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Environmental factors affect efficacy of some essential oils and resveratrol to control growth and ochratoxin A production by *Penicillium verrucosum* and *A. westerdijkiae* on wheat grain

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ABSTRACT

This study determined the efficacy of three essential oils (ESO; bay, clove and cinnamon oil) and the antioxidant resveratrol (0-500 µg g^{-1}) on control of growth and ochratoxin A (OTA) production by *Penicillium verrucosum* Dierckx and *Aspergillus westerdijkiae* Frisvad & Samson *(=A. ochraceus Wilhem)* under different water activity (a_w, 0.90, 0.95, 0.995), and temperature (15, 25°C) conditions on irradiated wheat grain with retained germinative capacity. The best treatment (resveratrol) was tested on natural grain. The ED_{50} values for growth inhibition by ESOs were 200-300 µg g^{-1} at the a_w and temperatures tested. For resveratrol this varied from <50 µg g^{-1} at 0.90-0.95 a_w and >350 at 0.995 a_w at both temperatures. The ED_{50} values for OTA control were slightly lower than for control of growth, with approx 200 µg g^{-1} required for the ESOs and 50-100 µg g^{-1} of the antioxidant, at 0.90/0.95 a_w and both temperatures. In wet grain (0.995 a_w) higher concentrations were required. For growth there were statistically significant effects of single, two and three way interactions between treatments except for concentration x temperature and concentration x temperature x essential oil/antioxidant treatment. For OTA control, statistically significant treatments were a_w, temperature x a_w, concentration x temperature, treatment x concentration, and three way interaction of concentration x a_w x treatment for *P. verrucosum* and *A. westerdijkiae*. Subsequent studies were done with the best treatment (resveratrol, 200 µg g^{-1}) on natural wheat grain with either *P. verrucosum* or *A. westerdijkiae* at 0.85-0.995 a_w and 15/25^{o}C over 28 days storage. This showed that the populations of the mycotoxigenic species and OTA contamination could be reduced by >60% by this treatment at the end of the storage period.

1. Introduction

The contamination of cereals with ochratoxin (OTA) occurs predominantly post-harvest, and is caused by *Penicillium verrucosum*, especially in northern Europe where cooler damp harvesting conditions exists (Magan & Olsen, 2004). Inefficiently dried grain can result in pockets of growth by *P. verrucosum* in storage (Magan et al., 2003; Magan et al., 2004). There has thus been interest in determining potential treatments which could effectively control this important mycotoxigenic species. While moist grain is sometimes treated with aliphatic acid-based treatments, these are often fungistats, and require very efficient coverage to be effective. Sub-optimal concentrations can result in a stimulation of OTA production by *P. verrucosum* strains (Arroyo et al., 2005).

Recent studies have attempted to determine the efficacy of extracts from selected plants as antimicrobial and antifungal agents. Some studies have shown that specific essential oils can control spore germination and growth of mycotoxigenic spoilage fungi. For example, screening of essential oils suggested that oregano and thyme essential oils inhibited the growth of *Aspergillus niger* van Tieghem, *Aspergillus westerdijkiae* and *Aspergillus flavus* Link (Magan *et al*., 2004). Antioxidants have also been demonstrated to inhibit fungal growth and some are already used as preservatives in food. Thompson (1994) studied the effects of esters of p-hydroxybenzoic acid (paraben) on the growth of three mycotoxigenic fungi. Butyl and propyl parabens were the most effective, completely inhibiting mycelial growth at 1.0-2.0 mM concentration. However, the impact of environmental factors were not considered in these previous studies. Screening of about 25 essential oils showed that cinnamon leaf, clove bud and thyme were the most effective essential oils in vitro over a range of environmental factors in controlling growth of *P. verrucosum* and *A. westerdijkiae* (Cairns et al., 2003; Hope et al., 2003, 2005). Studies of antioxidants have shown that some

parabens and resveratrol, an extract of grape skin, were effective at controlling a range of mycotoxigenic species on maize (Fanelli et al., 2003). However, detailed studies of efficacy of these essential oils and antioxidants for controlling *P. verrucosum* and *A. westerdijkiae* and OTA production on wheat grain under different environmental conditions has not been determined. Cairns-Fuller et al. (2005) detailed the optimum and marginal environmental conditions for growth and OTA production by *P. verrucosum* on wheat grain while Linblad et al. (2004) determined the environmental conditions and contamination levels which may lead to OTA contamination above the legislative limit. The ecological conditions for growth and production of OTA by *A. westerdijkiae* has been previously identified and were different from *P. verrucosum* (Ramos et al., 1998).

The objectives of this study were to (a) examine the efficacy of cinnamon leaf, clove bud and thyme essential oils and the antioxidant resveratrol against *P. verrucosum* and *A. westerdijkiae* and OTA production on irradiated wheat grain under different environmental conditions (15, 25^{o}C; 0.995-0.90 a_w); (b) determine ED_{50} values for control; and (c) investigate treatment of naturally contaminated stored wheat grain with the best treatment (resveratrol) to examine control of *P. verrucosum* and *A. westerdijkiae*, total fungal populations and OTA contamination at 0.995-0.85 a_w and the two temperatures.

2. Materials and Methods

2.1 Fungal species and isolates

A strain of *P. verrucosum* (OTA11) isolated from wheat grain and with a known history of OTA production was used in this study (Linblad et al., 2004; Cairns-Fuller et al., 2005). This was kindly supplied by Dr. M. Olsen, National Food Administration, Uppsala, Sweden. An OTA producing isolate of *Aspergillus westerdijkiae (=A. ochraceus;*

IBT21991) was kindly supplied by Dr. P.V. Nielsen, Technical University of Denmark, Lyngby, Denmark.

2.2 Studies on gamma irradiated wheat grain

Studies were carried out with gamma irradiated (12kGy) wheat grain with retained germinative capacity which was modified to 0.90, 0.95 and 0.995 a_w by the addition of sterile water using a moisture adsorption curve (Cairns-Fuller et al., 2005). The treatments were mixed regularly and stored at 4^{o}C for 24-36 hrs to allow equilibration. The essential oils (F.D. Copeland & Sons, Ltd., London) were dissolved in 10ml methanol to produce final concentrations of 5, 50, 150 and 500 µg g^{-1} grain (w/w). The control was treated with methanol only. The resveratrol treatment consisted of using Resvin, a commercial product containing 10% w/w concentration of resveratrol. This was dissolved in ethanol and 0, 50 and 150 µg g^{-1} treatment concentrations prepared. The grain treatments were equilibrated at 4^{o}C for 72 hrs and shaken regularly to ensure a good even distribution of treatments. The a_w of treatments was checked with an Aqualab (CX-2, Decagon Devices Inc, U.S.A.). Grain was placed in monolayers in 90 mm Petri dishes and centrally inoculated with 50 µl of 1 x 10^6 spores ml^{-1} of *P. verrucosum* or *A. westerdijkiae* obtained from a 10 days old wheat (2%) agar culture medium. The experiments were carried out with 3 replicates per treatment and repeated twice. The Petri plates were placed in humidity chambers in which glycerol/water solutions were used to maintain the equilibrium relative humidity the same as the treatment aw levels. These were incubated at 15 and 25^{o}C for up to 28 days. The colonisation rates were measured regularly by taking two measurements at right angles to each other. The slopes of the regression lines were used to plot the relationship between treatment conditions and environmental conditions. The control colonisation rates were used to determine the

relative concentrations of the essential oils and resveratrol to inhibit growth and OTA production by 50% (ED_{50} values).

2.3 In situ effect of resveratrol on fungal populations and OTA control in stored wheat grain inoculated with P. verrucosum *and* A. westerdijkiae *under different environmental conditions*

Sub-samples of freshly harvested winter wheat grain (1000g) were placed in flasks. Water was added to obtain the required water activity levels (0.80, 0.85, 0.90, 0.95 and 0.995 a_w) and treated with resveratrol to obtain a final concentration of 200 µg g^{-1}. The treatment was dissolved in 10 ml ethanol before adding to the grain. The control treatments were all treated with 10 ml ethanol only. 100 g sub-samples of treated wheat grain were placed in solid culture vessels (Magenta, Sigma Ltd, U.K.). The treated wheat grain was then inoculated with 1 ml of 10^2 spores of *P. verrucosum* or *A. westerdijkiae* and mixed thoroughly before incubation in controlled environment chambers which were maintained at the same equilibrium relative humidity conditions as the grain treatments. They were all incubated at 15 and 25^{o}C for up to 28 days. Experiments were carried out with five replicates per treatment and carried out twice.

Samples were removed after 7 and 28 days for fungal population assessment. They were assessed by serial dilution using three replicate sub-samples of 1 g of wheat grain in 9 ml sterile water (+ 0.01% Tween 80) on malt extract and DYSG media at the same a_w as the treatment. The latter medium is selective for *P. verrucosum* and distinguishes the mycotoxigenic species from other Penicillia by the characteristic brick red reverse. *A. westerdijkiae* was identified by morphological and sporulation characteristics. Samples were extracted for OTA after 28 days.

2.4 Ochratoxin extraction and analysis

The experiment was terminated at 28 days and the samples dried at 50^{o}C for 48 hrs. The samples were milled and a 20g sub-sample extracted with 50 ml of methanol and analysed for OTA using HPLC and fluorescence detection. The sample was shaken for 24 hrs at 110 rpm and 25^{o}C in the dark. Samples were then all filtered through filter paper (Whatman, No. 4) containing 5-10 g of Celite 545 (Aldrich Chemical Co., U.K.) for clean up of the sample and to improve final extract. From each sample 1ml was removed and centrifuged at 1100 rpm for 15 mins for final purification. The supernatant of each sample was removed and placed in HPLC amber vials for analyses.

The HPLC system used consisted of a Millipore Waters 600E system controller, a Millipore 712 WISP autosampler and a Millipore Waters 470 scanning fluorescence detector (Millipore Corporation Massachusetts USA)(excitation 330 nm, emission 460 nm). The samples were separated using a C18 Luna Spherisorb ODS2 column (150 x 4.6mm, 5µm) (Phenomenex, Macclesfield, U.K.), with a guard column of the same material. Run time for samples was 12 minutes with OTA being detected at about 5.75 minutes. The flow rate of the mobile phase (acetonitrile:water:acetic acid; 57:41:2) was 1 ml min^{-1}. Standards used were 50-1200 ng ml^{-1}. The recovery rate was 78% for wheat grain with a limit of detection of <0.01 µg OTA g^{-1} medium, based on a signal to noise ratio of 3:1. Analysis of the results was carried out on a computer with Kroma systems 2000 software (Bio-tek Instruments, Milan, Italy).

2.5 Statistical analyses of data

All data was subjected to statistical analyses. Two and three-way interactions were examined using Analysis of Variance and the software programme Minitab 13.32 (Minitab Inc, USA.). Where appropriate LSD between treatments were calculated at the P=0.05 level.

3. RESULTS

3.1 Effect of essential oils and resveratrol on colonisation of layers of wheat grain

Figure 1 shows an example of the efficacy of the treatments on growth rate of *P. verrucosum* and *A. westerdijkiae* in relation to up to 500 µg g^{-1} at 0.95 and 0.90 a_w, and 15 and 25^oC. Generally, growth was relatively unaffected by 50 µg g^{-1} of essential oils, while at this concentration, resveratrol sometimes gave >50% inhibition of mycelial colonisation of wheat grain. However, 500 µg g^{-1} of all treatments was required for >90% inhibition of growth. Table 1 shows the ED_{50} concentrations of treatments required to inhibit colonisation of the grain by both *P. verrucosum* and *A. westerdijkiae.* For growth there were statistically significant effects of single, two and three way interactions between treatments except for concentration x temperature and concentration x temperature x essential oil/antioxidant treatment.

3.2 Effects of essential oils and resveratrol on OTA production

Figure 2 shows an example of the efficacy of treatments on OTA production at 0.95 a_w and 15 and 25^oC after 28 days incubation for both *P. verrucosum* and *A. westerdijkiae.* The trend in control of OTA is similar to that for growth. However, resveratrol was able to completely inhibit OTA at 500 µg g^{-1} for *P. verrucosum*. Only the lowest concentration (50 µg g^{-1}) appeared to stimulate OTA production by *A. westerdijkiae*. Overall, resveratrol was more effective than the three essential oils in controlling OTA production. Table 2 confirms that the efficacy of resveratrol (ED_{50} values) was greater than the essential oil treatments under most of the treatment conditions tested.

3.3 Control of P. verrucosum, A. westerdijkiae *and OTA production with resveratrol in natural stored wheat grain*

The efficacy of resveratrol on the total populations of fungi and *P. verrucosum* or *A. westerdijkiae* over a range of a_w conditions are shown in Figure 3. The total fungal populations increased with stored grain wetness from 0.85 to 0.995 a_w. The presence of the inoculum increased the overall populations of fungi in some cases. Both the total fungal populations, and that of *P. verrucosum* and *A. westerdijkiae* were significantly inhibited by treatment with resveratrol over the storage time of 28 days when compared with the control treatments. Overall, >60% inhibition of *P. verrucosum* and *A. westerdijkiae* populations occurred by treatment with resveratrol. Statistical analyses showed that a_w, temperature, time and resveratrol all significantly affected *P.verrucosum* and *A. westerdijkiae* populations.

The effect of treatments on OTA production after 28 days storage at 15 and 25^{o}C for both *P. verrucosum* and *A. westerdijkiae* are shown in Table 3. The highest OTA content was found in the 0.95 a_w treatments, regardless of whether inoculum of *P. verrucosum* or *A. westerdijkiae* were used. Grain treated with resveratrol had significantly less OTA than the untreated controls. Again OTA contamination was reduced by > 60% in most of the treatment conditions. Statistical analyses showed that a_w, temperature, time and treatment and interactions between these factors all significantly affected OTA content. The statistical analyses for one data set is shown in Table 4.

4. DISCUSSION

This study has shown that the three essential oils are not as effective as the antioxidant resveratrol in controlling growth of *P. verrucosum and A.westerdijkiae* colonising wheat grain. The ED_{50} values confirm that for controlling both growth and OTA

production by these two species is more efficient with the antioxidant. *A. westerdijkiae* is mainly isolate from warmer climates and thus grew better at 25^{o}C than *P. verrucosum* (Magan and Aldred, 2006). It was noticeable that at 50 µg ml^{-1} there was often some stimulation of OTA production by both essential oil and the antioxidant treatments. However, when higher concentrations were used >50% control was achieved, especially with the resveratrol treatment. The a_w conditions used in these studies represent the important range where poorly dried conditions can lead to spoilage post-harvest.

Inhibition of growth and toxin production do not always occur together. For example, previous studies with *Fusarium culmorum* (W.G.Smith) and *Fusarium graminearum* Schwabe showed that growth was significantly inhibited by 500 µg g^{-1} cinnamon oil at 0.955a_w/25°C yet toxin production was enhanced (Hope et al., 2005). This is similar to the results of Magan et al. (2002) who found that suboptimal levels of fungicides stimulated DON production by *F. culmorum* in wheat grain. The additional stress of the fungicidal agents combined with water stress may stimulate mycotoxin production as a defence reaction. Further, stress conditions can be imposed by sub-optimal fungicides alone (Magan and Aldred, 2007). This was supported by Kang et al. (2001) who demonstrated that DON levels in the cell walls, cytoplasm, mytochondria and vacuoles of the hyphae of *F. culmorum* was significantly higher than the control for growth that occurred in the presence of the fungicides metaconazole and tebuconazole (20 µg ml^{-1}).

In this study only resveratrol was examined in naturally contaminated wheat grain. These studies were carried out at a single concentration of resveratrol to determine the short term efficacy over the whole water content range representative of poorly stored grain. By addition of either mycotoxigenic species it was possible to ensure that contamination and OTA was produced in the wheat grain. The total populations of fungi were reduced by the presence of resveratrol significantly, often by about 1-3 log CFU. However, the CFUS of the

mycotoxigenic moulds were reduced by about 1-2 logs. It appeared to be more effective against *P. verrucosum* than *A. westerdijkiae.* Fanelli et al. (2003) examined antioxidants such as resveratrol on *P. verrucosum* and *Fusarium graminearum* and OTA and Deoxynivalenol, respectively. They found that resveratrol at 230 µg g^{-1} and BHA at 0.2% were very effective in inhibiting 90% of OTA production at 0.95 and 0.85 a_w on sterilised wheat grain. This also mirrored the ergosterol content used as a biomass marker. On natural wheat grain they found that efficacy of 23 ppm was more effective at 0.85 than 0.95 a_w.

Studies have been conducted to determine the antimicrobial effect of essential oil active components. However, this has resulted in conflicting results. Limonene and terpinene are the two main components of carrot seed oil. The inhibition of *Aspergillus parasiticus* Speare was found to be higher for the components than for the complete oil (Batt et al., 1983). In contrast the oils derived from orange or lemon peel were more effective at controlling growth and aflatoxin production than was d-limonene the main constitute of the two peel oils (Alderman & Marth, 1976). Cinnamic aldehyde and eugenol, the major constituents of cinnamon and clove oils were found to inhibit mould growth (Bullerman et al., 1977). Paster et al. (1994) found that thyme essential oil was more inhibitory than the individual components. It was suggested that essential oil activity resulted mainly from synergistic or cumulative effects existing between the components. However, more information on the mode of action of such oils is required to identify such possible synergism.

Other anti-oxidants (BHA, propyl paraben) have been also shown to be effective against *Fusarium* Section Liseola species. Etcheverry et al. (2002) found that BHA and propyl paraben inhibited *Fusarium verticillioides and F. proliferatum* growth and production of fumonisins. Subsequent studies also suggested that using low concentrations of two antioxidants may have a synergistic inhibitory effect on growth of these fumonisin producing

species (Reynoso et al., 2002). Recent studies by Passone et al. (2007) suggest that binary and tertiary mixtures of analytical and industrial grade parabens were effective against *A. flavus* and aflatoxin production in short term storage of peanuts. The mechanism of action of antioxidants for inhibiting mycelial growth of toxigenic fungi is however not clear. It is known that the effectiveness of the parabens increases with an increase in the chain length of the ester group (Thompson et al., 1993; Thompson, 1994). Khan et al. (2001) suggested that propyl paraben and BHA appear to work mainly at the cell membrane level eliminating the pH related component of the protomotive force and affecting energy transduction and substrate transport. BHA has also been shown to have a direct effect on the mitochondrial electron chain of trypanosomes, thus inhibiting respiration.

Resveratrol appears to be more effective than parabens. The fact that industrial grade antioxidants may have as good efficacy as analytical grade chemicals (Passone et al., 2007) suggests that opportunities do exist for the utilization of such chemicals. The cost of resveratrol is becoming cheaper as efficiency of extraction and production methods improve. This suggests that antioxidants such as resveratrol, perhaps of industrial quality, may have good applications in both raw commodities destined for human consumption directly and for animal feed preservation. Resveratrol is also recommended as a health supplement for some groups of consumers and thus may ultimately be a more acceptable additive than some of the aliphatic acids employed at present.

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Table 1. The ED_{50} concentrations ($\mu g\ g^{-1}$) of essential oils and resveratrol required for control of *Penicillium verrucosum* and *Aspergillus westerdijkiae* colonisation of layers of gamma irradiated wheat grain under different water activity and temperature treatments

	P. verrucosum					
Temperature (^oC)	15			25		
Water activity	0.90	0.95	0.995	0.90	0.95	0.995
Treatment						
Clove oil	250	230	320	220	190	260
Cinnamon oil	200	220	280	210	185	380
Thyme oil	260	235	320	260	185	385
Resveratrol	10	40	360	40	20	380
	A. westerdijkiae					
Clove oil	210	310	280	365	260	160
Cinnamon oil	210	270	190	325	220	155
Thyme oil	190	210	190	260	215	140
Resveratrol	60	180	190	150	110	140

Table 2. The ED_{50} concentrations (μg kg^{-1}) of essential oils and resveratrol required for inhibition of ochratoxin A production by *Penicillium verrucosum* and *Aspergillus westerdijkiae* on treated gamma irradiated wheat grain under different water activity and temperature treatments

	P. verrucosum					
Temperature (°C)	15			25		
Water activity	0.90	0.95	0.995	0.90	0.95	0.995
Treatment						
Clove oil	240	210	295	210	230	160
Cinnamon oil	260	210	295	210	230	210
Thyme oil	180	325	320	210	200	210
Resveratrol	25	80	180	90	80	215
	A. westerdijkiae					
Clove oil	225	150	275	215	200	150
Cinnamon oil	105	200	185	200	180	160
Thyme oil	60	145	120	140	150	160
Resveratrol	10	100	110	30	130	130

Table 3. Effect of treatment of natural grain with resveratrol (200 µg g^{-1}) after inoculation with *P. verrucosum* or *A. westerdijkiae* (1 ml of 10^2) on ochratoxin A (µg kg^{-1}) in stored wheat grain at different water activities and temperatures for 28 days.

Temperature (°C)	15				
Water activity	0.995	0.95	0.90	0.85	0.80
Grain + *P. verrucosum*	21.2	121.8	81.6	29.7	1.7
Grain + resveratrol + *P. verrucosum*	19.5	38.8	19.4	4.8	0
Grain + *A. westerdijkiae*	7.6	94.5	57.8	5.9	0
Resveratrol + *A. westerdijkiae*	7.9	41.3	18.2	6.1	0

Temperature (°C)	25				
Water activity	0.995	0.95	0.90	0.85	0.80
Grain + *P. verrucosum*	11.8	157.4	104.6	56.3	9.8
Grain + resveratrol + *P. verrucosum*	8.6	88.3	32.7	19.6	2.8
Grain + *A. westerdijkiae*	20.2	124.8	76.1	13.7	3.8
Resveratrol + *A. westerdijkiae*	5.2	53.9	31.6	1.9	1.1

Table 4. Statistical analyses and significance of inoculam species (*P. verrucosum* or *A. westerdijkiae*; 1ml 10^2 spores), treatment with 200 µg g^{-1} resveratrol, water activity (0.995-0.80), and temperature (15, 25^{o}C) on ochratoxin A in inoculated and stored wheat grain.

Factors	**DF**	**MS**	**F-value**	**P-value**
Water activity (aw)	4	31995.5	1027.4	<0.001
Fungal species (SP)	1	5122.1	164.5	<0.001
Treatment (T)	1	24825.6	797.2	<0.001
Temperature (temp)	1	4013.6	128.9	<0.001
Aw x SP	4	499.7	16.1	<0.001
Aw x T	4	5176.4	166.4	<0.001
Aw x temp	4	937.9	30.1	<0.001
SP x T	1	1128.5	36.2	<0.001
SP x temp	1	83.3	2.7	0.106
T x temp	1	158.7	5.1	0.027
SP x aw x T	4	284.4	9.1	<0.001
SP x aw x temp	4	269.4	8.7	<0.001
T x aw x temp	4	39.8	1.3	0.286
SP x T x temp	1	245.6	10.3	<0.001
SP x T x temp x aw	4	201.5	6.5	<0.001
Residuals	80	31.1		

Figure legends

Figure 1. The efficacy of the different concentrations of essential oils and resveratrol on relative growth rate of *P. verrucosum* at (a) 0 0.95 and (b) 0.90 a_w at 15^{o}C, and of *A. westerdijkiae* at (c) 0.95 and (d) .90 a_w at 25^{o}C on wheat grain over a 28 day period. Bars indicate Least Significant Differences between treatments (P=0.05).

Figure 2. Effect of essential oil and resveratrol treatments on amounts of ochratoxin A production by *P. verrucosum* at (a) 0.95 and (b) 0.90 a_w and *A. westerdijkiae* at (c) 0.95 and (d) 0.90 a_w at 25^{o}C on wheat grain after 28 days incubation. Bars indicate Least Significant difference between treatments (P=0.05).

Figure 3. Effect of resveratrol (200 µg g^{-1}) on total fungal populations and that of *P. verrucosum* (a) and total fungal populations and that of *A. westerdijkiae* (b) over a range of water activity levels after storage for 28 days at 25^{o}C.

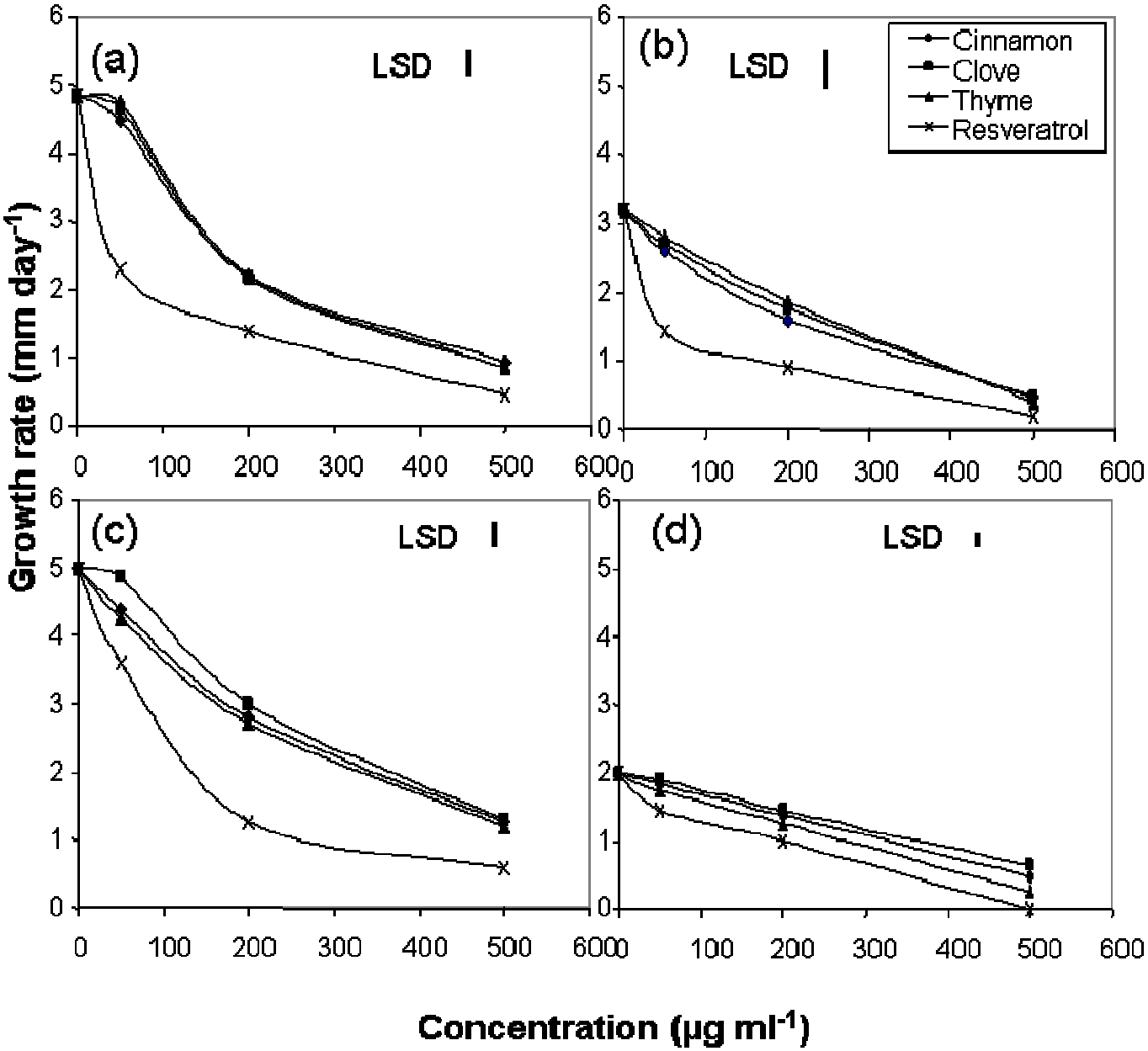


Figure 1. Aldred et al.

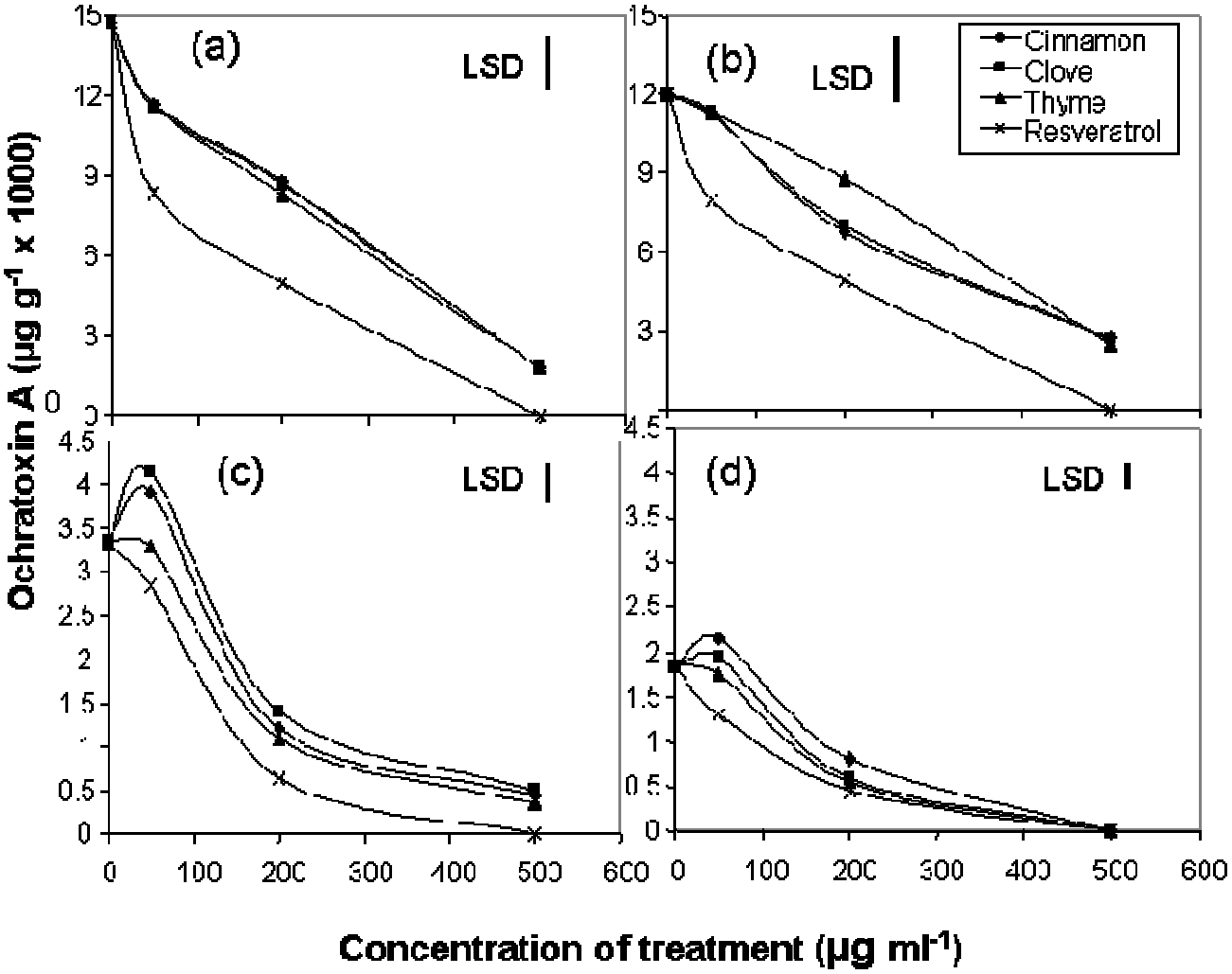


Figure 2. A drec e: al.

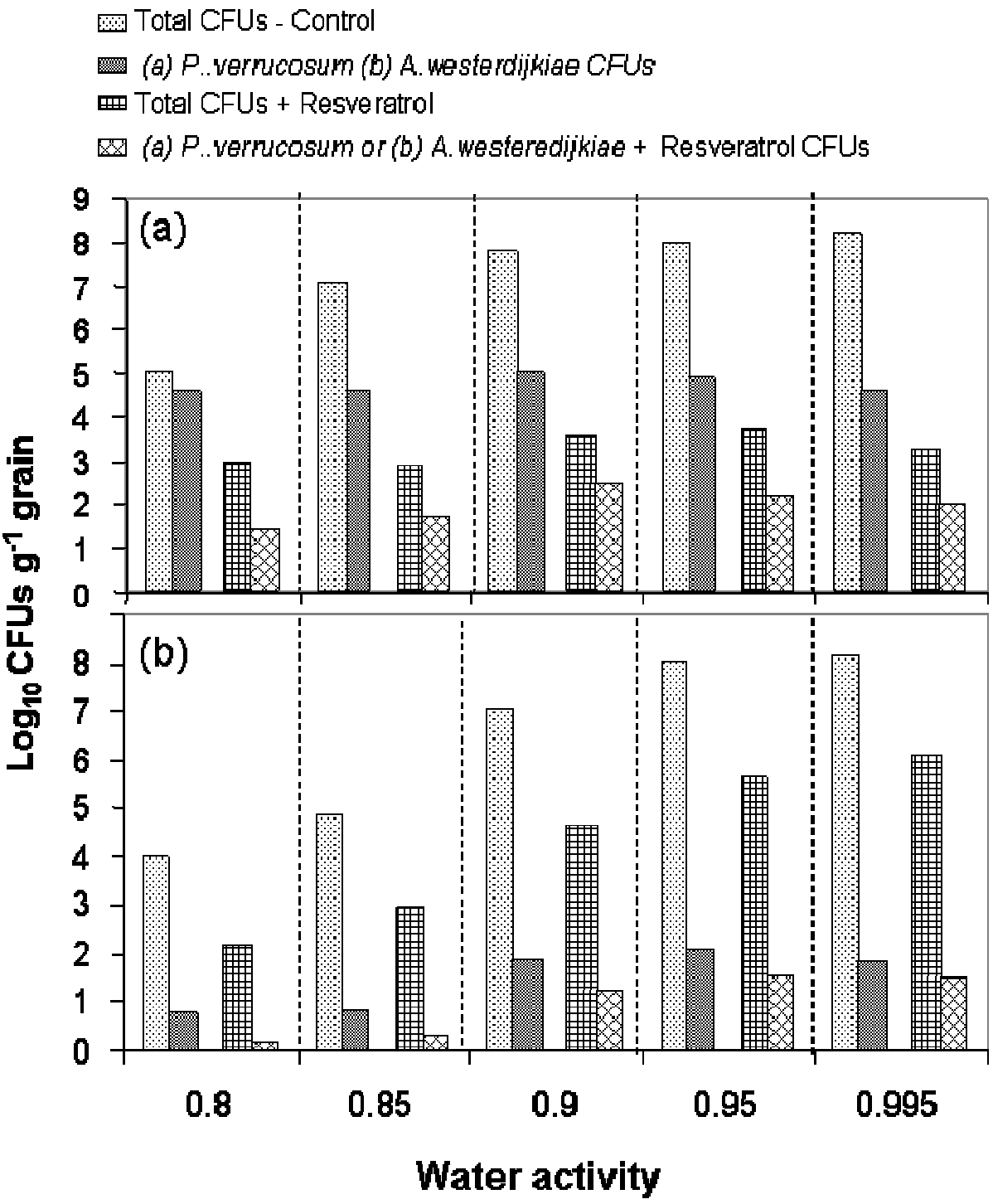


Figure 3. Aldred et al.



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Environmental factors affect efficacy of some essential oils and resveratrol to control growth and ochratoxin A production by Penicillium verrucosum and Aspergillus westerdijkiae on wheat grain.

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