fruit. Based on these studies and our findings, it may be possible that a combination of these factors might play an important role in the susceptibility of the mature rockmelon fruit resulting in postharvest losses.

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