CRANFIELD UNIVERSITY

APPLIED MYCOLOGY GROUP

INSTITUTE OF BIOSCIENCE AND TECHNOLOGY

PhD THESIS

Academic Year 2003-2004

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DYNAMICS AND CONTROL OF OCHRATOXIGENIC STRAINS OF *PENICILLIUM VERRUCOSUM* AND *ASPERGILLUS OCHRACEUS* IN THE STORED GRAIN ECOSYSTEM

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April 2004

This thesis is submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy

ABSTRACT

This study investigated the effect of interacting environmental factors on the ecophysiology of *Penicillium verrucosum* and *Aspergillus ochraceus* and ochratoxin A (OTA) production and potential for controlling growth and OTA production using essential oils and resveratrol on wheat grain. Two dimensional temporal profiles of growth and OTA production were constructed for the first time and showed that the water activity (a_w) ranges required for growth and OTA production were different *in vitro* and on wheat grain. Growth occurred over a wider a_w range than OTA production for *P. verrucosum* and *A. ochraceus* respectively regardless of the temperature tested. For example, *P. verrucosum* grew at 0.80–0.995 a_w on wheat grain however OTA production occurred between 0.85-0.995 a_w. *A. ochraceus* grew at 0.80-0.995 a_w on wheat grain however OTA production occurred between 0.85-0.995 a_w. Optimum OTA production was at intermediate a_w ranges (0.93-0.98 a_w) and increased with incubation period. Interestingly at sub-optimal a_w there was a stimulation in OTA production by *A. ochraceus* at 15-25 °C on wheat grain.

Carbon dioxide (CO_2) levels (25-50 %) interacted with a_w to influence growth and OTA production by *P. verrucosum* and *A. ochraceus*. As CO_2 levels increased germ tubes of conidia, growth and OTA production by *P. verrucosum* and *A. ochraceus* significantly decreased.

Interactions between a_w and temperature had a significant effect on competitiveness, growth and OTA production by *P. verrucosum* and *A. ochraceus* in interspecific interactions with six other spoilage fungi. Interactions were complex, changing with environmental conditions, resulting in a significant reduction of both growth and OTA production by *P. verrucosum* and *A. ochraceus in vitro* and *in situ*. An Index of Dominance (I_D) was used to compare interspecific interactions between *P. verrucosum*, *A. ochraceus* and other wheat spoilage fungi. *P. verrucosum* and *A. ochraceus* were both more competitive at intermediate a_w levels. *P. verrucosum* was more competitive at 15 °C, whereas *A. ochraceus* was more competitive at 25 °C. There was no direct

relationship between growth rates of *P. verrucosum* or *A. ochraceus* and their competitiveness. Niche overlap indices (NOIs) and niche maps were developed for the first time and showed that interspecific competitiveness was closely related to the number of nutritional carbon sources shared between spoilage fungi. Generally as a_w increased so did the niche sizes of all species tested. Interestingly, all *P. verrucosum* strains did not necessarily share the same ecological niche over all the conditions tested. There was no correlation between I_D and NOIs. I_D depended more on a_w and temperature than on niche overlap, implying that there may be some other factor influencing competitiveness.

Twenty-three essential oils and resveratrol were screened for their potential control to growth and OTA production by *P. verrucosum* and *A. ochraceus.* Resveratrol, cinnamon leaf, clove bud and thyme were able to significantly reduce growth and OTA production by >90 % *in vitro* and 80 % *in situ.* Resveratrol was the most effective treatment at controlling growth and OTA production. Resveratrol controlled fungal growth and OTA production by >60 % for up to 28 days on naturally contaminated wheat grain.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Professor Naresh Magan for his invaluable advice, guidance, encouragement and constant enthusiasm throughout this project.

I would also like to thank my second supervisor Dr. David Aldred for his patience, help and efficiency. I was so fortunate to have the best supervisors I could ever wish for. Thankyou!

I would like to acknowledge the excellent 'on-hand' technicians, Esther Baxter for her help particularly when I first arrived in the mycology laboratory and Alan Hilton for his training on using and fixing the GC and gas integrator systems.

Finally, I would like to thank my family for their encouragement, love and support throughout.

In memory of my Grandad

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CHAPTER 1

LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Grain entering store contains a wide variety of microorganisms including bacteria, yeasts and filamentous fungi. The numbers and variety are dependent on field climatic conditions and the harvest, drying and storage techniques employed (Magan & Lacey, 1986; Bruce & Ryniecki, 1991; Lacey & Magan, 1991; Magan *et al.*, 2003). Poor post-harvest management results in the initiation of fungal activity which can lead to undesirable effects in grain including discolouration, contribute to heating and losses in nutritional value, produce off-odours, losses in germination, deterioration in baking and milling quality and can result in contamination with mycotoxins (Magan *et al.*, 2003). It has been estimated that 10-30 % of grain during storage is rendered unusable due to poor post-harvest management (Harris & Lindblad, 1978; Sode *et al.*, 1995).

Penicillium verrucosum and *Aspergillus ochraceus* are found in small numbers in the field in temperate cereals but can flourish during storage of moist grain. They produce the mycotoxin, ochratoxin A (OTA) which is harmful to both animals and humans (International Agency for Research on Cancer, 1995; Creppy *et al.*, 1995; Creppy, 1999) and there has been recent concern regarding the daily intake of this toxin by humans (EC's Scientific Committee for Food, 1998).

Wallace & Sinha (1981) were the first to consider stored grain as a man-made ecosystem and use multivariate statistical approaches to examine the complex interactions between abiotic and biotic factors and identify the key parameters for safe storage (Magan *et al.*, 2003). The key parameters identified include grain type and quality, fungal populations and community structure, mycotoxin production, pest-infestation and time the grain is kept during storage. The most important environmental factors include water availability, temperature and intergranular gaseous composition which all interact to influence the rate of fungal spoilage and the production of mycotoxins which can contaminate stored grain (Figure 1.1). These interactions need to be understood to enable control measures to be effectively employed.

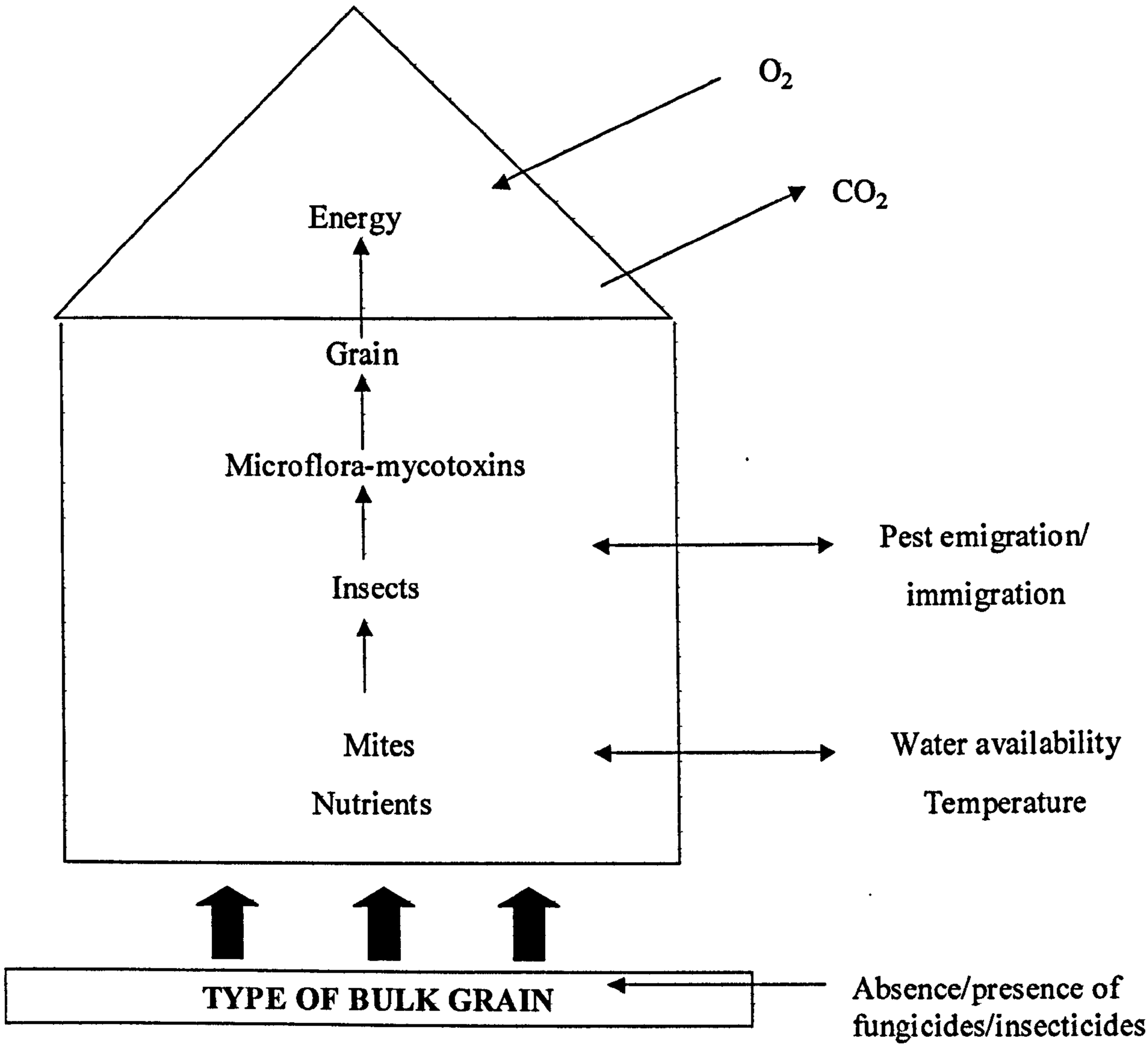


Figure 1.1 Diagrammatic representation of grain ecosystem with interacting abiotic and biotic factors (adapted from Sinha, 1995; Magan *et al.*, 2003).

Storage fungi such as *P. verrucosum* and *A. ochraceus* are commonly controlled using synthetic preservatives; however, many of these substances cause side effects including carcinogenicity, teratogenicity and residual toxicity (Foegeding & Busta, 1991; Basilico & Basilico, 1999). In the past decade, research has focused on the use of natural preservatives (Paster *et al.* 1995; Hope & Magan, 2002), which are perceived as raising fewer concerns amongst consumers and regulatory bodies or within the food industry (Dillon & Board, 1994; Nychas, 1995; Lopez-Malo *et al.*, 1997; Basilico & Basilico, 1999).

This study investigated how the key parameters of water availability, temperature, time, intergranular gaseous compositions and interactions with other wheat mycoflora interact to influence growth and OTA production by isolates of *P. verrucosum* and *A. ochraceus.* The potential for using essential oils and resveratrol for prevention and control of growth and OTA during storage conditions were also evaluated.

1.2 CONCEPT OF WATER AVAILABILITY

There has been much documentation regarding the fundamental requirement for water for microbial growth (Scott, 1957; Ayerst, 1969; Cooke & Whipps, 1993, Magan, 1997).

Water is probably the most important single limiting factor affecting colonisation of cereal grain. Its availability governs whether a fungal spore can germinate and how quickly it can become established; its metabolic rate, respiratory activity and rate of growth and the extent to which heat is released through respiration. This in turn may alter the range of microorganisms that can grow (Lacey & Magan, 1991).

Water availability is usually determined by the water content of the product when it is placed in store although it can be modified by exchange of moisture with the atmosphere or by leakage of rainwater into the store. Water content is determined in terms of percentage moisture content, based on the ratio of the dry weight to the wet weight (expressed either on a wet weight or dry weight basis), but it gives little indication of the amount of water available for fungal growth.

Not all water surrounding grain is equally accessible to microorganisms. Some is bound to grain constituents via strong hydrogen bonding, initially in a monolayer and is referred to as water of constitution. Outside this layer, water is more and more weakly bound, the greater the depth of molecules separating the molecule from the grain constituents. Eventually the water molecules become freed from chemical binding but are held in micropores with dimensions at least equal to the water molecule. These fill with increasing water content and low molecular weight compounds may dissolve to give additional osmotic effects. As the pores fill, water they contain becomes more and more available to microorganisms until the grain is saturated and water is freely available (Lacey & Magan, 1991).

The most used measure of the availability of water to microorganisms is the ratio of the vapour pressure of the water in the substrate to that of pure water at the same temperature and pressure. This is referred to as water activity (a_w) or when it is expressed as a percentage as the equilibrium relative humidity (ERH) (Ayerst, 1965).

$$A_w = p/p_o = \text{ERH}\ (\%)/100 \qquad \text{(Labuza, 1974)}$$

Where p = vapour pressure of water in solution or solid substrate

p_o = vapour pressure of pure water at experimental temperature and pressure

ERH (%) = equilibrium relative humidity at which a solution or solid substrate neither gains nor loses moisture to the atmosphere.

A_w is a measure of the ability of water to evaporate from a substrate and humidify the immediate environment and is measured from 0-1.0, where 1.0 represents the a_w of pure water. The lower the a_w the less water is available to microorganisms. A_w is always measured at a constant temperature. A_w is related to temperature and for a given substrate and moisture content a_w will increase with increasing temperature. This is primarily the consequence of the general increase in thermal motion (Multon, 1988). An alternative measure of the a_w is that of water potential (ψ) which is measured in pascals (Pa) and measures the potential free energy in a system (relative to a hypothetical pool of pure free water of specific mass) (Magan & Lacey, 1988; Papendick & Mulla, 1988).

Pure free water is zero and water which is chemically or physically bound to the substrate at a lower water potential, i.e. negative. Microorganisms must use energy to raise the thermodynamic potential of the water and make it available. ψ and a_w are associated by the following formulae:

$$\Psi = (RT/V)\log_n a_w (+P)$$

Where R = the ideal gas constant

T = the absolute temperature

V = the volume of one mole of water

P = the atmospheric pressure

The relationship between a_w and water potential is summarized in Table 1.1.

A_w is widely used in the grain industry whereas ψ is more commonly used in soil microbiology. There has been pressure to adopt ψ more widely to unify the concept of water availability (Lacey & Magan, 1991). One of the advantages of ψ over a_w is that a_w represents only one component of ψ and varies with temperature, whereas ψ is expressed in the same units as osmotic potential of both substrate and cell and is independent of temperature. It can also be separated into its components osmotic, matrice and turgor potentials to indicate their relative importance.

1.2.1 Moisture sorption isotherms

Water content can be plotted against water activity (a_w) at a constant temperature to give a characteristic sigmoidal curve for a particular substrate. This is known as a moisture sorption isotherm. Moisture sorption isotherms are highly specific to substrate type and condition and are an important stage in manipulating a_w to investigate microbial growth and toxin production. For each harvest and each batch of grain a new moisture sorption isotherm was constructed due to variability in substrate type and differences in initial moisture contents.

Table 1.1 The relationship between water activity, equilibrium relative humidity and water potentials at 25 °C.

Water activity	E.R.H. (%)	Water potential (-MPa)
1.00	100	0
0.99	99	1.38
0.98	98	2.78
0.97	97	4.19
0.96	96	5.62
0.95	95	7.06
0.90	90	14.50
0.85	85	22.40
0.80	80	30.70
0.75	75	39.60
0.70	70	40.10
0.60	60	70.30

1.2.2 Effect of a_w on fungal growth

Fungi which invade grain are traditionally divided into two groups, namely 'field' or 'storage' fungi (Christensen & Kaufmann, 1969). 'Field' fungi, which invade temperate grains are mainly from the genera *Alternaria, Cladosporium, Fusarium* and *Dreschslera* which colonise grain before harvest and seldom grow during storage. Field fungi generally require >0.90 a_w for growth.

'Storage' fungi include species mainly from two genera: *Aspergillus* and *Penicillium.* These fungi are adapted to lower moisture conditions than field fungi. They are often widespread but are present in grains at low density before harvest but flourish under favourable conditions during storage. Grain may also be contaminated with storage fungi during harvest and when it is placed in contaminated stores (Flannigan, 1978; Lacey & Magan, 1991). During initial storage, fungal inoculum can become redistributed in grain; mechanical damage is also conducive to entry of spoilage fungi in insufficiently dried grain.

An intermediate group has been proposed since there is no clear distinction between field and storage fungi. This is demonstrated by *Fusarium* spp., which although traditionally categorised as 'field' fungi, can sometimes continue to develop in storage under certain condition (Pelhate, 1968). Generally provided grain is stored at <0.70 a_w then no grain spoilage will occur. However, since grain is often traded on a wet weight basis, inefficient drying systems can lead to fungal activity and mycotoxin production which renders grain useless for consumption.

1.3 FUNGAL INTERACTIONS

A_w and temperature interact with one-another to determine the range of microorganisms which can colonise a given substrate, their activity and contribution to spontaneous heating. Changing either temperature or a_w affects growth and may affect the ability of species to compete with one-another (Magan & Lacey, 1985a, 1985b; Marin *et al.*,

1998a, b, c). This in turn could have a serious impact on the production of OTA (Ramakrishna *et al.*, 1996; Lee & Magan, 1999a; Cairns *et al.*, 2003).

Freshly harvested grain is contaminated with 'field' and 'storage' fungi. Most grain fungi, including *Penicillium* and *Aspergillus* spp. grow between 10-40 °C with optima growth at 25-35 °C and are classified as mesophiles. Water availability and temperature not only interact to affect fungal development, but also plant growth and its subsequent susceptibility to fungal invasion both in the field and during storage.

Since wheat grain is colonised by a wide variety of microorganisms it is inevitable that intraspecific and interspecific interactions will occur depending on the nutritional status of the grain and the prevailing environmental conditions (Marin *et al.*, 1998a). The ability of *P. verrucosum* and *A. ochraceus* to colonise wheat grain during storage and produce the mycotoxin OTA suggest that they have strong competitive capabilities at specific environmental conditions. Spoilage fungi colonising grain employ different primary and secondary strategies to occupy grain during storage conditions. These may involve combative (C-selected) strategies which maximise occupation and exploitation of resources in relatively non-stressed and undisturbed conditions; stress (S-selected) strategies which have involved the development of adaptations which allow survival and endurance of continuous environmental stress; and ruderal (R-selected) strategies which involve a short life span with a high reproductive potential, which often allows success in severely disturbed but nutrient-rich conditions. These strategies can merge to form secondary strategies (C-R, S-R, C-S, C-S-R) (Cooke & Whipps, 1993). Prolific production of spores, quick germination of these, possession of extracellular enzymes and increased growth rates allow species to succeed in primary resource capture. Subsequent interactions between spoilage fungi result in combat, antagonism and niche overlap which all influence secondary resource capture. A summary of the three primary strategies employed by fungi to survive and prosper in different environments is shown in Figure 1.2.

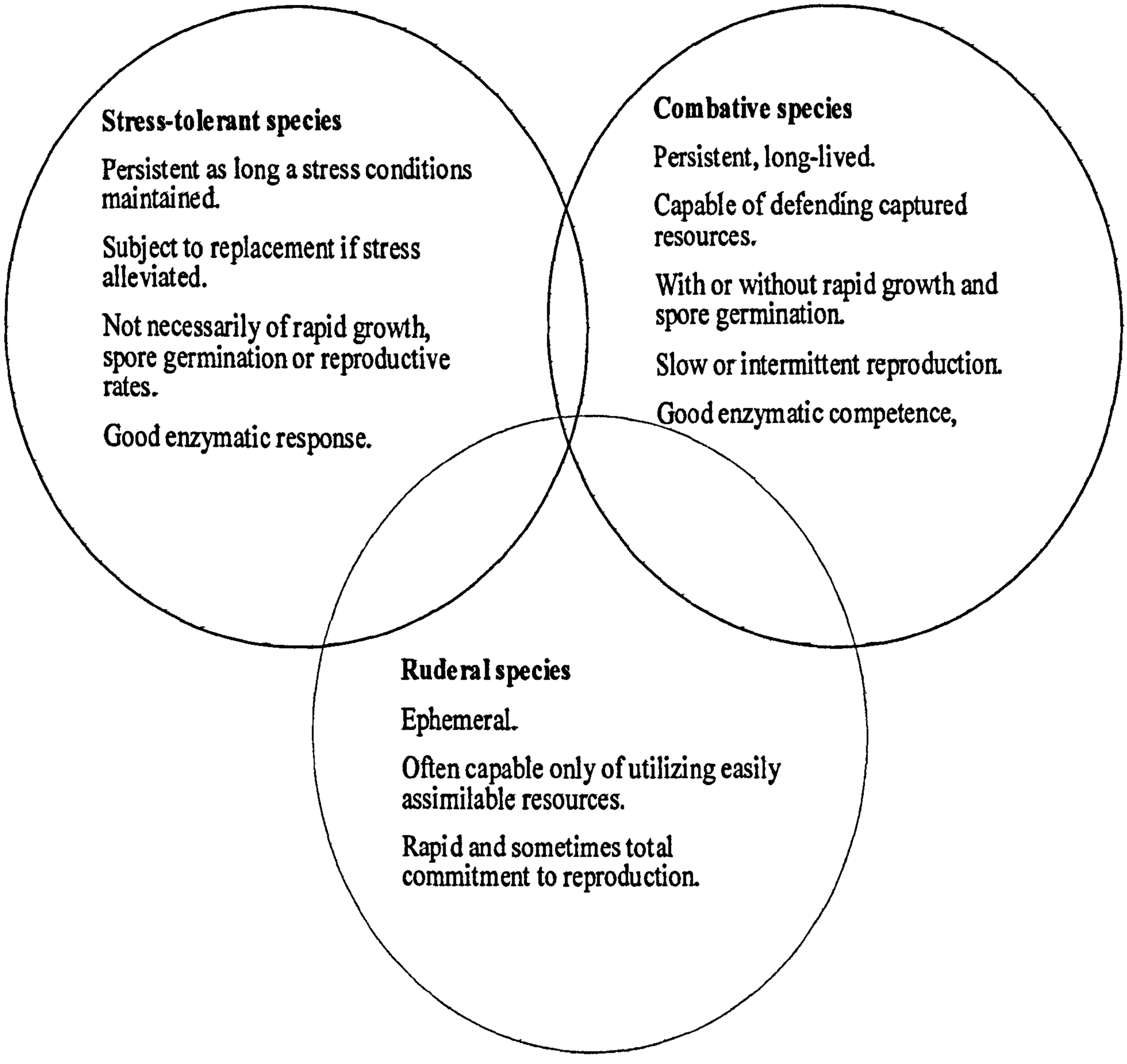


Figure 1.2 A summary of the three primary strategies employed by fungi to survive and prosper in different environments (Cooke & Rayner, 1984).

Water availability and temperature are probably the two most important abiotic factors influencing primary and secondary resource capture in the stored grain ecosystem. Changing either temperature or a_w affects growth and may affect the ability of species to compete with one another (Magan & Lacey, 1984a, b, 1985a; Marin *et al.,* 1998a) which could affect mycotoxin production (Ramakrishna *et al.,* 1996).

1.3.1 Measurement of competitive abilities of mycotoxigenic spoilage fungi

a) Index of Dominance

Attempts have been made to identify and score the range of interactions which can occur between species (Magan & Lacey, 1984b). Table 1.2 summarises the main types of interactions and the numerical scores given to try and understand competitiveness of individual species. The more dominant a fungus is over its neighbour the higher the score.

Magan & Lacey (1984b) demonstrated the impact of environmental conditions including temperature, a_w and the type of substrate on overall Index of Dominance (I_D) by addition of the competitive scores against other species. The I_D is used to compare the dominance of fungi under different environmental conditions from the same ecological niche. On maize grain, interactions and competition have been shown to markedly influence OTA production by *A. ochraceus* (Ramakrishna *et al.,* 1993, 1996; Lee & Magan, 2000). There have been no previous studies to examine the competitiveness of *P. verrucosum* and *A. ochraceus* against other spoilage fungi on wheat grain and the influence these interactions may have on OTA production.

Table 1.2 Descriptions and numerical values classifying interactions between fungi.

Classification description	**Numerical value**	**Interaction score ***
Mutual intermingling	1	1/1
Mutual inhibition on contact or space between colonies small (< 2mm)	2	2/2
Mutual inhibition at a distance	3	3/3
Inhibition of one organisms on contact, the inhibitor species continues to grow unchanged or at a reduced rate through the inhibited colony	4	4/0
Inhibition of one organism at a distance, the inhibitor species then continuing to grow through the resulting clear zone and the inhibited colony, perhaps at a reduced rate.	5	5/0

* The highest number is always given to the more dominant species

b) Niche overlap indices

Alternative approaches have been utilised to try and understand the relative competitiveness of different species within fungal communities colonising grain. Wilson & Lindow (1994a, b) working with biocontrol systems, suggested that the co-existence of microorganisms particularly on plant surfaces may be mediated via nutritional resource partitioning. Thus *in vitro* carbon utilization patterns could be used to determine niche overlap indices (NOI) and, thus the level of ecological similarity. Based on the range of similar carbon sources utilised and those unique to an individual isolate of species they suggested NOI values >0.9 were indicative of co-existence between species in an ecological niche, whereas scores <0.9 indicated occupation of separate niches. This approach has been adapted for stored grain ecosystems. Environmental factors including water availability and temperature can be used in conjunction with carbon source utilisation patterns to determine the level of co-existence or dominance of species in a stored grain niche (Marin *et al.*, 1998c; Lee & Magan, 1999b). These variables may be critical in understanding the conditions which allow some fungi to become dominant in a specific niche which in turn could have a serious impact on mycotoxin production (Marin *et al.*, 1998b). Lee & Magan (1999b) showed that changing either a_w or temperature modified the total number of carbon sources used (niche size) by *A. ochraceus* and other species, based on the Biolog plate spectrum consisting of 95 carbon sources and the 18 major carbon sources present in maize. With freely available water (0.995 a_w) the NOI for *A. ochraceus* changed against *Aspergillus flavus*, *Aspergillus niger* and *Eurotium* spp., where it had NOI <0.9. The capacity to utilise unusual nutrient sources at lowered a_w may enable some species, such as *E. repens*, to become dominant by assimilating carbon sources not available to *A. ochraceus*. Competitiveness is not solely dependent on the assimilation of carbon sources but rather a combination of factors including growth rates, metabolite production, niche overlap and interactions with environmental conditions (Lee & Magan, 1999b). No studies have examined the competitiveness of *P. verrucosum* against other wheat-spoilage fungi in the wheat grain ecosystem.

1.3.2 Hydrolytic enzyme production

There are biochemical tests available for assessing fungal presence such as chitin and ergosterol analysis, however, these are often time-consuming (Jain & Lacey, 1991). It has been suggested that the production of specific hydrolytic enzymes by spoilage fungi on temperate cereals can act as a good indicator of initiation of moulding in grain post-harvest (Magan, 1993).

Fungi produce an array of hydrolytic enzymes including cutinases, pectinases, cellulases and proteases (Knogge, 1996) which enable them to invade cereal grain and plant tissue. There are three main types of enzyme required for hydolysis of cellulose to glucose, namely endoglucanases, exoglucanases and β-glucosidases. The production of these enzymes is crucial for fungal colonisation of stored seeds.

Chromogenic 4-nitrophenyl substrates have been successfully used as indicators of fungal activity (Stevens & Relton, 1981, Jain *et al.*, 1991, Marin *et al.*, 1998d). Stevens & Relton (1981) and Jain *et al.* (1991) found the largest quantities of enzymes produced by *Aspergillus, Penicillium* and *Eurotium* spp. to be N-acetyl-β-glucosaminidase and α-D-galactosidase. Jain *et al.* (1991) showed that *Aspergillus, Penicillium* and *Eurotium* spp. produce β-glucosidase and N-acetyl-β-glucosaminidase, while only some produce α-galactosidase, β-galactosidase and α-gluosidase during colonisation of barley/wheat grain. Nearly all showed no β-glucoronidase, α-mannosidase and α-fucosidase activity.

Since hydrolytic enzymes play an important role in the colonisation and growth of a fungus on a specific substrate (Marin *et al.*, 1998d; Keshri & Magan, 2002), their production could be instrumental in helping to clearly distinguish between the activities of different mycotoxigenic species.

1.4 CONTROLLED ATMOSPHERIC STORAGE

During respiration of damp grain, oxygen is utilised and carbon dioxide produced according to the equation:

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6H_2O + 6CO_2 + 2835KJ$$

Moulds are classified as obligate aerobes but the amount of oxygen required for growth is often overestimated. Concentrations of oxygen and carbon dioxide in the intergranular atmosphere are important in determining fungal colonisation of grain during storage. In airtight storage, damp grain and contaminant fungi are allowed to respire resulting in oxygen utilisation and an accumulation in carbon dioxide. This inhibits fungal growth and toxin production and reduces the activity of insects which can spread fungal spores. This approach has been used on farms to store damp grain in sealed and unsealed silos (Hyde, 1974).

The effects of modified oxygen and carbon dioxide concentrations on the growth of fungi has been studied, but mainly only at a constant temperature (Follstad, 1966; Tabak & Cooke, 1968; McCarter, 1980; Pelhate, 1980). Magan & Lacey (1984c) investigated the effects of growth of some spoilage fungi in atmospheres containing 0.14–21 % O_2 and 0.03-15 % CO_2 on wheat-based media at various water activities (0.90-0.98 a_w). They found that concentrations of 5–10 % CO_2 stimulated growth of some species at 0.98 a_w but at lower a_w growth was decreased under these conditions. Changing either a_w or gas composition increased the lag phase for growth but combining these factors had a more than additive effect on lag phase periods, indicating synergism. Paster *et al.* (1995) studied the effects of different atmospheres on OTA production by *Aspergillus ochraceus* Wilhelm over 14 days on a solid synthetic media (Paster & Chet, 1980). Results showed that OTA was completely inhibited at high levels of CO_2 (30 %) and growth was completely inhibited at 80 % CO_2. High concentrations of CO_2 were not lethal to the fungus, and after removing the colonies from this treatment, normal growth and OTA production occurred.

There have been no studies to-date on the combined effects of a_w, temperature and varying levels of carbon dioxide on growth and OTA production by *P. verrucosum* and *A. ochraceus* on wheat grain. Previously, modification of atmospheres using CO_2 and O_2 concentrations have shown promise in controlling aflatoxin production in maize grain (Wilson *et al.*, 1975; 1976). However, removal from the modified atmospheres led to deterioration and further aflatoxin production.

1.5 MYCOTOXINS

Mycotoxins are 'naturally produced secondary metabolites produced by fungi that evoke a toxic response when introduced in low concentration to higher vertebrates and other animals by a natural route. Some mycotoxins have multiple effects and may cause phytotoxic and antimicrobial syndromes in addition to animal toxicity' (Bennett, 1987). The most important mycotoxins are aflatoxins, ochratoxin A, fumonisins, trichothecenes and zearalenon. Table 1.3 lists the major mycotoxins and toxin producing fungi from corn, cereals, soybeans, peanuts and other products and some of their effects on animals. The toxicity and carcinogenicity of many of these mycotoxins and their potential to contaminate foods and animal feedstuffs is a cause of serious concern globally, both from a food safety and food trade standpoint. Most cereals, including wheat, are susceptible to contamination by mycotoxins due to growth of *Aspergillus, Penicillium* and *Fusarium* spp. The extent and degree of contamination is highly dependent on the prevailing climatic conditions and hence mycotoxin prevalence tends to be seasonal. As these fungi are natural contaminants of wheat grain, exposure to them cannot be completely prevented. However, it has become necessary to reduce their intake as much as possible and many countries have legislation setting maximum limits for specific mycotoxins in raw materials and processed foods.

Table 1.3 Major mycotoxins and toxin producing fungi from corn, cereals, soybeans, peanuts and other products and some of their effects on animals (Jacobsen *et al.* 1993).

Genus	Toxin	Example of Fungal Source	Feeds or Food Effect	Possible Effects on Animals
Aspergillus species	(primary) Aflatoxins B1, B2, G1 and G2 B2a, G2a, M1 and M2 are metabolites and seldom present in grain; M1 and M2 are important contaminants in milk.	*A. flavus* and *A. parasiticus*	Cereal grains, peanuts, soybean and other foods	Hepatoxin; carcinogenic; reduced growth; haemorrhagic enteritis; suppression of natural immunity to infection; decreased production of meat, milk and eggs.
	Ochratoxins (nephrotoxins)	*A. ochraceus* and *P. verrucosum*	Cereal grain	Toxin to kidneys and liver; poor feed conversion, reduced growth rate. General unthriftness; reduced immunity to infection.
	Sterigmatocystin	*A. nidulans* and *A. versicolor*	Cereal grains	Toxaemia; carcinogenic.
	Tremorgenic toxin	*A. flavus, P. cyclopium* and *P. palitans*	Cereal grains, soybeans, peanuts and other food feeds	Tremors and convulsions.
Penicillium species	(primary) Luteoskyrin	*P. islandicum*	Rice	Tremors and convulsions.
	Patulin	*P. urticae, P. expansum, P. claviforme* and *A. clavatus*	Cereal grains, apple products	Haemorrhages of lung and brain; endema toxic to kidneys; possible carcinogen.
	Rubratoxin	*P. rubrum*		Liver damage and haemorrhage.
	Citrinin	*P. citrinum*		Kidney damage.

Table 1.3 (continued) Major mycotoxins and toxin producing fungi from corn, cereals, soybeans, peanuts and other products and some of their effects on animals (Jacobsen *et al.* 1993).

Genus	Toxin	Example of Fungal Source	Feeds or Food Effect	Possible Effects on Animals
Fusarium species	Zearalenone (estrogenic syndrome)	*F. graminearum*, *F. tricinctum* and *F. moniliforme*	Cereal grains	Hyperoestrogenism, infertility, stunting and death.
	Zearalenol Emetic or feed refusal (vomitoxin), deoxynivalenol, similar symptoms caused by nivalenol	*F. graminearum*, *F. culmorum*	Cereal grains	Food refusal, reduction in weight, reproductive dysfunctions, immune suppression.
	Other trichothecenes (T2 HT2) monoacetoxyscripenol or MAS, Diactoxyscripenol or DAS	*F. graminearum*, *F. tricinctum*, *F. poae* and *F.*sporotrichioides	Cereal grains	Inflammation of GI tract and possible haemorrhage; edema; vomiting and diarrhoea; infertility; degradation of bone marrow; weight reduction; slow growth; sterility.
	Fumonisin B1, B2	*F. moniliforme*	Corn	Leucoencephalomalacia, 'blind staggers' in horses.
Ergot	Ergopeptines	*Claviceps purpurea*	Cereal grains	Vasoconstriction, loss of extremities (i.e. ears, arms etc.).
	Ergovaline	*Acremonium coenophialwn*	Fescue	Reduced weight gain, abortion, poor survivability of offspring, fescue foot.

1.5.1 Ochratoxin A

OTA is found as a contaminant of a wide range of commodities including cereals, cereal products, dried vine fruit, coffee and coffee products, wine, grape juice, beer, cocoa and cocoa products and spices. It was first isolated by Van der Merwe *et al.* (1965) in cultures of *Aspergillus ochraceus*. Figure 1.3 shows the chemical structure of OTA.

In the following years, this toxin was found in the culture media of various species of *Aspergillus* and *Penicillium* (Pitt, 1987; International Agency for Research on Cancer, 1995). The two most important species which produce OTA in grain during storage are *Aspergillus ochraceus* and *Penicillium verrucosum*. *P. verrucosum* is reported almost exclusively from grain in temperature zones (Pitt, 1987; Frisvad & Lund, 1993; Elmholt & Hestberg, 1999; Scudamore *et al.,* 1999) whereas *Aspergilli* predominate in semi-tropical areas (Lillehoj & Elling, 1983). Although our knowledge on the fungi producing mycotoxins is increasing, many reports in the literature on these metabolites are unfortunately based on incorrect classification of the fungi and this has caused a lot of confusion. OTA was originally reported to be produced by *P. viridicatum* and this view prevailed for over a decade. It is now accepted that isolates that have been regarded as *P. viridicatum* but produced OTA are actually *P. verrucosum* (Pitt, 1987; Frisvad & Filtenborg, 1989; Frisvad, 1995). This is the only species of this genus which produces OTA.

A large number of cereal samples have been screened for fungal species and isolates that produce OTA. The results obtained from the EU funded project within the Fifth Framework (QLK-CT-1999-00433) indicated that *P. verrucosum* was the predominant producer in cereals in Europe. However, due to a limited number of samples obtained from southern Europe other less important producing species cannot be totally excluded.

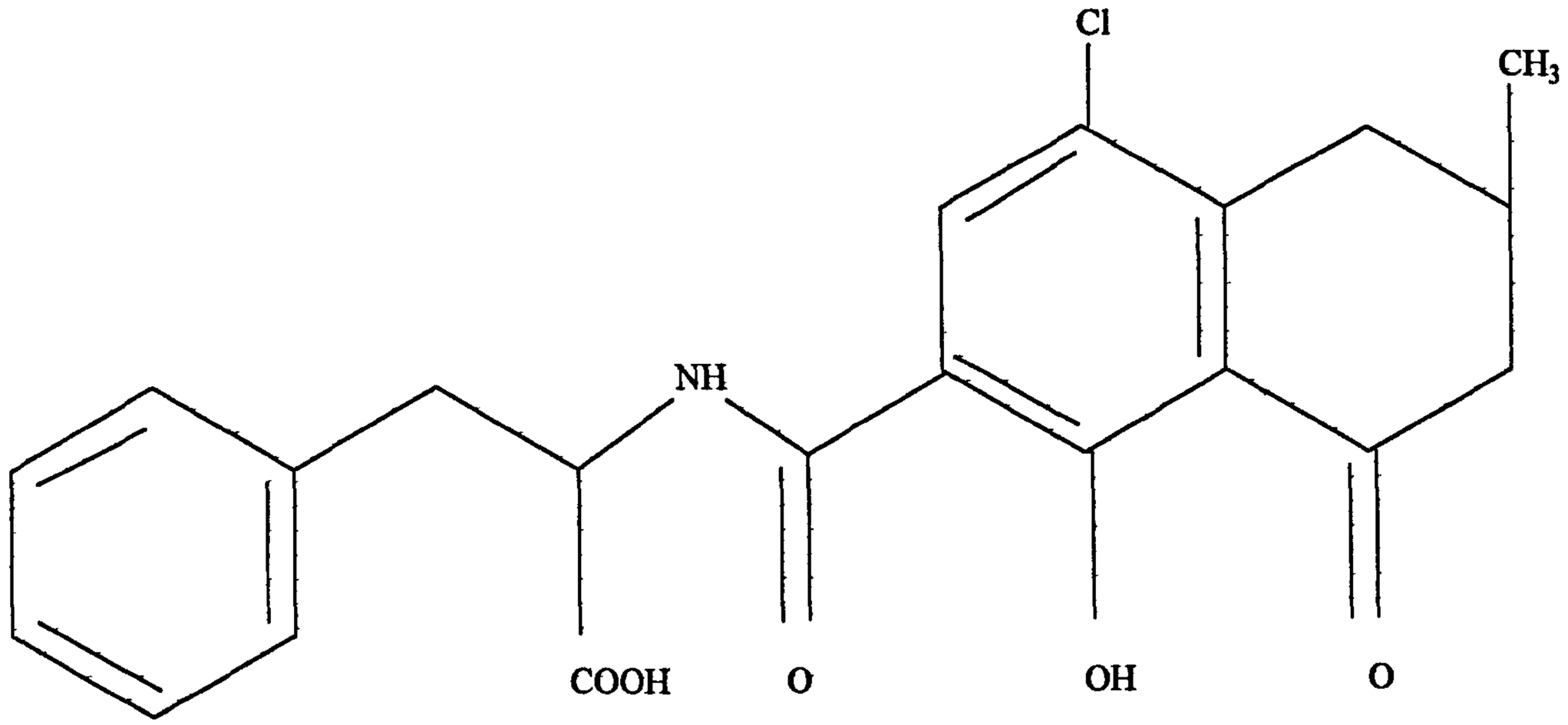


Figure 1.3 Chemical structure of ochratoxin A

In humans, OTA is believed to be a genotoxic carcinogen and so it is imperative that concentrations reaching consumers are kept as low as possible. In laboratory studies it has been shown to be carcinogenic to mice and rats (National Toxicology Program, 1989). OTA is frequently found in the blood of humans suggesting continuous and widespread exposure (Jörgenson *et al.,* 1996). This toxin is also believed to have been involved in the aetiology of Balkan Endemic Nephropathy in Balkan countries (Pohland *et al.,* 1992; Pohland, 1993; Kuiper-Goodman, 1995). There is no evidence to suggest that humans have adapted to the presence of mycotoxins (Kuiper-Goodman, 1995). Furthermore, due to the stability of OTA, it may pass through industrial processing into consumer products such as bread, without a large reduction in toxin (Scott *et al.,* 1972; Elling *et al.,* 1975; Krogh *et al.,* 1993). OTA is therefore of considerable concern from a human health perspective.

1.5.2 Ochratoxin A in food: a regulatory perpective

OTA has been implicated as a cause of kidney damage in humans and is considered by the UK committees on Carcinogenicity and Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COC/COM) to be potentially genotoxic to humans (COC/COM, 1992). The UK committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (COT) has recommended that OTA concentrations in food should be reduced to the lowest technologically achievable amount (COT, 1993). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) reviewed OTA in 1995 and again in 2001 when they retained the provisional weekly intake of 100 ng kg^{-1} body weight (JECFA, 2001). OTA was considered by the EC's Scientific Committee for Food (SCF) in 1998 which concluded that it would be prudent to reduce exposure of OTA as much as possible and proposed a tolerable daily intake (TDI) of less than 5 ng kg^{-1} bodyweight (SCF, 1998). In European legislation (EC) No 472/2002 dated 12 March 2002 maximum allowed levels of OTA in some foods were established. These are summarised in Table 1.4.

In the most recent assessment of OTA among European consumers (SCOOP, 2002) and in earlier investigations, cereals have been found to be the most important dietry source of OTA, contributing between 50–80 % of the intake.

Table 1.4 European legislation for maximum levels of ochratoxin A allowed in various foods (European legislation No 472, 2002).

	Product	Maximum levels (μg kg $^{-1}$ or ppb)
2.2.	OCHRATOXIN A	
2.2.1	Cereals (including rice and buckwheat) and derived cereal products	
2.2.1.1.	Raw cereal grains (including raw rice and buckwheat)	5
2.2.1.2.	All products derived from cereals (including processed cereal products and cereal grains intended for direct human consumption)	3
2.2.2.	Dried vine fruit (currants, raisins and sultanas)	10
2.2.3.	Green and roasted coffee and coffee products, Wine, beer, grape juice, cocoa and cocoa products and spices	-

1.5.3 Factors which affect fungal growth and mycotoxin production

The formation of mycotoxins is closely linked to fungal growth. Without growth mycotoxin production will not normally occur. Mycotoxins are usually produced in the late exponential or early stationary phase of growth. The presence of mycotoxigenic fungi in a product does not automatically indicate the presence of mycotoxins. Toxins however may persist long after vegetative growth has occurred and the moulds have died (Northolt, 1979).

Factors which affect fungal growth and mycotoxin production in storage include water availability, temperature, time, intergranular gaseous composition, grain cultivar, mechanical damage to the grain, invertebrate vectors, composition of the substrate, fungal abundance, prevalence of toxigenic strains and species, spore loads, use of chemical preservatives and interactions with other microorganisms. Each toxigenic fungus has different water, temperature, oxygen and nutritional requirement for growth and development and these may be different to that required for mycotoxin production (Lacey & Magan, 1991).

The large number of environmental variables and combinations which affect mycotoxin contamination of cereals in storage has created problems in establishing cause-effect relationships. Many authors have suggested that there are differences in mycotoxin accumulation between different cultivars of the same crop species. This indicates that some properties of the cultivar influence its resistance to fungal infection, or the ability of the attacking fungi to produce mycotoxins (Axberg *et al.*, 1997). It has been shown that durum wheat (*Triticum durum*) can contain up to three times more OTA than products of various other wheat cultivars (Baumann & Zimmerli, 1988). The growth of fungi and their production of OTA is closely related to water availability and temperature during the growth, harvest and storage of cereals. Important factors which affect the water availability in cereals during storage are the climatic conditions during growth and harvest, and the harvesting, drying and storage techniques used (Bruce & Ryniecki, 1991; Lacey & Magan, 1991). It has been observed that OTA levels vary from year to year depending on the climate and storage condition. In Sweden, an increase in OTA levels in

cereals and in human blood have been linked to wet harvest years (Ősen *et al.*, 1993; Thuvander *et al.*, 2001).

1.6 HARVESTING, MECHANICAL DAMAGE, STORAGE AND INSECT VECTORS

1.6.1 Harvesting and mechanical damage

Most cereal is harvested by using a combine (a combination of cutting and threshing). Before harvesting with a combine, the grain should be ripe and dried down to approximately 15–18 % moisture. Grain with a higher moisture content is not able to be stored safely unless artificially dried immediately after harvest.

During harvest, fungal inoculum is redistributed throughout the grain, whether it be directly from the soil, air or wheat itself and further inoculum is reintroduced. There is further contamination from residues trapped within the combine harvester even from previous harvest years (Banks *et al.*, 2002). Wheat grain can be damaged by the combine itself, or by rodents, birds or insects which can break the outer seed coat and facilitate infection. Furthemore, insect movement can also aid in the spreading of inoculum.

1.6.2 Drying and storage practices

The term 'storage' is often used to encompass drying, cooling and storage of grain (Bruce & Ryniecki, 1991). In Northern Europe during harvest the weather is normally wet and therefore it is necessary to dry grain artificially before storage. Problems may arise when the amount of grain harvested exceeds the amount the drying plants can process. This means that large amounts of grain are temporarily stored in moist conditions for days or even weeks without cooling or ventilation prior to drying. It is imperative that efficient drying, cooling and storage of grain are employed immediately after harvest to ensure that its moisture content is sufficiently low to suppress fungal metabolism and prevent mycotoxin production.

Grain can be dried using air at near-ambient or high temperatures resulting in **'near-ambient air'** or **'hot air'** drying.

In **'near ambient'** drying, air is blown vertically through the bed of grain which can be up to 3 m in depth. This depth is dependent on the moisture content of the grain and the volume flow of air which when combined, determine the time taken for the grain to dry. The drying process can take up to several weeks. The configuration of drying patterns and progress of drying are dependent upon the type of grain being dried, initial moisture content of the grain, condition and amount of air being forced through the grain and uniformity of air distribution within the bulk grain (Jayas *et al.*, 2003). There are distinct levels of zones of dry, drying and wet grain created in the bulk (Figure 1.4). Grain on the upper surface is wettest for the longest period of time, and therefore it is more susceptible to fungal growth. 'Near ambient' drying is predominantly used in the UK. Sometimes, the drying power of the ambient air is insufficient to dry the grain, and on these occasions there is a need for a heated electric fan.

'Hot air' dryers use air temperatures of 40-100 °C to dry grain and has the advantage of the process being independent of the weather. Air at these high temperatures has a greater capacity for holding moisture, so much less air is required per unit of grain than the near-ambient drying system. When using hot air for drying grain, grain should not be heated above the maximum allowed temperature for a particular end use (Table 1.5). If the wheat grain is heated too quickly, stress cracks can form in the grain, thereby increasing susceptibility to fungal attack. If the wheat grain is overheated, then the relationship between water availability and water content can be altered, perhaps leading to increased susceptibility to moulding at given water contents (Tuite & Foster, 1963). There are two different methods for heated-air drying:

-Batch, where the dryer is emptied between batches. This system is often mobile. Moisture removal depends on the drying time, which can be altered to reach a moisture level safe for storage. Cooling time can be set independently of drying time. Some batch dryers mix and re-circulate the grain while running to give more uniform drying.

-Continuous, where grain is dried without re-circulation. Plant is usually fixed. The moisture removal depends on the drying time, i.e. time to pass through the heated air section, which is determined by the grain discharge rate. This rate determines cooling time so extra cooling may be necessary if grain discharge is high (Central Science Laboratory, 1999).

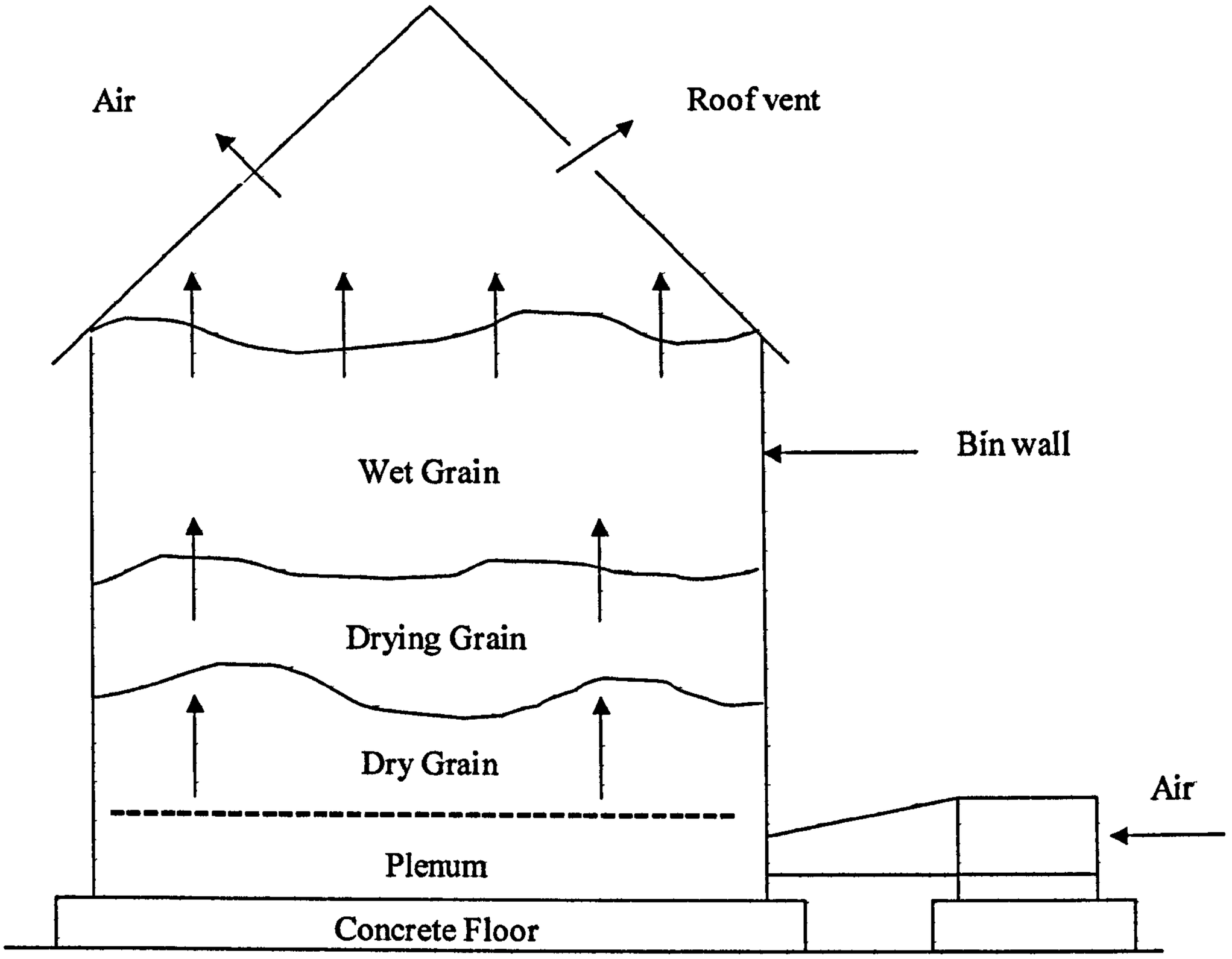


Figure 1.4 Components of a near-ambient grain drying system and the movement of the drying front (Pabis *et al.*, 1998)

Table 1.5 Maximum safe temperatures (°C) of heated air entering grain for drying for various end uses (Hall, 1980)

Crop	End use		
	Seed	Commercial use[a]	Animal feed[b]
Ear corn	43	54	82
Shelled corn	43	54	82
Wheat	43	60	82
Oats	43	60	82
Barley	41	41	82
Sorghum	43	60	82
Soybeans	43	49	
Rice	43	43	
Peanuts	32	32	

[a]Higher air temperatures than those listed may be used when the grain is dried under carefully controlled conditions so that the maximum temperature of the kernels, at any time, does not exceed those listed in the table.

1.6.3 Storage of grain

Provided grain is dry and cool and placed in a weather-proof and rodent-proof structure, it can usually be stored for long periods without suffering quality loss from fungal deterioration or insect feeding such as lowered germination, increased free fatty acids and other biochemical changes (Tipples, 1995). Grain initially containing 12.6 % water (0.60 a_w) has been safely stored experimentally for up to 16 years, during which water content increased by 0.7 % (Pixton, 1967; Pixton *et al.*, 1972, 1975). However, if grain is stored in large bulks without aeration it will be indirectly affected by the weather. The bulk will retain the heat it had at harvest at the centre (Jayas *et al.*, 1994) and reflect external ambient temperatures near the periphery (Jayas *et al.*, 2003). In temperate climates particularly northern Europe, grain can remain as warm (15-20 °C) throughout the winter even though the outside temperature can be as low as 0 °C. Air convection currents carry moisture to the top-centre of the bulk which can result in localised grain spoilage and mycotoxin production (Abramson, 1991).

1.6.4 Relationship between insects and mycotoxin producing fungi in stored grain

Insects are an important common problem in stored grain ecosystems. They may grow at water availabilities lower than that required for fungal growth. Insect activity can produce heat and water enabling fungi to grow resulting in 'hot spots,' which can lead to complete spoilage of grain (Magan *et al.*, 2003). Temperatures can reach as high as 60 °C which although are not high enough to cause combustion of the grain (McLean, 1989) can spoil the germ and create conditions in which thermophilic organisms can thrive. There are two causes of 'hot spot' formation, namely 'dry' and 'damp' grain heating.

'Dry' grain heating occurs when grain is too dry to allow metabolic heating from its microflora (<15 % m.c. at 70 % E.R.H.), but where insects can breed. Insect activity causes a small increase in temperature which increases both the metabolism of the insect and the population's growth rate thereby continuously increasing the amount of metabolic heat produced (Howe, 1962; Sinha & Wallace, 1965). The metabolic heat accumulates faster than it can disperse creating a hot spot. Vertical heat transfer caused

by convection is frequently accompanied by migration of active species (Cooke & Armitage, 2002).

'Damp' grain heating is caused by metabolic heat from high levels of fungi and is associated with grain at >15 % m.c. Condensation of the metabolic water at surfaces increases the spread of fungi and the associated heating. Damp grain heating may follow from insect-induced heating as hot air condenses on cool grain at the grain surface and can produce much higher temperatures.

Not only can insects contribute to deterioration of the grain via spontaneous heating (Sinha & Wallace, 1966), but they can damage the wheat through feeding and thereby increasing fungal infection (Wallace & Sinha, 1981); carrying spores on their bodies (Dix, 1984; Dunkel, 1988); faecal material may provide substrates for colonisation (Wallace & Sinha, 1981); and production of inhibitory secretions (Van Wyck *et al.*, 1959; Armitage & George, 1986). Fungi may either attract or inhibit insects or mites, perhaps through mediation of volatiles or mycotoxins (Van Wyck *et al.*, 1959; Wright *et al.*, 1976; Wright *et al.*, 1980a, b) and may provide them with food (Wallace & Sinha, 1981; Armitage & George, 1986).

Despite the interactions between insects and ochratoxigenic fungi in stored grain ecosystems, there have been no recent studies of this relationship. Dix (1984) found that *Penicillium* spp. and *A. flavus* were associated with *Sitophilus zeamais*. As adults they carried a high density of spores without succumbing to aflatoxicoses. Dunkel (1988) examined the effect of varying concentrations of OTA, rubratoxin B, citrinin and patulin on larval weight and development time of three different insect species at between 0-1000 ppm concentration. Larval weight of *T. confusum* was only significantly affected by rubratoxin B, citrinin and patulin at 1000 ppm concentration, with little or no effect on adult emergence. Only one of the tree insect pests, namely *Attagenus megatoma* was significantly affected at 100-1000 ppm concentrations. More studies of the interactions between insect pests and spoilage fungi are necessary in order to increase our understanding and improve post-harvest management of stored grain ecosystems.

1.7 OTHER METHODS FOR CONTROLLING GROWTH AND OCHRATOXIN A PRODUCTION BY *PENICILLIUM VERRUCOSUM* AND *ASPERGILLUS OCHRACEUS* DURING GRAIN STORAGE.

1.7.1 Irradiation

Gamma irradiation allows sterilisation of grain without destroying its germinative capacity and with minimal chemical damage. *Aspergillus* and *Penicillium* are killed by 1.2 kGy, Mucoraceae require 6 kGy and *Fusarium*, yeasts and *Bacillus* spp. require up to 12 kGy (Cuero *et al.,* 1986). It has been suggested that low doses of irradiation may result in an increase in mycotoxin production, although this point has been argued and may depend partly on other factors such as differences in irradiation conditions or water content (Applegate & Chipley, 1973; Chang & Markakis, 1982), or stimulation of non-toxigenic isolates to produce toxin (Jemmali & Guilbot, 1969; Applegate & Chipley, 1979).

Whilst irradiating wheat grain with 12 kGy kills *Aspergillus* and *Penicillium* species, the presence of OTA is not eliminated. Furthermore, irradiating wheat grain is expensive and therefore uneconomical and is unacceptable from a consumer perspective.

1.7.2 Modified atmospheric storage

Sulphur dioxide (SO_2) and ammonia (NH_3) have also been used to manipulate the atmosphere in grain stores both alone and in combination. When used separately, both inhibit spore germination or mycelial growth of contaminant fungi with small doses and have been used in low temperature stores (Ecknoff *et al.*,1979; 1983). In combination, it has been reported that mould growth is stimulated. It has been suggested that this is due to the *in situ* formation of ammonium sulphate when the NH_3 is added to SO_2. Ammonium sulphate may actually promote growth because it can serve as a nitrogen source. Ecknoff *et al.* (1983) reported that in laboratory tests in which NH_3 was applied to corn followed by SO_2 moulds grew faster than in the untreated control. Furthermore, it was found that there was a larger diversity of fungi present in the control than in the

treated bins which consisted mainly of *Penicillium* spp. It has been suggested that the treatment initially suppresses the indigenous mycoflora so that when the treatment begins to fail, the most resilient and opportunistic fungi dominate, in this case the *Penicillium* spp. Single-organism cultures can grow faster than mixed cultures because of the lack of competition and depending on the organism may result in greater toxin production than in mixed cultures (Ecknoff, 1983).

1.7.3 Synthetic preservatives

Storage fungi are commonly controlled using synthetic preservatives (mainly low molecular weight organic acids), but most of these are associated with side-effects including carcinogenicity, teratogenicity and residual toxicity (Wurtzen *et al.*, 1986; Verhagen *et al.*, 1991; Basilico & Basilico, 1999; Milos, 2000) and there is worldwide trend towards limiting their use in grain (Paster *et al.*, 1995). Butlylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and propyl paraben are examples of some synthetic preservatives which have been found to be effective inhibitors of mycelial growth (Thompson, 1991; Thompson, 1992; Torres *et al.*, 2003). The phenolic antioxidant BHA has been shown to prevent germination of conidia of *Aspergillus* spp. on PDA at concentrations of >200 $\mu g\ ml^{-1}$. Thompson (1993) studied the effects of esters of p-hydroxybenzoic acid (Paraben) on the growth of three mycotoxigenic fungi and found that butyl and propyl paraben were the most effective causing complete mycelial inhibition at concentrations of 1.0-2.0 mM. The effectiveness of parabens was shown to increase with chain length of the ester group, however its mechanism of action is unclear. Two hypotheses have been proposed; depression of intracellular pH by ionization of the undissociated acid molecule or distribution of substrate transport by alteration of cell membrane permeability. Propyl paraben and BHA appear to work mainly at the cell membrane eliminating the pH component of the promotive 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, thereby inhibiting respiration (Khan *et al.*, 2001).

During the past few years, interest has grown regarding the use of new antimicrobials for controlling fungal growth in food and feed (Gould, 1995; Nychas, 1995). Natural antimicrobial systems include herbs, plants and spices (or their natural components) which are traditional ingredients and flavour enhancers (López-Malo *et al.*, 1998). Their use is perceived as raising few concerns amongst consumers, regulatory agencies or within the food industry (Dillon & Board, 1994).

Many antimicrobial compounds are identified as secondary metabolites mainly of terpenoid or phenolic biosynthetic nature. They may be categorized into four groups; phenols, phytoalexins, organic acids or essential oil components (Smid & Gorris, 1999).

1.7.4 Phenolic compounds

Phenolic compounds are compounds containing an aromatic ring bearing one or more hydroxy substituents. Phenolic compounds are usually conjugated to sugars (Gould, 1996a, b). They contribute towards the defence mechanism of plants against invading microorganisms (Smid & Gorris, 1999). Russell & Chopra (1990) reported that these molecules affect microbial cell membranes or inhibit the germination of spores. Phenolic compounds contain a wide range of antimicrobials including those from essential oils (Nakatani, 1994) and phytoalexins (Smid & Gorris, 1999).

1.7.5 Organic acids

Propionic acid is found naturally in foods but in trace elements (Smid & Gorris, 1999). It has been used widely on farms. However, in grain inadequately treated with propionic acid there has been reports of contamination with aflatoxin (Hacking & Bigg, 1979). Recent studies investigating the effects of propionic acid on growth and fumonisin production at various temperatures and water availability showed that to some extent depending on water availability and temperature growth can be controlled. However, there was little effect on fumonisin production (Marin *et al.*, 1999; Marin *et al.*, 2000). For successful mould control, sufficient propionic acid must be applied for the water content of the grain, and treatment needs to be uniform without untreated or under-treated

pockets which could allow the growth of propionate-tolerant organisms. Furthermore, small concentrations of propionate have been shown to stimulate aflatoxin production by *A. flavus.* (Al-Hilli & Smith, 1979). Although sorbic acid has been used less than propionic acid, it is insecticidial and fungistatic so provides a useful alternative to propionic acid (Lacey & Magan, 1991).

1.7.6 Phytoalexins

Phytoalexins are host-synthesised, low molecular weight, broad-spectrum compounds produced by plants in response to microbial infection. One example of a phytoalexin is the protein chitinase. Chitinases target chitin, a major components of the cell wall of most fungi and also of the skeletal structure of most invertebrates such as insects and mites. As vertebrates and higher plants do not contain chitin they should be unaffected by this protein. There is therefore potential for using chitinases as antimicrobial agents in stored grain ecosystems. The disadvantage of using phytoalexins in practice has been limited since it has been found that high concentrations are usually required for a moderate antimicrobial effects (Gould, 1996; Smid & Gorris, 1999). However, recently there has been much interest surrounding resveratrol. It is a polyphenolic compound with antioxidant and antimicrobial properties (Pinto *et al.,* 1999; Fremont, 2000; Miura *et al.,* 2000), so far used as a dietry integrator and naturally found in plants such as *Polygonum cuspidatum* and *Vitis vinifera* (Fanelli *et al.*, 2003). It is a phytloalexin in grapes which is synthesised in response to stress factors such as UV exposure or attack by fungal pathogens. It has also been found to have anti-cancerous properties inducing cell differentiation and apoptosis in cancerous cells (Pervaiz, 2001). Promising results have been obtained using resveratrol to suppress OTA production by *A. ochraceus* at 0.95 a_w in maize and on wheat grain (Fanelli *et al.,* 2003). Future use of phytoalexins may lie in the development of analogues with higher specific activity and reduced toxicity (Smid & Gorris, 1999).

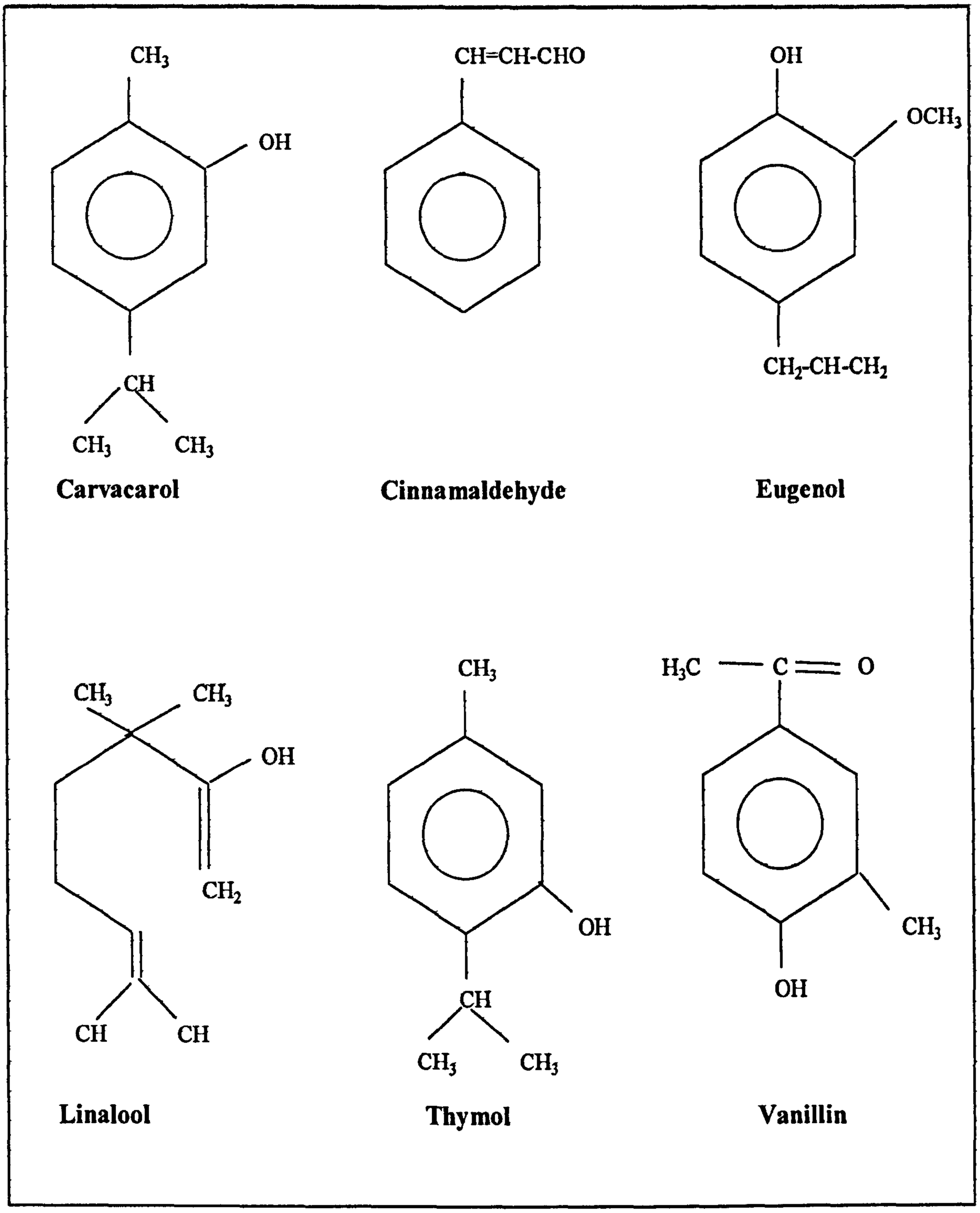
1.7.7 Essential oils

Essential oils are volatile products of plant secondary metabolism which in many cases are biologically active with antimicrobial, antioxidant and bioregulatory properties (French, 1985; Caccioni *et al.,* 1995a, b; Yin & Tsao, 1999; Hope *et al.,* 2002). Essential oils are mostly soluble in alcohol and to a limited extent in water. They consist of esters, aldehydes, ketones and terpenes. To date, essential oils of oregano, thyme, basil, garlic, onion and cinnamon have been reported as some of those with the greatest antimicrobial effectiveness (Bilgrami *et al.,* 1992; Paster *et al.,* 1995; Ozcan, 1998; Basilico & Basilico, 1999; Yin & Tsao, 1999; Cairns & Magan, 2002; Hope *et al.,* 2002).

Phenolic compounds are probably the major type of essential oil components (Sinha, 1990; Smid & Gorris, 1999). Figure 1.5 shows some of the most active phenolic compounds of essential oils. Essential oil components with a wide range of antimicrobial effects include thymol from thyme and oregano, carvacrol from oregano (Madsen & Bertelsen, 1995), cinnamaldehyde from cinnamon and eugenol from clove (Cold, 1996; Consentino *et al.,* 1999; Smid & Gorris, 1999).

Paster *et al.* (1995) investigated the effects of different concentrations of oregano (*Origanum vulgare)* and thyme (*Coridothymus capitatus)* on mycelia and spores of *A. flavus, A. niger* and *A. ochraceus* and 'normal' flora on wheat grains. They found that when these oils were used as fumigants, oregano inhibited mycelial growth of fungi at MIC 2.0 µl L^{-1} and spore production was inhibitied at 2.0-2.5 µl L^{-1}. Thyme inhibited mycelial growth at 4.0 µl and was fungistatic to spores at 3.0 µl L^{-1}.

Basilico *et al.* (1999) investigated the effects of oregano *(Origanum vulgare),* mint (*Menta arevensis),* basil *(Ocimum basilicum),* sage *(Salvia officinalis)* and coriander *(Corianrum sativum)* on mycelial growth and OTA production by *A. ochraceus* NRRL 3174 on a yeast-extract-sucrose medium (YES). Results showed that sage and coriander showed no effect on growth or OTA production. However, at 1000 ppm oregano and mint completely inhibited fungal growth and OTA production for up to 21 days. Basil was effective for only 7 days. At 750 ppm, oregano was completely effective for up to 14 days, whereas mint allowed fungal growth but no OTA production for up to 14 days. At 500 ppm, there was no evident inhibition by any of the tested essential oils.



Carvacarol **Cinnamaldehyde** **Eugenol**

Linalool **Thymol** **Vanillin**

Figure 1.5 Phenolic structures of active components of some essential oils.

Several studies have been carried out on the inhibitory effects of essential oil components (Sinha & Gulati, 1990; Mahmoud, 1994; Saxena & Mathela, 1996; Lachowicz *et al.*, 1998), however, the exact role of these components on the overall antimicrobial activity of the oil is unclear. The components may act synergistically to bring about antimicrobial action, or they may act antagonistically and lead to growth stimulation (French, 1985). Limone and terpinene are the two main components of carrot seed oil. Batt *et al.* (1983) showed that the inhibition of *A. parasiticus* was found to be higher for the components than the essential oil. Alderman & Marth (1976) showed the oils derived from orange and lemon peel were more effective at controlling growth and aflatoxin production than d-limonene, the main constituent of orange and lemon peel. Paster *et al.* (1995) found that thyme essential oil was more inhibitory than the individual components.

There has been much speculation regarding the mode of action of essential oils. Prindle & Wright (1977) reported that the mode of action of phenolics as antimicrobial agents was concentration dependent. Conner & Beuchat (1984) suggested that the antimicrobial activity of essential oils could be the result of damage to enzymatic cell systems including those associated with energy production and synthesis of structural compounds. Nychas (1995) indicated that phenolics could denature the enzymes responsible for spore germination or interfere with amino acids involved in germination.

Few studies have investigated the effects of essential oils on growth and OTA production by ochratoxigenic strains of *P. verrucosum* and *A. ochraceus* (Basilico & Basilico, 1999). Furthermore, many studies have not taken into account the effect that environmental factors such as temperature and water availability may have on the effectiveness of essential oils in relation to fungal growth and OTA production. In most studies where antimicrobial activity has been studied the technique has involved incorporation of the essential oil into the growth medium. When the essential oil has been tested with food less efficacy has been observed. It has been suggested that this is due to specific components of the food product such as proteins and fats, binding to the essential oil, inactivating them (McNeil & Chmidt, 1993; Smid & Gorris, 1999).

If essential oils were to be use in stored grain ecosystems certain variables would have to be considered. These include variables in the yield of the active compounds or total oil with the plant genotype and with differences in extraction procedures. Also, variations are to be expected in essential oils composition of the same species according to geographical location, environmental and agronomical conditions as well as differences in essential oils content with diurnal rhythm. Essential oils or their active components are not in a ready-to use-form and many parameters need to be standardised if essential oils were to be used in practice (Smid & Gorris, 1999). Although the compounds in the essential oils such as eugenol from clove, thymol from thyme possess antioxidative properties, their aromatic character could limit their use as antioxidants in wheat grain (Madsen & Bertelsen, 1995).

1.7.8 Biological Control of Spoilage in Moist Cereals

Recent studies in Sweden have attempted to prevent poor quality cereals and potential contamination with mycotoxins by using antagonistic and competitive yeasts such as *Pichia anomala* which can be inoculated onto wheat, barley, or oats prior to storage. This yeast effectively colonises the stored cereal and prevents spoilage moulds from causing deterioration in feed grain quality. *P. roqueforti* growth has shown to be controlled by *P. anomola* in moist cereals (Petersson and Schnurer, 1998). Recent studies indicate that antagonistic yeasts such as *P. anomala* may also affect OTA production accumulation by *P. verrucosum* in cool temperature climates (Petersson *et al.*, 1998). The use of biological control agents is a promising area for future research of natural inoculant treatments, especially feedstuffs free of mycotoxin contaminations (Magan *et al.*, 2004).

1.8 Hazard Analysis Critical Control Point (HACCP) - An Integrated Approach

HACCP (Hazard Analysis Critical Control Point) is the name given to a system of controls specifically designed to prevent safety problems. It is normally applied to food manufacture, and represents a proactive system of preventive actions rather than a reliance on periodic inspection, end point testing and reactive responses to problems. The HACCP system is composed of seven principles outlined in Table 1.6.

Table 1.6 The seven principles of Hazard Analysis Critical Control Point (HACCP).

Principle number	Description
1	Assess the hazard associated with growing, harvesting, raw materials, ingredients, processing, manufacturing, distribution, marketing, preparation and consumption of food.
2	Determine Critical Control Points (CCPs) required to control the identified hazards.
3	Establish the critical control limits that must be met at each identified CCP.
4	Establish procedures to monitor CCPs.
5	Establish corrective action to be taken when there is a deviation identified by monitoring a CCP.
6	Establish effective record-keeping systems that document the HACCP plan.
7	Establish procedures for verification that the HACCP system is working properly.

(National Advisory Committee on Microbiological criteria for Food, 1990)

Unfortunately, the presence of OTA may be unavoidable because it is a naturally occurring substance and spores of *P. verrucosum* and *A.ochraceus* are ubiquitous. One way to manage the risks associated with OTA contamination is by using the HACCP approach.

In today's global market, the grain industry is usually divided into separate entities that include, but are not limited to agriculture, storage and transportation, process and post-process distribution. Due to this, it is important to consider every stage in the chain from field to storage, to consumer (Lopez-Garcia, 1991). Each stage in the process should be assessed in terms of:

a) the role it plays in contributing to the risk of OTA production
b) what aspects within that stage contribute to increasing the risk of OTA contamination
c) the steps which can be taken within any particular stage that could reduce the risk of unacceptable contamination

Each stage should be used in conjunction with GMP (Good Manufacturing Practices) and/or GMP (Good Agricultural Practices) (Lopez Garcia, 1991). The preparation of suitably detailed and comprehensive procedural and record keeping documentation is of paramount importance for the running of a successful HACCP scheme. The application of a holistic HACCP plan for OTA control in an entire commodity supply train will inevitably involve complex and extensive documentation. When control systems to minimise the risk of OTA are developed and introduced, each country needs to take into account such factors as climate, farming systems, pre- and post-harvest technologies available, public health significance of OTA, availability of sampling and analytical resources and economic factors (Park *et al.*, 1999). The information from this project will be used to help construct a HACCP scheme to control growth of *P. verrucosum* and *A. ochraceus* and OTA production.

1.9 AIMS AND OBJECTIVES OF THE PROJECT

A. ochraceus and *P. verrucosum* are common contaminants of wheat grain during storage conditions and are responsible for large losses in yield and grain quality and contamination with OTA. Since *P. verrucosum* and *A. ochraceus* are ubiquitous it is impossible to remove them completely from grain during storage.

The overall objectives of this project were to a) identify the key abiotic and biotic factors and their interactions with other wheat spoilage fungi on growth and OTA production by *P. verrucosum* and *A. ochraceus* and b) assess the potential for control of growth and OTA production by *A. ochraceus* and *P. verrucosum* using natural antimicrobial agents in the form of essential oils and the phytoalexin, resveratrol, over a wide range of environmental conditions.

To achieve this the following studies were carried out:

1. Comparison of growth and OTA production by strains of *P. verrucosum* and *A. ochraceus* isolates under different a_w and temperature conditions.

2. Investigate the effects of competition against other wheat spoilage fungi on competitiveness, growth and OTA production by *P. verrucosum* and *A. ochraceus* under different abiotic conditions.

3. Investigate the effects of varying intergranular gaseous compositions on germination, growth and OTA production by *P. verrucosum* and *A. ochraceus* at different water availabilities.

4. Examine ecological competition of the OTA producing species by examining niche overlap dominance for *P. verrucosum, A. ochraceus* and other wheat-spoilage fungi.

5. Screen twenty-three food grade essential oils for their effectiveness at controlling growth of *P. verrucosum* and *A. ochraceus* isolates.

6. Study the most effective essential oils identified from the initial screen in more detail *in vitro* on a wheat-based media and *in situ* on γ-irradiated wheat grain. Determine their efficacy for control of growth and OTA production by *P. verrucosum* and *A. ochraceus*.

7. Investigate the ability of resveratrol to control growth and OTA production by *P. verrucosum* and *A. ochraceus* under different environmental conditions on irradiated wheat grain.

8. Investigate the ability of essential oils and resveratrol to control fungal populations and OTA levels in naturally contaminated wheat grain.

Figure 1.6 summarises the components of this work, their order and interaction

OCHRATOXIN PRODUCING ISOLATES

➢***Penicillium verrucosum***

➢***Aspergillus ochraceus***

PHASE I

ECOPHYSIOLOGY-ABIOTIC FACTORS

➢Effect of a_w x temperature interactions on

•Growth *in vitro* and *in situ*

•OTA production *in vitro* and *in situ*

➢Effect of a_w x gas composition interactions on

•Conidial germ tube lengths, growth and OTA production *in vitro* and *in situ*

•Growth and OTA production *in vitro* and *in situ*

ECOLOGY-FUNGAL INTERACTIONS

➢Other wheat-spoilage fungi

➢Interactions *in vitro* and *in situ*

• Indices of Dominance

•Niche overlap indices

•Colonisation

•OTA production

PHASE II

CONTROL STRATEGIES

➢Screening of twenty-three essential oils for their effectiveness at controlling growth *in vitro*

➢Use of the most effective essential oils and resveratrol for control of growth and OTA production over a range of a_w x temperature regimes *in vitro* and *in situ*

➢Effect of resveratrol for control of total fungal populations, *A. ochraceus, P. verrucosum* and OTA production on naturally contaminated wheat grain over a range of a_w x temperature regimes

➢PROPOSAL OF CRITICAL CONTROL LIMITS WITHIN THE WHEAT GRAIN PRODUCTION CHAIN

➢RECOMMENDATION OF AN ESSENTIAL OIL / RESVERAROL AS A POST-HARVEST ANTIFUNGAL

Figure 1.6 Flow diagram of the experimental work carried out in this thesis.

CHAPTER 2

MATERIALS AND METHODS

2.1 FUNGAL SPECIES AND CULTURE MAINTENANCE

The fungal species used in this study, together with their origin and Culture Collection Codes are shown in Table 2.1.

Spore suspensions were initially prepared for all species using 7-10 day old cultures grown on MEA at 0.995 a_w and 25 °C. Spores were suspended in a sterile 10 % glucose (Sigma), 90 % distilled H_2O solution including 0.9 % NaCl (technical, BDH) and 0.1 % Tween 80 (Sigma), before being transferred aseptically into sterile 1 ml Eppendorf tubes and frozen to –80 °C until required. Purity of storage cultures was verified by streak plating and microscopic analysis.

During the duration of the project, all species were periodically sub-cultured on MEA (every 2-3 months) and on a wheat-based medium in order to have a constant supply of fresh cultures. These cultures were used for all experiments over this 2-3 months.

2.2. SUBSTRATES FOR FUNGAL GROWTH

2.2.1 Wheat-based media

The 2 % wheat-based media was prepared by using 2 % (w/v) of milled wheat and 1.5 % (w/v) agar (Oxoid, technical agar no. 3) in distilled water. This was autoclaved for 15 minutes at 121 °C and 1 atm. The sterilised agar was allowed to cool to approximately 50 °C before being aseptically poured into sterile 90 mm diameter Petri dishes. Media had to be agitated whilst pouring to ensure that insoluble particles were distributed evenly. Plates were kept at 4 °C in sealed polyethylene bags for a maximum period of 21 days.

2.2.2 Malt extract agar (MEA)

Commercial MEA (Merck) was made up using the supplier recommendations. Once the components were mixed, the substrate was autoclaved and poured as mentioned previously. Petri plates were kept at 4 °C for a maximum period of 21 days.

Table 2.1 Fungal species used including their origin and Culture Collection Codes.

SPECIES	ORIGIN	CODE
Alternaria tenuissima	T.U. Denmark	IBT8320
Aspergillus ochraceus Wilhelm	Mixed feed or cereal (Spain)	IBT21991
Eurotium repens de Bary	Almond cake (Denmark)	IBT18000
Fusarium culmorum Link	Wheat (UK)	CC171
Fusarium poae Link	Wheat (UK)	
Penicillium aurantiogriseum Dierckx var. *aurantiogriseum*		
Penicillium verrucosum Dierckx	Wheat (UK)	IBT22625
Penicillium verrucosum Dierckx	Wheat (UK)	IBT22626
Penicillium verrucosum Dierckx	Wheat (Sweden)	OTA11

2.2.3 γ-irradiated wheat grain

Wheat grain was irradiated at 12 kGrays of gamma irradiation and stored aseptically at 4 °C. The grain contained no fungal infection or contamination but had retained germinability.

2.2.4 Water activity modification of media, γ-irradiated and natural wheat grain

To study the effect of environmental conditions on fungal ecophysiology and growth, wheat-based media was modified with glycerol (BDH) prior to autoclaving to obtain different a_w levels (Dallyn & Fox, 1980). The a_w levels were kept constant throughout experiments by keeping the same a_w treatments in closed polyethylene bags.

A moisture adsorption curve was constructed for wheat grain by adding known amounts of sterile distilled water to 10 g sub-samples of wheat grain in sterile Universal bottles. These were shaken vigorously and stored at 4 °C for 48 hours before equilibration at 25 °C. Sub-samples of each treatment were placed in an Aqualab (model CX-2, Washington, USA) to determine the a_w. The remaining samples were oven dried to obtain the moisture content (m.c.). The data was plotted as moisture content (m.c.) against a_w, and grams of added water against a_w. The latter adsportion curve was used to accurately control treatment conditions for subsequent experiments. Wheat grain was adjusted to the required a_w by aseptically adding calculated amounts of sterile distilled water using the moisture adsorption curve. For each harvest a new adsorption curve was constructed. The grain in all studies was equilibrated for 72 hours at 4 °C and shaken by hand several times per day. The a_w levels of grain treatments were kept constant by enclosing plates of the same a_w in closed containers with a beaker of 500 ml glycerol/water solution at the same a_w as the experimental treatment. This was to maintain the equilibrium relative humidity (ERH) of the atmosphere the same as the grain treatment. The a_w of all media and grain treatments were confirmed by measurement in an Aqualab (model CX-2, Washington, U.S.A).

2.3 TEMPORAL GROWTH AND OCHRATOXIN A PRODUCTION STUDY ON A 2% WHEAT BASED MEDIA AND ON γ-IRRADIATED WHEAT GRAIN

The objective of this study was to investigate the potential of ochratoxigenic strains of *P. verrucosum* and *A. ochraceus* to grow and produce ochratoxin A (OTA) over a range of a_w, temperatures and time intervals on a wheat-based media and γ-irradiated wheat grain over periods of 56 days. This study enabled a greater understanding of how the key parameters of a_w and temperature interact to determine the minimum and optimum threshold conditions required for growth and toxin production.

Wheat-based media and γ-irradiated wheat grain (approximately 500 g for each a_w condition) were modified to 0.80, 0.85, 0.90, 0.95 and 0.995 a_w respectively and placed into 90 mm diameter sterile Petri plates. The irradiated wheat grain treatments were placed into 90 mm Petri plates to form a monolayer in each case (approximately 20 g $plate^{-1}$).

A 1 x 10^6 spore ml^{-1} was prepared for *P. verrucosum* (strains IBT22625, IBT22626 and OTA11) and *A. ochraceus* (IBT21991) respectively from 7-10 day old cultures grown on MEA at 0.995 a_w. Using a sterile loop each Petri plate was centrally inoculated. For γ-irradiated grain experiments only *A. ochraceus* (IBT21991) and the most ochratoxigenic *P. verrucosum* strain (OTA11) were used as derived from the agar experiment. Incubation was at 7-56 days at 10, 15 °C or 25 °C respectively. These were the only temperatures used for all experiments as this project was concerned only with temperate cereals. All treatments were carried out in triplicate and repeated to confirm results. Storage of agar plates and grain was as described previously.

At regular intervals during the incubation period, growing colonies were measured with the aid of a bifocal microscope. Two diameters were obtained from each colony; the growth rates (mm d^{-1}) were calculated by linear regression of colony radius against time for each strain at each set of conditions tested. After the set incubation

periods, plates were destructively sampled and analysed for OTA using the method outlined in section 2.4.

2.4 EXTRACTION AND QUANTIFICATION OF OCHRATOXIN A

2.4.1 Extraction of ochratoxin A from agar media

For extraction of OTA a 20 g sample of the agar medium was cut into small pieces and extracted with 50 ml methanol (Sigma, UK). The sample was shaken at 110 rpm for 1 hour at 25 °C. Coloured samples were filtered through filter paper (no. 4; Whatman, UK) and 1 g of filter agent, Celite® 545 (Aldrich Chemical Company Inc., UK). From each sample, 1 ml was removed and centrifuged at 1100 rpm for 15 minutes for final purification. The supernatant of each sample was removed and placed in an HPLC amber glass vial for HPLC analysis.

2.4.2 Extraction of ochratoxin A from wheat grain

Samples were first dried for 24 hours at 50 °C. The dry samples were then milled. A 20 g sub-sample of the milled grain was then extracted with 50 ml methanol. The sample was shaken at 110 rpm for 24 hours at 25 °C. All samples were filtered through filter paper (No. 4; Whatman, UK) and 1 g of filter agent, Celite® 545 (Aldrich Chemical Company Inc., UK). 1 ml of the sample was removed and centrifuged at 1100 rpm for 15 minutes for final purification. The supernatant of each sample was removed and placed in an HPLC amber glass vial for HPLC analysis.

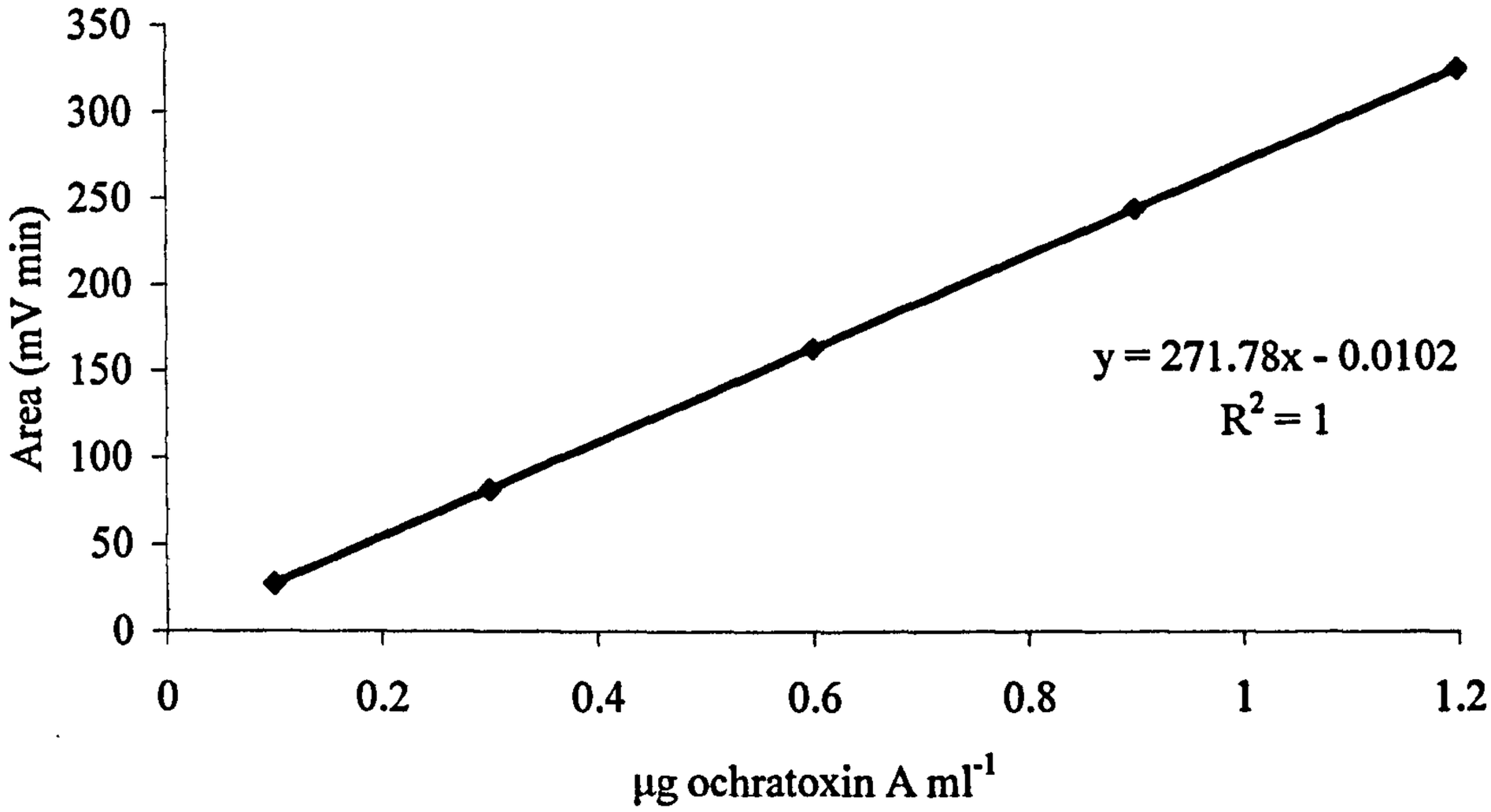
2.4.3 Quantification of ochratoxin A for agar media and wheat grain samples

OTA production was measured using High Performance Liquid Chromatography (HPLC). The HPLC equipment was composed of a pump (Waters 600E System controller), an integrator (Waters 712E) and a fluorescence detector (Waters 470). Conditions for OTA detection and quantification were as follows:

Mobile Phase	Acetonitrile (57%): Water (41%): Acetic acid (2%) (Fisher, HPLC grade)
Column	5µl C18 (150 x 4.6 mm) (Phenomenex Luna)
Pre column	Security Guard provided with 4 x 3mm cartridges (Phenomenex Luna)
Excitation	330 nm
Emission	460 nm
Flux	1 ml min^{-1}
Volume of sample injected	50 µl
Toxin standards	50, 100, 300, 500, 600, 1000 and 1200 ng ml^{-1} (methanol solutions)
Retention time	5.75 minutes
Run time	30 minutes
Limit of detection	< 1 µg ml^{-1}
Analytical software	Kroma 2000

The recovery rate of OTA in agar was 95 % and in wheat grain was 85 %. A calibration curve of OTA (µg ml^{-1}) versus peak area (mV min) was made for each sample run. Figure 2.1 shows the calibration curves obtained for agar and grain respectively.

A



B

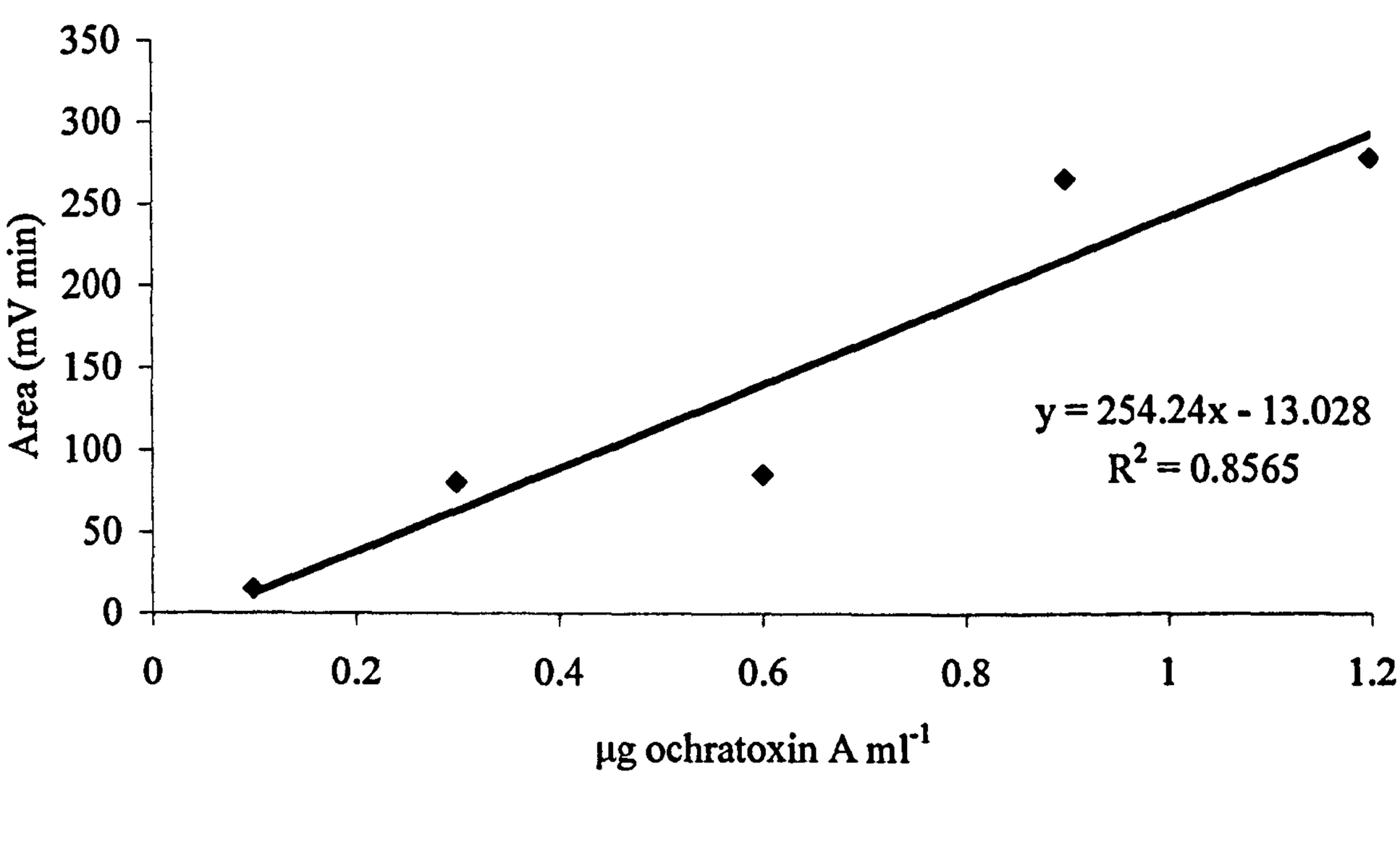


Figure 2.1 Examples of calibration curves obtained of OTA (µg ml^{-1}) versus peak area (mV min) in A) agar and B) grain.

2.5 GAS COMPOSITION AND GERMINATION OF OCHRATOXIGENIC SPECIES AND STRAINS

The objective of this study was to determine how the key parameters of a_w and intergranular gaseous composition interact to influence germination, growth and OTA production by *P. verrucosum* and *A. ochraceus*. The isolates tested were *P. verrucosum* (IBT22625, IBT22626 and OTA11) and *A. ochraceus* (IBT21991). Spore suspensions containing 1 x 10^6 spores ml^{-1} were prepared from 7-10 day old cultures grown on 2 % wheat-based media, 0.995 a_w at 25 °C. Growth, OTA production and germination of all species was analysed at three different water activities (0.995, 0.95 and 0.90 a_w) at three different gas compositions (air, 25 % CO_2 and 50 % CO_2). Table 2.2 shows the various gas compositions used.

Experiments were carried out at 25 °C in chambers of 36 L volume. One tube was attached to each end to allow the passage of gas through the chamber. For growth and OTA studies, fungi were centrally inoculated using either a 1 x 10^6 spore ml^{-1} suspension of *P. verrucosum* (OTA11) or *A. ochraceus* (IBT21991) onto 90 mm diameter Petri dishes containing 2 % wheat-based media or γ-irradiated wheat grain modified to 0.90, 0.95 and 0.995 a_w. For germination studies, 200 µl of a 1 x 10^6 spores ml^{-1} suspension was spread plate onto wheat-based media of the desired a_w.

Flasks containing 500 ml of appropriate glycerol/water solutions were placed in the chambers. Figure 2.2 shows a summary of the contents of a typical chamber. Chambers were flushed with the relative gas mixtures for 20 minutes at 3 L min^{-1} and then sealed. Nitrogen/air mixtures were used to decrease O_2 and CO_2/air mixtures to increase CO_2 concentrations. Gases were bubbled through the flask containing 500 ml of the appropriate glycerol/water solutions to humidify the gas to the required a_w. Figure 2.3 shows the gas blender system used. To ensure the proportion of gases programmed to enter the chambers was what was actually going in, CO_2 and O_2 levels were verified using a Carlo Erba model GC-8340 gas chromatograph (Carlo Erba Instruments, Hemel Hempstead, UK). Figure 2.4 shows the gas chromatograph used. Colonies were measured daily, using a bifocal microscope to assess initial growth and determine growth rates over 28 days. OTA production was measured at 7 day intervals over the 28 day duration. Germination was analysed microscopically at 24, 36 and 48 hours by staining a sample of each plate with lactophenol blue (Magan, 1988). All experiments were carried out in triplicate.

Table 2.2 Summary of gas compositions used in experiments.

Gas Composition	Oxygen (%)	Carbon dioxide (%)	Nitrogen (%)
Air	16	0	84
25% CO_2	16	25	59
50% CO_2	16	50	34

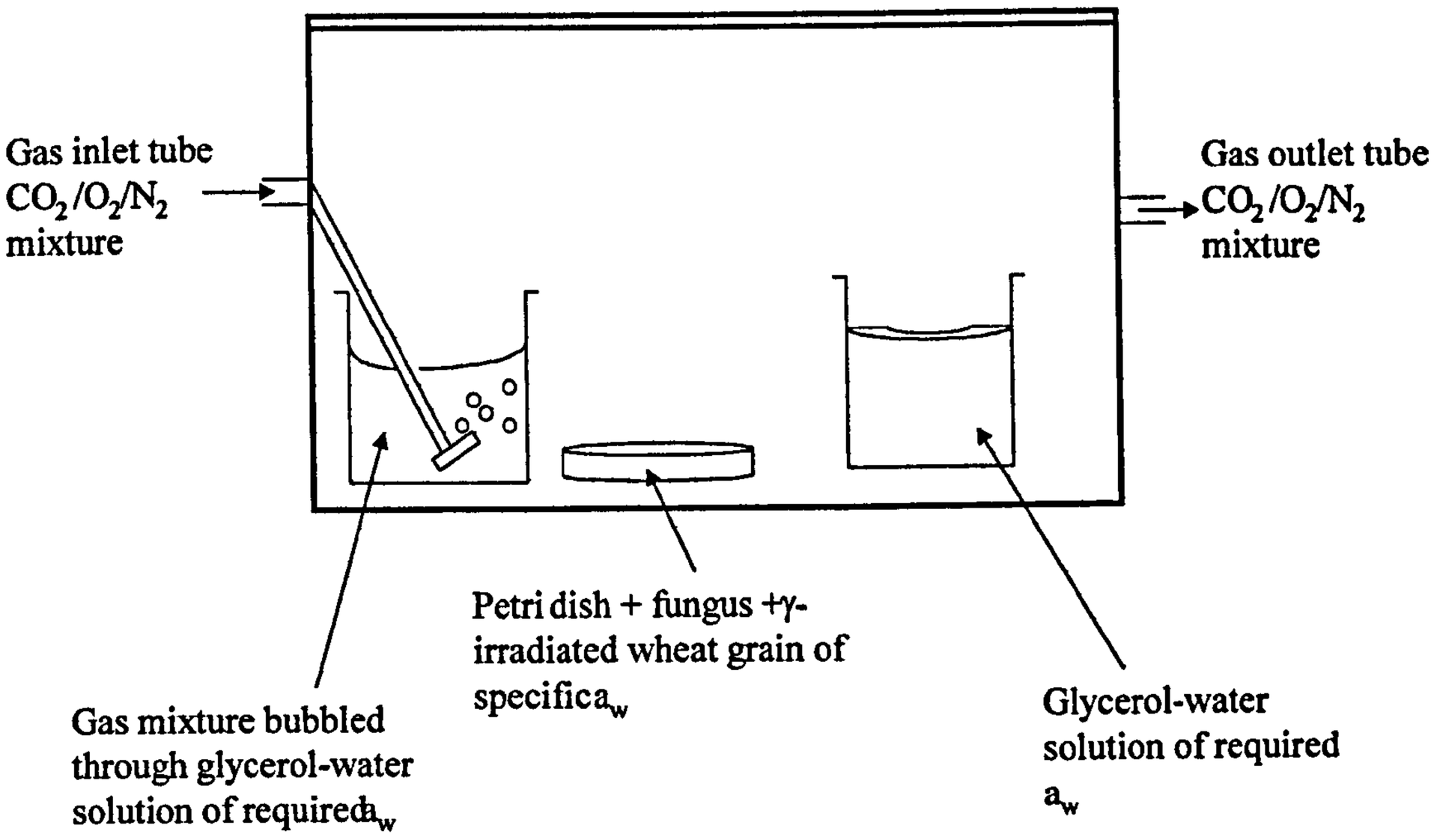


Figure 2.2 Experimental system used to study the effects of gas composition and water activity on germination and growth of fungi.

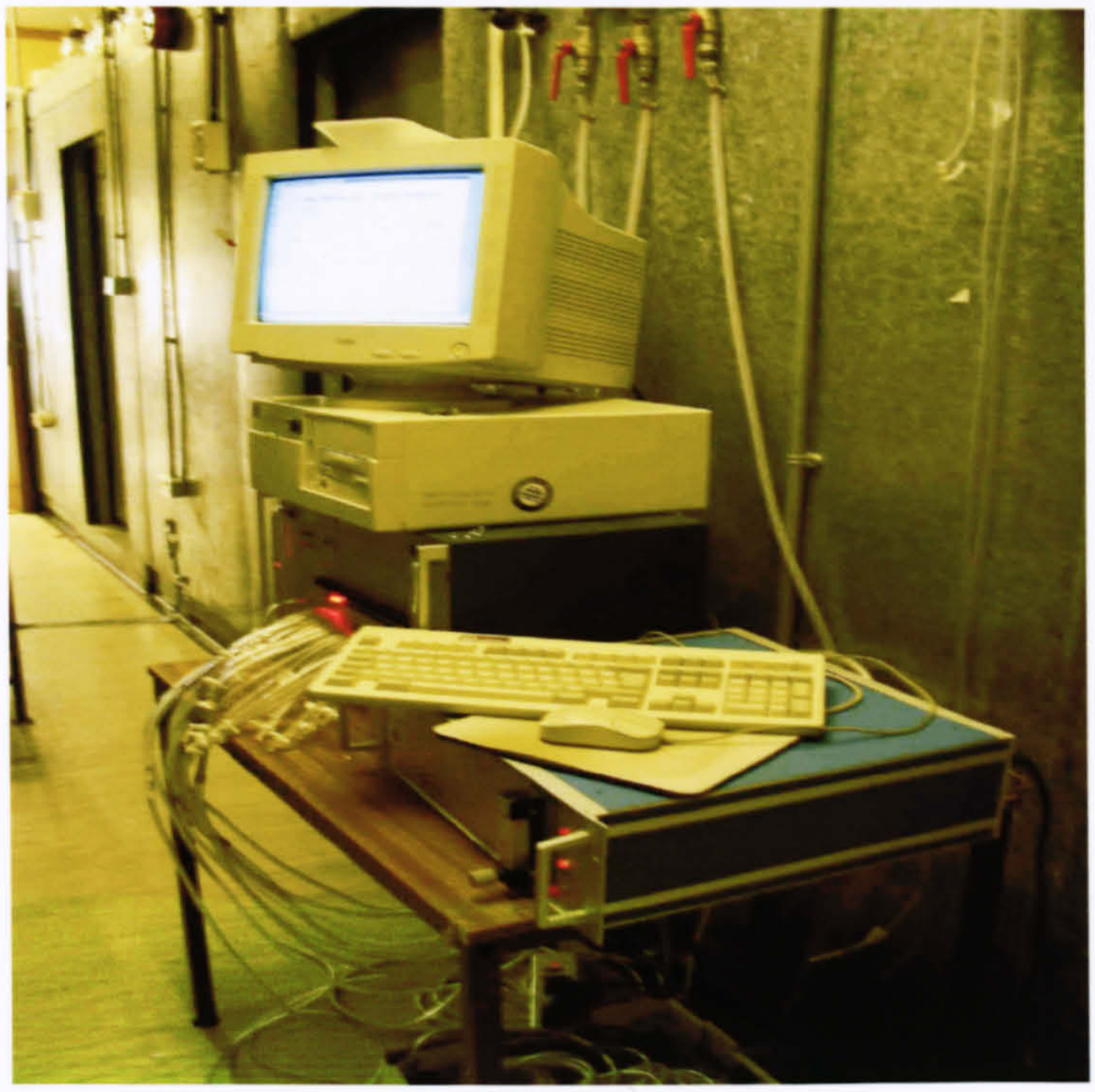


Figure 2.3 Gas blender system used to modify the compositions of carbon dioxide, oxygen and nitrogen in each of the nine treatment chambers.

Figure 2.4 Gas chromatograph used for analysing the carbon dioxide and oxygen levels from the outlet tube of each of the nine treatment containers.

2.6 FUNGAL INTERACTIONS AND INDEX OF DOMINANCE

The objective of this study was to examine the effect of a_w (0.90, 0.95 and 0.995 a_w) and temperature (15 °C and 25 °C) on growth and OTA production by *P. verrucosum* (OTA11) and *A. ochraceus* (IBT21991) when interacting with other wheat spoilage fungi on a wheat-based substrate and on wheat grain. These studies were carried out to evaluate the competitiveness of these ochratoxigenic species against other competitors in the stored grain ecosystem.

Spore suspensions containing 1 x 10^6 spores ml^{-1} were prepared for *P. verrucosum*, *A. ochraceus, F. poae, F. culmorum, E. repens, P. aurantiogriseum* and *A. tenuissima* using 7-10 day old cultures grown on 2 % wheat agar, 0.995 a_w at 25 °C. Spores were suspended in a sterile 10 % glucose (Sigma), 90% H_2O solution containing 0.9 % NaCl (technical, BDH) and 0.1 % Tween 80 (Sigma).

The 2 % wheat-based media and the γ-irradiated wheat grain were modified to 0.90, 0.95 and 0.995 a_w respectively. The a_w was confirmed in an Aqualab (model CX-2, Washington, USA).

For interaction studies, *P. verrucosum/A. ochraceus* and the interacting species were inoculated 2.5 cm apart at 15 °C and 4 cm apart at 25 °C. At regular intervals during the incubation period, colonies of *P. verrucosum* and *A. ochraceus* were measured with the aid of a bifocal microscope. Two diameters were obtained from each colony; the growth rates (mm d^{-1}) were calculated by linear regression of colony radius against time for each fungus at each set of conditions tested. During the incubation period, the interactions between mycelia of dual cultures were examined regularly both macroscopically and with the aid of a bifocal microscope. Each interaction was given a score based on mutual intermingling (1-1), mutual antagonism on contact (2-2), mutual antagonism at a distance (3-3), dominance of one species on contact (4-0) and dominance at a distance (5-0) (Magan & Lacey, 1984a). In the case of the dominant interactions, the higher score was always awarded to the most competitive fungus. The scores of each species were totalled for each a_w x temperature condition to obtain an overall Index of Dominance (I_D) under different steady-state environmental conditions.

Controls were individual cultures inoculated centrally with *P. verrucosum* or *A. ochraceus* only.

Experiments were carried out at 15 °C or 25 °C in triplicate. For agar studies, plates of the same a_w were sealed in polyethylene bags for up to 56 days. For irradiated wheat grain studies, plates were placed in sealed chambers with glycerol/water solutions at the same a_w as the treatments for 28 days. After the incubation periods, plates were analysed for OTA production using the method detailed in section 2.4.

2.7 NICHE SIZE AND NICHE OVERLAP INDICES

The objective of this study was to investigate the effects of environmental factors on *in vitro* carbon source utilisation patterns and niche overlap indices (NOIs) for the OTA producing isolates *A. ochraceus* (IBT21991) and *P. verrucosum* (OTA11) in relation to other spoilage fungi. This provided an insight into why some species are more competitive than others under certain conditions. This component was considered complementary to the I_D studies.

A biolog plate (GN Microplates, BIOLOG, Inc., CA, USA) is commercially available which can be used to determine the range of carbon sources utilised by fungi. The plate contains a range of carbohydrates, amino acids, carboxylic acids, amines and amides and miscellaneous carbon sources. Only the 18 major carbon sources present in wheat were taken into account. These were dextrin, D-fructose, D-galactose, α-D-glucose, D-melobiose, D-raffinose, sucrose, D-alanine, L-alanine, L-aspartic acid, L-glutamic acid, L-histidine, L-leucine, L-phenylalanine, L-proline, D-serine, L-serine and L-threonine. Previously, modifications of environmental factors including water availability and temperature were used successfully in conjunction with carbon source utilisation patterns to determine the level of co-existence or dominance of species in a stored grain niche (Marin *et al.*, 1998c; Lee & Magan, 1999b)

Spores of all species were washed three times in sterile water by centrifugation. Spores were suspended in 0.25 mol l^{-1} 2-(N-morpholino)ethanosulphonic acids (MES, Sigma)

buffered at pH 5.5. The solutions were modified to 0.93 and 0.995 a_w using the ionic solute NaCl instead of glycerol as it was one of the carbon sources used in the Biolog test plates. The final concentration of the spore suspensions was 1 x 10^6 spores ml^{-1}. A 100 µl fraction was placed in each well of a Biolog plate to determine the carbon sources utilised by the fungi. Plates were incubated at 15 or 25 °C for 14 days in sealed containers with a 500 ml beaker of glycerol/water solution of the same a_w as the treatment. Plates were observed microscopically at 48 hour intervals (Marin *et al.*, 1998c).

The NOI was determined as follows (Wilson & Lindow, 1994a):

$$NOI = \frac{\text{No. of C sources in common between two fungi}}{\text{Total no. of C sources utilised by test fungus}}$$

NOI values >0.9 indicated co-existence between species in an ecological niche, whereas score <0.9 indicated occupation of separate niches (Wilson & Lindow, 1994a, b). In this study, *P. verrucosum* (OTA11) and *A. ochraceus* (IBT21991) respectively were used as the test fungi and the range of nutritional substrates used were compared. The NOI values were compared for each set of carbon sources and for each set of a_w x temperature conditions used.

2.8 EFFECT OF ESSENTIAL OILS ON GROWTH AND OCHRATOXIN A PRODUCTION

2.8.1 Initial disc diffusion screening assay

The objective of this study was to determine whether essential oils or resveratrol could be used to control growth and OTA production by *P. verrucosum* and *A. ochraceus* during wheat storage.

A total of twenty-four essential oils were screened in this study (kindly supplied by F.D.Copeland and Sons Ltd., London). Table 2.3 lists the range used.

Table 2.3 List of essential oils screened.

Code	Essential Oil
H83T	Oil of basil (methyl chavicol type)
H681T	Oil of aniseed Chinese
H682T	Oil of basil (linalool type)
H683T	Oil of cinnamon leaf
H684T	Oil of bay west Indian
H685T	Oil of clove bud
H686T	Oil of clove leaf cleaned
H687T	Oil of eucalyptus citriodora
H688T	Oil of sweet fennel
H689T	Oil of ginger Chinese
H691T	Oil of lime
H692T	Oil of marjoram
H693T	Oil of nutmeg
H694T	Oil of orange
H695T	Oil of mandarin
H696T	Oil of peppermint
H697T	Oil of rosemary
H698T	Oil of spearmint
H699T	Oil of thyme
H701T	Oil of grapefruit
H702T	Oil of pine sylvestris
H703T	Oil of sage
H704T	Oil of lemongrass
H705T	Oil of lemon

For each species, a 200 µl spore suspension containing 1 x 10^6 spore ml^{-1} was spread plate onto 2 % wheat-based medium at 0.995 a_w. Four sterile filter paper discs (Whatmann, 6 mm diameter) were placed in each quadrant of the inoculated plates.

The twenty-four essential oils were diluted in methanol (1:10) (Sigma, UK). Each treatment plate was inoculated with one essential oil by aliquoting 100 µl of the dilution onto each of the three discs. 100 µl of methanol was placed on the fourth disc, which acted as the control. Incubation was at 25 °C and diametrical zones of inhibition were measured after 48 hours. The essential oils most effective against the strains of *P. verrucosum* (IBT22625, IBT22626 and OTA11) and *A. ochraceus* (IBT21991) were studied in greater detail for the ability to inhibit growth. The most effective essential oils were those which had a mean radial zone of inhibition >5 mm.

2.8.2 Detailed screening of the most effective essential oils *in vitro*

The detailed screen consisted of a temporal growth study of *P. verrucosum* (OTA11) and *A. ochraceus* (IBT21991) on a 2 % wheat-based medium supplemented with different concentrations of essential oils (0, 50, 150 and 500 µg g^{-1} in 10 ml methanol) on media adjusted to 0.901, 0.955 and 0.995 a_w respectively by using glycerol-water solutions (Dallyn & Fox, 1980). The essential oils were added to the molten media (50 °C) prior to pouring. Control plates were prepared using 10 ml of methanol only. The a_w was confirmed by measurement in an Aqualab, (CX-2; Decagon Devices Inc., USA). Plates were inoculated centrally using a 1 µl sterile loop with a 10^6 spores ml^{-1} spore suspension. All experiments were carried out in triplicate. Incubation was at 15 and 25 °C for 56 days in sealed polyethylene bags. Colony diameters were measured regularly by taking two measurements at right angles to one another. The slopes of the regression lines of the linear portions of the radial extension rates were used for calculating growth rates.

2.8.3 Detailed screen of reveratrol and the most effective essential oils for controlling growth and ochratoxin A production by *Penicillium verrucosum* and *Aspergillus ochraceus* on irradiated wheat grain.

γ-irradiated wheat grain was adjusted to 0.90, 0.95 and 0.995 a_w respectively by aseptically adding calculated amounts of sterile distilled water using a moisture adsorption curve. The seeds were treated with Resvin® a commercial product containing resveratrol at 10 % w/w concentration. Resvin® was solubilised in ethanol to produce a final treatment concentration of 0, 50 or 150 μg g^{-1} resveratrol (w/w) in the grain. The control was treated with ethanol only. Essential oils were dissolved in 10 ml of methanol to produce final concentrations of 0, 50, 150 or 500 μg g^{-1} in the grain (w/w). The control was treated with methanol only. The grain was equilibrated for 72 hours at 4 °C and shaken by hand several times per day. The a_w of all grain treatments was confirmed by measurement in an Aqualab, (CX-2; Decagon Devices Inc., USA). Grain was aseptically placed in 90 mm diameter Petri dishes to form a monolayer. Petri plates containing grain were centrally inoculated with 1 x 10^6 spores ml^{-1} of *P. verrucosum* (OTA11) and *A. ochraceus* (IBT21991) obtained from 7-10 day old cultures grown on wheat based medium. Experiments were carried out in triplicate in closed containers with each treatment in a separate container. Beakers of glycerol/ water mixtures of the same a_w as the treatment were placed in the containers to maintain the equilibrium relative humidity (Dallyn & Fox, 1980). Incubation was at 15 and 25 °C for 28 days. Colony diameters were measured regularly by taking two measurements at right angles to one another. The slopes of the regression lines of the linear portions of the radial extension rates were used for calculating growth rates. At the end of the incubation period, samples were analysed for OTA production.

2.9 EFFECT OF RESVERATROL ON THE NATURAL FUNGAL POPULATIONS AND OCHRATOXIN A PRODUCTION IN NATURALLY CONTAMINATED WHEAT GRAIN AT DIFFERENT ENVIRONMENTAL CONDITIONS

The objective of this study was to determine the effect of resveratrol on naturally contaminated wheat grain. This was achieved using an *in situ* study to determine the efficacy of resveratrol on the inhibition of fungi and OTA production on wheat grain spiked with *P. verrucosum* (OTA11) and *A. ochraceus* (IBT21991).

400 g of wheat grain was placed into flasks. Resveratrol was dissolved in 10 ml ethanol to obtain a final concentration of 200 μg g^{-1}. The control was treated with 10 ml ethanol only. This solution was then placed into known amounts of sterile water to adjust the a_w to 0.80, 0.90, 0.95 and 0.995 a_w respectively. This was added to the grain. The flasks were equilibrated at 4 °C with periodic shaking by hand to ensure equal distribution over the grains. 100 g sub-samples were then placed into solid culture vessels (Magenta; Sigma). Each flask was inoculated with either 1 ml of 10^2 spore suspension of *P. verrucosum* or *A. ochraceus*. The culture vessels were shaken by hand to ensure equal distribution of the inoculum throughout the grain. Treatments of the same a_w were placed in sealed containers with beakers of glycerol/water solutions of the same a_w. Samples were incubated for 21 days at 15 or 25 °C respectively. At 7 day intervals, three replicates from each treatment were taken from each container. 1 g of each sub-sample was used for determining fungal populations, including *P. verrucosum* and *A. ochraceus* colony forming units (CFU) using serial dilutions, and ten grains per replicate were directly plated to assess fungal populations. The reminder was used for OTA extraction and analysis.

For serial dilutions, 1 g of grain was shaken in 9 ml sterile water with 0.1% Tween 80. A serial dilution series was prepared for concentrations of 10^{-1} to 10^{-5}. 100 μl of each dilution was spread-plate onto MEA plates with 0.1 % chloramphenicol and DYSG media at the same a_w as the treatments. DYSG media was used as the selective media

for distinguishing between *P. verrucosum* and other *Penicillium* spp. Colonies of *P.verrucosum* are 'brick' red on the reverse of the Petri plate whereas other *Penicillium* spp. are colourless. For the direct plating, ten grains of each sample were placed equal distances apart on the MEA + 0.1 % chloramphenicol plates and DYSG medium incubated at the same temperature as the treatments. After 5-7 days the colonies were counted and where possible identified to genus level.

2.10 ANALYSIS OF DATA

Data input, data handling/manipulation, linear regression, and graph plotting was carried out using Microsoft Excel 2002 (Microsoft Co.) ANOVA was performed using Minitab 13.32 (Minitab Inc.).

CHAPTER 3

RESULTS

3.1 TWO DIMENSIONAL PROFILES OF GROWTH AND MYCOTOXIN PRODUCTION BY *PENICILLIUM VERRUCOSUM* AND *ASPERGILLUS OCHRACEUS* ON A 2 % WHEAT-BASED MEDIA AND γ-IRRADIATED WHEAT GRAIN.

If growth and mycotoxin production by *Penicillium verrucosum* and *Aspergillus ochraceus* are to be effectively controlled, then the impact of key environmental parameters and their interactions need to be understood. This information is important for determining critical control points in HACCP schemes and in the timing of a suitable control treatment in the stored grain ecosystem.

3.1.1 Effect of water availability and temperature on growth and ochratoxin A production by *Penicillium verrucosum* on a 2% wheat-based media.

Figures 3.1 and 3.2 show the radial extension rates (K_r) obtained for three different isolates of *Penicillium verrucosum* (strains IBT22625, IBT22626 and OTA11) on 2 % milled wheat medium over a range of water activities at 15 and 25 °C respectively. All isolates grew faster at 25 than 15 °C over the complete range of water activities tested (0.80-0.995 a_w). Optimum growth was at 0.98 a_w regardless of the temperature tested. All *P. verrucosum* isolates grew over a narrower range of water activities at 15 °C (0.85-0.995 a_w) than at 25 °C (0.80-0.995 a_w). Statistical analysis (Table 3.1) shows that a_w, temperature, *P. verrucosum* strain and their interactions significantly affected growth.

Figures 3.3 and 3.4 show OTA production by the three strains of *P. verrucosum* after 56 days on 2 % milled wheat medium at the same temperatures. Optimum toxin production was at 0.95 a_w regardless of the temperature tested and was greater at 25 than 15 °C. All *P. verrucosum* isolates produced OTA over a narrower range of water activities than those required for growth. At 25 °C growth occurred over the complete range of water activities tested (0.80-0.995 a_w), however there was no OTA produced on 2 % milled wheat medium at <0.85 a_w. At 15 °C growth occurred at 0.85-0.995 a_w, however only strain OTA11 was able to produce OTA over this range of water activities. Strains IBT22626 and IBT22625 were only able to produce OTA between 0.90-0.995 a_w. This shows that key environmental parameters allowing OTA production varies between strains of the same species. This is confirmed by statistical analysis (Table 3.2). A_w, temperature and strain used were significant but two and three way interactions were not.

Overall, toxin production was greater for strain OTA11 over the complete range of conditions tested and was chosen as the only *P. verrucosum* isolate to be investigated in further detail. Figures 3.5 and 3.6 show the temporal changes in OTA levels determined for strain OTA11 over time for up to 56 days. OTA production was greater at 25 than at 15 °C and generally increased as incubation time increased. There was no OTA production up to 21 days regardless of a_w or the temperature tested on 2 % milled wheat medium. Statistical analysis (Table 3.3) shows that a_w, temperature, incubation time and their interactions significantly affected OTA production.

3.1.2 Effect of water availability and temperature on growth and ochratoxin A production by *Penicillium verrucosum* on γ-irradiated wheat grain.

Figure 3.7 shows the mean radial extension rates (K_r) determined for *P. verrucosum* strain OTA11 over a range of water activities (0.80-0.995 a_w) at 10, 15 and 25 °C respectively on γ-irradiated wheat grain. Growth rates were fastest at 0.95 a_w regardless of the temperature tested. These results are in contrast to those on 2 % wheat agar where growth was fastest at 0.98 a_w. At 15 and 25 °C growth occurred over the complete range of water activities tested. At 10 °C growth occurred over a narrower range of water activities and was completely inhibited at $\leq$ 0.87 a_w. Growth was always fastest at 25 followed by 15 and 10 °C regardless of the water activity tested. ANOVA (Table 3.4) shows that all experimental factors and their interactions had a significant effect on growth. Plates 3.1 and 3.2 compare the growth of *P. verrucosum* growing on 2 % wheat agar and γ-irradiated wheat grain.

Figures 3.8-3.10 show the temporal production of OTA by *P. verrucosum* strain OTA11 on γ-irradiated wheat grain at 10, 15, and 25 °C respectively over 56 days. No OTA production occurred at 7 days regardless of temperature or water activity treatment. OTA production was greatest at 25 followed by 15 and 10 °C over the entire range of water activities tested regardless of incubation time. Optimum OTA production occurred after 56 days at 0.93-0.95 a_w for all temperatures tested. ANOVA (Table 3.5) showed that all experimental factors and their interactions significantly affected OTA production. Figure 3.11 compares OTA production by *P. verrucosum* after 56 days over the range of water activities tested at 10, 15 and 25 °C respectively. ANOVA (Table 3.6) showed that at this incubation time, all experimental factors and their interactions significantly affected OTA production.

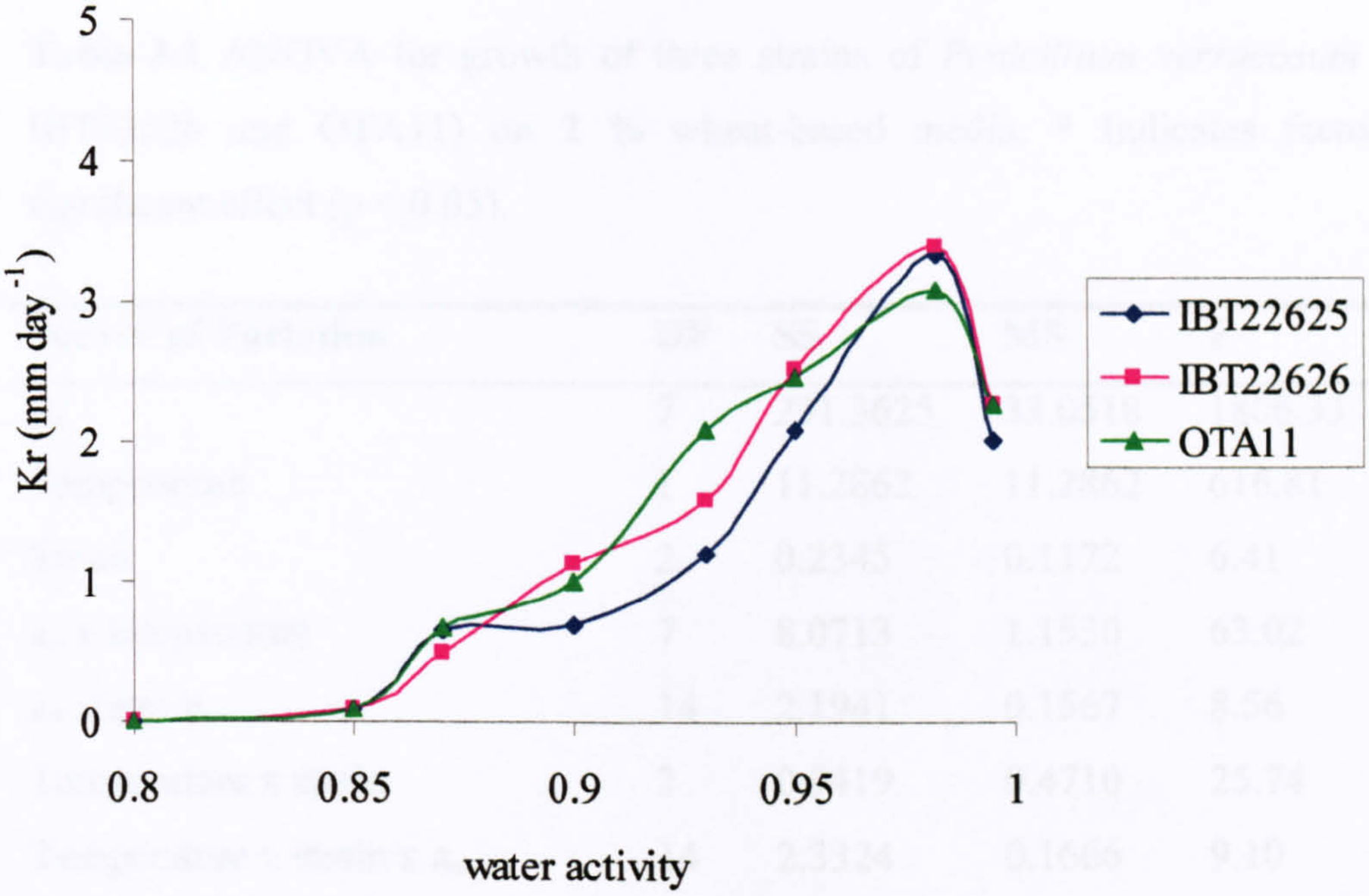


Figure 3.1 Growth of three strains of *Penicillium verrucosum* (IBT22625, IBT22626 and OTA11) on 2 % wheat-based media at various water activities at 15 °C. Least Significant Difference (LSD) at $p < 0.05$ is 0.06.

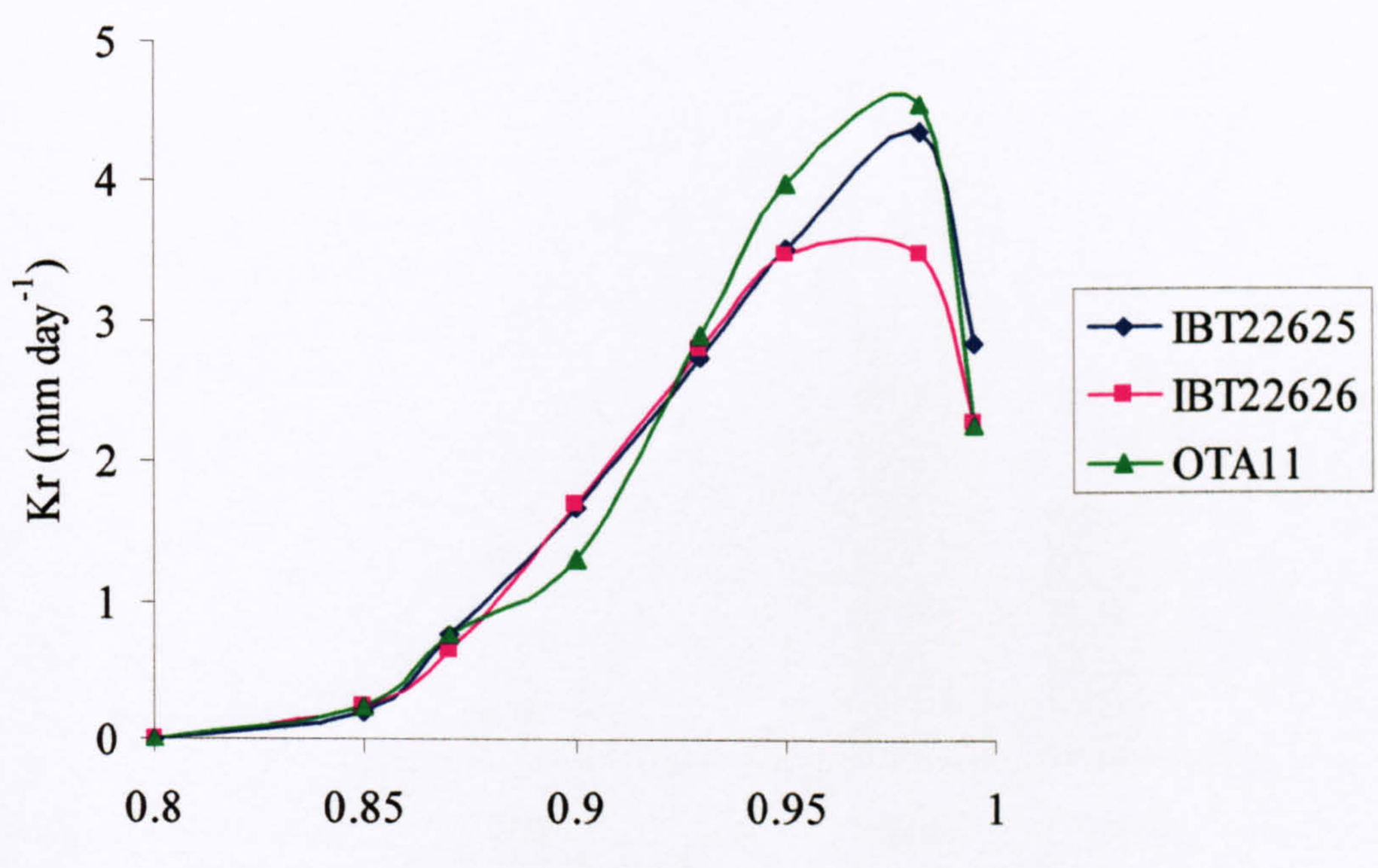


Figure 3.2 Growth of three strains of *Penicillium verrucosum* (IBT22625, IBT22626 and OTA11) on 2 % wheat-based media at various water activities at 25 °C. Least Significant Difference (LSD) at $p < 0.05$ is 0.028.

Table 3.1 ANOVA for growth of three strains of *Penicillium verrucosum* (IBT22625, IBT22626 and OTA11) on 2 % wheat-based media. * Indicates factor elicited a significant effect ($p < 0.05$).

Source of Variation	DF	SS	MS	F	P
a_w	7	231.3625	33.0518	1806.33	<0.001*
Temperature	1	11.2862	11.2862	616.81	<0.001*
Strain	2	0.2345	0.1172	6.41	0.002*
a_w x temperature	7	8.0713	1.1530	63.02	<0.001*
a_w x strain	14	2.1941	0.1567	8.56	<0.001*
Temperature x strain	2	0.9419	0.4710	25.74	<0.001*
Temperature x strain x a_w	14	2.3324	0.1666	9.10	<0.001*
Residual	96	1.7566	0.0183		
Total	143	258.1794			

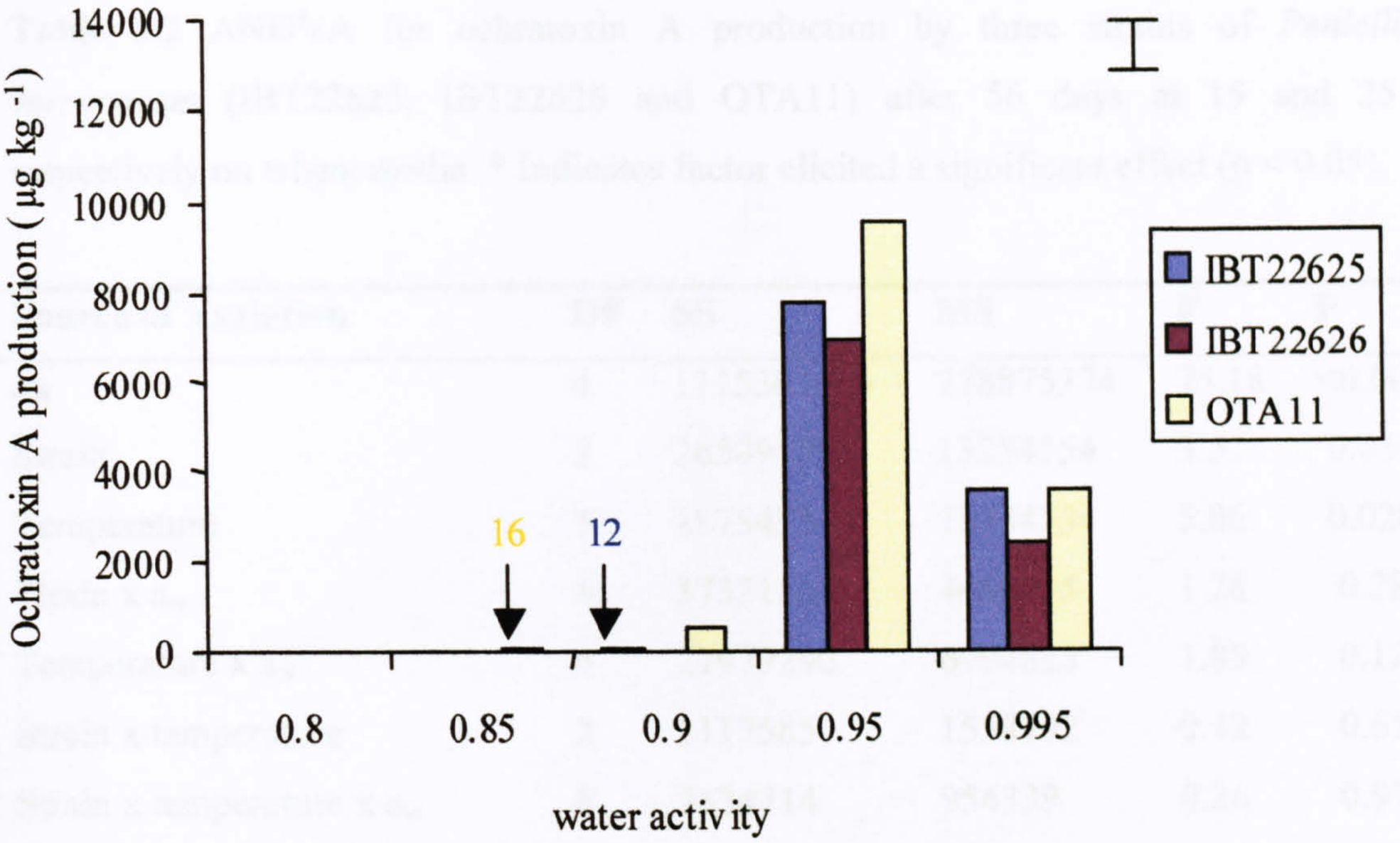


Figure 3.3 Ochratoxin A production by three strains of *Penicillium verrucosum* (IBT22625, IBT22626 and OTA11) on 2 % wheat-based media at various water activities at 15 °C after 56 days. Bar indicates Least Significant Difference (LSD) at $p < 0.05$.

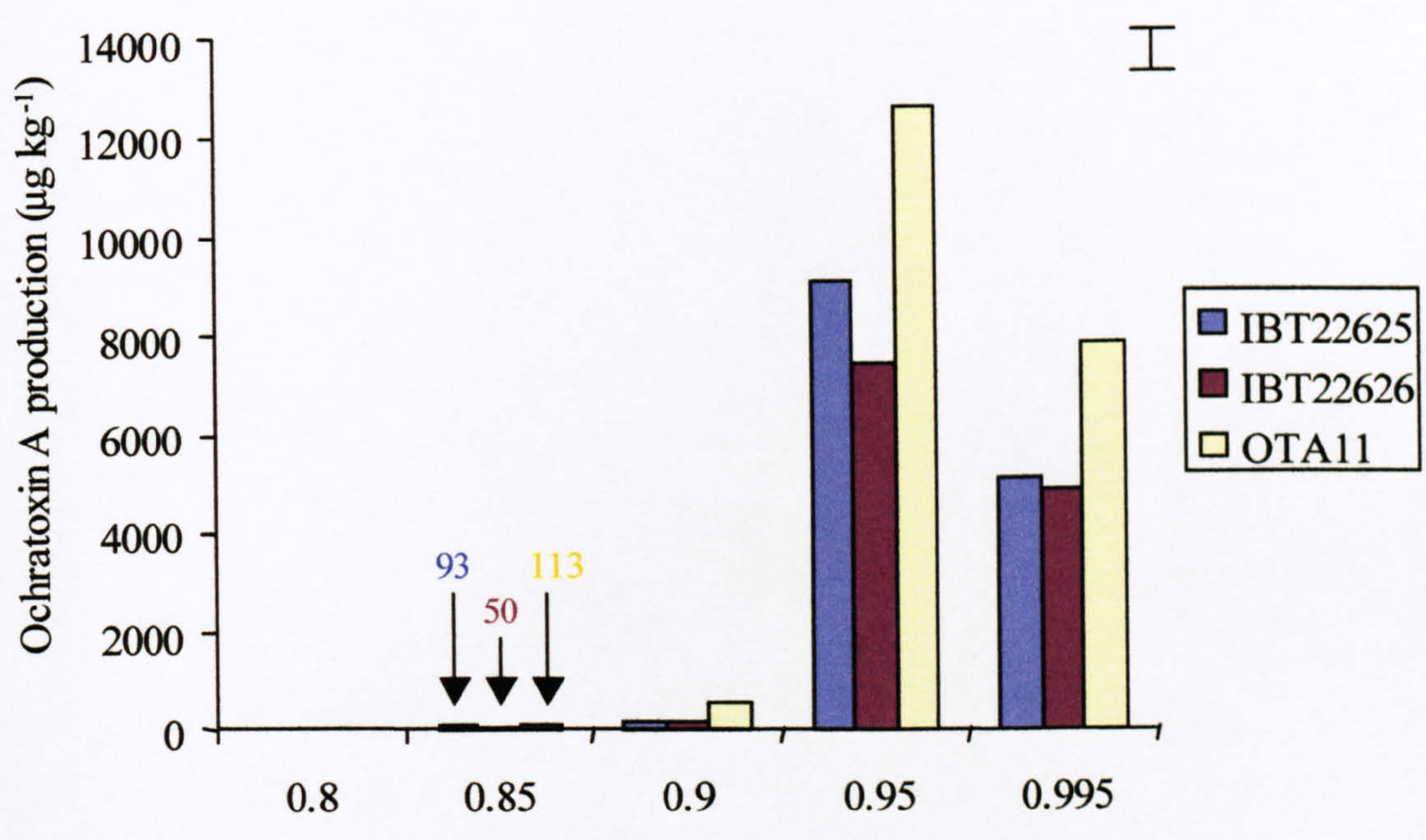


Figure 3.4 Ochratoxin A production by three strains of *Penicillium verrucosum* (IBT22625, IBT22626 and OTA11) on 2 % wheat-based media at various water activities at 25 °C after 56 days. Bar indicates Least Significant Difference (LSD) at $p < 0.05$.

Table 3.2 ANOVA for ochratoxin A production by three strains of *Penicillium verrucosum* (IBT22625, IBT22626 and OTA11) after 56 days at 15 and 25 °C respectively on wheat media. * Indicates factor elicited a significant effect ($p < 0.05$).

Source of Variation	DF	SS	MS	F	P
aw	4	1115501494	278875374	75.18	<0.001*
Strain	2	26509109	13254554	3.57	0.034*
Temperature	1	18754734	18754734	5.06	0.028*
Strain x a_w	8	37351162	4668895	1.26	0.282
Temperature x a_w	4	27979290	6994823	1.89	0.125
Strain x temperature	2	3117585	1558792	0.42	0.659
Strain x temperature x a_w	8	7634714	954339	0.26	0.977
Residual	60	222560626	3709344		
Total	89	1459408714			

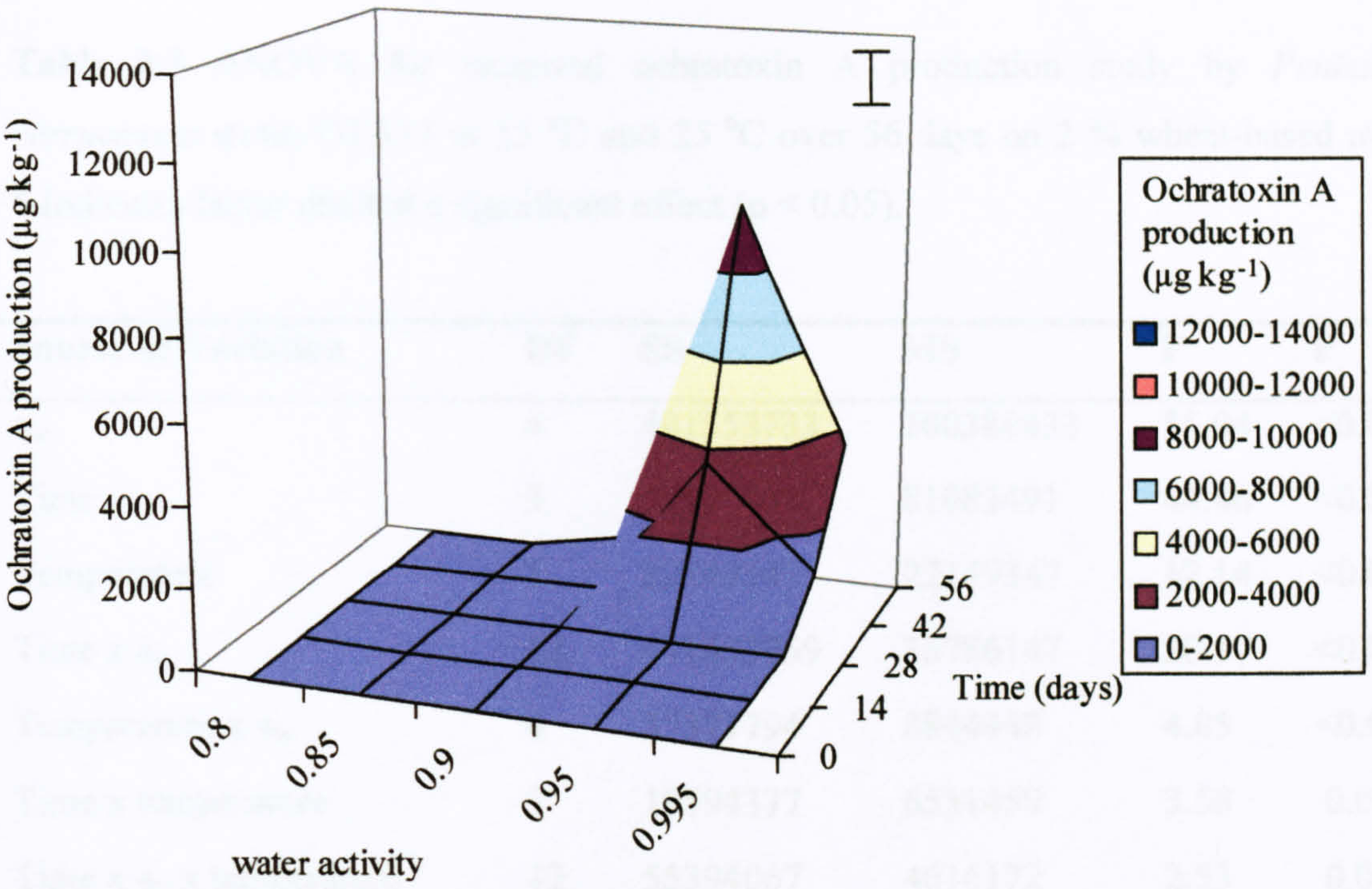


Figure 3.5 Ochratoxin A production by *Penicillium verrucosum* strain OTA11 on 2 % wheat–based media at various water activities at 15 °C over 56 days. Bar indicates Least Significant Difference (LSD) at $p < 0.05$

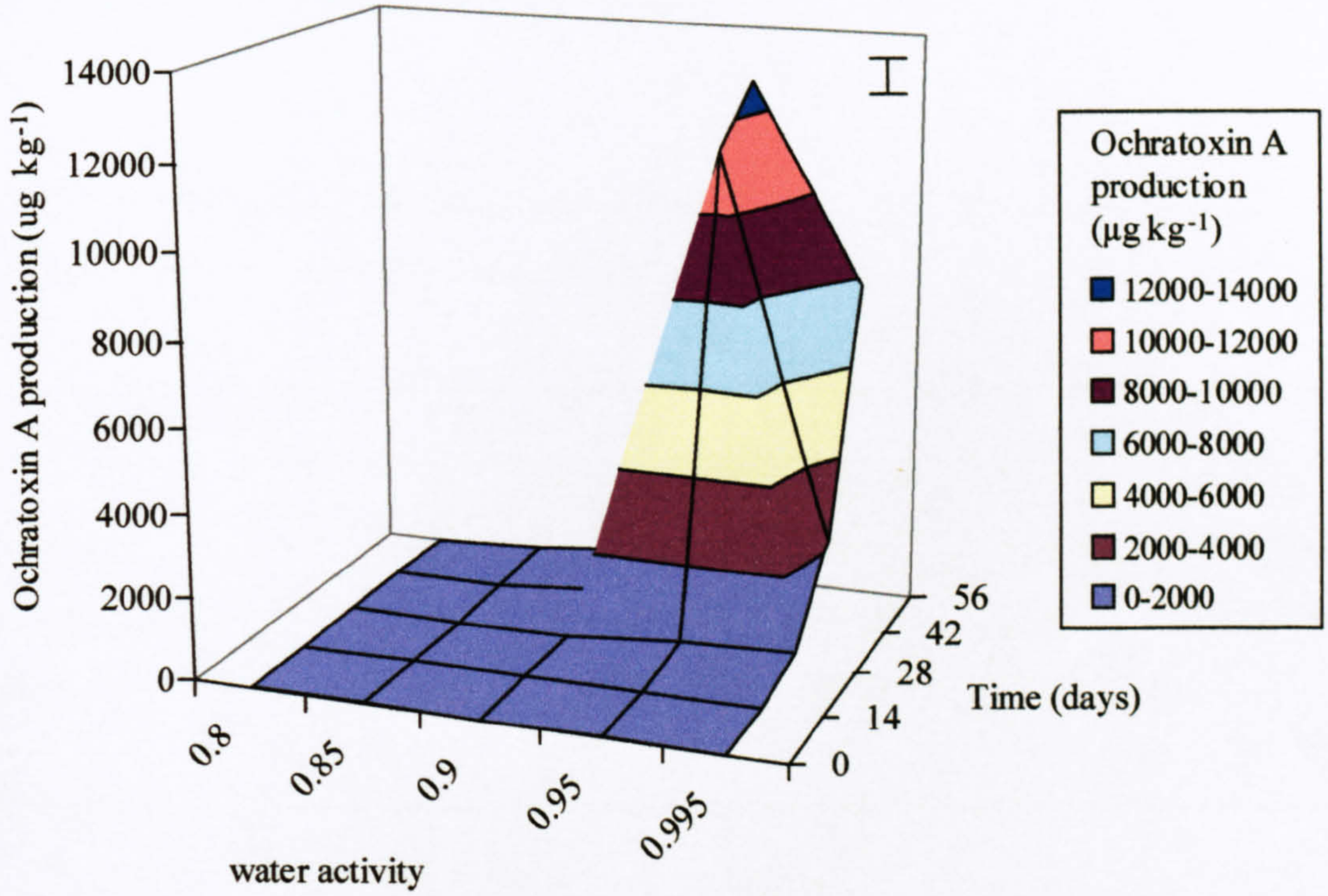


Figure 3.6 Ochratoxin A production by *Penicillium verrucosum* strain OTA11 on 2 % wheat-based media at various water activities at 25 °C over 56 days. Bar indicates Least Significant Difference (LSD) at $p < 0.05$

Table 3.3 ANOVA for temporal ochratoxin A production study by *Penicillium verrucosum* strain OTA11 at 15 °C and 25 °C over 56 days on 2 % wheat-based media. * Indicates factor elicited a significant effect ($p < 0.05$).

Source of Variation	DF	SS	MS	F	P
a_w	4	401553733	100388433	55.04	<0.001*
Time	3	243250472	81083491	44.46	<0.001*
Temperature	1	22149347	22149347	12.14	<0.001*
Time x a_w	12	441433759	36786147	20.17	<0.001*
Temperature x a_w	4	35377794	8844448	4.85	<0.001*
Time x temperature	3	19594377	6531459	3.58	0.017*
Time x a_w x temperature	12	55394067	4616172	2.53	0.007*
Residual	80	145905519	1823819		
Total	119	18368678			

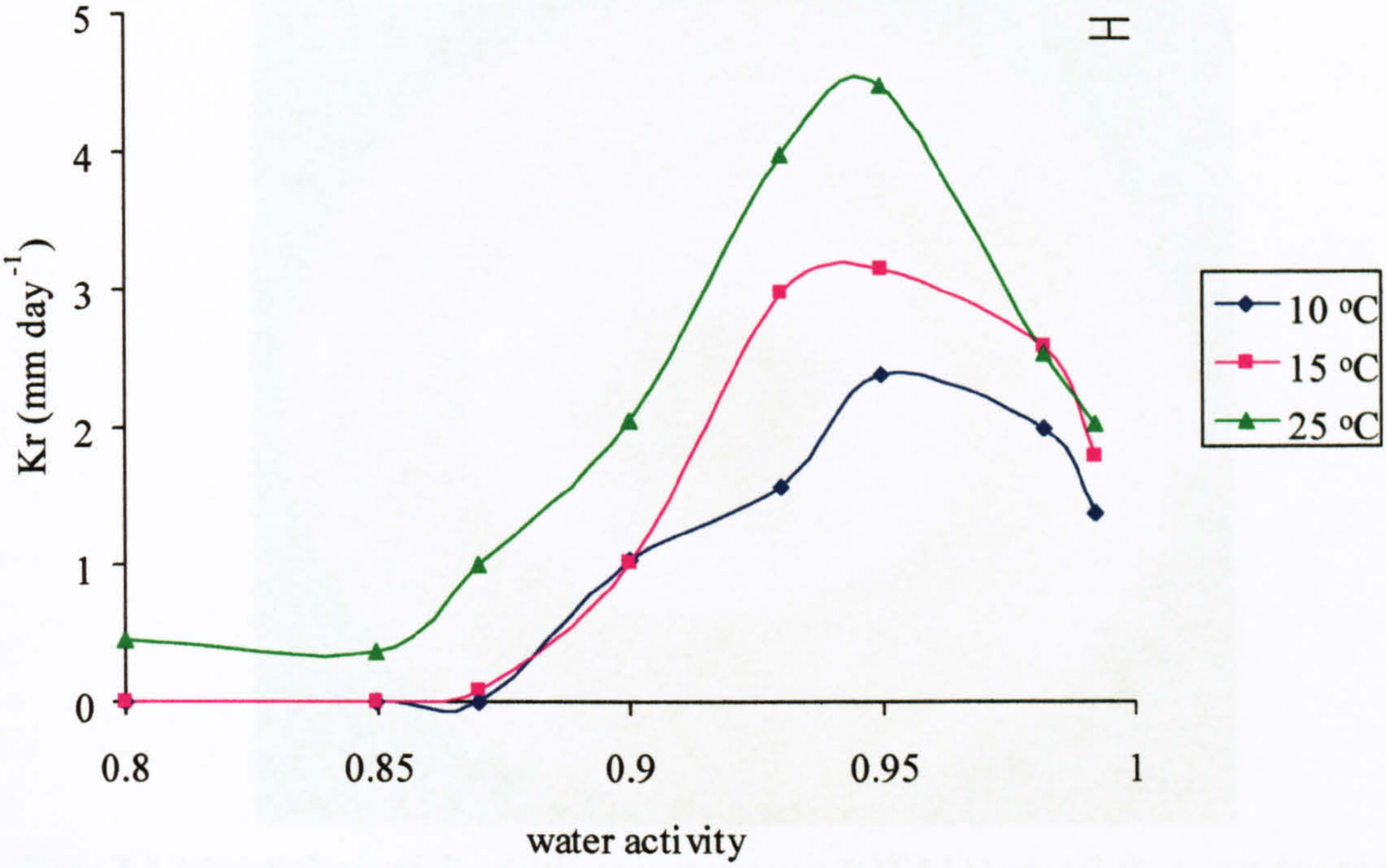


Figure 3.7 Growth of *Penicillium verrucosum* strain OTA11 on wheat grain adjusted to various water activities and incubated at 10, 15 and 25 °C respectively. Bar indicates Least Significant Difference (LSD) at $p < 0.05$.

Table 3.4 ANOVA for *Penicillium verrucosum* (OTA11) growth on γ-irradiated wheat grain. * Indicates factor elicited a significant effect ($p < 0.05$).

Source of Variation	DF	SS	MS	F	P
a_w	7	99.5948	14.2278	253.55	<0.001*
Temperature	2	13.9549	6.9775	123.34	<0.001*
a_w x temperature	14	7.6612	0.5472	9.75	<0.001*
Residual	48	2.6935	0.0561		
Total	71	123.9045			

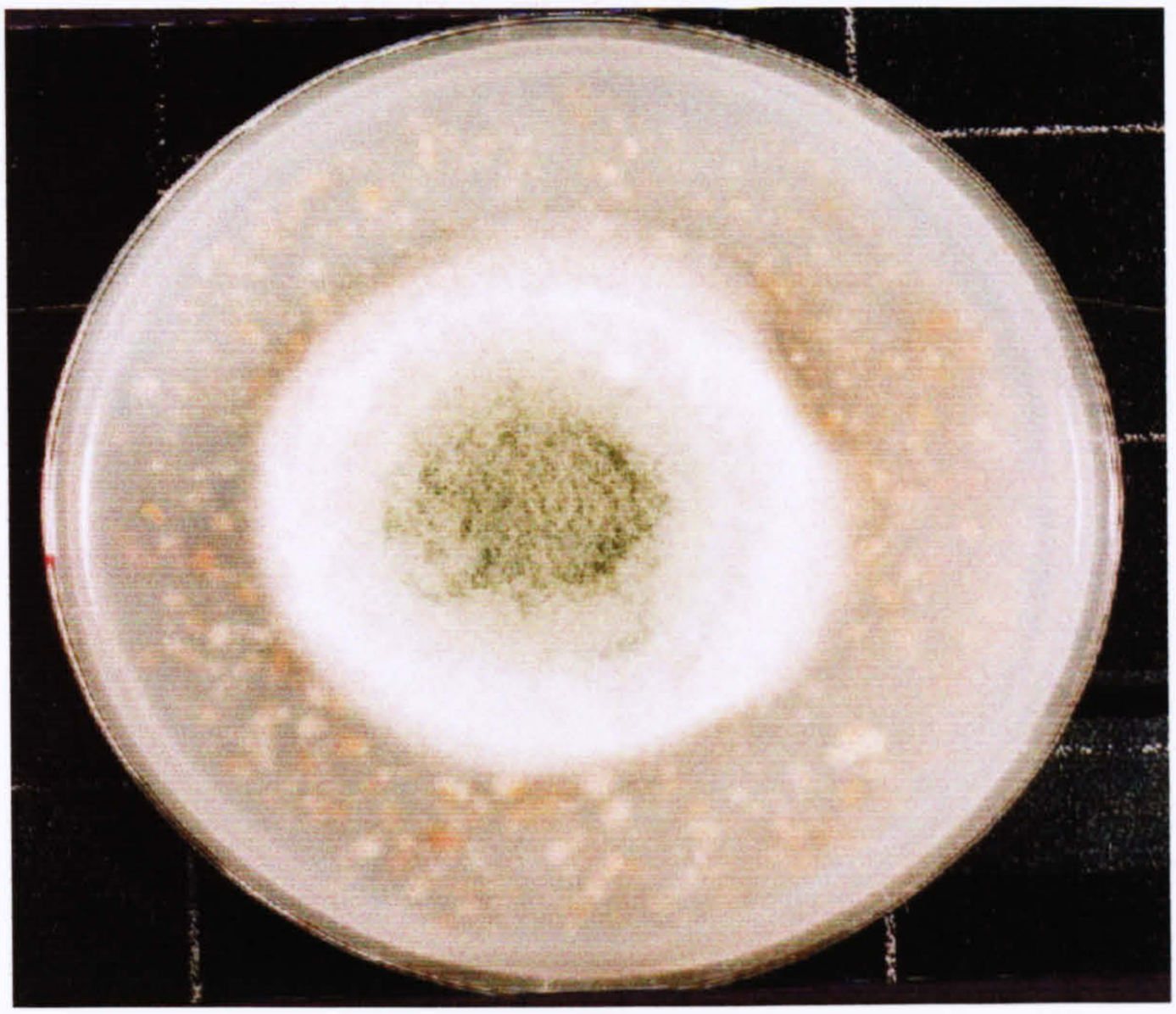


Plate 3.1 Morphology of *Penicillium verrucosum* (OTA11) on a 2 % wheat-based media after 7 days at 0.982 a_w and 15 °C.

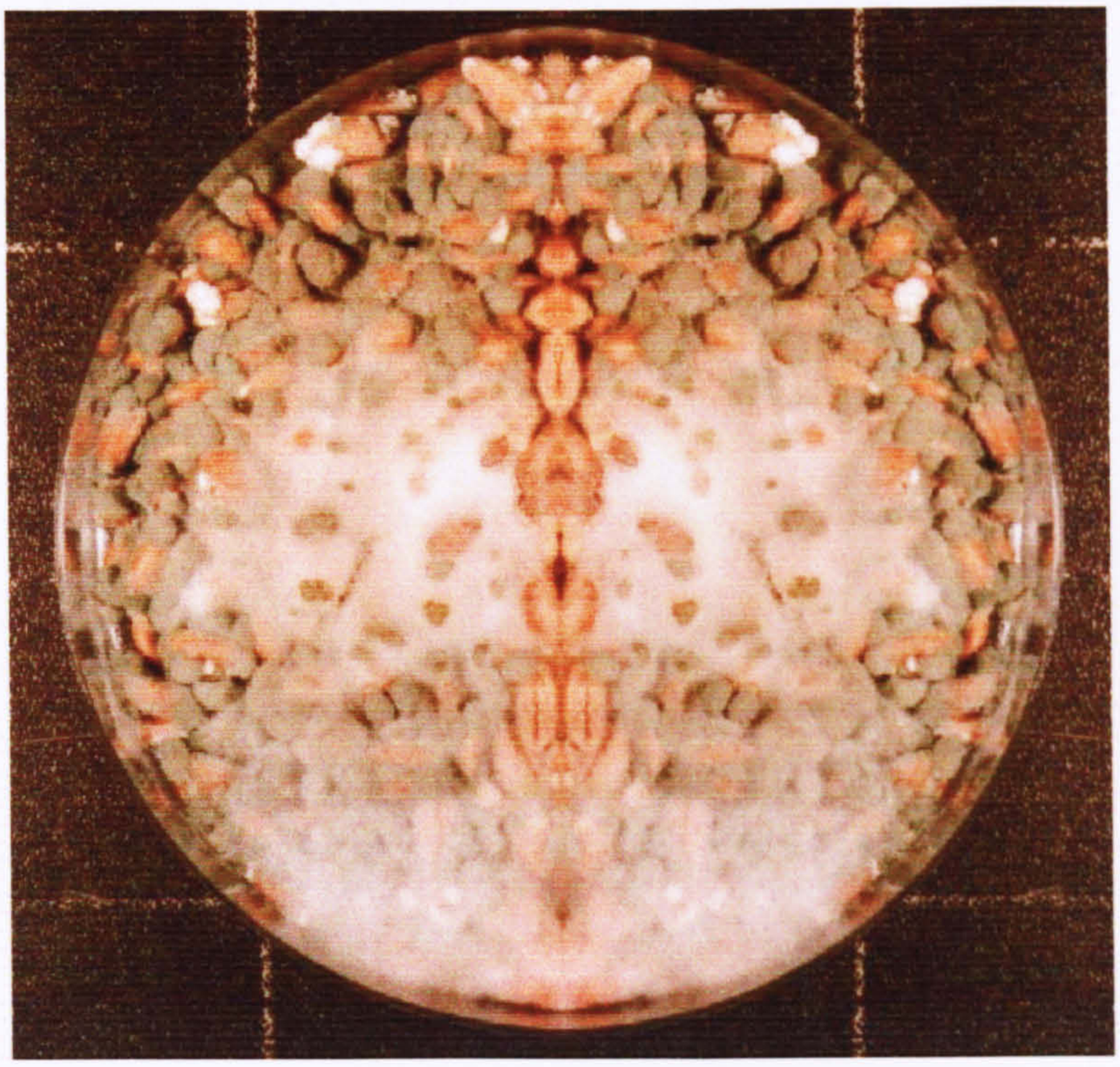


Plate 3.2 *Penicillium verrucosum* growing on γ-irradiated wheat grain after 7 days at 0.95 a_w and 25 °C.

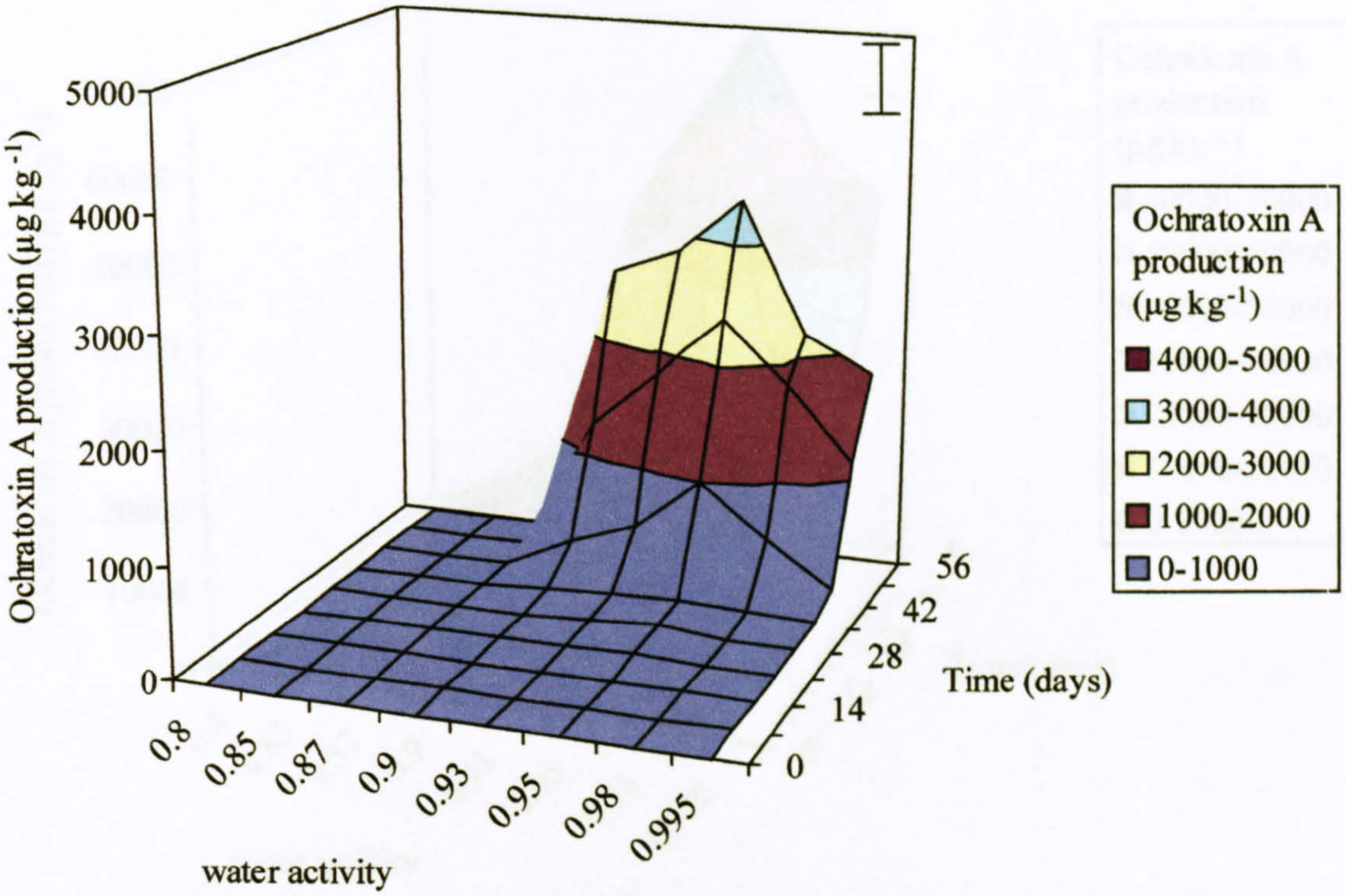


Figure 3.8 Ochratoxin A production by *Penicillium verrucosum* strain OTA11 on γ-irradiated wheat grain at various water activities at 10 °C over 56 days. Bar indicates Least Significant Difference (LSD) at $p < 0.05$.

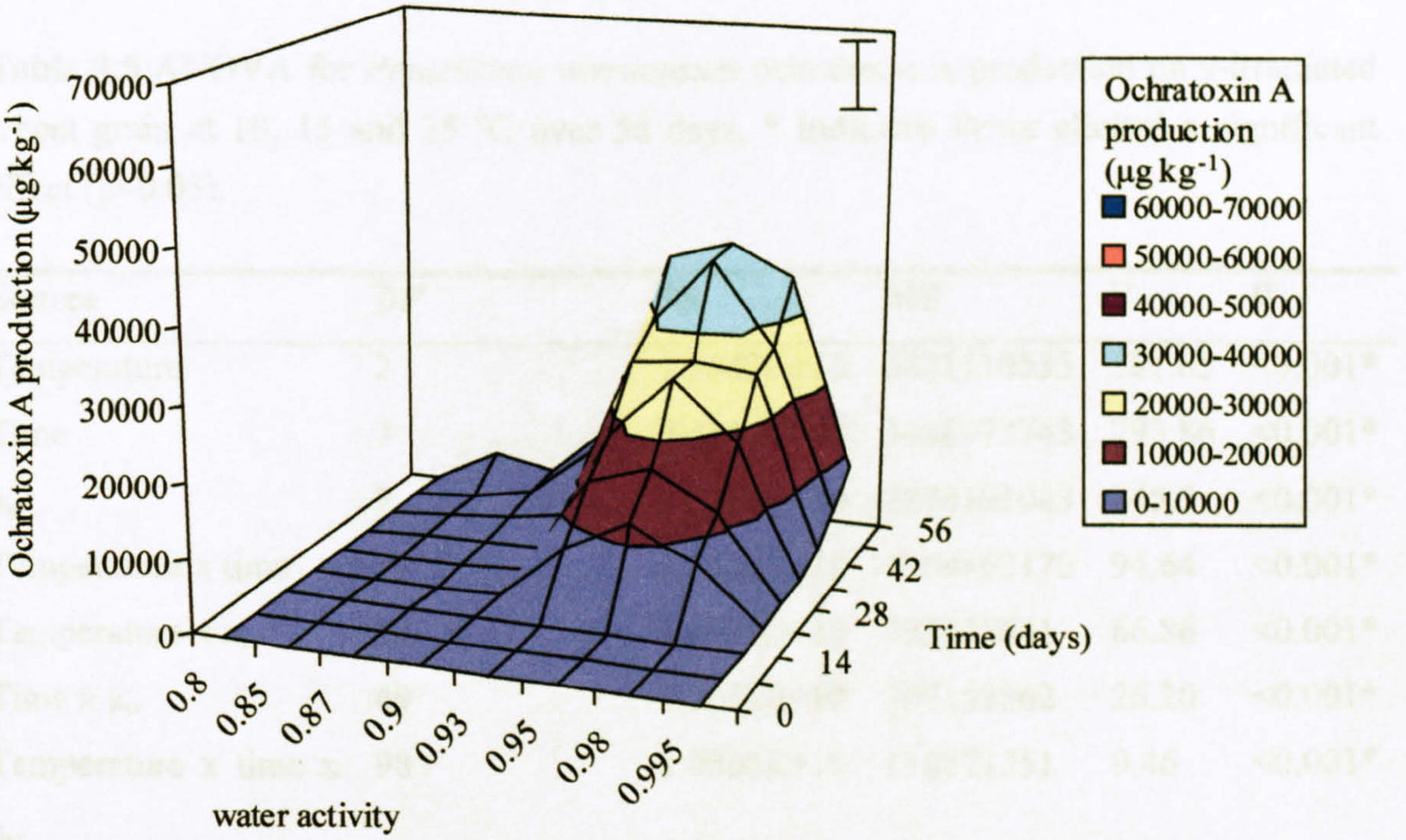


Figure 3.9 Ochratoxin A production by *Penicillium verrucosum* strain OTA11 on γ-irradiated wheat grain at various water activities at 15 °C over 56 days. Bar indicates Least Significant Difference (LSD) at $p < 0.05$.

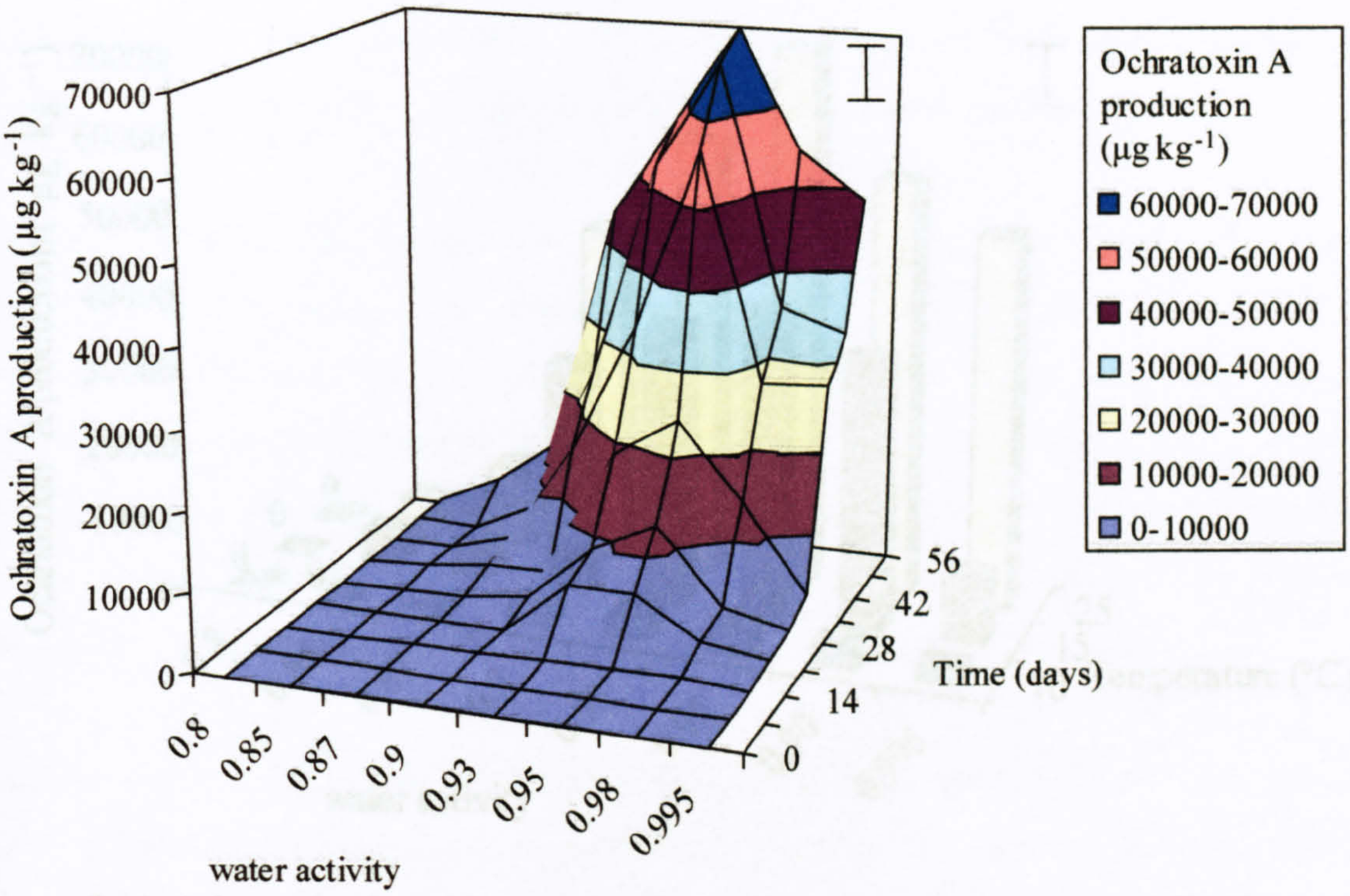


Figure 3.10 Ochratoxin A production by *Penicillium verrucosum* strain OTA11 on γ-irradiated wheat grain at various water activities at 25 °C over 56 days. Bar indicates Least Significant Difference (LSD) at $p < 0.05$.

Table 3.5 ANOVA for *Penicillium verrucosum* ochratoxin A production on γ-irradiated wheat grain at 10, 15 and 25 °C over 56 days. * Indicates factor elicited a significant effect ($p<0.05$).

Source	DF	SS	MS	F	P
Temperature	2	1.3642E+10	6821110535	581.85	<0.001*
Time	7	2.4115E+10	3444972743	293.86	<0.001*
a_w	7	2.0147E+10	2878101043	245.5	<0.001*
Temperature x time	14	1.5532E+10	1109462170	94.64	<0.001*
Temperature x a_w	14	1.0974E+10	783835641	66.86	<0.001*
Time x a_w	49	1.5051E+10	307162362	26.20	<0.001*
Temperature x time x a_w	98	1.0865E+10	110871251	9.46	<0.001*
Residual	3.84045×10^{21}				
Total	5751.1483E+11				

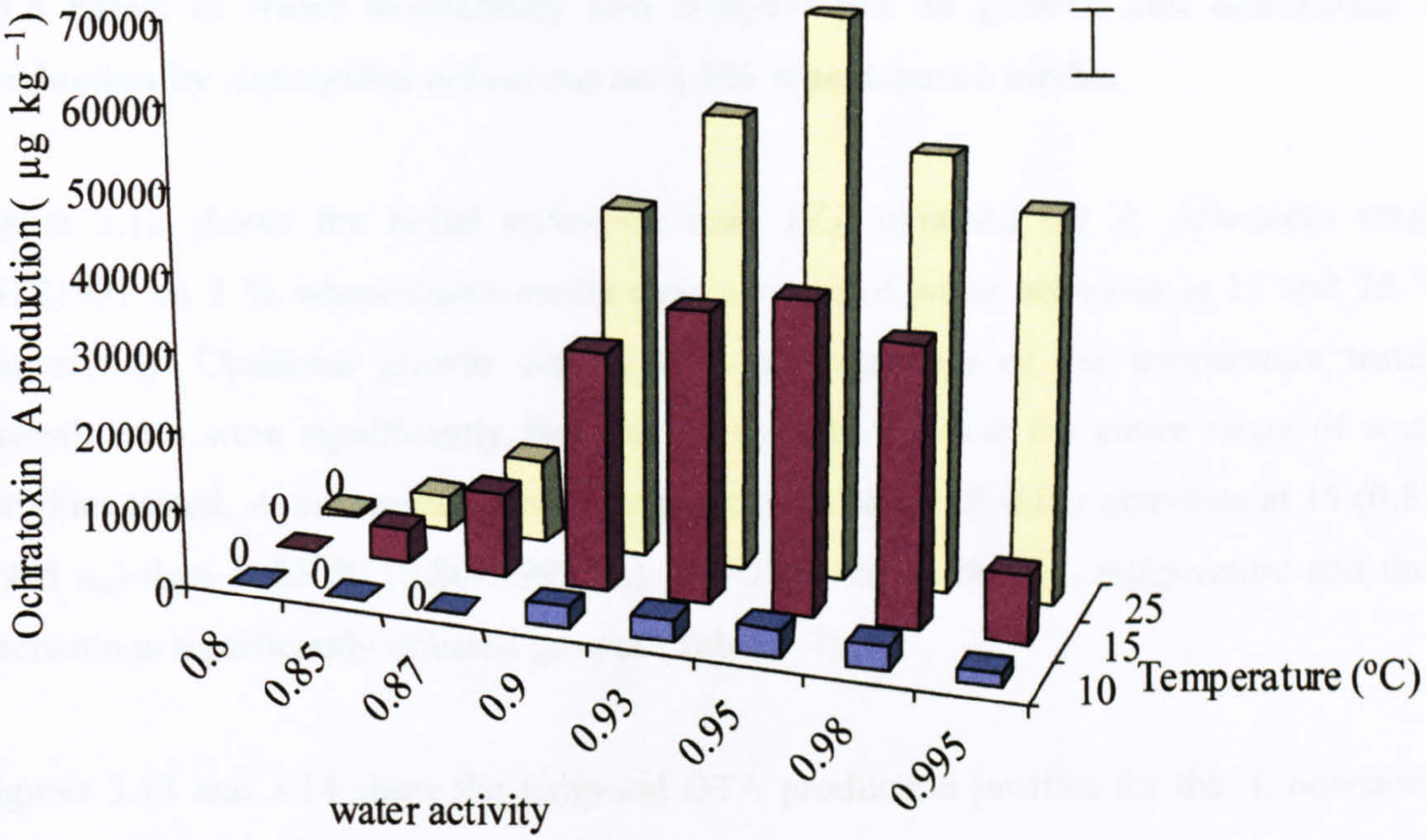


Figure 3.11 Ochratoxin A production by *Penicillium verrucosum* strain OTA11 on γ-irradiated wheat grain at various water activities at 10, 15 and 25 °C after 56 days. Bar indicates Least Significant Difference (LSD) at $p < 0.05$.

Table 3.6 ANOVA for ochratoxin A production by *Penicillium verrucosum* on γ-irradiated wheat grain at 10, 15 and 25 °C after 56 days. * Indicates factor elicited a significant effect ($p < 0.05$).

Source	DF	SS	MS	F	P
Temperature	2	1.4475E+10	7237617476	669.76	<0.001*
a_w	7	1.29474E+10	1849511450	171.15	<0.001*
Temperature x a_w	14	764449156	546035112	50.83	<0.001*
Residual	48	518698880	10806227		
Total	71	3.5585E+10			

3.1.3 Effect of water availability and temperature on growth and ochratoxin A production by *Aspergillus ochraceus* on a 2% wheat-based media.

Figure 3.12 shows the radial extension rates (K_r) obtained for *A. ochraceus* strain IBT21991 on 2 % wheat-based media over a range of water activities at 15 and 25 °C respectively. Optimum growth was at 0.98 a_w regardless of the temperature tested. Growth rates were significantly faster at 25 than 15 °C over the entire range of water activities tested. *A. ochraceus* grew over a narrower range of water activities at 15 (0.87-0.995 a_w) than at 25 °C (0.80-0.995 a_w). ANOVA shows that a_w, temperature and their interactions significantly affected growth (Table 3.7).

Figures 3.13 and 3.14 show the temporal OTA production profiles for the *A. ochraceus* strain incubated at 15 and 25 °C respectively. Optimum OTA production was at 0.95 a_w regardless of the temperature tested. Toxin production was greater at 25 than 15 °C over the entire range of water activities tested and generally increased as incubation time increased. There was no OTA production up to 21 days regardless of a_w or the temperature tested on 2 % wheat-based media. Toxin production occurred over a narrower range of water activities than those required for growth. At 15 °C growth occurred over the range 0.87-0.995 a_w whereas OTA production occurred over the a_w range 0.90–0.995. At 25 °C growth occurred over the range 0.80-0.995 a_w whereas OTA production occurred over the a_w range 0.85–0.995. ANOVA shows that a_w and temperature and their interactions significantly affected OTA production (Table 3.8).

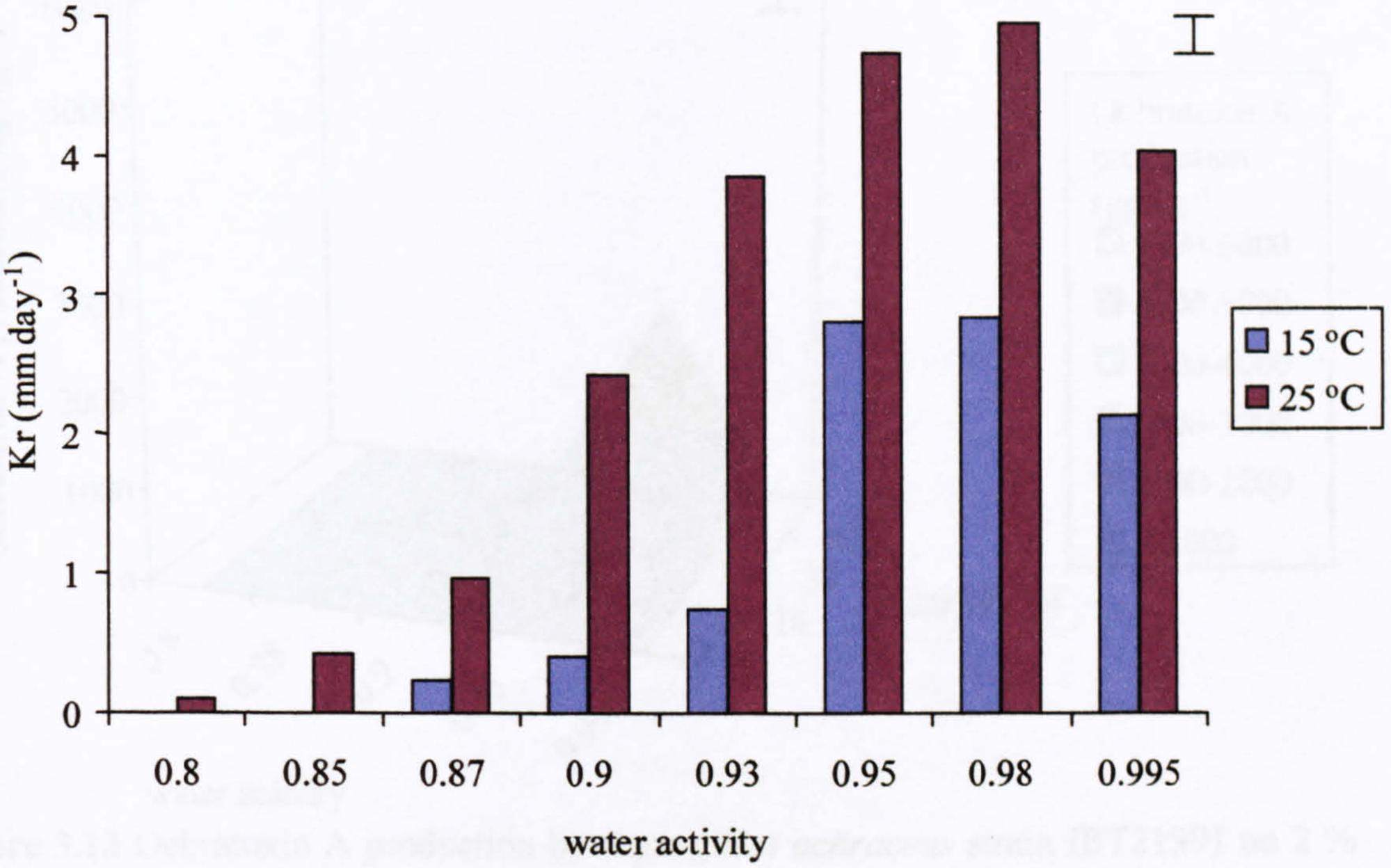


Figure 3.12 Growth of *Aspergillus ochraceus* strain IBT21991 on 2 % wheat-based media at various water activities and 15 and 25 °C respectively. Bar indicates Least Significant Difference (LSD) at $p < 0.05$.

Table 3.7 ANOVA for growth of *Aspergillus ochraceus* strain IBT21991 on 2 % wheat-based media. * Indicates factor elicited a significant effect ($p < 0.05$).

Source of Variation	DF	SS	MS	F	P
a_W	7	106.0224	15.1461	538.75	<0.001*
Temperature	1	27.8488	27.8488	990.59	<0.001*
a_W x temperature	7	11.6432	1.6622	59.16	<0.001*
Residual	32	0.8996	0.0281		
Total	47	146.4140			

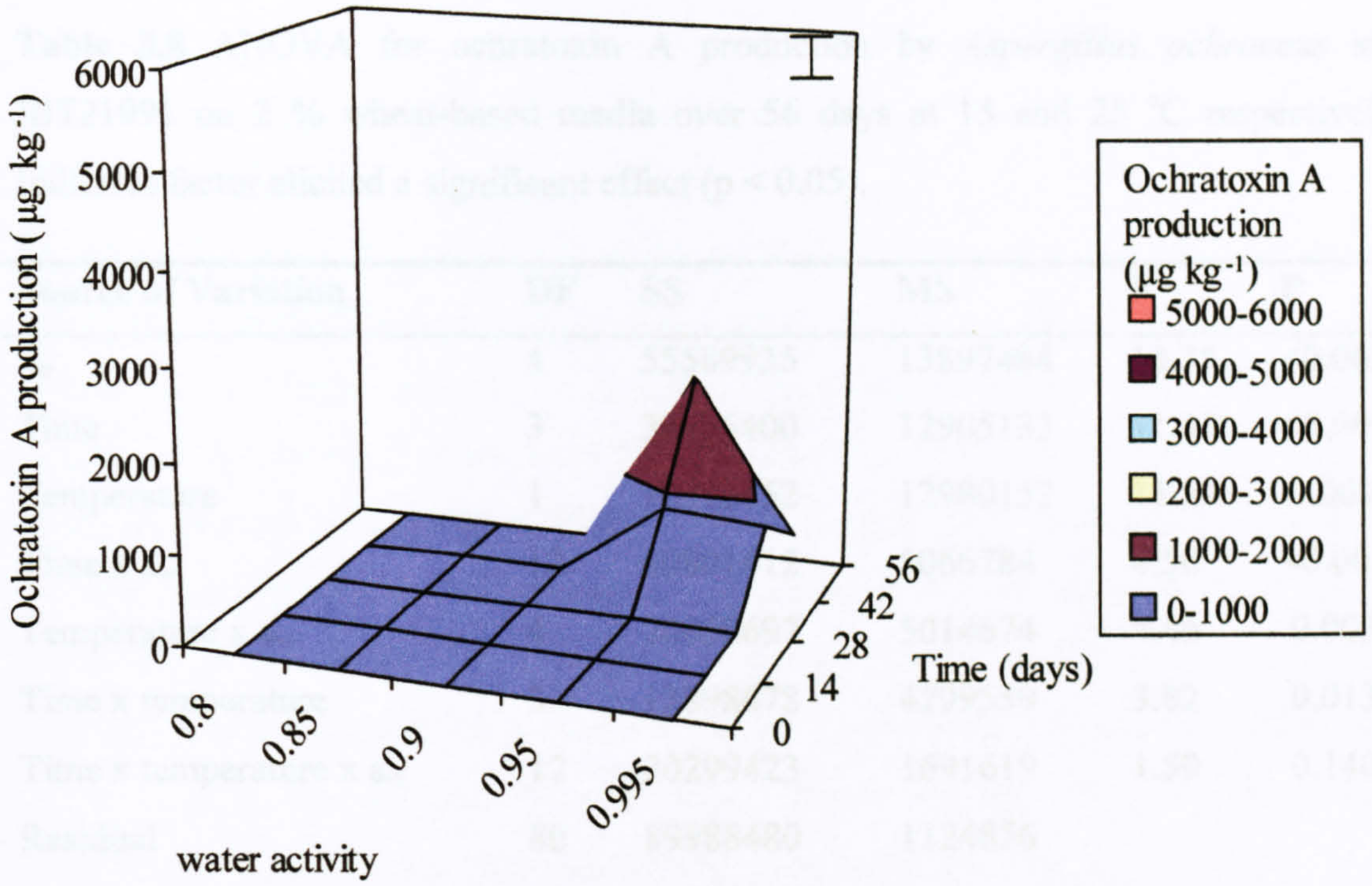


Figure 3.13 Ochratoxin A production by *Aspergillus ochraceus* strain IBT21991 on 2 % wheat-based media at various water activities at 15 °C over 56 days. Bar indicates Least Significant Difference (LSD) at $p < 0.05$.

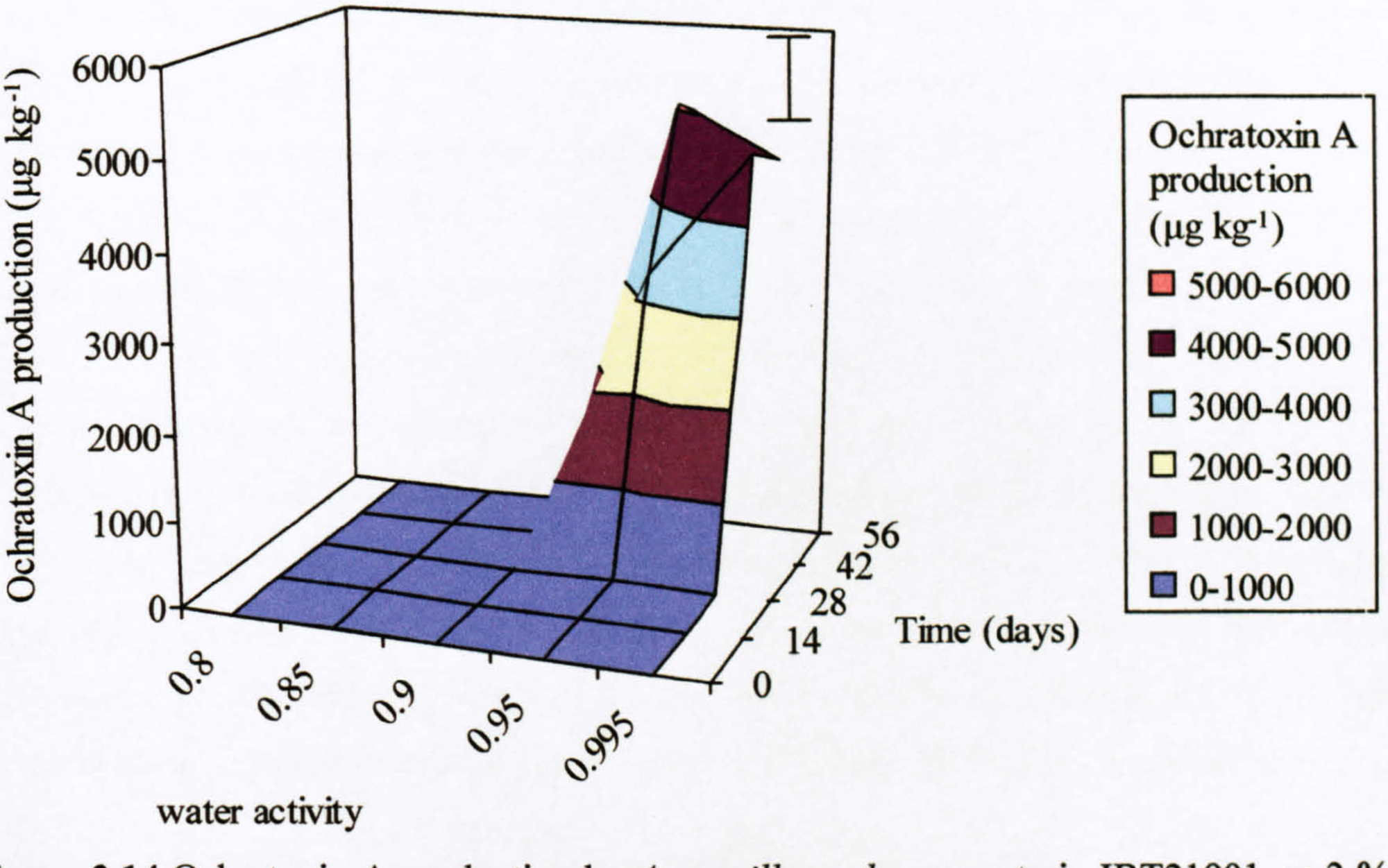


Figure 3.14 Ochratoxin A production by *Aspergillus ochraceus* strain IBT21991 on 2 % wheat-based media at various water activities at 25 °C over 56 days. Bar indicates Least Significant Difference (LSD) at $p < 0.05$.

Table 3.8 ANOVA for ochratoxin A production by *Aspergillus ochraceus* strain IBT21991 on 2 % wheat-based media over 56 days at 15 and 25 °C respectively. * Indicates factor elicited a significant effect ($p < 0.05$).

Source of Variation	DF	SS	MS	F	P
a_w	4	55589935	13897484	12.35	<0.001*
Time	3	38715400	12905133	11.47	<0.001*
Temperature	1	12980152	12980152	11.54	0.001*
Time x a_w	12	60801412	5066784	4.50	<0.001*
Temperature x a_w	4	20058697	5014674	4.46	0.003*
Time x temperature	3	12898678	4299559	3.82	0.013
Time x temperature x a_w	12	20299423	1691619	1.50	0.140
Residual	80	89988480	1124856		
Total	119	311332177			

3.1.4 Effect of water availability and temperature on growth and ochratoxin A production by *Aspergillus ochraceus* on wheat grain.

Figure 3.15 shows the radial extension rates (K_r) obtained for *A. ochraceus* strain IBT21991 on γ-irradiated wheat grain over a range of water activities at 10, 15 and 25 °C respectively. Optimum growth was at 0.95 a_w regardless of the temperature tested. These results are in contrast to those on 2 % wheat-based media where growth was fastest at 0.982 a_w. Growth was fastest at 25 followed by 15 and 10 °C respectively regardless of the water activity tested. As temperature decreased so did the range of water activities permitting growth. At 25 °C *A. ochraceus* was able to grow over the entire range of water activities tested (0.80-0.995 a_w). However, at 15 °C, there was no growth at <0.85 a_w and at 10 °C there was no growth at <0.90 a_w. Plates 3.3 and 3.4 compare growth of *A. ochraceus* on 2 % wheat-based media and γ-irradiated wheat grain.

Figures 3.16-3.18 show temporal OTA production by *A. ochraceus* on γ-irradiated wheat grain over a range of water activities (0.80-0.995 a_w) over 56 days at 10, 15 and 25 °C respectively. No OTA production occurred at 7 days regardless of temperature or water activity. Optimum OTA production was at 56 days at 0.95 a_w for all temperatures tested. Interestingly, at sub-optimal water activity levels (0.85-0.87 a_w) there was a stimulation in OTA production at 15 and 25 °C. This did not occur at 10 °C. OTA production was greatest at 25 followed by 15 and 10 °C respectively over the entire range of water activities tested regardless of the incubation period. Figure 3.19 compares OTA production by *A. ochraceus* after 56 days over the a_w range at 10, 15 and 25 °C respectively. OTA production occurred over a narrower range of conditions than those required for growth. At 25 °C OTA production occurred at 0.85-0.995 a_w whereas growth occurred between 0.80-0.995 a_w. At 10 °C OTA production occurred at 0.93-0.995 a_w whereas growth occurred between 0.90-0.995 a_w. ANOVA (Table 3.10) shows that all experimental factors and their interactions significantly affected OTA production.

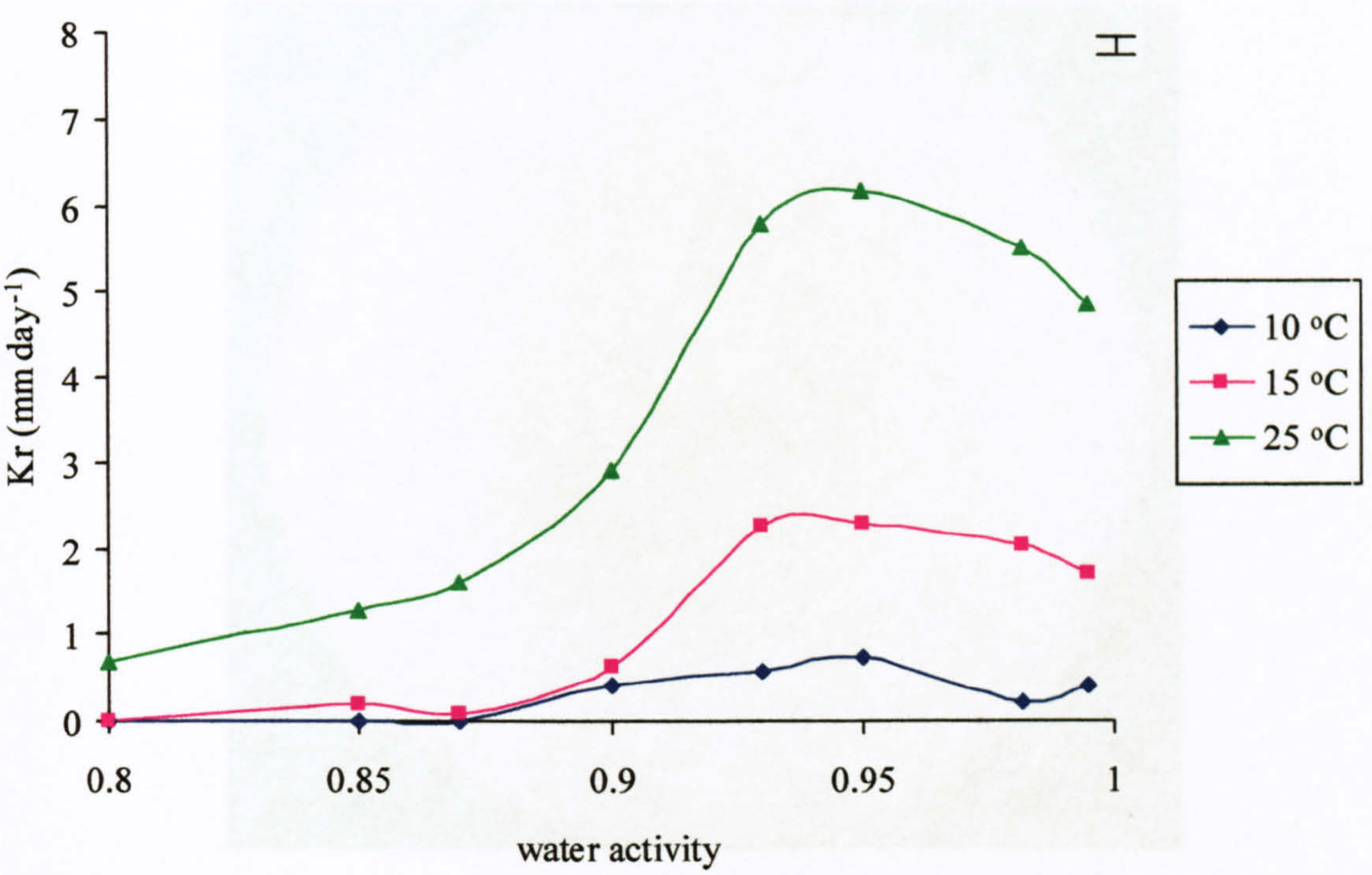


Figure 3.15 Growth of *Aspergillus ochraceus* strain IBT21991 on γ-irradiated wheat grain adjusted to various water activities and incubated at 10, 15 and 25 °C respectively. Bar indicates Least Significant Difference (LSD) at $p < 0.05$.

Table 3.9 ANOVA for *Aspergillus ochraceus* (IBT21991) growth on γ-irradiated wheat grain. * Indicates factor elicited a significant effect ($p < 0.05$).

Source of Variation	DF	SS	MS	F	P
a_w	7	85.428	12.204	293.40	<0.001*
Temperature	2	141.178	70.589	1697.07	<0.001*
a_w x temperature	14	43.085	3.078	73.99	<0.001*
Residual	48	1.997	0.042		
Total	71	271.689			

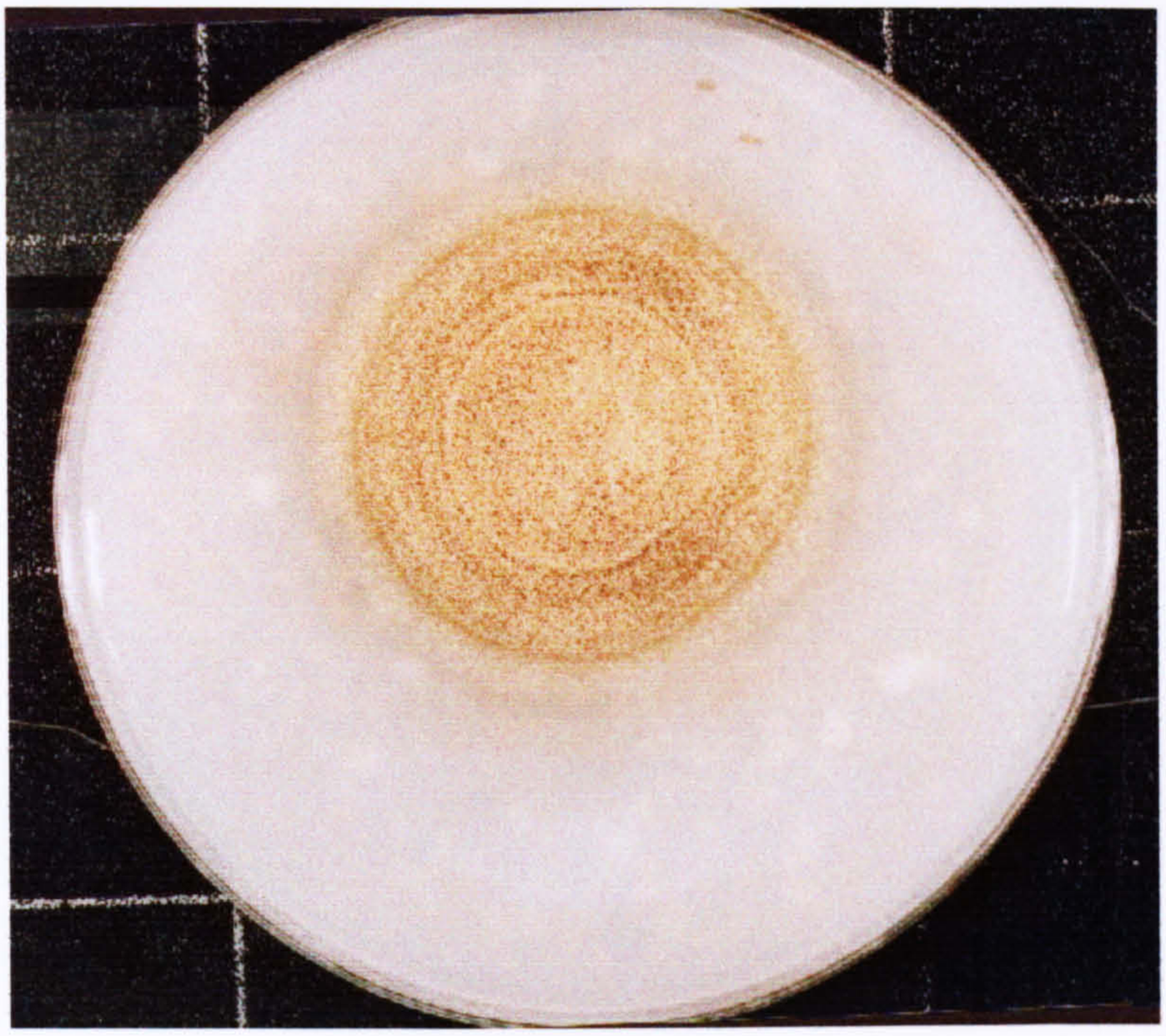


Plate 3.3 Morphology of *Aspergillus ochraceus* (IBT21991) on a 2 % wheat-based media after 7 days at 0.90 a_w and 15 °C.

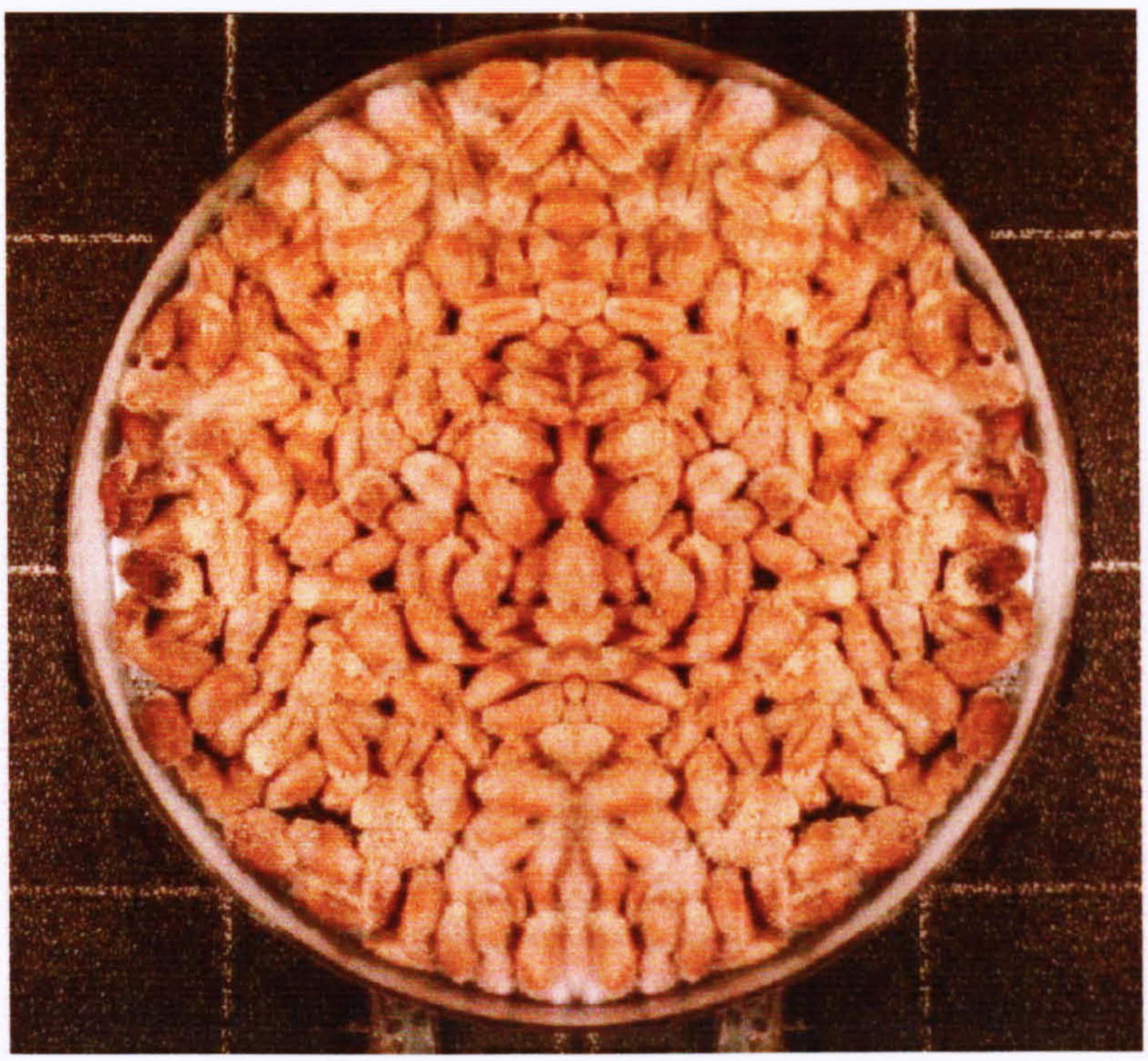


Plate 3.4 *Aspergillus ochraceus* growing on γ-irradiated wheat grain after 4 days at 0.95 a_w and 25 °C.

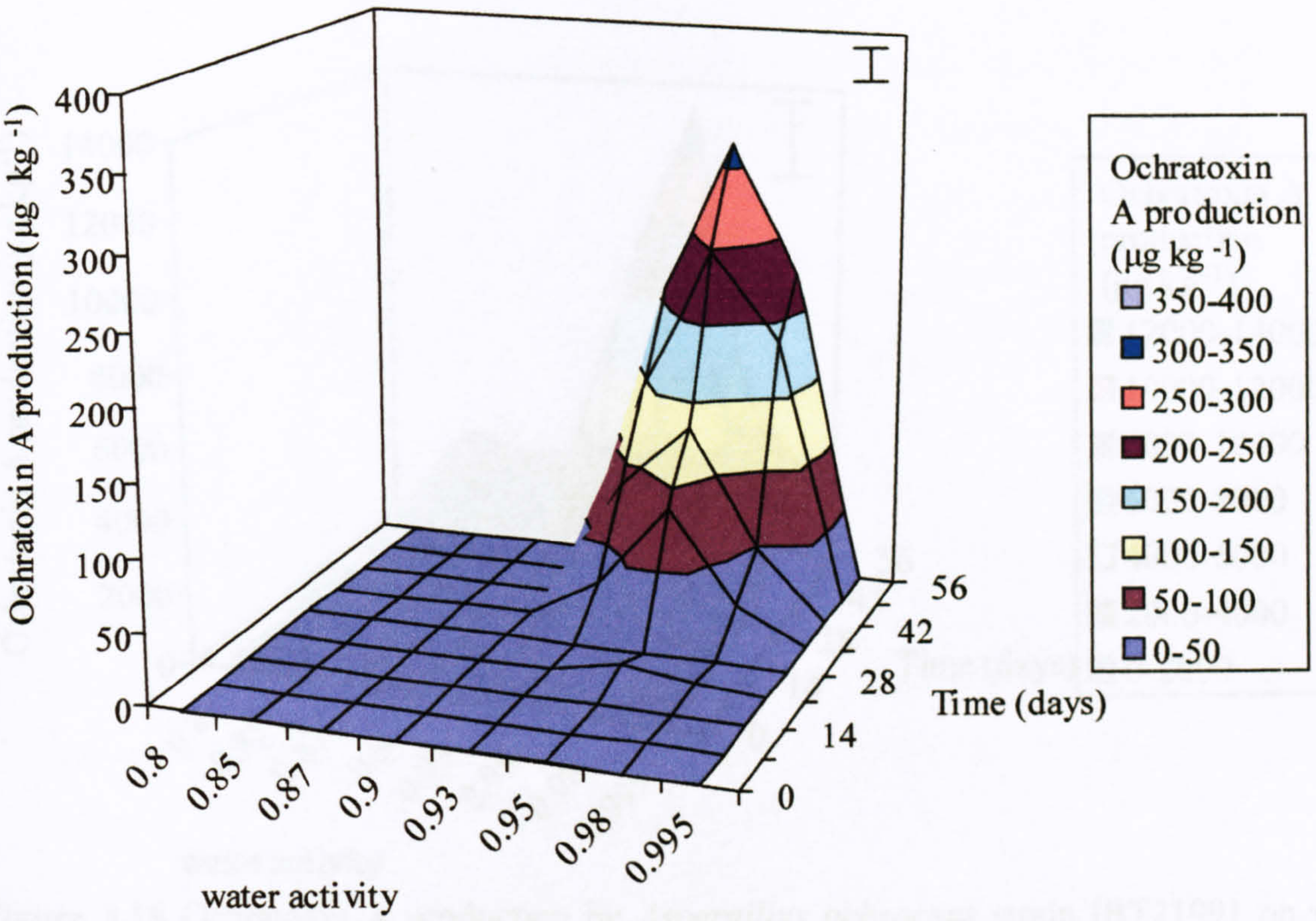


Figure 3.16 Ochratoxin A production by *Aspergillus ochraceus* strain IBT21991 on γ-irradiated wheat grain at various water activities at 10 °C over 56 days. Bar indicates Least Significant Difference (LSD) at $p < 0.05$.

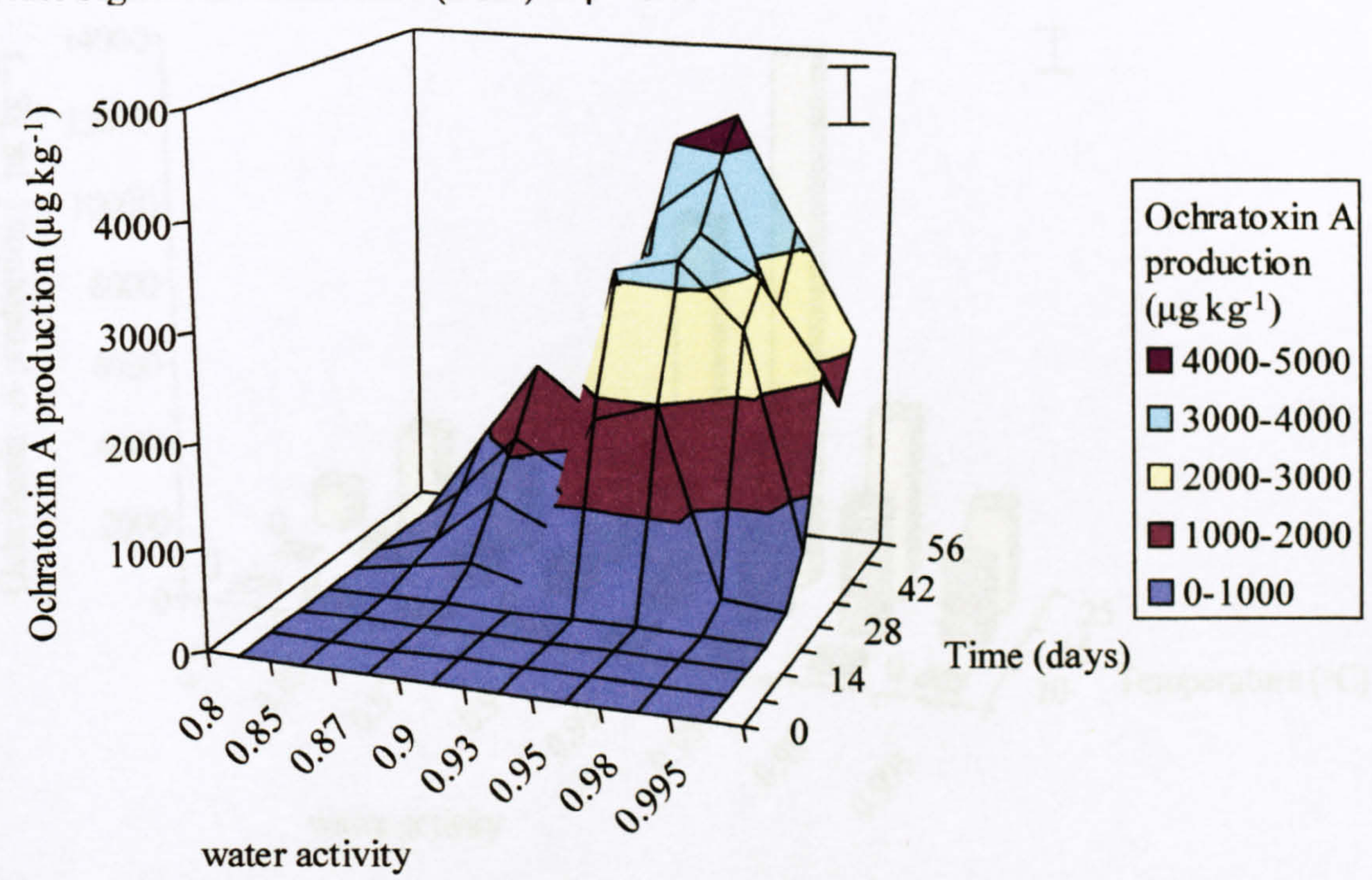


Figure 3.17 Ochratoxin A production by *Aspergillus ochraceus* strain IBT21991 on γ-irradiated wheat grain at various water activities at 15 °C over 56 days. Bar indicates Least Significant Difference (LSD) at $p < 0.05$.

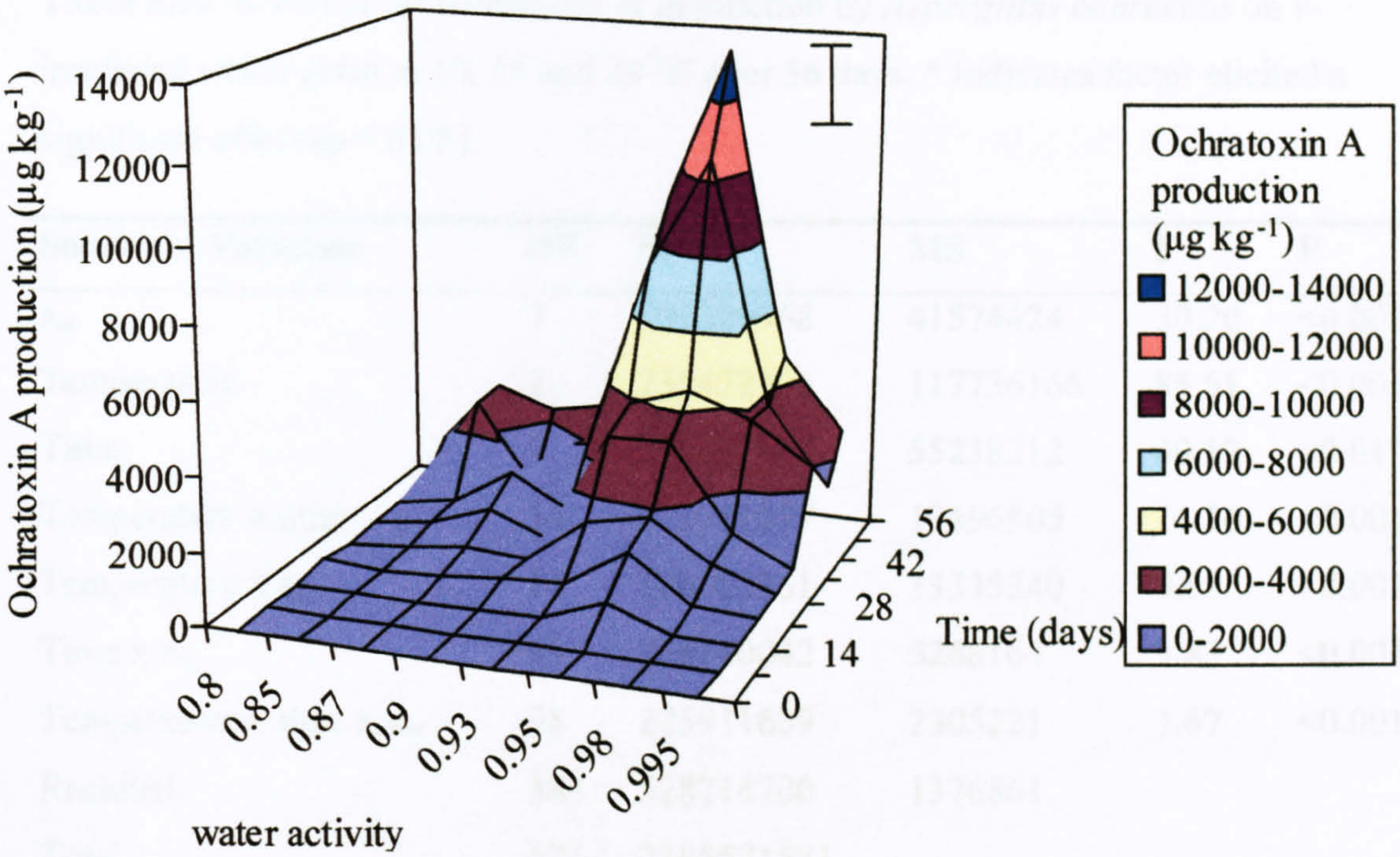


Figure 3.18 Ochratoxin A production by *Aspergillus ochraceus* strain IBT21991 on γ-irradiated wheat grain at various water activities at 25 °C over 56 days. Bar indicates Least Significant Difference (LSD) at $p < 0.05$.

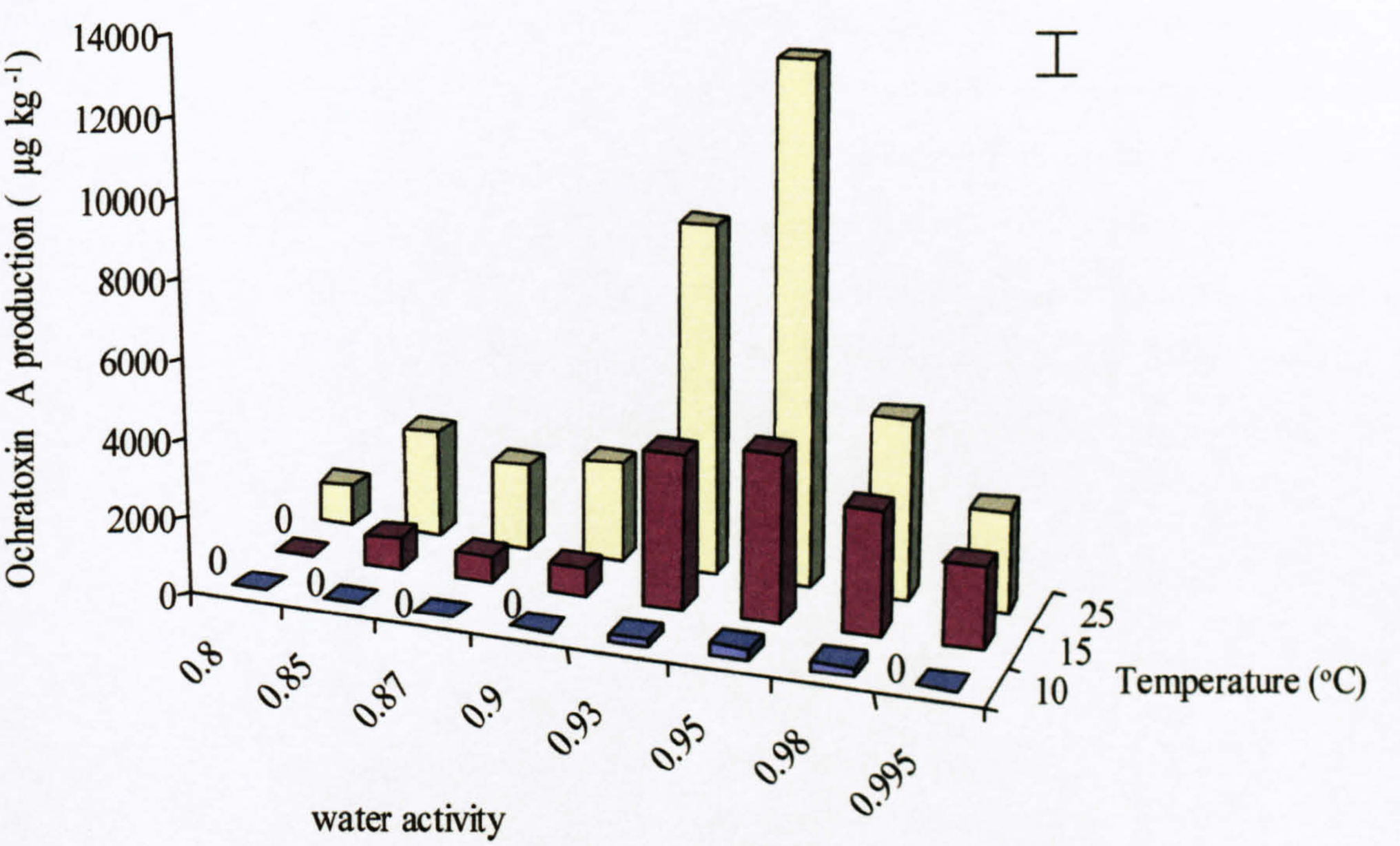


Figure 3.19 Ochratoxin A production by *Aspergillus ochraceus* strain IBT21991 on γ-irradiated wheat grain at various water activities at 10, 15 and 25 °C after 56 days. Bar indicates Least Significant Difference (LSD) at $p < 0.05$.

Table 3.10 ANOVA for ochratoxin A production by *Aspergillus ochraceus* on γ-irradiated wheat grain at 10, 15 and 25 °C over 56 days. * Indicates factor elicited a significant effect ($p < 0.05$).

Source of Variation	DF	SS	MS	F	P
a_w	7	291020968	41574424	30.20	<0.001*
Temperature	2	235472333	117736166	85.51	<0.001*
Time	7	386667486	55238212	40.12	<0.01*
Temperature x time	14	272951077	19496505	14.16	<0.001*
Temperature x a_w	14	186693361	13335240	9.69	<0.001*
Time x a_w	49	258140042	5268164	3.83	<0.001*
Temperature x time x a_w	98	225911609	2305221	1.67	<0.001*
Residual	384	528714700	1376861		
Total	575	2385571581			

3.2 GAS COMPOSITION AND WATER AVAILABILITY EFFECTS ON GERMINATION, GROWTH AND MYCOTOXIN PRODUCTION BY *PENICILLIUM VERRUCOSUM* AND *ASPERGILLUS OCHRACEUS*

During respiration of damp grain and contaminant fungi O_2 is utilised and CO_2 is produced. If air exchange is restricted this can lead to an accumulation of CO_2 in the intergranular atmosphere. This study examines for the first time the effects of varying carbon dioxide concentrations and a_w on germination, growth and OTA production by *P. verrucosum* and *A. ochraceus* on a 2 % wheat-based media and γ-irradiated wheat grain.

3.2.1 Effect of water availability and gas composition on germination and germ tube lengths by *Penicillium verrucosum* on 2 % wheat-based media

All spores germinated regardless of the treatments. Figure 3.20 shows the mean germ tube lengths of *P. verrucosum* conidia on 2 % wheat-based media at three different gas compositions (air, 25 % CO_2 and 50 % CO_2) and three different water activities (0.90, 0.95 and 0.995 a_w) at 25 °C after 36 hours. Germ tube lengths were longest in air, followed by 25 % CO_2 and 50 % CO_2 when compared with their respective water activities. Germ tube lengths were always longest at 0.95 a_w followed by 0.995 a_w and 0.90 a_w regardless of the gas composition tested. ANOVA (Table 3.11) shows that water activity and gas composition and their interactions significantly affected germ tube lengths. Plates 3.5 and 3.6 illustrate the effects of gas composition on germ tube lengths of germinating spore of *P. verrucosum* at 0.90 a_w.

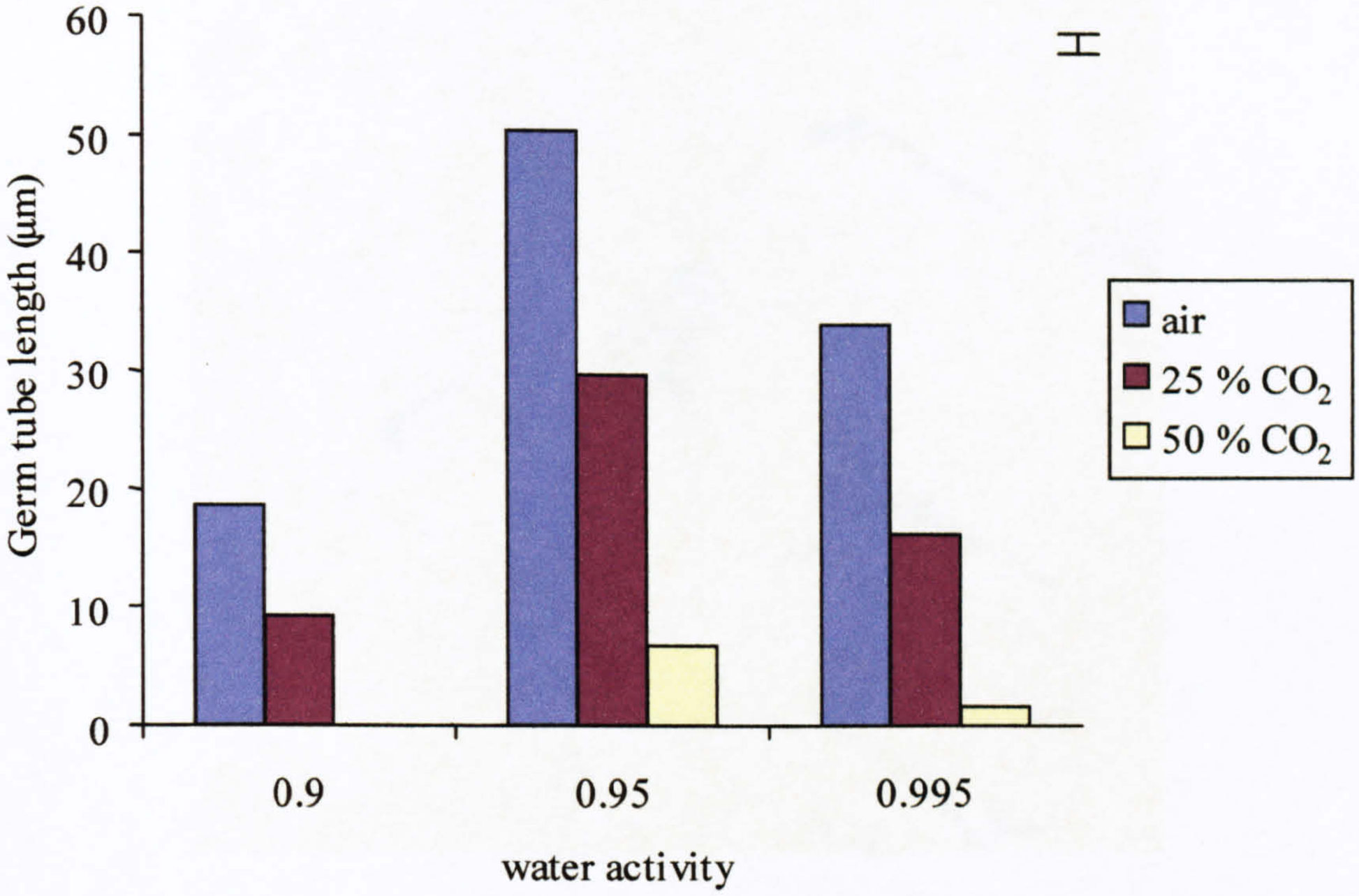


Figure 3.20 Germ tube lengths of conidia of *Penicillium verrucosum* (strain OTA11) on 2 % wheat-based media after 36 hours at various water activities and gas compositions. Bar indicates Least Significant Difference (LSD) at $p < 0.05$.

Table 3.11 ANOVA for germ tube lengths by *Penicillium verrucosum* (strain OTA11) on 2 % wheat-based media after 36 hours at various gas compositions and water activities at 25 °C. * Indicates factor elicited a significant effect ($p < 0.05$).

Source	DF	SS	MS	F	P
a_w	2	1739.85	869.93	146.80	<0.001 *
Gas treatment	2	4480.96	2240.48	378.08	<0.001 *
Gas treatment x a_w	4	477.26	119.31	20.13	<0.001 *
Residual	18	106.67	5.93		
Total	26	6804.74			

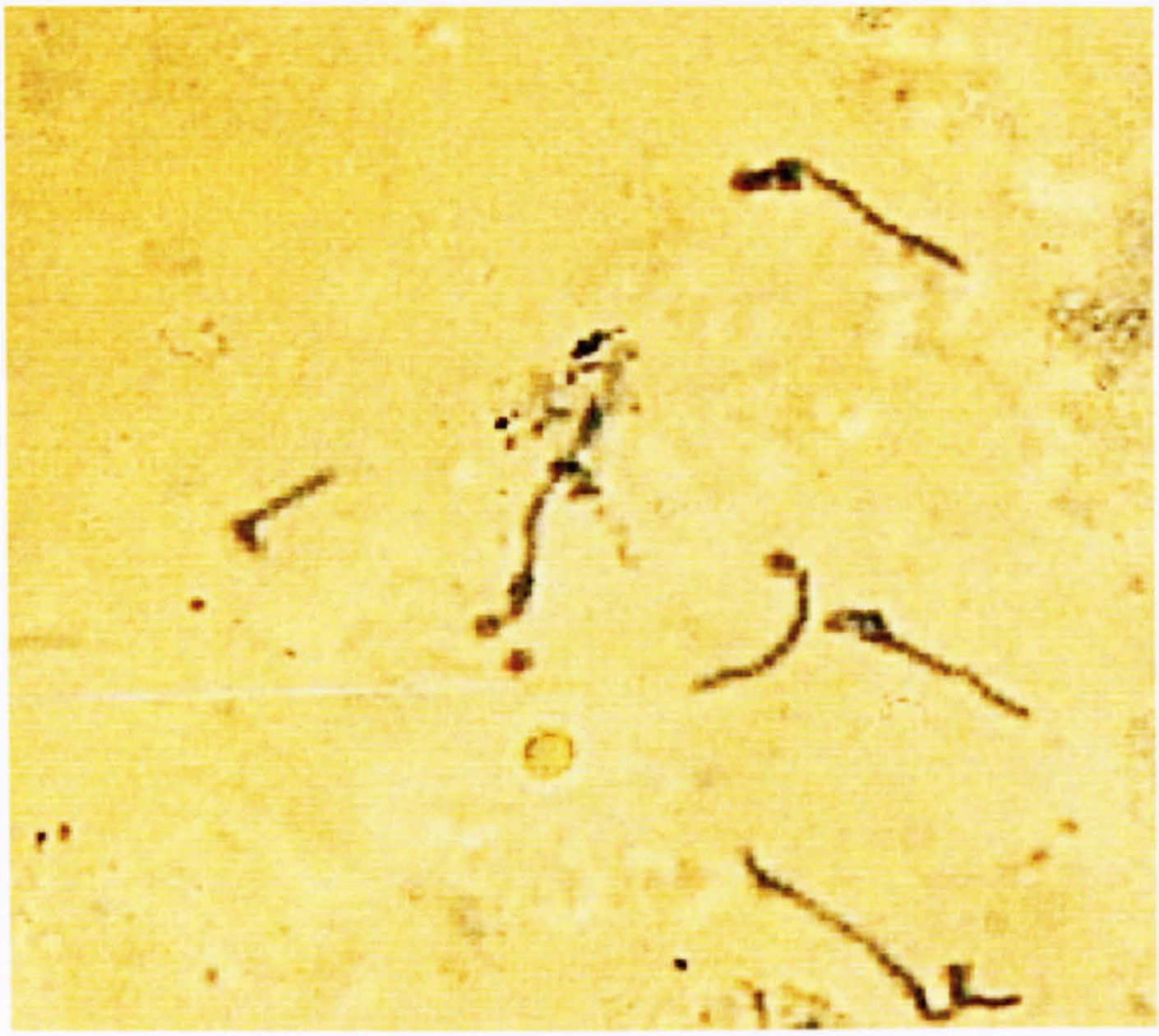


Plate 3.5 Germ tubes of conidia of *Penicillium verrucosum* on 2 % wheat-based media at 0.90 a_w and 25 °C after 36 hours in air. Magnification x 200.

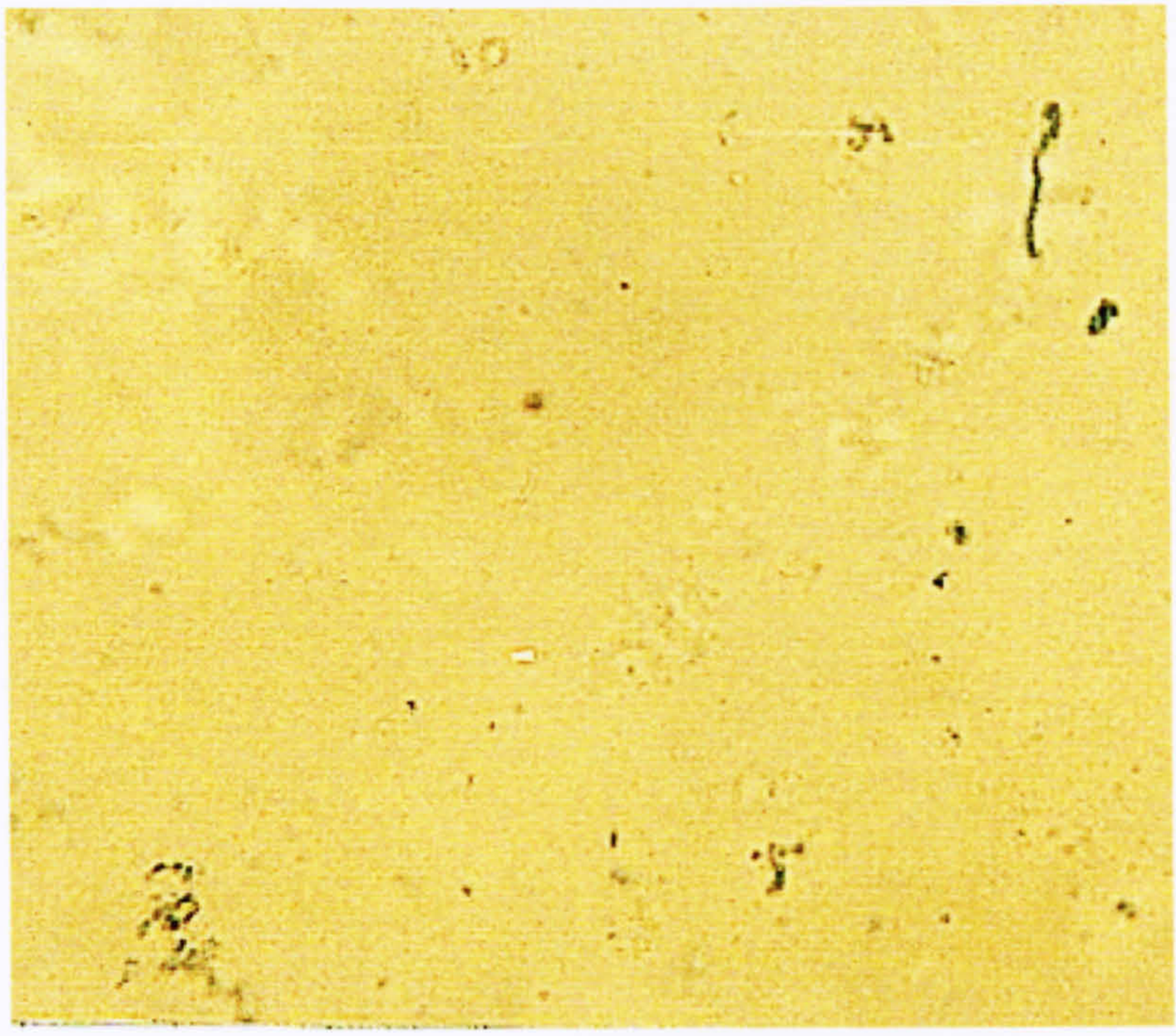


Plate 3.6 Germ tubes of conidia of *Penicillium verrucosum* on 2 % wheat-based media at 0.90 a_w and 25 °C after 36 hours at 25 % CO_2. Magnification x 200.

3.2.2 Effect of water availability and gas composition on growth and ochratoxin A production by *Penicillium verrucosum* on wheat agar.

Figure 3.21 shows the radial extension rates (K_r) by *P. verrucosum* on 2 % wheat-based media at three different gas compositions (air, 25 % CO_2 and 50 % CO_2) and three different water activities (0.90, 0.95 and 0.995 a_w) at 25 °C. Growth rates were fastest in air followed by 25 % CO_2 and 50 % CO_2 when compared with their respective water activities. Growth rates were fastest at 0.95 a_w followed by 0.995 a_w and 0.90 a_w regardless of the gas composition tested. When growth rates were compared with germ tube lengths (Figure 3.20) it was found that the longer the germ tube length, the faster the growth rate. ANOVA showed that water activity and gas composition and their interactions significantly affected growth (Table 3.14).

Figures 3.22 shows the effect of gas treatments on OTA production by *P. verrucosum* Regardless of the gas composition or water activity no toxin production occurred until 21 days incubation. Optimum OTA production was at 0.95 a_w irrespective of the gas composition tested. OTA production was greatest in air, followed by 25 % CO_2 and 50 % CO_2 respectively. After 56 days OTA production was suppressed by up to 92 % at 25 % CO_2 and up to 100 % at 50 % CO_2 when compared with their respective water activities in air. ANOVA (Table 3.13) shows that water activity, gas composition and incubation time and their interactions significantly affected OTA production.

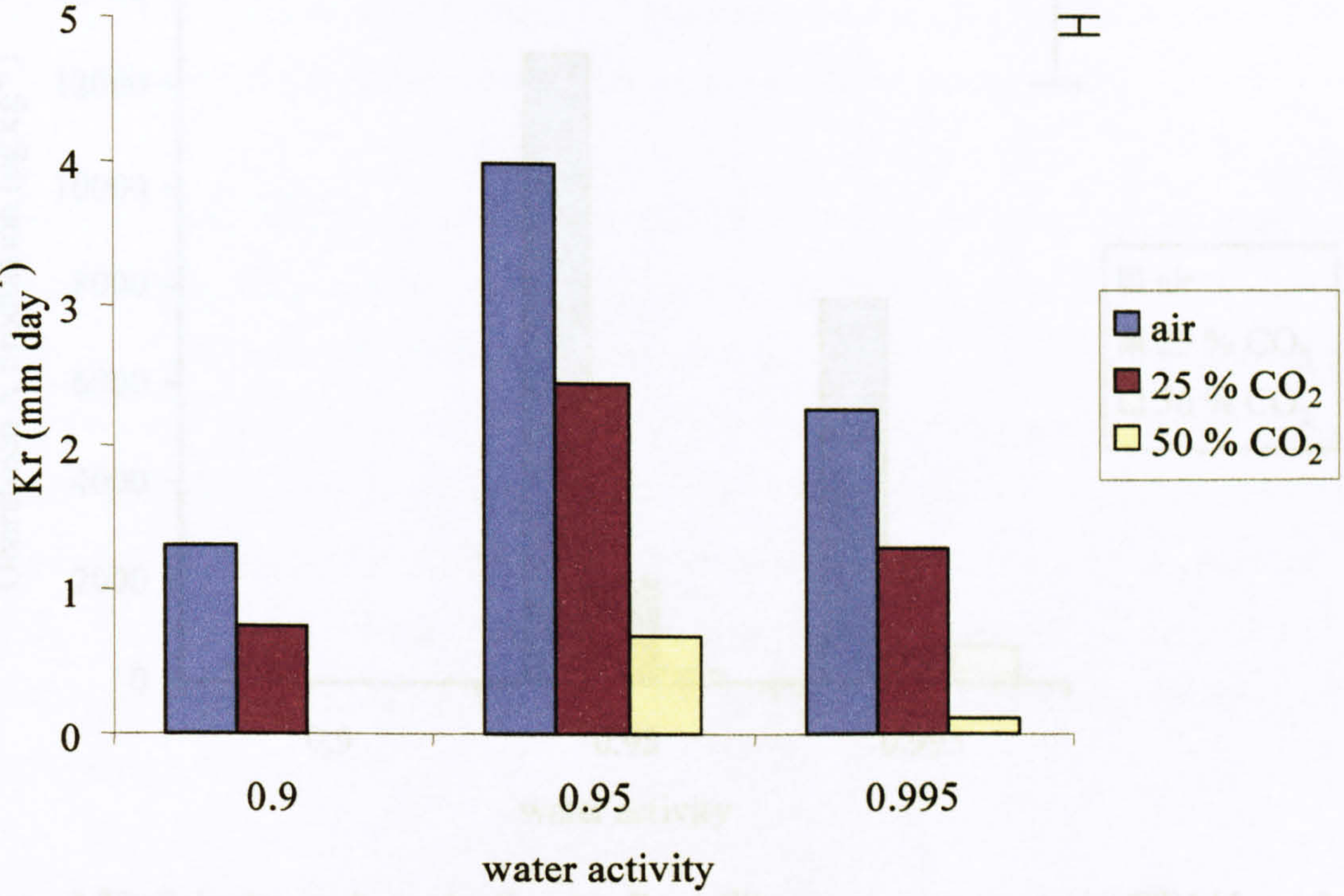


Figure 3.21 Growth of *Penicillium verrucosum* strain OTA11 on 2 % wheat-based media at various water activities and gas compositions at 25 °C. Bar indicates Least Significant Difference (LSD) at $p < 0.05$.

Table 3.12 ANOVA for *Penicillium verrucosum* (OTA11) growth on wheat media at various gas compositions. * Indicates factor elicited a significant effect ($p < 0.05$).

Source of Variation	DF	SS	MS	F	P
a_w	2	13.3616	6.6808	227.21	<0.001*
Treatment	2	22.8213	11.4107	388.07	<0.001*
a_w x treatment	4	3.0097	0.7524	25.59	<0.001*
Residual	18	0.5293	0.0294		
Total	26	39.7219			

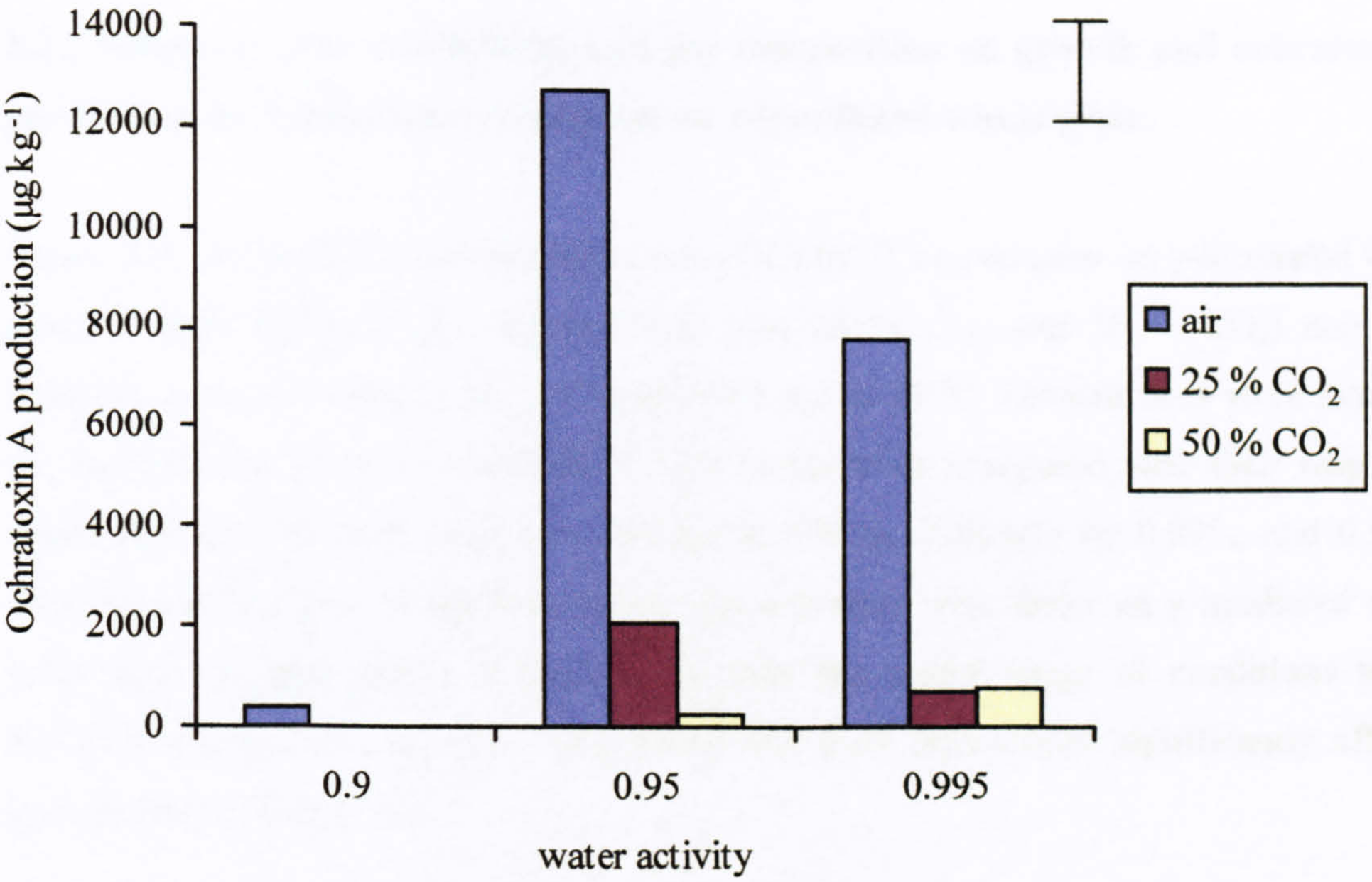


Figure 3.22 Ochratoxin A production by *Penicillium verrucosum* strain OTA11 on 2 % wheat-based media at various water activities and gas compositions at 25 °C after 56 days. Bar indicates Least Significant Difference (LSD) at $p < 0.05$.

Table 3.13 Ochratoxin A production by *Penicillium verrucosum* strain OTA11 on 2 % wheat-based media at various water activities and gas compositions at 25 °C over 56 days. * Indicates factor elicited a significant effect ($p < 0.05$).

Source of Variation	DF	SS	MS	F	P
a_W	2	79955080	39977540	13.54	<0.001*
Time	3	138773276	46257759	15.67	<0.001*
Gas treatment	2	153529573	76764787	26.00	<0.001*
Time x a_w	6	85140695	14190116	4.81	<0.001*
Gas treatment x a_w	4	101492335	25373084	8.59	<0.001*
Gas treatment x time	6	167995755	27999293	9.48	<0.001*
Gas treatment x time x a_w	12	113084949	9423746	3.19	<0.001*
Residual	72	212595967	2952722		
Total	107	1052567631			

3.2.3 Effect of water availability and gas composition on growth and ochratoxin A production by *Penicillium verrucosum* on γ-irradiated wheat grain.

Figure 3.23 shows the radial extension rates (K_r) by *P. verrucosum* on γ-irradiated wheat grain at three different gas compositions (air, 25 % CO_2 and 50 % CO_2) and three different water activities (0.90, 0.95 and 0.995 a_w) at 25 °C. Growth rates were fastest in air, followed by 25 % CO_2 and 50 % CO_2 levels when compared with their respective water activities. Growth rates were fastest at 0.95 a_w followed by 0.995_w and 0.90 a_w regardless of the gas composition tested. Growth rates were faster on γ-irradiated wheat grain than on agar media (Figure 3.21) over the entire range of conditions tested. ANOVA showed that a_w, gas composition and their interactions significantly affected growth rates (Table 3.14).

Figures 3.24-3.26 show temporal OTA production by *P. verrucosum* on γ-irradiated wheat grain in relation to the treatments at 25 °C over 28 days. No OTA production occurred at 7 days regardless of the gas composition tested. OTA production was greatest in air, followed by 25 % CO_2 and 50 % CO_2 levels respectively. Optimum OTA production was at 28 days at 0.95 a_w irrespective of the gas composition tested. Figure 3.27 compares the OTA production at 28 days for each of the different treatments. OTA production occurred over the entire range of water activities and gas compositions tested which is in contrast to agar studies where no OTA production occurred at 0.90 a_w and 50 % CO_2. ANOVA showed that a_w, gas composition and incubation time and their interactions significantly affected OTA production (Table 3.15).

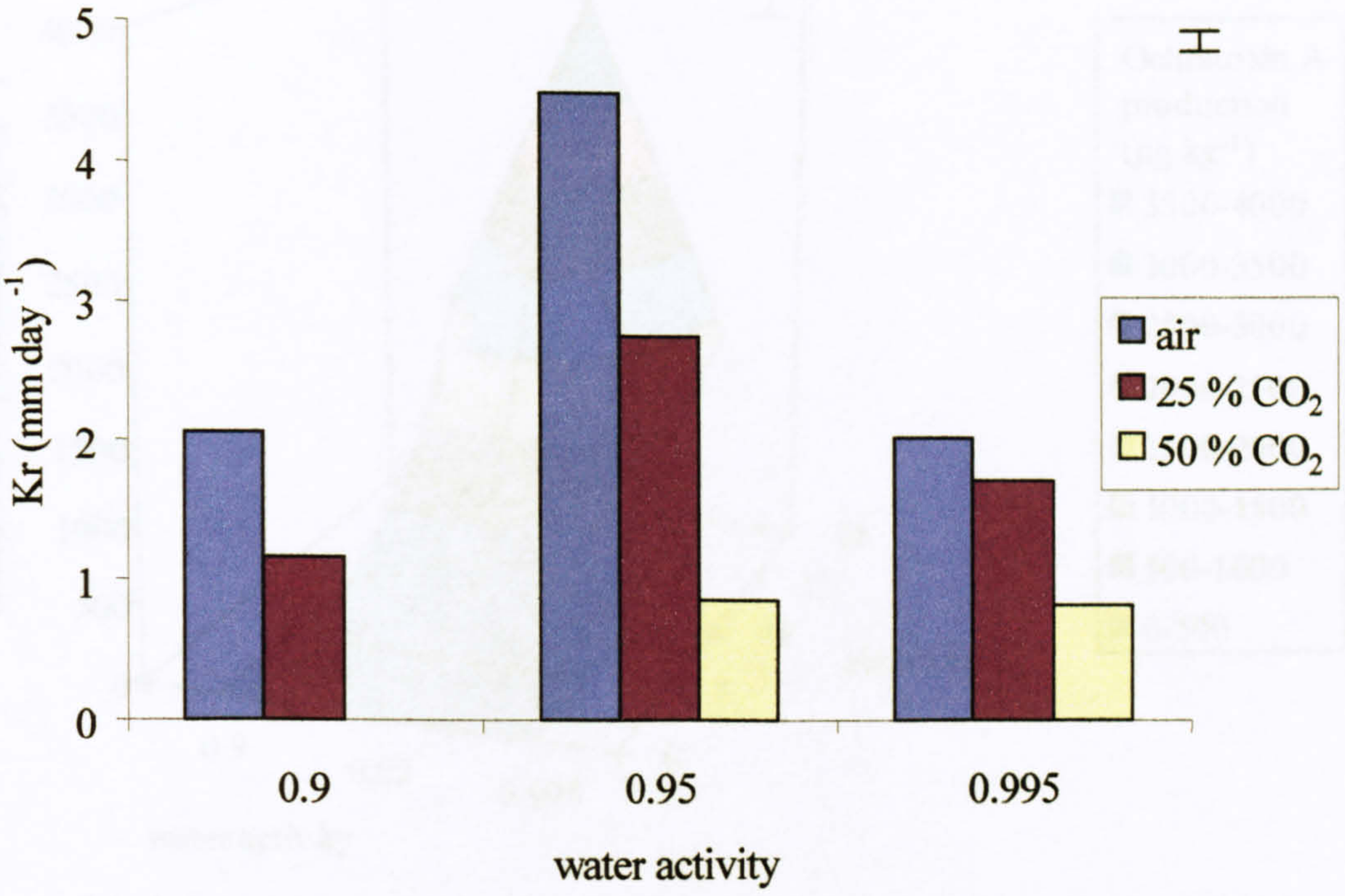


Figure 3.23 Growth of *Penicillium verrucosum* strain OTA11 on γ-irradiated wheat grain at various water activities and gas compositions at 25 °C. Bar indicates Least Significant Difference (LSD) at $p < 0.05$.

Table 3.14 ANOVA for *Penicillium verrucosum* (OTA11) growth on γ-irradiated wheat grain at various water activities and gas compositions. * Indicates factor elicited a significant effect ($p < 0.05$).

Source of Variation	DF	SS	MS	F	P
a_w	2	12.4424	6.2212	225.82	<0.001*
Gas treatment	2	22.4273	11.2136	407.04	<0.001*
a_w x gas treatment	4	6.5936	1.6484	59.83	<0.001*
Residual	18	0.4959	0.0275		
Total	26	41.9591			

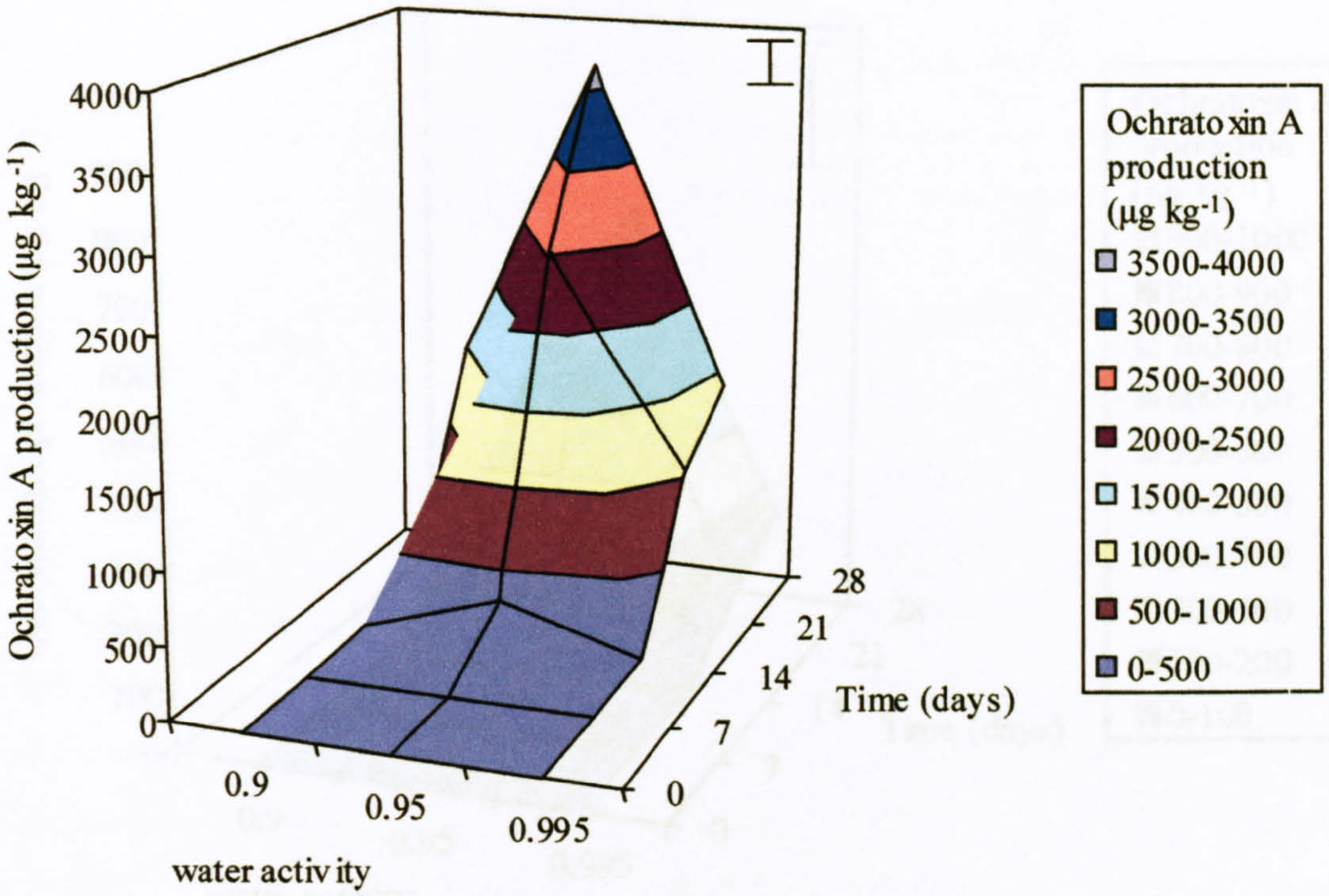


Figure 3.24 Ochratoxin A production by *Penicillium verrucosum* strain OTA11 on γ-irradiated wheat grain at various water activities in air at 25 °C over 28 days. Bar indicates Least Significant Difference (LSD) at $p < 0.05$.

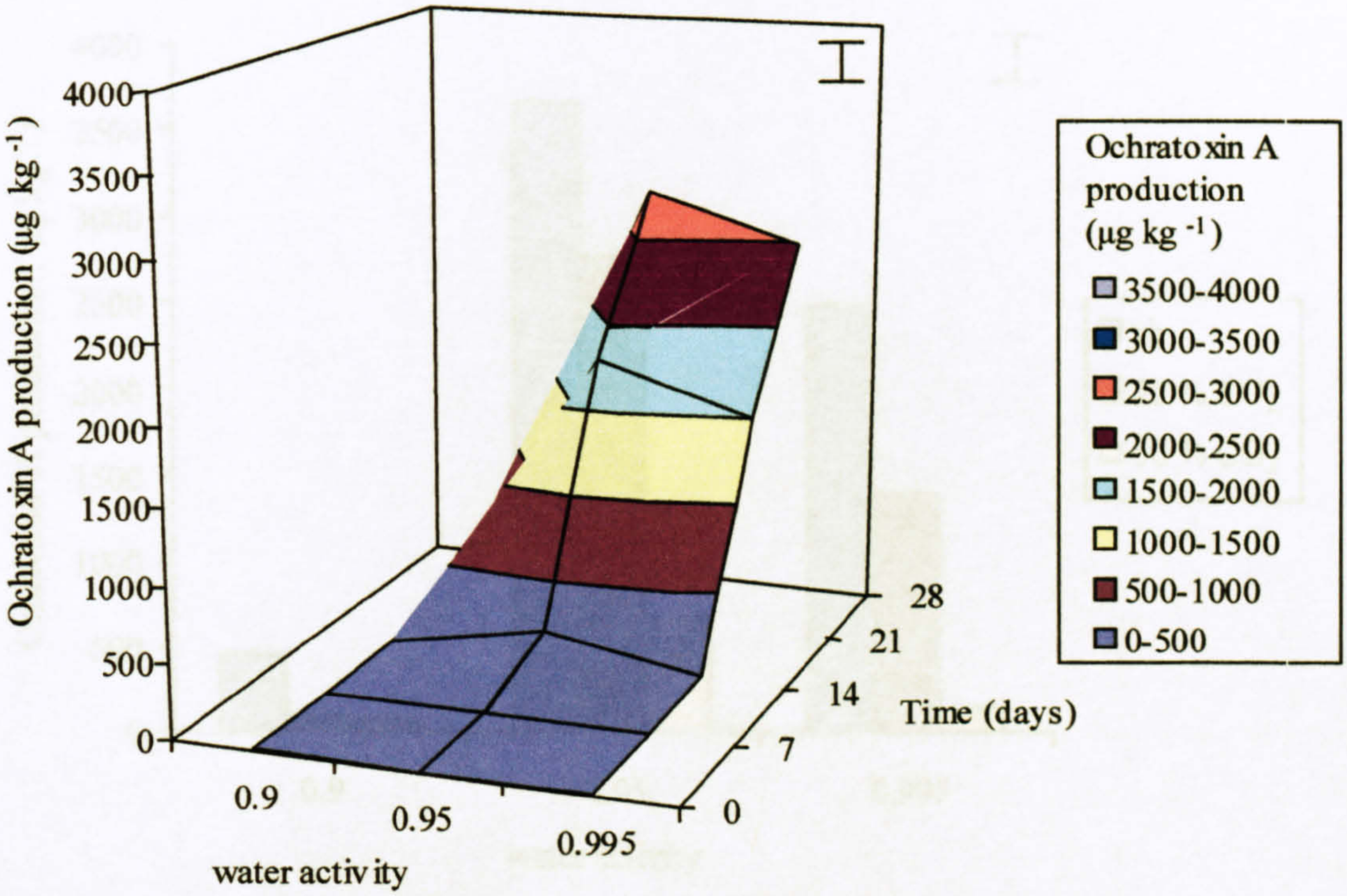


Figure 3.25 Ochratoxin A production by *Penicillium verrucosum* strain OTA11 on γ-irradiated wheat grain at various water activities at 25 % CO_2 at 25 °C over 28 days. Bar indicates Least Significant Difference (LSD) at $p < 0.05$.

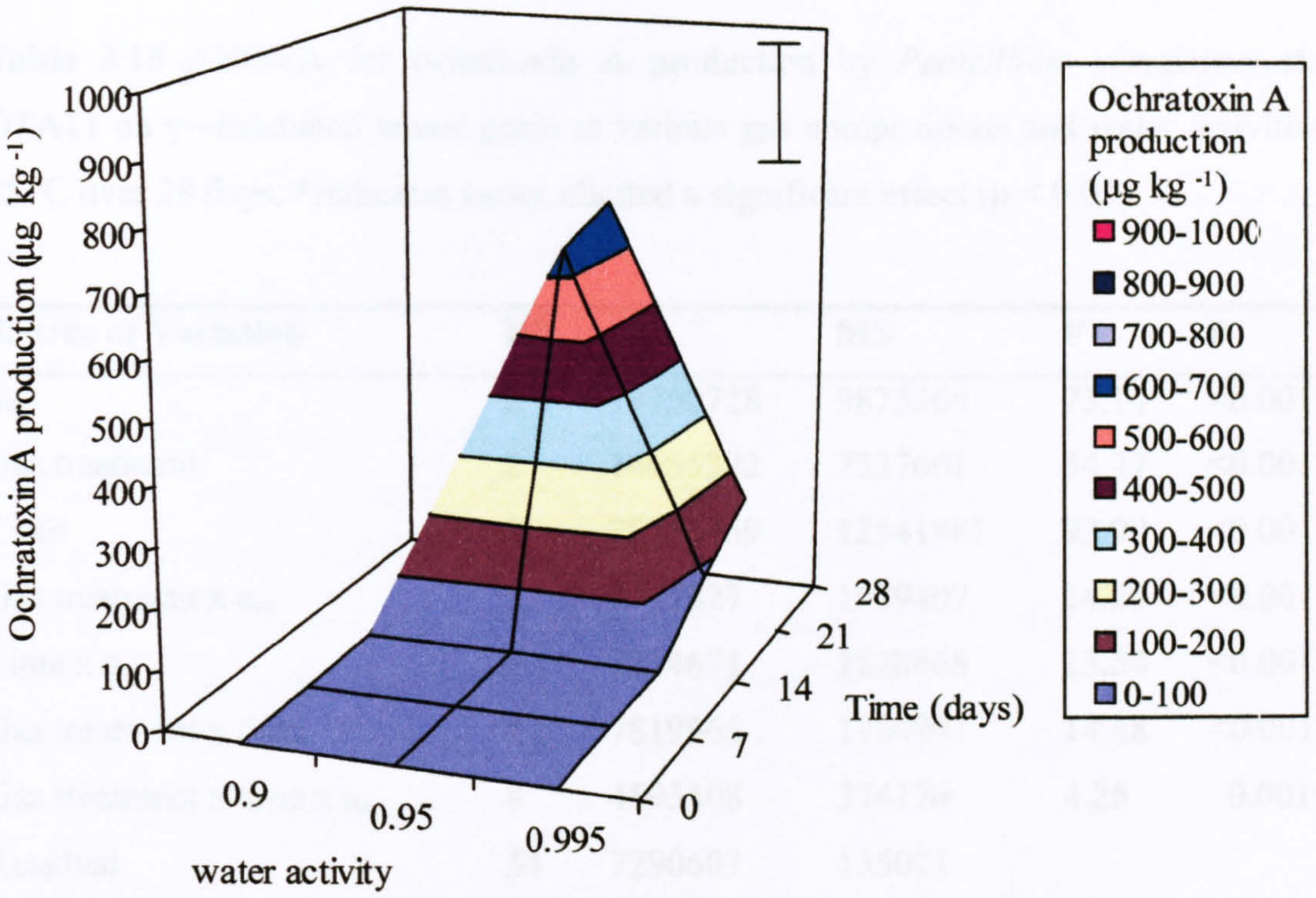


Figure 3.26 Ochratoxin A production by *Penicillium verrucosum* strain OTA11 on γ-irradiated wheat grain at various water activities at 50 % CO_2 at 25 °C over 28 days. Bar indicates Least Significant Difference (LSD) at $p < 0.05$.

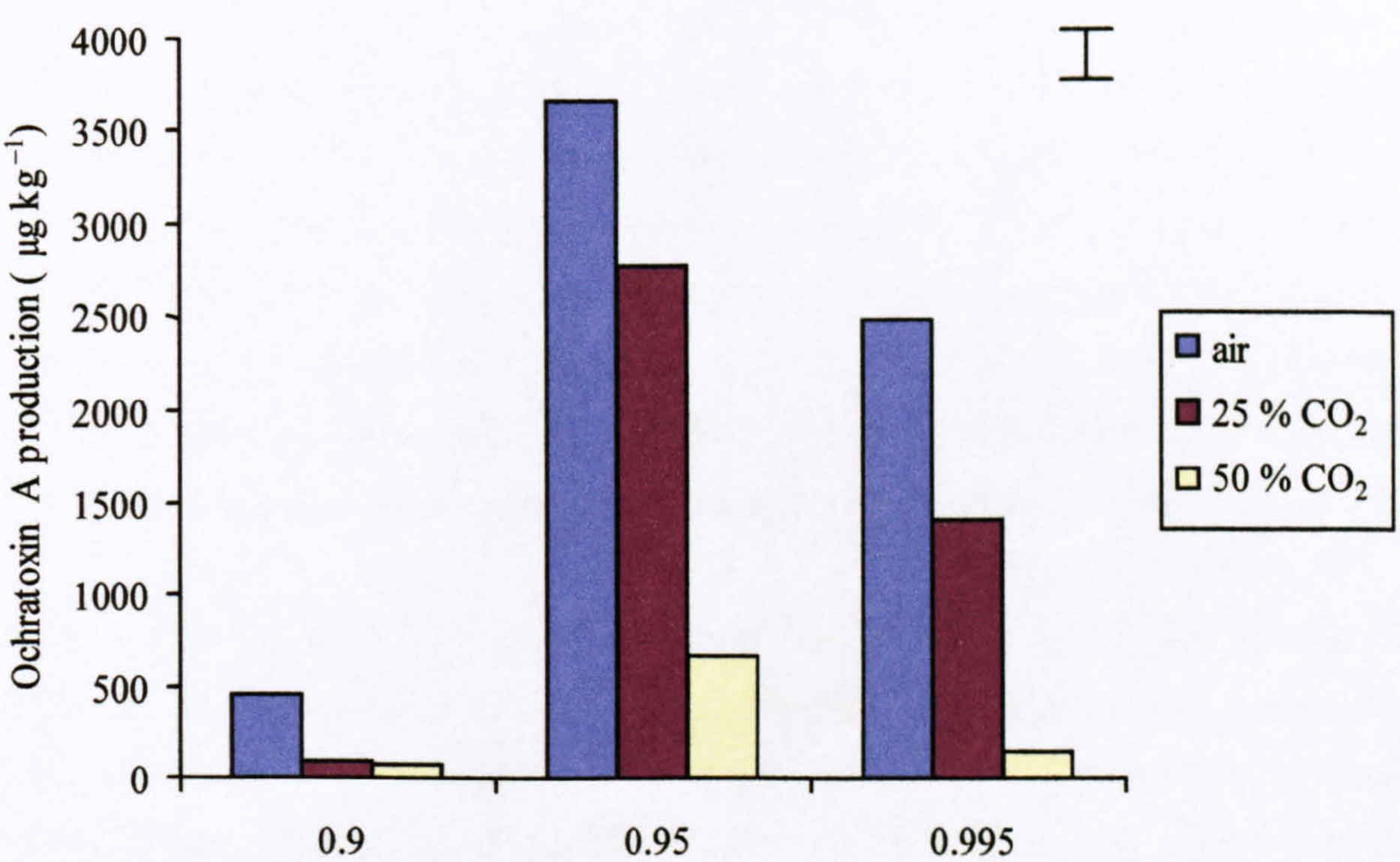


Figure 3.27 Ochratoxin A production by *Penicillium verrucosum* strain OTA11 on γ-irradiated wheat grain at various water activities and gas compositions at 25 °C after 28 days. Bar indicates Least Significant Difference (LSD) at $p < 0.05$.

Table 3.15 ANOVA for ochratoxin A production by *Penicillium verucosum* strain OTA11 on γ –irradiated wheat grain at various gas compositions and water activities at 25 °C over 28 days. *Indicates factor elicited a significant effect ($p < 0.05$).

Source of Variation	DF	SS	MS	F	P
a_w	2	19750728	9875364	73.14	<0.001*
Gas treatment	2	14655322	7327661	54.27	<0.001*
Time	2	25083969	12541981	92.90	<0.001*
Gas treatment x a_w	4	7717627	1929407	14.29	<0.001*
Time x a_w	4	7314671	1828668	13.54	<0.001*
Gas treatment x time	4	7819966	1954991	14.48	<0.001*
Gas treatment x time x a_w	8	4593408	574176	4.25	0.001*
Residual	54	7290603	135011		
Total	80	94226293			

3.2.4 Effect of water availability and gas composition on germination and germ tube lengths of conidia of *Aspergillus ochraceus* on 2 % wheat-based media

All spore of *A. ochraceus* germinated at the treatments, except at 50 % CO_2 and 0.90 a_w. Figure 3.28 shows the mean conidial germ tube lengths of *A. ochraceus* on agar media at the different gas compositions and a_w levels tested at 25 °C after 36 hours. Conidial germ tube lengths were longest in air, followed by 25 % CO_2 and 50 % CO_2 when compared with their respective water activities. Regardless of gas composition, germ tube lengths were longest at 0.95 a_w followed by 0.995 a_w and 0.90 a_w respectively. After 36 hours at 50 % CO_2 and 0.90 a_w conidial germination was completely inhibited whilst at 0.95 and 0.995 a_w mean germ tube lengths were suppressed by 80 % and 85 % respectively. ANOVA showed that water activity and gas composition and their interactions significantly affected germ tube lengths (Table 3.16).

3.2.5 Effect of water availability on growth and ochratoxin A production by *Aspergillus ochraceus* on 2 % wheat-based media

Figure 3.29 shows the radial extension rates (K_r) by *A. ochraceus* on agar in relation to the different gas compositions and a_w levels tested at 25 °C. Growth rates were fastest in air followed by 25 % CO_2 and 50 % CO_2. Growth rates were fastest at 0.95 a_w followed by 0.995 a_w and 0.90 a_w regardless of the gas composition tested. Regardless of the water activity tested, 25 % CO_2 suppressed growth of *A. ochraceus* by $\geq$ 50 % and at 50 % CO_2 growth of *A. ochraceus* was suppressed by $\geq$ 90 % at 0.95 and 0.995 a_w. At 0.90 a_w and 50 % CO_2 growth was completely inhibited. ANOVA showed that water activity and gas composition and their interactions significantly affected growth rates (Table 3.17).

Figures 3.30 shows OTA production by *A. ochraceus* on agar media in relation to the different gas compositions and a_w levels tested at 25 °C. Optimum OTA was produced at 0.95 a_w after 56 days irrespective of the gas composition tested. OTA production was greatest in air, followed by 25 % CO_2 and 50 % CO_2 respectively. After 56 days at 25 % CO_2 OTA production was suppressed by up to 70 % and at 50 % CO_2 OTA production was suppressed almost completely when compared with their respective water activities in air. ANOVA showed that water activity, gas composition and incubation time and their interactions significantly affected OTA production (Table 3.18)

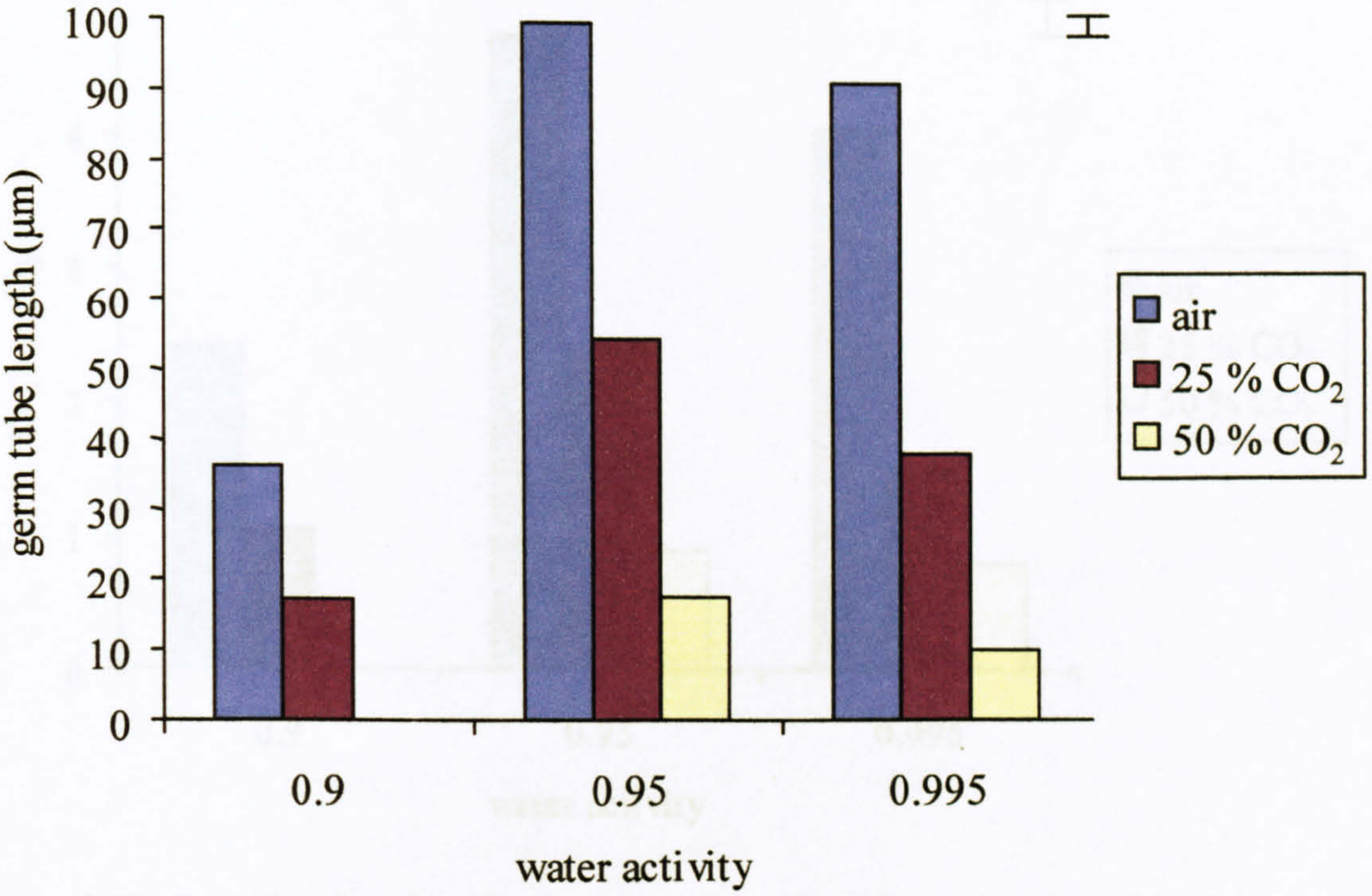


Figure 3.28 Germ tube lengths of *Aspergillus ochraceus* (strain IBT21991) on 2 % wheat-based media after 36 hours at various water activities and gas compositions. Bar indicates Least Significant Difference (LSD) at $p < 0.05$.

Table 3.16 ANOVA for germ tube lengths by *Aspergillus ochraceus* (strain IBT21991) on 2 % wheat-based media at 25 °C after 36 hours at various water activities and gas compositions. * Indicates factor elicited a significant effect ($p < 0.05$).

Source	DF	SS	MS	F	P
a_w	2	7379.5	3689.6	286.26	< 0.001 *
Gas treatment	2	19934.5	9967.3	773.32	< 0.001 *
a_w x gas treatment	4	2150.8	537.7	41.72	< 0.001 *
Residual	18	232.0	12.9		
Total	26	29696.5			

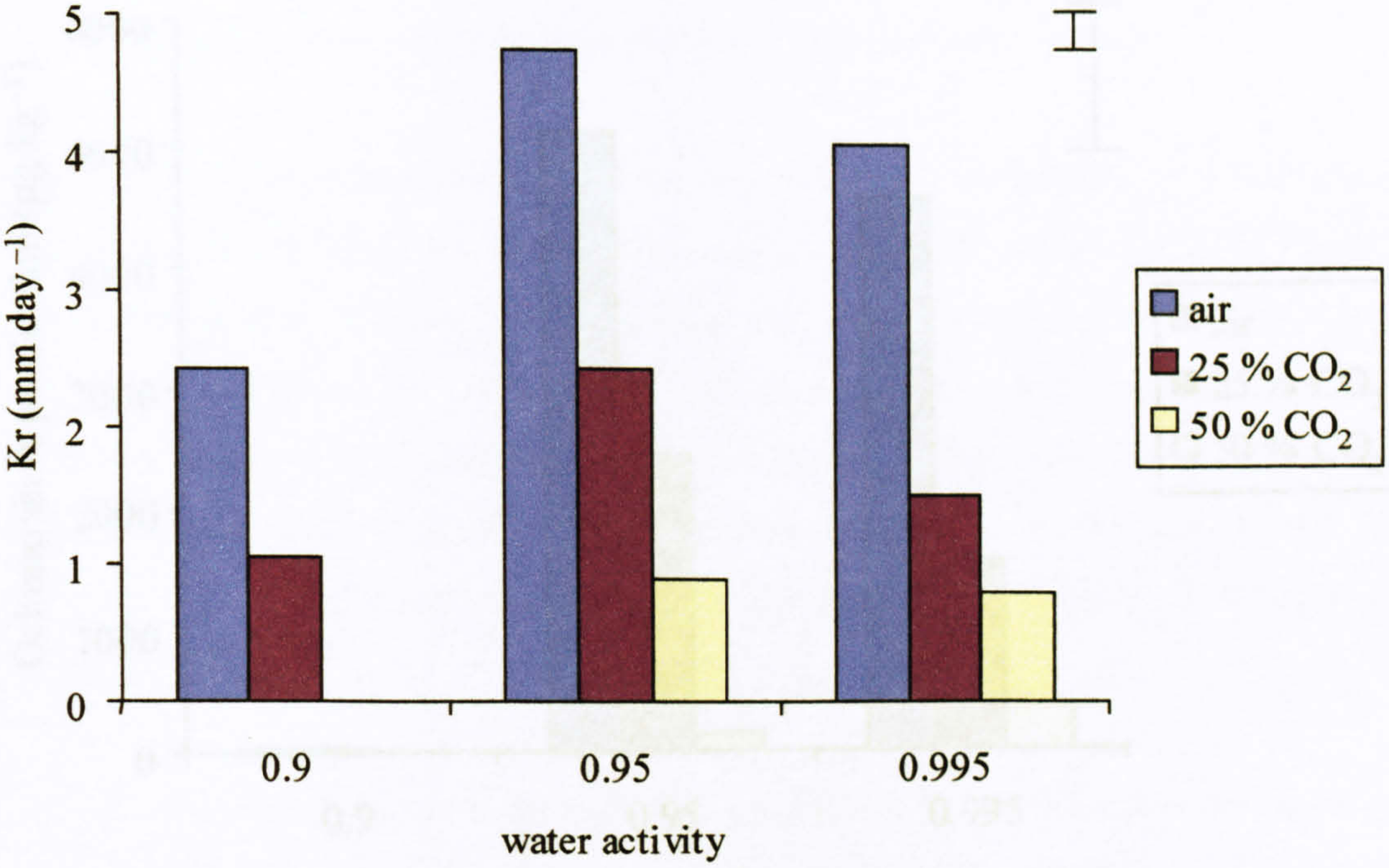


Figure 3.29 Growth of *Aspergillus ochraceus* strain IBT21991 on 2 % wheat-based media at various water activities and gas compositions at 25 °C. Bar indicates Least Significant Difference (LSD) at $p < 0.05$.

Table 3.17 ANOVA for *Aspergillus ochraceus* (IBT21991) growth on 2 % wheat-based media at various water activities and gas compositions. * Indicates factor elicited a significant effect ($p < 0.05$).

Source of Variation	DF	SS	MS	F	P
a_w	2	10.9057	5.4528	138.03	<0.001*
Temperature	2	46.2312	23.1156	585.15	<0.001*
Temperature x a_w	4	2.0667	0.5167	13.08	<0.001*
Residual	18	0.7111	0.0395		
Total	25	59.9147			

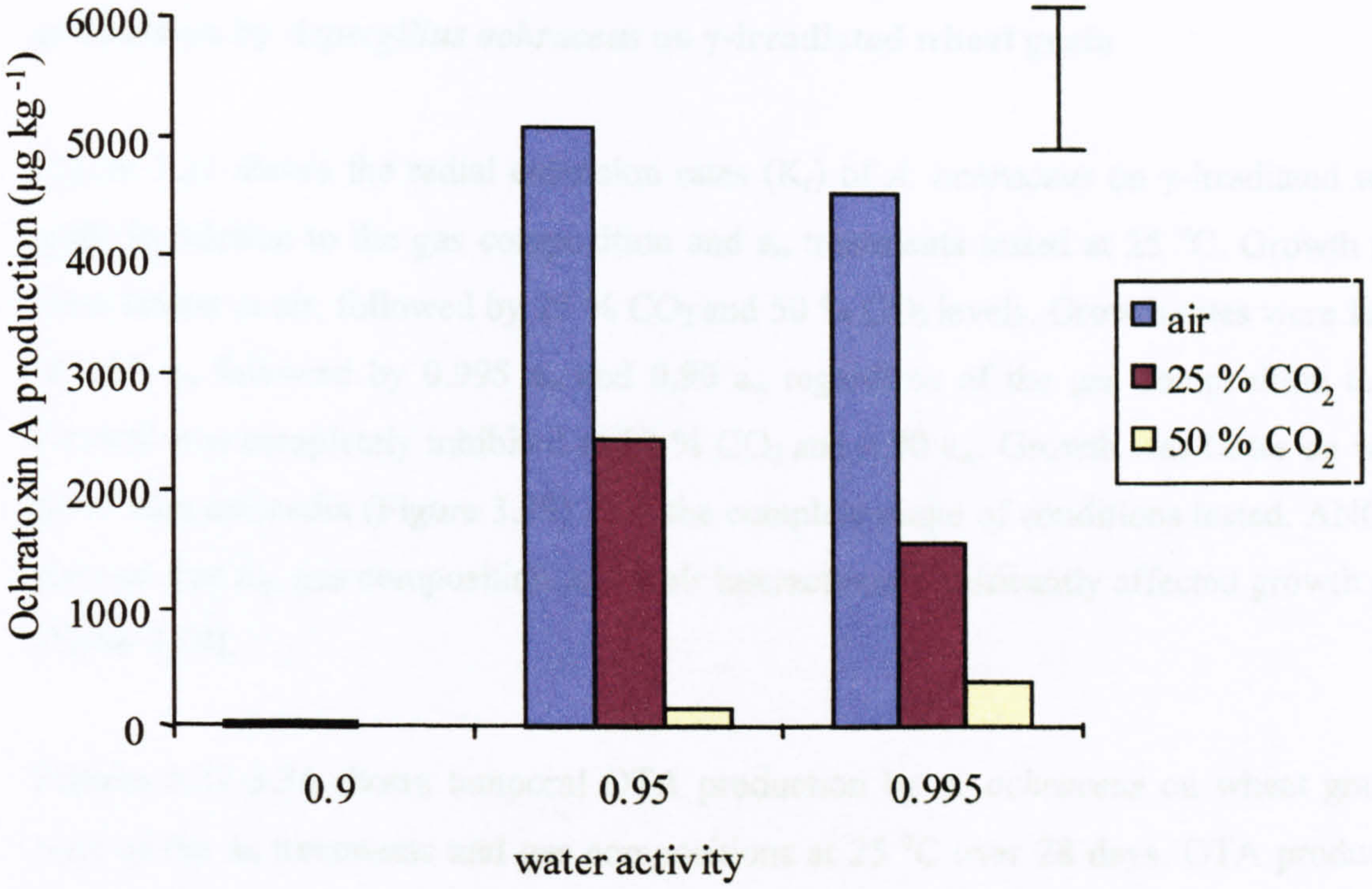


Figure 3.30 Ochratoxin A production by *Aspergillus ochraceus* strain IBT21991 on 2 % wheat-based media after 56 days at 25 °C at various gas compositions and water activities. Bar indicates Least Significant Difference (LSD) at $p < 0.05$.

Table 3.18 ANOVA for ochratoxin A production by *Aspergillus ochraceus* strain IBT21991 on 2 % wheat-based media over 56 days at 25 °C at various gas compositions and water activities. * Indicates factor elicited a significant effect ($p < 0.05$)

Source of Variation	DF	SS	MS	F	P
a_w	2	31732173	15866087	11.94	<0.001*
Time	3	43180884	14393628	10.83	<0.001*
Gas treatment	2	36616040	18308020	13.78	<0.001*
Time x a_w	6	24938960	4156496	3.13	0.009*
Gas treatment x a_w	4	18464768	4616192	3.47	0.012*
Gas treatment x time	4	18464768	4616192	3.47	0.012*
Gas treatment x time x a_w	12	30544279	2545357	1.92	0.046*
Residual	72	95652609	1328508		
Total	107	326423228			

3.2.6 Effect of water availability and gas composition on growth and ochratoxin A production by *Aspergillus ochraceus* on γ-irradiated wheat grain

Figure 3.31 shows the radial extension rates (K_r) of *A. ochraceus* on γ-irradiated wheat grain in relation to the gas composition and a_w treatments tested at 25 °C. Growth rates were fastest in air, followed by 25 % CO_2 and 50 % CO_2 levels. Growth rates were fastest at 0.95 a_w followed by 0.995 a_w and 0.90 a_w regardless of the gas composition tested. Growth was completely inhibited at 50 % CO_2 and 0.90 a_w. Growth was faster on wheat grain than on media (Figure 3.29) over the complete range of conditions tested. ANOVA showed that a_w, gas composition and their interactions significantly affected growth rates (Table 3.19).

Figures 3.32-3.34 shows temporal OTA production by *A. ochraceus* on wheat grain at each of the a_w treatments and gas compositions at 25 °C over 28 days. OTA production was greatest in air, followed by 25 % CO_2 and 50 % CO_2 levels. OTA production was greatest at 0.95 a_w followed by 0.995 a_w and 0.90 a_w regardless of the gas composition tested. No OTA production occurred at 7 days regardless of the treatment. No OTA production occurred at 50 % CO_2 and 0.90 a_w over the 28 day experimental period. Optimum OTA production occurred at 28 days at 0.95 a_w irrespective of the gas composition tested. Figure 3.35 compares OTA production at 28 days in relation to the different treatments. ANOVA showed that a_w, gas composition and incubation time and their interactions significantly affected OTA production (Table 3.20).

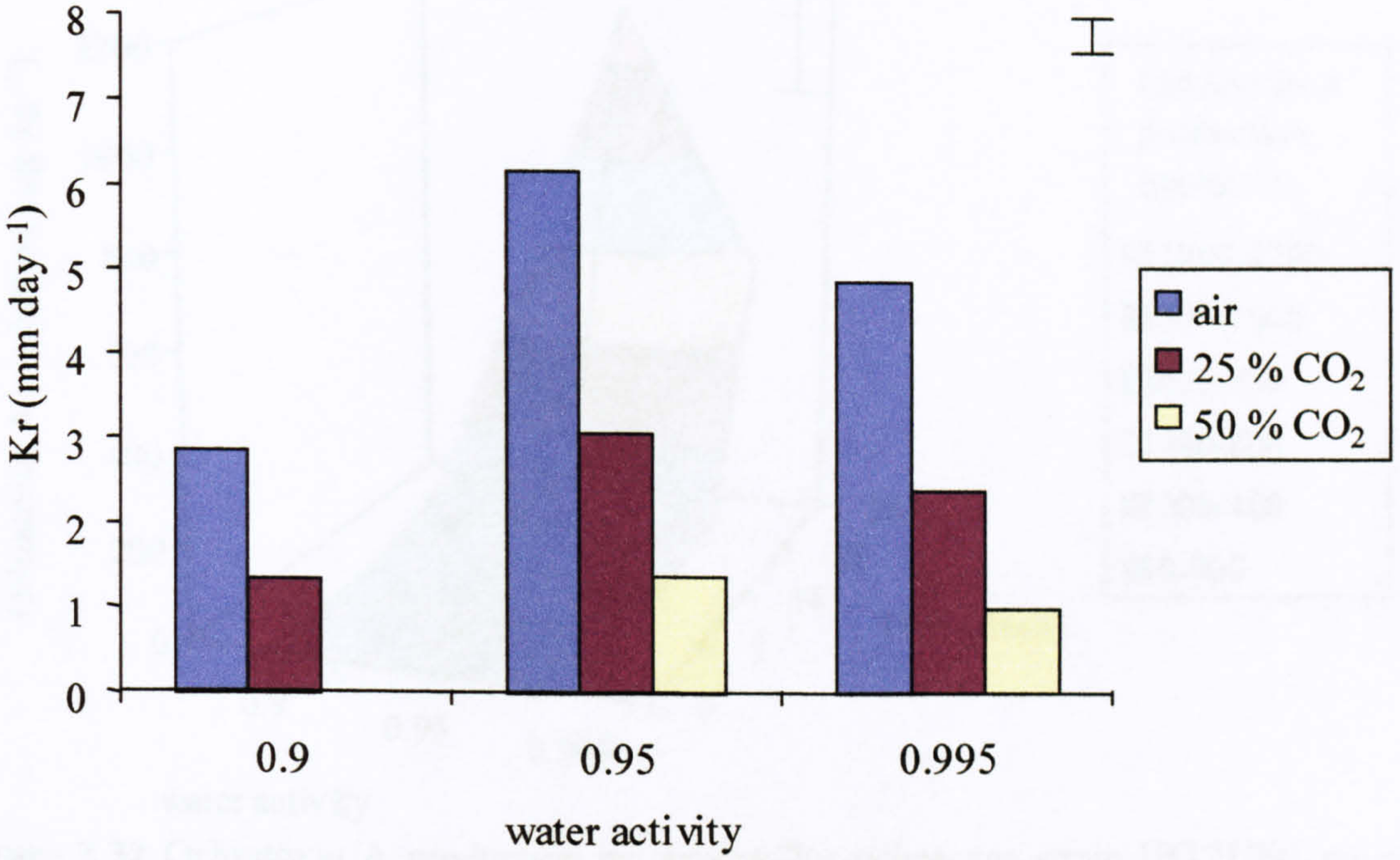


Figure 3.31 Growth of *Aspergillus ochraceus* strain IBT21991 on γ-irradiated wheat grain at various water activities and gas compositions at 25 °C. Bar indicate Least Significant Difference (LSD) at $p < 0.05$.

Table 3.19 ANOVA for *Aspergillus ochraceus* (IBT21991) growth on γ-irradiated wheat grain at various water activities and gas compositions. * Indicates factor elicited a significant effect ($p < 0.05$).

Source of Variation	DF	SS	MS	F	P
a_w	2	25.433	12.717	183.90	<0.001*
Gas treatment	2	66.716	33.358	482.40	<0.001*
a_w x gas treatment	4	3.130	0.782	11.31	<0.001*
Residual	18	1.245	0.069		
Total	26	96.523			

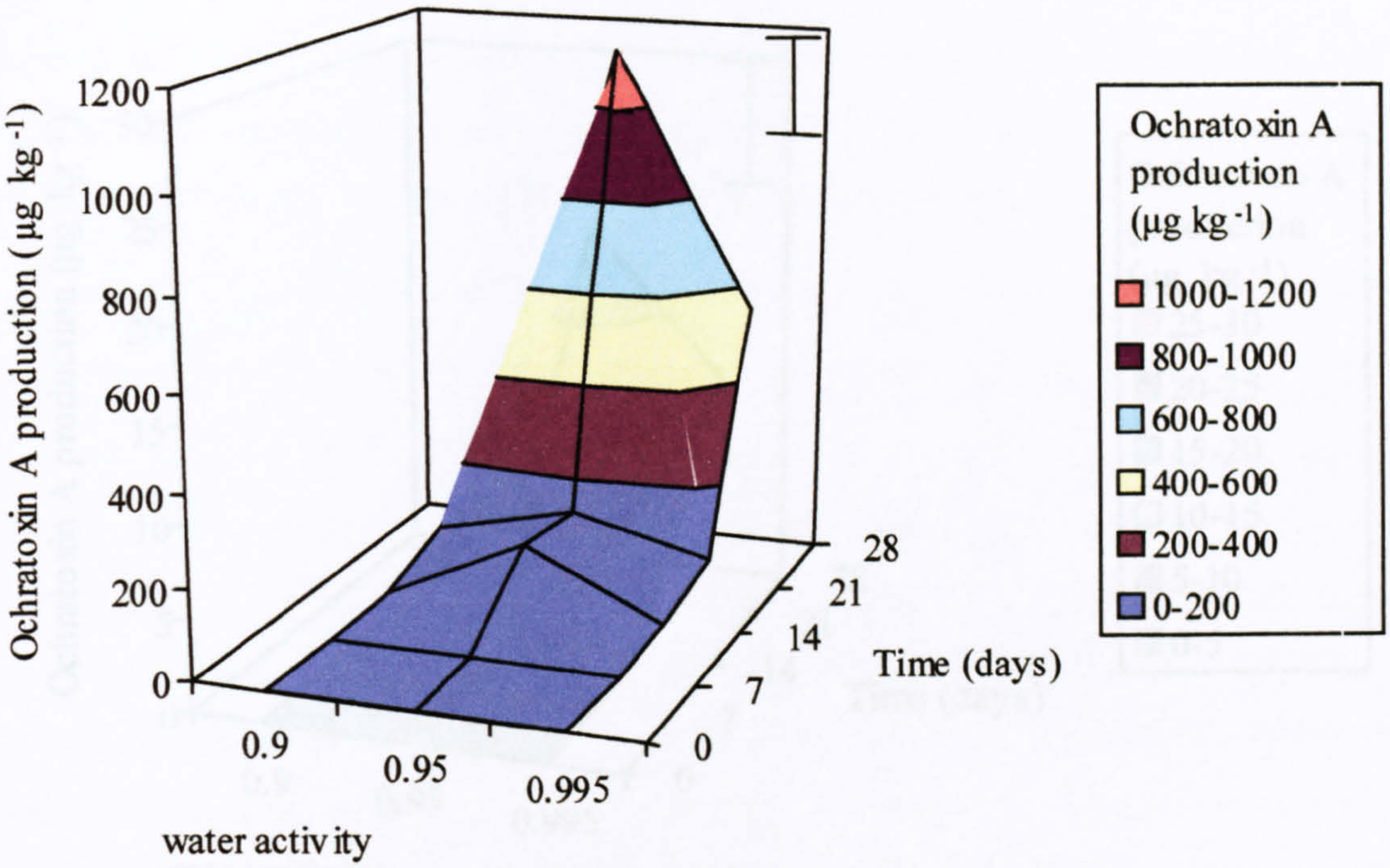


Figure 3.32 Ochratoxin A production by *Aspergillus ochraceus* strain IBT21991 on γ-irradiated wheat grain at various water activities in air at 25 °C over 28 days. Bar indicates Least Significant Difference (LSD) at $p < 0.05$.

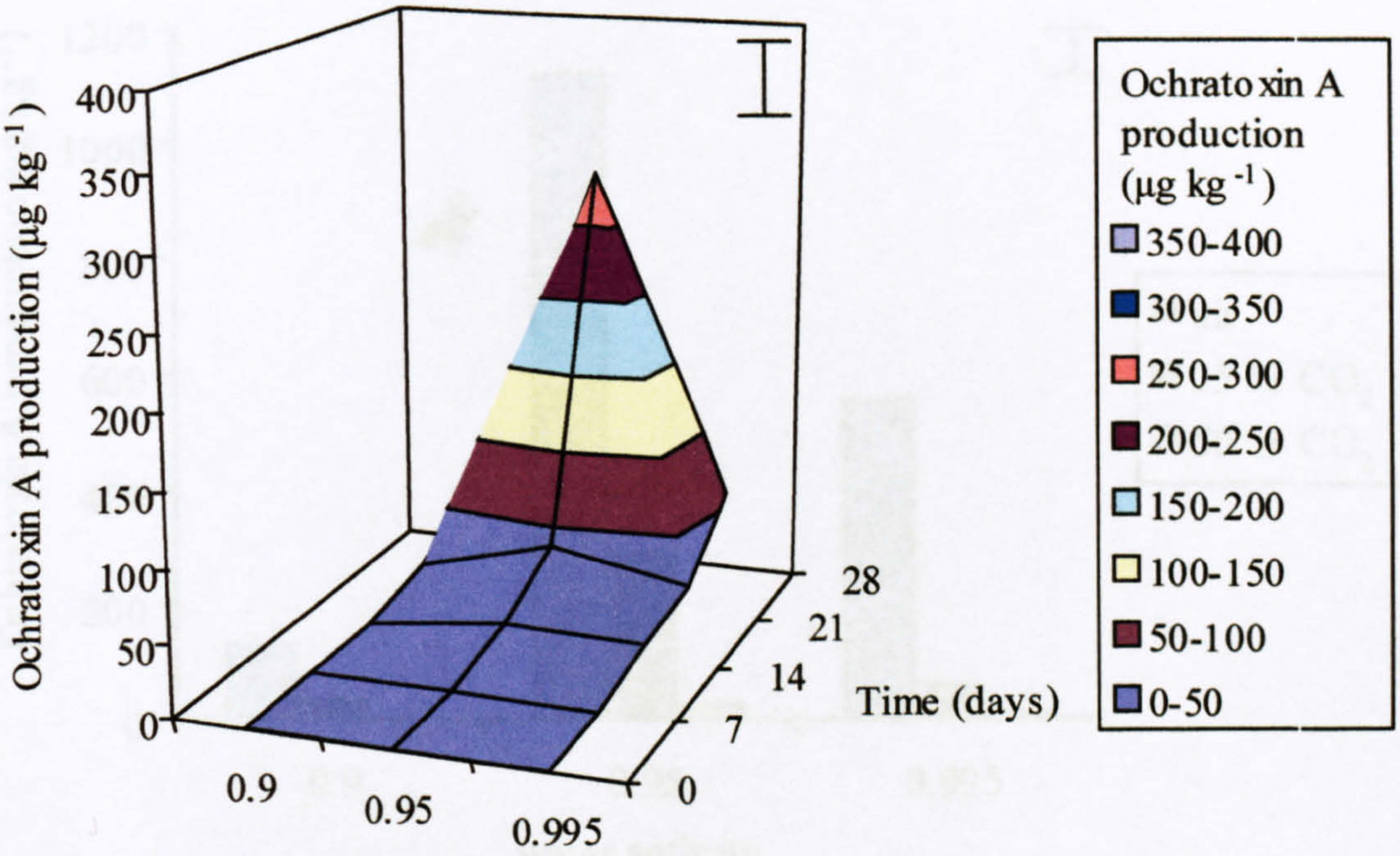


Figure 3.33 Ochratoxin A production by *Aspergillus ochraceus* strain IBT21991 on γ-irradiated wheat grain at various water activities at 25 % CO_2 at 25 °C over 28 days. Bar indicates Least Significant Difference (LSD) at $p < 0.05$.

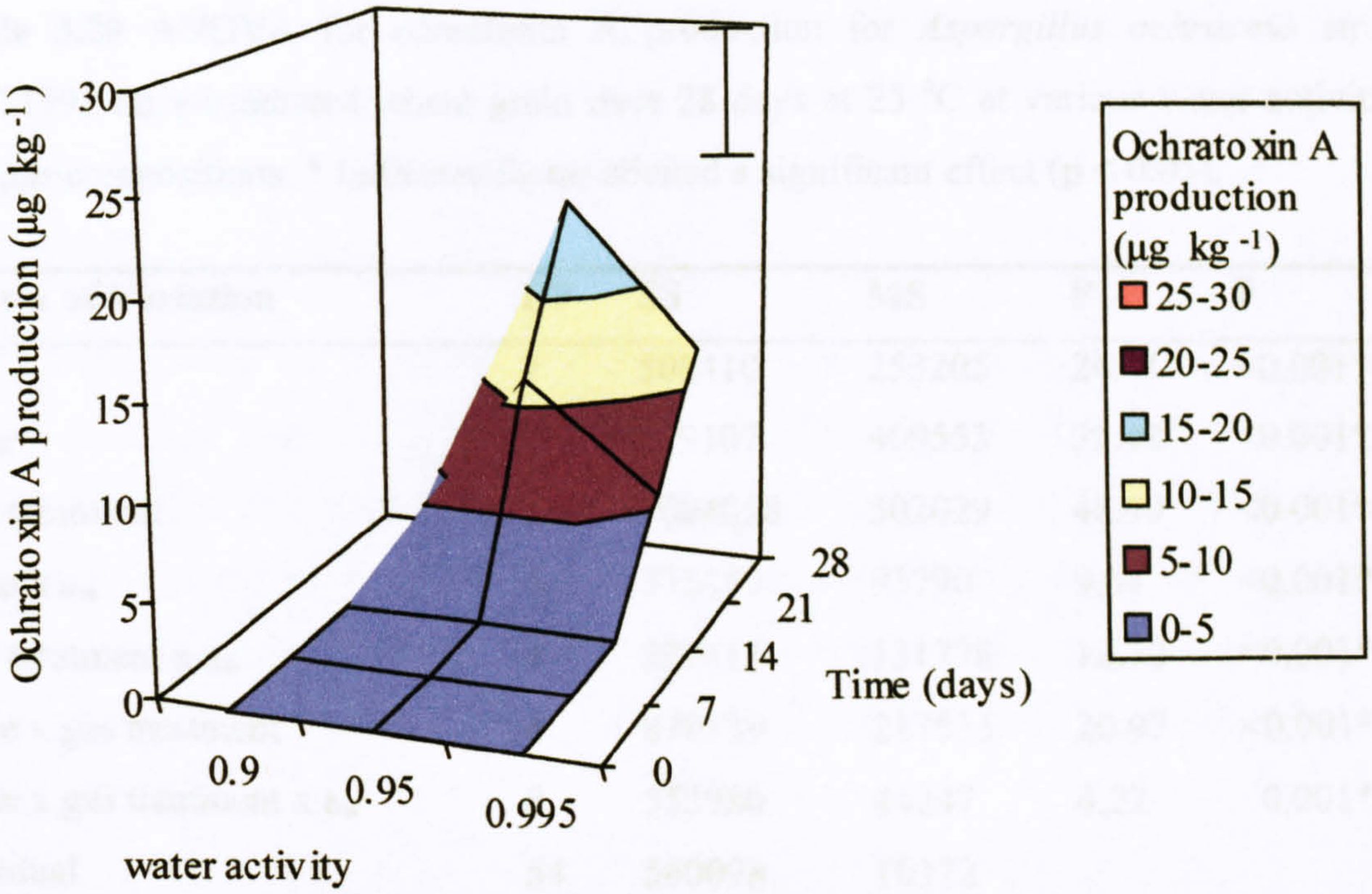


Figure 3.34 Ochratoxin A production by *Aspergillus ochraceus* strain IBT21991 on γ-irradiated wheat grain at various water activities at 50 % CO_2 at 25 °C over 28 days. Bar indicates Least Significant Difference (LSD) at $p < 0.05$.

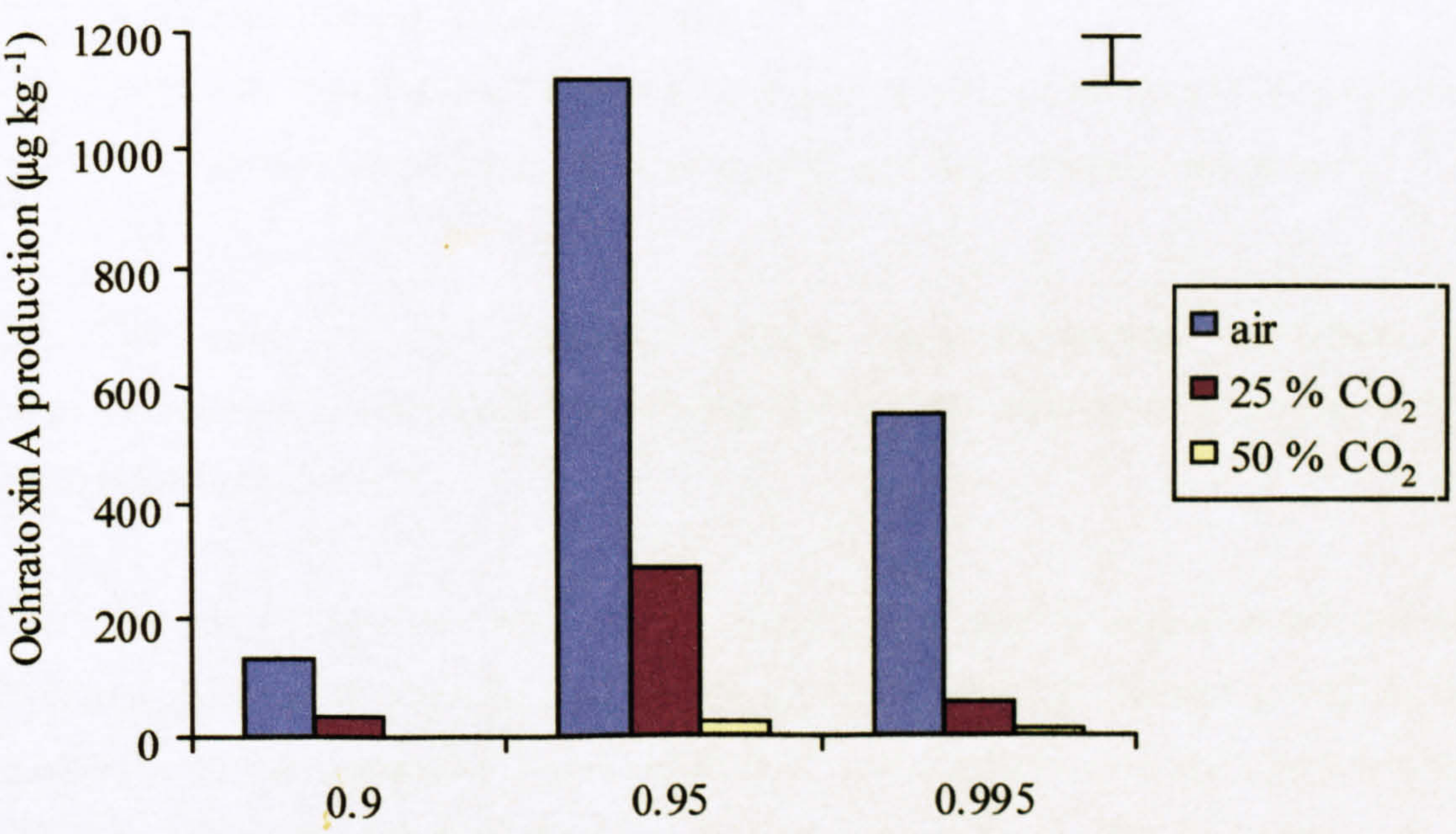


Figure 3.35 Ochratoxin A production by *Aspergillus ochraceus* strain IBT21991 on γ-irradiated wheat grain at 25 °C after 28 days at various water activities and gas compositions. Bar indicate Least Significant Difference (LSD) at $p < 0.05$.

Table 3.20 ANOVA for ochratoxin A production for *Aspergillus ochraceus* strain IBT21991 on γ-irradiated wheat grain over 28 days at 25 °C at various water activities and gas compositions. * Indicates factor elicited a significant effect ($p < 0.05$).

Source of Variation	DF	SS	MS	F	P
a_w	2	506410	253205	24.41	<0.001*
Time	2	819107	409553	39.49	<0.001*
Gas treatment	2	1004058	502029	48.40	<0.001*
Time x a_w	4	375159	93790	9.04	<0.001*
Gas treatment x a_w	4	527113	131778	12.70	<0.001*
Time x gas treatment	4	870139	217535	20.97	<0.001*
Time x gas treatment x a_w	8	353980	44247	4.27	0.001*
Residual	54	560098	10372		
Total	80	5016063			

3.3 THE EFFECT OF ENVIRONMENTAL FACTORS AND COMPETING MYCOFLORA ON COMPETITIVENESS, GROWTH AND OCHRATOXIN A PRODUCTION BY *PENICILLIUM VERRUCOSUM* AND *ASPERGILLUS OCHRACEUS*

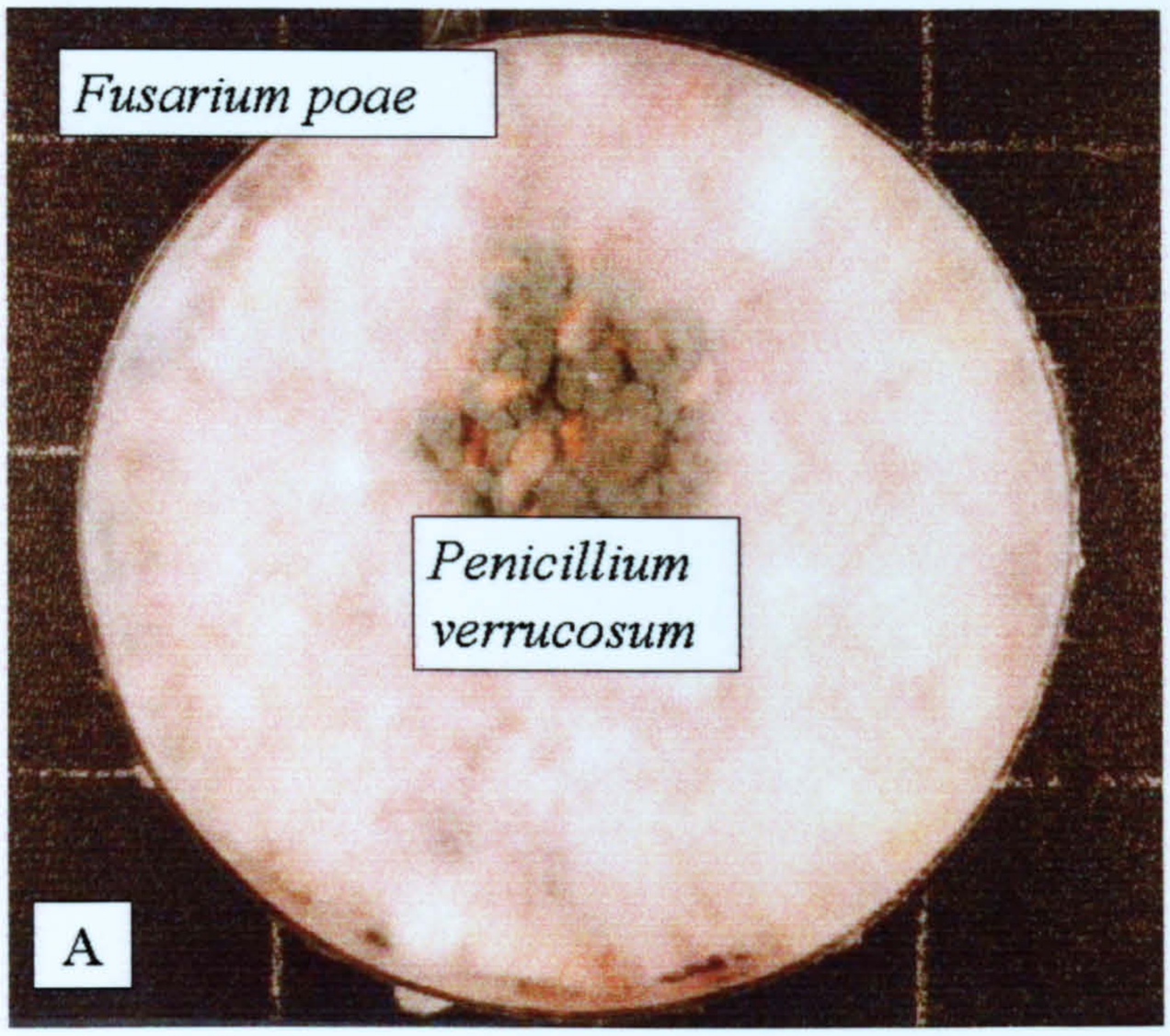
Fungi seldom occur on grain alone, but usually as a mixed consortium of yeasts, bacteria and filamentous fungi. It is therefore inevitable that interspecific and intraspecific interactions will occur depending on environmental conditions and the nutritional status of the grain (Magan *et al.*, 2003). Interactions between different fungi have been shown to have a significant impact on growth, toxin production and population structure on cereal grain (Magan & Lacey, 1984b; Marin *et al.* 1998b; Lee & Magan, 2000). The aims of this study were to:

1. Determine the competitiveness of *P. verrucosum* and *A. ochraceus* against a range of other wheat spoilage fungi on agar media and γ-irradiated wheat grain at various a_w x temperatures by examining macroscopic interactions and Indices of Dominance (Magan & Lacey, 1984a)
2. Investigate the effects of interspecific interactions on growth and OTA production by *P. verrucosum* and *A. ochraceus* in relation to the environmental factors.

3.3.1 The effect of environmental factors and competing mycoflora on competitiveness of *Penicillium verrucosum* on a 2 % wheat-based media and γ-irradiated wheat grain

Plates 3.7a) and b) show examples of dual culture plates used to determine macroscopic interactions between two species. Table 3.21 shows the effect of changes in both a_w and temperature on the interaction scores between *P. verrucosum* and other wheat spoilage fungi on a 2 % wheat-based media and γ-irradiated wheat grain. The first number of the interaction scores always represents that for *P. verrucosum.*

On a 2 % wheat-based media and γ-irradiated wheat grain *P. verrucosum* was consistently dominant on contact with *A. ochraceus, F. culmorum, A. tenuissima, E. repens* and *F. poae* at 0.90 a_w regardless of the temperature tested with scores of 4 consistently. However, at 0.95 and 0.995 a_w at 25 °C, *A. ochraceus, F. culmorum, F. poae* and *A. tenuissima* were all dominant over *P. verrucosum.* At 0.995 a_w and 15 °C, *P.verrucosum* was dominant over *E. repens* and mutually antagonistic with all other species tested with scores of 2 consistently. At 0.95 a_w and 15 °C, *P. verrucosum* was dominant over *E. repens* and *A. tenuissima* and mutually antagonistic with all other species tested. The sum of the Index of Dominance scores indicates that *P. verrucosum* was more competitive at 15 than 25 °C and at lower water activities (< 0.95 a_w) against the species tested.

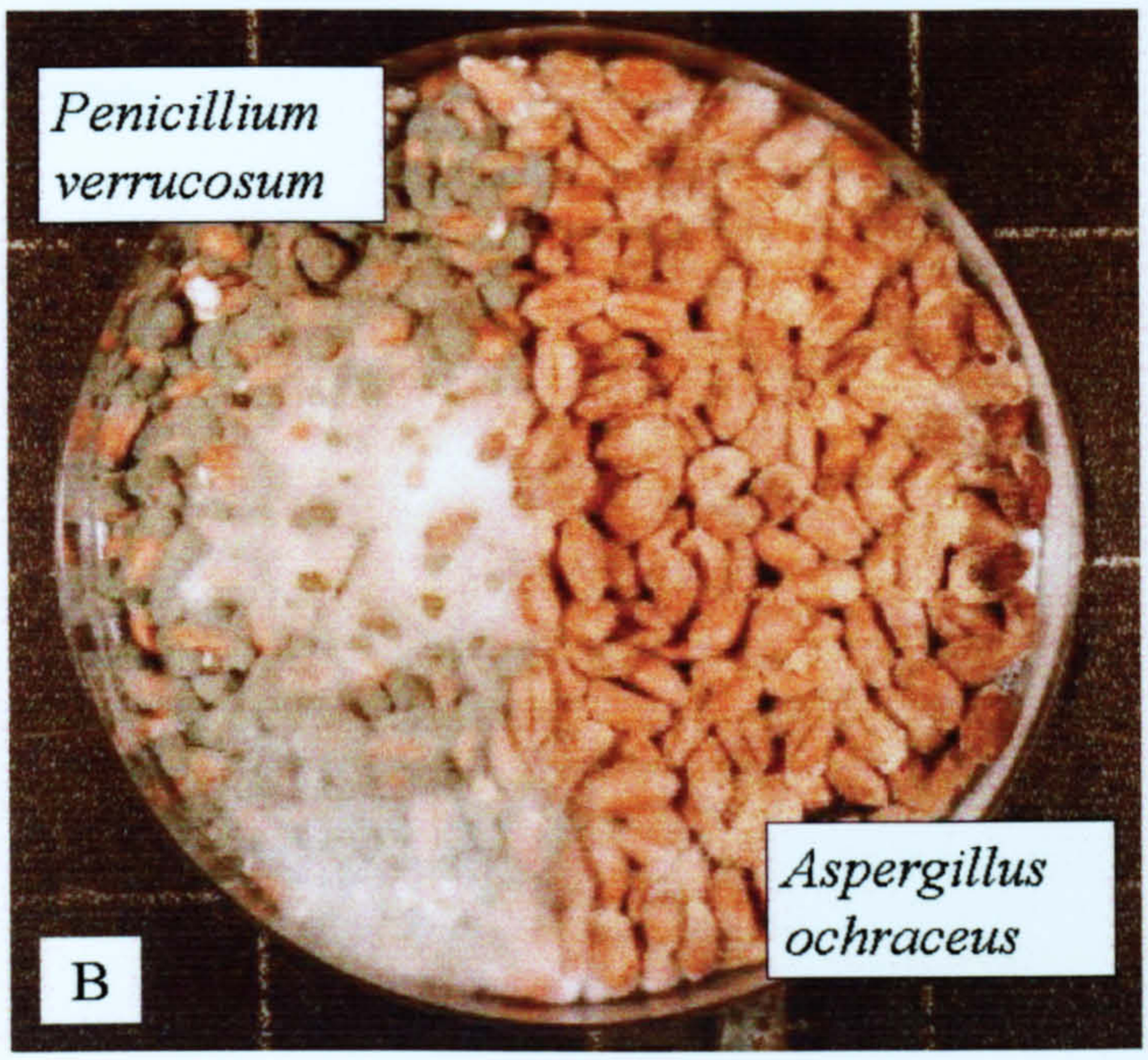


Type of interaction

Mutual intermingling

Interaction score

PV = 0 / FP = 4



Type of interaction

Mutual intermingling

Interaction score

PV=2 / AO = 2

Plate 3.7 A) Dominance of *Fusarium poae* (FP) over *Penicillium verrucosum* (PV) at 0.995 a_w and 25 °C. This is reflected in the interaction score by FP being awarded 4 and PV 0. The highest number is always given to the more dominant species. **B)** Mutual intermingling between *Aspergillus ochraceus* (AO) and *Penicillium verrucosum* (PV) at 0.995 a_w and 25 °C. This is an example of inhibition on contact which is reflected in the interaction score for both fungi of 2.

Table 3.21 Effect of water activity (a_w) and temperature on numerical interaction scores and Index of Dominance (I_D) for *Penicillium verrucosum* (OTA11) and other interacting species on a 2 % wheat-based media. Results were identical on γ-irradiated wheat grain.

Water activity (a_w)	Interacting species	15°C	25°C	Index of Dominance (I_D)
0.90	*Aspergillus ochraceus*	4/0	4/0	8/0
	Fusarium culmorum	4/0	4/0	8/0
	Fusarium poae	4/0	4/0	8/0
	Alternaria tenuissima	4/0	4/0	8/0
	Eurotium repens	4/0	4/0	8/0
	Penicillium aurantiogriseum	2/2	2/2	4/4
	I_D	**22/2**	**22/2**	**44/4**
0.95	*Aspergillus ochraceus*	2/2	0/4	2/6
	Fusarium culmorum	2/2	0/4	2/6
	Fusarium poae	2/2	0/4	2/6
	Alternaria tenuissima	4/0	0/4	4/4
	Eurotium repens	4/0	0/4	4/4
	Penicillium aurantiogriseum	2/2	2/2	4/4
	I_D	**16/8**	**2/22**	**18/30**
0.995	*Aspergillus ochraceus*	2/2	0/4	2/6
	Fusarium culmorum	2/2	0/4	2/6
	Fusarium poae	2/2	0/4	2/6
	Alternaria tenuissima	2/2	0/4	2/6
	Eurotium repens	4/0	2/2	6/2
	Penicillium aurantiogriseum	2/2	2/2	4/4
	I_D	**14/10**	**4/20**	**18/30**

Penicillium verrucosum (OTA11) score/other species score. NE, not examined.

3.3.2 Effect of interactions on growth and ochratoxin A production by *Penicillium verrucosum* on wheat-based media

Figure 3.36 shows the relative growth rates of *P. verrucosum* on a 2 % wheat-based media alone or when competing with six other spoilage fungi at two different water activities (0.95 and 0.995 a_w) and two different temperatures (15 and 25 °C). At 15 °C, growth rates were largely unaffected by the presence of all competing spoilage fungi regardless of the water activity tested. At 25 °C and 0.995 a_w growth of *P. verrucosum* was suppressed by *A. tenuissima, F. culmorum, F. poae, E. repens* and *A. ochraceus.* The presence of *P. aurantiogriseum* had no effect on the overall growth rate of *P.verrucosum* at this condition. At 25 °C and 0.95 a_w growth of *P. verrucosum* was largely unaffected by the presence of all competing fungi. ANOVA showed that overall a_w, temperature and the presence of other wheat-spoilage fungi and their interactions elicited a significant effect on growth rates of *P. verrucosum* (Table 3.22).

Figure 3.37 shows OTA production by *P. verrucosum* on agar media alone or when competing with six other spoilage fungi at two different water activities (0.95 and 0.995 a_w) and two different temperatures (15 and 25 °C) after 56 days. When *P. verrucosum* was paired with *A. tenuissima, F. culmorum or F. poae,* at both water activities and temperatures there was a significant decrease in OTA production. When *P. verrucosum* was paired with *A. ochraceus* at 0.95 a_w and 15 °C there was no significant effect on OTA production, however at 25 °C and 0.95 a_w the presence of *A. ochraceus* resulted in an increase in OTA production. When *P. verrucosum* was paired with *A. ochraceus* at 0.995 a_w and 15 °C there was an increase in OTA production but at 25 °C at this water activity there was a decrease in OTA production. ANOVA (Table 3.22) shows that overall a_w, temperature and the presence of other wheat-spoilage fungi and their interactions had a significant effect on OTA production by *P. verrucosum* Table 3.23 summarises the effects of interactions on OTA production by *P. verrucosum* over the complete range of conditions tested.

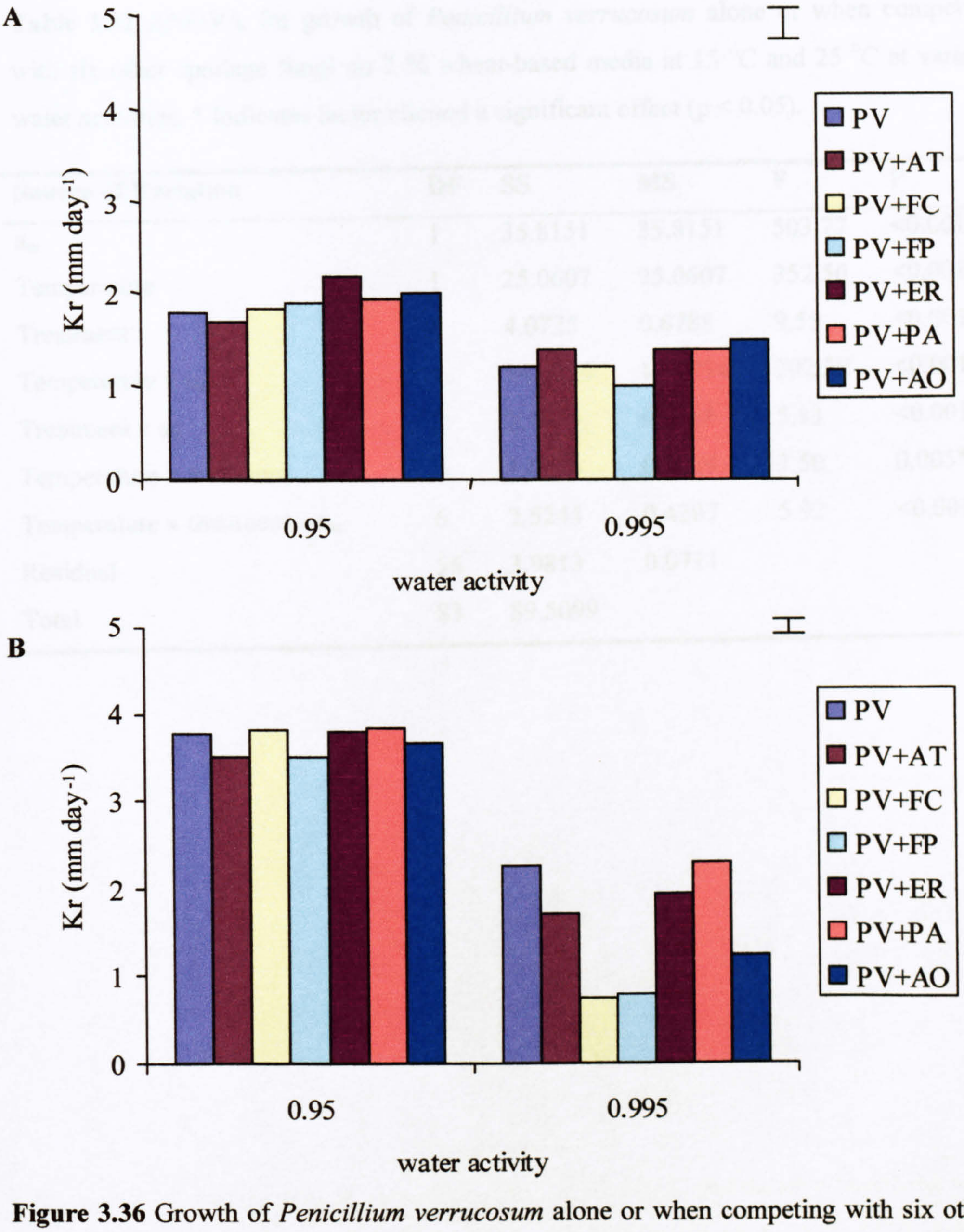


Figure 3.36 Growth of *Penicillium verrucosum* alone or when competing with six other spoilage fungi at A) 15 °C or B) 25 °C and two water activity (a_w, 0.95 and 0.995 a_w) conditions on 2 % wheat-based media. Key to interacting species: PV, *P. verrucosum* ; AT, *A. tenuissima* ; FC, *F. culmorum* ; FP, *F. poae* ; ER, *E. repens* ; PA, *P. aurantiogriseum;* AO, *A. ochraceus.* Bar indicates Least Significant Difference (LSD) at $p < 0.05$.

Table 3.22 ANOVA for growth of *Penicillium verrucosum* alone or when competing with six other spoilage fungi on 2 % wheat-based media at 15 °C and 25 °C at various water activities. * Indicates factor elicited a significant effect ($p < 0.05$).

Source of Variation	DF	SS	MS	F	P
a_w	1	35.8151	35.8151	503.77	<0.001*
Temperature	1	25.0607	25.0607	352.50	<0.001*
Treatment	6	4.0725	0.6788	9.55	<0.001*
Temperature x a_w	1	14.3754	14.3754	202.20	<0.001*
Treatment x a_w	6	2.1869	0.3645	5.13	<0.001*
Temperature x treatment	6	1.4936	0.2489	3.50	0.005*
Temperature x treatment x a_w	6	2.5244	0.4207	5.92	<0.001*
Residual	56	3.9813	0.0711		
Total	83	89.5099			

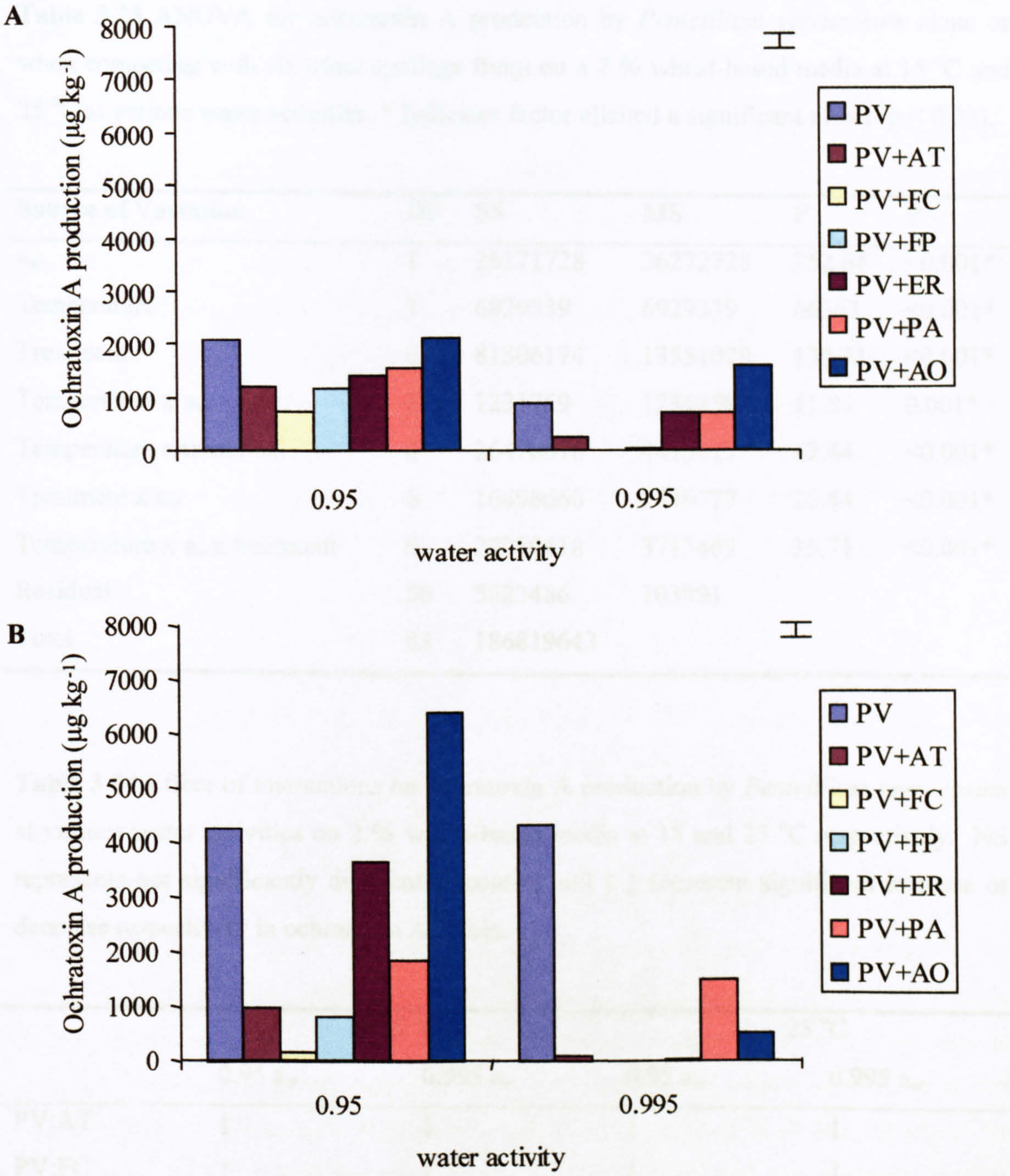


Figure 3.37 Ochratoxin A production by *Penicillium verrucosum* alone or when competing with six other spoilage fungi after 56 days at A) 15 °C or B) 25 °C and two water activity (a_w, 0.95 and 0.995 a_w) conditions on 2 % wheat-based media. Key to interacting species: PV, *P. verrucosum* ; AT, *A. tenuissima* ; FC, *F. culmorum* ; FP, *F. poae;* ER, *E. repens* ; PA, *P. aurantiogriseum;* AO, *A. ochraceus.* Bar indicates Least Significant Difference (LSD) at $p < 0.05$.

Table 3.23 ANOVA for ochratoxin A production by *Penicillium verrucosum* alone or when competing with six other spoilage fungi on a 2 % wheat-based media at 15 °C and 25 °C at various water activities. * Indicates factor elicited a significant effect ($p < 0.05$).

Source of Variation	DF	SS	MS	F	P
a_w	1	26271728	26272728	252.64	<0.001*
Temperature	1	6929339	6929339	66363	<0.001*
Treatment	6	81306174	13551029	130.31	<0.001*
Temperature x a_w	1	1231759	1231759	11.84	0.001*
Temperature x treatment	6	26478078	4413013	42.44	<0.001*
Treatment x a_w	6	16498660	2749777	26.44	<0.001*
Temperature x a_w x treatment	6	22280418	3713403	35.71	<0.001*
Residual	56	5823486	103991		
Total	83	186819643			

Table 3.24 Effect of interactions on ochratoxin A production by *Penicillium verrucosum* at various water activities on 2 % wheat-based media at 15 and 25 °C respectively. NS represents not significantly different to control and ↑ ↓ represent significant increase or decrease respectively in ochratoxin A levels.

	15 °C		25 °C	
	0.95 a_w	0.995 a_w	0.95 a_w	0.995 a_w
PV:AT	↓	↓	↓	↓
PV:FC	↓	↓	↓	↓
PV:FP	↓	↓	↓	↓
PV:ER	↓	NS	↓	↓
PV:PA	↓	NS	↓	↓
PV:AO	NS	↑	↑	↓

3.3.3 Effect of interactions on growth and ochratoxin A production by *Penicillium verrucosum* on γ-irradiated wheat grain

Figure 3.38 shows the relative growth rates of *P. verrucosum* on γ-irradiated wheat grain alone or when competing with six other spoilage fungi in relation to a_w and temperature. At 0.95 a_w and 15 °C or 25 °C growth rates were largely unaffected by the presence of all competing spoilage fungi. At 0.995 a_w and 15 °C, growth of *P. verrucosum* was suppressed when paired with *A. tenuissima, F. culmorum* or *F. poae* and unaffected when paired with *E. repens, A. ochraceus* or *P. aurantiogriseum.* At 0.995 a_w and 25 °C growth of *P. verrucosum* was suppressed when paired with *F. culmorum, F. poae, P. aurantiogriseum, E. repens* or *A.ochraceus.* ANOVA shows that overall a_w, temperature and the presence of other wheat-spoilage fungi and their interactions have a significant effect on growth rates of *P. verrucosum* on γ-irradiated wheat grain (Table 3.25).

Figure 3.39 shows OTA production by *P. verrucosum* on γ-irradiated wheat grain alone or when competing with six other spoilage fungi in relation to the treatments. When *P. verrucosum* was paired with *A. tenuissima, F. culmorum, F. poae, E. repens* or *P. aurantiogriseum* at 0.95 and 0.995 a_w levels at 15 °C there was a significant decrease in OTA production. At 0.95 a_w and 25 °C the presence of *F. culmorum, F. poae, E. repens, A. tenuissima* or *P.aurantiogriseum* resulted in a significant decrease in OTA production. When *P. verrucosum* was paired with *A. tenuissima, F. culmorum* or *F. poae* at 25 °C and 0.995 a_w, there was a significant reduction in OTA production, whereas when *P. verrucosum* was paired with *E. repens* or *P aurantiogriseum* there was no change in OTA production. When *P. verrucosum* was paired with *A. ochraceus* at both water activity and temperature treatments, there was an increase in OTA production. The ANOVA (Table 3.26) shows that overall a_w, temperature and the presence of other wheat-spoilage fungi and their interactions had a significant effect on OTA production by *P. verrucosum.* Table 3.27 summarises the effects of interactions on OTA production by *P. verrucosum* over the complete range of conditions tested.

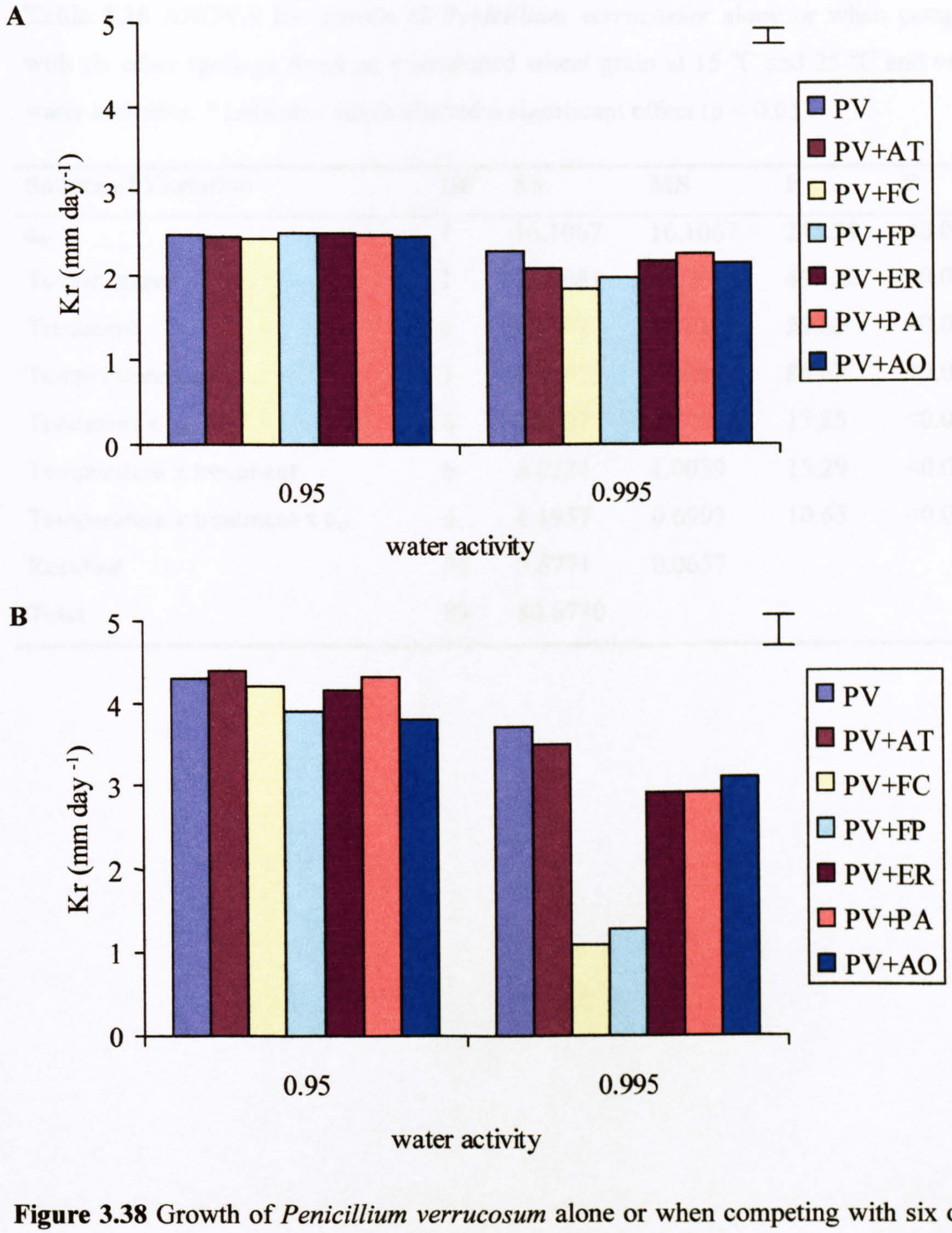


Figure 3.38 Growth of *Penicillium verrucosum* alone or when competing with six other spoilage fungi at A) 15 °C or B) 25 °C and two water activity (a_w, 0.95 and 0.995 a_w) conditions on γ-irradiated wheat grain. Key to interacting species: PV, *P. verrucosum* ; AT, *A. tenuissima* ; FC, *F. culmorum* ; FP, *F. poae* ; ER, *E. repens* ; PA, *P.aurantiogriseum;* AO, *A. ochraceus.* Bar indicates Least Significant Difference (LSD) at $p < 0.05$.

Table 3.25 ANOVA for growth of *Penicillium verrucosum* alone or when competing with six other spoilage fungi on γ-irradiated wheat grain at 15 °C and 25 °C and various water activities. * Indicates factor elicited a significant effect ($p < 0.05$).

Source of Variation	DF	SS	MS	F	P
a_w	1	16.1067	16.1067	245.29	<0.001*
Temperature	1	28.7081	28.7081	437.20	<0.001*
Treatment	6	9.3062	1.5510	23.62	<0.001*
Temperature x a_w	1	5.6252	5.6252	85.67	<0.001*
Treatment x a_w	6	7.0307	1.1718	17.85	<0.001*
Temperature x treatment	6	6.0234	1.0039	15.29	<0.001*
Temperature x treatment x a_w	6	4.1957	0.6993	10.65	<0.001*
Residual	56	3.6771	0.0657		
Total	83	80.6730			

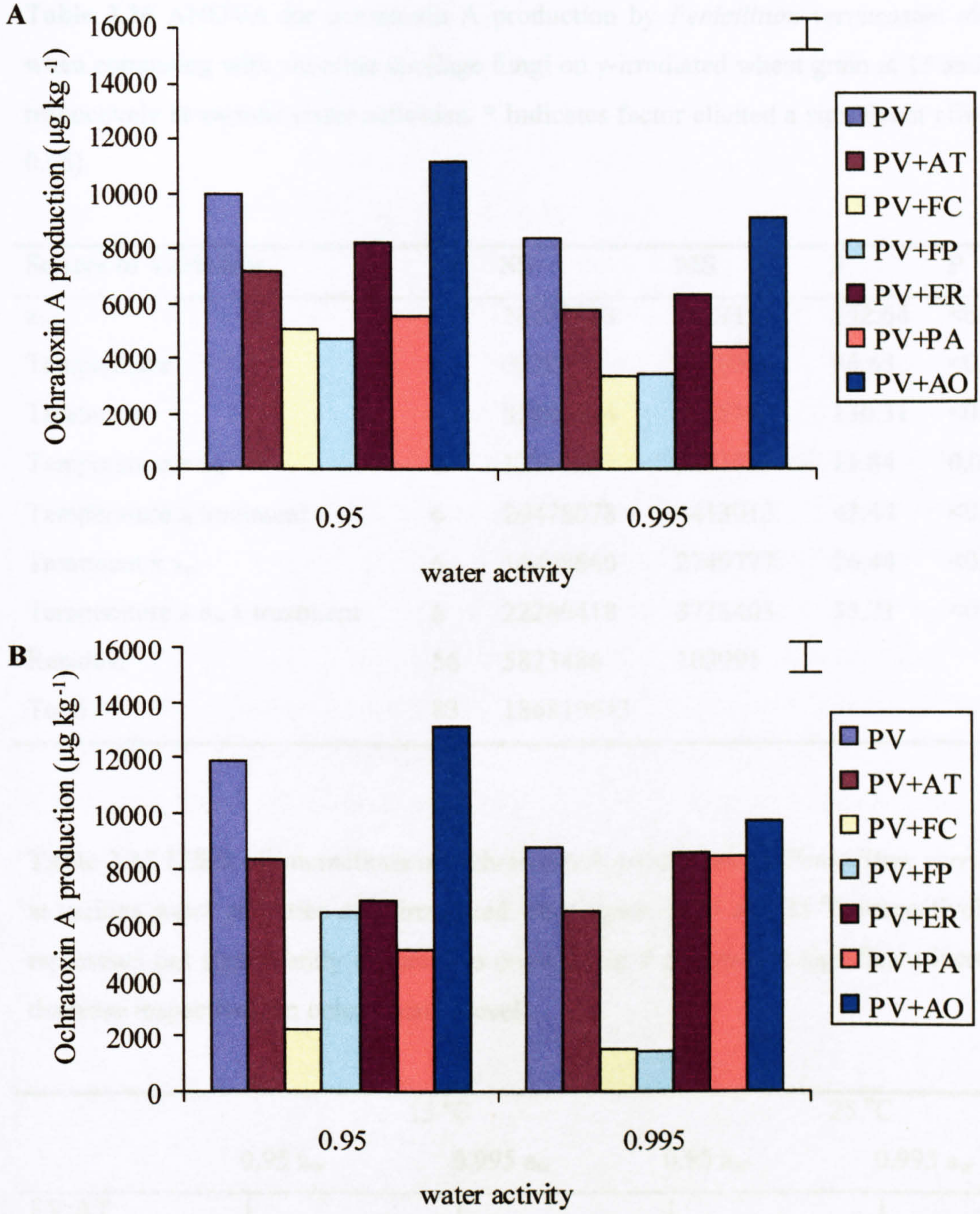


Figure 3.39 Ochratoxin A production by *Penicillium verrucosum* alone or when competing with six other spoilage fungi after 28 days at A) 15 °C or B) 25 °C and two water activity (a_w, 0.95 and 0.995 a_w) conditions on γ-irradiated wheat grain. Key to interacting species: PV, *P. verrucosum* ; AT, *A. tenuissima* ; FC, *F. culmorum* ; FP, *F. poae;* ER, *E. repens* ; PA, *P. aurantiogriseum;* AO, *A. ochraceus.* Bar indicates Least Significant Difference (LSD) at $p < 0.05$.

Table 3.26 ANOVA for ochratoxin A production by *Penicillium verrucosum* alone or when competing with six other spoilage fungi on γ-irradiated wheat grain at 15 and 25 °C respectively at various water activities. * Indicates factor elicited a significant effect ($p < 0.05$).

Source of Variation	DF	SS	MS	F	P
a_w	1	26271728	26271728	252.64	<0.001*
Temperature	1	6929339	6929339	66.63	<0.001*
Treatment	6	81306174	13551029	130.31	<0.001*
Temperature x a_w	1	1231759	1231759	11.84	0.001*
Temperature x treatment	6	26478078	4413013	42.44	<0.001*
Treatment x a_w	6	16498660	2749777	26.44	<0.001*
Temperature x a_w x treatment	6	22280418	3713403	35.71	<0.001*
Residual	56	5823486	103991		
Total	83	186819643			

Table 3.27 Effect of interactions on ochratoxin A production by *Penicillium verrucosum* at various water activities on γ-irradiated wheat grain at 15 and 25 °C respectively. NS represents not significantly different to control and ↑ ↓ represent significant increase or decrease respectively in ochratoxin A levels.

	15 °C		25 °C	
	0.95 a_w	0.995 a_w	0.95 a_w	0.995 a_w
PV:AT	↓	↓	↓	↓
PV:FC	↓	↓	↓	↓
PV:FP	↓	↓	↓	↓
PV:ER	↓	↓	↓	NS
PV:PA	↓	↓	↓	NS
PV:AO	↑	↑	↑	↑

3.3.4 The effect of environmental factors and competing mycoflora on competitiveness of *Aspergillus ochraceus* on a 2 % wheat-based media and γ-irradiated wheat grain

Table 3.28 shows the effect of changes in both a_w and temperature on the interaction scores between *A. ochraceus* and six other spoilage fungi on a 2 % wheat-based media and γ-irradiated wheat grain. The first number of the interaction scores always represents that for *A. ochraceus*.

On a 2 % wheat-based media and γ-irradiated wheat grain *A.ochraceus* was consistently dominant on contact with all species tested at 0.95 a_w and 25 °C with scores of 4 consistently. At 0.95 a_w and 15 °C, *A. ochraceus* was dominant against all species tested except *P. verrucosum* and *P. aurantiogriseum* where it was mutually antagonistic on contact. At 0.995 a_w and 25 °C, *F. culmorum, F. poae* and *A. tenuissima* were all dominant over *A. ochraceus*. However *A. ochraceus* was dominant at this condition over *P. verrucosum, P. aurantiogriseum* and *E. repens*. At 0.995 a_w and 15 °C, *P. verrucosum, P. aurantiogriseum, F. culmorum* and *F. poae* were all mutually antagonistic on contact with *A. ochraceus* whereas *A. tenuissima* was dominant over *A. ochraceus* and *A.ochraceus* was dominant over *E. repens*. The sum of the Index of Dominance scores indicates that *A. ochraceus* was more competitive at 25 °C at intermediate a_w against the species tested.

Table 3.28 Effect of water activity (a_w) and temperature on numerical interaction scores and Index of Dominance (I_D) for *Aspergillus ochraceus* (IBT21991) and other interacting species on a 2 % wheat-based media. Results were identical on γ-irradiated wheat grain

Water activity (a_w)	Interacting species	15°C	25°C	Index of Dominance (I_D)
0.90	*Penicillium verrucosum*	NE	NE	NE
	Fusarium culmorum	NE	NE	NE
	Fusarium poae	NE	NE	NE
	Alternaria tenuissima	NE	NE	NE
	Eurotium repens	NE	NE	NE
	Penicillium aurantiogriseum	NE	NE	NE
	I_D	**NE**	**NE**	**NE**
0.95	*Penicillium verrucosum*	2/2	4/0	6/2
	Fusarium culmorum	4/0	4/0	8/0
	Fusarium poae	4/0	4/0	8/0
	Alternaria tenuissima	4/0	4/0	8/0
	Eurotium repens	4/0	4/0	8/0
	Penicillium aurantiogriseum	2/2	4/0	6/2
	I_D	**20/4**	**24/0**	**44/4**
0.995	*Penicillium verrucosum*	2/2	4/0	6/2
	Fusarium culmorum	2/2	0/4	2/6
	Fusarium poae	2/2	0/4	2/6
	Alternaria tenuissima	0/4	0/4	0/8
	Eurotium repens	4/0	4/0	8/0
	Penicillium aurantiogriseum	2/2	4/0	6/2
	I_D	**12/12**	**12/12**	**24/24**

Aspergillus ochraceus (IBT21991) score/other species score. NE, not examined.

3.3.5 Effect of interactions on growth and ochratoxin A production by *Aspergillus ochraceus* on a 2 % wheat-based media

Figure 3.40 shows the relative growth rates of *A. ochraceus* on a 2 % wheat-based medium alone or when competing with six other spoilage fungi at different a_w x temperature conditions. At 15 °C and 0.95 a_w growth of *A. ochraceus* was unaffected when paired with *F. poae, A. tenuissima, F. culmorum* or *E. repens* and significantly reduced when paired with *P. aurantiogriseum* or *P. verrucosum*. At 25 °C and 0.95 a_w growth of *A. ochraceus* was significantly reduced when paired with *F. poae, F. culmorum, A. tenuissima* or *E. repens*. At 15 °C and 0.995 a_w growth of *A. ochraceus* was significantly reduced when paired with *A. tenuissima. F. culmorum, F. poae, P. aurantiogriseum* or *P. verrucosum* and unaffected when paired with *E. repens*. At 25 °C and 0.995 a_w growth of *A. ochraceus* was significantly reduced when paired with *A. tenuissima, F. poae* or *F. culmorum* and unaffected when paired with *P. aurantiogriseum, P. verrucosum* or *E. repens*. The ANOVA (Table 3.29) shows that a_w, temperature and the presence of other wheat-spoilage fungi and their interactions significantly affected growth rates.

Figure 3.41 shows OTA production by *A. ochraceus* on a 2 % wheat-based media alone or when competing with six other spoilage fungi. When *A. ochraceus* was paired with *A. tenuissima, F. culmorum, F. poae, E. repens* or *P. aurantiogriseum* there was a significant reduction in OTA production over the complete range of water activities and temperatures tested. At 0.95 a_w at both temperatures and 0.995 a_w at 15 °C there was a significant increase in OTA production when *A. ochraceus* was paired with *P. verrucosum*. However, at 0.995 a_w and 25 °C when *A. ochraceus* was paired with *P. verrucosum* there was a significant reduction in OTA production. The ANOVA (Table 3.30) shows that overall a_w, temperature and the presence of other wheat-spoilage fungi and their interactions significantly affected OTA production. Table 3.31 summarises the effects of interactions on OTA production by *A. ochraceus* over the complete range of conditions tested.

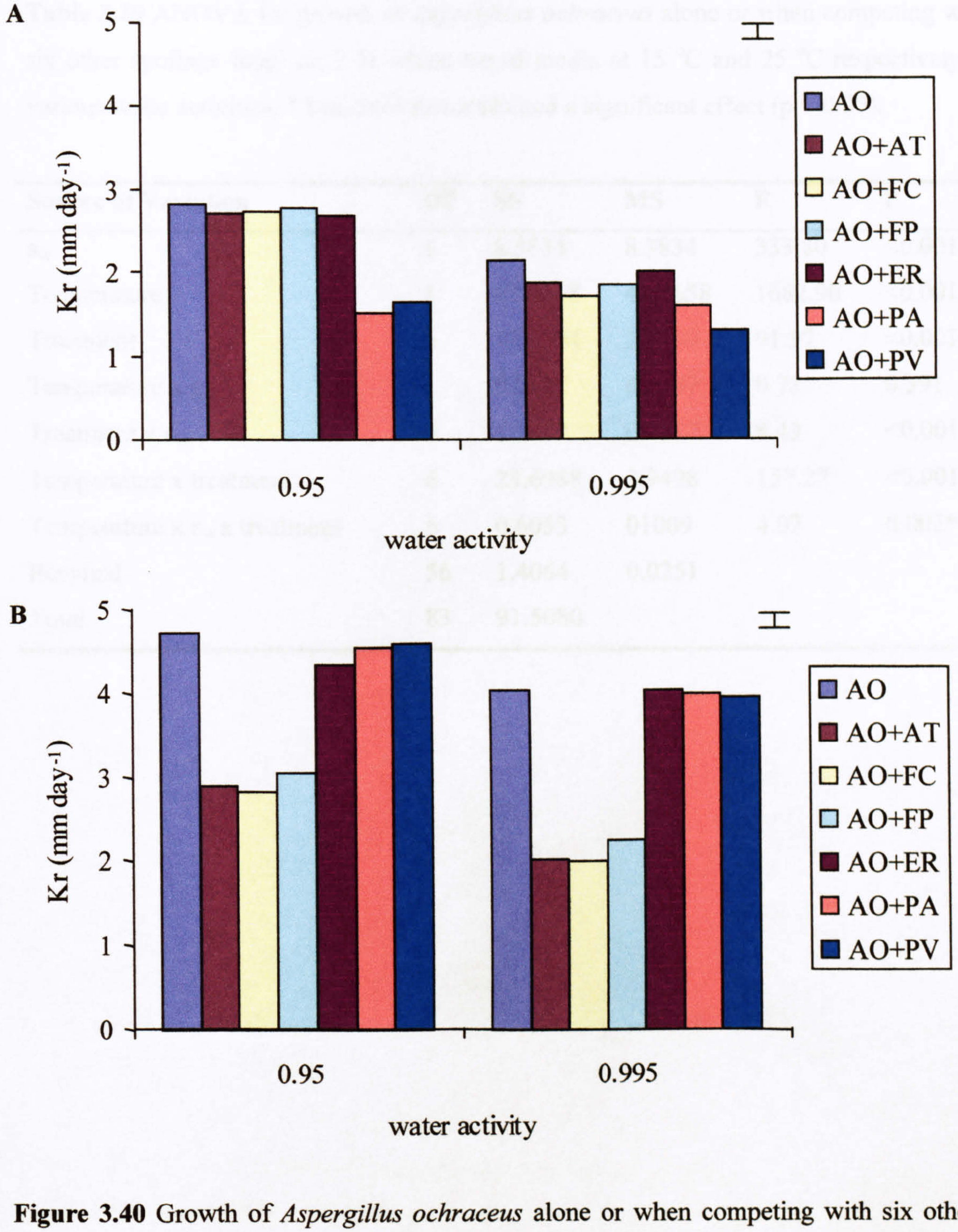


Figure 3.40 Growth of *Aspergillus ochraceus* alone or when competing with six other spoilage fungi at A) 15 °C or B) 25 °C and two water activity (a_w, 0.95 and 0.995 a_w) conditions on 2 % wheat-based media. Key to interacting species: PV, *P. verrucosum* ; AT, *A. tenuissima* ; FC, *F. culmorum* ; FP, *F. poae* ; ER, *E. repens* ; PA, *P. aurantiogriseum;* AO, *A. ochraceus.* Bar indicates Least Significant Difference (LSD) at $p < 0.05$.

Table 3.29 ANOVA for growth of *Aspergillus ochraceus* alone or when competing with six other spoilage fungi on 2 % wheat-based media at 15 °C and 25 °C respectively at various water activities. * Indicates factor elicited a significant effect ($p < 0.05$).

Source of Variation	DF	SS	MS	F	P
a_w	1	8.3834	8.3834	333.80	<0.001*
Temperature	1	42.2658	42.2658	1682.90	<0.001*
Treatment	6	13.8594	2.3099	91.97	<0.001*
Temperature x a_w	1	0.0187	0.0187	0.75	0.391
Treatment x a_w	6	1.2702	0.2117	8.43	<0.001*
Temperature x treatment	6	23.6988	3.9498	157.27	<0.001*
Temperature x a_w x treatment	6	0.6053	01009	4.02	0.002*
Residual	56	1.4064	0.0251		
Total	83	91.5080			

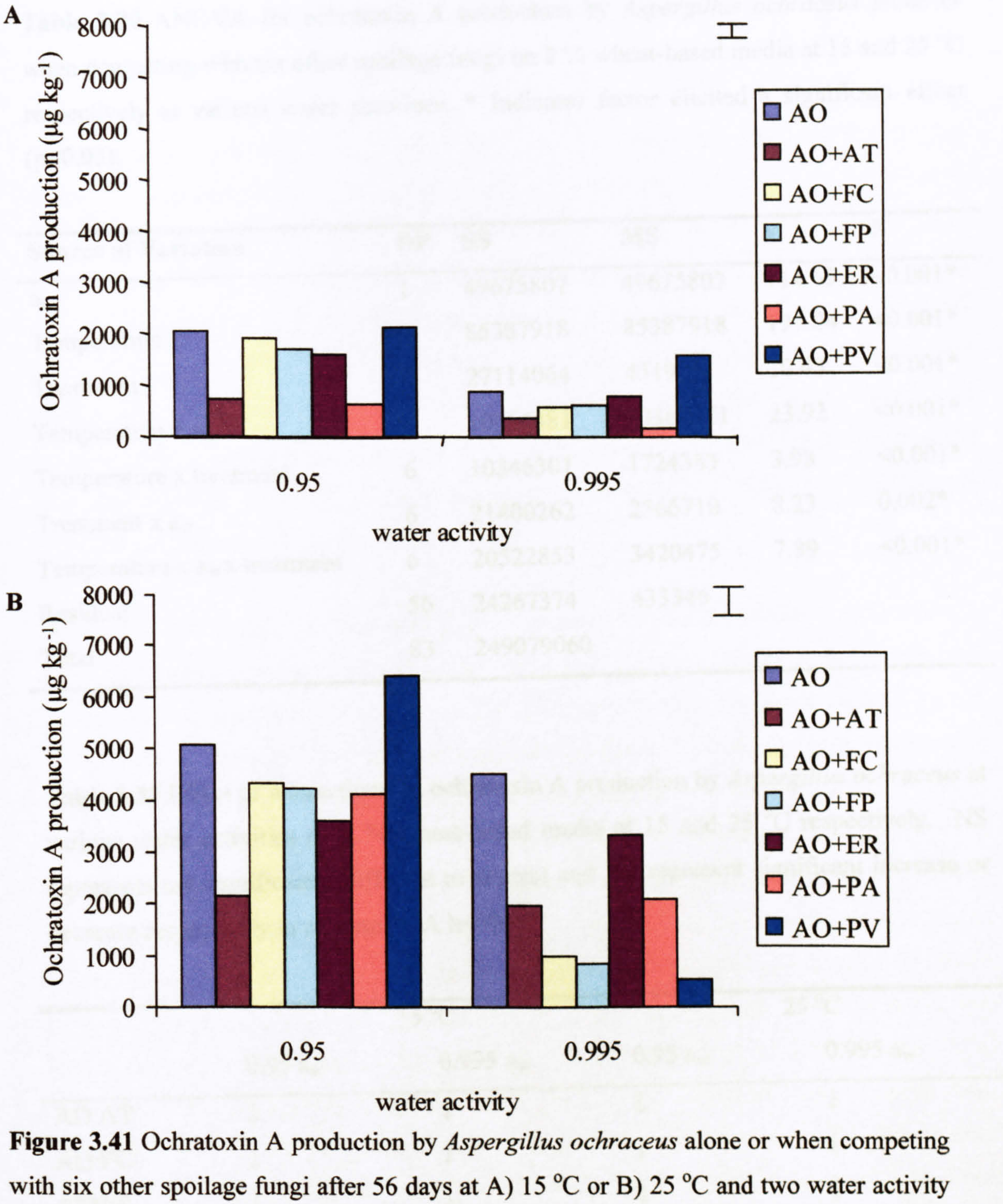


Figure 3.41 Ochratoxin A production by *Aspergillus ochraceus* alone or when competing with six other spoilage fungi after 56 days at A) 15 °C or B) 25 °C and two water activity (a_w, 0.95 and 0.995 a_w) conditions on 2 % wheat-based media. Key to interacting species: PV, *P. verrucosum* ; AT, *A. tenuissima* ; FC, *F. culmorum* ; FP, *F. poae* ; ER, *E. repens* ;

Table 3.30 ANOVA for ochratoxin A production by *Aspergillus ochraceus* alone or when competing with six other spoilage fungi on 2 % wheat-based media at 15 and 25 °C respectively at various water activities. * Indicates factor elicited a significant effect ($p<0.05$).

Source of Variation	DF	SS	MS	F	P
a_w	1	49675807	49675807	114.63	<0.001*
Temperature	1	85387918	85387918	197.04	<0.001*
Treatment	6	27114064	4519011	10.43	<0.001*
Temperature x a_w	1	10364481	10364481	23.92	<0.001*
Temperature x treatment	6	10346301	1724383	3.98	<0.001*
Treatment x a_w	6	21400262	2566710	8.23	0.002*
Temperature x a_w x treatment	6	20522853	3420475	7.89	<0.001*
Residual	56	24267374	433346		
Total	83	249079060			

Table 3.31 Effect of interactions on ochratoxin A production by *Aspergillus ochraceus* at various water activities on 2 % wheat-based media at 15 and 25 °C respectively. NS represents not significantly different to control and ↑ ↓ represent significant increase or decrease respectively in ochratoxin A levels.

	15 °C		25 °C	
	0.95 a_w	0.995 a_w	0.95 a_w	0.995 a_w
AO:AT	↓	↓	↓	↓
AO:FC	↓	↓	↓	↓
AO:FP	↓	↓	↓	↓
AO:ER	↓	↓	↓	↓
AO:PA	↓	↓	↓	↓
AO:PV	↑	↑	↑	↓

3.3.6 Effect of interactions on growth and ochratoxin A production by *Aspergillus ochraceus* on γ-irradiated wheat grain

Figure 3.42 shows the relative growth rates of *A. ochraceus* on γ-irradiated wheat grain alone or when competing with six other spoilage fungi in relation to a_w x temperature conditions. At 15 °C and 0.95 a_w growth rates by *A. ochraceus* were largely unaffected when paired with *A. tenuissima, F. culmorum, F. poae,* or *E. repens* and significantly reduced when paired with *P. aurantiogriseum* or *P. verrucosum.* At 0.95 a_w at 25 °C and 0.995 a_w at 15 and 25 °C growth rates of *A. ochraceus* were significantly reduced when paired with each of the six other wheat-spoilage fungi particularly when paired with *A. tenuissima, F. poae* or *F. culmorum*. ANOVA (Table 3.32) shows that a_w, temperature and the presence of other wheat-spoilage fungi and their interactions significantly affected growth rates of *A. ochraceus*.

Figure 3.43 shows OTA production by *A. ochraceus* on γ-irradiated wheat grain alone or when competing with the other spoilage fungi after 28 days. When *A. ochraceus* was paired with *A. tenuissima, F. culmorum, F. poae* or *P. aurantiogriseum* at 0.95 a_w and 15 °C there was a significant reduction in toxin production. However, when paired with *E. repens* there was no significant effect on toxin production. At 0.995 a_w and 15 °C there was no significant effect on OTA production when *A. ochraceus* was paired with *A. tenuissima, F. culmorum, F. poae, E. repens* or *P. aurantiogriseum.* At 0.95 a_w and 25 °C there was no significant effect on OTA production when *A. ochraceus* was paired with *A. tenuissima* or *F. culmorum* and a significant reduction in toxin production when *A. ochraceus* was paired *F. poae, E. repens* or *P. aurantiogriseum*. When *A. ochraceus* was paired with *A. tenuissima, E. repens* or *P. aurantiogriseum* at 0.995 a_w and 25 °C there was no significant effect on OTA production. However, when paired with *F. culmorum* and *F. poae* there was a significant reduction in OTA production. Regardless of the condition tested, OTA production significantly increased when *A. ochraceus* was paired with the other ochratoxigenic species *P. verrucosum.* ANOVA (Table 3.33) shows that overall a_w, temperature and the presence of other wheat-spoilage fungi and their interactions significantly affected OTA production by *A. ochraceus.* Table 3.34 summarises the effects of interactions on OTA production by *A. ochraceus* over the complete range of conditions tested.

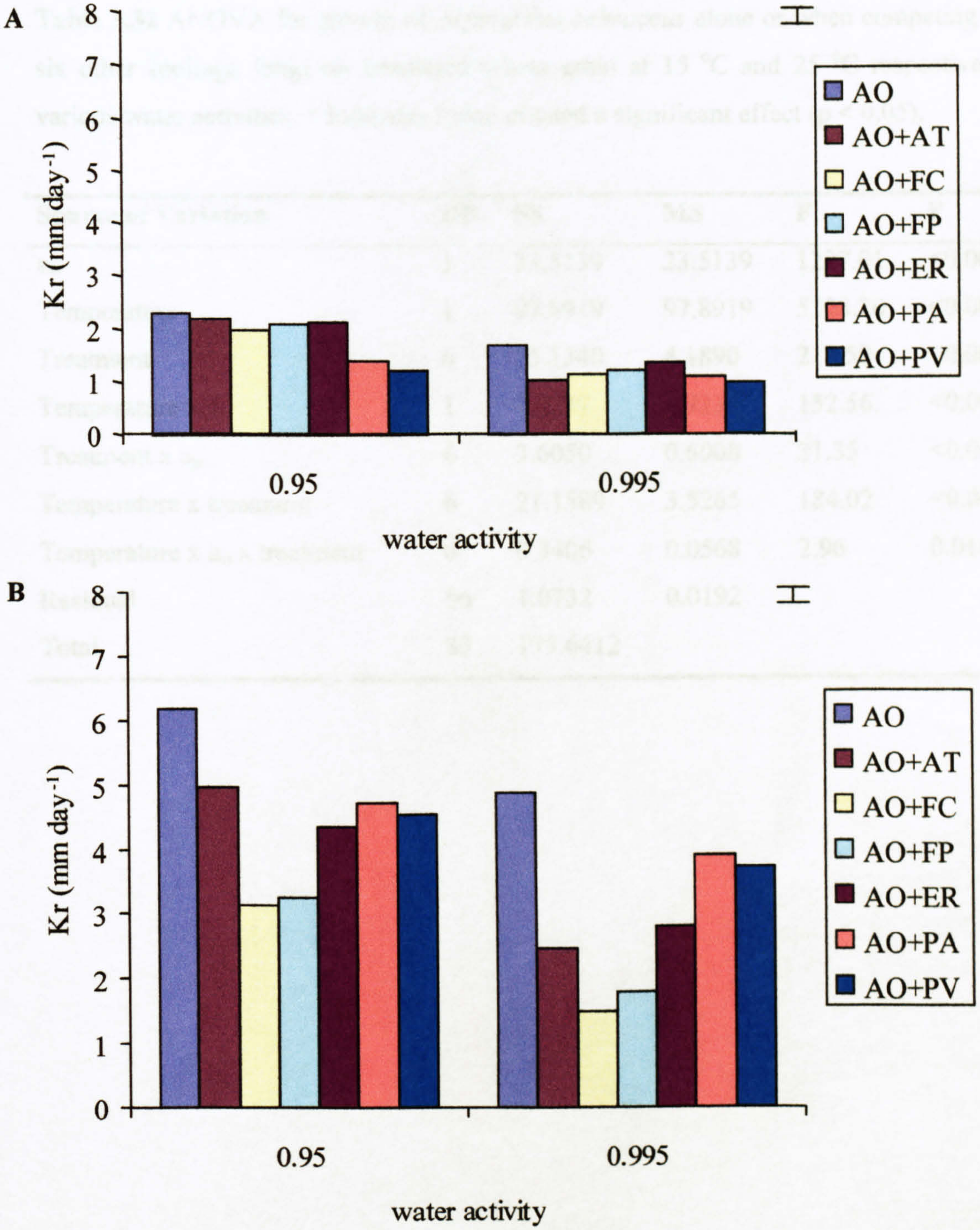
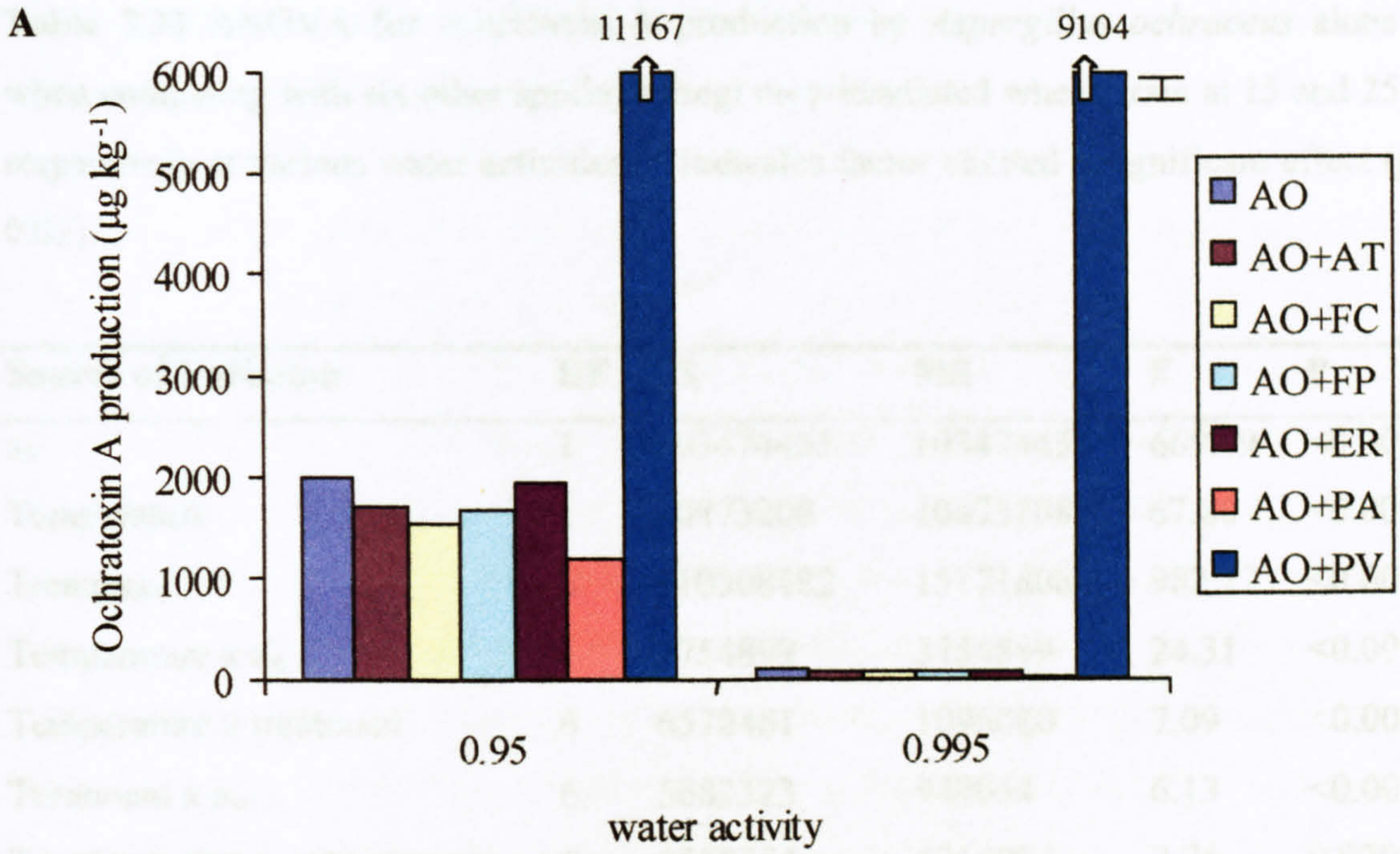


Figure 3.42 Growth of *Aspergillus ochraceus* alone or when competing with six other spoilage fungi at A) 15 °C or B) 25 °C and two water activity (a_w, 0.95 and 0.995 a_w) conditions on γ-irradiated wheat grain. Key to interacting species: PV, *P. verrucosum* ; AT, *A. tenuissima* ; FC, *F. culmorum* ; FP, *F. poae* ; ER, *E. repens* ; PA, *P. aurantiogriseum;* AO, *A. ochraceus.* Bar indicates Least Significant Difference (LSD) at $p < 0.05$.

Table 3.32 ANOVA for growth of *Aspergillus ochraceus* alone or when competing with six other spoilage fungi on irradiated wheat grain at 15 °C and 25 °C respectively at various water activities. * Indicates factor elicited a significant effect ($p < 0.05$).

Source of Variation	DF	SS	MS	F	P
a_w	1	23.5139	23.5139	1227.01	<0.001*
Temperature	1	97.8919	97.8919	5108.20	<0.001*
Treatment	6	25.1340	4.1890	218.59	<0.001*
Temperature x a_w	1	2.9237	2.9237	152.56	<0.001*
Treatment x a_w	6	3.6050	0.6008	31.35	<0.001*
Temperature x treatment	6	21.1589	3.5265	184.02	<0.001*
Temperature x a_w x treatment	6	0.3406	0.0568	2.96	0.014*
Residual	56	1.0732	0.0192		
Total	83	175.6412			



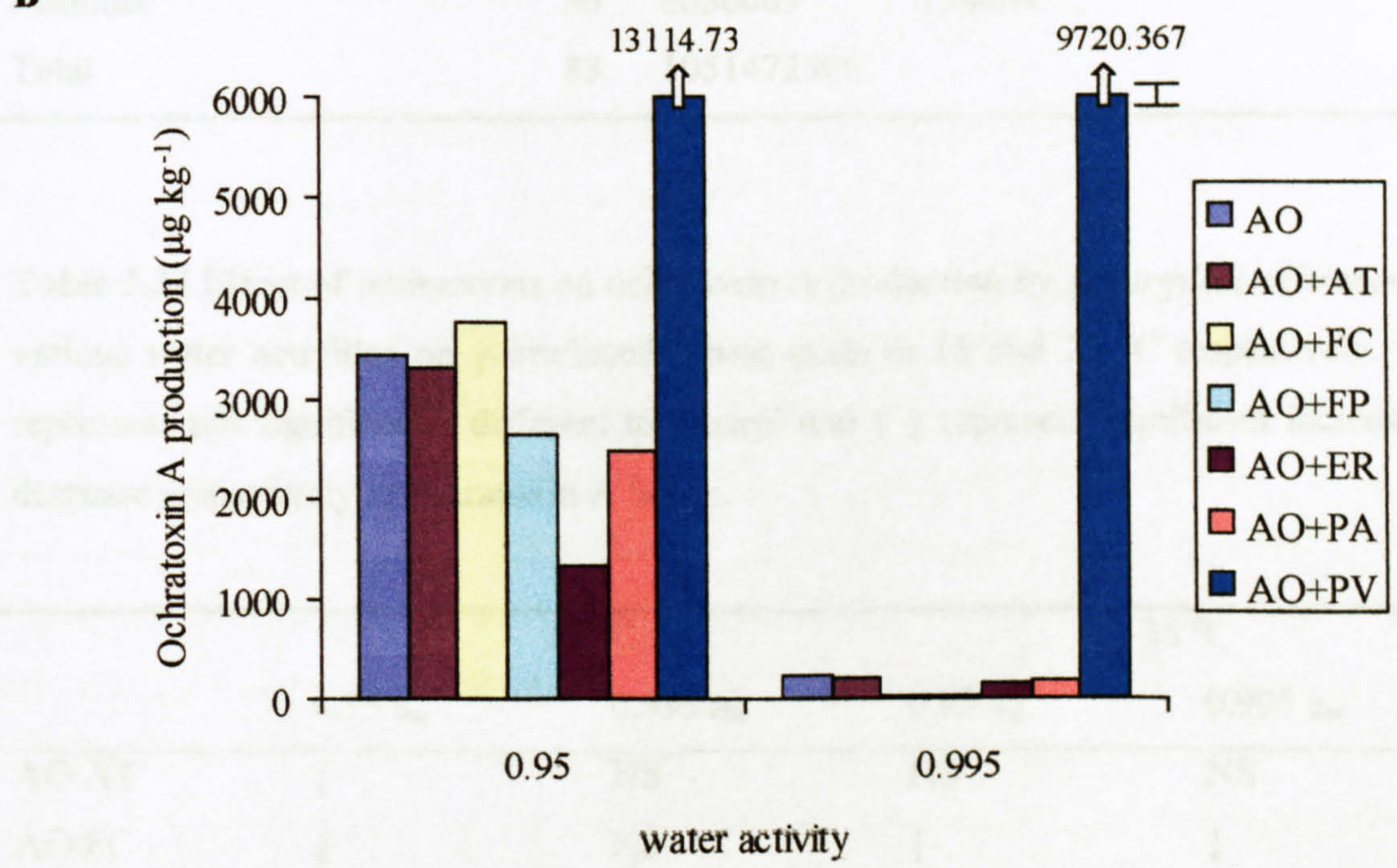


Figure 3.43 Ochratoxin A production by *Aspergillus ochraceus* alone or when competing with six other spoilage fungi after 28 days at A) 15 °C or B) 25 °C and two water activity (a_w, 0.95 and 0.995 a_w) conditions on γ-irradiated wheat grain. Key to interacting species: PV, *P. verrucosum* ; AT, *A. tenuissima* ; FC, *F. culmorum* ; FP, *F. poae* ; ER, *E. repens* ; PA, *P. aurantiogriseum;* AO, *A. ochraceus.* Bar indicates Least Significant Difference (LSD) at $p < 0.05$

Table 3.33 ANOVA for ochratoxin A production by *Aspergillus ochraceus* alone or when competing with six other spoilage fungi on γ-irradiated wheat grain at 15 and 25 °C respectively at various water activities. * Indicates factor elicited a significant effect ($p < 0.05$).

Source of Variation	DF	SS	MS	F	P
a_w	1	103474455	103474455	669.89	<0.001*
Temperature	1	10473208	10473208	67.80	<0.001*
Treatment	6	910308482	151718080	982.22	<0.001*
Temperature x a_w	1	3754899	3754899	24.31	<0.001*
Temperature x treatment	6	6570481	1095080	7.09	<0.001*
Treatment x a_w	6	5682323	947054	6.13	<0.001*
Temperature x a_w x treatment	6	2558454	426409	2.76	0.020*
Residual	56	8650003	154464		
Total	83	1051472306			

Table 3.34 Effect of interactions on ochratoxin A production by *Aspergillus ochraceus* at various water activities on γ-irradiated wheat grain at 15 and 25 °C respectively. NS represents not significantly different to control and ↑ ↓ represent significant increase or decrease respectively in ochratoxin A levels.

	15 °C		25 °C	
	0.95 a_w	0.995 a_w	0.95 a_w	0.995 a_w
AO:AT	↓	NS	NS	NS
AO:FC	↓	NS	↑	↓
AO:FP	↓	NS	↓	↓
AO:ER	NS	NS	↓	NS
AO:PA	↓	NS	↓	NS
AO:PV	↑	↑	↑	↑

3.4 CARBON SOURCE UTILISATION AND NICHE OVERLAP INDICES OF COMMON WHEAT-SPOILAGE FUNGI

The carbon source utilisation profiles were carried out at two water activities (0.93 and 0.995 a_w) and temperatures (15 and 25 °C) for all fungi used in the interaction experiments. The profiles were used to compare the total number of carbon sources utilised (niche size) for each fungus at each set of environmental conditions using the 18 key carbon sources found in wheat grain. These profiles were then used to calculate niche overlap indices as described in the Methodology (section 2.7). These indices were used as a measure of determining the level of co-existence or dominance in a specific niche with scores of >0.9 indicating co-existence between species in an ecological niche and scores of <0.9 indicating occupation of separate niches. It was hypothesised that fungi found to occupy a similar niche would also be those which attempted to exclude or dominate each other in interaction experiments.

3.4.1 Carbon source utilisation and niche overlap indices for *Penicillium verrucosum* against other wheat-spoilage fungi

Table 3.35 shows the niche sizes and niche overlap index scores determined from carbon source utilisation assays encompassing the 18 key carbon sources found in wheat grain at different environmental conditions. The target pathogen for niche overlap indices was *P. verrucosum* and therefore did not have a niche overlap index score. It can be seen that changing a_w or temperature altered the number of carbon sources utilised (niche size) by all species examined. Interestingly, carbon utilisation varied amongst *P. verrucosum* strains (IBT22625, IBT22626 and OTA11) at both temperatures and a_w levels tested. Generally as a_w increased so did the niche size for all species tested. The only exception to this was *E. repens* whose niche size decreased with an increase in a_w. It can be seen that temperature had a smaller effect on niche size than a_w.

Using the niche overlap index data, niche overlap maps were constructed by plotting $NOI_{(target\ pathogen)}$ against $NOI_{(competing\ species)}$. This allowed a comparison of the relationship

between each fungus and the target organism's niche. Figures 3.44 and 3.45 show niche overlap maps for *P. verrucosum* (the target pathogen) against other wheat-spoilage fungi. The maps are divided into four separate sections which show:

i. $NOI_{(target\ pathogen)}$ and $NOI_{(competing\ species)}$ = >0.9. Competing species occupies the same niche as the target pathogen: **Coexistence**

ii. $NOI_{(target\ pathogen)}$ and $NOI_{(competing\ species)}$ = <0.9. Competing species occupies separate niche to the target pathogen: **Occupation of separate niches.**

iii. $NOI_{(target\ pathogen)}$ = >0.9 and $NOI_{(competing\ species)}$ = <0.9. Not only can the competing species exist within the niche of the target pathogen but it can metabolise fewer carbon sources within this niche than the target pathogen: **Nutritional dominance of target pathogen over species.**

iv. $NOI_{(target\ pathogen)}$ = <0.9 and $NOI_{(competing\ species)}$ = >0.9. The competing species is able to utilise >90 % of the same carbon source as the target pathogen but is not restricted to the target pathogen's niche: **Nutritional dominance of species over target pathogen.**

Figure 3.44 shows that *P. verrucosum* (strain OTA11) occupied the same niche as *P. verrucosum* (strains IBT22625, IBT22626), *F. poae, F. culmorum* and *P. aurantiogriseum* but had nutritional dominance over *E. repens, A. ochraceus* and *A. tenuissima* at 15 °C and 0.93 a_w. *P. verrucosum* (strain OTA11) occupied the same niche as *E. repens* and *P. verrucosum* (strain IBT22625) at 0.93 a_w and 25 °C but had nutritional dominance over *A. ochraceus, A. tenuissima* and *P. verrucosum* (strain IBT22626). At 0.93 a_w and 25 °C, *F. culmorum, F. poae* and *P. aurantiogriseum* all had nutritional dominance over *P. verrucosum (*strain OTA11).

Figure 3.45 shows that at 15 °C and 0.995 a_w *P. verrucosum* (strain OTA11) occupied the same niche as *P. verrucosum* (strains IBT22626 and IBT22626), *A. ochraceus, F. poae, P. aurantiogriseum* and *A. tenuissima* and had nutritional dominance over *F. culmorum, E. repens* and *A. ochraceus.* At 25 °C and 0.995 a_w *P. verrucosum* (strain OTA11) occupied the same niche as *P. verrucosum* (strains IBT22625 and IBT22626), *F. poae, F. culmorum, A. ochraceus, A. tenuissima* and *P. aurantiogriseum* and had nutritional dominance over *E. repens.*

Table 3.35 Niche size and niche index results determined from carbon source utilisation assays encompassing the 18 key carbon sources found in wheat grain. Assays were carried out at two different a_w levels and incubated at 15 °C (A) or 25 °C (B) respectively. Target niche was that of *Penicillium verrucosum* strain OTA11.

A

	0.93 a_w			**0.995 a_w**		
	Niche Size	NOI $_{(P.V)}$	NOI $_{(spp.)}$	Niche Size	NOI $_{(P.V)}$	NOI $_{(spp.)}$
OTA11 (*P. verrucosum*)	17			18		
IBT22626 (*P. verrucosum*)	18	1	0.94	18	1	1
IBT22625 (*P. verrucosum*)	18	1	0.94	18	1	1
IBT21991 (*A. ochraceus*)	13	0.76	1	15	0.83	1
E. repens	15	0.88	1	14	0.78	1
F. culmorum	16	0.94	1	15	0.83	1
F. poae	16	0.94	1	18	1	1
P. aurantiogriseum	16	0.94	1	17	0.94	1
A. tenuissima	6	0.35	1	17	0.94	1

B

	0.93 a_w			**0.995 a_w**		
	Niche Size	NOI $_{(P.V)}$	NOI $_{(spp.)}$	Niche Size	NOI $_{(P.V)}$	NOI $_{(spp.)}$
OTA11 (*P. verrucosum*)	15			18		
IBT22626 (*P. verrucosum*)	13	0.87	1	17	0.94	1
IBT22625 (*P. verrucosum*)	16	1	0.94	18	1	1
IBT21991 (*A. ochraceus*)	13	0.87	1	18	0.83	1
E. repens	14	0.93	1	12	0.67	1
F. culmorum	18	1	0.83	18	1	1
F. poae	18	1	0.83	18	1	1
P. aurantiogriseum	15	1	0.83	18	1	1
A. tenuissima	7	0.54	1	17	0.94	1

Table 3.35 Niche size and niche index results determined from carbon source utilisation assays encompassing the 18 key carbon sources found in wheat grain. Assays were carried out at two different a_w levels and incubated at 15 °C (A) or 25 °C (B) respectively. Target niche was that of *Penicillium verrucosum* strain OTA11.

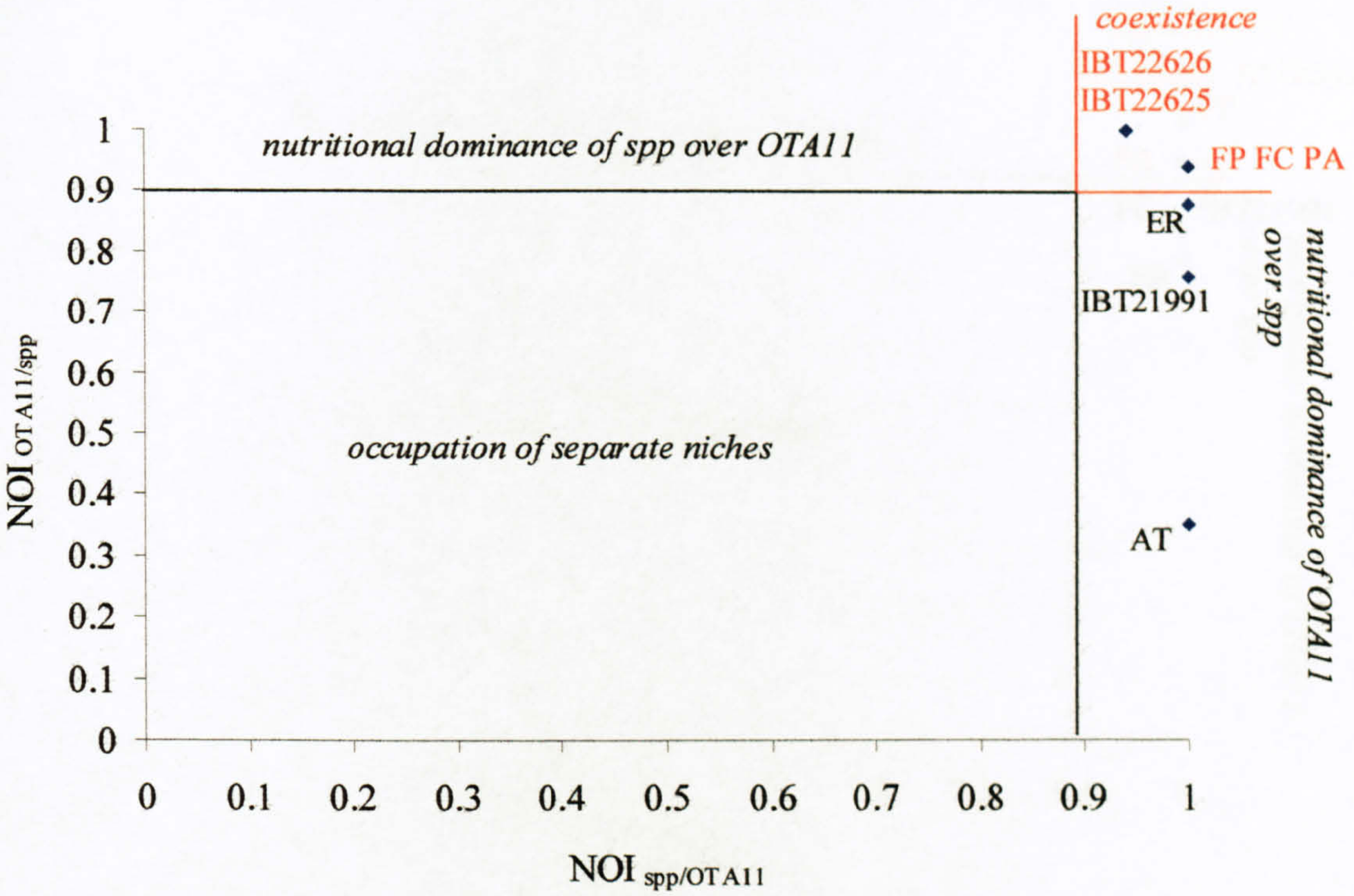
A

	0.93 a_w			**0.995 a_w**		
	Niche Size	NOI $_{(P.V)}$	NOI $_{(spp.)}$	Niche Size	NOI $_{(P.V)}$	NOI $_{(spp.)}$
OTA11 (*P. verrucosum*)	17			18		
IBT22626 (*P. verrucosum*)	18	1	0.94	18	1	1
IBT22625 (*P. verrucosum*)	18	1	0.94	18	1	1
IBT21991 (*A. ochraceus*)	13	0.76	1	15	0.83	1
E. repens	15	0.88	1	14	0.78	1
F. culmorum	16	0.94	1	15	0.83	1
F. poae	16	0.94	1	18	1	1
P. aurantiogriseum	16	0.94	1	17	0.94	1
A. tenuissima	6	0.35	1	17	0.94	1

B

	0.93 a_w			**0.995 a_w**		
	Niche Size	NOI $_{(P.V)}$	NOI $_{(spp.)}$	Niche Size	NOI $_{(P.V)}$	NOI $_{(spp.)}$
OTA11 (*P. verrucosum*)	15			18		
IBT22626 (*P. verrucosum*)	13	0.87	1	17	0.94	1
IBT22625 (*P. verrucosum*)	16	1	0.94	18	1	1
IBT21991 (*A. ochraceus*)	13	0.87	1	18	0.83	1
E. repens	14	0.93	1	12	0.67	1
F. culmorum	18	1	0.83	18	1	1
F. poae	18	1	0.83	18	1	1
P. aurantiogriseum	15	1	0.83	18	1	1
A. tenuissima	7	0.54	1	17	0.94	1

A



B

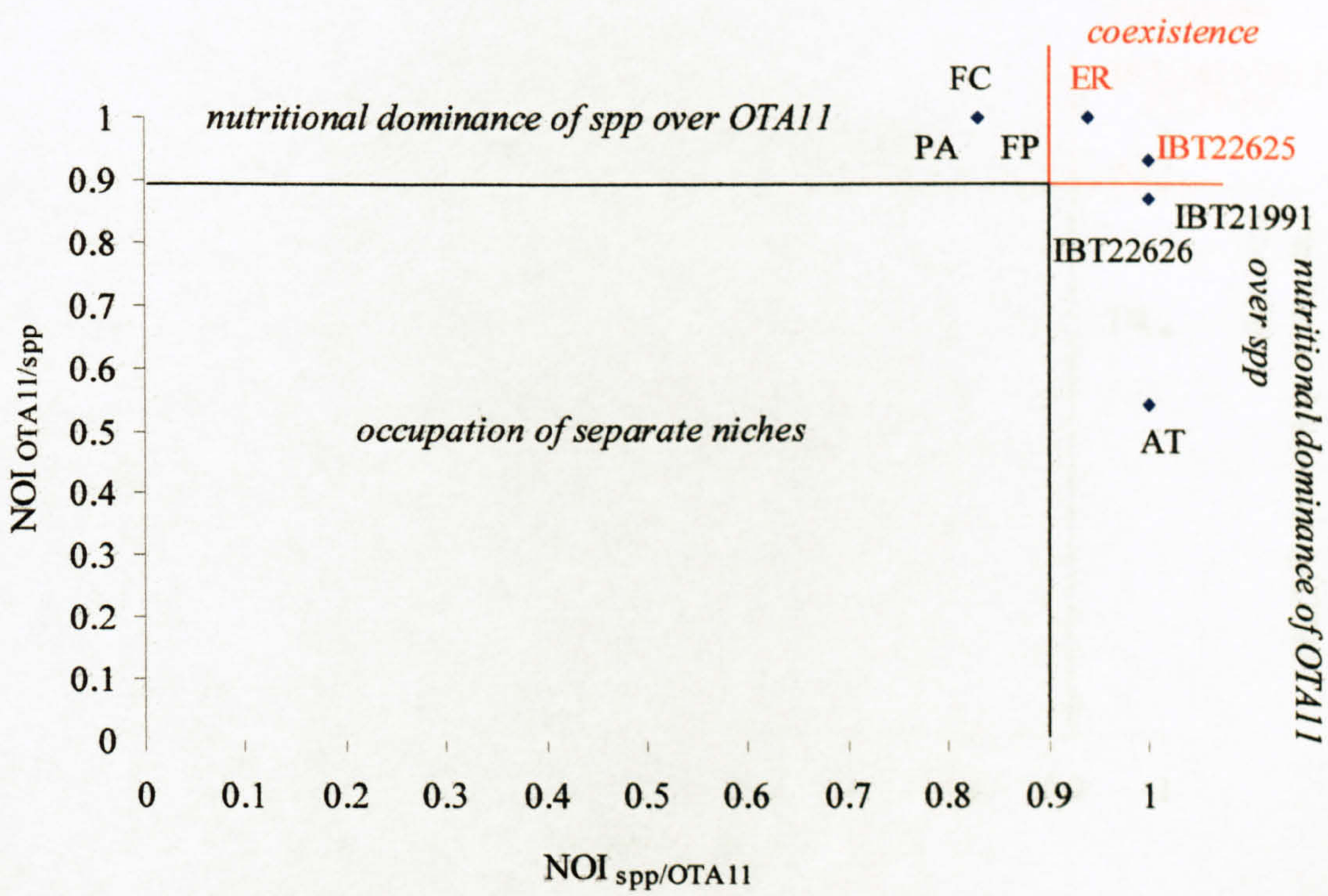


Figure 3.44 Niche map for *P. verrucosum* strain OTA11 encompassing the 18 key carbon sources found in wheat grain. Water activity was adjusted to 0.93 a_w and incubation was at 15 °C (A) or 25 °C (B) respectively. Species key: *P. verrucosum* (strains IBT22625, IBT22626), *A. ochraceus* (IBT21991), *E. repens* (ER), *F. culmorum* (FC), *F. poae* (FP), *P. aurantiogriseum* (PA) and *A. tenuissima* (AT).

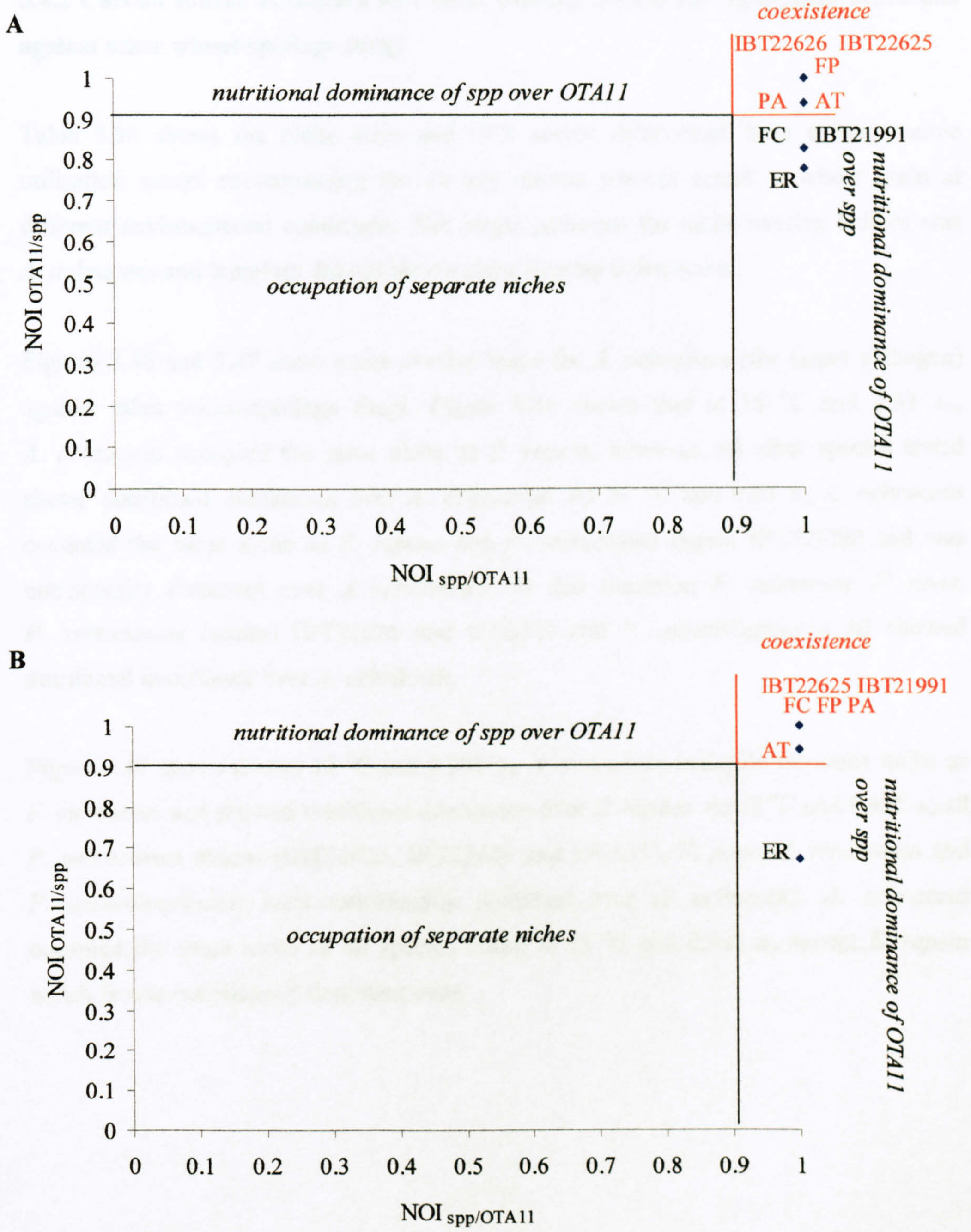


Figure 3.45 Niche map for *P. verrucosum* strain OTA11 encompassing the 18 key carbon sources found in wheat grain. Water activity was adjusted to 0.995 a_w and incubation was at 15 °C (A) or 25 °C (B) respectively. Species key: *P. verrucosum* (strains IBT22625, IBT22626), *A. ochraceus* (IBT21991), *E. repens* (ER), *F. culmorum* (FC), *F. poae* (FP), *P. aurantiogriseum* (PA) and *A. tenuissima* (AT).

3.4.2 Carbon source utilisation and niche overlap indices for *Aspergillus ochraceus* against other wheat-spoilage fungi

Table 3.36 shows the niche sizes and NOI scores determined from carbon source utilisation assays encompassing the 18 key carbon sources found in wheat grain at different environmental conditions. The target pathogen for niche overlap indices was *A. ochraceus* and therefore did not have a niche overlap index score.

Figures 3.46 and 3.47 show niche overlap maps for *A. ochraceus* (the target pathogen) against other wheat-spoilage fungi. Figure 3.46 shows that at 15 °C and 0.93 a_w, *A. ochraceus* occupied the same niche as *E. repens*, however, all other species tested shows nutritional dominance over *A. ochraceus*. At 25 °C and 0.93 a_w *A. ochraceus* occupied the same niche as *E. repens* and *P. verrucosum* (strain IBT22626) and was nutritionally dominant over *A. tenuissima*. At this condition *F. culmorum*, *F. poae*, *P. verrucosum* (strains IBT22626 and OTA11) and *P. aurantiogriseum* all showed nutritional dominance over *A. ochraceus*.

Figure 3.47 shows that at 15 °C and 0.995 a_w *A. ochraceus* occupied the same niche as *F. culmorum* and showed nutritional dominance over *E. repens*. At 15 °C and 0.995 a_w all *P. verrucosum* strains (IBT22625, IBT22626 and OTA11), *F. poae*, *A. tenuissima* and *P. aurantiogriseum* were nutritionally dominant over *A. ochraceus*. *A. ochraceus* occupied the same niche as all species tested at 25 °C and 0.995 a_w except *E. repens* which is was nutritionally dominant over.

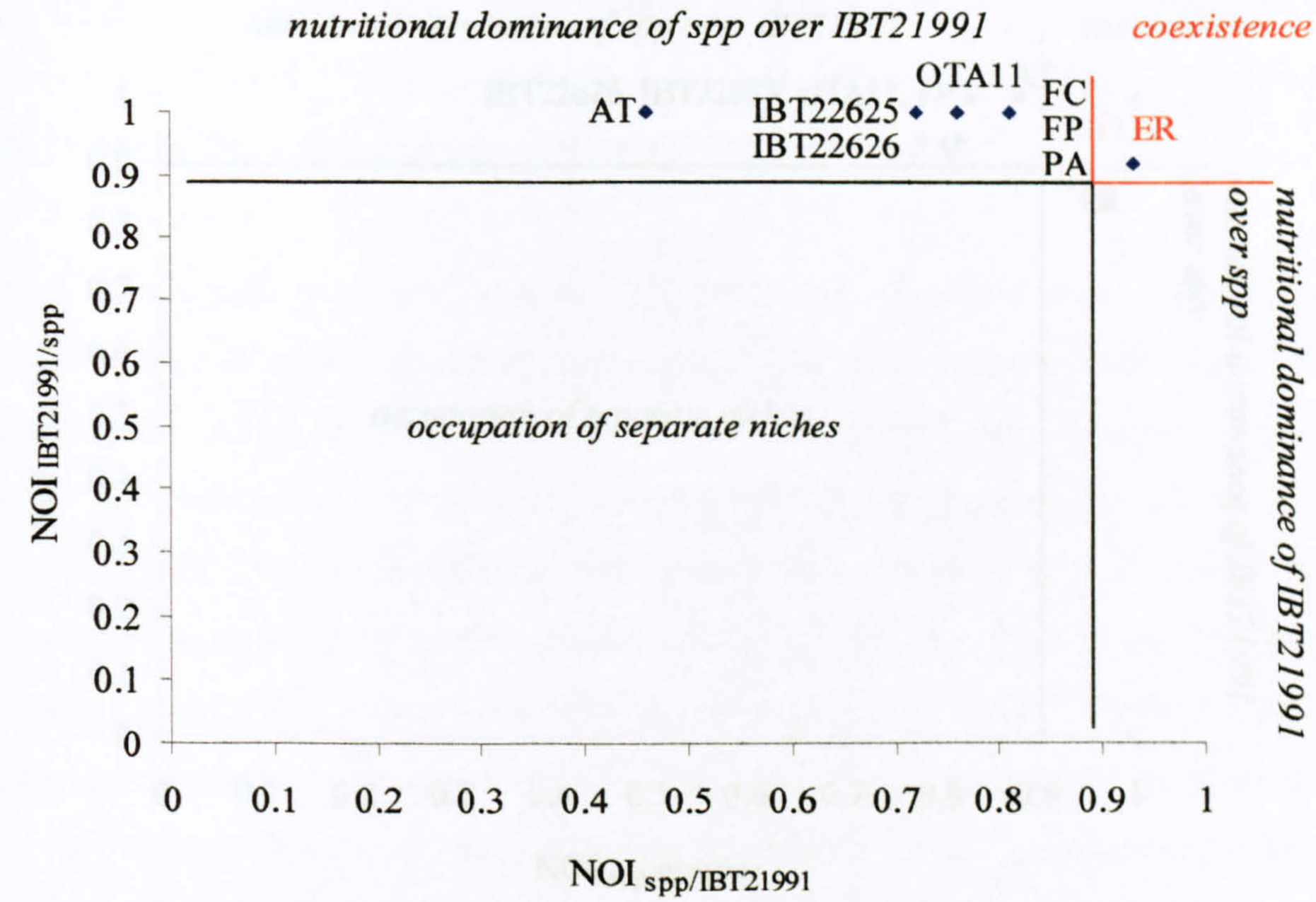
Table 3.36 Niche size and niche index results determined from carbon source utilisation assays encompassing the 18 key carbon sources found in wheat grain. Assays were carried out at two different a_w levels and incubated at 15 °C (A) or 25 °C (B) respectively. Target niche was that of *Aspergillus ochraceus* strain IBT21991.

A

	0.93 a_w			**0.995 a_w**		
	Niche Size	NOI $_{(A.O)}$	NOI $_{(spp.)}$	Niche Size	NOI $_{(A.O.)}$	NOI $_{(spp.)}$
IBT21991 (*A. ochraceus)*	13			15		
IBT22626 (*P. verrucosum*)	18	1	0.72	18	1	0.83
IBT22625 (*P. verrucosum*)	18	1	0.7	18	1	0.83
OTA11 (*P. verrucosum)*	17	1	0.76	18	1	0.83
E. repens	15	0.92	0.93	14	0.87	0.93
F. culmorum	16	1	0.81	15	1	1
F. poae	16	1	0.81	18	1	0.83
P.aurantiogriseum	16	1	0.81	17	0.93	0.82
A. tenuissima	6	0.46	1	17	1	0.88

B

	0.93 a_w			**0.995 a_w**		
	Niche Size	NOI $_{(A.O)}$	NOI $_{(spp.)}$	Niche Size	NOI $_{(A.O.)}$	NOI $_{(spp.)}$
IBT21991 (*A. ochraceus)*	13			18		
IBT22626 (*P. verrucosum*)	13	1	0.92	17	0.94	1
IBT22625 (*P. verrucosum*)	16	1	0.82	18	1	1
OTA11 (*P. verrucosum*)	15	1	0.87	18	1	1
E. repens	14	1	0.92	12	0.75	1
F. culmorum	18	1	0.72	18	1	1
F. poae	18	1	0.72	18	1	1
P.aurantiogriseum	15	1	0.87	18	1	1
A. tenuissima	7	0.39	1	17	0.94	1



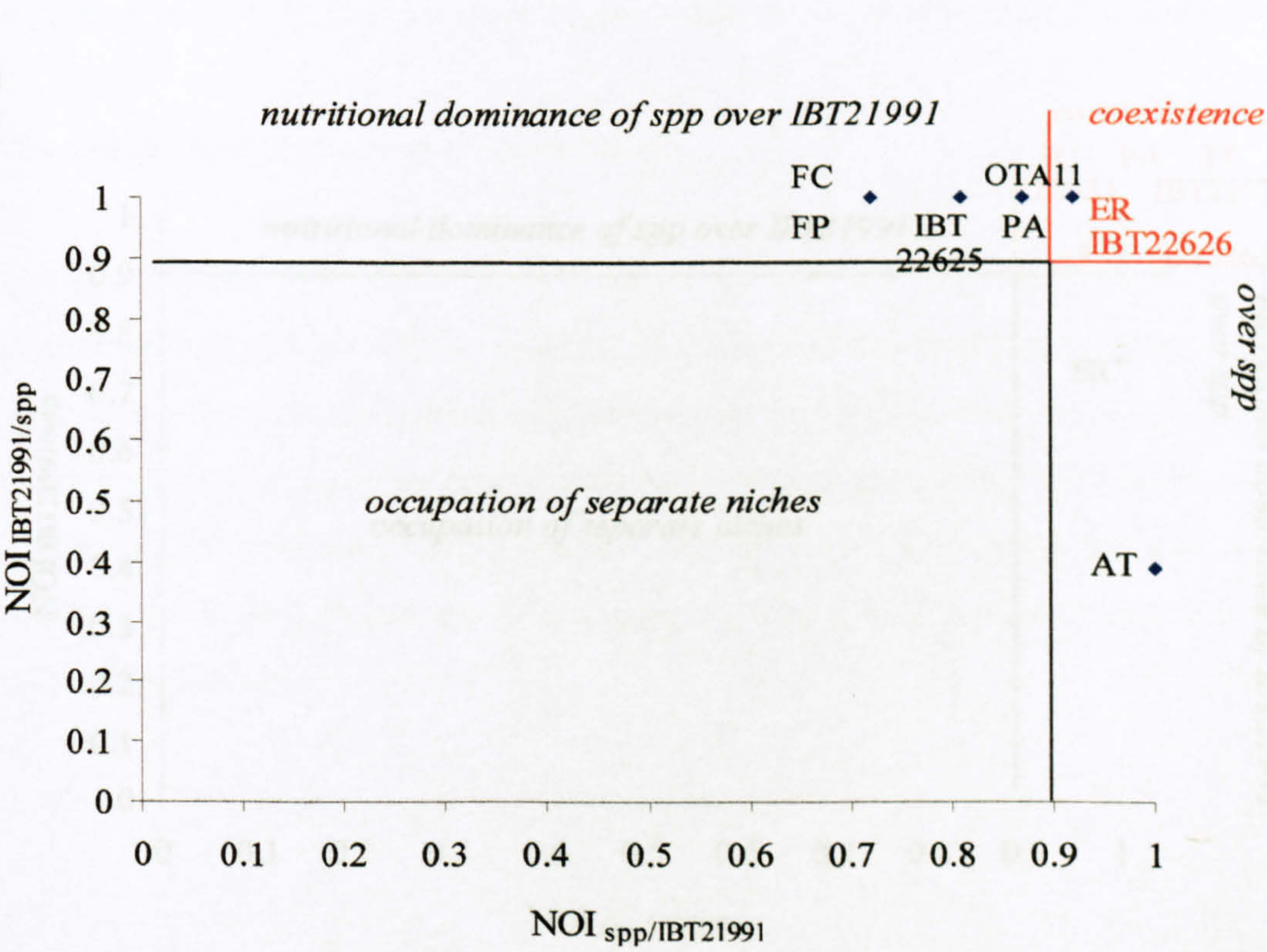


Figure 3.46 Niche map for *A.ochraceus* (IBT21991) encompassing the 18 key carbon sources found in wheat grain. Water activity was adjusted to 0.93 a_w and incubation was at 15 °C (A) or 25 °C (B) respectively. Species key: *P. verrucosum* (strains IBT22625, IBT22626, OTA11), *E. repens* (ER), *F. culmorum* (FC), *F. poae* (FP), *P. aurantiogriseum* (PA) and *A. tenuissima* (AT).

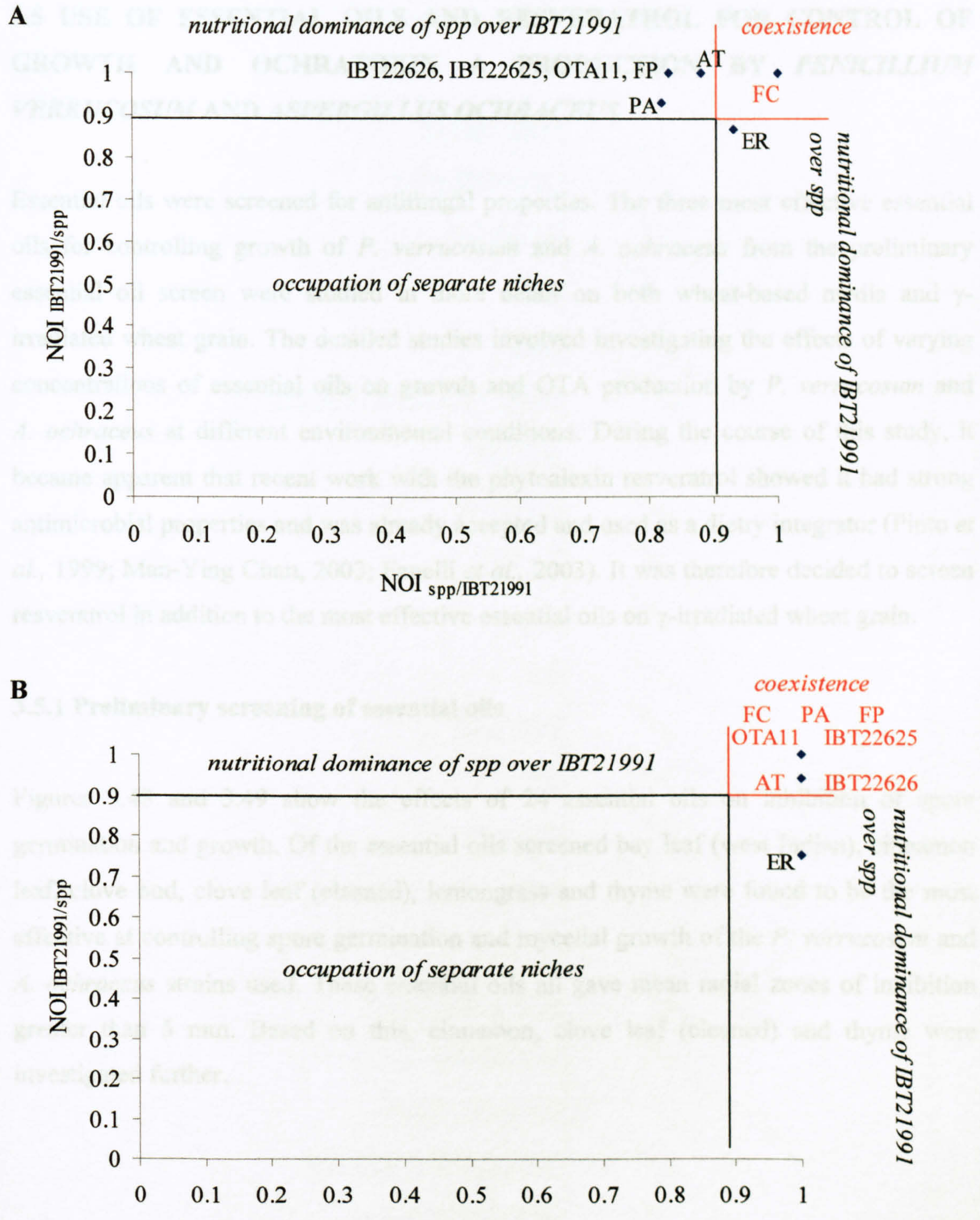


Figure 3.47 Niche map for *A.ochraceus* (IBT21991) encompassing the 18 key carbon sources found in wheat grain. Water activity was adjusted to 0.995 a_w and incubation was at 15 °C (A) or 25 °C (B) respectively. Species key: *P. verrucosum* (strains IBT22625, IBT22626, OTA11), *E. repens* (ER), *F. culmorum* (FC), *F. poae* (FP), *P. aurantiogriseum* (PA) and *A. tenuissima* (AT).

3.5 USE OF ESSENTIAL OILS AND RESVERATROL FOR CONTROL OF GROWTH AND OCHRATOXIN A PRODUCTION BY *PENICILLIUM VERRUCOSUM* AND *ASPERGILLUS OCHRACEUS*

Essential oils were screened for antifungal properties. The three most effective essential oils for controlling growth of *P. verrucosum* and *A. ochraceus* from the preliminary essential oil screen were studied in more detail on both wheat-based media and γ-irradiated wheat grain. The detailed studies involved investigating the effects of varying concentrations of essential oils on growth and OTA production by *P. verrucosum* and *A. ochraceus* at different environmental conditions. During the course of this study, it became apparent that recent work with the phytoalexin resveratrol showed it had strong antimicrobial properties and was already accepted and used as a dietry integrator (Pinto *et al.,* 1999; Man-Ying Chan, 2003; Fanelli *et al.,* 2003). It was therefore decided to screen resveratrol in addition to the most effective essential oils on γ-irradiated wheat grain.

3.5.1 Preliminary screening of essential oils

Figures 3.48 and 3.49 show the effects of 24 essential oils on inhibition of spore germination and growth. Of the essential oils screened bay leaf (west Indian), cinnamon leaf, clove bud, clove leaf (cleaned), lemongrass and thyme were found to be the most effective at controlling spore germination and mycelial growth of the *P. verrucosum* and *A. ochraceus* strains used. These essential oils all gave mean radial zones of inhibition greater than 5 mm. Based on this, cinnamon, clove leaf (cleaned) and thyme were investigated further.

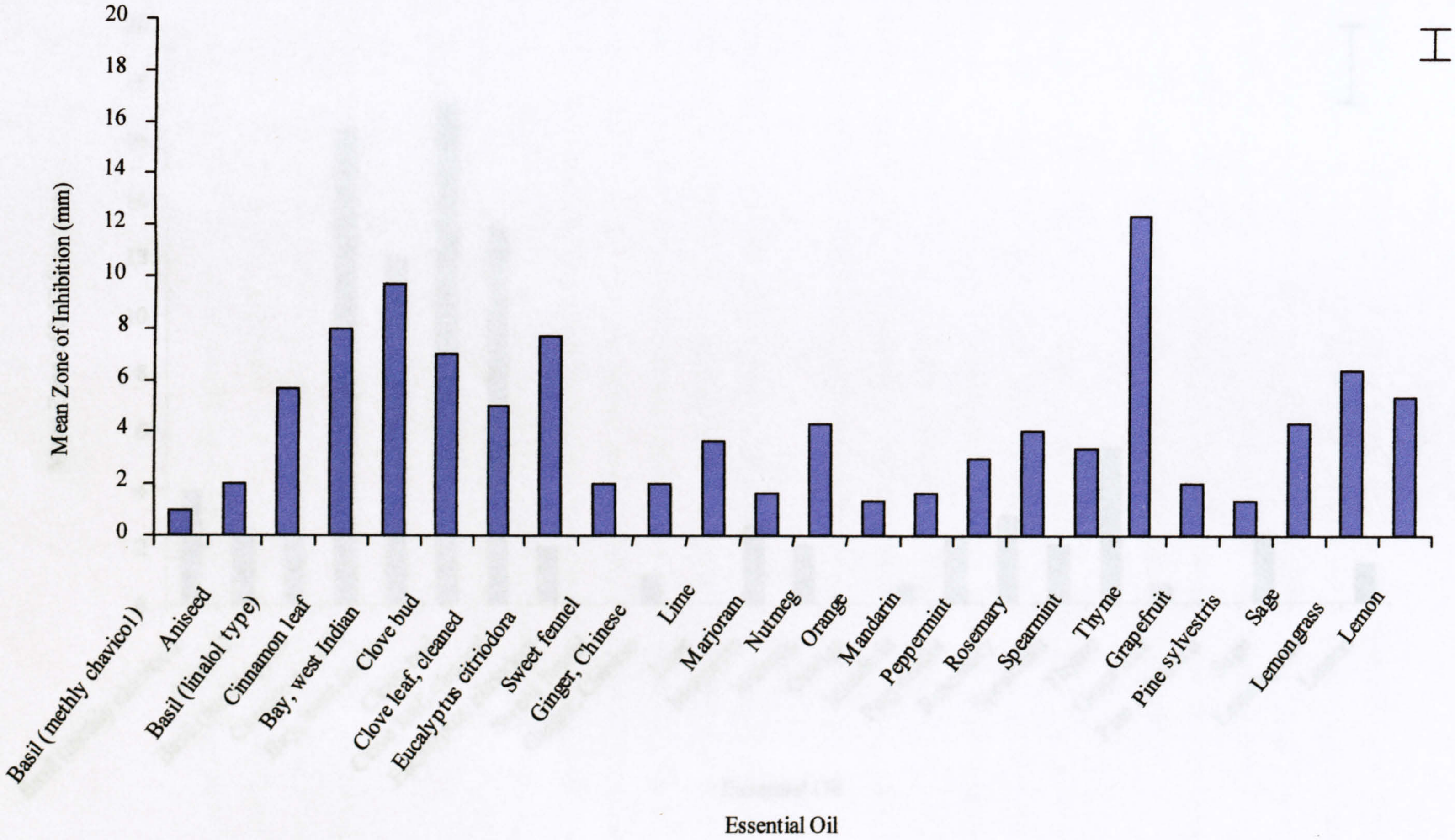


Figure 3.48 Zones of inhibition of *Penicillium verrucosum* (strain OTA11) when exposed to various essential oils diluted 1:10 methanol after 48 hours at 25 °C on a 2 % wheat-based media at 0.995 a_w. Bar indicates Least Significant Difference (LSD) at $p < 0.05$.

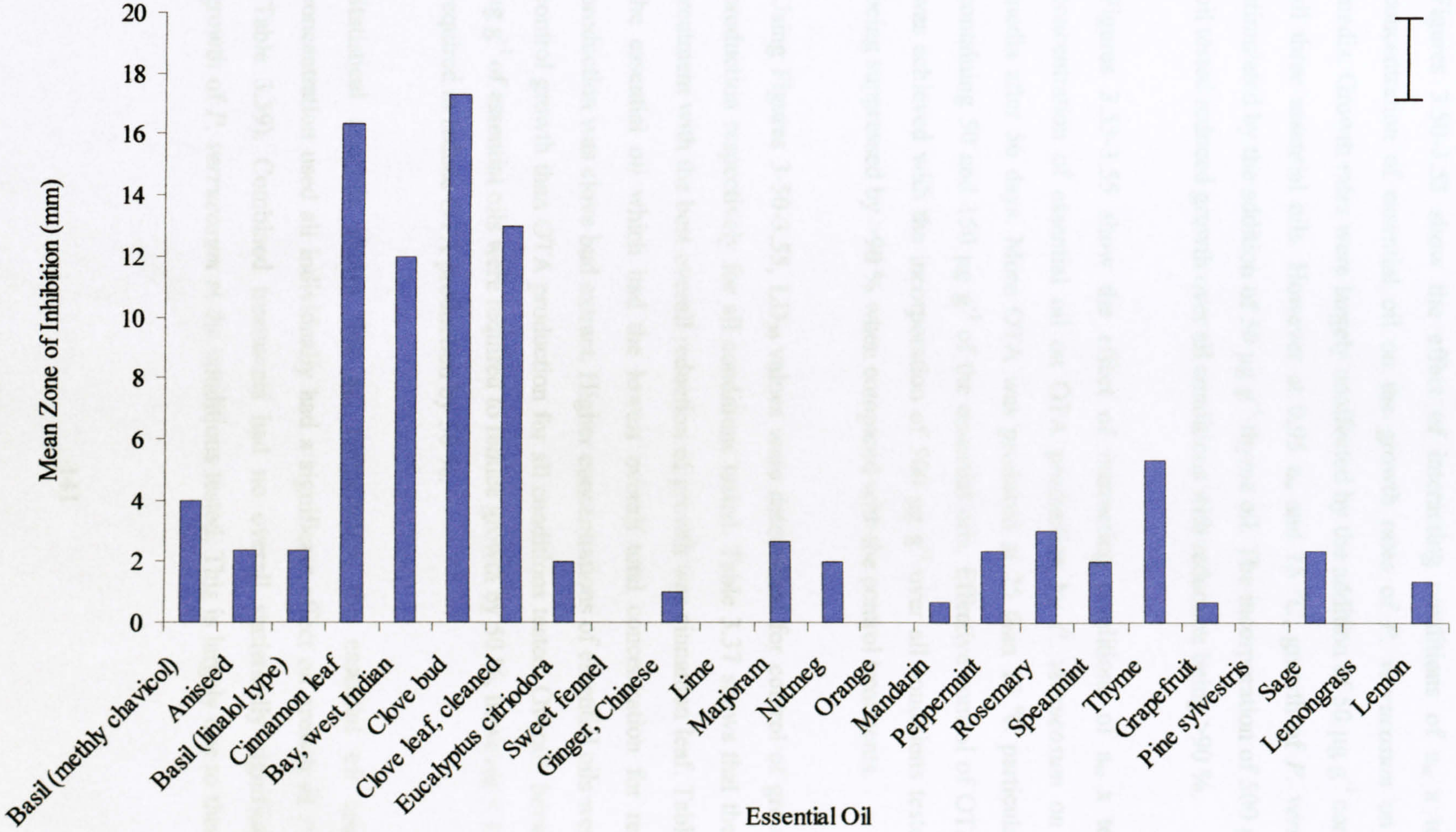


Figure 3.49 Zones of inhibition of *Aspergillus ochraceus* (strain IBT21991) when exposed to various essential oils diluted 1:10 methanol after 48 hours at 25 °C on a 2 % wheat-based media at 0.995 a_e. Bar indicates Least Significant Difference (LSD) at $p < 0.05$.

3.5.2 Detailed study on the efficacy of essential oils on growth and ochratoxin A production by *Penicillium verrucosum* on wheat-based media

Figures 3.50-3.53 show the effect of interacting conditions of a_w x temperature x concentration of essential oil on the growth rates of *P. verrucosum* on wheat-based media. Growth rates were largely unaffected by the addition of 50 μg g^{-1} concentration of all three essential oils. However at 0.95 a_w and 15 °C, growth of *P. verrucosum* was stimulated by the addition of 50 μg g^{-1} thyme oil. The incorporation of 500 μg g^{-1} of each oil tested reduced growth over all conditions with reduction being >90 %.

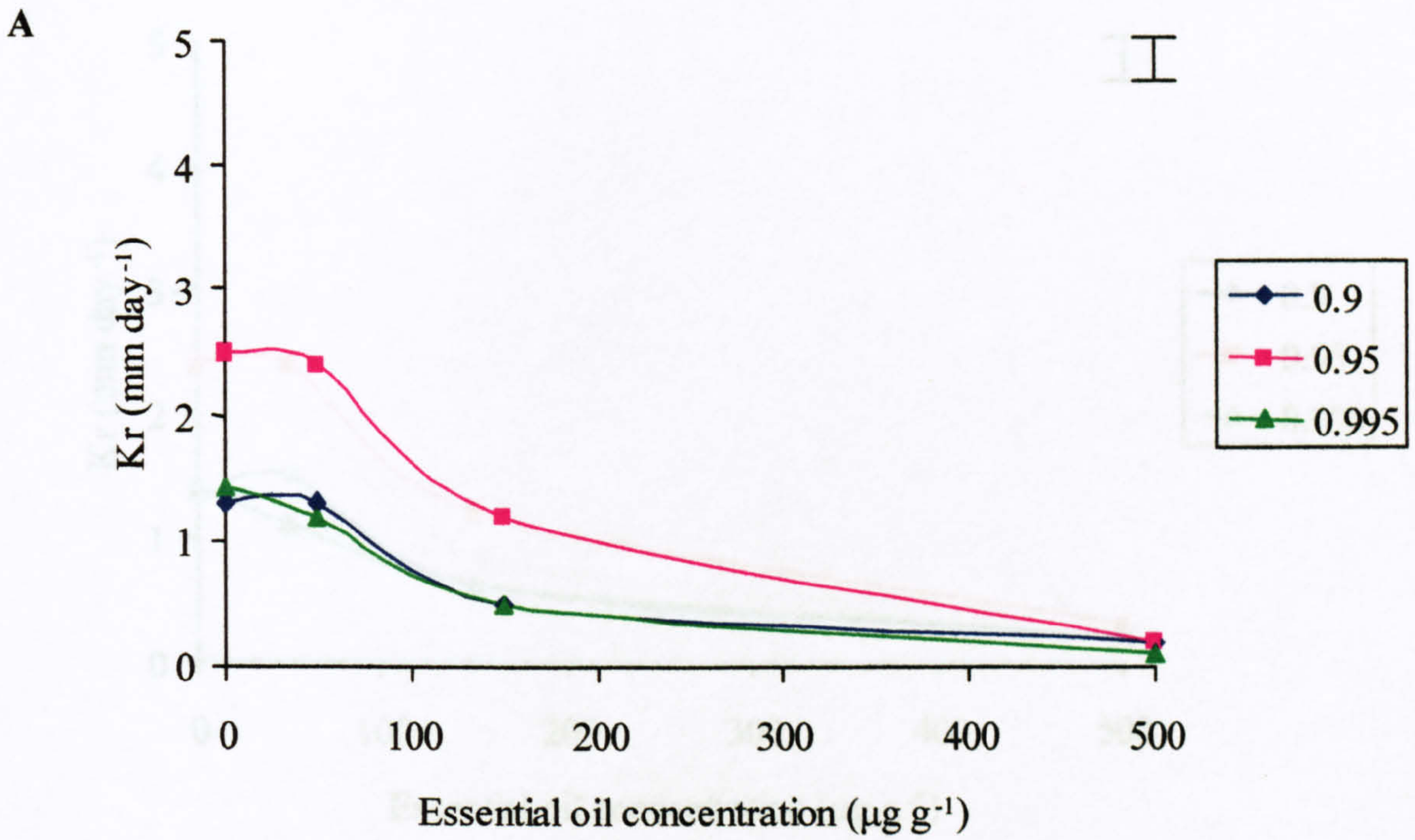
Figures 3.53-3.55 show the effect of interacting conditions of a_w x temperature x concentration of essential oil on OTA production by *P. verrucosum* on wheat-based media after 56 days. More OTA was produced at 25 than 15 °C particularly in media containing 50 and 150 μg g^{-1} of the essential oils. Effective control of OTA production was achieved with the incorporation of 500 μg g^{-1} over all conditions tested with OTA being suppressed by >90 % when compared with the control treatments.

Using Figures 3-50-3.55, LD_{50} values were determined for control of growth and OTA production respectively for all conditions tested. Table 3.37 shows that the essential oil treatment with the best overall reduction of growth was cinnamon leaf. Table 3.38 shows the essential oil which had the lowest overall total concentration for reducing OTA production was clove bud extract. Higher concentrations of essential oils were required to control growth than OTA production for all conditions tested. Overall, between 100–300 μg g^{-1} of essential oils were required to reduce growth by 50 %, however < 130 μg g^{-1} was required to reduce OTA production by 50 %.

Statistical analysis shows that a_w, temperature, the essential oil tested and the concentration used all individually had a significant effect on growth of *P. verrucosum* (Table 3.39). Combined treatments had no overall statistically significant effect on growth of *P. verrucosum* at the conditions tested. This is largely due to there being little

variation between treatments at 0.90 a_w regardless of temperature as there was very little growth to start with in the control treatments.

Statistical analysis shows that a_w, temperature and the concentration of essential oil tested all had a significant effect on OTA production by *P. verrucosum* (Table 3.40). There was no significant difference between the three essential oils tested on OTA production. Overall, these variables in combination had no significant effect on OTA production. This is largely due to there being no significant difference between the three essential oils tested. This is confirmed in Appendix I (Tables I.38-I.43).



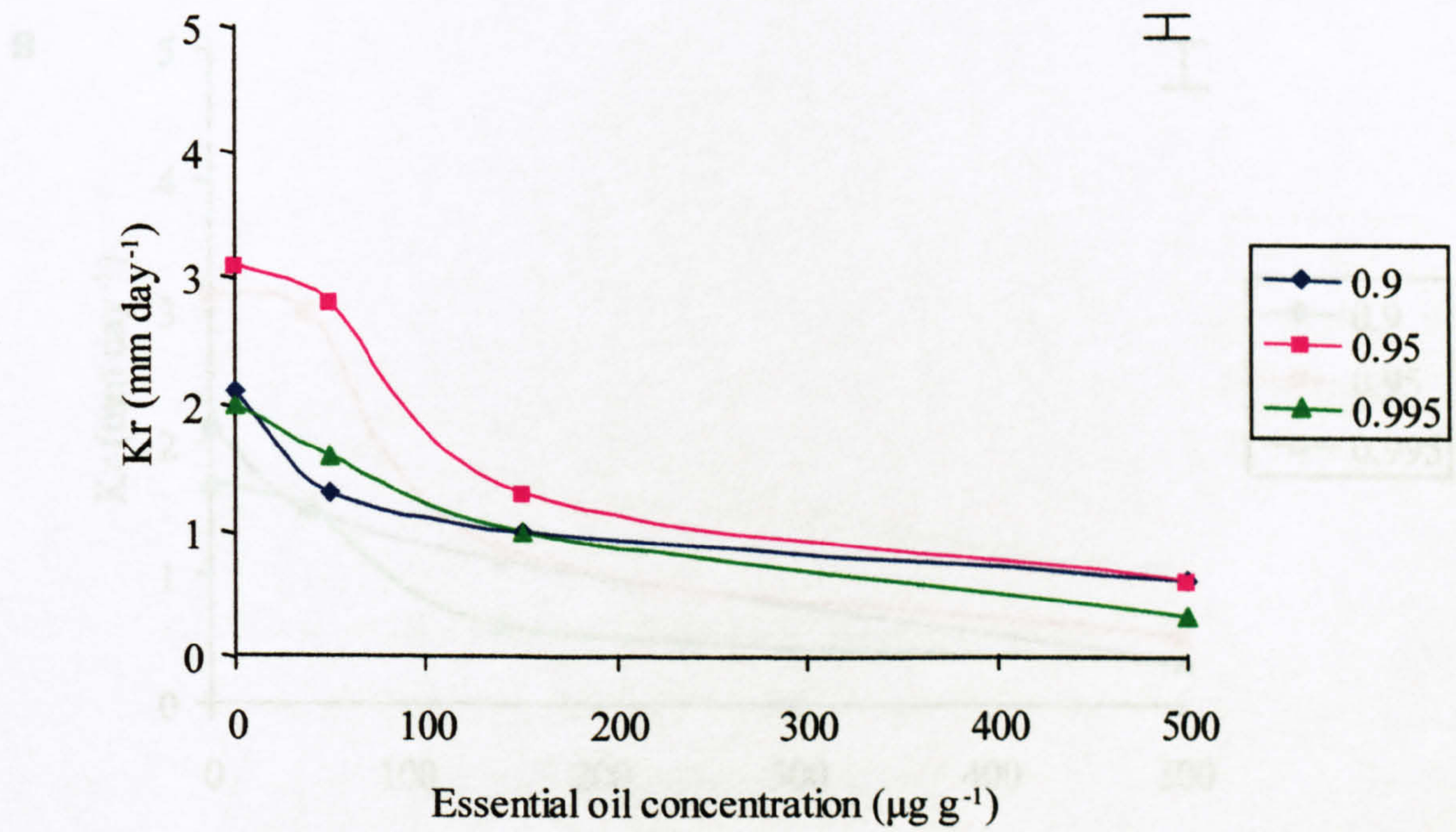
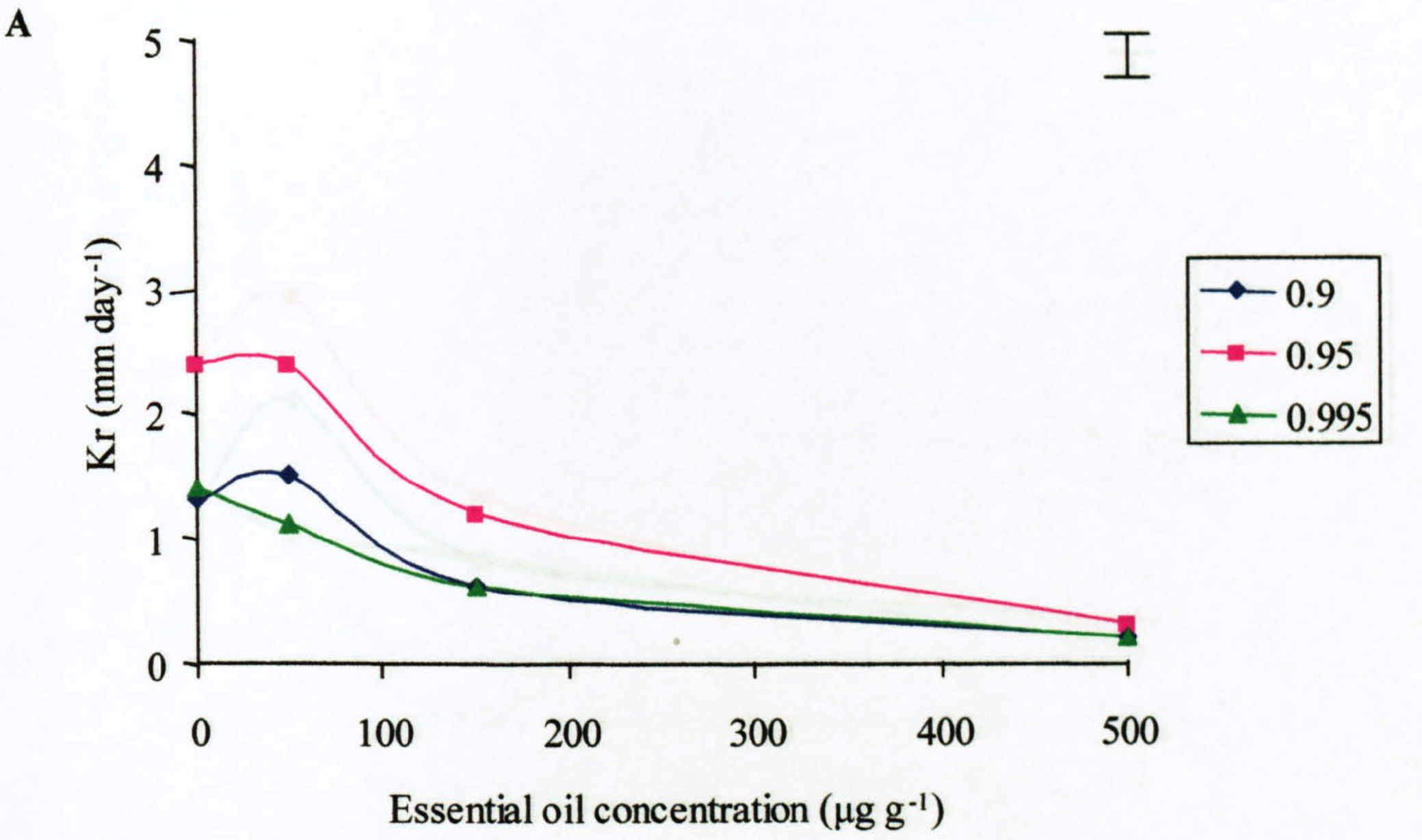


Figure 3.50 Radial extension rates of *Penicillium verrucosum* inoculated on a 2 % wheat-based media at different water activities (0.90, 0.95, 0.995 a_w) and supplemented with 0, 50, 150 or 500 ug g^{-1} of cinnamon essential oil at A) 15 °C or B) 25 °C respectively. Bars indicate Least Significant Difference (LSD) at $p < 0.05$.



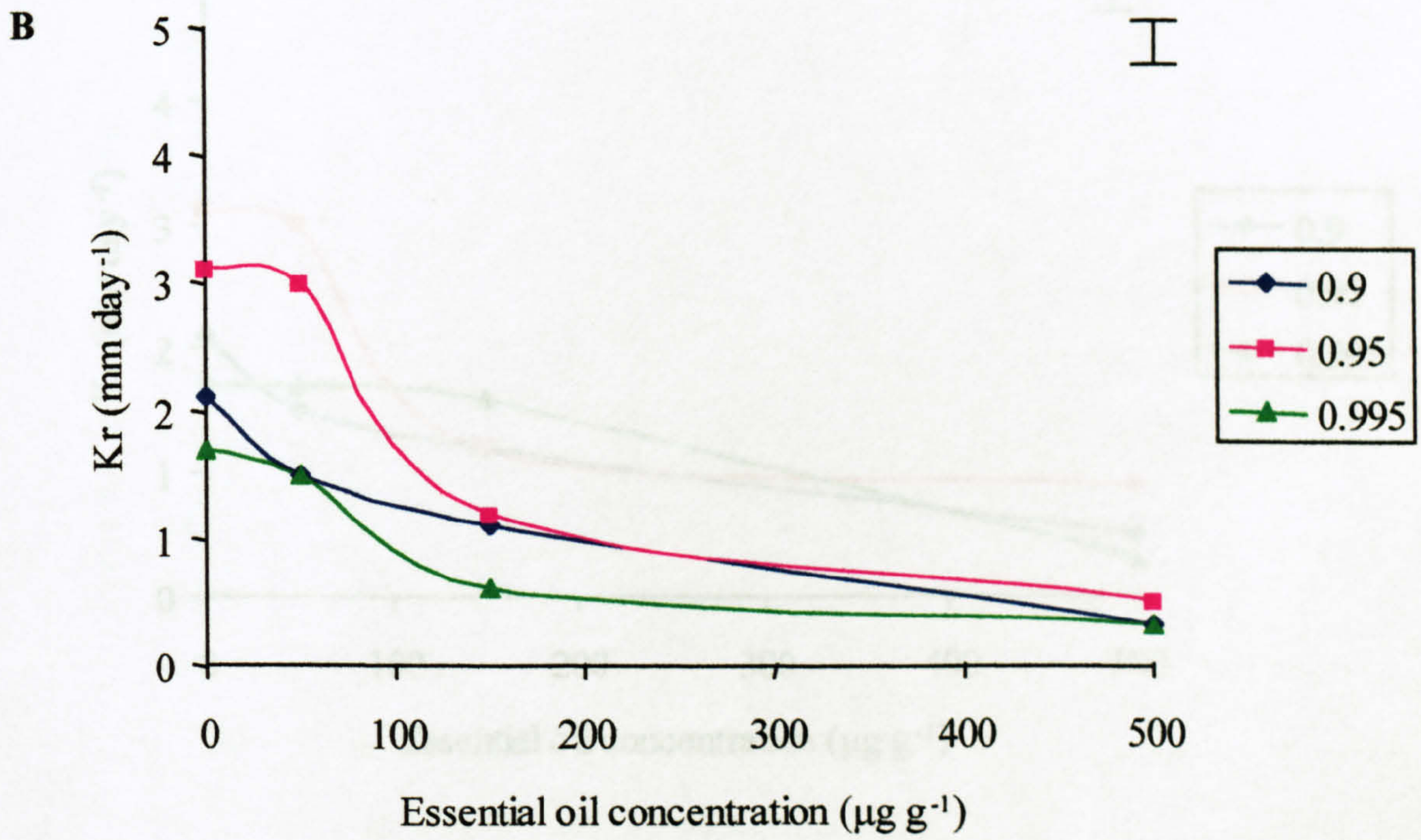


Figure 3.51 Radial extension rates of *Penicillium verrucosum* inoculated on a 2 % wheat-based media at different water activities (0.90, 0.95, 0.995 a_w) and supplemented with 0, 50, 150 or 500 ug g^{-1} of clove bud essential oil at A) 15 °C or B) 25 °C respectively. Bars indicate Least Significant Difference (LSD) at $p < 0.05$.

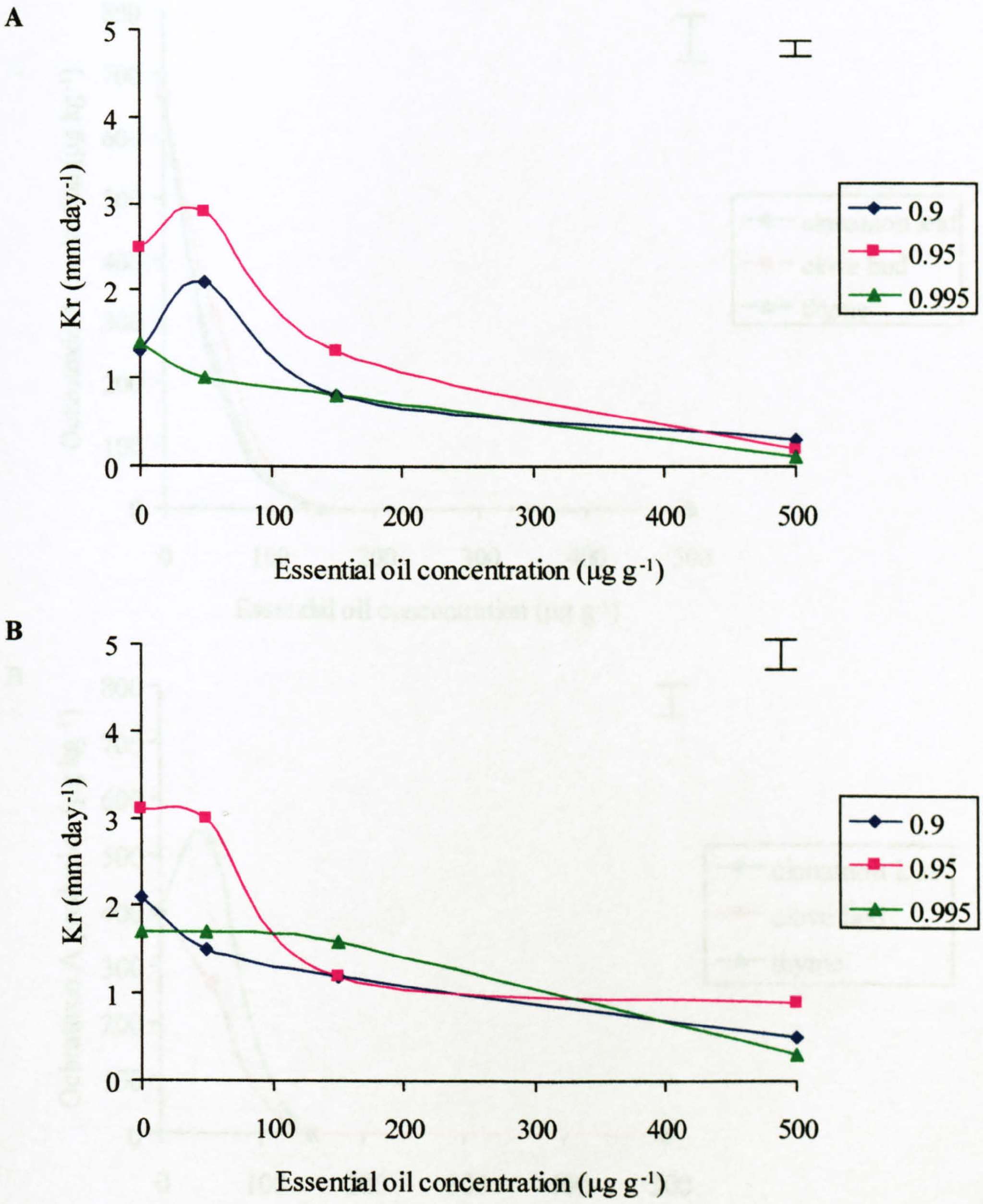


Figure 3.52 Radial extension rates of *Penicillium verrucosum* inoculated on a 2 % wheat-based media at different water activities (0.90, 0.95, 0.995 a_w) and supplemented with 0, 50, 150 or 500 ug g^{-1} of thyme essential oil at A) 15 °C or B) 25 °C respectively. Bars indicate Least Significant Difference (LSD) at $p < 0.05$.

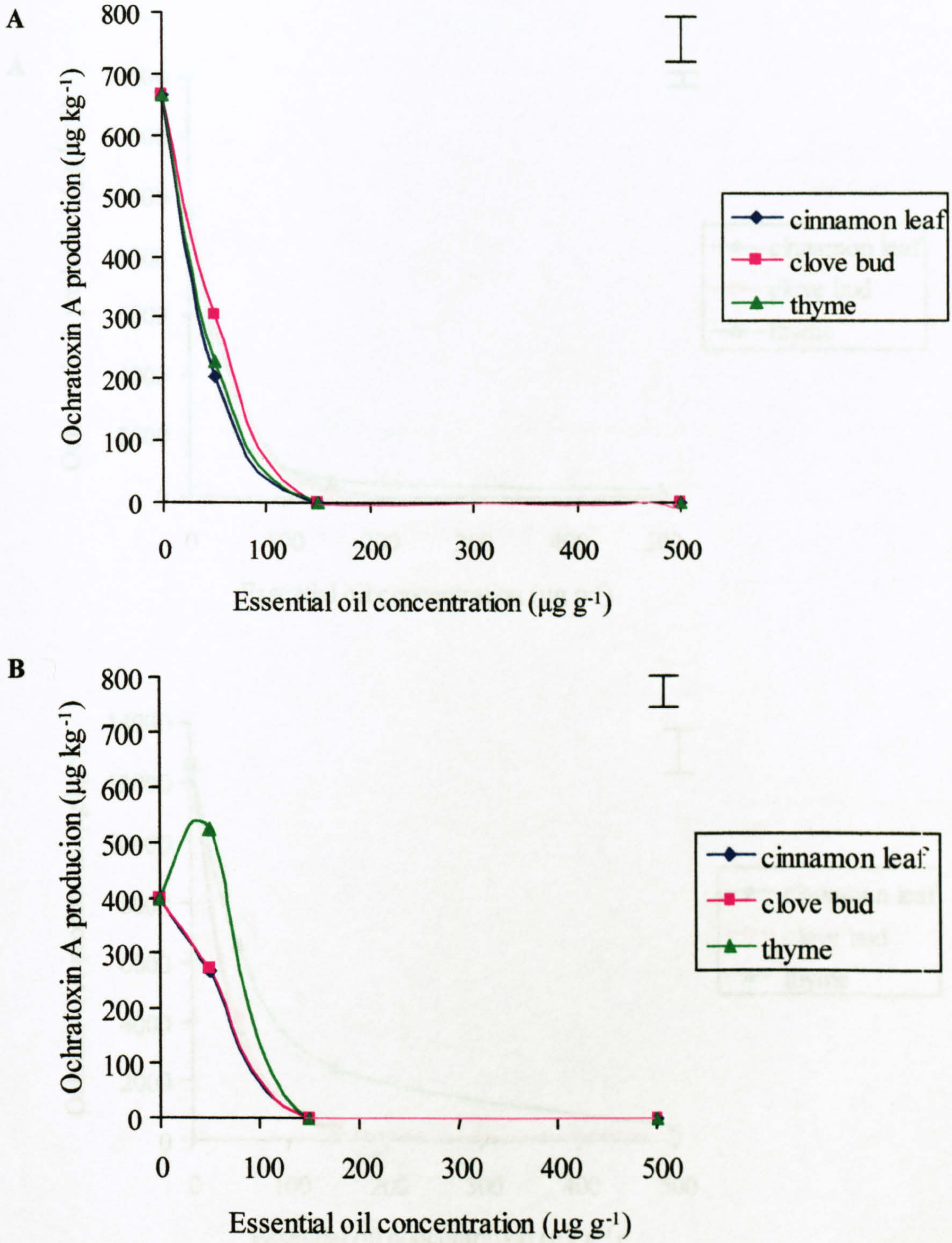


Figure 3.53 Effect of various concentrations of the essential oils cinnamon leaf, clove bud and thyme on ochratoxin A production by *Penicillium verrucosum* on a 2 % wheat-based media after 56 days at A) 0.90 a_w at 15 °C B) 0.90 a_w at 25 °C. Bars indicate Least Significant Difference (LSD) at $p < 0.05$.

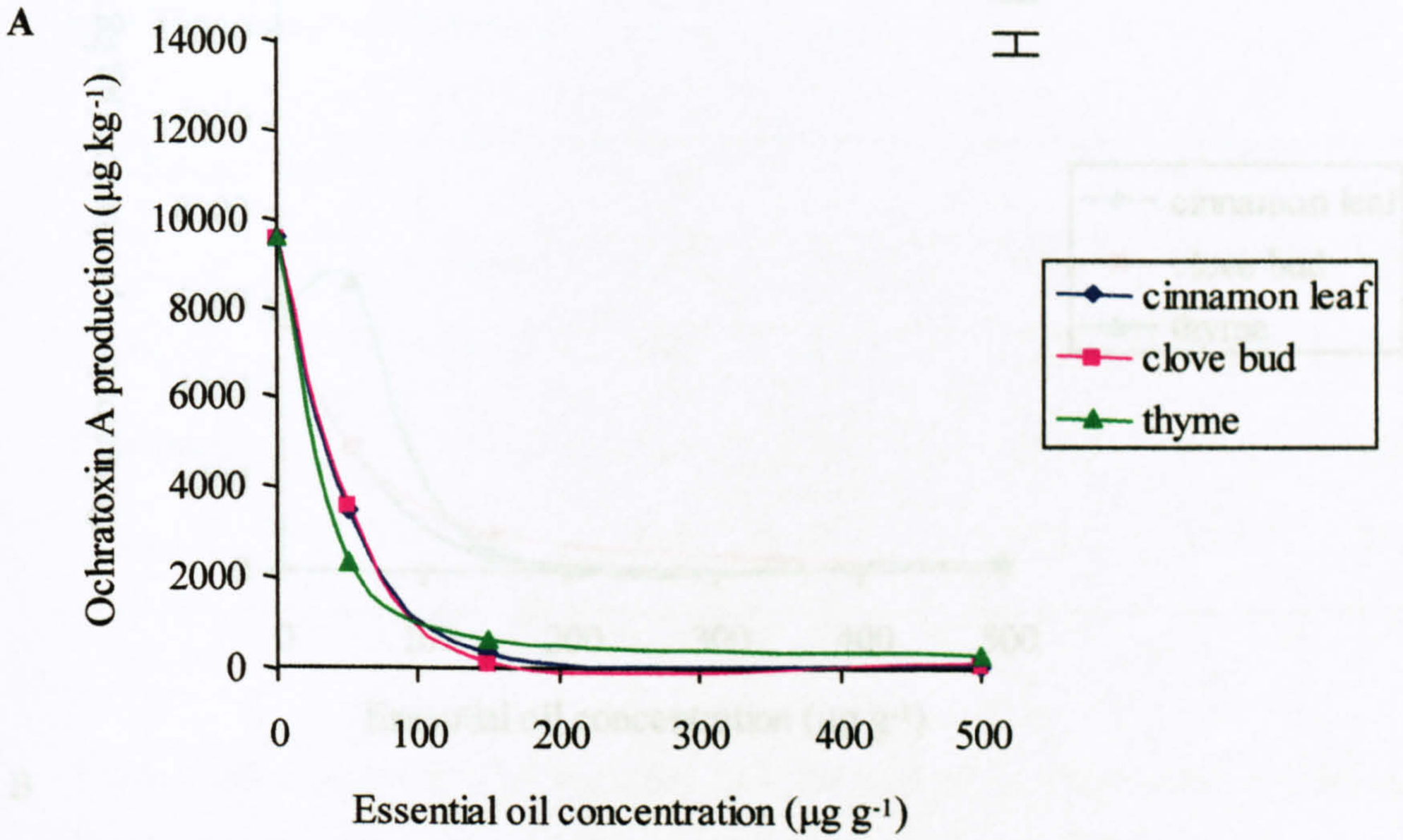


Figure 3.54 Effect of various concentrations of the essential oils cinnamon leaf, clove bud and thyme on ochratoxin A production by *Penicillium verrucosum* on a 2 % wheat-based media after 56 days at A) 0.95 a_w at 15 °C B) 0.95 a_w at 25 °C. Bars indicate Least Significant Difference (LSD) at $p < 0.05$.

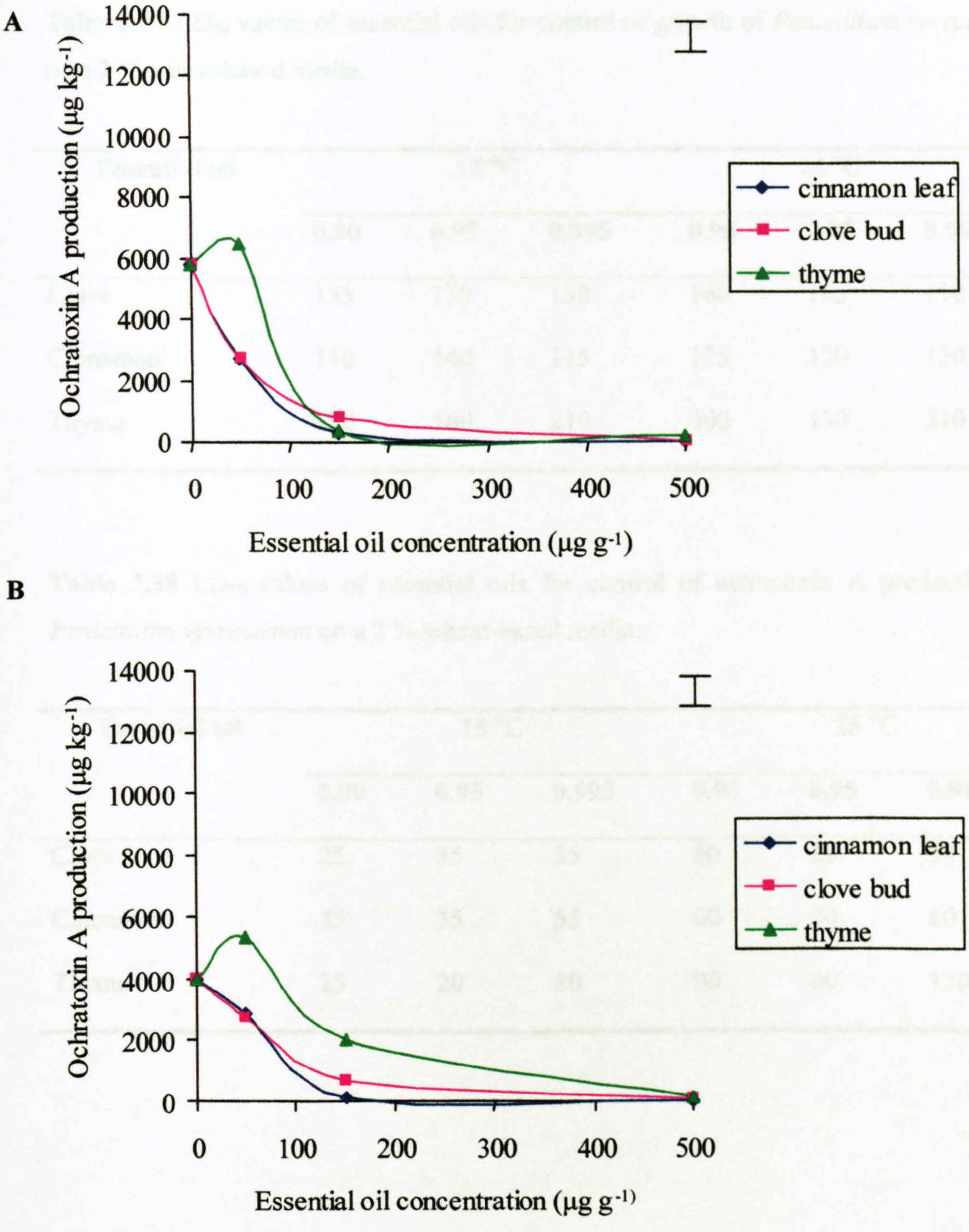


Figure 3.55 Effect of various concentrations of the essential oils cinnamon leaf, clove bud and thyme on ochratoxin A production by *Penicillium verrucosum* on a 2 % wheat-based media after 56 days at A) 0.995 a_w at 15 °C B) 0.995 a_w at 25 °C. Bars indicate Least Significant Difference (LSD) at $p < 0.05$.

Table 3.37 LD_{50} values of essential oils for control of growth of *Penicillium verrucosum* on a 2 % wheat-based media.

Essential oil	15 °C			25 °C		
	0.90	0.95	0.995	0.90	0.95	0.995
Clove	155	150	150	160	165	110
Cinnamon	110	140	115	155	120	150
Thyme	190	160	210	300	130	310

Table 3.38 LD_{50} values of essential oils for control of ochratoxin A production by *Penicillium verrucosum* on a 2 % wheat-based media.

Essential oil	15 °C			25 °C		
	0.90	0.95	0.995	0.90	0.95	0.995
Clove	25	35	55	60	20	80
Cinnamon	45	35	55	60	20	80
Thyme	25	20	80	90	60	130

Table 3.39 ANOVA for growth of *Penicillium verrucosum* on a 2 % wheat-based media at various water activities incorporated with cinnamon, clove or thyme essential oils at various concentrations at 15 or 25 °C respectively. * Indicates factor elicited a significant effect ($p < 0.05$).

Source of Variation	DF	SS	MS	F	P
a_w	2	22.3814	11.1907	213.35	<0.001*
Temperature	1	6.35274	6.3524	121.11	<0.001*
Treatment	2	0.7451	0.3725	7.10	0.001*
Concentration	3	99.8730	33.2710	634.69	<0.001*
Temperature x a_w	2	0.3256	0.1628	3.10	0.048*
Treatment x a_w	4	0.4884	0.1221	2.33	0.059
Concentration x a_w	6	16.6821	2.7803	53.01	<0.001*
Concentration x temperature	3	1.0222	0.3407	6.50	<0.001*
Treatment x concentration	6	0.5231	0.0872	1.66	0.134
Treatment x temperature	2	0.1034	0.0517	0.99	0.376
Concentration x a_w x temperature	6	2.2434	0.3739	7.13	<0.001*
Concentration x a_w x treatment	12	2.1146	0.1762	3.36	<0.001*
Temperature x a_w x treatment	4	0.3421	0.0905	1.73	0.147
Concentration x temperature x treatment	6	0.5047	0.0841	1.60	0.150
Concentration x a_w x temperature x treatment	12	0.7876	0.0656	1.25	0.254
Residual	144	7.5532	0.0525		
Total	215	162.0623			

Table 3.40 ANOVA for toxin production by *Penicillium verrucosum* after 56 days on a 2 % wheat-based media at various water activities incorporated with cinnamon, clove or thyme essential oils at various concentrations at 15 or 25 °C respectively. * Indicates factor elicited a significant effect ($p < 0.05$).

Source of Variation	DF	SS	MS	F	P
a_w	2	570721955	260360978	68.62	<0.001*
Temperature	1	46620279	46620279	12.29	0.001*
Treatment	2	12605923	6302962	1.66	0.194
Concentration	3	1118773194	372924398	98.28	<0.001*
Temperature x a_w	2	25355982	12677991	3.34	0.038*
Treatment x a_w	4	6719719	1679930	0.44	0.778
Concentration x a_w	6	642581130	107096855	28.22	<0.001*
Concentration x temperature	3	39322088	13107363	3.45	0.018*
Treatment x concentration	6	10314504	1719084	0.45	0.842
Treatment x temperature	2	7795569	3897785	1.03	0.361
Concentration x a_w x temperature	6	29240844	4873474	1.28	0.268
Concentration x a_w x treatment	12	10667258	888938	0.23	0.996
Temperature x a_w x treatment	4	4024279	1006070	0.27	0.900
Concentration x temperature x treatment	6	9084561	1514093	0.40	0.879
Concentration x a_w x temperature x treatment	12	4769161	397430	0.10	1.000
Residual	144	546396135	3794418		
Total	215	3034992582			

3.5.3 Detailed study on the efficacy of essential oils on growth and ochratoxin A production by *Penicillium verrucosum* on γ-irradiated wheat grain

Figures 3.56-3.59 show the effect of interacting conditions of a_w x temperature x concentration of essential oil or resveratrol treatments on growth of *P. verrucosum* on γ-irradiated wheat grain. Growth rates were generally unaffected by the addition of 50 μg g^{-1} concentration of all three essential oils and resveratrol However, thyme essential oil stimulated growth of *P. verrucosum* at 0.995 a_w at both temperatures tested and resveratrol stimulated OTA production at 0.995 a_w and 25 °C. The incorporation of 500 μg g^{-1} of each essential oil or resveratrol reduced growth rates over all conditions with reduction being more than 80 %.

Figures 3.60-3.63 show the effect of interacting conditions of a_w x temperature x concentration of essential oil or resveratrol on OTA production by *P. verrucosum* on γ-irradiated wheat grain after 28 days. Significantly more OTA was produced at 25 °C than 15 °C particularly on grain containing 50 μg g^{-1} and 200 μg g^{-1} concentration of treatments. OTA production was stimulated with the addition of 50 μg g^{-1} thyme at 0.995 a_w at both temperatures tested and resveratrol at 0.995 a_w and 25 °C. Effective control of OTA production was achieved with the incorporation of 500 μg g^{-1} over all conditions tested with OTA being suppressed by more than 80 % when compared with the control treatments.

Using Figures 3.56-3.63 LD_{50} values were determined for control of growth and OTA production respectively for all conditions tested. Table 3.41 and 3.42 show that resveratrol had the lowest overall total concentration for reducing growth and OTA production by 50 % respectively. Higher concentrations of essential oils and resveratrol were required for control of growth than OTA production for all conditions tested. Furthmore, higher concentrations of essential oils were required to reduce growth and OTA production by 50 % on γ-irradiated wheat grain than 2 % wheat-based media.

Statistical analysis shows that a_w x temperature x treatment x concentration had a significant effect on growth of *P. verrucosum* on γ-irradiated wheat grain (Table 3.43). Statistical analysis shows that overall a_w x temperature x treatment x concentration had no significant effect on OTA production (Table 3.44).

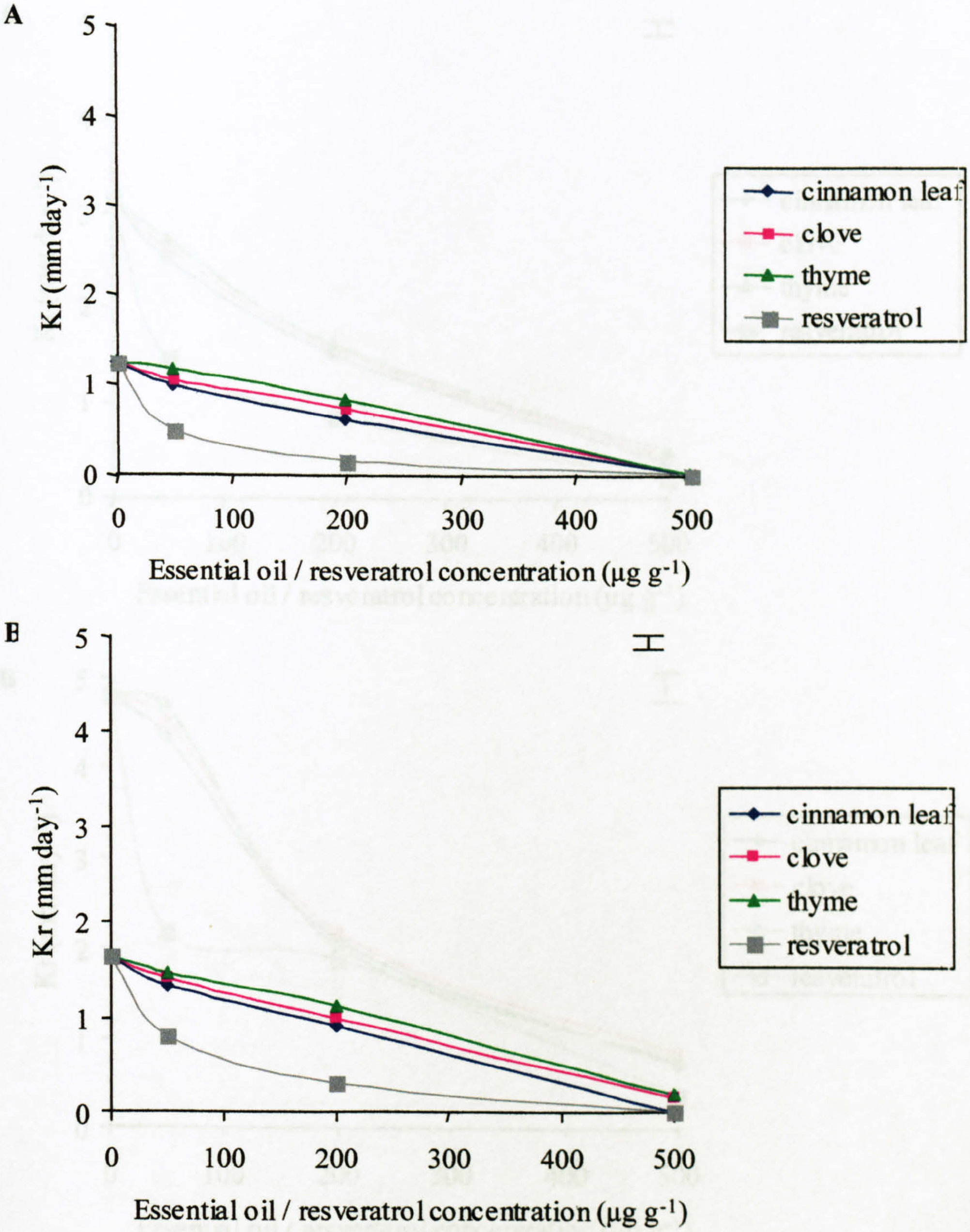


Figure 3.56 Effect of various concentrations of resveratrol and the essential oils cinnamon leaf, clove bud and thyme on growth of *Penicillium verrucosum* on γ-irradiated wheat grain at A) 0.90 a_w at 15 °C B) 0.90 a_w at 25 °C. Bar indicates Least Significant Difference (LSD) at $p < 0.05$. LSD for A) is 0.037.

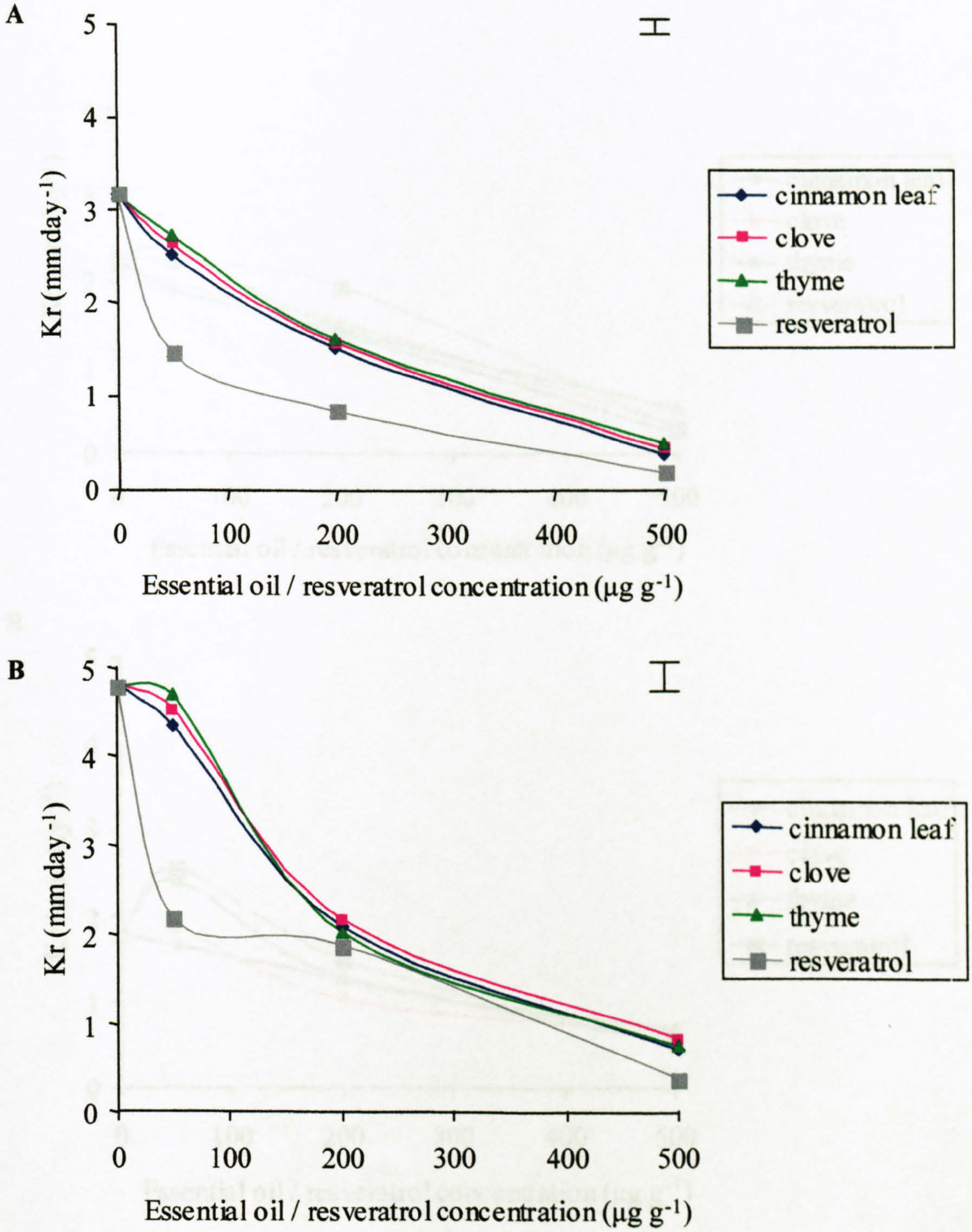
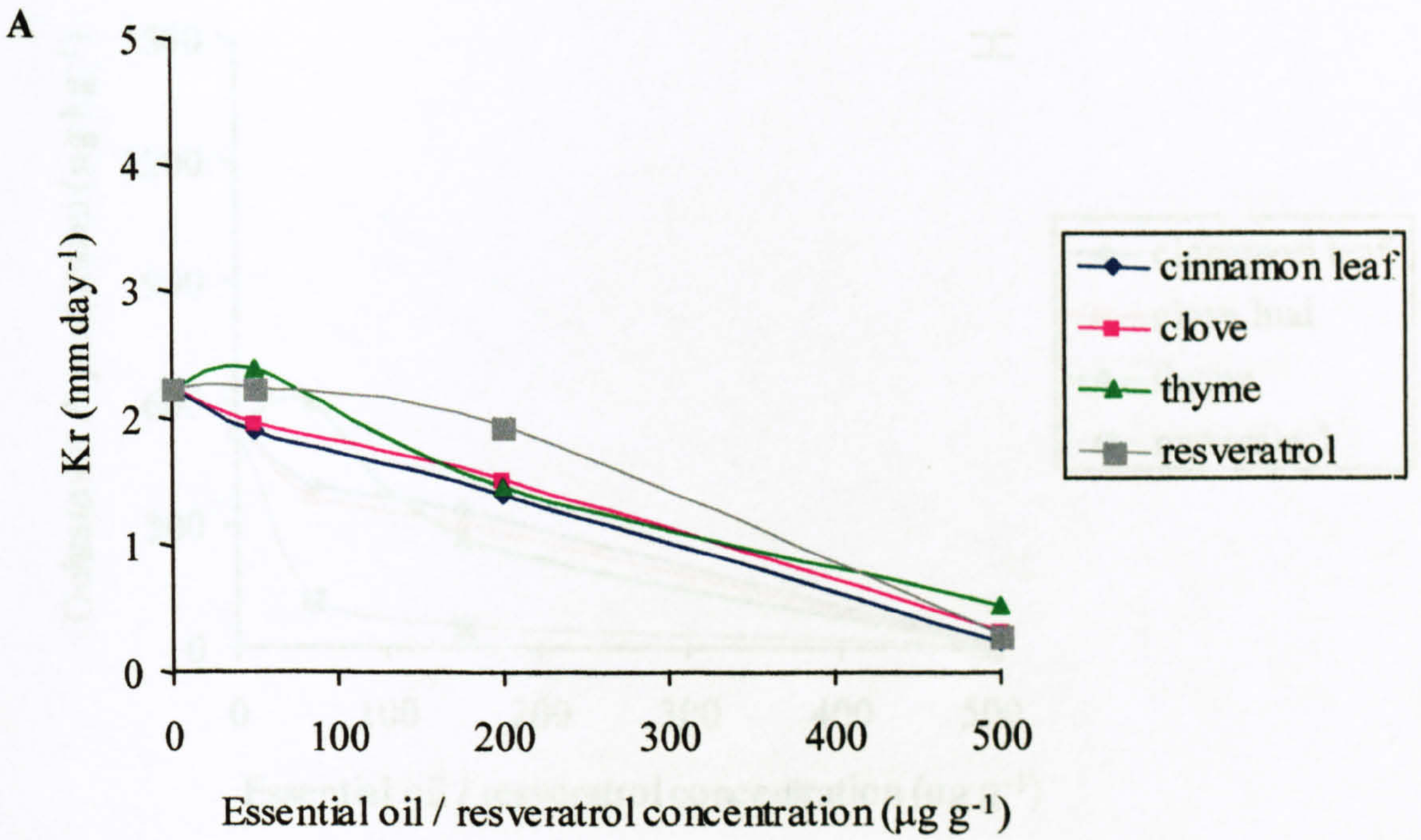


Figure 3.57 Effect of various concentrations of resveratrol and the essential oils cinnamon leaf, clove bud and thyme on growth of *Penicillium verrucosum* on γ-irradiated wheat grain at A) 0.95 a_w at 15 °C B) 0.95 a_w at 25 °C. Bars indicates Least Significant Difference (LSD) at $p < 0.05$.



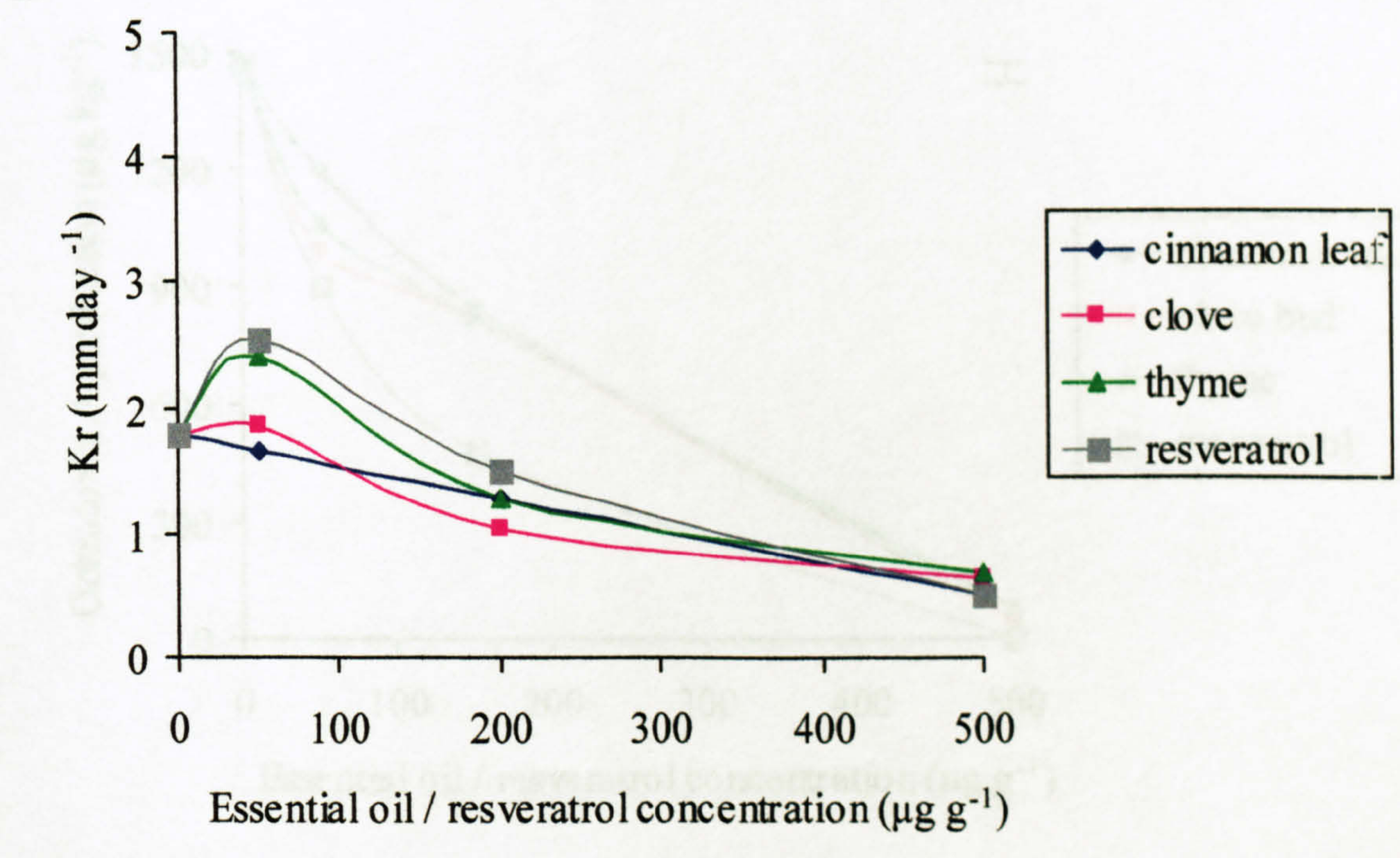


Figure 3.58 Effect of various concentrations of resveratrol and the essential oils cinnamon leaf, clove bud and thyme on growth of *Penicillium verrucosum* on γ-irradiated wheat grain at A) 0.995 a_w at 15 °C B) 0.995 a_w at 25 °C. Least Significant Differences (LSD) at $p < 0.05$ are A) 0.059 and B) 0.049.

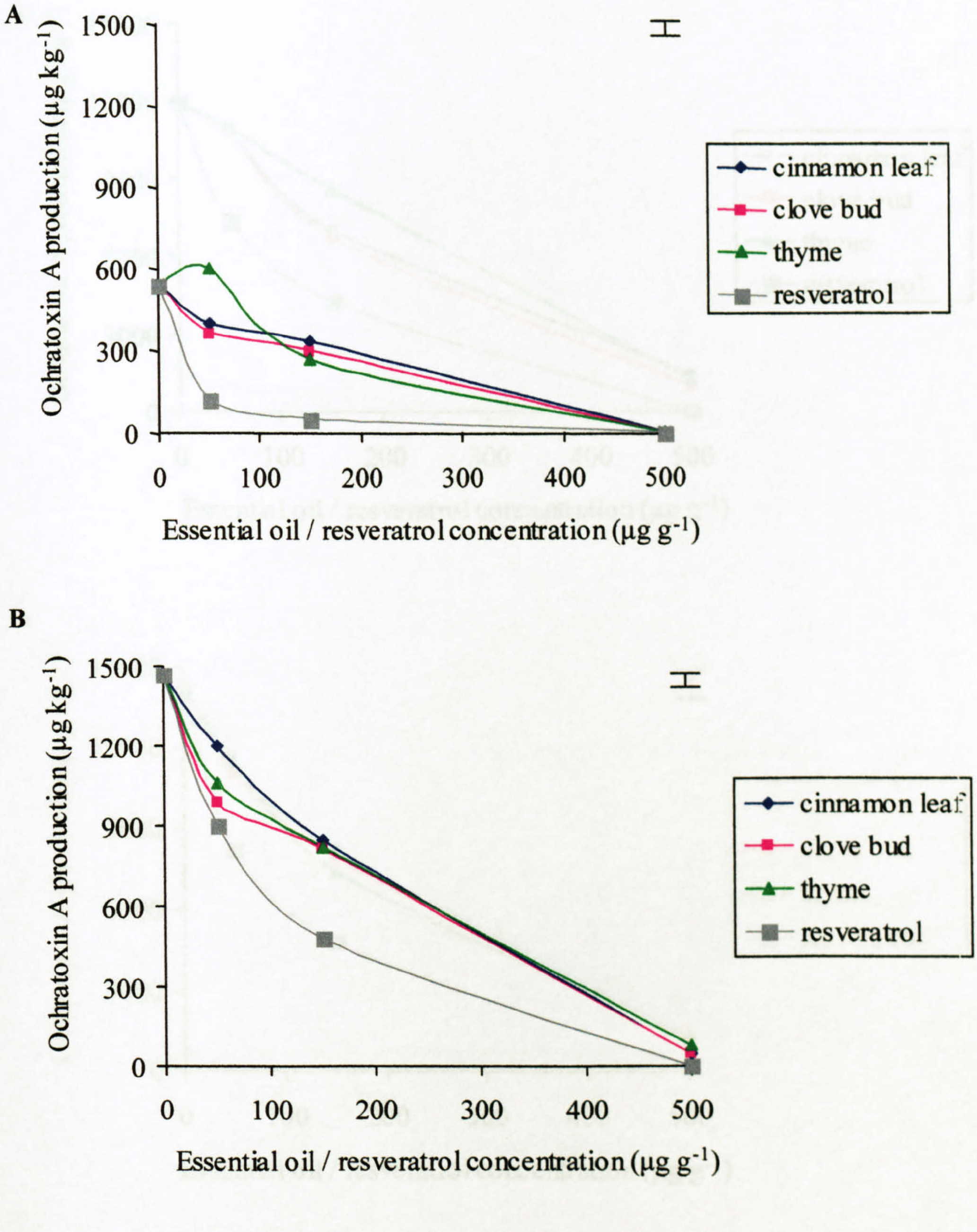


Figure 3.59 Effect of various concentrations of resveratrol and the essential oils cinnamon leaf, clove bud and thyme on ochratoxin A production after 28 days by *Penicillium verrucosum* on γ-irradiated wheat grain at A) 0.90 a_w at 15 °C B) 0.90 a_w at 25 °C. Bars indicate Least Significant Difference (LSD) at $p < 0.05$.

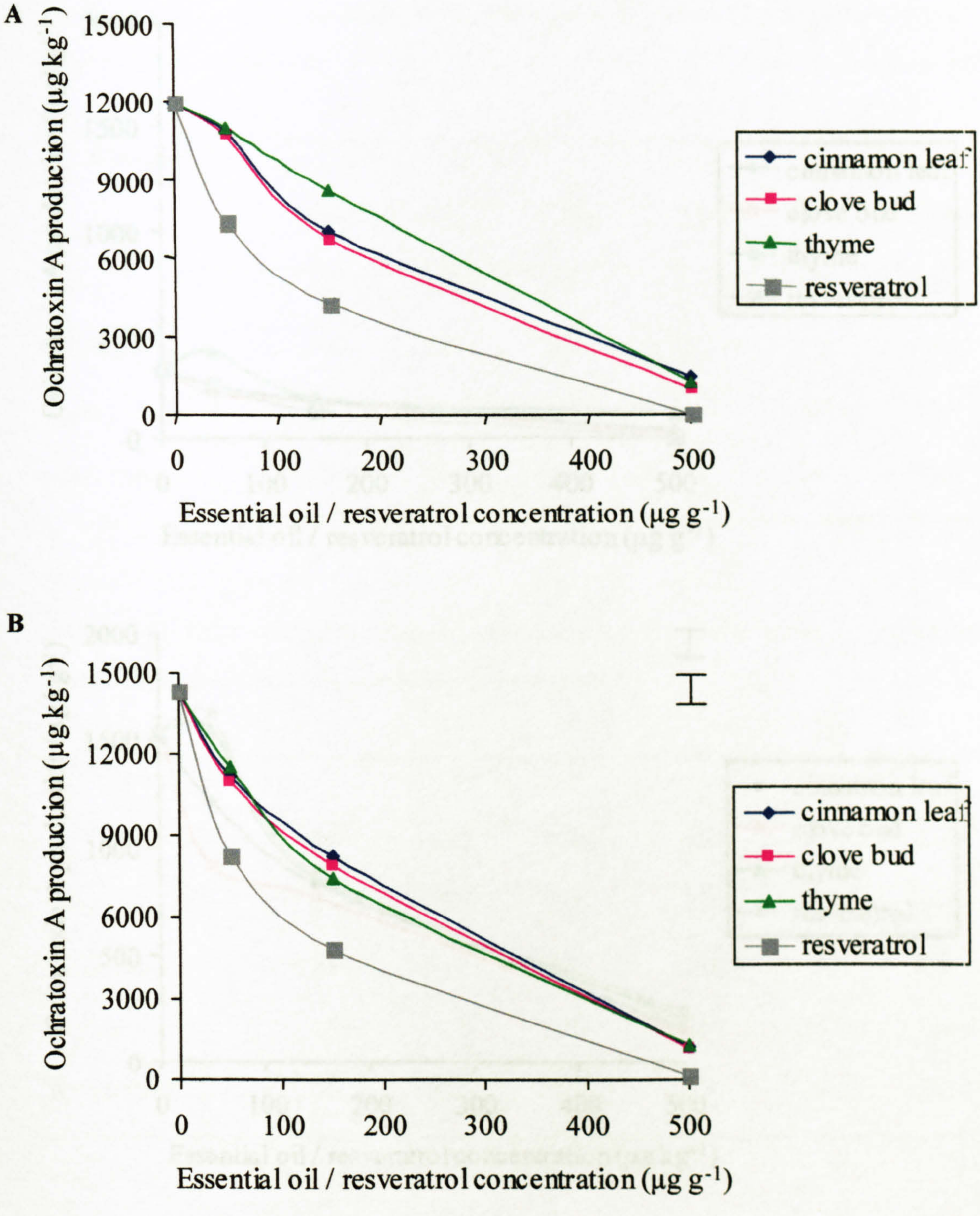
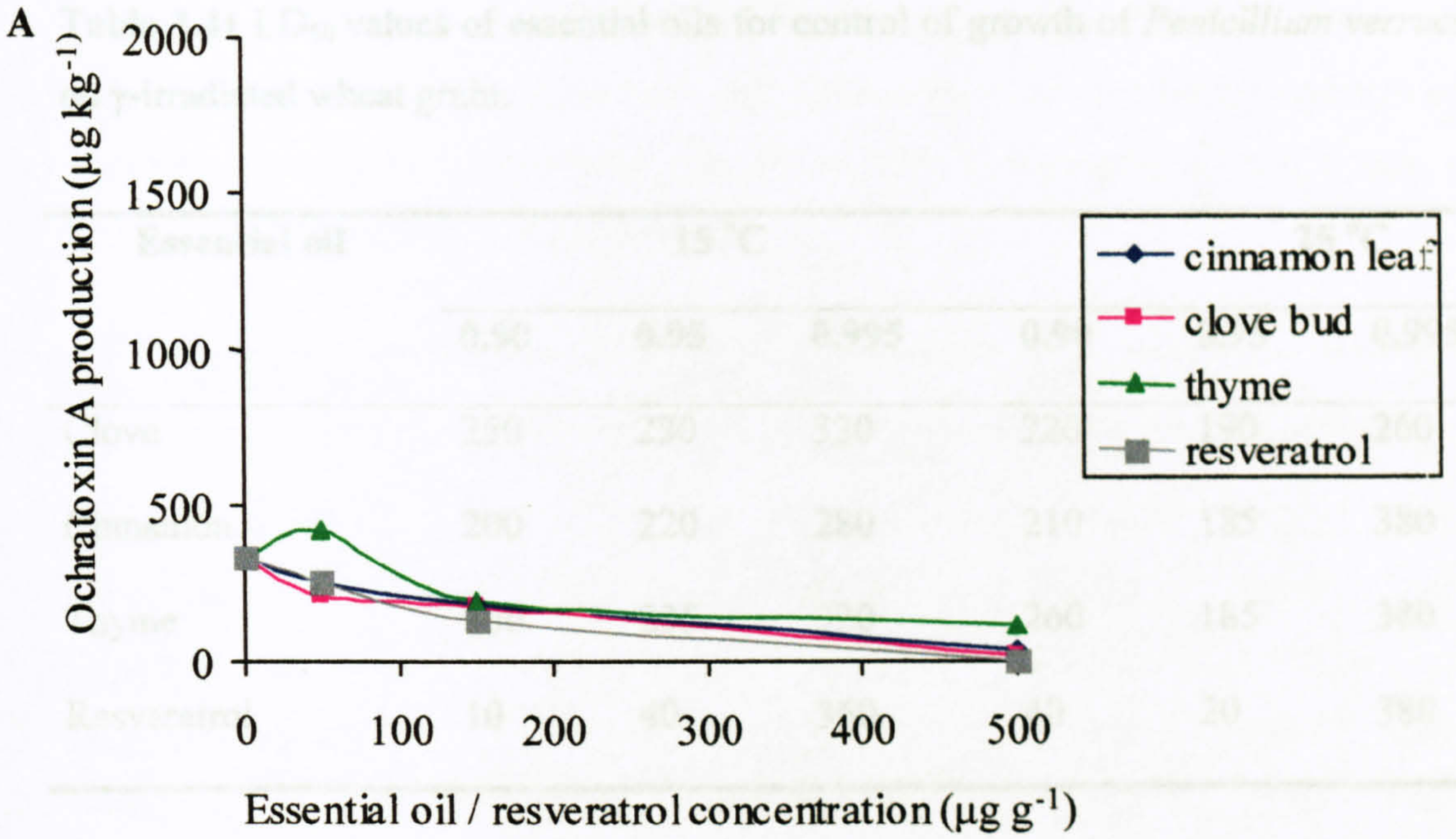


Figure 3.60 Effect of various concentrations of resveratrol and the essential oils cinnamon leaf, clove bud and thyme on ochratoxin A production after 28 days by *Penicillium verrucosum* on γ-irradiated wheat grain at A) 0.95 a_w at 15 °C B) 0.95 a_w at 25 °C. Bars indicate Least Significant Differences (LSD) at $p < 0.05$



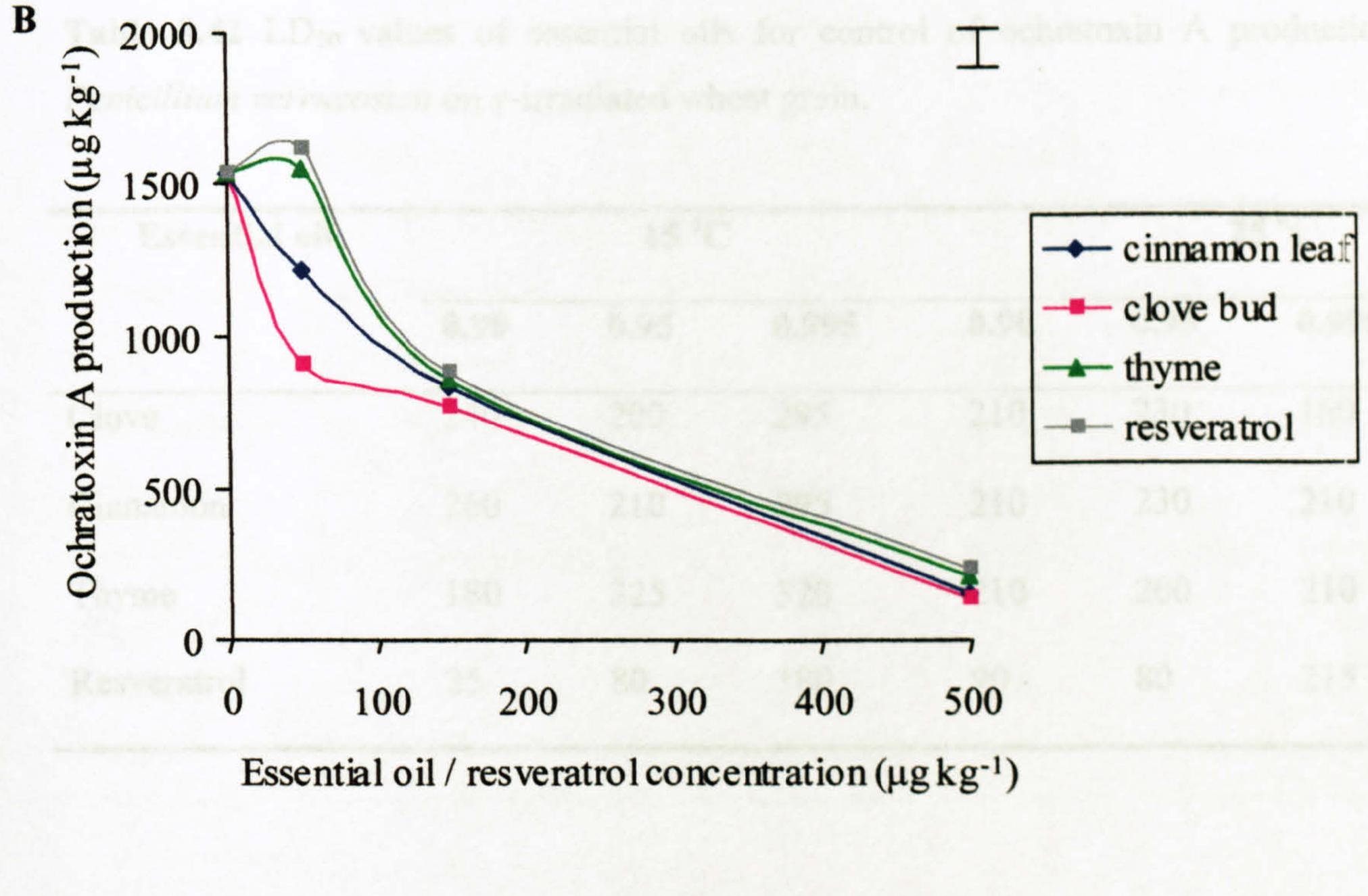


Figure 3.61 Effect of various concentrations of resveratrol and the essential oils cinnamon leaf, clove bud and thyme on ochratoxin A production after 28 days by *Penicillium verrucosum* on γ-irradiated wheat grain at A) 0.995 a_w at 15 °C B) 0.995 a_w at 25 °C. Bar indicates Least Significant Difference (LSD) at $p < 0.05$. LSD for A) is 15.22

Table 3.41 LD_{50} values of essential oils for control of growth of *Penicillium verrucosum* on γ-irradiated wheat grain.

Essential oil	15 °C			25 °C		
	0.90	0.95	0.995	0.90	0.95	0.995
Clove	250	230	320	220	190	260
Cinnamon	200	220	280	210	185	380
Thyme	260	235	320	260	185	380
Resveratrol	10	40	360	40	20	380

Table 3.42 LD_{50} values of essential oils for control of ochratoxin A production by *Penicillium verrucosum* on γ-irradiated wheat grain.

Essential oil	15 °C			25 °C		
	0.90	0.95	0.995	0.90	0.95	0.995
Clove	240	200	295	210	230	160
Cinnamon	260	210	295	210	230	210
Thyme	180	325	320	210	200	210
Resveratrol	25	80	180	90	80	215

Table 3.43 ANOVA for growth of *Penicillium verrucosum* on γ-irradiated wheat grain at various water activities incorporated with cinnamon, clove or thyme essential oils or resveratrol at various concentrations at 15 and 25 °C respectively. * Indicates factor elicited a significant effect ($p < 0.05$).

Source of Variation	DF	SS	MS	F	P
a_w	2	110.2901	55.1451	1250.79	<0.001*
Temperature	1	11.4068	11.4068	258.73	<0.001*
Treatment	3	4.3215	1.4405	32.67	<0.001*
Concentration	3	195.1155	65.0385	1475.19	<0.001*
Temperature x a_w	2	16.3982	8.1991	185.97	<0.001*
Treatment x a_w	6	6.0944	1.0157	23.04	<0.001*
Concentration x a_w	6	40.6531	6.7755	153.68	<0.001*
Concentration x temperature	3	0.0650	0.0217	0.49	0.688
Treatment x concentration	3	2.5707	0.8569	19.44	<0.001*
Treatment x temperature	9	3.7878	0.4209	9.55	<0.001*
Concentration x a_w x temperature	6	0.1859	0.0310	0.70	0.648
Concentration x a_w x treatment	6	7.7080	1.2847	29.14	<0.001*
Temperature x a_w x treatment	18	7.3967	0.4109	9.32	<0.001*
Concentration x temperature x treatment	9	0.2687	0.0299	0.68	0.729
Concentration x a_w x temperature x treatment	18	1.7366	0.0965	2.19	0.005*
Residual	192	8.4650	0.0441		
Total	287	416.4639			

Table 3.44 ANOVA for ochratoxin A production by *Penicillium verrucosum* after 28 days on γ-irradiated wheat grain incorporated with cinnamon, clove or thyme essential oils or resveratrol at various concentrations at 15 or 25 °C respectively. * Indicates factor elicited a significant effect ($p<0.05$)

Source of Variation	DF	SS	MS	F	P
a_w	2	2943718638	1471859319	3.99	0.020*
Temperature	1	1212191537	1212191537	3.29	0.071
Treatment	3	272883315	90961105	0.25	0.864
Concentration	3	6648321746	2216113815	6301	0.001*
Temperature x a_w	2	2610211296	1305105648	3.54	0.031*
Treatment x a_w	6	960462640	155077107	0.42	0.865
Concentration x a_w	6	5785720856	964286793	2.61	0.019*
Concentration x temperature	3	3384280435	1128093478	3.03	0.030*
Treatment x concentration	9	1260813770	140090419	0.38	0.944
Treatment x temperature	3	416218304	138739435	0.38	0.770
Concentration x a_w x temperature	6	6947492285	1157915381	3.14	0.006*
Concentration x a_w x treatment	18	2125182420	118065690	0.32	0.997
Temperature x a_w x treatment	6	748039918	119173320	0.32	0.924
Concentration x temperature x treatment	9	1085641938	120626882	0.33	0.965
Concentration x a_w x temperature x treatment	18	2284532362	126918465	0.34	0.995
Residual	192	7.0835E+10	36891390		
Total	287	1.0946E+11			

3.5.4 Detailed study on the efficacy of essential oils on growth and ochratoxin A production by *Aspergillus ochraceus* on wheat-based media

Figures 3.62-3.64 show the effect of interacting conditions of a_w x temperature x concentration of essential oil on the growth rates of *A. ochraceus* on wheat-based media. Growth rates were stimulated by the addition of 50 μg g^{-1} concentration of all three essential oils regardless of the a_w and temperature tested. The incorporation of 500 μg g^{-1} concentration of each essential oil tested reduced growth rates over all conditions with reduction being by >90 %.

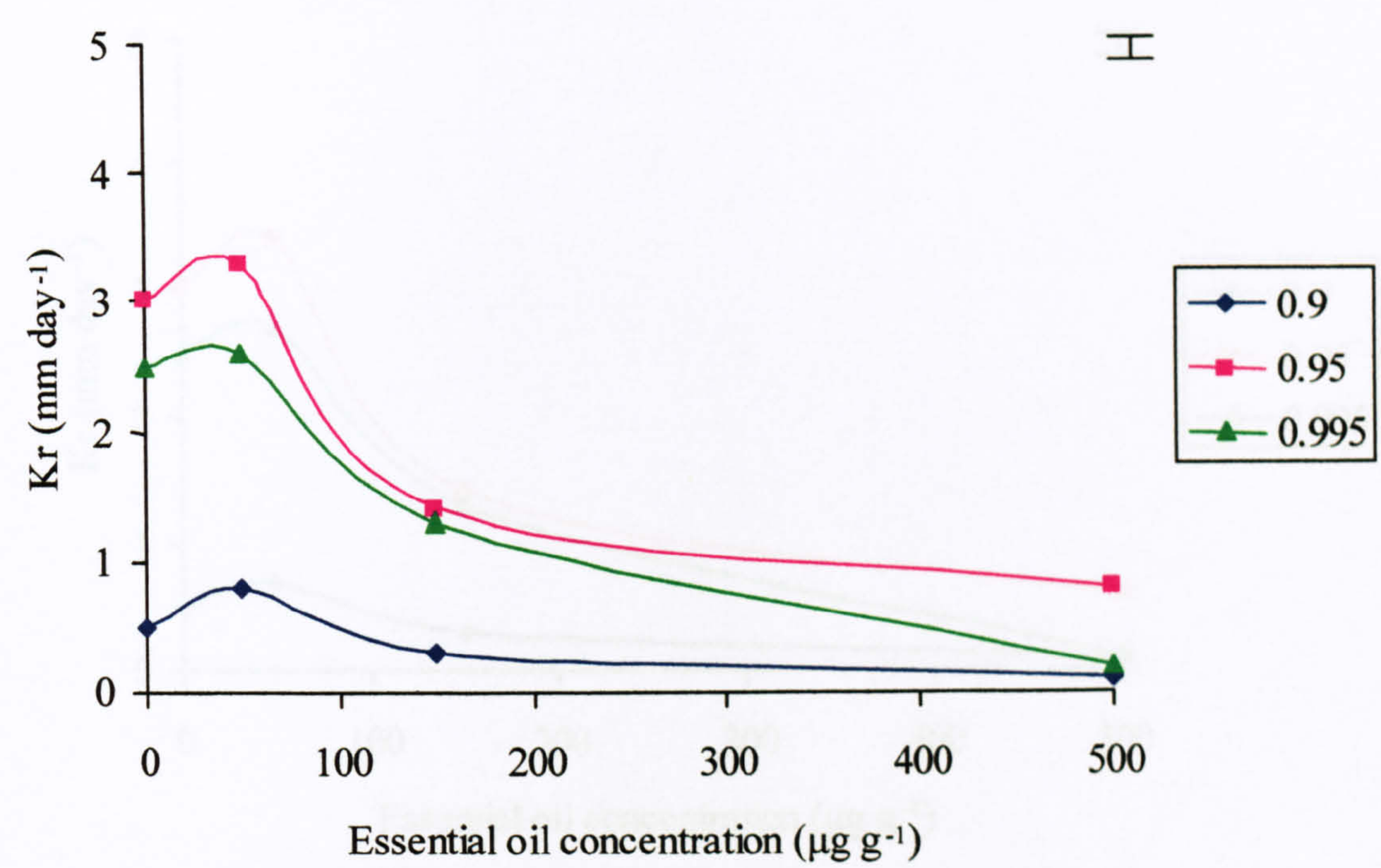
Figures 3.65-3.67 show the effect of interacting conditions of a_w x temperature x concentration of essential oil on OTA production by *A. ochraceus* on wheat-based media after 56 days. Significantly more OTA production was produced at 25 than 15 °C particularly in media containing 50 μg g^{-1} and 150 μg g^{-1} of the essential oils. OTA production was stimulated by 50 μg g^{-1} cinnamon essential oil at 0.95 a_w irrespective of the temperature tested. Effective control of OTA production was achieved with the incorporation of 500 μg g^{-1} over all conditions tested with OTA being suppressed by >90 % when compared with the control treatments.

Using Figures 3.62-3.67, LD_{50} values were determined for control of growth and OTA production respectively for all conditions tested. Table 3.45 and 3.46 show that the essential oil which had the lowest overall total concentration for reducing growth and OTA production respectively by 50 % was thyme oil. Higher concentrations of essential oils were required for control of growth than OTA production over the complete range of conditions tested. Overall between 115-200 μg g^{-1} of essential oils were required to reduce growth by 50 %. However 20-180 μg g^{-1} of essential oils were required to reduce OTA production by 50 %.

Statistical analysis shows that a_w, temperature, the essential oil tested and the concentration used had a significant effect on growth of *A. ochraceus* on a 2 % wheat-based media (Table 3.47). Combined these variables had no significant effect on growth

of *A. ochraceus* for the conditions tested. This is largely due to there being little variation between treatments at 0.90 a_w regardless of the temperature used as there was very little growth to start with in the control treatments.

Statistical analysis shows that a_w, temperature and the concentration of essential oil tested all had a significant effect on OTA production (Table 3.48). There was no significant difference between the three essential oils tested on OTA production. Overall, the interactions between all variables had no significant effect on OTA production which is largely due to there being no significant difference between the three essential oils tested.



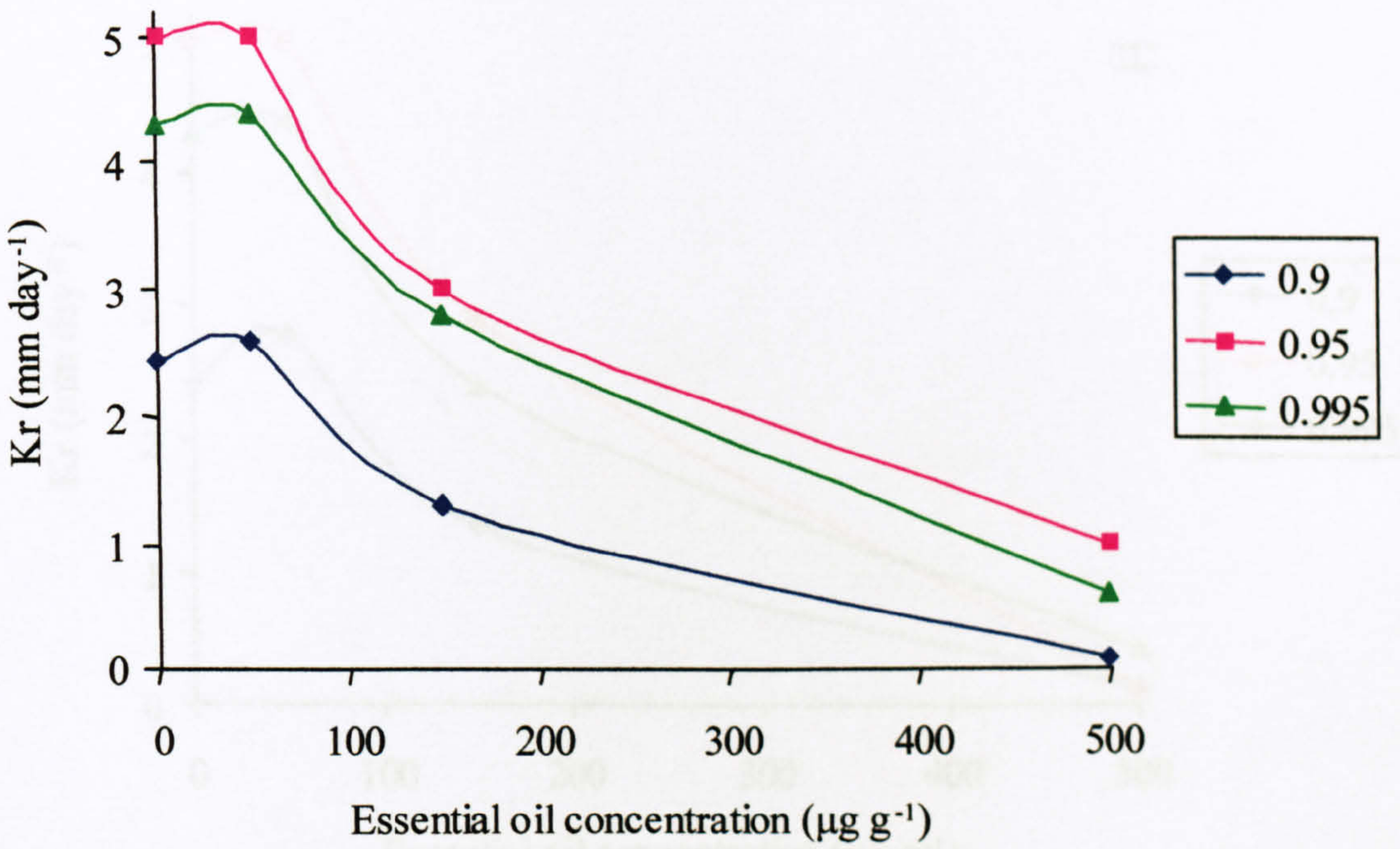


Figure 3.62 Radial extension rates of *Aspergillus ochraceus* inoculated on a 2 % wheat-based media at different water activities (0.90, 0.95, 0.995 a_w) and supplemented with 0, 50, 150 or 500 ug g^{-1} of cinnamon essential oil at A) 15 °C or B) 25 °C respectively. Bar indicates Least Significant Difference (LSD) at $p < 0.05$. LSD for B) is 0.0678364.

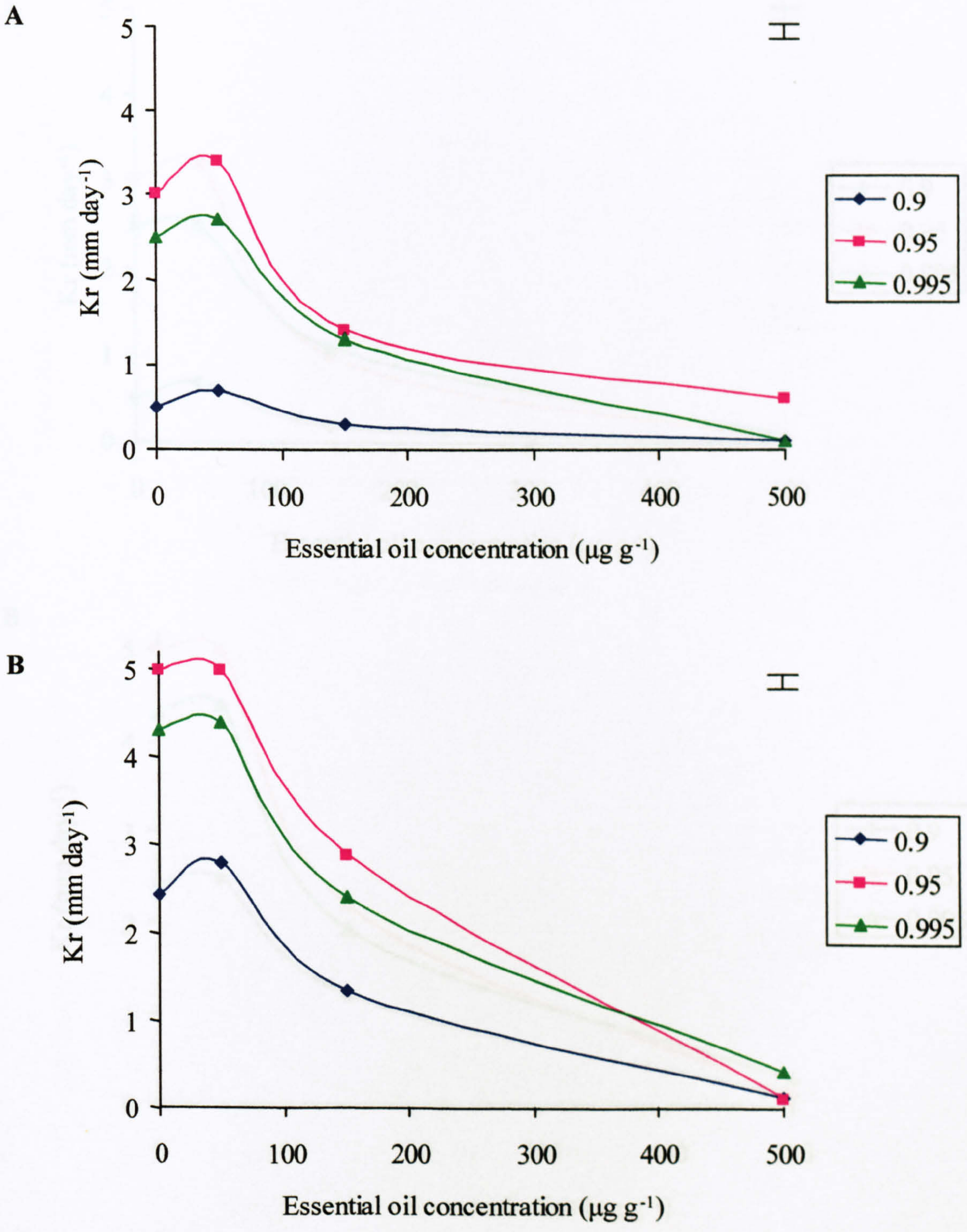


Figure 3.63 Radial extension rates of *Aspergillus ochraceus* inoculated on a 2 % wheat-based media at different water activities (0.90, 0.95, 0.995 a_w) and supplemented with 0, 50, 150 or 500 ug g^{-1} of clove bud essential oil at A) 15 °C or B) 25 °C respectively. Bars indicates Least Significant Difference (LSD) at $p < 0.05$.

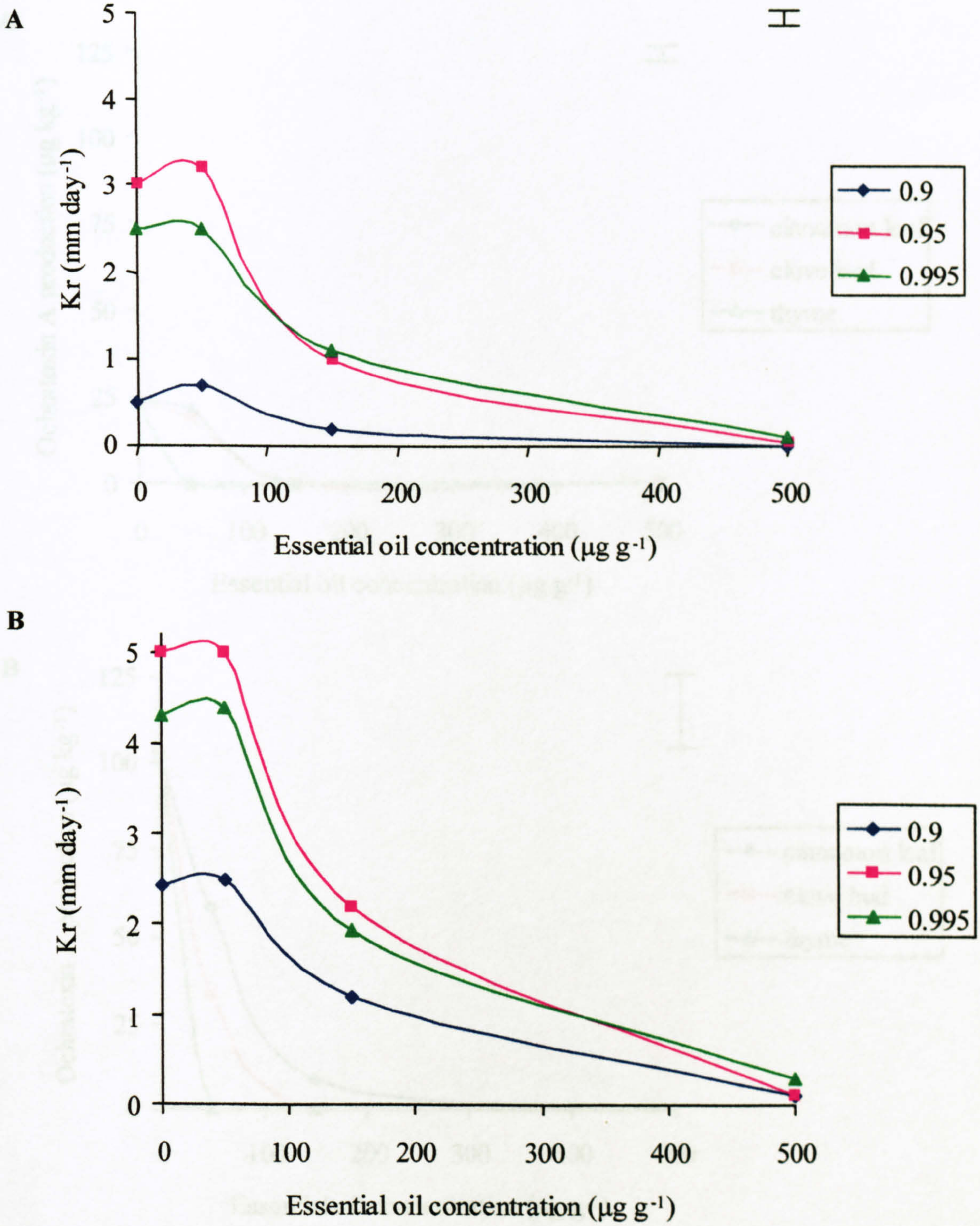


Figure 3.64 Radial extension rates of *Aspergillus ochraceus* inoculated on a 2 % wheat-based media at different water activities (0.90, 0.95, 0.995 a_w) and supplemented with 0, 50, 150 or 500 ug g^{-1} of thyme essential oil at A) 15 °C or B) 25 °C respectively. Bars indicate Least Significant Difference at $p < 0.05$. LSD for B) is 0.0602247.

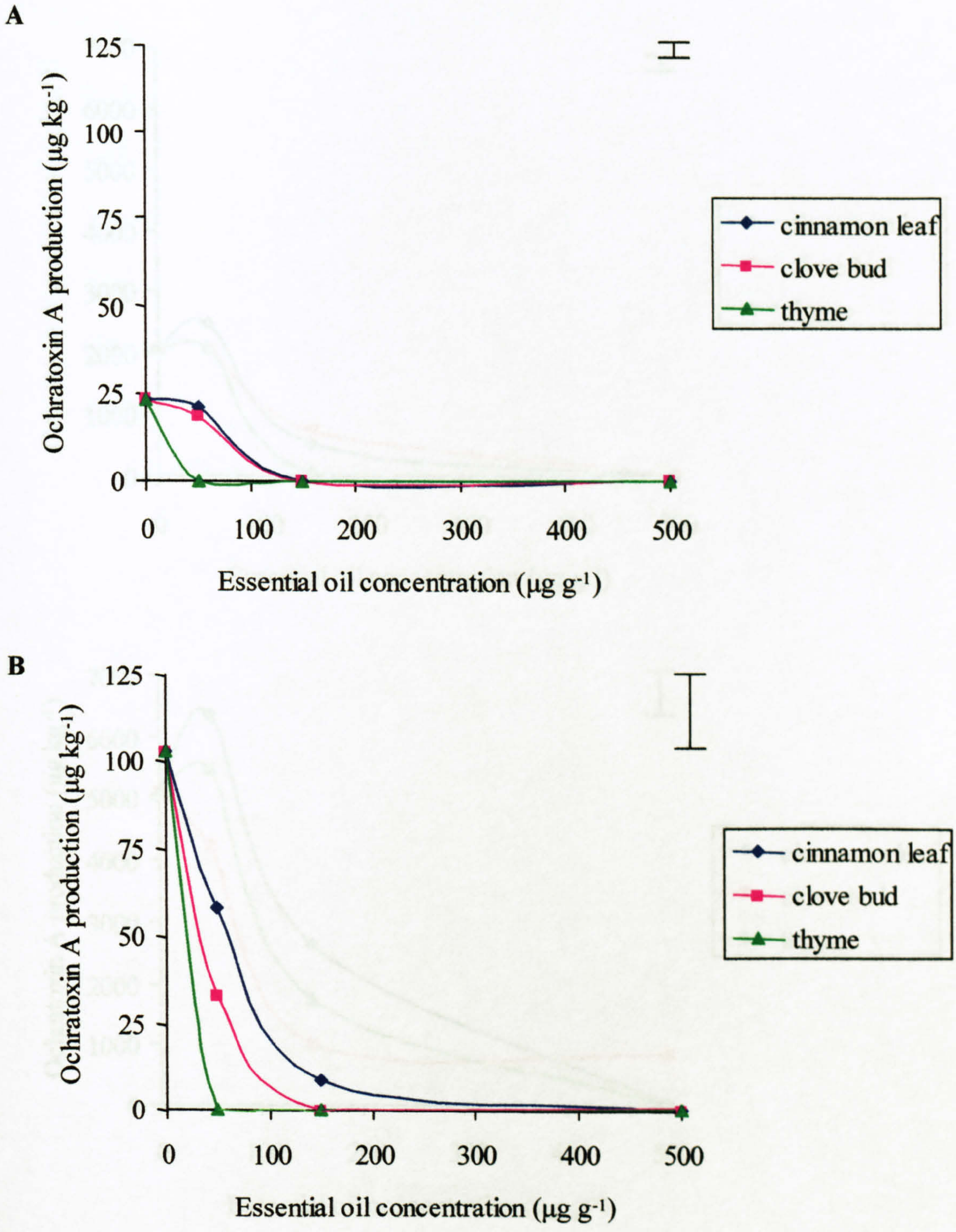


Figure 3.65 Effect of various concentrations of the essential oils cinnamon leaf, clove bud and thyme on ochratoxin A production by *Aspergillus ochraceus* on a 2 % wheat-based media after 56 days at A) 0.90 a_w at 15 °C B) 0.90 a_w at 25 °C. Bars indicate Least Significant Difference (LSD) at $p < 0.05$.

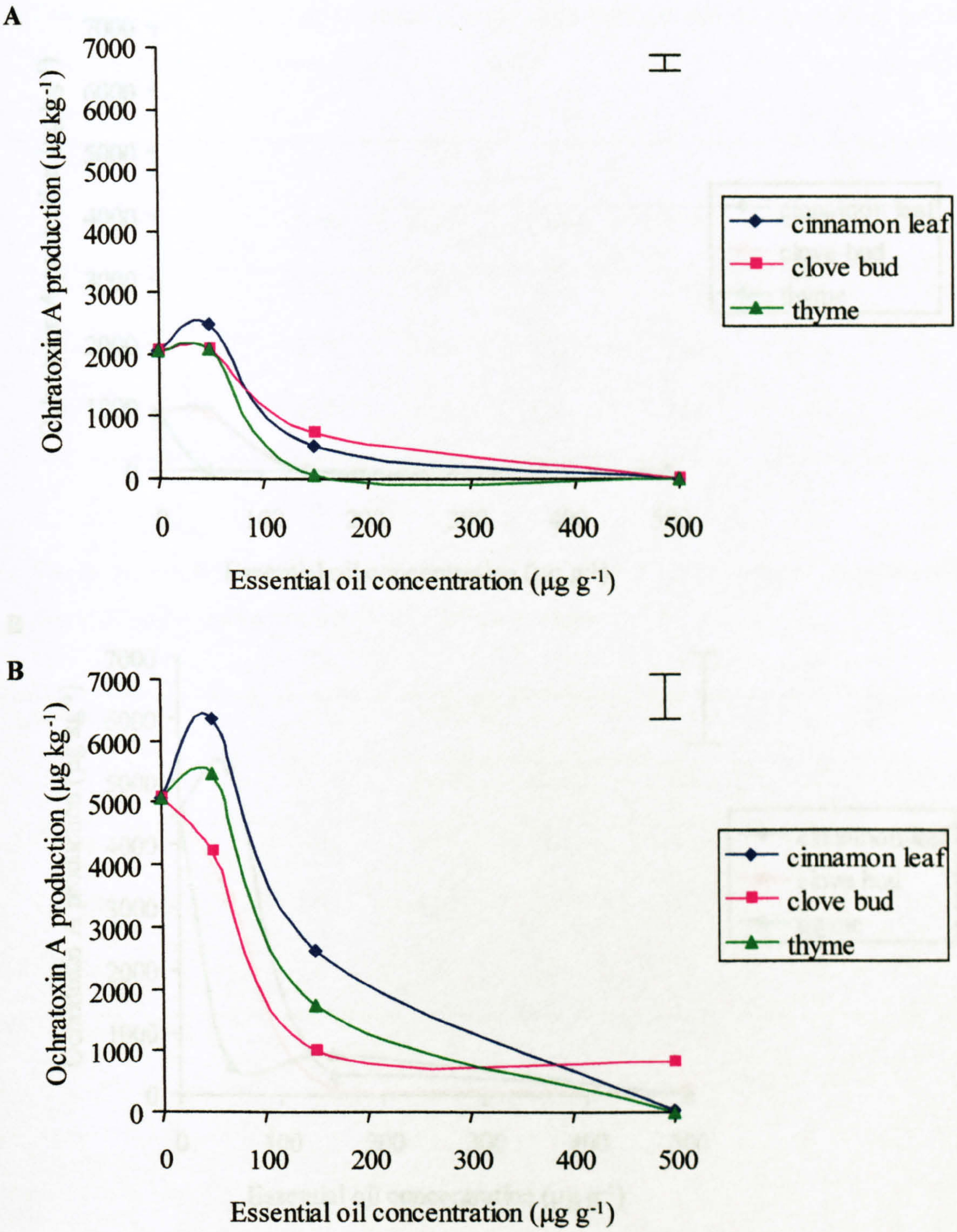


Figure 3.66 Effect of various concentrations of the essential oils cinnamon leaf, clove bud and thyme on ochratoxin A production by *Aspergillus ochraceus* on a 2 % wheat-based media after 56 days at A) 0.95 a_w at 15 °C B) 0.95 a_w at 25 °C. Bars indicate Least Significant Difference (LSD) at $p < 0.05$.

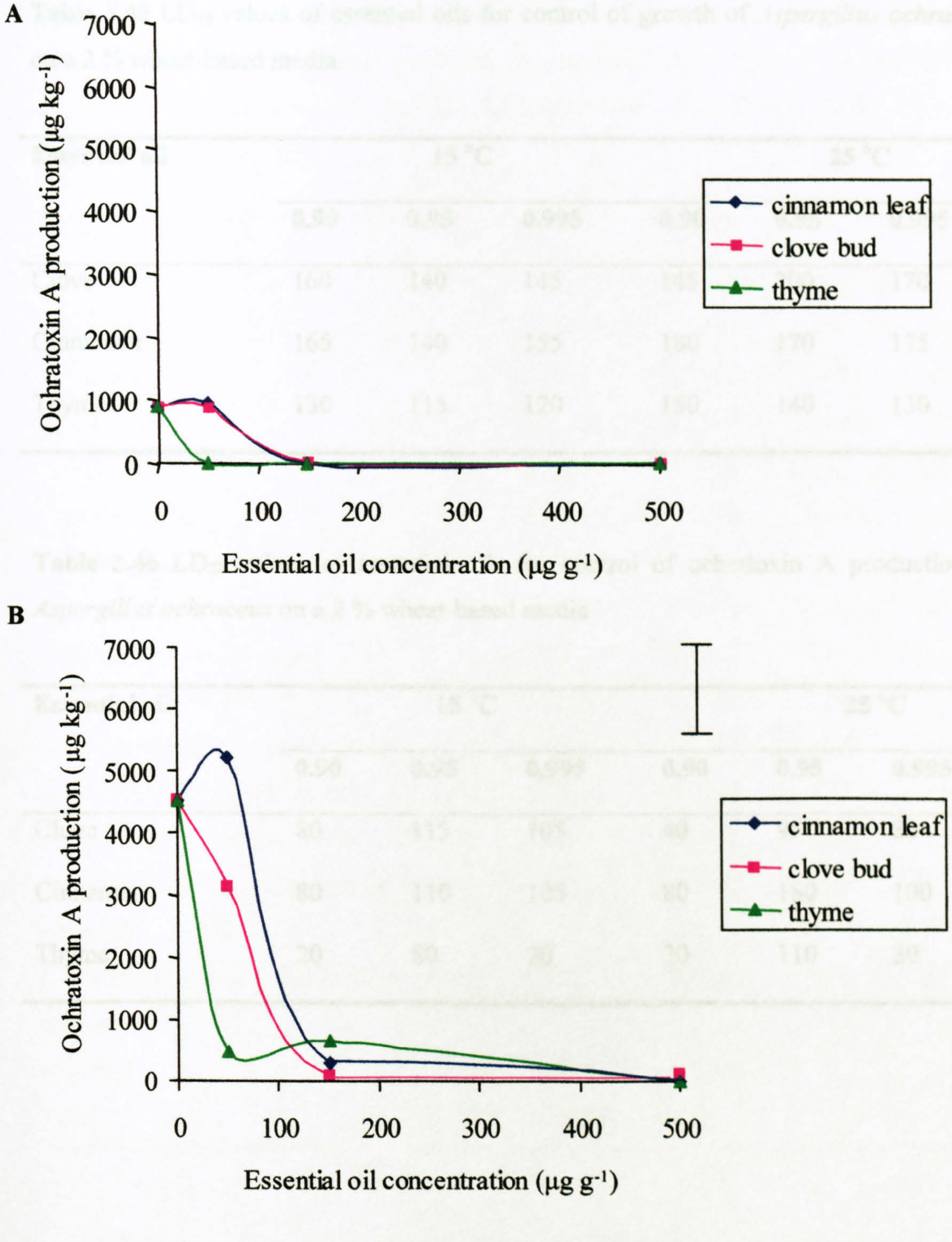


Figure 3.67 Effect of various concentrations of the essential oils cinnamon leaf, clove bud and thyme on ochratoxin A production by *Aspergillus ochraceus* on a 2 % wheat-based media after 56 days at A) 0.995 a_w at 15 °C B) 0.995 a_w at 25 °C. Bars indicate Least Significant Difference (LSD) at $p < 0.05$. LSD for A) 18.25.

Table 3.45 LD_{50} values of essential oils for control of growth of *Aspergillus ochraceus* on a 2 % wheat-based media

Essential oil	15 °C			25 °C		
	0.90	0.95	0.995	0.90	0.95	0.995
Clove	160	140	145	145	200	170
Cinnamon	165	140	155	180	170	175
Thyme	130	115	120	150	140	130

Table 3.46 LD_{50} values of essential oils for control of ochratoxin A production of *Aspergillus ochraceus* on a 2 % wheat-based media

Essential oil	15 °C			25 °C		
	0.90	0.95	0.995	0.90	0.95	0.995
Clove	80	115	105	40	95	80
Cinnamon	80	110	105	80	180	100
Thyme	20	80	20	30	110	30

Table 3.47 ANOVA for growth of *Aspergillus ochraceus* on 2 % wheat-based media at various water activities incorporated with cinnamon, clove or thyme essential oils at various concentrations at 15 and 25 °C respectively. * Indicates factor elicited a significant effect ($p<0.05$).

Source of Variation	DF	SS	MS	F	P
a_w	2	109.373	54.6866	1351.92	<0.001*
Temperature	1	89.4918	89.4918	2212.35	<0.001*
Treatment	2	1.7190	0.8595	21.25	<0.001*
Concentration	3	276.7834	92.2611	2280.81	<0.001*
Temperature x a_w	2	0.1800	0.0900	2.22	0.112
Treatment x a_w	4	0.7666	0.1916	4.74	0.001*
Concentration x a_w	6	30.6830	5.1138	126.42	<0.001*
Concentration x temperature	3	26.0410	8.6803	214.59	<0.001*
Treatment x concentration	6	1.1341	0.1890	4.67	<0.001*
Treatment x temperature	2	0.1117	0.0559	1.38	0.255
Concentration x a_w x temperature	6	0.6693	0.1116	2.76	0.014*
Concentration x a_w x treatment	12	1.1315	0.0943	2.33	0.009*
Temperature x a_w x treatment	4	0.1105	0.0276	0.68	0.605
Concentration x temperature x treatment	6	0.3032	0.0505	1.25	0.285
Concentration x a_w x temperature x treatment	12	0.3357	0.0280	0.69	0.759
Residual	144	5.8249	0.0405		
Total	215	544.6590			

Table 3.48 ANOVA for ochratoxin A production by *Aspergillus ochraceus* after 56 days on 2 % wheat-based media incorporated with cinnamon, clove or thyme essential oils at various concentrations at 15 or 25 °C respectively. * Indicates factor elicited a significant effect ($p < 0.05$).

Source of Variation	DF	SS	MS	F	P
a_w	2	144606641	72303321	42.09	<0.001*
Temperature	1	87751863	87751863	51.09	<0.001*
Treatment	2	4266463	2133231	1.24	0.292
Concentration	3	151508036	50502679	29.40	<0.001*
Temperature x a_w	2	45773569	22886785	13.32	<0.001*
Treatment x a_w	4	3281831	820458	0.48	0.752
Concentration x a_w	6	91513156	15252193	8.88	<0.001*
Concentration x temperature	3	52995734	17665245	10.28	<0.001*
Treatment x concentration	6	9618941	1603157	0.93	0.473
Treatment x temperature	2	1821260	910630	0.53	0.590
Concentration x a_w x temperature	6	25760197	4293366	2.50	0.025*
Concentration x a_w x treatment	12	15288147	1274012	0.74	0.709
Temperature x a_w x treatment	4	1907919	476980	0.28	0.892
Concentration x temperature x treatment	6	5349238	891540	0.52	0.793
Concentration x a_w x temperature x treatment	12	7844550	653712	0.38	0.969
Residual	144	247355103	1717744		
Total	215	896642649			

3.5.5 Detailed study of the efficacy of essential oils on growth and ochratoxin A production by *Aspergillus ochraceus* on γ-irradiated wheat grain

Figures 3.68-3.70 show the effect of interacting conditions of a_w x temperature x concentration of essential oil or resveratrol treatments on the growth of *A. ochraceus* on γ-irradiated wheat grain. Growth rates were stimulated by the addition of 50 μg g^{-1} concentration of cinnamon and clove bud essential oils at 0.90 a_w and 0.995 a_w irrespective of the temperature tested. The incorporation of 500 μg g^{-1} of each essential oil or resveratrol reduced growth rates over all conditions with reduction being more than 80 %.

Figures 3.71-3.73 show the effect of interacting conditions of a_w x temperature x concentration of essential oil or resveratrol on OTA production by *A. ochraceus* on γ-irradiated wheat grain after 28 days. Significantly more OTA was produced at 25 °C than 15 °C particularly on grain containing 50 μg g^{-1} and 200 μg g^{-1} concentration of treatments. OTA production was stimulated with the addition of 50 μg g^{-1} concentration of cinnamon at 0.95 a_w at both temperatures and at 0.995 a_w at 25 °C. OTA production was stimulated with the addition of 50 μg g^{-1} concentration of clove bud at 0.95 a_w at both temperatures and at 0.995 a_w at 15 °C. Effective control of OTA production was achieved with the incorporation of 500 μg g^{-1} of all treatments over all conditions tested with OTA being suppressed >80 %.

Using Figures 3.68-3.73, LD_{50} values were determined for control of growth and OTA production respectively for all conditions tested. Tables 3.49 and 3.50 show that resveratrol had the lowest overall total concentration for reducing growth and OTA production by 50 % respectively. Higher concentration of essential oils and resveratrol were required for control of growth than OTA production for all conditions tested. Higher concentrations of essential oils were required to reduce growth and OTA production by 50 % on γ-irradiated wheat grain than 2 % wheat-based media.

Statistical analysis shows that a_w, temperature, treatment, concentration and their interactions all had a significant effect on growth and OTA production by *A. ochraceus* on γ-irradiated wheat grain (Tables 3.51, 3.52).

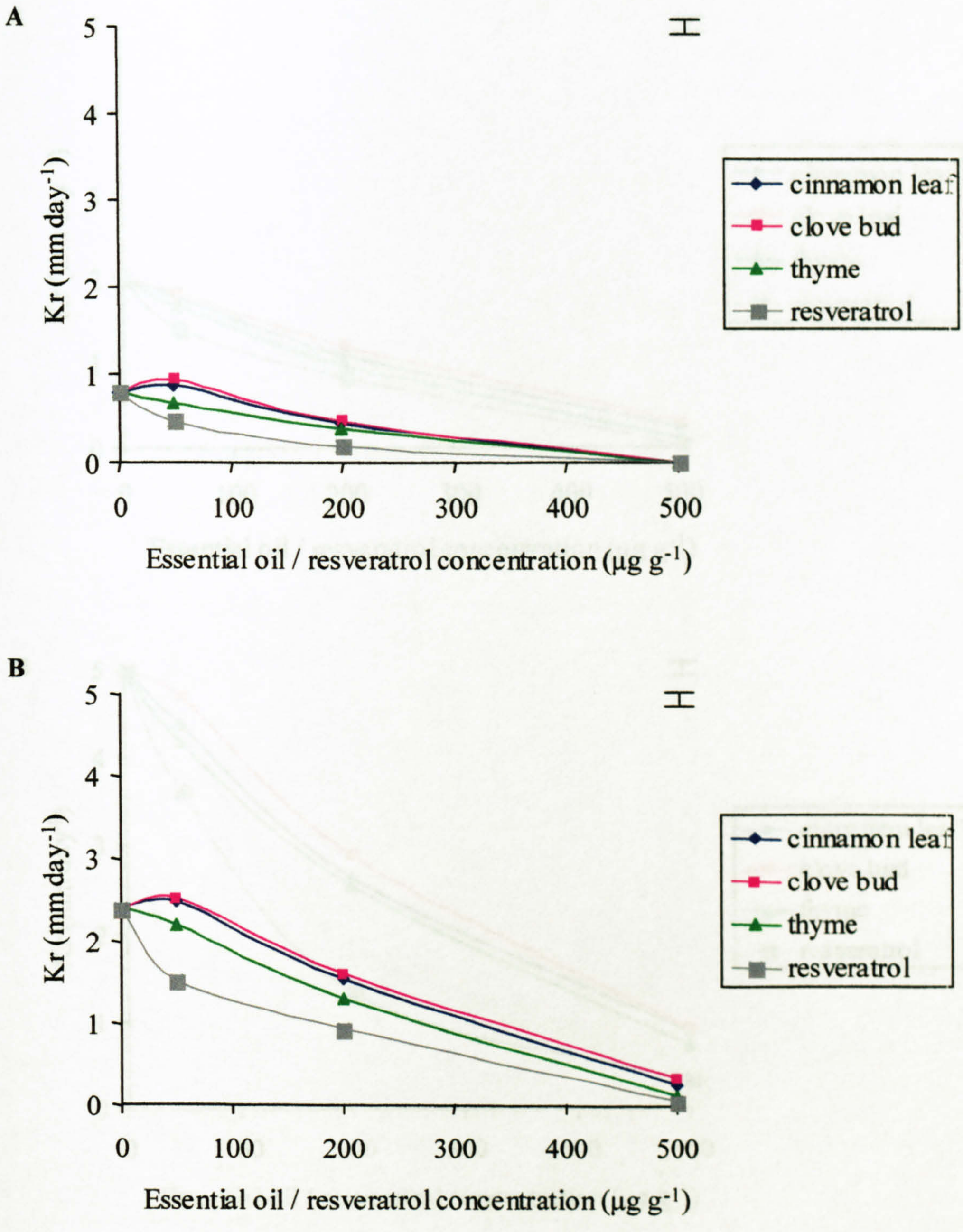


Figure 3.68 Effect of various concentrations of resveratrol and the essential oils cinnamon leaf, clove bud and thyme on growth of *Aspergillus ochraceus* on γ-irradiated wheat grain at A) 0.90 a_w at 15 °C B) 0.90 a_w at 25 °C. Bars indicate Least Significant Difference (LSD) at $p < 0.05$.

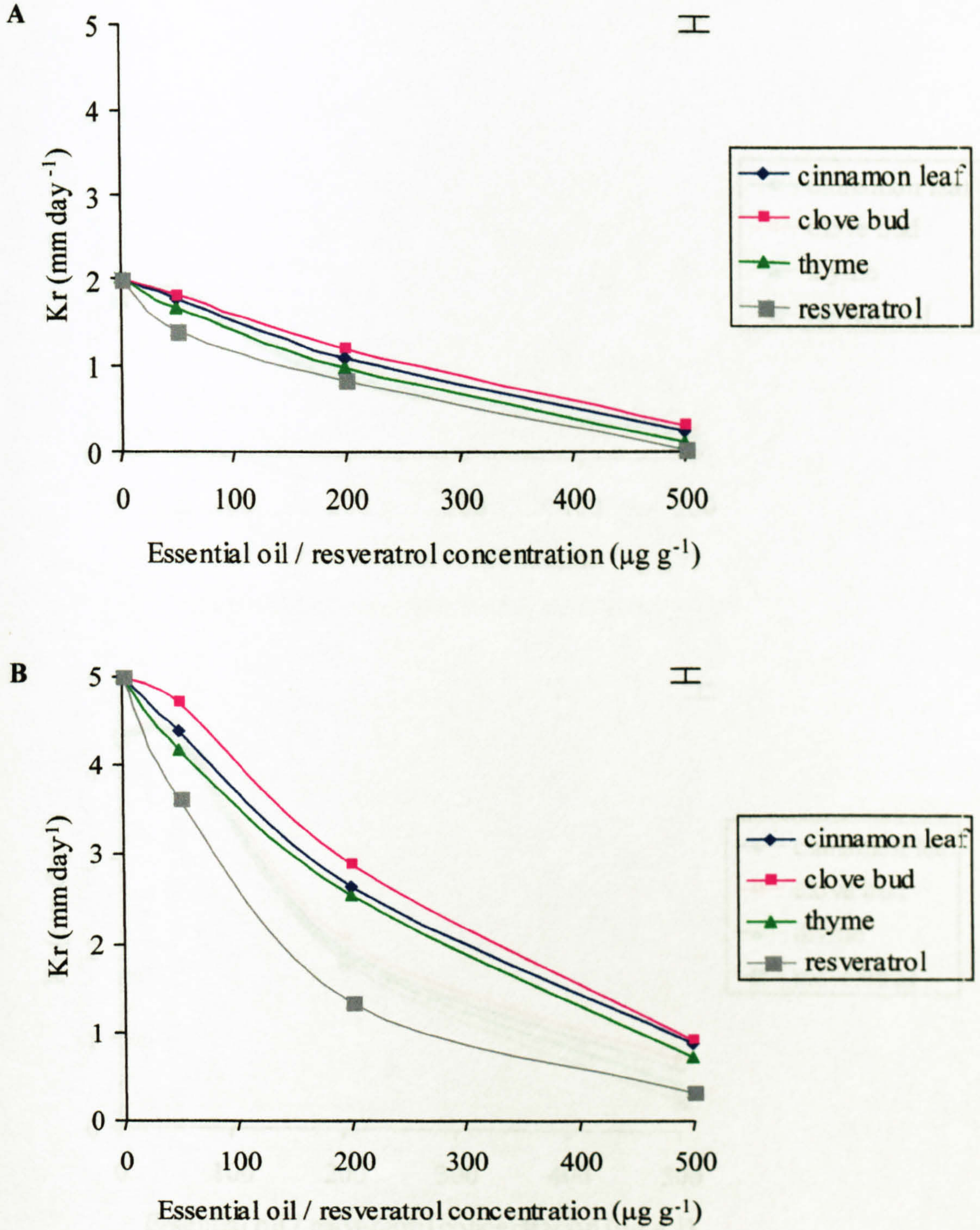


Figure 3.69 Effect of various concentrations of resveratrol and the essential oils cinnamon leaf, clove bud and thyme on growth of *Aspergillus ochraceus* on γ-irradiated wheat grain at A) 0.95 a_w at 15 °C B) 0.95 a_w at 25 °C. Bars indicate Least Significant Difference (LSD) at $p < 0.05$.

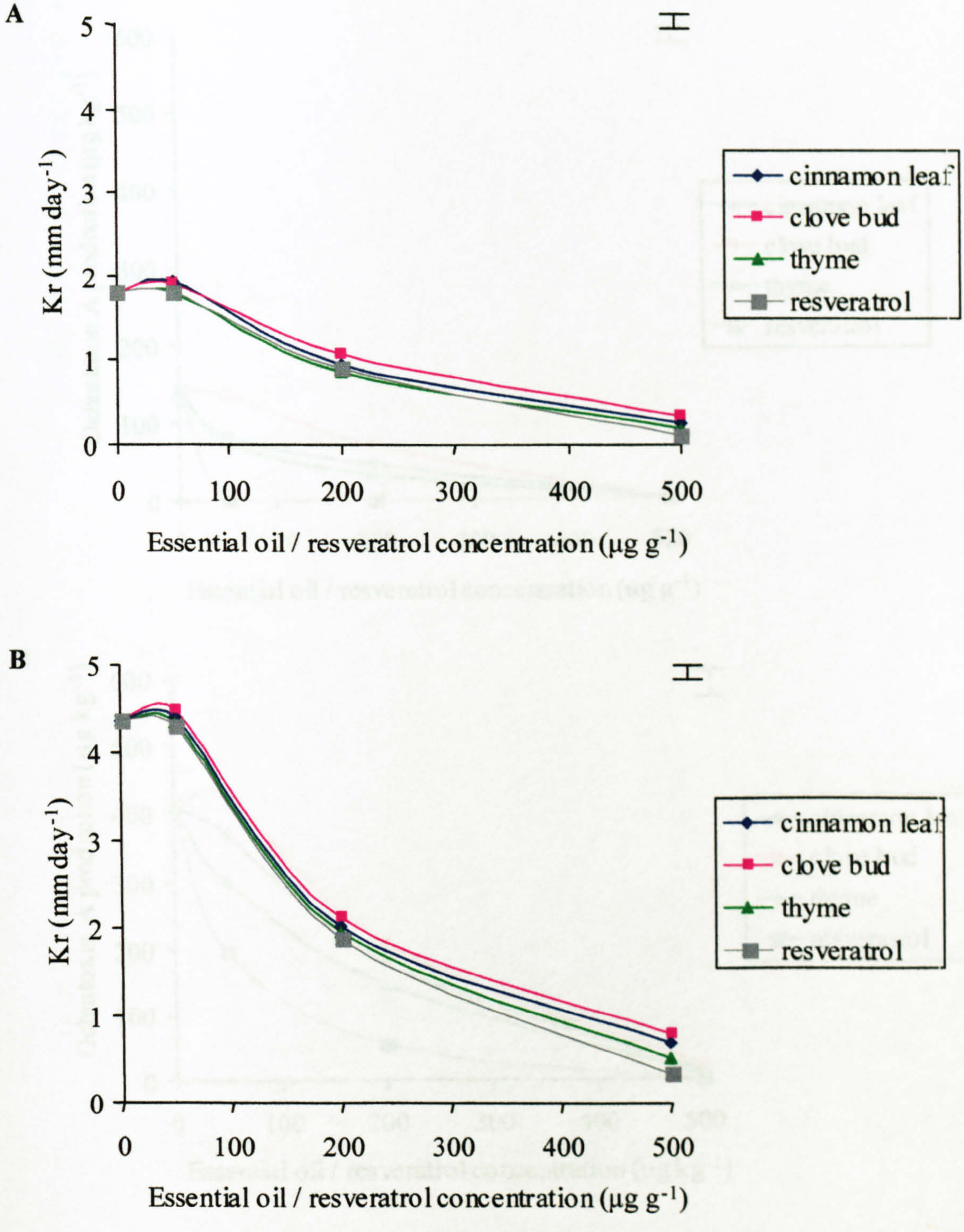


Figure 3.70 Effect of various concentrations of resveratrol and the essential oils cinnamon leaf, clove bud and thyme on growth of *Aspergillus ochraceus* on γ-irradiated wheat grain at A) 0.995 a_w at 15 °C B) 0.995 a_w at 25 °C. Bars indicate Least Significant Difference (LSD) at $p < 0.05$.

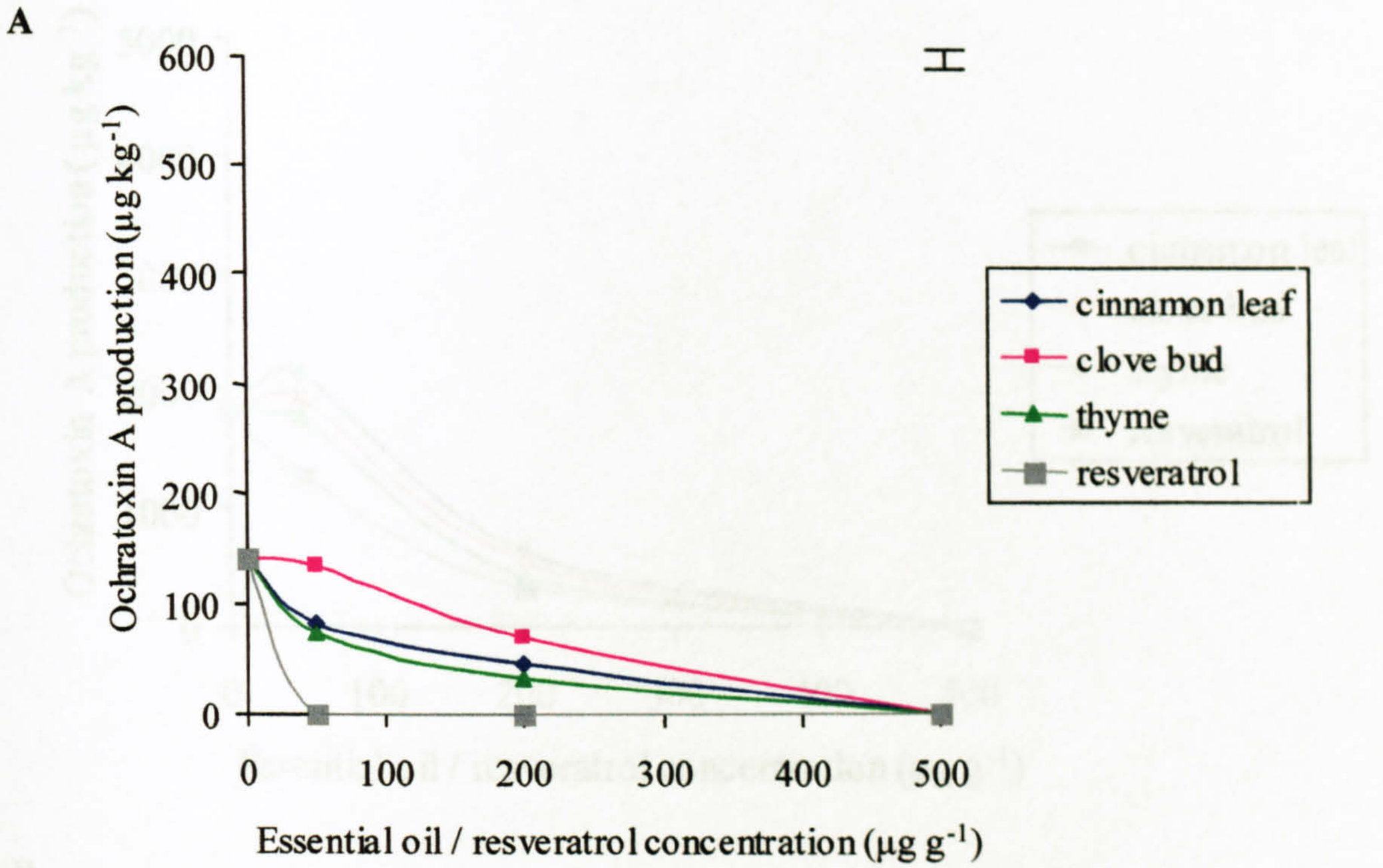
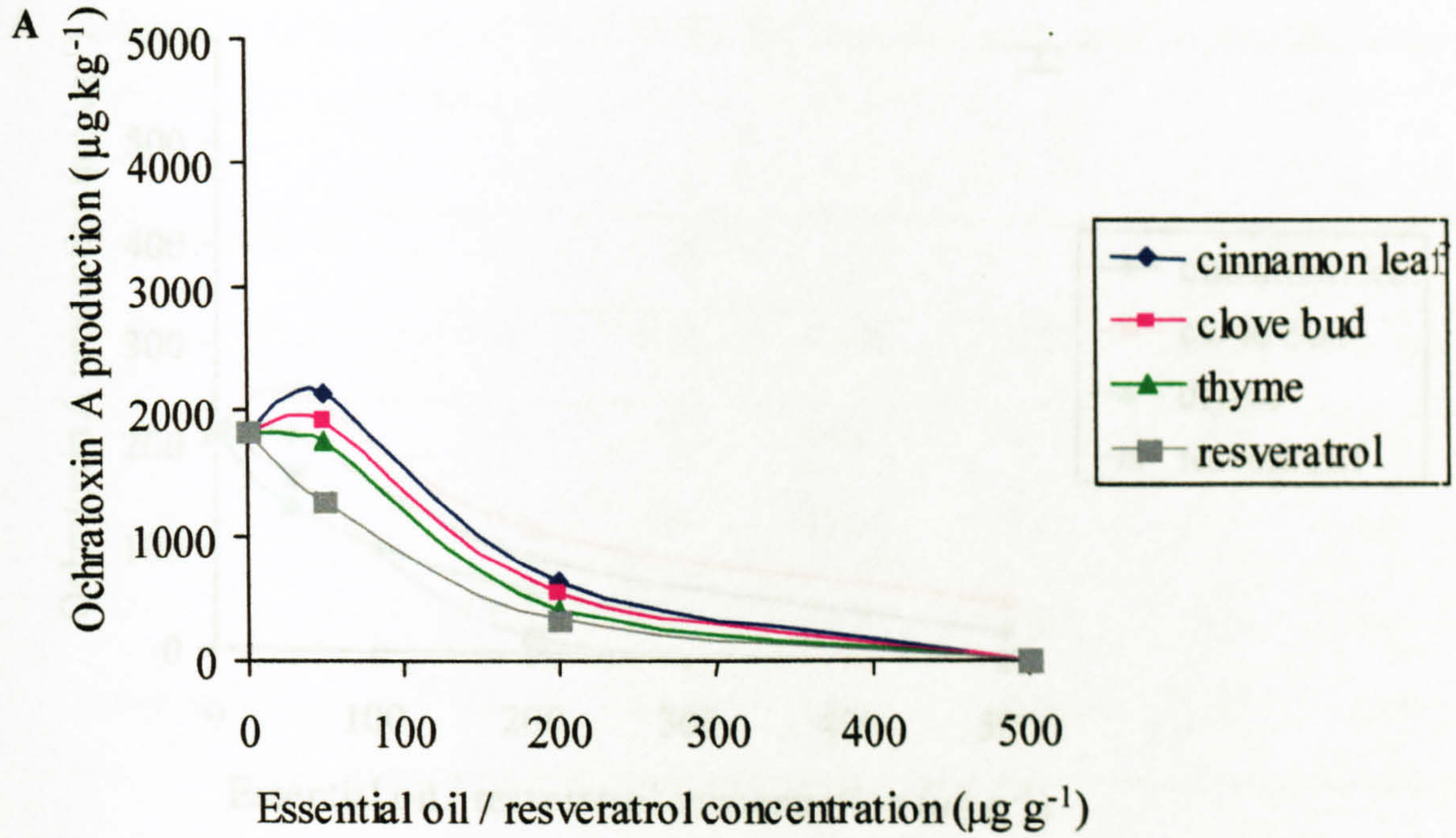


Figure 3.71 Effect of various concentrations of resveratrol and the essential oils cinnamon leaf, clove bud and thyme on ochratoxin A production by *Aspergillus ochraceus* on γ-irradiated wheat grain after 28 days at A) 0.90 a_w at 15 °C B) 0.90 a_w at 25 °C. Bars indicate Least Significant Difference (LSD) at $p < 0.05$.



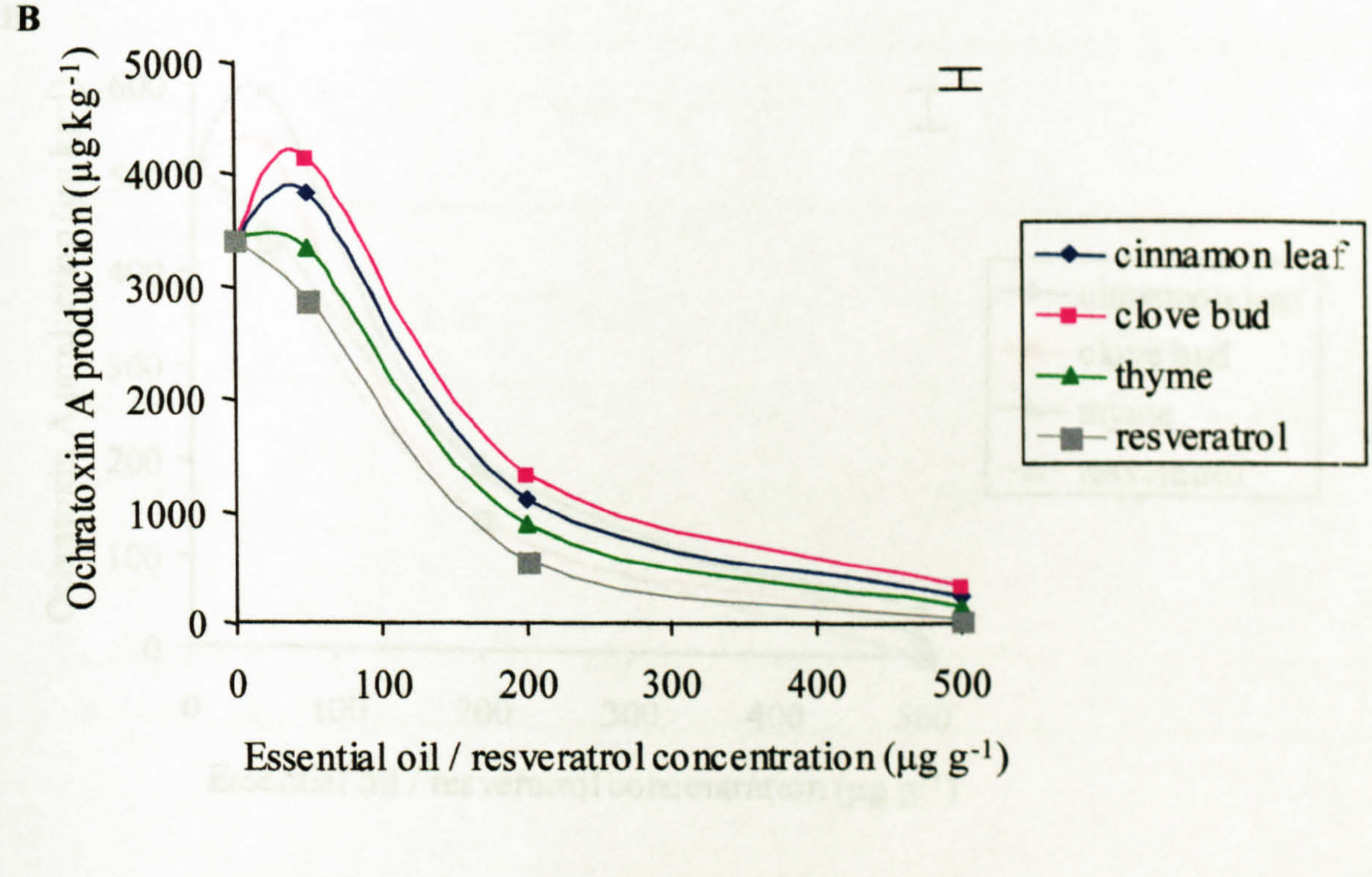
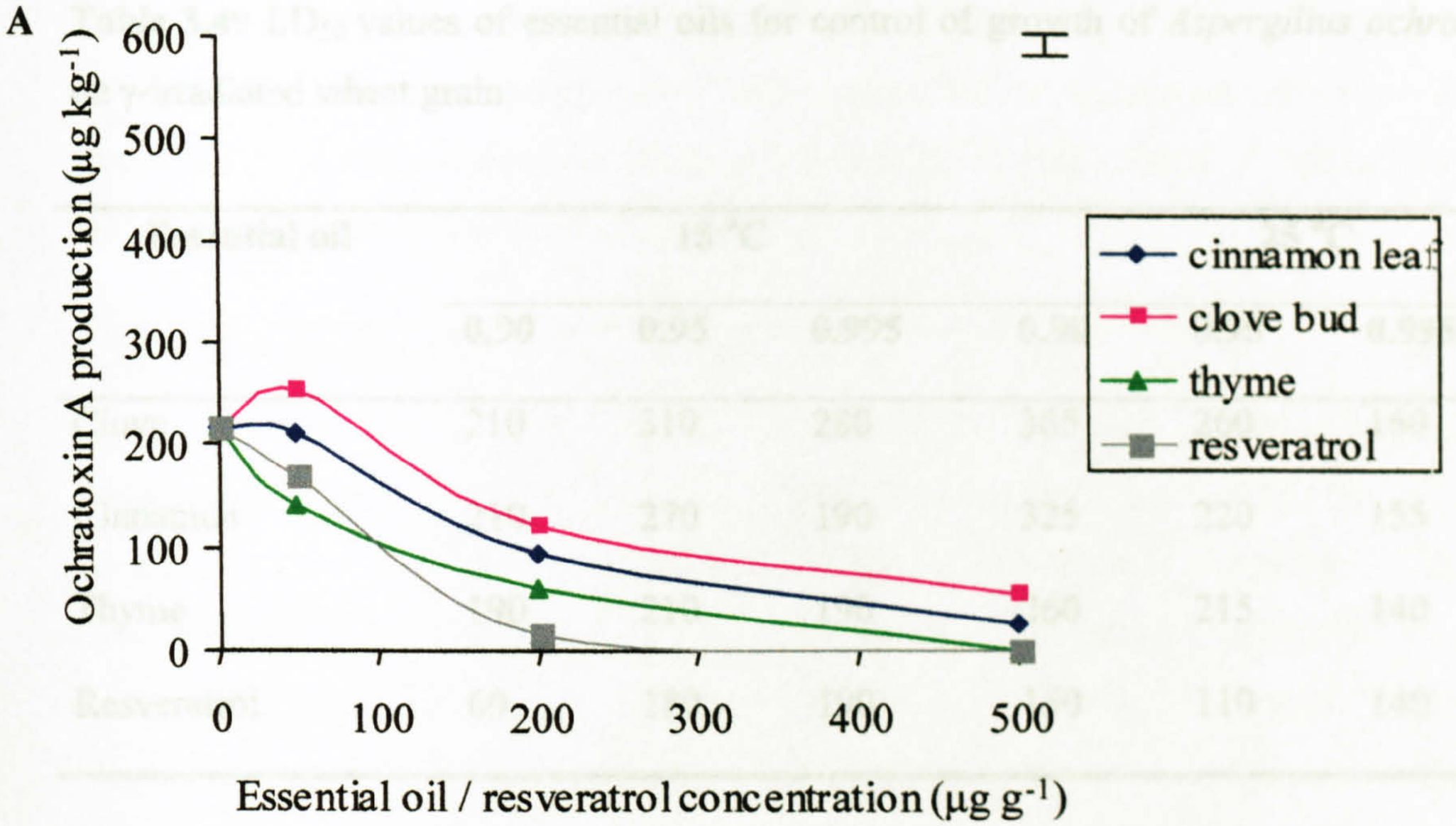


Figure 3.72 Effect of various concentrations of resveratrol and the essential oils cinnamon leaf, clove bud and thyme on ochratoxin A production by *Aspergillus ochraceus* on γ-irradiated wheat grain after 28 days at A) 0.95 a_w at 15 °C B) 0.95 a_w at 25 °C. Bar indicates Least Significant Difference (LSD) at $p < 0.05$. LSD for A) is 19.42.



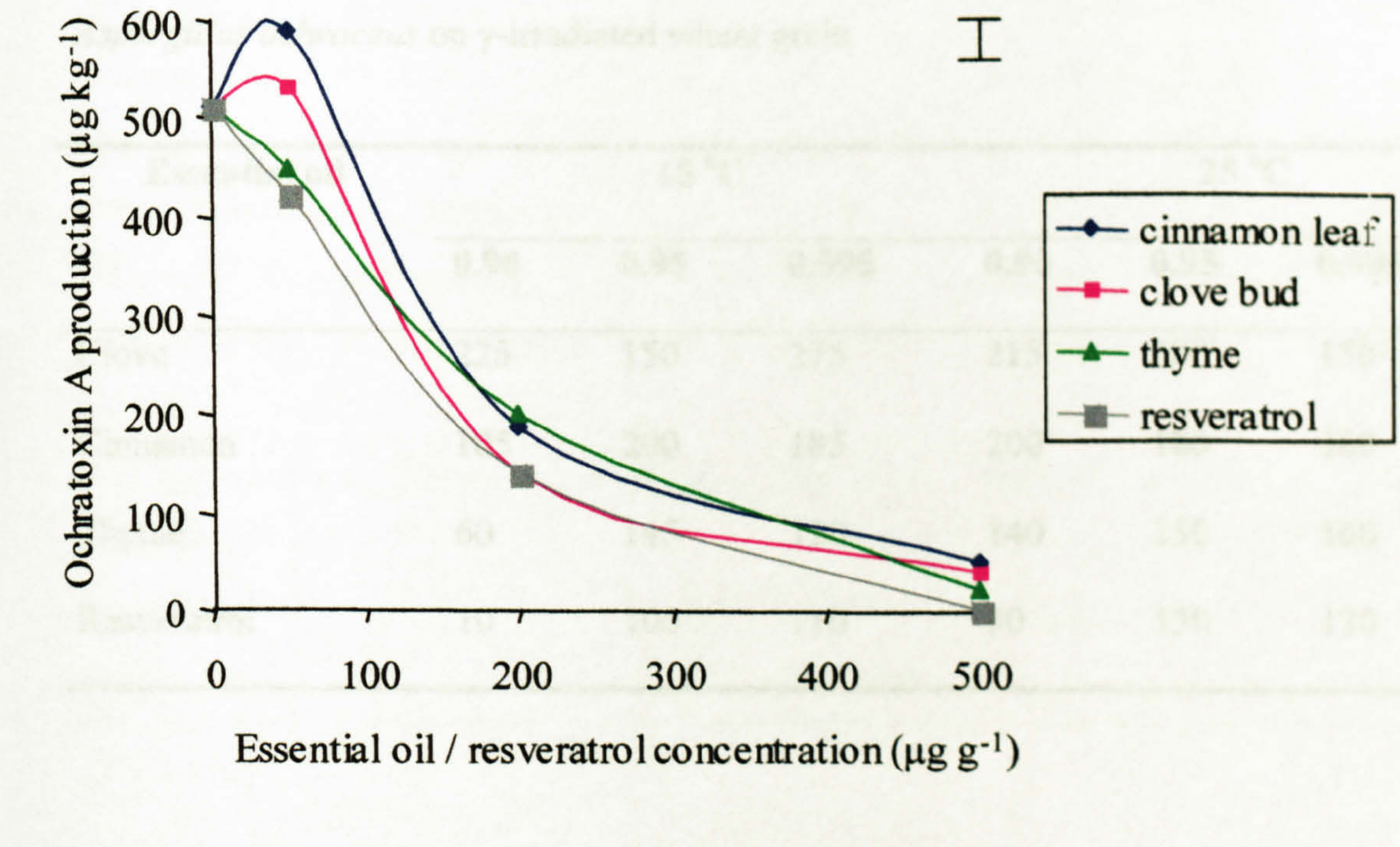


Figure 3.73 Effect of various concentrations of resveratrol and the essential oils cinnamon leaf, clove bud and thyme on ochratoxin A production by *Aspergillus ochraceus* on γ-irradiated wheat grain after 28 days at A) 0.995 a_w at 15 °C B) 0.995 a_w at 25 °C. Bars indicate Least Significant Difference (LSD) at $p < 0.05$.

Table 3.49 LD_{50} values of essential oils for control of growth of *Aspergillus ochraceus* on γ-irradiated wheat grain

Essential oil	15 °C			25 °C		
	0.90	0.95	0.995	0.90	0.95	0.995
Clove	210	310	280	365	260	160
Cinnamon	210	270	190	325	220	155
Thyme	190	210	190	260	215	140
Resveratrol	60	180	190	150	110	140

Table 3.50 LD_{50} values of essential oils for control of ochratoxin A production by *Aspergillus ochraceus* on γ-irradiated wheat grain

Essential oil	15 °C			25 °C		
	0.90	0.95	0.995	0.90	0.95	0.995
Clove	225	150	275	215	200	150
Cinnamon	105	200	185	200	180	160
Thyme	60	145	120	140	150	160
Resveratrol	10	100	110	30	130	130

Table 3.51 ANOVA for growth of *Aspergillus ochraceus* on γ-irradiated wheat grain at various water activities incorporated with resveratrol or cinnamon, clove or thyme essential oils at various concentrations at 15 and 25 °C respectively. * Indicates factor elicited a significant effect ($p<0.05$).

Source of Variation	DF	SS	MS	F	P
a_w	2	75.3979	37.6989	2898.03	<0.001*
Temperature	1	164.7536	164.7536	1.3E+04	<0.001*
Treatment	3	5.0051	1.6684	128.25	<0.001*
Concentration	3	270.6124	90.2041	6934.25	<0.001*
Temperature x a_w	2	8.3190	4.1595	319.75	<0.001*
Treatment x a_w	6	0.9869	0.1645	12.64	<0.001*
Concentration x a_w	6	23.0788	3.8465	295.69	<0.001*
Concentration x temperature	3	48.2185	16.0728	1235.59	<0.001*
Treatment x concentration	9	2.0962	0.2329	17.90	<0.001*
Treatment x temperature	3	1.0018	0.3339	25.67	<0.001*
Concentration x a_w x temperature	6	3.4757	0.5793	44.53	<0.001*
Concentration x a_w x treatment	18	1.1863	0.0659	5.07	<0.001*
Temperature x a_w x treatment	6	0.3459	0.0577	4.43	<0.001*
Concentration x temperature x treatment	9	0.4632	0.0515	3.96	<0.001*
Concentration x a_w x temperature x treatment	18	0.5211	0.0289	2.23	0.004*
Residual	192	2.4976	0.0130		
Total	287	607.9601			

Table 3.52 ANOVA for ochratoxin A production by *Aspergillus ochraceus* after 28 days on γ-irradiated wheat grain at various water activities incorporated with resveratrol or cinnamon, clove or thyme essential oils at various concentrations at 15 or 25 °C respectively. * Indicates factor elicited a significant effect ($p < 0.05$).

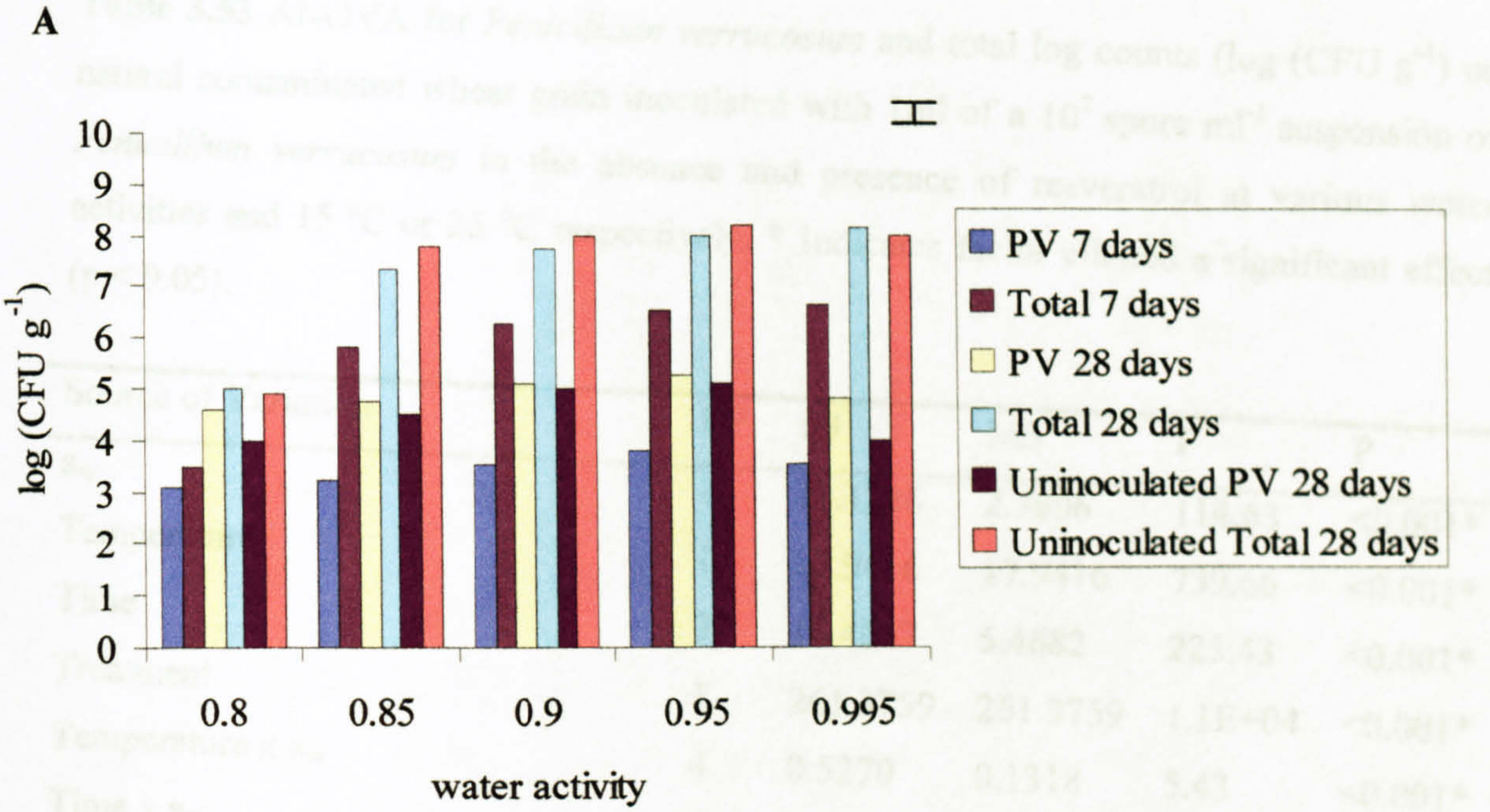
Source of Variation	DF	SS	MS	F	P
a_w	2	113992573	56996287	5658.59	<0.001*
Temperature	1	14082362	14082362	1398.10	<0.001*
Treatment	3	1873369	624456	62.00	<0.001*
Concentration	3	60262611	20087537	1994.29	<0.001*
Temperature x a_w	2	10865936	5432968	539.38	<0.001*
Treatment x a_w	6	1757450	29298	29.08	<0.001*
Concentration x a_w	6	64477478	10746246	1066.89	<0.001*
Concentration x temperature	3	6391443	2130481	211.51	<0.001*
Treatment x concentration	9	1829361	203262	20.18	<0.001*
Treatment x temperature	3	361446	120482	11.96	<0.001*
Concentration x a_w x temperature	6	4659220	776537	77.09	<0.001*
Concentration x a_w x treatment	18	1988894	110494	10.97	<0.001*
Temperature x a_w x treatment	6	615087	102514	10.18	<0.001*
Concentration x temperature x treatment	9	275608	30623	3.04	0.002*
Concentration x a_w x temperature x treatment	18	418837	23269	2.31	0.003*
Residual	192	1933926	10073		
Total	287	285785601			

3.6 THE EFFECT OF RESVERATROL ON NATURAL FUNGAL POPULATIONS AND OTA PRODUCTION IN WHEAT GRAIN

In section 3.5 the efficacy of essential oils and resveratrol as controls for growth and OTA production by *P. verrucosum* and *A. ochraceus* were evaluated. Resveratrol required the lowest concentration to reduce growth and OTA production by 50 % for both *P. verrucosum* and *A. ochraceus* and was therefore chosen to be studied in more detail on naturally contaminated wheat grain.

The impact of resveratrol on the populations of fungi in naturally contaminated wheat grain was assessed by comparing the colony forming units (CFUs) g^{-1} from wheat grain treated with resveratrol with that of untreated controls. Samples were destructively analysed by serial dilution using malt extract agar (MEA) and confirmed using DRYES media which was used as the selective media for distinguishing between *P. verrucosum* and other *Penicillium spp.* The changes in total fungal populations in relation to *P. verrucosum* and *A. ochraceus* caused by the different treatments, environmental conditions and time are shown in Figures 3.74 and 3.75 respectively. Statistical analysis showed that total populations and *P. verrucosum* and *A.ochraceus* populations increased significantly with time regardless of the treatment tested (Tables 3.53 and 3.54 respectively). *P. verrucosum* was most abundant at 0.95 a_w whereas *A. ochraceus* was most abundant at 0.95 a_w and 0.995 a_w. Total populations and those of *P. verrucosum* and *A. ochraceus* were inhibited by resveratrol when compared with the control treatments. Resveratrol was able to inhibit total fungal populations and those of *P. verrucosum* and *A. ochraceus* by >60 % under the majority of experimental conditions. Statistical analysis shows that a_w, temperature, time interval and the treatment tested had a significant effect on populations of *P. verrucosum* and *A. ochraceus*. Plates 3.8 and 3.9 compare and contrast the difference in naturally contaminated wheat grain treated with resveratrol in relation to the untreated control treatments. It can be seen that contamination of the grain is visibly less in the resveratrol treatment than that of the untreated sample.

OTA production was quantified for all treatments tested after 28 days incubation. Figure 3.76 shows that the highest OTA production was at 0.95 a_w irrespective of the inoculum used or the treatment tested. Grain inoculated with 1 x 10^2 ml g^{-1} *P. verrucosum* had a higher OTA content than that inoculated with 1 x 10^2 ml g^{-1} *A. ochraceus* regardless of temperature used. Grain treated with resveratrol had a lower OTA level over the entire range of conditions tested when compared with control treatments. Resveratrol was able to reduce OTA production by up to 60 % for the majority of conditions tested. Statistical analysis (Table 3.55) shows that a_w, temperature, time interval and the treatment tested had a significant effect on OTA levels.



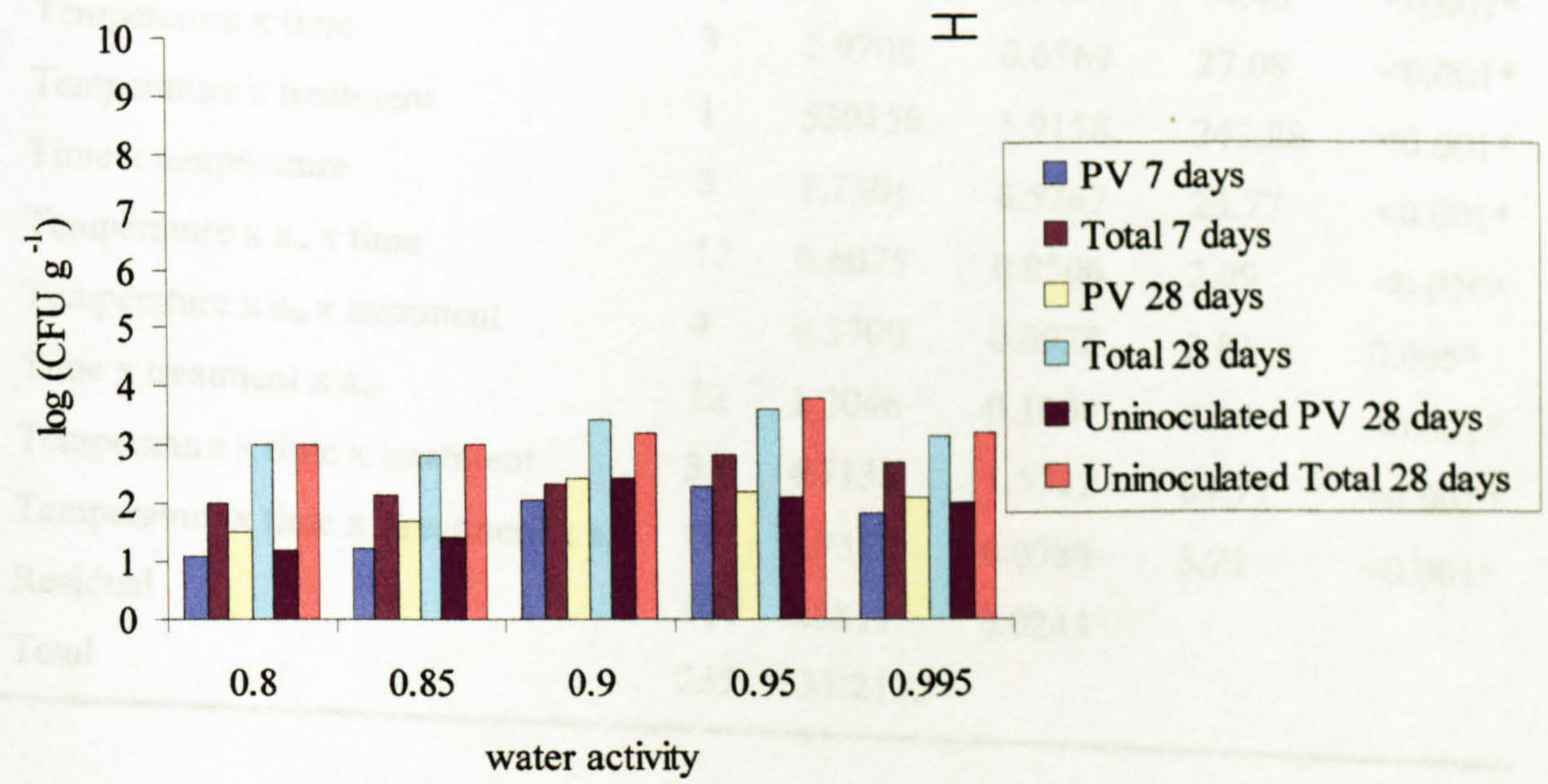


Figure 3.74 Population size of *Penicillium verrucosum* (PV) against total other species on naturally contaminated wheat grain at various water activities on grain inoculated with 1 ml of a 1 x 10^2 spores ml^{-1} suspension of *Penicillium verrucosum* at 25 °C at 7 days or 28 days for A) control B) 200 μg g^{-1} resveratrol. Bars indicate Least Significant Differences (LSD) at $p < 0.05$.

Table 3.53 ANOVA for *Penicillium verrucosum* and total log counts (log (CFU g^{-1}) on natural contaminated wheat grain inoculated with 1ml of a 10^2 spore ml^{-1} suspension of *Penicillium verrucosum* in the absence and presence of resveratrol at various water activities and 15 °C or 25 °C respectively. * Indicates factor elicited a significant effect ($p < 0.05$).

Source of Variation	DF	SS	MS	F	P
a_w	4	11.1226	2.7806	114.63	<0.001*
Temperature	1	17.9416	17.9416	739.66	<0.001*
Time	3	16.4047	5.4682	225.43	<0.001*
Treatment	1	261.3759	261.3759	1.1E+04	<0.001*
Temperature x a_w	4	0.5270	0.1318	5.43	<0.001*
Time x a_w	12	1.1096	0.0925	3.81	<0.001*
Treatment x a_w	4	1.4026	0.3607	14.46	<0.001*
Temperature x time	3	1.9708	0.6569	27.08	<0.001*
Temperature x treatment	1	539158	5.9158	243.88	<0.001*
Time x temperature	3	1.7301	0.5767	23.77	<0.001*
Temperature x a_w x time	12	0.6075	0.0506	2.09	<0.020*
Temperature x a_w x treatment	4	0.3700	0.0925	3.81	0.005*
Time x treatment x a_w	12	1.2046	0.1004	4.14	<0.001*
Temperature x time x treatment	3	4.7135	1.5712	64.77	<0.001*
Temperature x time x Treatment x a_w	12	0.9358	0.0780	3.21	<0.001*
Residual	160	3.8811	0.0243		
Total	239	331.2132			

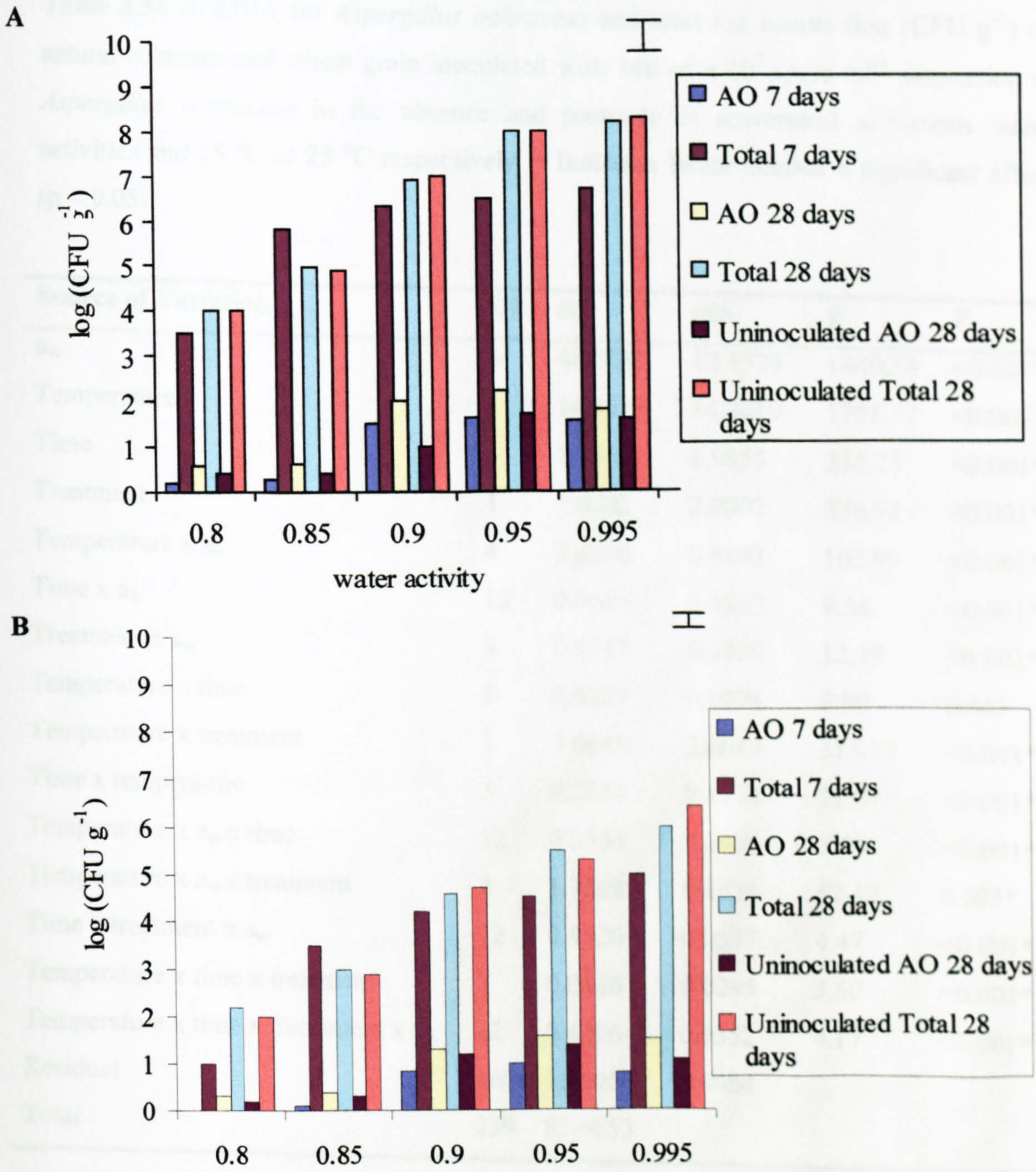


Figure 3.75 Population size of *Aspergillus ochraceus* (AO) against total other species on naturally contaminated wheat grain at various water activities inoculated with 1 ml of a 1 x 10^2 spores ml^{-1} suspension of *Aspergillus ochraceus* at 25 °C at 7 days or 28 days for A) control B) 200 μg g^{-1} resveratrol. Bars indicate Least Significant Differences (LSD) at $p < 0.05$.

Table 3.54 ANOVA for *Aspergillus ochraceus* and total log counts (log (CFU g^{-1}) on natural contaminated wheat grain inoculated with 1ml of a 10^2 spore ml^{-1} suspension of *Aspergillus ochraceus* in the absence and presence of resveratrol at various water activities and 15 °C or 25 °C respectively. * Indicates factor elicited a significant effect ($p < 0.05$).

Source of Variation	DF	SS	MS	F	P
a_w	4	48.6297	12.1574	1440.38	<0.001*
Temperature	1	14.3619	14.3619	1701.57	<0.001*
Time	3	5.9565	1.9855	235.24	<0.001*
Treatment	1	2.0002	2.0002	236.98	<0.001*
Temperature x a_w	4	3.4770	0.8693	102.99	<0.001*
Time x a_w	12	0.9683	0.0807	9.56	<0.001*
Treatment x a_w	4	0.4117	0.1029	12.19	<0.001*
Temperature x time	3	0.0227	0.0076	0.90	0.445
Temperature x treatment	1	2.6649	2.6649	315.73	<0.001*
Time x temperature	3	0.5334	0.1778	21.07	<0.001*
Temperature x a_w x time	12	0.5548	0.0462	5.48	<0.001*
Temperature x a_w x treatment	4	1.7698	0.4424	52.42	0.005*
Time x treatment x a_w	12	0.4526	0.0377	4.47	<0.001*
Temperature x time x treatment	3	0.0886	0.0295	3.50	<0.001*
Temperature x time x Treatment x a_w	12	0.4226	0.0352	4.17	<0.001*
Residual	160	1.3505	0.0084		
Total	239	83.6653			

Plate 3.8 Fungal contamination in naturally contaminated wheat grain adjusted to 0.95 a_w and incubated at 25 °C after 28 days after A) inoculation with 1ml of 10^2 spore ml^{-1} *Aspergillus ochraceus* and B) inoculation with 1ml of 10^2 spore ml^{-1} *Penicillium verrucosum*..

Plate 3.9 Fungal contamination in naturally contaminated wheat grain adjusted to 0.95 a_w and treated with 200 µg g^{-1} resveratrol after 28 days at 25 °C after inoculation with A) 1ml of 10^2 spore ml^{-1} *Aspergillus ochraceus* and B) 1ml of 10^2 spore ml^{-1} *Penicillium verrucosum*.

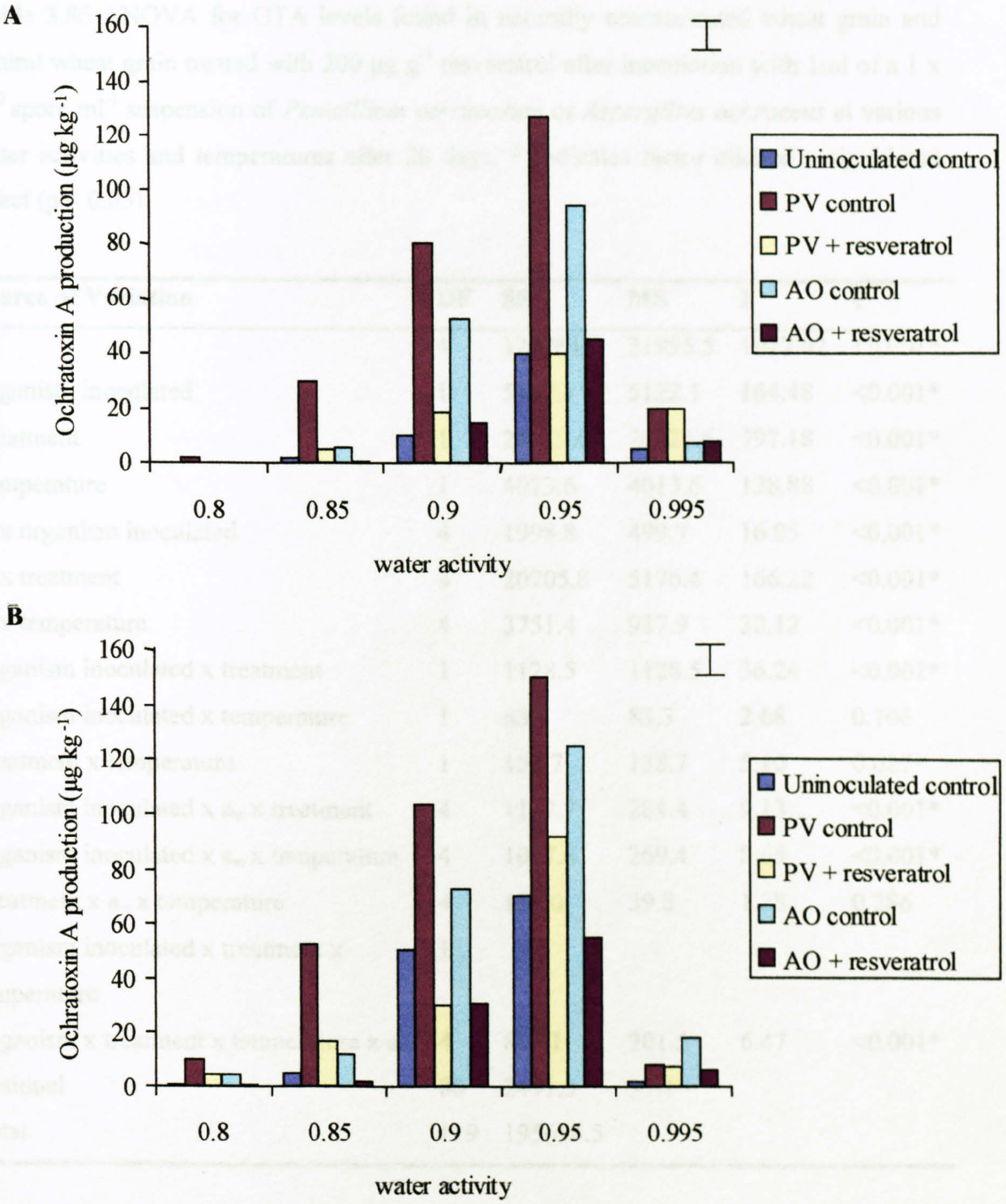


Figure 3.76 Ochratoxin A levels present in natural wheat grain inoculated with 1 ml of a 10^{2} spores ml^{-1} suspension of *Penicillium verrucosum* or *Aspergillus ochraceus* on untreated naturally contaminated wheat grain and grain treated with 200 µg g^{-1} of resveratrol after 28 days at A) 15 °C and B) 25 °C. Bars indicate Least Significant Difference (LSD) at $p < 0.05$.

Table 3.55 ANOVA for OTA levels found in naturally contaminated wheat grain and natural wheat grain treated with 200 μg g^{-1} resveratrol after inoculation with 1ml of a 1 x 10^2 spore ml^{-1} suspension of *Penicillium verrucosum* or *Aspergillus ochraceus* at various water activities and temperatures after 28 days. * Indicates factor elicited a significant effect ($p < 0.05$).

Source of Variation	DF	SS	MS	F	P
a_w	4	127981.9	31995.5	1027.42	<0.001*
Organism inoculated	1	5122.1	5122.1	164.48	<0.001*
Treatment	1	24825.6	24825.6	797.18	<0.001*
Temperature	1	4013.6	4013.6	128.88	<0.001*
a_w x organism inoculated	4	1998.8	499.7	16.05	<0.001*
a_w x treatment	4	20705.8	5176.4	166.22	<0.001*
a_w x temperature	4	3751.4	937.9	30.12	<0.001*
Organism inoculated x treatment	1	1128.5	1128.5	36.24	<0.001*
Organism inoculated x temperature	1	83.3	83.3	2.68	0.106
Treatment x temperature	1	158.7	158.7	5.10	0.027*
Organism inoculated x a_w x treatment	4	1137.7	284.4	9.13	<0.001*
Organism inoculated x a_w x temperature	4	1077.6	269.4	8.65	<0.001*
Treatment x a_w x temperature	4	159.0	39.8	1.28	0.286
Organism inoculated x treatment x temperature	1				
Organism x treatment x temperature x a_w	4	806.1	201.5	6.47	<0.001*
Residual	80	2491.3	31.1		
Total	119	195614.5			

CHAPTER 4

DISCUSSION

4.1 EFFECT OF ENVIRONMENTAL CONDITIONS ON GROWTH OF *PENICILLIUM VERRUCOSUM* AND *ASPERGILLUS OCHRACEUS.*

Growth studies are important because they provide a good indication of the colonisation potential and utilisation of the nutritional substrate. Since grain is colonised by a large number and variety of microorganisms, it is advantageous for a particular fungus to grow quickly so it can reach, colonise and utilise nutrients ahead of its competitors (Magan *et al.*, 2004). This study has developed for the first time detailed growth profiles at various temperatures, water activities and gas compositions for the wheat spoilage fungi *Penicillium verrucosum* and *Aspergillus ochraceus* on both wheat-based media and wheat grain. This clearly shows that environmental factors often have a significant impact on growth rates of both spoilage fungi. The interactions between these factors were often also found to be significant.

i) Temperature

Temperature has been shown to be a key environmental parameter affecting growth rates of spoilage fungi (Magan & Lacey, 1984a; Paster & Chen, 1980; Lacey & Magan, 1991; Ramakrishna *et al.*, 1993; Marin *et al.*, 1998a). Results show that temperature significantly affected growth of *P. verrucosum* and *A. ochraceus.* Ramos *et al.* (1998) showed that *A. ochraceus* grows faster on barley extract medium and barley grains at 30 °C, however, the present study only used temperatures <25 °C because these conditions were more relevant to temperate countries. Both fungi grew faster at 25 than 15 °C over the complete range of water activities tested on both wheat-based media and wheat grain. The effects of temperature on relative growth rates were most marked at intermediate water activity levels (0.93-0.98 a_w) on both substrates. As temperature decreased so did the range of water activities permitting growth. At 10 °C on wheat grain *P. verrucosum* was unable to grow at <0.87 aw although at 15 and 25 °C growth occurred over the entire range of water activities tested (0.80-0.995 a_w). *A. ochraceus* was able to grow over the entire range of water activities tested at 25 °C on irradiated wheat grain but was unable to grow at <0.85 a_w at 15 °C and <0.90 a_w at 10 °C. Similarly, Ramos *et al.*

(1998) observed that on a barley meal extract agar *A. ochraceus* isolates were unable to grow at higher water activity levels >0.90 a_w at 10 °C. Present results show that overall, *A. ochraceus* was more sensitive to temperature than *P. verrucosum.* The reduced impact of temperature on growth rates at sub-optimal water activity conditions <0.90 a_w is probably due to water activity being the most significant growth limiting factor.

ii) Water activity

Water activity has been shown to be a key environmental parameter affecting growth of spoilage fungi (Northolt, 1979; Magan & Lacey, 1984a; Lacey & Magan, 1991; Ramakrishna *et al.*, 1996; Marin *et al.*, 1998a). Many studies have been carried out on the effect of a_w on growth of fungal species. Results from these studies are however difficult to compare directly as they were generally undertaken on a wide range of nutritional media and carried out over a wide range of environmental conditions. Results from the present study show that at 15 °C *P. verrucosum* was unable to grow at <0.85 a_w on wheat-based media. However, at the same temperature on wheat grain growth occurred over the complete range of water activities tested (0.80-0.995 a_w). Results show that at 15 °C *A. ochraceus* was unable to grow at <0.87 a_w on a wheat-based media while at the same temperature on grain growth was inhibited at <0.85 a_w. This agrees with previous studies on other strains, which suggest that a_w minima for *A. ochraceus* may vary between 0.76-0.88 a_w depending on the substrate type (Christensen, 1962; Lopez & Christensen, 1967; Ramos *et al.*, 1998). Slight discrepancies between results could also be due to differences between strains of the same species. In the present study, *P. verrucosum* isolates varied in their ability to grow on a wheat-based media. Only *P. verrucosum* strain OTA11 was able to grow at 0.85 a_w and 15 °C. Ramos *et al.* (1998) observed variations in growth between *A. ochraceus* isolates on barley meal extract agar at different water activities. The present results show that optimum growth was at 0.95-0.982 a_w on wheat-based media and 0.93-0.95 a_w on grain for both *P. verrucosum* and *A. ochraceus* isolates regardless of the temperature tested. Ramos *et al.* (1998) noted that optimum growth by *A. ochraceus* was higher on barley extract agar than on barley grains. These intermediate water activities are most commonly found in damp, poorly

dried grain, which could explain the suitability of *P. verrucosum* and *A. ochraceus* in this particular ecological niche.

iii) Gas composition

Grain intergranular competition is an important parameter which influences fungal activity and rates of grain deterioration. Carbon dioxide concentrations have been shown to be important in determining levels of fungal colonisation in stored grain (Hyde & Oxley, 1960; Hyde, 1974; Magan & Lacey, 1984b). Despite this, it is surprising that relatively few studies have investigated the effects of carbon dioxide concentrations on growth of spoilage fungi and their interactions with other key environmental parameters such as water activity and temperature. Results from this study show that CO_2 concentrations and their interaction with water activity significantly affected germination and growth rates of *P. verrucosum* and *A. ochraceus* on a wheat-based media and wheat grain.

On wheat-based media, germination rates were fastest in air, followed by 25 % CO_2 and 50 % CO_2 when compared with their respective water activities for both *P. verrucosum* and *A. ochraceus*. These results complement growth rate studies which showed that growth of both fungi were more rapid in air, followed by 25 % CO_2 and 50 % CO_2 levels when compared with their respective water activities. At 25 % CO_2 levels on agar media, growth rates were reduced by up to 60 % for *P. verrucosum* and 80 % for *A. ochraceus* while on grain growth rates were reduced by up to 45 % for *P. verrucosum* and 66 % for *A. ochraceus*. These results suggest that substrate type may influence growth and sensitivity of *P. verrucosum* and *A. ochraceus* to varying CO_2 levels and care should be taken when extrapolating from *in vitro* to *in situ* studies. At 50 % CO_2 levels on agar media and wheat grain, growth of *P. verrucosum* and *A. ochraceus* was reduced by up to 100 %. These inhibitory concentrations were not lethal to the fungi and after removal of colonies from these conditions normal growth was observed to resume. Paster *et al.* (1983) reported that 80 % CO_2 completely inhibited growth of *A. ochraceus* on a solid synthetic media. However after removal of the colonies from these high CO_2 levels,

normal growth occurred. Overall, *A. ochraceus* was more sensitive to elevated CO_2 levels than *P. verrucosum.*

The present study showed that total inhibition of *A. ochraceus* isolates was dependent upon the water activity used as well as the CO_2 concentration. Total growth inhibition occurred at 50 % CO_2 and 0.90 a_w but growth by *P. verrucosum* and *A. ochraceus* was not inhibited at 50 % CO_2 with more available water (0.95 a_w or 0.995 a_w). Differences between the present results with those of Paster *et al.* (1983) may also be due to differences in sensitivity to CO_2 concentrations between strains of the same species. Growth of *P. verrucosum* and *A. ochraceus* isolates were always fastest at 0.95 a_w followed by 0.995 a_w and 0.90 a_w respectively for both fungi regardless of the substrate type or CO_2 level tested. Growth was decreased more when CO_2 levels were increased and water activity was altered from 0.95 to 0.995 to 0.90 a_w than when either was changed alone, although this effect was not synergistic. This agrees with previous findings by Magan &Lacey (1984b). They found that individually changing a_w or gas composition increased the lag phase for growth whilst combinations of these factors had a more additive effect on lag periods, indicating synergism. They also reported concentrations of 5-10 % CO_2 stimulated growth of some grain spoilage fungi although at 15 % CO_2 most species showed some level of inhibition depending on a_w level.

Although fungi are often regarded as obligate aerobes this data clearly shows that *P. verrucosum* and *A. ochraceus* can tolerate high carbon dioxide levels although growth initiation is considerably delayed, especially at low water activities. Furthermore, the use of modified atmospheres has shown promising results in controlling insects in stored products which would lead to an increase in the safe storage time of wheat grain (Harein & Press, 1968). The effects of sub-optimal CO_2 levels on fungal growth and grain mycoflora needs to be evaluated in further detail if elevated CO_2 is to be used in larger scale grain storage systems. Long-term data are needed to evaluate the practical use of environmental control using these factors and to assess the economics of such inputs (Magan & Lacey, 1984b).

iv) Substrate type

Substrate type affected the range and optimum conditions allowing growth of *P. verrucosum* and *A. ochraceus.* This study has shown that both *P. verrucosum* and *A. ochraceus* grew faster on layers of wheat grain than on wheat-based agar media over the range of water activities and temperatures tested. Both fungi grew over a greater range of water activities on wheat grain than on the media. It has been suggested that nutrient source can affect the minimal a_w for growth (Wearing & Burgess, 1972). This was similarly reported by Ramos *et al.* (1998) when growth of *A. ochraceus* was compared on barley extract agar and barley grain. It has been suggested that studies on artificial substrates may not accurately represent the real capacity to grow on a natural substrate (Wearing & Burgess, 1972). For this reason studies were carried out on both a wheat-based substrate and wheat grain with retained germinative capacity which unlike autoclaving, does not significantly affect the chemical composition of the grain.

4.2 EFFECT OF ENVIRONMENTAL PARAMETERS ON OCHRATOXIN A PRODUCTION BY *PENICILLIUM VERRUCOSUM* AND *ASPERGILLUS OCHRACEUS*

i) Effect of temperature x water activity interactions on ochratoxin A production by *Penicillium verrucosum* and *Aspergillus ochraceus.*

It has been shown that environmental factors such as a_w, temperature and CO_2 levels play a crucial role in determining levels of mycotoxin contamination of grain (Northolt, 1979; Paster *et al.*, 1998; Ramakrishna *et al.*, 1996; Marin *et al.*, 1998c; Lee & Magan, 1999a). Despite this, it is surprising that few studies have been carried out to determine the effects of key environmental factors such as water activity, temperature and gas composition at various time intervals on mycotoxin production by *P. verrucosum.* Extensive work has been carried out on the effects of water activity and temperature on aflatoxin, patulin and cyclopiazonic acid production (Northolt *et al.*, 1977; 1978) and more recently the effects of water activity and temperature on fumonisin production by *F. moniliforme* and

F. proliferatum infecting maize grain (Marin *et al.*, 1998a). The present work represents, for the first time, a full factorial experiment for temporal OTA production at various water activities and temperatures for *P. verrucosum* and *A. ochraceus* on wheat-based media and irradiated wheat grain. Gqaleni *et al.* (1997) suggested that a full factorial designed experiment is useful because it allows the analysis of a range of different factors simultaneously, is economical and saves time. Marin *et al.* (1998b) suggested that in mycotoxin studies it helps explain concentrations in natural substrates. The present data clearly shows that environmental factors have a significant effect on concentrations of OTA produced by *P. verrucosum* and *A. ochraceus.* The interactions between these factors were also often significant. This study not only establishes the range and optimum conditions which are conducive to OTA production but also the limits for OTA production.

On agar media all *P. verrucosum* strains produced most OTA at 0.95 a_w regardless of the temperature tested. The *P. verrucosum* strains produced significantly more OTA at 25 than 15 °C over the complete a_w range tested. OTA production varied significantly between strains of *P. verrucosum.* All isolates were able to produce OTA at between 0.90–0.995 a_w at 15 °C and 0.85-0.995 a_w at 25 °C respectively. However in addition *P. verrucosum* (strain OTA11) was able to produce OTA at 0.85 a_w and 15 °C. This strain produced significantly more OTA than the two others (IBT22625 and IBT22626) regardless of the water activity or temperature tested although growth by strain OTA11 was not always faster than the other strains tested. Ramos *et al.* (1998) observed variations between strains of *A. ochraceus* when grown on barley grains at various water activities and temperatures. The in-depth toxin profile of *P. verrucosum* on a wheat-based media shows that as incubation period increases up to 56 days so does toxin production over the complete range of a_w x temperatures tested. OTA production occurred over a narrower range of water activities than those required for growth. For example, at 25 °C on agar media OTA production occurred at 0.85–0.995 a_w whereas growth occurred at 0.80–0.995 a_w. OTA production was optimum at 0.95 a_w regardless of the temperature tested. This was also the water activity where growth was fastest. OTA

production and growth were both greater at 25 than 15 °C over the complete range of water activities tested.

On agar media, *A. ochraceus* produced most OTA at 0.95 a_w regardless of the temperature tested. *A. ochraceus* produced significantly more OTA a 25 than 15 °C over the entire range of water activities tested. As incubation period increased so did OTA production. OTA production was greatest after 56 days over the entire range of water activities tested. OTA production by *A. ochraceus* occurred over a narrower range of conditions than those which permitted growth. At 25 °C growth occurred between 0.80-0.995 a_w whereas OTA production occurred between 0.85-0.995 a_w. At 15 °C growth occurred between 0.87 - 0.995 a_w whereas OTA production occurred between 0.90- 0.995 a_w. Studies by Northolt *et al.* (1979) reported that a minimum of 0.83-0.87 a_w is required for toxin production by *A. ochraceus*. Northolt *et al.* (1979) reported that maximum toxin yields were at 0.99 a_w on a rich agar medium, however present results show maximum toxin yields were at 0.95 a_w on a wheat-based media irrespective of the temperature or time interval tested. Reasons for the differences between results could be due to the use of different substrates and different isolates used.

On wheat grain both *P. verrucosum* and *A. ochraceus* produced the greatest amount of OTA at 0.95 a_w and 25 °C. These were also the conditions optimal for growth. OTA production increased with incubation time for both species tested with optimum OTA production at the greatest time interval tested of 56 days. *P. verrucosum* strain OTA11 was more ochratoxigenic than *A. ochraceus* strain IBT21991 on wheat grain. *P. verrucosum* produced up to 70 000 μg kg^{-1} of OTA after 56 days at 0.95 a_w and 25 °C, whereas *A. ochraceus* produced up to 14 000 μg kg^{-1} of OTA at the same conditions. These amounts far exceed those stated in European legislation (EC) No 472/2002 dated 12 March 2002 which established a maximum level of 5 μg kg^{-1} in raw cereal grains and 3 μg kg^{-1} in all products derived from cereals. This highlights the importance of grain being dried immediately after harvest and stored in a cool dry weather-proof structure. Interestingly, at sub-optimal water activities (<0.87 a_w) there was a stimulation in OTA production by *A. ochraceus* at 15 and 25 °C on irradiated wheat grain. This was not

observed at 10 °C. This stimulation in OTA production was not mirrored by a stimulation in growth at <0.87 a_w. This stimulation in OTA production could have been due to a response to environmental stress and could be a survival strategy in a niche where water availability is scarce.

ii) Effect of water activity x gas compositions interactions on ochratoxin A production by *Penicillium verrucosum* and *Aspergillus ochraceus.*

Present results show that CO_2 levels interacted with water activity to influence OTA production by *P. verrucosum* and *A. ochraceus.* OTA production was greatest at 0.95 a_w followed by 0.995 a_w and 0.90 a_w regardless of the gas composition tested for both fungi tested. OTA production was greatest in air, followed by 25 % CO_2 and 50 % CO_2 levels respectively. These conditions were also optimal for growth. More OTA production was produced on irradiated wheat grain than on agar media for both *P. verrucosum* and *A. ochraceus.* OTA production was completely inhibited at 50 % CO_2 and 0.90 a_w for both fungi regardless of the substrate tested. These results are in contrast to those of Paster *et al.* (1983) who reported that OTA production by *A. ochraceus* was totally inhibited at high levels of CO_2 (>30 %) on a solid synthetic media after 14 days. Differences between results could be due to varying degrees of tolerance in CO_2 levels existing between strains of *A. ochraceus* species or substrate type could influence OTA production. Paster *et al.* (1983) carried out their experiments on a solid synthetic media, whereas the present study involved comparing OTA production on a wheat-based media and wheat grain respectively. Paster *et al.* (1983) may have allowed inadequate time for the development for OTA production at the high CO_2 levels. Present results show that high CO_2 levels (>25 %) delayed the onset of OTA production. The present results also show that a_w and CO_2 interacted to influence OTA production by both *P. verrucosum* and *A. ochraceus.* Although 50 % CO_2 completely inhibited OTA production by both fungi at 0.90 a_w, toxin production still occurred at 50 % CO_2 levels at 0.95 a_w and 0.995 a_w respectively. OTA production is affected more when CO_2 levels increase and when a_w is altered from 0.95 a_w to 0.995 a_w to 0.90 a_w than when either is altered separately although this effect was not synergistic.

In summary it can be shown that OTA production is greatest at intermediate a_w levels regardless of temperature and so it is crucial to avoid any delay before harvested grain is dried. Any delay could allow establishment of *P. verrucosum* and *A. ochraceus* and concomitant OTA production. Subsequent stable storage conditions which prevent initiation and growth of spoilage fungi such as *P. verrucosum* and *A. ochraceus* are necessary for long-term safe storage.

iii) Effect of substrate type on ochratoxin A production by *Penicillium verrucosum* and *Aspergillus ochraceus*

Substrate type had an influence on the range of conditions permitting OTA production. Both *P. verrucosum* and *A. ochraceus* produced more OTA on irradiated wheat grain than on wheat-based media over the range of water activities and temperatures tested. Similarly Marin *et al.* (1998a) observed differences in fumonisin production when *F. proliferatum* and *F. moniliforme* were grown on different grain substrates. *P. verrucosum* and *A. ochraceus* produced OTA over a narrower range of conditions than those required for growth. At 15 °C on wheat grain *P. verrucosum* grew at 0.80-0.995 a_w whereas OTA production occurred at 0.85-0.995 a_w and at 10 °C. *P. verrucosum* grew at 0.87-0.995 a_w whereas OTA production occurred at 0.90–0.995 a_w. At 15 °C on irradiated wheat grain *A. ochraceus* grew at 0.85–0.995 a_w whereas OTA production occurred at 0.90-0.995 a_w and at 10 °C on irradiated wheat grain *A. ochraceus* grew at 0.90–0.995 a_w whereas OTA production occurred at 0.93–0.995 a_w. These results are in contrast to those of Häggblom (1983) who reported that no OTA production was produced at 10 °C by *A. ochraceus* on autoclaved barley grains. In this study wheat grain was used whereas Häggblom (1983) used autoclaved barley grains. Possible differences between results could also be due to differences in OTA production between strains of the same species making results difficult to compare.

4.3 COMPETITIVENESS AND THE EFFECT OF COMPETING MYCOFLORA ON GROWTH AND OCHRATOXIN A PRODUCTION BY *PENICILLIUM VERRUCOSUM* AND *ASPERGILLUS OCHRACEUS*

The study of fungal interactions has attracted interest in recent years because mycotoxin production by toxigenic species in stored grain can be enhanced or inhibited by other species which also colonise the grain. It has been suggested that production of aflatoxins and cyclopiazonic acid by *A. flavus* might allow it to colonise more substrate and aid in its survival in a particular ecological niche. It may thus modify the competitive ability of competing fungi and other microorganisms and inhibit their invasion of already colonised substrate (Gqualeni *et al.* 1997). This hypothesis has also been used to explain why *Fusarium spp.* produce fumonisins (Marin *et al.*, 1998a, b) and may be extended to explain why *P. verrucosum* and *A. ochraceus* produce OTA. When discussing fungal competition, two aspects must be taken into account: primary resource capture and combat (Cooke & Whipps, 1993). Prolific production of spores, quick germination of these, possession of appropriate extracellular enzymes and increased growth rates allow species to succeed in primary resource capture. However, hyphal interactions reflect the ability of a species for combat and can be important when the density of the initial inoculum is high. Cuero, Smith & Lacey (1987) showed that aflatoxin production by *Aspergillus flavus* was increased by *Bacillus amyloliquefaciens* and *Hyphopichia burtonii* when grown on irradiated maize and rice grains under certain environmental conditions. Work with *Fusarium spp.* on maize grain has shown that fumonisin production has been stimulated by interactions with *A. ochraceus* particularly at 0.98 a_w (Marin *et al.*1998b). Stimulation could have been due to *A. ochraceus* attempting to maintain occupation of nutrient areas and preemptive exclusion of competitors from the niche occupied (Lee & Magan, 1999).

This present study has shown that both interaction type, I_D, growth rates and OTA production are critically dependent on environmental conditions such as a_w and temperature as well as the species tested. *P. verrucosum* is more competitive in terms of I_D values at 15 than 25 °C, particularly at lowered a_w (<0.95 a_w). Although *P. verrucosum*

is known to produce the secondary metabolite OTA as well as other mycotoxins, it did not have high I_D scores, in contrast with *Penicillium brevicompactum* which produces mycophenolic acid (Magan & Lacey, 1984). The effects of growth showed that overall, regardless of temperature x a_w conditions, the actual colonisation rate of *P. verrucosum* was reduced by the competing fungi in dual culture at 25 °C at 0.95 and 0.995 a_w. This reduction resulted in a significant decrease in OTA production. This was particularly apparent when *P. verrucosum* was competing with *F. culmorum* and *F. poae* at 0.995 a_w and 25 °C There was no direct relationship between growth rates of *P. verrucosum* and its competitiveness which is similar to that found previously (Magan & Lacey, 1984a, 1985; Whipps & Magan, 1987; Whipps & Hocking, 1993; Marin *et al.* 1998a).

A. ochraceus was more competitive in terms of I_D at 25 than 15 °C particularly at intermediate a_w levels, i.e. 0.95 a_w, when compared with the other species tested. Although *A. ochraceus* produces OTA, it did not have a high I_D scores. Regardless of temperature x a_w conditions, the actual growth rates of *A. ochraceus* was reduced by competing fungi in dual culture, particularly at 0.995 a_w and 25 °C. This resulted in a significant decrease in OTA production and was particularly apparent when *A. ochraceus* was paired with *F. culmorum, A. tenuissima* or *F. poae* at 0.995 a_w and 25 °C. There was no direct relationship between growth rates of *A. ochraceus* and its competitiveness. Lee and Magan (2000) investigating the competitive ability of *A. ochraceus* on a maize grain came to similar conclusions.

Ecological similarity and coexistence between microbial species has usually been examined in relation to the suitability of biological control agents for control of pathogens on plant surfaces (Wilson & Lindow, 1994a,b). By understanding niche overlap indices, this could help explain why some species become more competitive than others under certain steady-state environmental conditions. Niche overlap indices compare the carbon sources that are able to be metabolised by different species. If either of the fungi has 90 % or more compatible carbon sources, they will occupy the same nutritional niche (Wilson & Lindow, 1994a, b). A fungus is assumed to attempt to exclude or inhibit other fungi and microorganisms which share the same nutritional

niche. It was hypothesised that fungi found to occupy a similar niche would also be those which attempted to exclude or dominate each other in interaction experiments. Fungi that produce more passive interactions (e.g. mutual intermingling) in dual culture are expected to occupy separate nutritional niches, as species utilising different nutritional sources pose less threat to one another than those assimilating the same sources.

This study showed that the number of carbon sources utilised varied with water availability and temperature for all species tested implying that the amount of niche overlap changes with environmental conditions. Generally as water availability increased so did the niche size of all species tested. The niche maps presented in this thesis allow the relationship between one species and the target pathogen in this case *P. verrucosum* or *A. ochraceus* respectively to be concisely determined. Interestingly, all *P. verrucosum* strains tested did not necessarily share the same ecological niche over all conditions tested, indicating strains of the same species do not necessarily share the same ecological niche as one another.

There was no correlation between I_D and niche overlap indices. I_D depended more on a_w and temperature than on niche overlap, implying that there may be some other factor other than carbon source which is influencing competitiveness. This has been found previously with maize spoilage fungi (Marin *et al.* 1998c). It is more likely that competitiveness depends upon a combination of factors including carbon source utilisation, growth rates, metabolite production, niche overlap and interactions with environmental conditions (Lee & Magan, 1999b, Magan *et al.*, 2004).

In this study, an equal inoculum of each fungal species was used to allow species an equal chance to become dominant. In practice, grain placed in storage is contaminated with several field fungi and smaller numbers of storage fungi (Sinha, 1973; Magan & Lacey, 1984). Also storage conditions are in a state of flux and are affected by the different abiotic and biotic factors. These factors make it difficult to interpret and understand why certain species become dominant in specific niches and predict the role of OTA production.

4.4 USE OF ESSENTIAL OILS AND RESVERATROL FOR CONTROL OF GROWTH AND OCHRATOXIN A PRODUCTION BY *PENICILLIUM VERRUCOSUM* AND *ASGPERGILLUS OCHRACEUS*

Screening of essential oils for effective control of growth and OTA production has seldom been examined in conjunction with environmental conditions, particularly temperature and water activity, despite these factors having a critical influence on growth and mycotoxin production (Magan *et al.*, 1997; Marin *et al.*, 1998, 2000; Lee & Magan, 2000).

This study has shown that potential exists for controlling growth and OTA production by *P. verrucosum* and *A. ochraceus* species using food-grade essential oils over a range of a_w and temperature conditions. However, results showed that only a few of the essential oils tested were effective in controlling growth and OTA production by *P. verrucosum* and *A. ochraceus*. The efficacy of these treatments were however affected by environmental conditions. Overall, 500 μg g^{-1} of the cinnamon, clove leaf (cleaned) and thyme essential oils significantly reduced growth of both species by >90 % on wheat-based media and 80 % on wheat grain. OTA production by both species was controlled by >90 % on agar media and 85 % wheat grain over the complete range of a_w levels, regardless of the temperature tested. Results indicate that higher concentrations of essential oils were required to control growth than OTA production over the range of environmental conditions tested. Higher concentrations of essential oils were required to control growth and OTA production on irradiated wheat grain than on wheat-based media for both *P. verrucosum* and *A. ochraceus*. This implies that care must be taken when extrapolating from *in vitro* to *in situ* studies with such antimicrobial compounds. Sub-optimal concentrations of essential oils were sometimes found to enhance growth and OTA production by both *P. verrucosum* and *A. ochraceus*. The stimulation of OTA production at low concentrations of essential oils has previously been demonstrated with low doses of fungicide applied to grain to control growth and deoxynivalenol production by *F. culmorum* (Magan, 2002). It is possible that under these conditions active growth is still occurring and the imposition of stress due to biocide application combined with

environmental stress could result in a survival or defence response by enhanced production of the mycotoxin. In contrast, at higher concentration (500 μg g^{-1}) where almost complete inhibition of growth was achieved, mycotoxin production was significantly controlled. Thus, there could be ecological reasons for these differences. Previously, Lord *et al.* (1982) expressed concern about potential problems that could arise from using fungistatic preservatives, especially when irregular distribution occurs, which in turn to could lead to an increase in mycotoxin production. If essential oils were to be used in stored grain, effective application would be needed to ensure good coverage of grain.

Recent studies have suggested that different strains of the same fungus may have varying tolerance to essential oils. This in turn could influence the efficacy of the best essential oil treatments in the stored-grain ecosystem. Matamoros-Leon *et al.* (1999) demonstrated that the antifungal activity of potassium sorbate was enhanced by other antimicrobial compounds such as vanillin. It has been suggested that combinations of essential oils and antioxidants could be evaluated for their effectiveness against growth and OTA production by isolates of *P. verrucosum* and *A. ochraceus.* Some studies have been carried out using the active components of essential oils but results are often conflicting. Batt *et al.* (1983) showed that *Aspergillus parasiticus* was inhibited more when only the active components of carrot seed oil (limonene and terpinene) were used than that of the complete oil. In contrast, Alderman and Marth (1976) reported that the oils derived from orange or lemon peel were more effective at controlling growth and aflatoxin production than d-limonene, the main component of the two peel oils. More information on the mode of action employed by essential oils is required if they were to be used in the stored grain ecosystem.

Overall, resveratrol was the most effective antifungal agent at controlling growth and OTA production by *A. ochraceus* and *P. verrucosum* over the entire range of conditions tested on irradiated wheat grain, especially at lower water activities <0.95 a_w. Recently, there has been much interest surrounding the phytoalexin resveratrol. It is known to have antioxidant and antimicrobial properties (Pinto *et al.*, 1991; Fremont, 2000) and it is

already accepted and used as a dietry integrator and is known to have anti-cancerous properties including cell differentiation and apoptosis in cancerous cells (Pervaiz, 2001). At intermediate and low water activities 500 μg g^{-1} resveratrol completely suppressed OTA production by *P. verrucosum* and *A. ochraceus* on wheat grain. In very moist grain (0.995 a_w) and some temperatures, resveratrol was less effective and inhibited growth of *P. verrucosum* and *A. ochraceus* by only about 70 % and OTA production by up to 75 %.

4.5 EFFECT OF RESVERATROL ON TOTAL FUNGAL POPULATIONS, *PENICILLIUM VERRUCOSUM* AND *ASPERGILLUS OCHRACEUS* AND OCHRATOXIN A PRODUCTION ON NATURALLY CONTAMINATED WHEAT GRAIN

This study complements previous work in which the inhibitory effects of resveratrol on growth and OTA production by *P. verrucosum* and *A. ochraceus* were demonstrated over a wide range of water activites (0.80-0.995 a_w) at 15 and 25 °C respectively. This work focused on the biotic factors, i.e. the influence of natural mycoflora of wheat, on *P. verrucosum* and *A. ochraceus* development and its relationship with resveratrol, a_w and temperature. For the first time the selective effect of resveratrol on *P. verrucosum* and *A. ochraceus* populations in relation to total mycoflora and the effect this has on OTA production were evaluated.

The impact of resveratrol on total mycoflora populations in relation to *P. verrucosum* and *A. ochraceus* populations in naturally contaminated wheat grain was determined by comparing these populations (CFUs) from wheat grain treated with 200 μg g^{-1} resveratrol with that of untreated controls. In this study, a_w was shown to be the most significant factor influencing total mycoflora and *P. verrucosum* and *A. ochraceus* populations respectively. *P. verrucosum* was most abundant at 0.95 a_w whereas *A. ochraceus* was most abundant at 0.95 a_w and 0.995 a_w. Total populations and those of *P. verrucosum* and *A.ochraceus*, increased significantly with time regardless of the treatment tested. The highest OTA production was at 0.95 a_w irrespective of the inoculum used or the treatment tested. Grain inoculated with 1 x 10^2 ml g^{-1} *P. verrucosum* had a higher OTA content than

that inoculated with 1 x 10^2 ml g^{-1} *A. ochraceus* regardless of temperature used. This complements the two-dimensional profile studies on wheat grain which revealed that *P. verrucosum* was more ochratoxigenic than *A. ochraceus.* Results showed that significantly less OTA production was produced on naturally contaminated wheat grain than on irradiated wheat grain inoculated with a single species. This is largely due to there being no competition between *P. verrucosum* and *A. ochraceus* respectively on irradiated wheat grain whereas on natural wheat grain the natural mycoflora was exerting a selective effect on growth and OTA production by *P. verrucosum* and *A. ochraceus* respectively.

Total mycoflora, *P. verrucosum* and *A. ochraceus* populations were inhibited by resveratrol when compared with the control treatments. Low populations of *P. verrucosum* were present at 0.995 a_w particularly at 25 °C and these were complemented by low concentrations of OTA. These results should be considered together with those obtained in I_D interactions which determined that *P. verrucosum* was not very competitive with other wheat-spoilage fungi at high a_w, i.e. 0.995 a_w

Resveratrol was able to inhibit fungal populations, *P. verrucosum* and *A. ochraceus* by >60 % under the majority of experimental conditions. Grain treated with resveratrol had a lower OTA level over the entire range of conditions tested when compared with control treatments. Resveratrol was able to reduce OTA production by up to 70 % for the majority of conditions tested. Although resveratrol has been shown to be less effective at controlling growth and OTA production at high a_w, *P. verrucosum* and *A. ochraceus* were largely uncompetitive at these conditions with other wheat-spoilage fungi which is illustrated in the low OTA production at high a_w in control treatments.

4.5.2 Cost Benefit Analysis

One of the main concerns regarding treatments of grain using new antifungals is the cost of such a treatment. Resveratol was the most effective antimicrobial tested at controlling growth and OTA production by *P. verrucosum* and *A. ochraceus* as well as controlling

fungal contamination. This treatment reduced fungal populations and toxin production by >90 % in 100 g grain samples of naturally contaminated wheat grain. The cost of resveratrol in relation to the best essential oils treatments determined from *in vitro* studies are illustrated below:

a) Cost of resveratrol is £400 kg^{-1}. To treat 1000 kg with 200 µg g^{-1} would cost £80
b) Cost of cinnamon leaf oil is £50 kg^{-1}. To treat 1000 kg with 200 µg g^{-1} would cost £10
c) Cost of clove oil is £35 kg^{-1}. To treat 1000 kg with 200 µg g^{-1} would cost £7
d) Cost of clove oil is £40 per kg^{-1}. To treat 1000 kg with 200 µg g^{-1} would cost £8

Although the cost of resveratrol is much higher than the other treatments it was shown to be significantly more effective at controlling fungal growth and OTA production by *P. verrucosum* and *A. ochraceus.* In order to reduce this cost, future use may lie in the development of analogues with higher specific activity.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

5.1 CONCLUSIONS

The following conclusions can be drawn based on the experimental work of this study:

1. Environmental factors (a_w, temperature and gas composition) and their interactions had a significant effect on germ tube lengths, growth rates, OTA production and interspecific interactions.

2. Intermediate water activities (0.93 a_w–0.98 a_w) produced the fastest growth rates for both *Penicillium verrucosum* and *Aspergillus ochraceus*. Growth was fastest at 25 °C followed by 15 °C and 10 °C respectively over the entire range of a_w tested.

3. Two dimensional profiles for *P. verrucosum* and *A. ochraceus* for the first time showed that

   a) OTA production was greatest between 0.93–0.98 a_w comparable with grain storage conditions

   b) the faster the growth rate of *P. verrucosum* the greater the OTA production

   c) the optimum conditions for OTA production were not necessarily those for optimum growth by *A. ochraceus*. OTA production by *A. ochraceus* was stimulated at sub-optimal water activities (0.85– 0.87 a_w)

   d) OTA production was greatest at 25 °C followed by 15 °C and 10 °C respectively for both fungi

   e) growth occurred over a wider a_w range than that for OTA production. For example on wheat grain both fungi grew at 0.80-0.995 a_w whereas OTA production occurred between 0.85-0.995 a_w

   f) the range of a_w levels permitting growth and OTA production became narrower as temperature decreased

   g) growth and OTA production was faster on wheat grain than on wheat-based media over the complete range of conditions tested

   h) *A. ochraceus* was more sensitive to temperature than *P. verrucosum*.

4. Gas composition interacted with a_w to determine conidial germ tube lengths, growth and OTA production by *P. verrucosum* and *A. ochraceus*. Results showed that:

   a) as CO_2 increased, conidial germ tube lengths, growth and OTA production decreased

   b) at 50 % CO_2 levels and 0.90 a_w growth and OTA by both fungi were completely inhibited.

5. Interspecific interactions had a significant effect on growth rates, OTA production and competitiveness of *P. verrucosum* and *A. ochraceus* under different environmental conditions. Results showed that:

   a) interactions often caused a significant reduction in growth and OTA production, but the interaction effects often depended on the environmental conditions

   b) both fungi were more competitive at intermediate a_w levels, i.e. 0.95 a_w *P. verrucosum* was more competitive at 15 °C whereas *A. ochraceus* was more competitive at 25 °C.

6. Niche overlap indices (NOIs) and niche maps were developed for the first time and showed that

   a) interspecific competitiveness was closely related to the number of nutritional carbon sources shared between spoilage fungi

   b) as a_w increased so generally did the niche sizes of all species tested

   c) all *P. verrucosum* strains tested did not necessarily share the same ecological niche to one another over range of a_w and temperatures used.

7. Twenty-three essential oils were screened for their ability to control growth of *P. verrucosum* and *A. ochraceus*. The most effective essential oils were found to be thyme, cinnamon leaf and clove bud.

8. The phytoalexin resveratrol and the essential oils cinnamon leaf, clove bud and thyme were able to control growth and OTA production by

P. verrucosum and *A. ochraceus* at > 90 % *in vitro* on wheat agar and > 80 % *in situ* on irradiated wheat grain.

9. Sub-optimal concentrations often caused a stimulation in growth and OTA production over the complete range of environmental conditions tested *in vitro* on wheat agar and *in situ* on irradiated wheat grain.

10. Resveratrol was the most effective treatment at controlling growth and OTA production identified from *in situ* studies. Resveratrol reduced *P. verrucosum* *A. ochraceus* and total fungal populations and OTA production by > 60 % in tests on naturally contaminated wheat grain.

5.2 FURTHER WORK

Possible further work based on the findings of this study could involve:

1. The use of computer modeling to predict *P. verrucosum* and *A. ochraceus* infections and OTA production based on data for a_w, temperature, gas composition and presence of fungal mycoflora on wheat grain. This could prove a useful tool in a HACCP approach to ensuring safe grain storage.

2. Investigate the effects of sub-optimal CO_2 concentrations on growth and OTA production by *P. verrucosum* and *A. ochraceus in situ.* The effects of varying CO_2 levels on *P. verrucosum, A. ochraceus,* total fungal species and associated OTA production on naturally contaminated wheat grain could be examined.

3. Investigate the effects of environmental conditions on enzyme production by *P. verrucosum* and *A. ochrceus* to investigate whether the production of specific enzymes play a role in growth and OTA production.

4. Investigate economics of resveratrol application for controlling *P. verrucosum* or *A. ochraceus* and OTA production on other cereals, and whether resveratrol can be used economically for practical use in grain storage.

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APPENDIX I

Table I.1 ANOVA for ochratoxin A production by three strains of *Penicillium verrucosum* (IBT22625, IBT22626 and OTA11) on wheat media at 15 °C. * Indicates factor elicited a significant effect (P<0.05).

Source of Variation	DF	SS	MS	F	P
a_w	4	432389779	108097445	76.02	<0.001*
Strain	2	5947021	2973510	2.09	0.141
Strain x a_w	8	9509719	1188715	0.84	0.579
Residual	30	42659193	1421973		
Total	44	490505711			

Table I.2 ANOVA for ochratoxin A production by three strains of *Penicillium verrucosum* (IBT22625, IBT22626 and OTA11) on wheat media at 25 °C. * Indicates factor elicited a significant effect (P<0.05).

Source of Variation	DF	SS	MS	F	P
a_w	4	711092674	177773169	29.64	<0.001*
Strain	2	23679519	11839759	1.97	0.156
Strain x a_w	8	35475951	4434494	0.74	0.656
Residual	30	179902196	5996740		
Total	44	950150339			

Table I.3 ANOVA for *Penicillium verrucosum* (strain OTA11) ochratoxin A production on 2% wheat agar over 56 days at 15 °C Indicates factor elicited a significant effect (P<0.05).

Source of Variation	DF	SS	MS	F	P
a_w	4	99759711	24939928	723.63	<0.001*
Time	3	70923062	23641021	685.94	<0.001*
Time x a_w	12	140074388	11672866	338.69	<0.001*
Residual	40	1378600	34465		
Total	59	312135761			

Table I.4 ANOVA for *Penicillium verrucosum* (strain OTA11) ochratoxin A production on 2% wheat agar over 56 days at 25 °C * Indicates factor elicited a significant effect (P<0.05).

Source of Variation	DF	SS	MS	F	P
a_w	4	337171815	84292954	23.33	<0.001*
Time	3	191921788	63973929	17.71	<0.001*
Time x a_w	12	356753438	29729453	8.23	<0.001*
Residual	40	144526919	3613173		
Total	59	1030373961			

Table I.5 ANOVA for *Penicillium verrucosum* (strain OTA11) ochratoxin A production on irradiated wheat grain over 56 days at 10 °C. * Indicates factor elicited a significant effect (P<0.05).

Source of Variation	DF	SS	MS	F	P
a_w	7	19303782	2757683	78.83	<0.001*
Time	7	62110185	8872884	253.65	<0.001*
Time x a_w	49	44544821	909078	25.99	<0.001*
Residual	128	4477556	34981		
Total	191	130436344			

Table I.6 ANOVA for *Penicillium verrucosum* (strain OTA11) ochratoxin A production on irradiated wheat grain over 56 days at 15 °C. * Indicates factor elicited a significant effect (P<0.05).

Source of Variation	DF	SS	MS	F	P
a_w	7	1.1706E+10	1672227244	225.09	<0.001*
Time	7	6888365581	984052226	132.46	<0.001*
Time x a_w	49	6419401375	131012273	17.64	<0.001*
Residual	128	950917251	7429041		
Total	191	2.5964E+10			

Table I.7 ANOVA for *Penicillium verrucosum* (strain OTA11) ochratoxin A production on irradiated wheat grain over 56 days at 25 °C. * Indicates factor elicited a significant effect (P<0.05).

Source of Variation	DF	SS	MS	F	P
a_w	7	3.2679E+10	4670971974	168.58	<0.001*
Time	7	1.9396E+10	2770787398	100.01	<0.001*
Time x a_w	49	1.9452E+10	396983514	14.33	<0.001*
Residual	128	354631252	27705564		
Total	191	8.5091E+10			

Table I.8 ANOVA for *Aspergillus ochraceus* ochratoxin A production on 2 % wheat-baesd media over 56 days at 15 °C only. * Indicates factor elicited a significant effect (P<0.05).

Source of Variation	DF	SS	MS	F	P
a_w	4	5291718	1322929	6.76	<0.001*
Time	3	3741222	1247074	6.38	<0.001*
Time x a_w	12	6976136	581345	2.97	<0.001*
Residual	40	7823297	195582		
Total	59	23832373			

Table I.9 ANOVA for *Aspergillus ochraceus* ochratoxin A production on 2 % wheat-baesd media over 56 days at 15 °C only. * Indicates factor elicited a significant effect (P<0.05).

Source of Variation	DF	SS	MS	F	P
a_w	4	70356914	17589228	8.56	<0.001*
Time	3	47872856	15957619	7.77	<0.001*
Time x a_w	12	74124698	6177058	3.01	<0.001*
Residual	40	82165184	2054130		
Total	59	274519651			

Table I.10 ANOVA for *Aspergillus ochraceus* ochratoxin A production on irradiated wheat grain over 56 days at 10 °C only. * Indicates factor elicited a significant effect (P<0.05).

Source of Variation	DF	SS	MS	F	P
a_w	7	239298	34185	253.07	<0.001*
Time	7	273627	39090	289.38	<0.001*
Time x a_w	49	423523	8643	63.99	<0.001*
Residual	128	17290	135		
Total	191	953738			

Table I.11 ANOVA for *Aspergillus ochraceus* ochratoxin A production on irradiated wheat grain over 56 days at 15 °C only. * Indicates factor elicited a significant effect (P<0.05).

Source of Variation	DF	SS	MS	F	P
a_w	7	128031373	182901.96	87.09	<0.001*
Time	7	103937940	14848277	70.70	<0.001*
Time x a_w	49	78688216	1605882	7.65	<0.001*
Residual	128	26880641	210005		
Total	191	337537170			

Table I.12 ANOVA for *Aspergillus ochraceus* ochratoxin A production on irradiated wheat grain over 56 days at 25 °C only. * Indicates factor elicited a significant effect (P<0.05).

Source of Variation	DF	SS	MS	F	P
a_w	7	373502761	53357537	13.61	<0.001*
Time	7	531347892	75906842	19.36	<0.001*
Time x a_w	49	404939918	8264080	2.11	<0.001*
Residual	128	501816769	3920444		
Total	191				

Table I.13 ANOVA for ochratoxin A production by *Penicillium verrucosum* on 2 % wheat-based media at various gas compositions and water activities at 25 °C. * Indicates factor elicited a significant effect (P<0.05).

Source of Variation	DF	SS	MS	F	P
a_w	2	107461874	53730939	6.81	0.006*
Time	2	242527541	121263770	15.36	<0.001*
Time x a_w	4	129287121	32321780	4.09	0.016*
Residual	18	142120218	7895563		
Total	26	321396755			

1.14 ANOVA for ochratoxin A production by *Penicillium verrucosum* in irradiated wheat grain over 28 days at 25 °C in air only. * Indicates factor elicited a significant effect (P<0.05).

Source of Variation	DF	SS	MS	F	P
a_w	2	14155008	7077504	31.29	<0.001*
Time	2	19094653	9547326	42.21	<0.001*
Time x a_w	4	5534157	1383539	6.12	0.003
Residual	18	4071090	226172		
Total	26	42854909			

1.15 ANOVA for ochratoxin A production by *Penicillium verrucosum* in irradiated wheat grain over 28 days at 25 °C and 25 % CO_2 only. * Indicates factor elicited a significant effect (P<0.05).

Source of Variation	DF	SS	MS	F	P
a_w	2	12455325	6227662	477.66	<0.001*
Time	2	13320063	6660031	510.82	<0.001*
Time x a_w	4	5943546	1485887	113.97	<0.001*
Residual	18	234683	13038		
Total	26	31953616			

I.16 ANOVA for ochratoxin A production by *Penicillium verrucosum* in irradiated wheat grain over 28 days at 25 °C and 50 % CO_2 only. * Indicates factor elicited a significant effect ($P<0.05$).

Source of Variation	DF	SS	MS	F	P
a_w	2	858022	429011	2.59	0.103
Time	2	2	244609	1.48	0.255
Time x a_w	4	4	430376	0.65	0.635
Residual	18	18	2984830		
Total	26	26			

I.17 ANOVA for ochratoxin A production by *Penicillium verrucosum* in irradiated wheat grain over 28 days at 25 °C for all three gas treatments. * Indicates factor elicited a significant effect ($P<0.05$).

Source of Variation	DF	SS	MS	F	P
a_w	2	15117804	7558902	162.16	<0.001*
Gas treatment	2	17578592	8789296	188.56	<0.001*
Gas treatment x a_w	4	8521856	2130464	45.71	<0.001*
Residual	18	839028	46613		
Total	26	42057280			

I.18 ANOVA for ochratoxin A production by *Aspergillus ochraceus* on 2 % wheat-based media over 56 days at 25 °C for all three gas treatments. * Indicates factor elicited a significant effect ($P<0.05$).

Source of Variation	DF	SS	MS	F	P
a_w	2	32896454	16448227	3.54	0.051
Gas treatment	2	42319129	21159564	4.55	0.025*
Gas treatment x a_w	4	2140362	5352590	1.15	0.365
Residual	18	83656632	4647591		
Total	26				

I.19 ANOVA for ochratoxin A production by *Aspergillus ochraceus* on irradiated wheat grain over 28 days at 25 °C in air only. * Indicates factor elicited a significant effect (P<0.05).

Source of Variation	DF	SS	MS	F	P
a_w	2	983205	491602	17.92	<0.001*
Time	2	1611497	805748	29.38	<0.001*
Time x a_w	4	662090	165523	6.03	0.003*
Residual	18	493709	27428		
Total	26	3750501			

I.20 ANOVA for ochratoxin A production by *Aspergillus ochraceus* on irradiated wheat grain over 28 days at 25 °C for 25 % CO_2 treatment only. * Indicates factor elicited a significant effect (P<0.05).

Source of Variation	DF	SS	MS	F	P
a_w	2	49829	24915	6.83	0.006*
Time	2	77248	38624	10.59	0.001*
Time x a_w	4	66746	16687	4.57	<0.001
Residual	18	665674	3649		
Total	26	259498			

I.21 ANOVA for ochratoxin A production by *Aspergillus ochraceus* on irradiated wheat grain over 28 days at 50 °C for 25 % CO_2 treatment only. * Indicates factor elicited a significant effect (P<0.05).

Source of Variation	DF	SS	MS	F	P
a_w	2	488.30	244.15	6.14	0.009*
Time	2	500.50	250.25	6.30	0.008*
Time x a_w	4	301.92	75.48	1.90	0.154
Residual	18	18	715.38	39.74	
Total	26	2006.09			

I.22 ANOVA for growth of *Penicillium verrucosum* when competing in dual culture with six other spoilage fungi on agar at 15 °C. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Interactions	6	0.83893	0.13982	2.02	0.092
a_w	1	2.40482	2.40482	35.25	<0.001*
Interactions x a_w	6	1.40893	0.23482	3.44	0.011*
Residual	28	1.91000	0.06821		
Total	41	6.56268			

I.23 ANOVA for growth of *Penicillium verrucosum* when competing in dual culture with six other spoilage fungi on agar at 25 °C. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Interactions	6	4.7272	0.7879	10.65	<0.001*
a_w	1	47.7859	47.7857	645.97	<0.001*
Interactions x a_w	6	3.3024	0.5504	7.44	<0.001*
Residual	28	2.0713	0.0740		
Total	41				

I.24 ANOVA for ochratoxin A production *Penicillium verrucosum* when competing in dual culture with six other spoilage fungi on agar at 15 °C. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Interactions	6	9596807	1599468	23.61	<0.001*
a_w	1	8063119	8063119	119.02	<0.001*
Interactions x a_w	6	479357	79893	1.18	0.346
Residual	28	1896955	67748		
Total	41	20036239			

I.25 ANOVA for ochratoxin A production *Penicillium verrucosum* when competing in dual culture with six other spoilage fungi on agar at 25 °C. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Interactions	6	0.26348	0.04391	2.04	0.093
a_w	1	1.31737	1.34737	62.58	<0.001*
Interactions x a_w	6	0.20208	0.3368	1.56	0.194
Residual	28	0.60285	0.02153		
Total	41				

1.27 ANOVA for ochratoxin A production *Penicillium verrucosum* when competing in dual culture with six other spoilage fungi on agar at 15 °C. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Interactions	6	206263659	34377277	72.18	<0.001*
a_w	1	26753749	2675749	56.18	<0.001*
Interactions x a_w	6	1502850	250475	0.53	0.784
Residual	28	13335164	476256		
Total	41	247855422			

1.28 ANOVA for ochratoxin A production by *Penicillium verrucosum* when competing in dual culture with six other spoilage fungi on agar at 25 °C. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Interactions	6	15.0662	2.5110	22.87	<0.001*
a_w	1	20.3845	20.3845	185.66	<0.001*
Interactions x a_w	6	11.0243	1.8374	16.73	<0.001*
Residual	28	3.0743	0.1098		
Total	41	49.5492			

I.29 ANOVA for ochratoxin A production by *Penicillium verrucosum* when competing in dual culture with six other spoilage fungi on irradiated wheat grain at 15 °C. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Interactions	6	206263659	34377277	72.18	<0.001*
a_w	1	26753749	26753749	56.18	<0.001*
Interactions x a_w	6	1502850	250475	0.53	0.784
Residual	28	13335164	476256		
Total	41	247855422			

I.30 ANOVA for ochratoxin A production by *Penicillium verrucosum* when competing in dual culture with six other spoilage fungi on irradiated wheat grain at 15 °C. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Interactions	6	400005577	66667596	33.57	0.007*
a_w	1	17115180	17115923	8.62	<0.001*
Interactions x a_w	6	78779890	13129982	6.61	<0.001*
Residual	28	55605958	1985927		
Total	41	551506605			

I.31 ANOVA for growth by *Penicillium verrucosum* when competing in dual culture with six other spoilage fungi on grain at 25 °C. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Interactions	6	32.2875	5.3812	751.14	<0.001*
a_w	1	4.5973	4.5973	641.72	<0.001*
Interactions x a_w	6	0.4028	0.0671	9.37	<0.001*
Residual	28	0.2006	0.0072		
Total	41	37.4882			

I.32 ANOVA for growth of *Aspergillus ochraceus* when competing in dual culture with six other spoilage fungi on agar at 15 °C. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Interactions	6	4.92741	4.92741	604.29	<0.001*
a_w	1	3.28049	0.54675	67.07	<0.001*
Interactions x a_w	6	0.99731	0.166622	20.39	<0.001*
Residual	28	0.22824	0.1002		
Total	41	9.43345			

I.33 ANOVA for growth of *Aspergillus ochraceus* when competing in dual culture with six other spoilage fungi on agar at 15 °C. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Interactions	6	43.0124	7.1687	237.56	<0.001*
a_w	1	21.5102	21.5102	712.83	<0.001*
Interactions x a_w	6	2.9483	0.4914	16.28	<0.001*
Residual	28	68.3159			
Total	41	9.43345			

I.34 ANOVA for ochratoxin A production by *Aspergillus ochraceus* when competing in dual culture with six other spoilage fungi on agar at 15 °C. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Interactions	6	8990246	1498374	50.33	<0.001*
a_w	1	7329530	7329530	246.20	<0.001*
Interactions x a_w	6	1330436	221739	7.45	<0.001*
Residual	28	833572	29770		
Total	41	18483784			

I.35 ANOVA for ochratoxin A production by *Aspergillus ochraceuus* when competing in dual culture with six other spoilage fungi on agar at 25 °C. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Interactions	6	52710758	52710758	62.98	<0.001*
a_w	1	28470119	4745020	5.67	0.001*
Interactions x a_w	6	40592679	6765446	8.08	<0.001*
Residual	28	23433802	836992		
Total	41	145207358			

I.36 ANOVA for ochratoxin A production by *Aspergillus ochraceuus* when competing in dual culture with six other spoilage fungi on grain at 15 °C. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Interactions	6	28789797	28789797	250.08	<0.001*
a_w	1	442101872	73683645	640.05	<0.001*
Interactions x a_w	6	855545	142591	1.24	0.317
Residual	28	3223424	115122		
Total	41	474970637			

I.37 ANOVA for ochratoxin A production by *Aspergillus ochraceuus* when competing in dual culture with six other spoilage fungi on grain at 25 °C. * Indicates factor elicited a significant effect (P<0.05)

Source	DF	SS	MS	F	P
Interactions	6	520561592	73325998	378.46	<0.001*
a_w	1	73325998	86760265	447.79	<0.001*
Interactions x a_w	6	4926933	821156	4.24	0.004
Residual	28	5425009	193750		
Total	41	604239532			

I. 38 ANOVA for ochratoxin A production by *P. verrucosum* in the presence of various essential oils and concentrations at 0.90 a_w and 15 °C on agar. * Indicates factor elicited a significant effect (P<0.05)

Source	DF	SS	MS	F	P
Treatment	2	*4067*	*2034*	*0.09*	*0.910*
Concentration	3	2668441	889480	41.41	<0.001*
Treatment x concentration	6	12202	2034	0.09	0.996
Residual	24	515560	21482		
Total	35	3200270			

I. 39 ANOVA for ochratoxin A production by *P. verrucosum* in the presence of various essential oils and concentrations at 0.90 a_w and 25 °C on agar. * Indicates factor elicited a significant effect (P<0.05)

Source	DF	SS	MS	F	P
Concentration	3	32057	16029	0.57	0.571
Treatment	2	1288519	429506	15.36	<0.001*
Treatment x concentration	6	96171	16029	0.57	0.748
Residual	24	671257	27969		
Total	35	2088004			

I. 40 ANOVA for ochratoxin A production by *P. verrucosum* in the presence of various essential oils and concentrations at 0.95 a_w and 15 °C on agar. * Indicates factor elicited a significant effect (P<0.05)

Source	DF	SS	MS	F	P
Treatment	2	217331	108665	0.59	0.563
Concentration	3	526350761	175450254	949.06	<0.001*
Treatment x concentration	6	3314578	552430	2.99	0.025*
Residual	24	4436833	184868		
Total	35	534319503			

I. 41 ANOVA for ochratoxin A production by *P. verrucosum* in the presence of various essential oils and concentrations at 0.95 a_w and 25 °C on agar. * Indicates factor elicited a significant effect (P<0.05)

Source	DF	SS	MS	F	P
Treatment	2	13925261	6962630	1.20	0.320
Concentration	3	874490499	291496833	50.09	<0.001*
Treatment x concentration	6	10810404	1801734	0.31	0.926
Residual	24	139659012	5819126		
Total	35	1038885177			

I. 42 ANOVA for ochratoxin A production by *P. verrucosum* in the presence of various essential oils and concentrations at 0.995 a_w and 15 °C on agar. * Indicates factor elicited a significant effect (P<0.05)

Source	DF	SS	MS	F	P
Treatment	2	9056561	4528281	1.14	0.336
Concentration	3	121498490	40499497	10.23	<0.001*
Treatment x concentration	6	20218314	3369719	0.85	0.544
Residual	24	95059670	3960820		
Total	35	245833036			

I. 43 ANOVA for ochratoxin A production by *P. verrucosum* in the presence of various essential oils and concentrations at 0.995 a_w and 25 °C on agar. * Indicates factor elicited a significant effect (P<0.05)

Source	DF	SS	MS	F	P
Treatment	2	8925366	4462683	14.90	0.165
Concentration	3	102543792	34181264	14.90	<0.001*
Treatment x concentration	6	8957748	492958	0.65	0.689
Residual	24	55046741	2293614		
Total	35	175473647			

I. 44 ANOVA for ochratoxin A production by *A. ochraceus* in the presence of various essential oils and concentrations at 0.90 a_w and 15 °C on agar. * Indicates factor elicited a significant effect (P<0.05)

Source	DF	SS	MS	F	P
Treatment	2	196.06	98.03	8.63	0.002*
Concentration	3	3458.67	1152.89	101.48	<0.001*
Treatment x concentration	6	588.17	98.03	8.63	<0.001*
Residual	24	272.67	11.36		
Total	35	4515.56			

I. 45 ANOVA for ochratoxin A production by *A. ochraceus* in the presence of various essential oils and concentrations at 0.90 a_w and 25 °C on agar. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Treatment	2	1681	840	030	0.746
Concentration	3	62264	20765	7.34	<0.001*
Treatment x concentration	6	3606	601	0.21	0.969
Residual	24	67887	2829		
Total	35	135439			

I. 46 ANOVA for ochratoxin A production by *A. ochraceus* in the presence of various essential oils and concentrations at 0.95 a_w and 15 °C on agar. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Treatment	2	331972	165986	0.96	0.398
Concentration	3	25014011	8338004	48.09	<0.001*
Treatment x concentration	6	750822	125137	0.72	0.636
Residual	24	4160850	173369		
Total	35	30257655			

I. 47 ANOVA for ochratoxin A production by *A. ochraceus* in the presence of various essential oils and concentrations at 0.95 a_w and 25 °C on agar. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Treatment	2	3314048	1657024	45.40	0.280
Concentration	3	167991563	55997188	1.34	<0.001*
Treatment x concentration	6	88215156	1470253	1.19	0.344
Residual	24	29600673	1233361		
Total	35	209727800			

I. 48 ANOVA for ochratoxin A production by *A. ochraceus* in the presence of various essential oils and concentrations at 0.995 a_w and 15 °C on agar. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Treatment	2	438547	219274	140.58	<0.001*
Concentration	3	5409562	1803187	1156.05	<0.001*
Treatment x concentration	6	1258521	209754	134.48	<0.001*
Residual	24	37435	1560		
Total	35				

I. 49 ANOVA for ochratoxin A production by *A. ochraceus* in the presence of various essential oils and concentrations at 0.995 a_w and 25 °C on agar. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Treatment	2	123296264	41098755	4.62	0.011*
Concentration	3	7191028	3595514	0.40	0.672
Treatment x concentration	6	27265823	4544304	0.51	0.794
Residual	24	213487985	8895333		
Total	35	35	37124110		

I. 50 ANOVA for growth by *P. verrucosum* in the presence of various essential oils and concentrations at 0.90 a_w and 15 °C on grain. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Treatment	2	123296264	41098755	4.62	0.011*
Concentration	3	7191028	3595514	0.40	0.672
Treatment x concentration	6	27265823	4544304	0.51	0.794
Residual	24	213487985	8895333		
Total	35	35	37124110		

I. 51 ANOVA for growth by *P. verrucosum* in the presence of various essential oils and concentrations at 0.90 a_w and 25 °C on grain. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Treatment	3	0.79431	0.26477	30.94	<0.001*
Concentration	3	10.28678	3.42893	400.69	<0.001*
Treatment x concentration	9	0.79649	0.08850	10.34	<0.001*
Residual	32	0.27384	0.00856		
Total	47	12.15142			

I. 52 ANOVA for growth by *P. verrucosum* in the presence of various essential oils and concentrations at 0.95 a_w and 15 °C on grain. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Treatment	3	1.2667	0.4222	4.44	0.01*
Concentration	3	15.7696	5.2565	55.26	<0.001*
Treatment x concentration	9	0.8552	0.0950	1.00	0.461
Residual	32	3.0440	0.0951		
Total	47	20.9354			

I. 53 ANOVA for growth by *P. verrucosum* in the presence of various essential oils and concentrations at 0.95 a_w and 25 °C on grain. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Treatment	3	2.5787	0.8596	19.71	<0.001*
Concentration	3	51.9348	17.3116	396.93	<0.001*
Treatment x concentration	9	1.8041	0.2005	4.60	0.001*
Residual	32	1.3956	0.0436		
Total	47	57.7132			

I. 54 ANOVA for growth by *P. verrucosum* in the presence of various essential oils and concentrations at 0.995 a_w and 15 °C on grain. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Treatment	3	4.7956	1.5975	20.00	<0.001*
Concentration	3	124.8736	41.6245	521.03	<0.001*
Treatment x concentration	9	7.7086	0.8565	10.72	0.001*
Residual	32	2.5564	0.0799		
Total	47	139.9313			

I. 55 ANOVA for growth by *P. verrucosum* in the presence of various essential oils and concentrations at 0.995 a_w and 25 °C on grain. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Treatment	3	0.4484	0.1495	6.73	<0.001*
Concentration	3	27.3247	9.1082	410.36	<0.001*
Treatment x concentration	9	0.7145	0.0794	3.58	0.001*
Residual	32	0.7103	0.0222		
Total	47	29.1979			

I. 56 ANOVA for ochratoxin A production by *P. verrucosum* in the presence of various essential oils and concentrations at 0.90 a_w and 15 °C on grain. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Treatment	3	203570	67857	88.72	<0.001*
Concentration	3	1868101	622700	814.14	<0.001*
Treatment x concentration	9	281137	31.237	40.84	0.001*
Residual	32	24475	765		
Total	47	2377284			

I. 57 ANOVA for ochratoxin A production by *P. verrucosum* in the presence of various essential oils and concentrations at 0.90 a_w and 25 °C on grain. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Treatment	3	217951	72650	37.94	<0.001*
Concentration	3	12840508	4280169	2235.37	<0.001*
Treatment x concentration	9	206973	22997	12.01	0.001*
Residual	32	61272	1915		
Total	47	13326703			

I. 58 ANOVA for ochratoxin A production by *P. verrucosum* in the presence of various essential oils and concentrations at 0.95 a_w and 15 °C on grain. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Treatment	3	37381179	12460393	14.71	<0.001*
Concentration	3	837743079	279247693	329.66	<0.001*
Treatment x concentration	9	23306737	2589637	3.06	0.001*
Residual	32	27106499	847078		
Total	47	925537494			

I. 59 ANOVA for ochratoxin A production by *P. verrucosum* in the presence of various essential oils and concentrations at 0.95 a_w and 25 °C on grain. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Treatment	3	28343815	84447938	3.82	0.019*
Concentration	3	1158448306	386149435	156.24	<0.001*
Treatment x concentration	9	16122149	1791350	0.72	0.683
Residual	32	79088903	2471528		
Total	47	1282003173			

I. 60 ANOVA for ochratoxin A production by *P. verrucosum* in the presence of various essential oils and concentrations at 0.995 a_w and 15 °C on grain. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Treatment	3	54483	18161	12.55	<0.001*
Concentration	3	605885	201962	139.60	<0.001*
Treatment x concentration	9	47672	5297	3.66	0.003*
Residual	32	46296	1447		
Total	47	754335			

I. 61 ANOVA for ochratoxin A production by *P. verrucosum* in the presence of various essential oils and concentrations at 0.995 a_w and 15 °C on grain. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Treatment	3	396927	132309	1.28	0.298
Concentration	3	12892209	4297403	41.59	<0.001*
Treatment x concentration	9	607305	67478	0.744	
Residual	32	3306364	103324		
Total	47				

I. 62 ANOVA for ochratoxin A production by *P. verrucosum* in the presence of various essential oils and concentrations at 0.995 a_w and 25 °C on grain. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Treatment	3	28343815	84447938	3.82	0.019*
Concentration	3	1158448306	386149435	156.24	<0.001*
Treatment x concentration	9	16122149	1791350	0.72	0.683
Residual	32	79088903	2471528		
Total	47	1282003173			

I. 63 ANOVA for ochratoxin A production by *A. ochraceus* in the presence of various essential oils and concentrations at 0.90 a_w and 15 °C on grain. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Treatment	3	0.25695	0.08565	21.83	<0.001*
Concentration	3	4.94232	1.64744	419.95	<0.001*
Treatment x concentration	9	0.30090	0.03343	8.52	<0.001*
Residual	32	012553	0.00392		
Total	47	5.62570			

I. 64 ANOVA for ochratoxin A production by *A. ochraceus* in the presence of various essential oils and concentrations at 0.90 a_w and 25 °C on grain. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Treatment	3	1.7417	0.5806	87.03	<0.001*
Concentration	3	36.5001	12.1667	1823.87	<0.001*
Treatment x concentration	9	1.1289	0.1254	18.80	<0.001*
Residual	32	0.2135	0.0067		
Total	47				

I. 65 ANOVA for ochratoxin A production by *A. ochraceus* in the presence of various essential oils and concentrations at 0.95 a_w and 15 °C on grain. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Treatment	3	0.5150	0.1717	30.21	<0.001*
Concentration	3	23.1714	7.7238	1359.02	<0.001*
Treatment x concentration	9	0.2069	0.0230	4.05	<0.001*
Residual	32	0.1819	0.0057		
Total	47	24.0752			

I. 66 ANOVA for ochratoxin A production by *A. ochraceus* in the presence of various essential oils and concentrations at 0.95 a_w and 25 °C on grain. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Treatment	3	4.3709	1.4570	28.70	<0.001*
Concentration	3	133.4712	44.4904	876.37	<0.001*
Treatment x concentration	9	2.3690	0.2362	5.18	<0.001*
Residual	32	1.6245	0.0508		
Total	47	141.8356			

I. 67 ANOVA for ochratoxin A production by *A. ochraceus* in the presence of various essential oils and concentrations at 0.995 a_w and 25 °C on grain. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Treatment	3	0.1224	0.0408	17.81	<0.001*
Concentration	3	21.9398	7.3133	3191.25	<0.001*
Treatment x concentration	9	0.0747	0.0083	3.62	<0.001*
Residual	32	0.0733	0.0023		
Total	47	22.2103			

I. 68 ANOVA for ochratoxin A production by *A. ochraceus* in the presence of various essential oils and concentrations at 0.995 a_w and 25 °C on grain. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Treatment	3	0.3326	0.1109	12.72	<0.001*
Concentration	3	125.3606	41.7869	4794.54	<0.001*
Treatment x concentration	9	0.1865	0.0207	2.38	<0.001*
Residual	32	0.2789	0.0087		
Total	47	126.156			

I. 69 ANOVA for growth by *A. ochraceus* in the presence of various essential oils and concentrations at 0.90 a_w and 15 °C on grain. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Treatment	3	15870	5290	50.28	<0.001*
Concentration	3	131225	43742	415.76	<0.001*
Treatment x concentration	9	18991	2110	20.06	<0.001*
Residual	32	3367	105		
Total	47	169452			

I. 70 ANOVA for growth by *A. ochraceus* in the presence of various essential oils and concentrations at 0.90 a_w and 25 °C on grain. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Treatment	3	68945	22982	9.35	<0.001*
Concentration	3	116274	33758	158.24	<0.001*
Treatment x concentration	9	62402	6934	2.82	<0.001*
Residual	32	78616	2457		
Total	47	1376236			

I. 71 ANOVA for growth by *A. ochraceus* in the presence of various essential oils and concentrations at 0.95 a_w and 15 °C on grain. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Treatment	3	554393	184798	78.45	<0.001*
Concentration	3	30531422	10177141	4320.17	<0.001*
Treatment x concentration	9	829513	92168	39.13	<0.001*
Residual	32	75383	2356		
Total	47	31990712			

I. 72 ANOVA for growth by *A. ochraceus* in the presence of various essential oils and concentrations at 0.95 a_w and 25 °C on grain. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Treatment	3	3897212	1299071	28.80	<0.001*
Concentration	3	111464208	33821403	749.82	<0.001*
Treatment x concentration	9	3532021	392447	8.70	<0.001*
Residual	32	1443400	45105		
Total	47	110336842			

I. 73 ANOVA for growth by *A. ochraceus* in the presence of various essential oils and concentrations at 0.995 a_w and 15 °C on grain. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Treatment	3	30236	10079	33.90	<0.001*
Concentration	3	322254	107418	361.62	<0.001*
Treatment x concentration	9	16893	1877	6.31	<0.001*
Residual	32	9513	297		
Total	47	378896			

I. 74 ANOVA for growth by *A. ochraceus* in the presence of various essential oils and concentrations at 0.995 a_w and 25 °C on grain. * Indicates factor elicited a significant effect (P<0.05).

Source	DF	SS	MS	F	P
Treatment	3	40695	13565	1.34	<0.001*
Concentration	3	2175369	725126	71.70	<0.001*
Treatment x concentration	9	52881	5876	0.58	<0.001*
Residual	32	323647	10114		
Total	47	2592591			

APPENDIX II

List of Publications

Arroyo, M., Cairns, V. & Magan, N. (2001). Impact of preservatives and environmental factors on growth and ochratoxin A production by *Aspergillus ochraceus* and *Penicillium verrucosum* strains on wheat substrates. *British International Symposium on Bioactive Fungal Metabolites: Impact and Exploitation,* Swansea, UK, 22-27 April 2001.

Cairns, V., Hope, R. & Magan, N. (2001). Impact of essential oils on growth and ochratoxin A production by *Penicillium verrucosum* and *Aspergillus ochraceus* in a wheat-based substrate. In Advances in Stored Product Protections, p.479-485. Edited by Credland, P.F., Armitage, D.G., Bell, C.M., Cogan, P.M. & Highley, E. Cambridge: Cabi Publishing.

Cairns, V. & Magan, N. (2002). Impact of essential oils and environmental factors on growth and ochratoxin A production by *Penicillium verrucosum* on a wheat-based substrate. *Second Postgraduate Research Conference,* Cranfield University, Silsoe, UK, 22 June 2001.

Cairns, V. & Magan, N. (2002). Environmental factors and competing mycoflora affect growth and ochratoxin A production by *Penicillium verrucosum* on wheat grain. 7th International Mycological Congress, Oslo, Norway. Abstract 251.

Cairns, V. & Magan, N. (2003). Environmental factors and competing mycoflora affect growth and ochratoxin production by *Penicillium verrucosum* on wheat grain. *Aspects of Applied Biology* 68, 81-90.

Magan, N., Hope, R., Cairns, V. & Aldred, D. (2003). Post-harvest fungal ecology: impact of fungal growth and mycotoxin accumulation in stored grain. *European Journal of Plant Pathology.* In Press.

(Several Journal papers in preparation)