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Rye Bran Alkylresorcinols Inhibit Growth of *Penicillium expansum* and *Neofabraea perennans In Vitro* and *In Vivo* on Different Apple Cultivars

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Summary

Apples (*Malus domestica* Borkh.*)* are highly susceptible to fungi causing rots, especially in organic orchards where these rots cause major economic losses. Since the use of conventional fungicides is prohibited or restricted in many countries and in organic production, altogether, one alternative is to make use of bioactive compounds that can inhibit fungal growth on apples. Antifungal effects of alkylresorcinols (ARs), isolated from rye bran, were used in two different bioactive emulsions and tested *in vitro* as well as *in vivo* against two fungi, *Penicillium expansum* and *Neofabraea perennans*, which cause important storage diseases in apple. For both fungi, application of AR emulsions produced a 50 % reduction in mycelial growth and spore germination. The *in vivo* tests were carried out on wound-inoculated fruit of four apple cultivars, 'Aroma', 'Ingrid Marie', 'Frida' and 'Gloster'. Spraying the inoculated fruit with ARs decreased lesion area diameter caused by *P. expansum* with 17–52 % and lesion area diameter caused by *N. perennans* with 31–77 %, depending on the apple cultivar. For each fungus, the inhibitory effect of ARs was stronger in those apple cultivars that appear to have the highest level of disease tolerance, i.e. the smallest lesions on the control fruit. The level of sun exposure of the fruit had neither significant influence on the decay nor in the inhibitory effect obtained with the postharvest application of ARs.

Key words. blue mould – bull's eye rot – postharvest decay

Introduction

Apple (*Malus domestica* Borkh.) fruits show relatively high susceptibility to fungal fruit decay because of their low pH, high moisture content and beneficial nutrient composition (Moss 2002; Spadaro et al. 2004). The fungal diseases infesting fruits have increased over the world during the latest decades, due to the global temperature increase (Sholberg and Haag 1996; Amiri et al. 2008). Among the 90 different postharvest phytopathogens that have been described in apples (Davey et al. 2007), the most important fungi in Northern Europe are *Neofabraea* (*N. alba* and *N. perennans*), *Colletotrichum acutatum*, *Penicillium expansum*, *Botrytis cinerea*, and *Monilia sp.* (Palm and Kruse 2005; Weber and Palm 2010).

Neofabraea spp. is an important cause of postharvest fruit damage around the world (Lennox et al. 2004), and causes about half of the damage in Swedish apple storage houses (Tahir, unpublished). The fruit is infested during the growth phase in the orchard. The spores remain dormant in the lenticels or in physical wounds in the apple skin until the fruit reaches a certain stage in maturity during storage. At some point, the spores start to grow and produce brownish lesions that increase in size and number, and eventually cause rotting of the entire fruit (Weibel et al. 2005).

P. expansum is essentially a wound-mediated pathogen (Li and Xiao 2008). The symptoms are most common towards the end of the storage period when the fruits are more physiologically mature. Infested parts of the fruit develop lesions that first are brownish with white spores and later turn bluish green. The decayed tissue is watery with a translucent appearance and can easily be separated from the healthy tissue (Onions 1998).

Application of synthetic fungicides is still the most common method for protection of apples from fungal storage diseases. Due to perceived threats involving high residual toxicity and environmental pollution, postharvest

application of chemicals is not permitted in some countries while preharvest use is severely restricted (Janisiewicz and Korsten 2002). Therefore, alternative strategies against postharvest decay are explored, like biological pesticides (Morales et al. 2010), physical stress (Saftner et al. 2003) and natural bioactive materials (Neri et al. 2009).

Phenolic lipids constitute a highly diversified group of compounds derived from mono- and dihydroxyphenols, i.e., phenol, catechol, resorcinol, and hydroquinone. Two resorcinols characterised in mango, 5-(12-cis-heptadecenyl) resorcinol and 5-n-pentadecylresorcinol, have been shown to inhibit the growth of *Alternaria alternata* (Droby et al. 1987) and *Colletotrichum gloeosporioides* (Hassan et al. 2007) in immature mango fruit. Alkylresorcinols (ARs), a naturally occurring type of phenolic lipids present in the outer layer of cereal grains, have been shown to possess antibacterial and antifungal activity. A solution with 10 mg mL^{-1} ARs extracted from rye and dissolved in acetone has been shown to inhibit the *in vitro* growth of bread moulds *Aspergillus parasiticus* and *Penicillium chrysogenum* (Reiss 1989). In another *in vivo* study, significant inhibition of disease symptoms on apples experimentally infected with *P. expansum* was obtained after spraying the infected apples with several emulsions containing 0.025 % (m/v) ARs isolated from rye bran (Dey et al. 2013).

The objectives of this study were to assess antifungal activity of two AR emulsions, selected in the previous study (Dey et al. 2013), on the mycelial growth of *N. perennans* and *P. expansum in vitro*, and to study their inhibitory effect on postharvest decay (bull's eye rot and blue mold) in four apple cultivars ('Aroma', 'Ingrid Marie', 'Frida' and 'Gloster') that are commonly grown in the Nordic countries.

Materials and Methods

Preparation of AR emulsions

Alkylresorcinols (ARs) were isolated from rye bran (particle size 0.5–1 mm and containing 9 % of water as measured after freeze drying, Nord Mills, Sweden) by a fractionated supercritical fluid of carbon dioxide extraction as described earlier (Dey and Mikhailopulo 2009; Dey et al. 2013), used for preparation of bioactive emulsions. Ten grams of material were loaded in a thimble (100 mL of empty volume) and extracted with a two-step process. Conditions for the first step were set to: temperature 70 °C and time 2 h, and the co-solvent rate (ethanol) was 0.06 mass% of carbon dioxide flow rate. For the second step, the temperature was decreased to 45 °C, while extraction time was increased to 4 h, and ethanol to 10 mass% of carbon dioxide flow rate. In both steps, flow rate of CO_2 was 10 g min^{-1}, and the CO_2 pressure was kept at 250 bar. The carbon dioxide ($\geq$ 99.998 %) used for extraction was from AGA Gas (Sweden). The ARs concentration was quantified using the Fast Blue B colorimetric method (Landberg et al. 2009) with the synthetic standards for the standard curve monitoring of the optical changes on a spectrophotometer DU 800 (Beckman Coulter, Lab Vision, Sweden). ARs were identified and quantified first by colorimetric spectrophotometry, followed by preparative high pressure liquid chromatography (HPLC), solid phase extraction (SPE) and high performance thin layer chromatography (HPTLC) to check the quality and to determine the exact amount of pure AR. A known amount of AR dissolved in ethanol was transferred to 50 ml Falcon tubes and placed in a desiccator, then attached to a vacuum system to remove the ethanol. The two most promising AR emulsions according to our previous results (Dey et al. 2013) were then prepared. Both of these emulsions contained 0.025 % (m/v) ARs, since no difference on the inhibitory activity on *P. expansum* growth was observed by using 0.025 or 0.05 % concentration of ARs. Presumably, optimization of other ingredients such as solvents, emulsifiers and stabilizers may be more critical. These emulsions also contained 0.2 % (m/v) Tween 20, 1 % (m/v) trioleate (Sigma-Aldrich, USA), 2 % (m/v) oleylalcohol (FlukaChemika, Germany) and 2 % (m/v) PEG400 (Kebo Lab, Sweden). In addition, the first emulsion (ARs 1) contained 0.1 % xanthan gum (SIA Glass, Sweden) and 5 % (m/v) $CaCl_2$ (Merck, Germany) while the second emulsion (ARs 11) contained 0.2 % (m/v) xanthan gum and 5 % (m/v) NaCl (Merck, Germany). De-ionized water was added and adjusted to 100 ml. To dissolve the ARs, 0.5 % (m/v) Synperonic 91/6 (MB-Sveda, Sweden) and polydimethylsiloxan copolymer 'Q2–5247' (PDMS) (Dow Corning, USA) were used in emulsions ARs 1 and ARs11, respectively. Each suspension was mixed at high speed (13.500 rpm) for 1 min, using the homogenizer Ultra Turrax T25 (Janke& Kunkel GmbH & Co, Staufen, Germany). Prepared samples were kept at 8 °C for 2–3 days before being used. The emulsion was warmed up at 37 °C for 30 min, vigorously shaken and transferred to a spray gun, and then sprayed onto the apple fruit or the nutrient agar.

Fruit harvesting and determination of maturity

Four cultivars, 'Aroma', 'Ingrid Marie', 'Frida' and 'Gloster', were harvested at the experimental orchard in Balsgård, the Swedish University of Agricultural Sciences, during two seasons 2011–2012. A set of 15 fruit per cultivar, in three replicates, was used to determine fruit maturity. Fruit firmness and soluble solids content (SSC) were estimated by penetrometer (model FT-327, Effigy, Alfonsine, Italy, plunger diameter 11.1 mm, depth 7.9 mm) and refractometer (Atago, Japan), respectively. Starch index (SI) was evaluated by dipping slices of apple, cut at the equator, into iodine solution (15 g potassium iodide and 6 g iodine L^{-1}) for 1 minute, and using a grading scale where 1 = full staining (unripe) and 10 = no staining (severely overripe). The Streif index was calculated as (firmness $(SSC \times SI)^{-1}$). The colour of the sun-exposed and the shaded side of the

fruit, respectively, was measured as colour index (CI), using a Chromameter CR200 with 8 mm diameter window (Minolta Ltd. Osaka, Japan). Measurements were conducted on 3 spots on each fruit side (sun-exposed and shaded, respectively), and expressed as CI.

$$CI = (1000 \times a)\ (L * b)^{-1},$$

where (+a) indicates red colour, (−a) green colour, (+b) yellow colour, (−b) blue colour and (L) is the luminosity from white to black.

Isolation of fungi

N. perennans and *P. expansum* were isolated from apples harvested in a commercial orchard in 2010, and kept in cold-storage for several months until fruit rot symptoms developed. Fruit surface was sterilized for 30 s with 70 % ethanol and rinsed with sterile distilled water. Small sections from the growing margin of the fruit rot lesion were plated on acidified potato-dextrose agar (PDA) for *P. expansum* and on 1 % (m/v) malt extract agar (MEA) for *N. perennans*, and subsequently held at 24 °C for two weeks. Pure cultures were obtained by transferring the hyphal tips to new PDA and MEA plates respectively and incubating at 20 °C for 3 weeks. Thereafter, the pathogens were maintained separately as pure cultures at 4 °C. Conidial suspensions of *P. expansum* and *N. perennans* were prepared by removing spores of the two pathogens from the surface of the cultures on PDA and MEA, respectively, and suspending these in 5 mL sterile distilled water containing 0.05 % (v/v) Tween 80. The suspensions were filtered through four layers of sterile cheesecloth to remove any adhering mycelia, and spore concentrations were determined with a hemacytometer and adjusted to $1x10^5$ conidia mL^{-1}. These suspensions were used to test the antifungal effect of ARs *in vivo*.

In vivo testing of AR antifungal effects

After harvesting, apples were randomly divided into 8 sets per cultivar, with 15 fruits in each set, and three replicates. The apples were rinsed in ethanol (70 %) then in sterilized water to remove possible remains of fungicides and wild fungi, wounded twice with a nail (4 mm diameter and 4 mm depth) with one wound on the sun-exposed side and another on the shaded side, and inoculated by pipetting 20 μL of freshly prepared conidial pathogen suspension ($1x10^5$ conidia mL^{-1}) into each of the wound sites. Four sets were inoculated with conidial suspensions of *P. expansum* and the other four sets with conidial suspensions of *N. perennans*. One inoculated set per pathogen was left untreated as a control, another set was sprayed with 10 ml distilled water (30 °C), and the remaining two sets were sprayed with 10 ml of warm emulsions (30 °C) containing ARs 1 and ARs 11, respectively, 3 h after inoculation. Inoculated and treated fruits were stored in a cold storage room at 2 °C and 90 % RH in ambient atmosphere for 12 weeks. At the end of the storage period, decay on fruit inoculated with *P. expansum* was evaluated directly while fruit inoculated with *N. perennans* were transferred to a plastic chamber (18 ± 2 °C and 80 % RH) for one week before evaluations. Decay severity was estimated as surface decay lesion diameter. The inhibition level was calculated as percent related to lesion diameter on non-treated fruit. Possible impact of fruit side (sun-exposed or shaded) on lesion diameter was also investigated. A subjective assessment of fruit odour, appearance and colour was made. Three cultivars; 'Aroma', 'Ingrid Marie' and 'Frida' were inoculated with both fungi, while 'Gloster' was only inoculated with *P. expansum*.

Testing of AR antifungal effects on mycelial growth and pathogenicity

A set of three Petri dishes (90 mm in diameter), containing 10 ml potato dextrose agar (PDA), was prepared. After autoclaving and agar solidification, 5 ml ARs 1 was added to one of the plates and 5 ml of ARs 11 to another plate while the third plate was left blank as a control. Plates were incubated at 4 °C for 3 h. From actively growing *P. expansum* cultures, a mycelium plug (10 mm in diameter) was transferred to each of the three plates. Another set of three Petri dishes, containing 10 ml PDA, was also prepared. A mycelium plug of *P. expansum* was transferred to each of the three dishes as above. After 3 h, the first and second Petri dishes were sprayed with 5 ml ARs 1 and ARs 11, respectively, while the third Petri dish was left unsprayed as a control. The same procedure as described above was repeated, replacing PDA with 1 % malt extract agar (MEA), and using mycelium from *N. perennans* instead. Dishes were sealed with parafilm and incubated for 21 days at 20 °C. The inhibitory effect of ARs was expressed as percentage of mycelial growth relative to control plates. Each test was performed three times in three replicates.

Thereafter, conidia were scraped off from each plate and suspended in 5 mL sterile distilled water containing 0.05 % (v/v) Tween 80. The suspensions were filtered through four layers of sterile cheesecloth to remove any adhering mycelia, and spore concentrations were determined with a hemacytometer under a microscope (10 × 40 magnifications) and adjusted to $1x10^5$ conidia mL^{-1} for both pathogens. Five fruits of 'Aroma' per plate, in three replicates (in total 90 fruit per pathogen) were washed in ethanol (70 %) and sterilized water, wounded twice with a nail (4 mm diameter and 4 mm depth), one wound on each sun-exposed and shaded side, and inoculated by pipetting 20 μL of freshly prepared conidial pathogen suspension ($1x10^5$ conidia mL^{-1}) into each of the wound sites. Fruits were incubated for one week at 20 °C (covered with wet filter paper), before lesion area on each fruit was measured to reveal the effect of AR emulsions on fungal pathogenicity. The AR inhibitory effect was expressed

as percentage of lesion diameter on treated fruit compared to control fruit.

To evaluate possible effects of ARs on the spore germination of these two pathogens, 12 fruits of 'Aroma', in three replicates (total 48 fruits), with visible *P. expansum* conidia from spontaneous infection, were collected from a storage house, and divided into three groups. The first and second groups were sprayed with 10 ml ARs 1 and ARs 11, respectively, while the third group was left untreated. All fruits were left at 20 °C for 24 h. Conidia were scraped off from the lesion area on these fruits, deposited into glass tubes (1 fruit each), and adjusted to a concentration of $1x10^5$ conidia per ml using a hemacytometer. From each tube (replicate), 20 μL of the suspension was placed on a water agar plug (3x3 cm), put on a glass slide and kept in a petri dish lined with a wet filter paper before incubation at 20 °C for 48 h. Conidia germination in each suspension was evaluated by a hemacytometer under a microscope (1 × 40 magnification). The effect of ARs was determined as percentage of germinated spores in the suspension from treated fruits compared to the number of germinated spores in the suspension from untreated fruit. The same procedure was repeated with 12 additional 'Aroma' fruits, in three replicates (total 48 fruits), with visible *N. perennans* conidia.

Statistics

A completely randomized design was used for all parts of this study. Percentual inhibition of fungi pathogenicity and mycelial radial growth, respectively, was calculated as $((\text{control} - \text{treatment}) \times \text{control}^{-1}) \times 100$ and subjected to one-way analysis of variance (ANOVA) for each fungus separately. Data from *in vivo* testing were analysed by multi-way ANOVA, with lesion diameter as dependent variable and treatment, fruit side and season as independent variables. Means were separated by Tukey's HSD test, at $P = 0.05$. Statistical analysis was carried out with Minitab ver. 16 (Minitab Inc., State College, PA, USA).

Results

Fruit quality at harvest

All cultivars were picked at the same maturity stage (suitable for commercial harvest) according to starch degradation and the Streif index to avoid maturation-related effects on susceptibility to fungal decay. The fruit showed a clear difference between the coloration on sun-exposed and shaded side, respectively (Table 1).

Effect of AR emulsions on mycelial growth and fungal pathogenicity

The pre-inoculation inclusion of AR emulsions into the PDA and MEA plates, inhibited mycelial growth rate significantly ($P < 0.05$) for both *P. expansum* and *N. perennans*. Compared to the control plates with no ARs, mycelial radial growth of the first pathogen was 22 % lower, and no differences were observed between the antifungal effects of the two AR emulsions on this pathogen (Table 2). By contrast, only ARs 1 caused a significant inhibition of the growth of *N. perennans* mycelium, resulting in a 46 % decrease (Table 2).

The inhibitory effect was improved by spraying the AR emulsions onto the plates 3 h after inoculation. The restriction of mycelial radial growth was 71 and 50 %, respectively, for *P. expansum*, and 77 and 43 %, respectively, for *N. perennans* after spraying with ARs 1 and ARs 11, respectively (Table 2). Thus, ARs 1 showed a significantly ($P < 0.05$) higher effect on both pathogens (Table 2).

Our results also indicate that AR emulsions can inhibit fungal virulence significantly ($P < 0.05$). Conidia of *P. expansum* showed 62 % and 43 % lower germination when they were harvested from apples treated with ARs 1 and ARs 11, respectively, compared to untreated fruit (Table 2). No significant difference was noted between the two ARs emulsions. This positive inhibitory effect was also found on *N. perennans*. Conidia of this pathogen showed 74 % and

Table 1. Fruit maturity stage and colour at harvesting (n = 15 fruits).

Season	Cultivar	Harvest date	Starch index	Streif index[x]	Coloration (CI colour index[y])	
					Sun-exposed	Shaded side
2011	'Aroma'	Sept 10	4.1	0.17	4.4	−4.0
	'Ingrid Marie'	Sept 22	3.9	0.16	11.6	8.2
	'Frida'	Sept 26	4.1	0.17	9.3	6.8
	'Gloster'	Nov 11	4.7	0.17	8.9	5.0
2012	'Aroma'	Sept 18	4.4	0.16	17.8	−3.1
	'Ingrid Marie'	Oct 09	4.3	0.15	48.6	−5.0
	'Frida'	Oct 18	4.2	0.16	25.2	−1.1

[x] Streif index = firmness × (soluble solids concentration × starch index)$^{-1}$; [y] CI = (1000 × a) (L × b)$^{-1}$ where a = red colour, b = yellow colour and L = luminosity.

Table 2. Antifungal effect of Alkylresorcinols (ARs) emulsions on the mycelial radial growth of *Penicillium expansum* and *Neofabraea perennans* on potato-dextrose agar (PDA) and malt extract agar (MEA) plates, respectively.

Pathogen	Treatment	Emulsions were added into the plates 3 h before inoculation		Emulsions were sprayed onto the plates 3 h after inoculation		Germination of conidia (%)
		Lesion area diameter (mm)	Inhibition (%)	Lesion area diameter (mm)	Inhibition (%)	
P. expansum	ARs 1	69.0 b	24.0 a	24.3 c	71.0 a	37.0 b
	ARs 11	72.0 b	21.0 a	41.7 b	50.0 b	55.0 b
	Control	90.7 a		83.3 a		97.5 a
N. perennans	ARs 1	31.3 b	46.0 a	10.3 c	77.0 a	22.0 c
	ARs 11	42.0 ab	27.5 b	25.3 b	43.0 b	57.0 b
	Control	58.0 a		44.3 a		85.0 a

a, b, c: values with different letters within a column and pathogen are significantly different at $P < 0.05$. n = 9 plates.
Control: no treatment at all.

33 % lower germination when they were scraped from treated fruit with ARs 1 and ARs 11 respectively compared to the germination of conidia that were scraped from untreated fruit. ARs 1 had significantly ($P < 0.05$) higher effectiveness compared with ARs 11 (Table 2).

Inoculation of apples with conidia that had been isolated from ARs-sprayed PDA and MEA plates produced smaller lesion areas in comparison with inoculating apples with conidia isolated from non-treated plates. The inhibitory effect was shown as a 40 % smaller lesion area on *P. expansum*-inoculated fruit and a 45 % smaller lesion area on *N. perennans*-inoculated fruit. The inhibitory effect of ARs 1 on the virulence of *P. expansum* conidia was significantly higher ($P < 0.05$) than the effect of ARs 11 whereas no difference was found between the effects of the two AR emulsions on the virulence of *N. perennans* (Fig. 1). By contrast, the addition of AR emulsions to the plates before inoculating them did not affect the virulence, neither of *P. expansum* nor of *N. perennans* (data not shown).

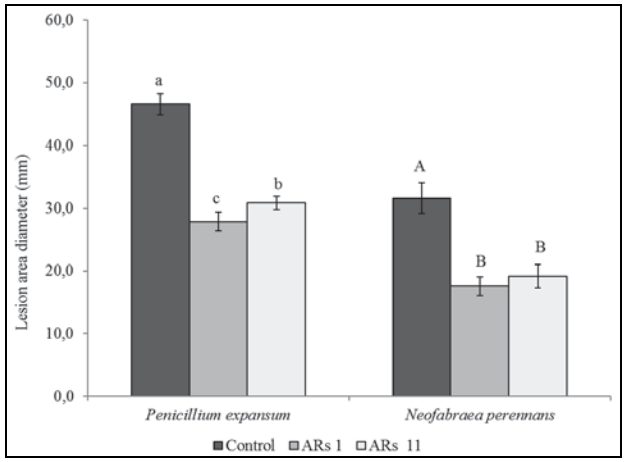


Fig. 1. Effect of AR emulsions on the pathogenicity of *P. expansum* and *N. perennans*, indicated as lesion area on inoculated 'Aroma' apples. The inoculums were extracted from PDA and MEA plates that had been sprayed with AR emulsions after inoculation by transferring the hyphal tips of the two fungi, respectively. Values with the same letter are not significantly different by Tukey´s test at $P < 0.05$, bars represent SD. Control: no treatment at all.

In vivo effect of AR emulsions

'Aroma' apples showed the lowest resistance to *P. expansum,* followed by 'Ingrid Marie' and 'Frida', while 'Gloster' apples showed the highest resistance to this fungus (Fig. 2; $P < 0.05$). By contrast, 'Aroma' apples were significantly ($P < 0.05$) less susceptible to storage decay caused by *N. perennans*, compared with 'Ingrid Marie' and 'Frida' apples (Fig. 2). Different cultivars also showed different responses to the antifungal effect of ARs. AR emulsions inhibited decay produced due to artificial inoculation with *P. expansum* by 17, 28, 39 and 52 % in 'Aroma', 'Ingrid Marie', 'Frida' and 'Gloster' apples, respectively, in comparison with untreated fruit when calculated on mean values across emulsions and years (Fig. 3; $P < 0.05$). Fungal decay caused by artificial inoculation with *N. perennans* was similarly inhibited by AR emulsions, with 77, 74 and 31 % in 'Aroma', 'Ingrid Marie' and 'Frida', respectively, in comparison with untreated fruit (Fig. 3).

There were significant differences ($P < 0.05$) between the effectiveness of the two AR emulsions. ARs 1 showed greater antifungal effect than ARs 11 against *P. expansum* in 'Aroma' and 'Ingrid Marie' apples but not in the other two cultivars. Lesion area diameter thus decreased from 50 and 33 mm in untreated 'Aroma' and 'Ingrid Marie' apples, respectively, to 37 and 20 mm when using ARs 1, and to 45 and 28 mm when using ARs 11 (Table 3). Application of ARs 1 showed a greater effect on 'Aroma' also when inoculated with *N. perennans*; lesion area diameter

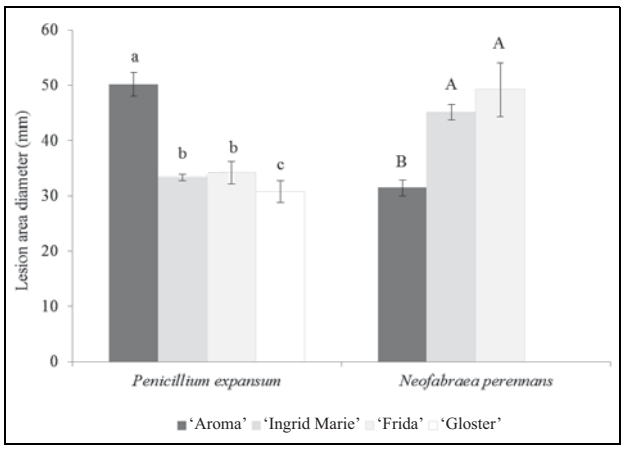


Fig. 2. Susceptibility of different apple cultivars to fungal decay caused by wounding fruit and inoculation with *P. expansum* or *N. perennans* (2011–2012 average). 'Gloster' was not included in *N. perennans* test. Values with the same letter are not significantly different by Tukey´s test at P < 0.05, bars represent SD.

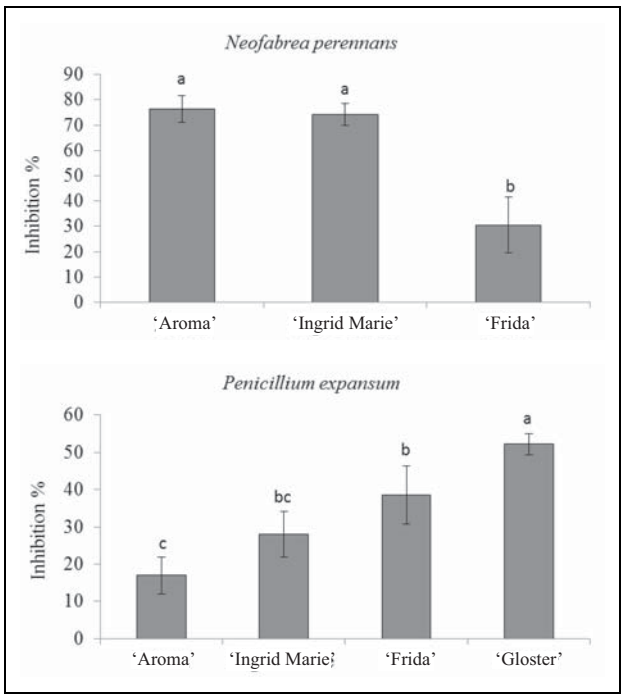


Fig. 3. Comparison between the responses of various apple cultivars to the antifungal effect of the two AR emulsions during cold storage. The inhibition level (average of the two ARs) was estimated as a percentage related to lesion diameter on untreated fruit. Values with the same letter are not significantly different by Tukey´s test at P < 0.05, bars represent SD.

was reduced from 31 mm in untreated fruit to 2 mm in fruit treated with ARs 1 *vs.* 11 mm in fruit treated with ARs 11 (Table 3). Use of ARs 1 resulted in smaller lesions in all the other cultivars as well, but the differences were not significant.

Lesions caused by *N. perennans* were somewhat smaller on the sun-exposed side of the fruit compared to on the shaded side but a significant difference (about 20 %) was encountered only for 'Aroma'. No effect of sun exposure on lesion size in *P. expansum*-inoculated fruit was observed (Table 3). Also, no significant interactions (at P = 0.05) were found between fruit side and treatment.

The AR inhibitory effect on *P. expansum* was somewhat higher in 2011 than in 2012, in 'Frida'. However, no significant interactions between season and treatment were

Table 3. Average lesion area diameter after 12 weeks in cold storage (2 °C and 90 % RH) in different apple cultivars (harvested 2011 and 2012) that had been artificially inoculated with *Penicillium expansum* (*P. e*) and *Neofabraea perennans* (*N.p*) and treated with alkylresorcinols (ARs) emulsions. In the last two rows, mean lesion diameter (across all treatments) is reported separately for the sun-exposed and the shaded side of the fruit, respectively.

Treatments	'Ingrid Marie'		'Frida'		'Aroma'		'Gloster'
	P. e	*N. p*	*P. e*	*N. p*	*P. e*	*N. p*	*P. e*
10 ml ARs 1	20.0 c	9.6 b	20.0 b	30.3 b	37.5 b	1.0 c	11.6 b
10 ml ARs 11	28.1 b	13.6 b	22.1 b	32.7 ab	45.2 a	14.0 b	16.6 b
10 ml warm water	35.0 a	46.7 a	35.1 a	50.0 a	46.5 a	32.1 a	29.6 a
Control	33.0 a	45.2 a	34.2 a	49.0 a	50.3 a	31.0 a	30.8 a
Exposed side	29.0 a	39.0 a	26.5 a	39.0 a	44.2 a	17.5 b	21.4 a
Shaded side	29.0 a	42.0 a	29.5 a	42.0 a	45.7 a	21.5 a	22.9 a
Treatment × side	ns^{x}	ns	ns	ns	ns	*	ns
Treatment × season	ns	ns	ns	ns	ns	ns	ns

Values which are an average of two years, followed by the same letter are not statistically different at P ≤ 0.05. ns^{x}: Non-significant and significant, respectively, at P ≤ 0.05. N = 60 fruit. All inoculated fruit were infested. Control: no treatment

found (Fig. 3 and Table 3). Post-storage assessment of fruit quality did not show any unacceptable effects of the AR treatment on odour, appearance and colour (data not shown).

Discussion

To provide further information on the antifungal properties of alkylresorcinols, two emulsions, which had been selected in a previous study (Dey et al. 2013), were tested in this study against two of the economically most important fungi, *P. expansum* and *N. perennans*, both of which cause high postharvest losses in various apple cultivars. Based on data from fungal decay lesions produced on inoculated fruit and conidia germination as well as *in vitro* investigation (mycelial radial growth), the two fungi were clearly inhibited by AR emulsions, with *N. perennans* overall exhibiting a higher sensitivity than *P. expansum*. For both fungi, a more than 50 % decline in the virulence and storage rot symptoms on inoculated fruit was thus noted after spraying the conidia with ARs. These results indicate that application of ARs has potential to act as an antifungal agent. Our results are in agreement with previous investigations of the antifungal properties of ARs on *Aspergillus parasiticus* and *Penicillium chrysogenum* (Reiss 1989), and on *Alternaria alternata* and *Colletotrichum gloeosporioides* (Droby et al. 1987; Hassan et al. 2007). However, the effective concentration of ARs in the tested emulsions in our study was only 0.025 instead of 1.000 % in previous work (Reiss 1989) thus making a large-scale industrial use more economically feasible.

The pre-incubation of ARs on agar plates for 3 h before inoculation may cause diffusion of ARs into the agar, decreasing the concentration and thus lowering the inhibition of spore germination and fungal growth in comparison with instead spraying ARs onto the plates. It is also possible that the ARs is bound up or broken down in agar over time, thereby reducing the effectiveness if applied before the fungus is present.

The antifungal activities of ARs have been attributed to membrane-modulating properties or/and to antioxidant effects of the phenolic hydrogen (Kozubek and Tyman 1999). The inhibition of fungal growth and spore germination may also arise from antioxidant activity of the phenolic hydrogen group (Reiss 1989; Zainuri et al. 2013). Overall, ARs 1 had even *in vitro* a stronger effect compared with ARs 11. This may be due to the lower mw of Synperonic 91/6 in comparison with PDMS, which may have improved the ARs exposure towards the fungi.

Considerable variation was found between the four investigated apple cultivars for lesion diameter, both in control fruit and in AR-treated fruit. Previous studies have also indicated that apple cultivars differ in tolerance to fungal decay due to their genetic properties, with 'Gloster' being more tolerant and 'Aroma' less tolerant than the other two when inoculated with *P. expansum* (Ahmadi-Afzadi et al. 2013). The relatively high content of triterpenes in 'Gloster' compared to 'Aroma' and 'Ingrid Marie' apples (data not published yet) might be the reason for the higher tolerance of 'Gloster' in this experiment. In the present study, the inhibitory effect of ARs was stronger in those apple cultivars that appear to have the highest level of disease tolerance for that particular fungus, i.e. the smallest lesions on the control fruit. Thus, 'Gloster' again showed the highest response to the AR antifungal effect on *P. expansum*-inoculated fruit among the investigated cultivars; while 'Aroma' and 'Ingrid Marie' showed the highest response on *N. perennans*-inoculated fruit ('Gloster' was not inoculated with this fungus).

Previous studies have reported that the sun-exposed side of the apple fruit, which has a higher content of anthocyanin and wax, has a lower sensitivity to fungal decay compared to the shaded side (Tahir et al. 2009). More ambiguous data have, however, also been reported, with results in opposite directions for different cultivars (Ahmadi-Afzadi et al. 2013). In spite of some, mostly non-significant differences, our present results could not confirm the hypothesis that sun-exposure might enhance the response to ARs.

Based on the high level of fungal decay inhibition and the absence of any adverse effects like unacceptable odour or skin discoloration, use of ARs as a postharvest natural 'fungicide' should be further investigated, using different AR concentrations and both wounded and unwounded fruit of the most susceptible apple varieties. In the next step, we also intend to study the effects of preharvest application of ARs in a commercial apple orchard, their effect on other pathogens, any requirement to use additives in the formulation, residues on or in the fruits and their behaviour in cold respective ULO storage.

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