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Preformed antifungal compounds in strawberry fruit and flower tissues

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Abstract

Antifungal activity against the pathogen, *Botrytis cinerea*, and a bioassay organism, *Cladosporium cladosporioides*, declined with advancing strawberry fruit maturity as shown by thin layer chromatography (TLC) bioassays. Preformed antifungal activity was also present in flower tissue. The fall in fruit antifungal compounds was correlated with a decline in natural disease resistance (NDR) against *B. cinerea in-planta*. Crude extracts of green stage I fruit (7 days after anthesis) contained at least two preformed antifungal compounds (R_f = 0.44 and 0.37) that were not present in white and red stage fruit. These compounds were shown with TLC reagent sprays to be neither phenolics nor alkaloids. Positive reactions to Ehrlich's reagent suggested that R_f = 0.37 was a terpene. Most antifungal activity was found in the achenes of green stage I fruit. However, antifungal activity was found in all tissue types (viz. pith, cortex, epidermis) of green stage I fruit. TLC bioassays revealed that all fruit stages yielded antifungal activity at the origin (R_f = 0.00). The approximate area of fungal inhibition at the origin in green stage 1 fruit extracts was 1.87-fold and 1.73-fold greater than in white and red stages, respectively. TLC reagent sprays showed that the antifungal compound(s) at origin included phenolics. This

observation is consistent with previous reports that phenolic compounds in strawberry fruit are inhibitory to *B. cinerea*.

Keywords: Botrytis cinerea, grey mould, natural disease resistance, phytoanticipin, thin layer chromatography bioassay

1. Introduction

Strawberry fruit (*Fragaria ananassa* Duch.) vary in their inherent susceptibility to *Botrytis cinerea* Pers. (Teleomorph: *Botryotinia fuckeliana* (de Bary) Whetzel) according to their physiological status (Gilles, 1959) and genotype (Daugaard et al., 1999; Hébert et al., 2002). However, no strawberry cultivar is highly resistant to grey mould. *B. cinerea* tends to infect inflorescences in the field, but extensive fruit decay is only usually seen following harvest after when the fruit has reached and passed full harvest maturity (Powelson, 1960; Bristow et al., 1986). Therefore, *B. cinerea* generally remains quiescent until either physiochemical defences and/or stimulation in the host fall or rise, respectively, to allow invasion to continue. The inherent natural disease resistance (NDR) of strawberry fruit probably declines during fruit development and senescence, including during postharvest storage. Between flowering and fruit senescence there is evidently a period of relatively high resistance when grey mould development is rare.

Authors have attributed variation in NDR in strawberry fruit to skin strength (Gooding, 1976), fruit tissue firmness (Barritt, 1980) and flower susceptibility (Bristow et al., 1986). As with many other fruit patho-systems, the lack of nutritional requirements for the pathogen, activation of fungal pathogenicity factors and the presence/decline of preformed or induced antifungal compounds during fruit development (Prusky, 1996) may also influence NDR in strawberry fruit. However, relatively little work has been undertaken to characterise and identify preformed (phytoanticipin) and/or induced (phytoalexin) compounds with activity against pathogens (Table 1) or pests (Luczynski et al., 1990) of strawberry. With a view to better characterising NDR in strawberry fruit, experiments were performed to elucidate NDR in strawberry flowers and fruit. These involved determining the presence and partial identity of constitutive antifungal compounds during

development. The presence of antifungals in different parts of the strawberry flower and fruit was also investigated.

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2. Materials and methods

2.1. Plant material

Cold-stored maiden-year A+ grade strawberry cv. Elsanta plants were supplied by KG Fruits Ltd. (Kent, UK). The plants were grown in a glasshouse according to Terry and Joyce, 2000 in 1 litre capacity pots containing peat and expanded polystyrene (10 + 1 parts by volume). Temperatures ranged from 20 to 40°C during the day and 5 to 20°C during the night in spring (March-May) 2000.

2.2. Inoculum preparation

Single-spored isolates of the pathogen, *B. cinerea*, and the bioassay organism, *Cladosporium cladosporioides* (Fres.) de Vries, were recovered from naturally infected strawberry cv. Elsanta fruit (Terry et al., 2003). They were cultured in 9 cm diameter Petri plates on ½ strength PDA (potato dextrose agar; 19.5 g l^{-1} distilled water) (Oxoid Ltd., Berkshire, UK) at 22 ±1 and 20 ±1°C, respectively. Streptomycin (1.0 mg ml^{-1}) was added to the ½ PDA to inhibit potential growth of bacteria. The cultures were subjected to diurnal (12 h d^{-1}) UV-A lighting to induce sporulation.

2.3. Fruit sampling, inoculation and disease assessment

Twenty-two fruit each of green stage I (7 days after anthesis; DAA), white (21 DAA) and red (28 DAA) stages of maturity (Culpepper et al., 1935) with no signs of fungal infection were harvested at random. DAA was monitored following the tagging of flowers at anthesis. Fruit were wound inoculated by application of a 15 µl drop of a 4 day-old *B. cinerea* conidial suspension (2 x 10^4 conidia ml^{-1}) to the fruit shoulder (Adikaram et al., 2002). Non-inoculated fruit were used as controls. Fruit

were held in the dark at 5°C and 95 to 100% relative humidity (RH) in individual closed but vented polystyrene containers in a completely randomised design (Terry and Joyce, 2000). Disease severity resulting from inoculation or natural infection was assessed as the percentage area of each fruit covered by grey mould, and was recorded daily. 10% disease severity was defined as the first instance disease was visible on surface of fruit.

2.4. Thin layer chromatography

Glass-backed TLC plates (20 x 10 or 20 x 20 cm) coated with silica gel 60 or silica gel 60 F_{254} (Merck, Darmstadt, Germany) were used. The plates were spotted using a 5 or 10 μl micro-pipette with 5-100 μl of resuspended crude or sequentially extracted strawberry fruit and flower extracts made at 0.2 ml g^{-1} fresh weight (FW) (Droby et al., 1986). The protein synthesis inhibitor cycloheximide (0.5 mg ml^{-1}) and extracting solvents (99% v/v) were used as positive and negative controls, respectively. TLC plates were developed in either one or two dimensions (1-D, 2-D) at approximately 22°C in a TLC tank (20 x 20 x 10 cm) lined with filter paper to create a solvent saturated atmosphere. 1-D TLCs were developed either in 100 ml of running solvents comprised of hexane: ethyl acetate: methanol (A = 60:40:1; B = 60:40:10; C = 60:40:20; D = 60:40:30 v/v/v) (Terry et al., 2003) or in the organic phase of ethyl acetate: benzene: ethanol (4:1:1, v/v/v; Mussell and Staples, 1971). 2-D TLCs (Wedge and Nagle, 2000) were developed in solvent system A (1-D) and then solvent system D (2-D). Developed 1-D and 2-D chromatograms were air-dried and then either used for antifungal bioassays or sprayed with chemical reagents.

2.5. TLC bioassay

Developed chromatograms were sprayed with spore suspensions of either *C. cladosporioides* (2 x 10^7 spores ml^{-1}) or *B. cinerea* (2 x 10^6 conidia ml^{-1}) in Czapek Dox nutrient solution, respectively (Zainuri et al., 2001; Adikaram et al., 2002). Plates were incubated at 100% RH and 20°C for 3 to 6 days. Zones of fungal inhibition, where mycelial growth was absent, indicated the presence of antifungal activity (Klarman and Stanford 1968). Weak antifungal activity was defined as areas

with less dense lighter coloured mycelium from positive control (extracting solvent only). The retention factor (R_f) and areas of inhibition were measured and recorded.

2.6. Antifungal identification

Detection procedures for compounds on 1 and 2-D TLCs included inspection under visible and ultraviolet (254 and 336 nm) light before and during fuming with 98% v/v ammonia. After examination, ammonia was removed from plates by air drying for 1h. Replicate plates were sprayed with 10% w/v phosphomolybdic acid (PMA) in ethanol, and heated to about 100°C for 2 min (Terry et al., 2003). PMA is a general visualisation reagent for oxidizable compounds. More chemical class specific visualisation reagents were used for detection of phenolics (Folin-Ciocalteu; diazotized sulfanilic acid), alkaloids (Dragendorff's) (Waterman and Mole, 1994; Terry et al., 2003) and terpenes (Ehlrich's; Vincent et al., 1999). The R_f and colour of detected spots were recorded and compared to zones of fungal inhibition on duplicate bioassay chromatograms.

2.7. Crude extraction of whole strawberry fruit at different development stages

Whole green I, white and red stage strawberry fruit without calyxes (50 g FW) were randomly harvested, immediately snap frozen in liquid nitrogen and stored at -18°C until use. Each sample extract was prepared according to Terry et al. (2003). Briefly, fruit was ground in liquid nitrogen and added into 99% (v/v) ethanol at 3 ml g^{-1} FW. The mixture was homogenised, filtered, and then concentrated in a rotary evaporator (Buchi Rotovapor, Büchi Labortechnik AG, Flawil, Switzerland) under vacuum of 0.6 kPa at 40°C to approximately one third of the original volume. The concentrated extract was then partitioned twice with equal volumes of 99% (v/v) dichloromethane using a separating funnel. The lower dichloromethane layers were pooled and dried by adding 6 g of anhydrous $MgSO_4$. This mixture was then filtered, evaporated to dryness and resuspended in 99% (v/v) ethanol at 0.2 ml g^{-1} FW (Droby et al., 1986). The experiment was repeated twice to reduce possible variations in fruit chemistry.

2.8. Crude extraction of different tissues from whole strawberry fruit at different development stages

Green stage I, white and red strawberry fruit were harvested and dissected into pith, corticular parenchyma, epidermal tissue without achenes and achenes. Each tissue type (10 g FW) from each fruit stage and whole fruit were extracted as described above for whole fruit. The experiment was repeated twice.

2.9. Sequential extraction and column chromatography of green stage I tissues

Green stage I achenes (35 g FW; 10.12 g DW), whole fruit without achenes (75 g FW; 8.12 g DW) and whole fruit with achenes (125 g FW; 15.92 g DW) were sequentially extracted with solvents of increasing polarity at 3 ml g^{-1} FW (hexane, ethyl acetate and ethanol (99% v/v)). 1-D TLCs run in solvent system B were performed on each extract (0.2 ml g^{-1} FW). On the basis of similar/identical patterns on the TLC plate to treatment with PMA reagent, hexane and ethyl acetate fractions of achenes (50; 119 mg extract DW) and whole fruit with achenes (74; 83 mg extract DW) were each combined to increase the amount of material in preparation for purification. .

Hexane and ethyl acetate dry extracts were each chromatographed in a glass column of 7.5 cm length and 2 cm diameter packed with Silica gel 60 (Fluka, St. Louis, USA) using three solvent combinations of increasing polarity. The extracts were first dissolved in the minimum volume of hexane: ethyl acetate (80: 20 v/v) and then layered on top of the column pre-equilibrated with the same solvent. One thousand drop fractions of the column eluate were collected using a Pharmain LKB-Redifrac (Amersham BioSciences, Bucks., UK). The sequential solvent systems used for elution (40 ml) were hexane: ethyl acetate (60: 40 v/v), hexane: ethyl acetate: methanol 40:60:1 (v/v/v) and (20: 80: 1; v/v/v). Eighteen and 43 fractions were obtained from hexane and ethyl acetate combined extracts, respectively. These fractions were reduced to 3 and 7 fractions, respectively, by pooling on the basis of similar TLC behaviour to PMA run in solvent B. 1D-TLC bioassays were performed on pooled fractions run in solvent B.

2.10. Sequential extraction of green stage I achenes

Green stage I fruit were randomly harvested and their achenes removed. Achenes (10 g FW; 2.89 g DW) were prepared and sequentially extracted in ethyl acetate and ethanol as described above and then used for TLC bioassays.

2.11. Sequential extraction of different strawberry flower development stages

Strawberry flowers at white bud, full bloom and post-anthesis development stages (Jarvis and Borecka, 1968) were randomly harvested and prepared as previously described for fruit. Besides whole flowers, receptacle tissue including pistils from each of the 3 flower stages were also selected and prepared as described above. Each sample (10 g FW) was sequentially extracted in ethyl acetate and ethanol (99% v/v) as previously described and the extracts were used for TLC bioassays.

2.12. Data analysis and presentation

Disease severity data were analysed by using ANOVA using Genstat 5 Version 4.1. Where significant differences among treatments were obtained, mean separation was by LSD ($P = 0.05$).

3. Results

3.1. Disease severity

Disease expression studies at 5°C suggested that the natural disease resistance of strawberry cv. Elsanta fruit declined during fruit development (Table 2). The time to 10% disease severity for green stage I fruit was significantly ($P < 0.001$) longer than for white and red stages. Control and inoculated green stage I fruit were approximately 1.5 and 3 times more resistant in terms of time to 10% disease severity to grey mould after harvest than red fruit. In contrast to white and red stage fruit, *B. cinerea* conidia were never produced on green stage I fruit. Rather a mass of white mycelium was observed. Thus, normal development of the fungus was also inhibited on green stage I fruit. Natural infection was always caused by *B. cinerea*. Time to 10% disease severity was significantly shorter ($P < 0.001$) for inoculated fruit than

control fruit. There was no significant ($P > 0.05$) interaction between fruit stage and inoculation. Thus, fruit development stage effects were similar for both ± inoculation treatments. No difference was observed in the morphology of grey mould infections between inoculated and control fruit of the same development stage.

INSERT TABLE 2

3.2. Preformed antifungals in strawberry fruit tissue extracts of different developmental stages

Crude ethanol extracts of green stage I, white and red stage strawberry cv. Elsanta fruit contain varying degrees of antifungal activity as shown by 1-D TLC bioassay (Plate 1). Green stage I fruit extracts contained at least two colourless preformed antifungal compounds (R_f 0.44 and 0.37) that were not present in white and red stage fruit (Table 3). Negative TLC plate reactions to UV-C, phenolic and Dragendorff's reagent sprays suggest that these compounds were neither phenolics nor alkaloids, respectively (data not shown). Low intensity to but positive reactions of R_f 0.37 to Ehrlich's reagent suggested that it may be a terpene.

INSERT PLATE 1
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Crude extracts of white and red fruit contained a yellow-coloured non-polar preformed antifungal compound (R_f 0.86) of weak intensity that was not found in green stage I fruit (Table 3). All fruit maturity stages showed antifungal activity at the origin of TLC bioassay (Plate 1). The approximate area of fungal inhibition at the origin in green stage I fruit extracts (R_f 0.13-0.00) was 1.87 and 1.73-fold greater than in white and red fruit extracts, respectively (Plate 1; Table 3). Positive reactions to UV-C (dark), Folin Ciocalteu (dark blue) and sulfanilic acid (brown) reagent sprays suggest that origin compound(s) contain phenolics with antifungal properties against *C. cladosporioides* and *B. cinerea* (data not shown).

3.3. Distribution of preformed antifungals in green stage I fruit tissue

TLC bioassays of extracts from various green stage I fruit tissues revealed that greatest antifungal activity was in the achenes (Table 4). None-the-less, antifungal activity was found in all tissue types; the pith, cortex and epidermis of green stage I fruit. The total area of fungal inhibition for green stage I achenes crude extracts was at least 1.4-fold greater than for other tissue types. All tissues showed similar antifungal profiles to those seen in whole green I fruit crude extracts (Plate 1 and Table 3).

INSERT TABLE 4

B. cinerea and *C. cladosporioides* were both inhibited by antifungal compounds separated in all running solvent systems (data not shown). Fungal growth inhibition by extracts from green stage I fruit achenes was evident for 20, 10 and 5 μl aliquots of crude extract (data not shown). The water content of achenes is almost one third that of other green stage I strawberry fruit tissue. However, on a dry weight basis achenes contained more antifungal compounds compared to whole fruit, pith, cortex and epidermis. 1 and 2-D *C. cladosporioides* TLC bioassays revealed that green stage I fruit achenes may contain at least eight preformed antifungal compounds (Table 5; data not shown) with two compounds at R_f 0.42 and 0.36 (Table 5) apparently corresponding to R_fs at 0.44 and 0.37 in whole green stage I extracts (Plate 1 and Table 3). Antifungal activity was observed at origin on TLC plates for white and red stage achene tissue (Table 5). However, when run in an organic phase of ethyl acetate: benzene: 50% (v/v) ethanol (4:1:1) (Mussell and Staples, 1971), antifungal activity against *B. cinerea* was observed at R_f 0.76-0.58 for green stage I achenes (Table 6).

INSERT TABLE 5
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Green stage I fruit achenes sequentially extracted in ethyl acetate or ethanol displayed very different TLC behaviour to PMA reagent spray and bioassay. Sequential extraction and column chromatography of green stage I tissues confirmed

that antifungal activity was only in ethyl acetate (Table 7) and ethanol fractions. The ethyl acetate fraction showed similar but much less intense antifungal activity at R_fs 0.54, 0.48, 0.28, 0.19 and 0.10 to the previous achene extract bioassays run in solvent B (60:40:10; v/v/v) (Table 7) when applied at 30 µl (0.2 ml g^{-1} FW). In contrast to previous TLC bioassay results with achene extracts (Table 5), strong inhibition was not seen at lower applications of 10 and 20 µl aliquots. No antifungal activity in ethyl acetate fraction was observed at the origin. However, more polar phenolic compounds extracted in ethanol fraction revealed antifungal activity at origin.

INSERT TABLE 7

3.4. Preformed antifungal compounds in strawberry flower tissue extracts at different developmental stages

Flower stages sequentially extracted in ethyl acetate and ethanol showed similar TLC behaviour to both PMA reagent spray and in bioassays (data not shown) as seen in sequentially extracted green stage I fruit achenes (Table 7 and Table 8). Whole flowers at post-anthesis displayed greater antifungal activity at R_fs 0.38, 0.29, 0.21, 0.10, 0.00 than white bud and full bloom stages (Table 8). Whole flower ethyl acetate fractions had greater antifungal activity than pistils and receptacle tissue in all flower stages. There was no difference in the antifungal activity (R_f 0.00) of ethanol fraction between whole flower stages and flower tissues (Table 8).

INSERT TABLE 8

4. Discussion

4.1. Disease expression study

Natural disease resistance declined during fruit development (Table 2). Enhanced symptom expression with increasing strawberry cv. Elsanta fruit maturity confirms similar findings by Gilles (1959) and Powelson (1960) for other cultivars. The differences in time to 10% disease severity score and in the growth habit of *B.*

cinerea between different fruit development stages may be due to resistance factors and/or availability of nutrients (Prusky, 1996).

4.2. *Preformed antifungals in strawberry fruit tissue extracts of different developmental stages*

Antifungal activity of strawberry cv. Elsanta crude and sequential extracts declined with increasing fruit development stage. Greater antifungal activity was shown in green stage I fruit (R_f 0.37, 0.44, 0.13-0.00) as compared to white and red stage fruit extracts (R_f 0.00). The antifungal zone at R_f 0.37 may correspond to one or more of the three triterpene wound-induced 'phytoalexins', euscaphic acid, tormentic acid and myrianthic acid (compounds 1-3), previously identified through NMR spectral analysis in green stage strawberry cv. Hokowase fruit (Hirai et al., 2000). However, it is unlikely that the observed antifungals in present study were induced as strawberry fruit were not subjected to biotic stress treatment after harvest.

The compounds R_f 0.44 and/or R_f 0.37 may be similar to preformed antifungal compounds found at R_f 0.66 and 0.56 (hexane: ethyl acetate; 80: 20 v/v) in strawberry leaves (Vincent et al., 1999). These compounds conferred resistance to *C. fragariae*, and were undetectable by UV or anisaldehyde spray reagent. Anisaldehyde reacts with a variety of natural chemicals, including terpenes. TLC bioassays revealed that the concentrations and presence of antifungal compounds varied between anthracnose resistant and susceptible cultivars (Vincent et al., 1999). It was suggested that anthracnose resistance in strawberry leaves may depend upon the concentrations of these preformed antifungals at R_f 0.56 and 0.66 and also a third phytoalexin-like compound at R_f 0.32. This third compound that was only induced in the resistant cv. Sweet Charlie.

Mussell and Staples (1971) discovered two phytoalexin-like compounds (A, R_f 0.52; B, R_f 0.39) in strawberry roots. These compounds were induced by challenging cv. Surecrop plants that were resistant to red stele disease with *Phytophthora fragariae*. Inoculation of susceptible cv. Blakemore did not result in appearance of these compounds. However, another preformed antifungal compound (denoted C, R_f 0.84) was found in extracts of both cultivars regardless of whether or not they were inoculated. Using TLC bioassays, both phytoalexin-like compounds (A, B) and preformed compound C were inhibitory to *Cladosporium cucumerinum*. Compounds

A and B extracted from TLC plates and introduced into cultures of *P. fragariae* proved to be inhibitory to mycelial growth. Compound C was not inhibitory to *P. fragariae*. Identities of these three compounds were not determined.

Hirai *et al*. (2000) proposed from TLC analysis that inducible triterpenes found in green stage strawberry cv. Hokowase fruit which might confer resistance to *Colletotrichum fragariae* probably correspond to the phytoalexin-like compounds A and B found in strawberry cv. Surecrop roots (Mussell and Staples, 1971). In addition, compounds A and B were apparently not derived from phenylpropanoid pathway as phenylalanine-ammonia lyase (PAL) activity remained constant even after infection. This observation suggests that fruit may produce similar antifungal compounds to those in the roots. In the present study, one or more of the compounds found may be similar to the preformed and/or phytoalexin-like antifungal compounds discovered in strawberry roots (Mussell and Staples, 1971), leaves (Vincent et al., 1999) and fruit (Hirai et al., 2000).

TLC reagent sprays and bioassays indicate that green stage I fruit contain more phenolic compounds with antifungal activity than white and red stage fruit. This observation is consistent with previous research showing that a decline in phenolics during strawberry fruit development (Table 1) or after harvest (Jiang et al., 2001) is correlated to decreased NDR to *B. cinerea* and other postharvest diseases.

Jersch *et al*. (1989) found no evidence that green stage strawberry cv. Senga Sengana fruit contained preformed or inducible antifungal compounds when tested in agar or ELISA plate bioassays using *B. cinerea* or *Cladosporium herbarum*. They suggested that a decline in proanthocyanin (PA) concentration during fruit development governs *B. cinerea* quiescence through removing inhibition of pathogen-derived polygalacturonase (PG). PA was shown *in-vitro* to inhibit PG produced by *B. cinerea*. PG is one of the key enzymes involved in fungal pathogenicity (Labavitch et al., 1998). Resistance of immature strawberry fruit to external infection was correlated with pronounced deposition of PA in the epidermal layer (Jersch et al., 1989). The overall PA content in immature fruits was negatively correlated with mycelial development on inoculated fruit. PA concentration was higher in less susceptible strawberry cultivars. Harris and Dennis (1982) demonstrated that PG produced by *B. cinerea* was rapidly inactivated by naturally occurring phenolics in infected strawberry cv. Cambridge Favourite fruit. Extracts of more resistant green

stage strawberry fruit infected with *B. cinerea* showed approximately twice the endo-PG activity of extracts of white and red stage fruit.

Hébert et al. (2001) and Di Venere et al. (1998) confirmed that increased resistance to *B. cinerea* was positively correlated with PA concentration. However, Hébert et al. (2001; 2002), using a similar bioassay methodology to Jersch et al. (1989), found that aqueous extracts of immature strawberry cv. Chandler fruit did have direct antifungal activity against *B. cinerea* conidial germination and mycelial growth. Cultivars with higher concentrations of free and bound catechin, epicatechin and gallic acid were more resistant to *B. cinerea*. A decline in NDR was correlated with a decrease in specific proanthocyanins with antifungal activity during fruit development. Thus, the phenolic compounds at the origin on TLC bioassays of strawberry cv. Elsanta fruit extracts (Plate 1; Table 3) probably contain catechin, epicatechin and gallate.

4.3. Distribution of preformed antifungals in green stage I fruit tissue

Extracts of green stage I tissues revealed that greatest antifungal activity was in the achenes as compared to other tissue types. El Ghaouth et al. (1991) reported that aqueous extracts of achenes from half-red cv. Chandler fruit inhibited radial growth of *B. cinerea*, but not *Rhizopus stolonifer*, in Petri plate bioassays. However, aqueous extracts from receptacle tissue without achenes did not inhibit the growth of either fungus. Achenes appeared to contain more constitutive and potentially antifungal glucanohydrolase enzyme activity than did receptacle tissue. The increased susceptibility of ripe-stage strawberries to *B. cinerea* may be explained in part by reduced chitinase, β-1,3-glucanase and lysozyme enzyme activities in receptacle tissue. Gilles (1959) showed that sterilised strawberry juice from ripe fruit markedly stimulated *B. cinerea* conidial germination. It is not clear how the presence of antifungal compounds in the achenes of different fruit development stages may influence NDR of the fruit as their release may be hindered by the achene pericarp.

4.4. Preformed antifungal compounds in strawberry flower tissue extracts at different developmental stages

Extracts of whole strawberry cv. Elsanta flowers at post-anthesis showed greater antifungal activity than white bud and full bloom stages (Table 8). However, the susceptibility of flowers to *B. cinerea* increases with age to a maximum at petal-fall (Hennebert and Gilles, 1958). It is possible that antifungal compounds in strawberry flowers may play a role in initiating *B. cinerea* quiescence, as *B. cinerea* tends to infect strawberries at flowering (Powelson, 1960). However, more research is required to establish the exact role that these compounds may have in NDR during initial infection of strawberry flowers by *B. cinerea*.

5. Conclusion

Strawberry cv. Elsanta fruit and flowers contain preformed antifungal compounds which differ markedly in number and activity during flower and fruit development. The greatest antifungal activity was found in green stage I fruit extracts, especially in achenes. These observations indicate that NDR against *B. cinerea* depends on the initial presence and subsequent decline in preformed antifungal compounds during flower and fruit development. Full characterisation of preformed antifungal compounds in strawberry flower and fruit tissue and the elucidation of pathways involved in their biosynthesis is still required. More detailed information will allow for precise definition of the role antifungal compounds play in strawberry fruit defence against *B. cinerea,* other fungal diseases, and also pests. In turn, such knowledge may enable strategies to enhance the levels of these compounds in strawberry (Joyce and Johnson, 1999), through environmental manipulation, genetic transformation, conventional breeding and/or preharvest treatment with elicitors/plant activators (Terry and Joyce, 2000; Adikaram et al., 2002). In addition, an increase in the concentration of these antifungal compounds, many of which are phenolics, may lead to increased health benefits for consumers (Törrönen and Määttä, 2002).

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List of Plates

Plate 1: 1-D TLC (*C. cladosporioides*) bioassay of crude ethanol extract partitioned into an organic dichloromethane phase of green stage I (lane 1), white (lane 2) and red (lane 3) cv. Elsanta fruit (100 µl spot; 0.2 ml g^{-1} FW) and run in hexane: ethyl acetate: methanol (60:40:1 v/v/v). Dotted line = origin. Dashed line = solvent front. Similar results were seen for replicate TLC plates (data not shown).

List of Tables

Table 1: Preformed and inducible compounds with antifungal activity in strawberry plant and fruit tissues.

Table 2: Times to 10 grey mould disease severity of strawberry cv. Elsanta fruit at different development stages ± *B. cinerea* inoculation held at 5°C.

Table 3: Areas (cm^2) of fungal inhibition on 1-D TLC *C. cladosporioides* bioassay plates for the crude ethanol extract of green stage I, white and red strawberry cv. Elsanta fruit (100 µl spot; 0.2 ml g^{-1} FW) run in hexane: ethyl acetate: methanol (60:40:1 v/v/v).

Table 4: Areas (cm^2) of fungal inhibition on 1-D TLC *C. cladosporioides* bioassay plates for crude ethanol extract of green stage I strawberry cv. Elsanta whole fruit, pith, cortex, epidermis and achene fruit tissues (20 µl spots, 0.2 ml g^{-1} FW) run in hexane: ethyl acetate: methanol (60:40:1 v/v/v)

Table 5: Areas (cm^2) of fungal inhibition on 1-D TLC *C. cladosporioides* bioassay plates for achenes of crude ethanol extract of green I, white and red stage cv. Elsanta fruit (20 µl spot; 0.2 ml g^{-1} FW) run in hexane: ethyl acetate: methanol (60:40:1 v/v/v).

Table 6: Areas (cm^2) of fungal inhibition on 1-D TLC *B. cinerea* bioassay plates for crude ethanol extract of green stage I strawberry cv. Elsanta whole fruit, pith, cortex, epidermis and achene fruit tissues (20 µl spots, 0.2 ml g^{-1} FW) run in organic phase of ethyl acetate; benzene; 50% ethanol (v/v) (4:1:1 v/v/v)

Table 7: 1-D TLC *C. cladosporioides* bioassay and phosphomolybdic acid (PMA) R_f values of sequentially fractionated green I fruit tissues extracts (20 µl spots; 0.2 ml g^{-1} FW) run in hexane: ethyl acetate: methanol (60:40:10 v/v/v).

Table 8: 1-D TLC (*C. cladosporioides*) bioassay and phosphomolybdic acid R_f of sequential extracts (20 µl) of whole flower and pistils and receptacle tissue of white bud

(WB), full bloom (FB) and post-anthesis (PA) flower stages run in hexane: ethyl acetate: methanol (60:40:10 v/v/v).

1
2
3

1

Cultivar	Tissue	Chemical	Preformed or Inducible	Pathogen	Reference
Deutch Evern	green fruit	N.I.	preformed	*Botrytis cinerea*	Gilles (1959)
Surecrop	roots	N.I. [A]	preformed and inducible	*Phytophthora fragaria*; *Cladosporium cucumerinum*	Mussell and Staples (1971)
Blackmore			preformed	as above	
Howard and Surecrop type varieties (n = 18)	roots	quercetin	preformed	*Phythium irregulare*; *Rhizoctonia solani*; *Alternaria alternata*	Nemec (1973; 1976)
Senga Sengana	green fruit	proanthocyanins (flavan-3-ol dimers and oligomers)	preformed	*B. cinerea*	Jersch et al. (1989)
Chandler	half-red fruit achenes	N.I.	preformed	*B. cinerea*	El Ghaouth et al. (1991)

Hybride	leaves	catechin	induced	*Mycosphaerella fragariae*	Feucht et al. (1992)
Clea and Pajaro	various fruit development stages	proanthocyanins and catechins	preformed	*B. cinerea*	Di Venere et al. (1998)
Chandler	leaves	fragarin (316 Da)	preformed	*Collectrichum actutatum*; *C. fragariae*; *C. gleosporioides*	Filippone et al. (1999)
Chandler and Sweet Charlie	leaves	N.I.	preformed and inducible	*C. fragariae*	Vincent et al. (1999)
Houkouwase (same as Hokowase; Hancock, 1999)	green fruit	triterpenes (euscaphic, tormentic and myrianthic acids)	inducible	*Colletotrichum musae*[B]	Hirai et al. (2000)
Morioka-16 and	leaves	catechin	preformed	*A. alternata*	Yamamoto et al.

Hokowase					(2000)
Chandler, Seascape, Sweet Charlie and Annapolis	various fruit development stages	proanthocyanins (catechin, epicatechin, gallic acid)	preformed	*B. cinerea*	Hébert et al. (2001; 2002)
Elsanta	green fruit	N.I	preformed and induced	*B. cinerea*; *Cladosporium cladosporioides*	Adikaram et al. (2002)

2 N.I. = not identified
3 [A] = evidence that phytoalexins-like compounds were not derived from phenylpropanoid pathway (Mussell and Staples, 1971)
4 [B] = used as bioassay instead of *Colletotrichum fragariae*

5 Table 2

6

Fruit Development stage	Time to 10% disease severity (days)	
	+ Inoculation[A]	Control[B]
Green stage I	25.9	32.1
White	19.6	27.4
Red	8.7	19.5
Column means	*18.3*	*26.3*

7 [A]Mean separation by LSD [P = 0.05] = 2.9 d

8 [B]Mean separation by LSD [P = 0.05] = 2.4 d

9 Table 3

R_f value	Fruit Development Stage		
	green stage I	white	red
0.86	---[A]	~1.26[B]	~1.26[B]
0.44	1.96	---	---
0.37	3.85	---	---
0.13-0.00[C]	13.27	1.26 (R_f 0.00)	3.85 (R_f 0.00)
Total area	19.08	2.52	5.11

10 [A] = no inhibition zone

11 [B] = weak antifungal activity

12 [C] = overlapped R_f

13

14

15 Table 4

16

R_f value	Green I tissue type				
	whole	pith	cortex	epidermis	achenes
0.50	2.49	---	3.39	---	2.49
0.42	3.39	1.73	1.73	---	1.73
0.37	---[A]	2.49	1.73	4.42	4.99
0.10	---	---	---	---	0.62
0.00	0.62	0.62	1.11	1.11	3.39
Total area	6.50	4.84	7.96	5.53	13.22

17 ---[A] = no inhibition zone

18

19 Table 5

20

Antifungal R_f	Fruit Development Stage		
	green I	white	red
0.57	0.34	---[A]	---
0.52	0.77	---	---
0.44	2.74	---	---
0.36	1.57	---	---
0.26	0.34	---	---
0.22	0.34	---	---
0.10	0.24	---	---
0.00	1.60	0.24	0.24
Total area	7.94	0.24	0.24

21 cycloheximide (0.5 mg ml^{-1}), R_f = 0.05 (1.36); ethanol (99% v/v) = no R_f

22 ---[A] = no inhibition zone

23 Table 6

24

R_f value	Green I tissue type				
	whole	pith	cortex	epidermis	achenes
0.70	1.65	1.06	1.06	1.06	7.98
0.58	1.06	1.06	1.06	1.06	---[B]
Total area	2.71	2.12	2.12	2.12	7.98

25 ---[B] = overlapped R_f

26 Table 7

27

Pooled fractions	Extract dry weight (mg)	R_f values	
		PMA	Antifungal
2,3[B]	71	0.81	---[A]
7-9[B]	37	0.64	---
9-14[C]	18	0.70, 0.63	0.59, 0.49
22-23[C]	3	0.30	0.28
24-27[C]	16	0.30, 0.36	0.28
28[C]	17	0.36, 0.30, 0.12, 0.09	0.28
29-34[C]	30	0.12	0.28, 0.19
36-39[C]	9	0.00	---
40-43[C]	98	0.00	---

28 [A] = no inhibition zone

29 [B]combined hexane extract (dry weight before and after purification = 124 and 116 mg)

30 [C]combined ethyl acetate extract (dry weight before and after purification = 202 and 191

31 mg)

32

33

Table 8

Ethyl acetate						Ethanol					
Whole flower			Pistils and receptacle			Whole flower			Pistils and receptacle		
WB	FB	PA	WB	FB	PA	WB	FB	PA	WB	FB	PA
0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83
0.70	0.70	0.70	0.70	0.70	0.70	0.68	0.68	0.68	0.68	0.68	0.68
0.60	0.60	0.60	0.60	0.60	0.60	0.58	0.58	0.58	0.58	0.58	0.58
0.49	0.49	0.49	--- [A]	---	---	---	---	---	---	---	---
0.38[B]	0.38[B]	0.38[B]	---	0.38[B]	---	---	---	---	---	---	---
0.29[B]	0.29[B]	0.29[B]	0.29	0.29	0.29	---	---	---	---	---	---
0.26	0.26	0.26	---	---	---	---	---	---	---	---	---
0.21[B]	0.21[B]	0.21[B]	---	---	---	0.18	0.18	0.18	0.18	0.18	0.18
0.10[B]	0.10[B]	0.10[B]	0.10	0.10	0.10	---	---	---	---	---	---
0.05	0.05	0.05	0.05	0.05	0.05	0.04	0.04	0.04	0.04	0.04	0.04
0.00[B]	0.00[B]	0.00[B]	0.00[B]	0.00[B]	0.00[B]	0.00[B]	0.00[B]	0.00[B]	0.00[B]	0.00[B]	0.00[B]

[A] = no zone

[B] = compounds with antifungal activity