Cranfield University

PhD Thesis

IMPACT OF ENVIRONMENTAL AND PLANT FACTORS ON *ASPERGILLUS* SECTION *FLAVI* ISOLATED FROM MAIZE IN ITALY

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Abstract

The exceptional hot weather conditions in Italy during the summer of 2003 resulted in an optimal environment for the development of *Aspergillus* section *Flavi* in maize and, consequently, for aflatoxin production. This caused significant contamination, above the EU legal limits, for maize destined to feed and food.

This study was focused to define the distribution of these fungi in Italy for the first time. In all the sampled regions of Northern Italy, *A. flavus* was present. Ecological studies were conducted and this defined the cardinal conditions of water availability (0.83-0.99 a_w), temperature (15-45°C) and gas composition (CO_2<50%) for sporulation, growth and aflatoxin B_1 production. Since in the field *A. flavus* does not occur alone, possible interactions with the fumonisin producing species *Fusarium verticillioides* was examined by using carbon source utilisation patterns and niche overlap indices. *F. verticillioides* was a better competitor over the range 0.93-0.98 a_w and temperature of 20°C while *A. flavus* dominated at 0.98 a_w and 30°C.

Inoculum concentration (10^1-10^7 CFUs ml) affected infection efficiency, with a low percentage of kernels becoming infected with up to 10^5 conidia mL^{-1}, and early maize growth stages were more susceptible. A total of 34 maize hybrids were screened for resistance to *A. flavus* and aflatoxin production and this showed that about 40% of these showed promise.

The data sets obtained in this study wil provide a powerful basis for the development of a Decision Support System to minimize aflatoxins in maize.

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Abbreviations

AF: aflatoxin

AFs: aflatoxins

AFB1: aflatoxin B1

AFB2: aflatoxin B2

AFG1: aflatoxin G1

AFG2: aflatoxin G2

AFM1: aflatoxin M1

AFM2: aflatoxin M2

a_w: water activity

BCA: Biocontrol Agent

CFU: colony forming unit

ppb: parts per billion

ng/g: nanograms/gram

CHAPTER 1

Introduction and Literature Review

1. INTRODUCTION

The exceptional hot weather conditions registered in Italy during the summer of 2003 (Figure 1.1) caused optimal environmental conditions for the development of *Aspergillus flavus* in maize and, consequently, for aflatoxin production. This resulted in high contamination of maize destined for food and feed.

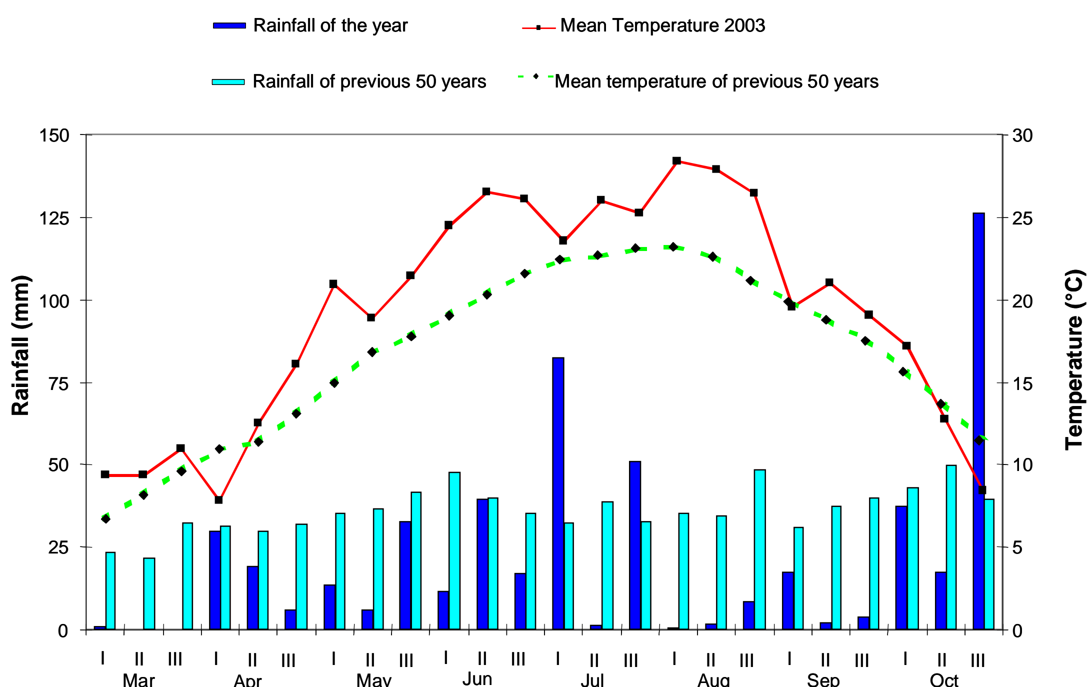


Figure 1.1 – Meteorological data recorded in 2003 at the Weather-Station CRA-U09 Unità di ricerca per la maiscoltura – Bergamo (Research Unit on maize – Bergamo).

Maize is a main component in the diet of cows bred for milk production. Every year 30.2 millions litres of milk are produced in Italy by 500.000 dairies located all over the peninsula. In 2003, an important part of the national milk production had aflatoxin M_1 content higher than the EU limit (0.05 ppb, EU Regulation 1881/2006) sometimes reaching levels of 0.1 ng/g (ppb). The

economic losses were great both for breeders and for cheese producers and many problems were created in the relationship with consumers (Piva et al., 2006).

This unusual situation increased the interest of scientists and others concerned with milk production and the processing chain, towards *A. flavus*, in particular the ecological needs of this fungus for development in the field and aflatoxin production.

1.1 THE ORIGIN OF MAIZE

Maize (*Zea mays*) is one of the most widely distributed food plants in the world. It is grown from 58 °N in Canada and Russia to 40 °S in South America. It is cultivated from below sea level to altitudes exceeding 3500 m (Bradburn et al., 1993). Its name is a Native American word that literally means “that which sustains life”. This cereal, in fact, is able to provide nutrients for humans and animals, also serving as a basic raw material for the production of starch, oil and protein, alcoholic beverages and food sweeteners (FAO, 1992). Furthermore, in recent years maize has been used for the production of bio-fuel and this new usage has increased the demand of this cereal (FAO, 2007).

Zea mays, from the botanical point of view, belongs to the grass family (Gramineae) and is a tall annual plant with an extensive fibrous root system. It is a cross pollinating species with female (ear) and male (tassel) flowers in separate places on the same plant. The grain develops in the ears, or cobs,

often one on each stalk (FAO, 1992). The kernels may be of different colours such as red or black but principally they are white or yellow. Grain may be of different types distinguished by variable colours and differences in the amount of chemical compounds stored in the kernel.

At the end of the fifteenth century, after the discovery of the American continent by Christopher Columbus, maize was introduced into Europe through Spain and spread through the warmer climates of the Mediterranean and later to northern Europe.

1.2 THE MAIZE PLANT

The maize plant may be defined as a metabolic system whose end product is mainly starch deposited in specialized organs, the maize kernels. The development of the plant may be divided into two physiological stages:

- the vegetative stage where different tissues develop and differentiate until the flower structures appear;
- the reproductive stage that begins with the fertilization of the female structures, which will develop into ears and grains.

The maize plant and its main parts is shown in Figure 1.2. The plant develops morphological characteristics and differences in the vegetative and reproductive stages (Table 1.1) (FAO, 1992).

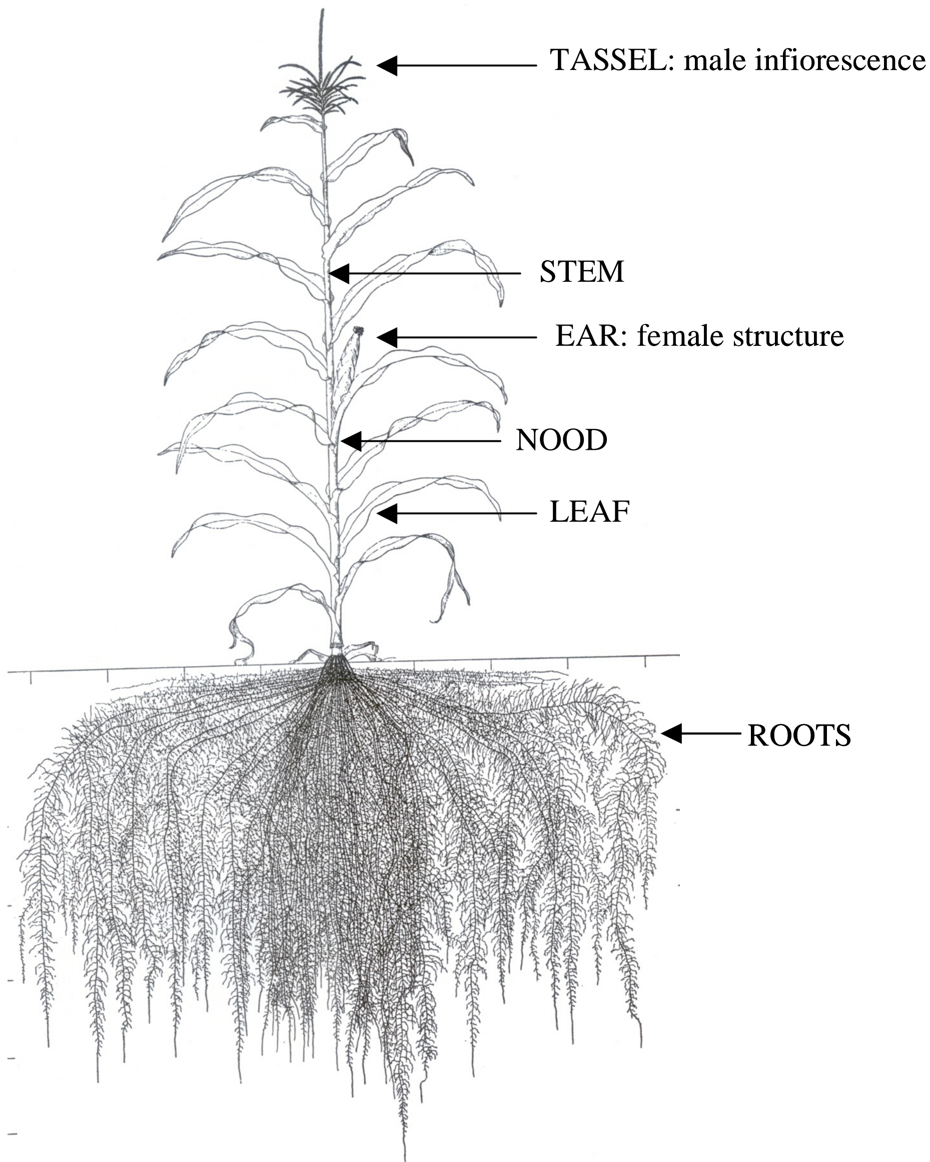


Figure 1.2 – Structure of a maize plant.

Table 1.1- Phenological growth stages and BBCH-identification keys of maize (Weber and Bleiholder, 1990; Lancashire et al., 1991).

Code	Description
Principal growth stage 0: Germination	
00	Dry seed (caryopsis)
01	Beginning of seed imbibition
03	Seed imbibition complete
05	Radicle emerged from caryopsis
06	Radicle elongated, root hairs and /or side roots visible
07	Coleptile emerged from caryopsis
09	Emergence: coleoptile penetrates soil surface (cracking stage)
Principal growth stage 1: Leaf development 1, 2	
10	First leaf through coleoptile
11	First leaf unfolded
12	2 leaves unfolded
13	3 leaves unfolded
1 .	Stages continuous till . . .
19	9 or more leaves unfolded
Principal growth stage 3: Stem elongation	
30	Beginning of stem elongation
31	First node detectable
32	2 nodes detectable
33	3 nodes detectable
3 .	Stages continuous till . . .
39	9 or more nodes detectable3
Principal growth stage 5: Inflorescence emergence, heading	
51	Beginning of tassel emergence: tassel detectable at top of stem
53	Tip of tassel visible
55	Middle of tassel emergence: middle of tassel begins to separate
59	End of tassel emergence: tassel fully emerged and separated
Principal growth stage 6: Flowering, anthesis	
61	Male: stamens in middle of tassel visible; Female: tip of ear emerging from leaf sheath
63	Male: beginning of pollen shedding; Female: tips of stigmata visible
65	Male: upper and lower parts of tassel in flower; Female: stigmata fully emerged
67	Male: flowering completed; Female: stigmata drying
69	End of flowering: stigmata completely dry
Principal growth stage 7: Development of fruit	
71	Beginning of grain development: kernels at blister stage, about 16% dry matter
73	Early milk
75	Kernels in middle of cob yellowish-white (variety-dependent), content milky, about 40% dry matter
79	Nearly all kernels have reached final size
Principal growth stage 8: Ripening	
83	Early dough: kernel content soft, about 45% dry matter
85	Dough stage: kernels yellowish to yellow (variety dependent), about 55% dry matter
87	Physiological maturity: black dot/layer visible at base of kernels, about 60% dry matter
89	Fully ripe: kernels hard and shiny, about 65% dry matter
Principal growth stage 9: Senescence	
97	Plant dead and collapsing
99	Harvested product

The morphology or architecture of the plant has also suffered evolutionary pressures that resulted in great variability in the number, length and width of leaves, plant height, position of ears, number of ears per plant, maturation cycles, grain types and number of rows of grain, among many other characteristics. This variability is of great value in improving the productivity of the plant and specific organic components of the grain. The main yield components include the number and weight of grains (FAO, 1992).

Maize kernels develop through accumulation of the products of photosynthesis, root absorption and metabolism of the maize plant on the female inflorescence (ear). This structure may hold from 300 to 1000 single kernels depending on the number of rows, diameter and length of the cob. The maize kernel is known botanically as a caryopsis and Figure 1.3 shows the four major physical structures of the kernel: the pericarp, hull or bran; the germ or embryo; the endosperm; and the tip cap (dead tissue found where the kernel joins the cob). The weight distribution of the different parts of the maize kernel is shown in Table 1.2.

Table 1.2 - Weight distribution of main parts of the kernel

Structure	**Percent weight distribution**
Pericarp	5-6
Aleurone	2-3
Endosperm	80-85
Germ	10-12

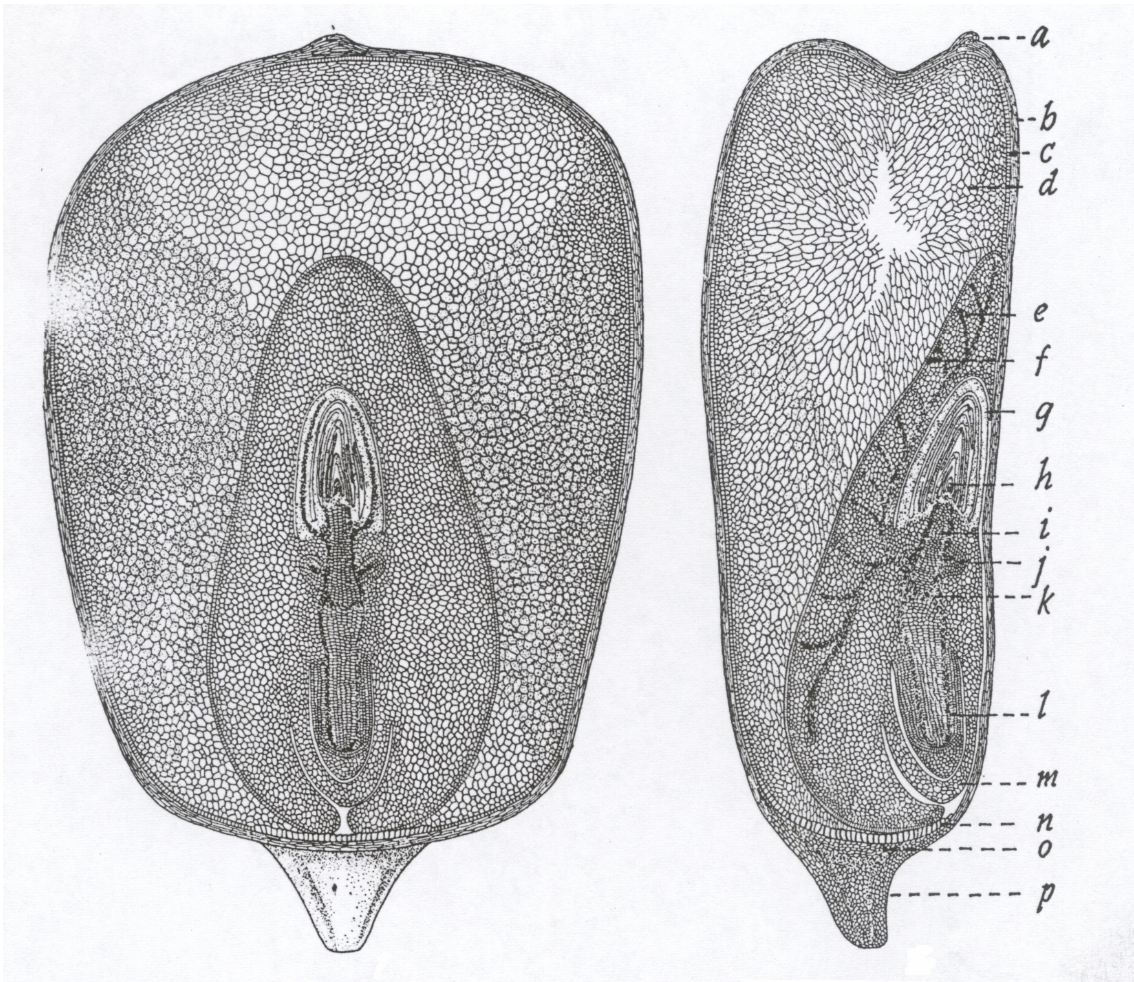


Figure 1.3 – Maize kernel structure: a) silk scar; b) pericarp; c) aleurone; d) endosperm; e) scutellum; f) glandular layer of scutellum; g) coleoptile; h) plumule with stem and leaves; i) first internode; j) lateral seminal root; k) scutellar node; l) primary root; m) coleorhiza; n) basal conducting cells of endosperm; o) brown abscission layer; and p) pedicel or flower stalk.

1.3 WORLD PRODUCTION

World maize production increased from 1979-1981 to 1987: the land area planted with maize increased from 105 million ha in 1961 to about 127 million ha in 1987. Although part of the increase resulted from additional land area planted, significant increases in production resulted from genetic improvement

and more efficient technological field practices and fertilizer applications, as well as from the introduction of new, more highly productive varieties (FAO, 1992). Since 1960 in the USA and later in Europe, maize hybrids started to be commonly cultivated because of their positive characteristics, in particular their higher productivity and their better product respect to ‘traditional’ maize.
The developing countries have wider areas given to maize cultivation than developed countries, but yield in the latter is about four times higher; while most of the production in developing countries is for human consumption, in the developed world it is mainly for industrial use and animal feed. Recent data shows that the world production of maize is almost stable in years(Table 1.3). In 2006 it registered a low decrement but in 2007 the production of maize increased in most countries also as a response of an incremented demand of this cereal for bio-fuel (FAO, 2007).

Considering the consumption of maize, some differences may be presupposed in future years regarding the use of this cereal. It is probable that an increase in the industrial sector of USA will occur, especially for the production of ethanol. Maize used for the production of alcohol has increased by 70% with respect to the 1990s. In the future, the commercial cultivation of maize will increase towards North and Central America, the Middle East and Africa, but the demand for this cereal will probably be reduced in the Far East (Zuppiroli and Mancini, 2002).

Table 1.3 – Maize production in the most important areas of the world (millions of tons) (FAO, 2007).

Country	2005	2006	2007 forecasted
EU[1]	134.5	127.3	136
Baltic countries	7.1	6.9	4.4
USA	299.1	280.4	354.0
Canada	25.2	23.3	28.1
Mexico	25.8	28.2	30.3
Argentina	24.5	18.3	26.5
Brazil	37.7	45.0	53.6
China	150.4	156.7	159.3
Indonesia	12.5	11.6	12.4
India	33.4	32.1	34.4
North Africa	11.7	12.5	10.8
Sub-Sahara countries	39.8	43.2	41.2

[1] EU-25 in 2005 and 2006; EU-27 in 2007

Maize is a very important crop for Italy, mainly in the north where 89% of the growing area is placed. Around 82% of yearly production is destined to animal feed, 4% to human food, as kernels in different ripening stages or milled products (gritz and flour), 12% is used for starch production and 2% for other destinations (ISTAT, 2005; www.istat.it). The annual budget of the maize commodity is 600 MEuros and about 2000 operators are involved in the maize chain.

1.4 THE *ASPERGILLUS FLAVUS* GROUP

a) *Aspergillus* section *Flavi*

Members of the *Aspergillus flavus* group are very widely distributed in nature. They are regularly isolated from soils, particularly those from tropical and subtropical areas, from forage and decaying vegetation, from stored seeds and grains and from various types of food products. They contribute to decomposition processes and some of them are pathogenic to insects and, for example, *A. flavus* and *A. parasiticus*, to higher animals including man (Raper and Fennell, 1965).

A. flavus (Plate 1-A) and *A. parasiticus* are closely related fungi which can contaminate primary agricultural products in the field, during harvest, in storage, and during processing (Diener et al., 1987). Strains with shorter stalks, borne from the substrate and bearing persistently yellow-green heads were placed in the *A. flavus* series and segregated as two species: *A. flavus* Link and *A. parasiticus* Speare. The two species were differentiated, in part, by their colour and relative conidiophore lengths, but primarily by the character of their sterigmata: *A. flavus* was typically biseriate and *A. parasiticus* uniseriate (Plate 1-B; Raper and Fennell, 1965). Researchers have frequently failed to distinguish between the two species in their research, Kurtzman et al. (1987) addressed this problem through comparisons of deoxyribonucleic acid (DNA) relatedness and found sufficiently high complementarity among the two taxa to conclude that they were conspecific. In fact it has been demonstrated that the

A. flavus cluster is 96% identical to that of *A. parasiticus* (Cary and Ehrlich, 2006).

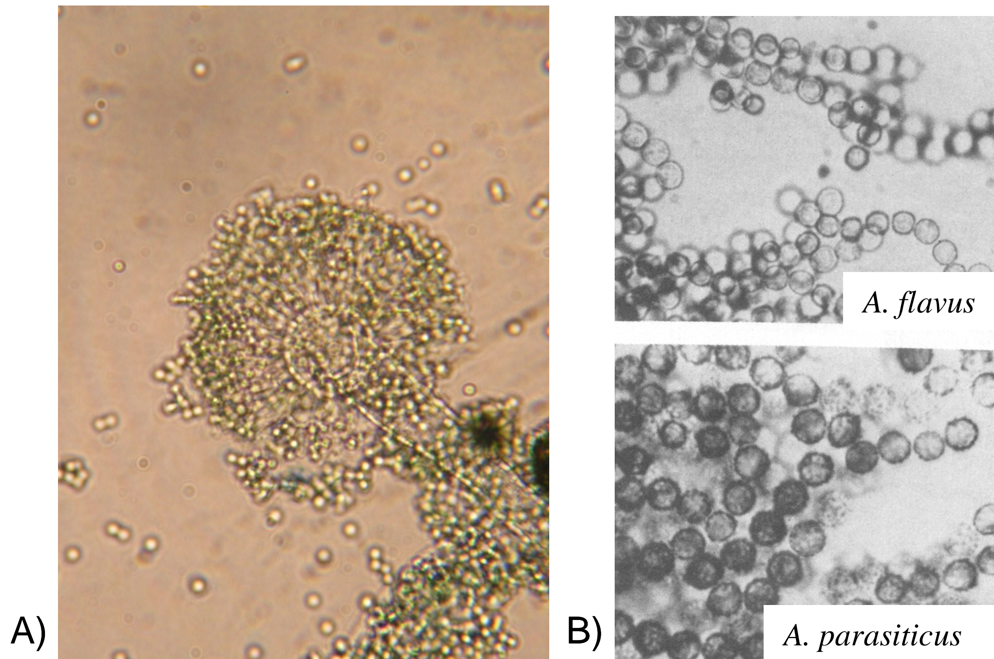


Plate 1.1 – Photomicrograph of: (A) conidiophore of *A. flavus* (Photo by P. Giorni) and (B) conidia of *A. flavus* and *A. parasiticus* (Photo by Raper and Fennell, 1965).

There is also another characteristic that helps distinguish between these two species: *A. parasiticus* appears to be adapted to a soil environment, being prominent in peanuts, whereas *A. flavus* seems adapted to the aerial and foliar environment, being dominant in corn, cottonseed, and tree nuts (Diener *et al.*, 1987).

b) aflatoxins

Aflatoxins acquired their names from the blue or green fluorescence that they exhibit when exposed to ultraviolet light (366 nm) on silica gel thin layer chromatograms (Hartley et al., 1963). In addition, aflatoxin M1 and M_2 have been identified in the milk of dairy cows consuming AFB_1 and AFB_2 from contaminated groundnut meal (van Egmond, 1989) (Figure 1.4).

The production of mycotoxins can be useful to separate strains of the *Aspergillus flavus group*. It is now generally accepted that *A. flavus* usually only produces aflatoxin B_1 (AFB_1) and aflatoxin B2 (AFB_2), but is also capable of synthesising cyclopiazonic acid, a mycotoxin confirmed as being present in the batch of contaminated groundnuts which killed turkey poults in 1960 (Turkey ‘X’ disease) (Smith, 1997). On the other hand, *A. parasiticus* often produces all four of the primary aflatoxins: this group of mycotoxins comprises aflatoxin B_1, B_2, G_1 and G_2 (Diener et al., 1987; D’Mello and McDonald, 1997). However, recent studies demonstrated that certain strains of *A. flavus* can also be able to produce AFG_1 and AFG_2. For example in a study with *A. flavus* isolates from Africa and America, it was found that from 40 to 100% of African strains were able to produce also AFG_1 depending on the media used while none of the American strains were able to (Cotty and Cardwell, 1999).

However, in both species of *Aspergillus* section *Flavi*, there are strains that are non-aflatoxigenic (Smith and Moss, 1985). *A. flavus* and *A. parasiticus* species develop when conditions such as temperature and humidity/water

activity favour their proliferation. Both temperature and water activity generally interact in the promotion of mycotoxin synthesis (Smith and Moss, 1985).

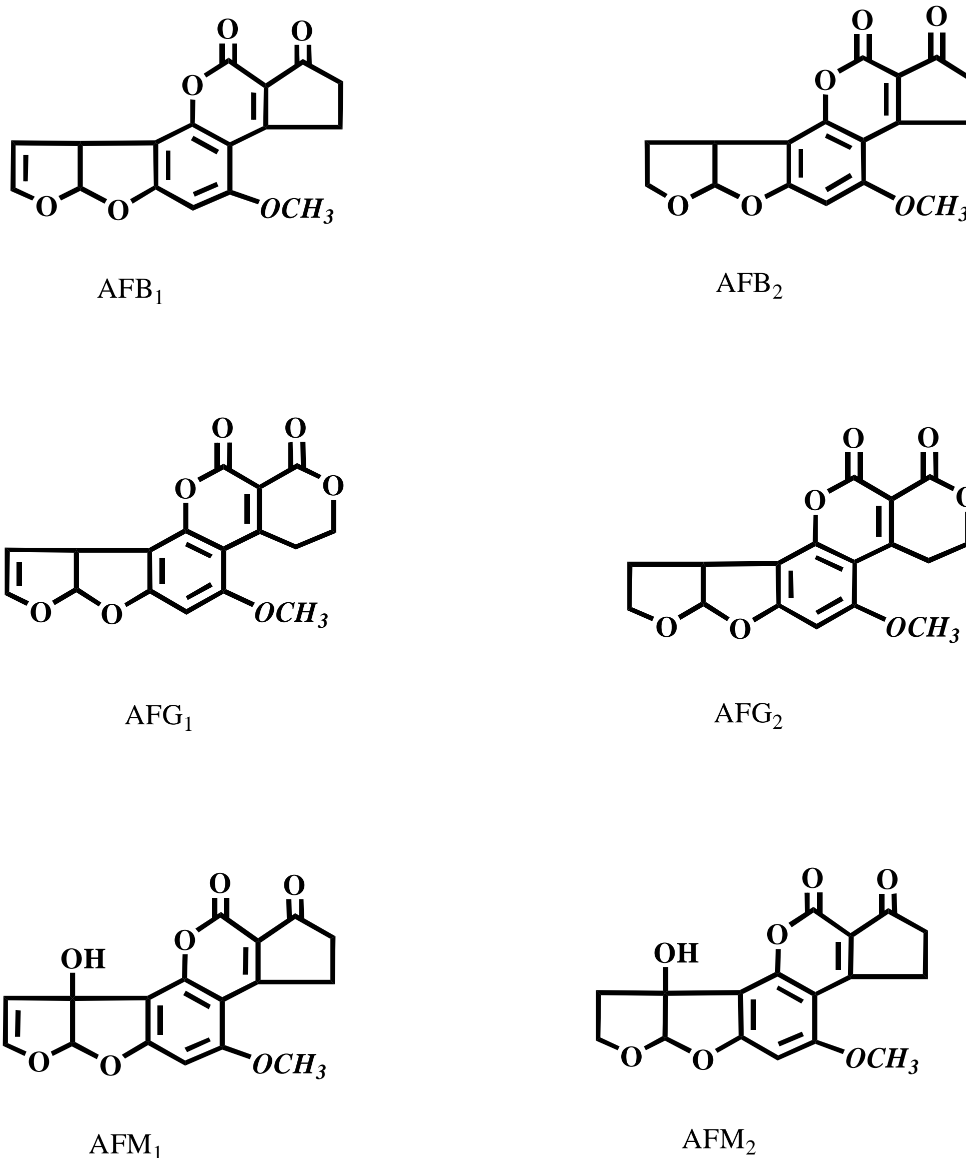


Figure 1.4 - Chemical structure of aflatoxins

There are many gaps in the understanding of the coordinated global regulation of toxin formation, of the signal transduction pathways underlying primary and secondary metabolism, of the biotic and abiotic factors that affect

toxin formation, and of the interactions of mycotoxigenic fungi and their host plants during infection (Bhatnagar et al., 2006). There are many theories about the meaning of aflatoxin production by fungi but nothing has been clearly demonstrated. Aflatoxins could be a defence response by fungi to stress, a way to protect fungi from UV damage, by-products of primary metabolism, necessary to increment fungal fitness or able to provide protection from predators for reproductive structures such as conidia and sclerotia (Cary and Ehrlich, 2006; Magan and Aldred, 2007).

Studies determined that aflatoxins are synthesized by a polyketide metabolic pathway and that genes of both *A. flavus* and *A. parasiticus* linked to the aflatoxin biosynthetic pathway are clustered (Bhatnagar et al., 2003; Chang et al., 2004).

It has been established that the most significant environmental factors able to influence aflatoxin synthesis are carbon and nitrogen sources, pH, temperature, water activity and plant metabolites, as volatile aldehydes of corn leaves (Bhatnagar et al., 2003; Calvo et al., 2002; Zaika and Buchanan, 1987).

Aflatoxin biosynthesis seems to be regulated by simple carbohydrates, such as glucose and sucrose, and pools of amino acids in the plant, dependent on nitrogen (Thapar, 1988).

Although fungi can grow over a wide pH range, it has been established that aflatoxin synthesis optimally occurs in the pH range of 3.4-5.5. Neither high temperature nor drought stress alone can lead to increased concentration of aflatoxins (Mehan et al., 1988) even if it has been established that high

maximum and high minimum daily temperatures are more important for aflatoxin production than humidity or average precipitation during the same period (Bhatnagar et al., 2006).

1.5 TOXICITY OF AFLATOXINS

Aflatoxins may act as acute toxins (Platonow, 1964), carcinogens (Platonow, 1964; Wogan et al., 1971), teratogens (Ellis and di Paolo, 1967) and mutagens (Ong, 1975; Wong and Hsieh, 1976).

Animals demonstrate varying susceptibilities to aflatoxin toxicity, which may be attributed to genetic (species, sex, breed and strain), physiological (age, nutrition, other diseases, presence of other toxins) and environmental (climate, husbandry, management) factors (Bradburn et al., 1993). Aflatoxins are primarily potent hepatotoxins, causing aflatoxicoses in humans and animals. Aflatoxicosis primarily attacks the liver causing necrosis, cirrhosis and carcinomas, and it does cause other health effects. Acute symptoms include vomiting, abdominal pain, pulmonary edema, convulsions, coma, and cerebral edema (USDA, 2004). They occur in farm animals, both as a chronic disease characterised by an impairment of resistance and immune responsiveness, which results in a reduction in growth rate and feed efficiency; and as acute poisoning characterised by severe clinical disease, liver tumours, and death (Logrieco et al., 2003).

For humans, aflatoxin is predominantly perceived as an agent promoting liver cancer, although lung cancer is also a risk among workers handling

contaminated grain (Kelly et al., 1997). The risk of cancers due to the exposure to aflatoxin is well established (Gorelick et al., 1993) and is based on the cumulative lifetime dose (Williams et al., 2004). However, the possible role of the immune system with respect to the incidence, severity and outcome of infectious diseases in developing countries leads to expect that aflatoxin may also affect the epidemiology of many diseases and health risks in those countries where the toxin is uncontrolled (Williams et al., 2004). In particular, it has been observed a strong synergy between aflatoxin and hepatitis B and C virus (Groopman, 1993) and also with the degree of stunting and underweight in young children (Gong et al., 2002).

Because of their mutagenic, teratogenic, and carcinogenic potency, aflatoxins are classified within Group 1, as compound carcinogenic to humans (IARC, 1993).

Regarding the toxicity of cyclopiazonic acid (CPA), it has been considered to be involved in human intoxications, referred to as Kodua poisoning (Urano et al., 1992)). The toxicology of CPA in animals has been demonstrated in rats, chicken, mice, dogs and pigs (Nishie et al., 1985). Effects observed include degeneration and necrosis of the liver, lesions of the myocardium, decreased weight gain, vomiting, and several neurotoxic symptoms like opisthotonus, hyperaesthesia, hypokinesis and convulsions (Kuilman-Wahls et al., 2002). However, conflicting results have been published on the mutagenicity of CPA (Wehner et al., 1978; Soreson et al., 1984).

The European Community has established maximum levels of aflatoxin presence in food and feed. These limits have been established to provide an adequate margin of safety to protect human and animal health (Table 1.4).

Table 1.4 – Maximum levels of aflatoxins in foodstuffs (EC regulation 1881/2006)

Commodity	**Maximum levels (µg/Kg – ppb)**		
	B_1	**B_1+B_2+G_1+G_2**	**M_1**
Groundnuts to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs	8.0	15.0	-
Nuts to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs	5.0	10.0	-
Groundnuts and nuts and processed products thereof, intended for direct human consumption or use as an ingredient in foodstuffs	2.0	4.0	-
Dried fruit to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs	5.0	10.0	-
Dried fruit and processed products thereof, intended for direct human consumption or use as an ingredient in foodstuffs	2.0	4.0	-
Maize to be subjected to sorting or other physical treatment before human consumption or use as an ingredient in foodstuffs	5.0	10.0	-
All cereals and all products derived from cereals, including processed cereal products	2.0	4.0	-
Raw milk, heat-treated milk and milk for the manufacture of milk-based products	-	-	0.050
Following species of spices: *Capsicum spp.* (dried fruits thereof, whole or ground, including chillies, chilli powder, cayenne and paprika); *Piper spp.* (fruits thereof, including white and black pepper); *Myristica fragrans* (nutmeg); *Zingiber officinale* (ginger); *Curcuma longa* (turmeric)	5.0	10.0	-
Processed cereal-based foods and baby foods for infants and young children	0.10	-	-
Infant formulae and follow-on formulae, including infant milk and follow-on milk	-	-	0.025
Dietary foods for special medical purposes intended specifically for infants	0.10	-	0.025

1.6 MAIZE DISEASE DEVELOPMENT

The extent and severity of infectious maize diseases depend on the presence of a virulent pathogen, the proper air and soil environment, and the susceptibility of the maize host. Insects or other vectors are necessary to spread the pathogen and time required to reach values of the different characteristic parameters able to influence infection is also important (Diener et al., 1987). These factors must be present and “in balance” for an infectious disease to develop (Shurtleff, 1980). The relationship among these conditions is shown in Figure 1.5.

Maize may be subject to infectious and non-infectious diseases. The first ones are caused by fungi, bacteria, phytoplasmas, viruses and nematodes while the second ones are caused by an excess, deficiency or imbalance of soil nutrients or water, extreme soil acidity or alkalinity, very high or low temperatures, air pollutants or by mechanical, chemical or other injuries. Fungi cause the majority of infectious diseases of maize including the rusts, smuts, downy mildews, most rots, spots and blights, and deformations (Shurtleff, 1980).

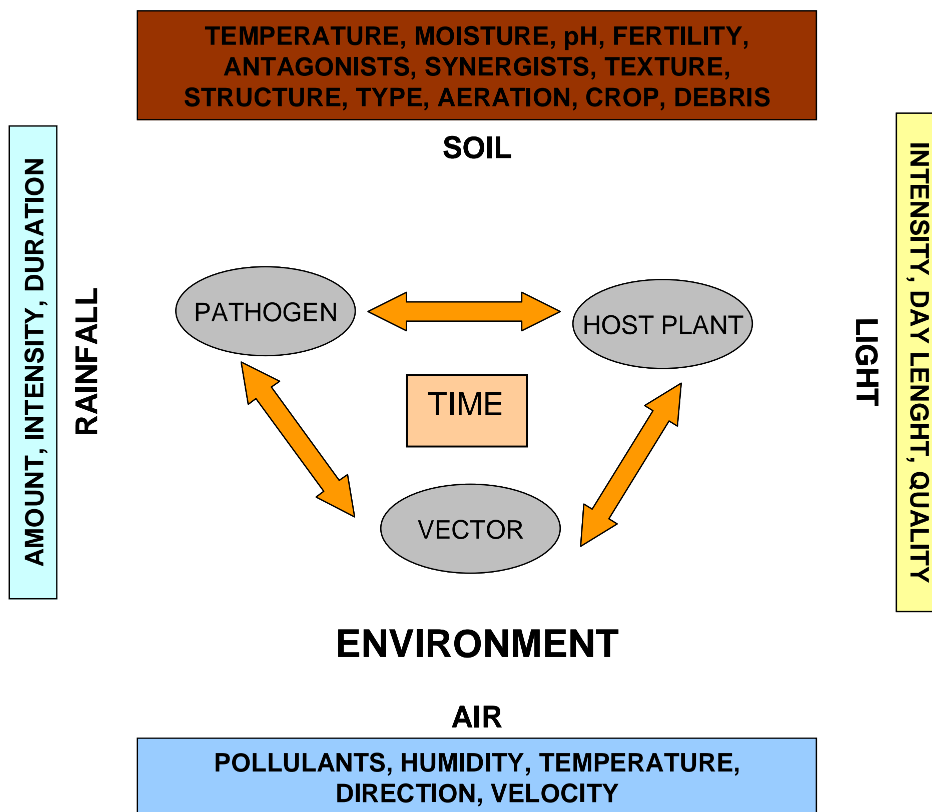


Figure 1.5 – Environmental influences on infectious plant diseases (Shurtleff, 1980).

1.6.1 *Aspergillus* ear rot

Aspergillus flavus and *A. parasiticus* are plant pathogens able to develop both on living tissues and on decaying plant and animal debris. The populations of these organisms on plant and in the soil are dependent upon how well they can compete with the other microflora present (Payne, 1998).

In particular, the host-parasite relationship of *A. flavus* with maize has been studied extensively, especially in the United States where this infection is a chronic problem.

Corn kernels become colonized with *A. flavus* early after silking (Jones et al., 1980; Payne, 1992). The fungus can be brought to the kernels surfaces by insects or can colonize silk tissues and grow down into the ear (Jones et al., 1980, Marsh and Payne, 1984; Payne et al., 1988). Although much of the hyphal growth appears on the surface of the silks, *A. flavus* can penetrate through the silks directly or through cracks and intercellular gaps (Payne, 1998). The fungus colonizes the silks first, then the glumes (by the milk stage), the kernel surfaces and, rarely, the cob pith (Marsh and Payne, 1984).
Colonization of the silks and kernel surfaces occurs soon after silking and may continue and increase throughout the season; although colonization of kernel surfaces by *A. flavus* may be extensive, internal infection is usually low (Marsh and Payne, 1984).

The infection cycle of maize by *A. flavus* is summarized in Figure 1.6.
A. flavus can overwinter in soil as mycelia or conidia (Angle et al., 1989); it can also produce sclerotia able to germinate on the soil surface (Wilson et al., 1989). The two major factors that influence soil populations of this fungus are soil temperature and moisture (Payne, 1998). *A. flavus* can grow at temperatures from 12 to 48°C and at water potentials as low as – 35 MPa (0.77 a_w) (Klich et al., 1994).

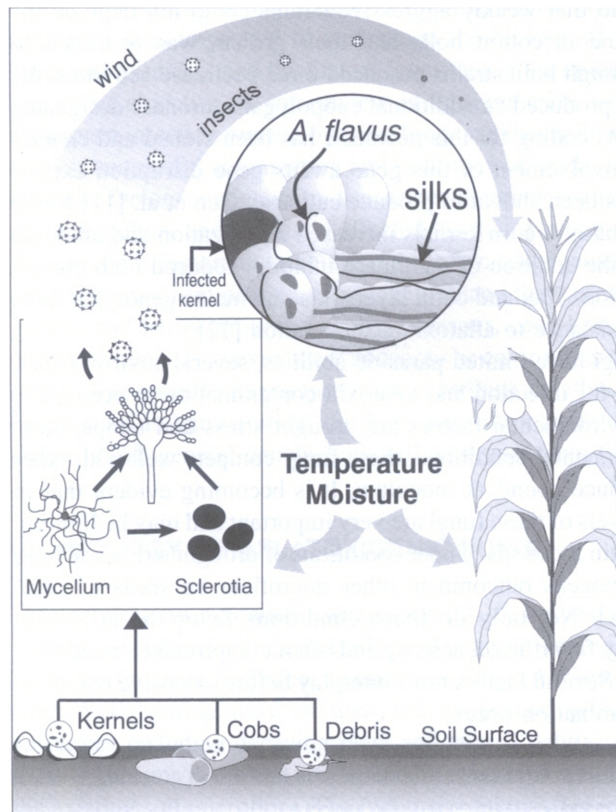


Figure 1.6 - Schematic representation of the ways of contamination by *A. flavus* in field (Payne, 1998).

Under conditions of high temperature and low water activity, *A. flavus* becomes very competitive and may become the dominant fungal species in the soil (Payne, 1998).

A. flavus has no known sexual stage and conidia are assumed to be the primary inoculum. From soil, the airborne conidia are deposited on the silks and kernels, and dispersed by wind and insects as reported in Figure 1.6 (Payne, 1998).

1.7 FACTORS INFLUENCING THE INFECTION BY *A. FLAVUS* OF MAIZE AND MYCOTOXIN PRODUCTION

Several factors may influence the presence of *Aspergillus* species on maize and their capacity to produce mycotoxins. They can be divided into biological, physical and chemical factors (D'Mello et al., 1997).

1.7.1 Biological factors

a) Fungal interactions

Maize is susceptible to infection of different mycotoxigenic fungi. In particular, it is infected by a range of different fusaria, including *F. graminearum, F. verticillioides, F. proliferatum, F. subglutinans* as well as by *Aspergillus* section *Flavi* and *A. ochraceus.* The dominant mycotoxigenic species is strictly related to meteorological conditions in the growing area.

F. verticillioides appears to compete with *A. flavus* on the corn ear; they can be dominant in years with temperate weather and with high temperature and drought stress respectively. In fact, years in which aflatoxin contamination is a serious problem are characterized as having above-average temperatures and below-average rainfall (Payne, 1998).

Wicklow et al. (1988) showed that *F. verticillioides* could interfere with infection and aflatoxin accumulation in developing maize seeds. Hill et al. (1985) also showed a negative correlation between the presence of *A. flavus* and *F. verticillioides*.

In experiments with maize grains at 18°C, *A. flavus* was dominant against *A. ochraceus* at high a_w (0.99), but it was not competitive at lower a_w levels (0.95). However, at 30°C *A. flavus* was dominant at all the a_w levels tested. Regarding the production of mycotoxins, at 18°C it was not competitive but at 30°C and 0.95 a_w aflatoxin production was dominant over ochratoxin production by *A. ochraceus*. At higher a_w, no significant differences in mycotoxin production between these fungi were observed (Lee and Magan, 2000).

b) Insects and kernel damage

A major factor in the epidemiology of *A. flavus* is the physical damage of the kernels resulting from invertebrate activity, mechanical damage from farm equipment, bird damage and from a variety of environmental factors (Bradburn et al., 1993). However, *A. flavus* has been shown to be able to colonize external silks, to grow down internal silks and to infect developing kernels free of insect injury (Fennell et al., 1977; Jones et al., 1980).

Although damage is not a prerequisite for aflatoxin formation, the incidence of *A. flavus* and levels of aflatoxin contamination were higher in damaged kernels (Diener et al., 1987).

Insects may contribute to the infection of kernels in four ways:

1) transport primary inoculum to the ears: insects may be conveyors of fungal spores but the ear may already be infected with *A. flavus* as a result of high spore loads during the receptive period for silk infestation;

2) move inoculum from the silks into the ear;

3) disseminate inoculum within the ear;

4) facilitate colonization and infection of kernels by injuring the kernels: insects are able to facilitate the infection process by wounding intact tissue and providing more infection sites. As demonstrated by Widstrom (1979) wounding may also allow kernels to dry down to moisture levels that support the growth of *A. flavus* and subsequent aflatoxin production.

Interestingly, Marsh and Payne (1984) mapped the distribution of *A. flavus* in two groups of naturally infected corn ears, one apparently free of insect damage and one with insect damage. In some cases there was colonization without insect injury and conversely, there was insect injury without colonization. Probably, under favourable environmental conditions, *A. flavus* is an aggressive pathogen and insects are not required to infect the ear, distribute it within the ear, or provide a site for the entry of the fungus. In contrast, when the environmental conditions are less favourable for *A. flavus*, only a few kernels may be colonized in the absence of insect injury (Payne, 1998).

1.7.2 Physical factors

A wide variety of interacting physical factors may affect mycotoxin production in the field and during storage. The most important are temperature, water activity (a_w) and relative humidity. Many *in vitro* studies have shown the optimal conditions for growth and aflatoxin production of *A. flavus* strains from different parts of the world.

a) Temperature

Optimal temperatures for *A. flavus* development and aflatoxin production are different.

Aspergillus strains are able to grow over a wide temperature range. The general consensus as shown by Northolt and van Egmond (1981) is that optimal growth of *A. flavus* on most substrates occurs over a range of 19-35°C; with minimal and maximal temperatures of 12 and 43°C respectively.

Regarding aflatoxin, Northolt et al. (1977) showed that the optimum temperature for the production of AFB_1 ranged from 24 to 32°C depending on the substrate. Other investigations found a range of 20-35°C (Schindler et al., 1967; Diener and Davis, 1968; Trenk and Hartman, 1970; Detroy et al., 1971; Boller and Schroeder, 1974; Reiss, 1975; Northolt et al., 1976). Generally, 28°C seems to be the optimum for aflatoxin production (Scott et al., 1970; Sanchis and Magan, 2004). Park and Bullerman (1983) found that no growth or aflatoxin production occurred at 5°C.

It is important to note that the length of incubation time can influence the level of toxin produced by *A. flavus*. In particular, Kheiralla et al. (1992) demonstrated that the greatest aflatoxin production was achieved at 30°C after 14 days incubation. Longer times resulted in a decrease of aflatoxin levels probably due to degradation or re-adsorption by the fungus (Kheiralla et al., 1992).

In the field, an important factor to consider is that fungi appear to be simultaneously synthesizing and degrading aflatoxins. Consequently, daily environmental changes could distinctly modify the extent of the two metabolic pathways concerned and so influence the final level of toxin (Schroeder and Hein, 1968; Stutz and Krumperman, 1976).

b) Water activity

Water activity (a_w) has a significant impact on growth and mycotoxin production. *Aspergillus* strains are able to grow and produce mycotoxins down to conditions of 0.73 and 0.85 a_w, respectively (Trucksess et al., 1988; Sanchis and Magan, 2004).Trucksess et al. (1988) inoculated several substrates (corn, soybeans and pinto beans) with *A. flavus* and incubated them at different a_w and temperature levels. At 16°C the fungus was able to grow but not produce aflatoxin on corn at 0.80 a_w, soybeans at 0.77 a_w and pinto bean at 0.85 a_w. For corn, the limiting a_w for *A. flavus* growth was 0.73 at 26 and 32°C. Aflatoxin production was essentially the same at 26 and 32°C with limiting a_w values in the range 0.85-0.89.

Later experiments showed that the germination of conidia of *A. flavus* was very rapid at >0.90 a_w with an almost linear increase with time when temperature was 25°C. However at lower level of a_w, germination was very slow (Marin et al., 1998b). The range of a_w conditions at optimal temperatures for germination were generally found to be wider than that for mycelial growth (Magan and Lacey, 1984; Marin et al., 1998b).

c) Chemical factors

There is relatively little data on the effects of fungicides on growth and aflatoxin production by *Aspergillus* species and, mainly, it is derived from *in vitro* studies.

Criseo et al. (1994) examined *in vitro* the influence of different concentrations of 5 inhibitors of mycelial growth on colony growth and aflatoxin production by several strains of *A. flavus* and *A. parasiticus*. Cycloheximide and mercuric chloride were the most effective in reducing fungal growth but they were able to enhance aflatoxin production. Biphenyl at high concentrations resulted in a reduction in both fungal growth and aflatoxin production while at low concentration the aflatoxin production was only delayed. Dichloran was not able to influence fungal growth, however, at high concentration, it inhibited aflatoxin production. Sodium desoxycholate reduced both fungal growth and aflatoxin production. Often with higher concentration of fungicides there was not a higher inhibition of fungal growth. Indeed sometimes, fungal growth was found to recommence after initial inhibition. (Criseo et al., 1994).

More recent studies demonstrated that fungi have a great capacity to adapt to fungicides creating some differences in the colonial morphology (Delen and Tosun, 1999). Prochloraz and imazalil seem to be two ergosterol biosynthesis inhibitors effective in reducing growth and aflatoxin formation by *A. flavus* and *A. parasiticus*. Increasing concentrations of these chemicals altered both conidial formation and aflatoxin biosynthesis, resulting in > 80% reduction in aflatoxin concentrations (Delen and Tosun, 1999).

Conventional methods of plant disease control with the use of fungicides and insecticides were ineffective in controlling *A. flavus* infection of corn when employed at concentrations that are both cost-effective and environmentally safe (Bhatnagar et al., 1993). As a result, conventional practices that are available may reduce aflatoxin concentrations in the field but these practices can involve substantial unacceptable cost to the grower (Brown et al., 1998).

When fungicides are used effectively to control fungal diseases of crop plants, then this risk is minimised (D'Mello and Macdonald, 1997). However, a number of *in vitro* studies show that the use of fungicides at sub-lethal concentrations may enhance mycotoxin production because of stress caused to the fungus. In Italy no fungicide applications are permitted for maize in field destined for feed for animals and for milk and cheese production.

1.8 POST-HARVEST MAIZE INFECTION BY *ASPERGILLUS* SPECIES

The quality of grains postharvest is influenced by a range of abiotic and biotic factors and can be studied as a stored grain ecosystem (Magan and Aldred, 2003). This ecosystem includes grain and contaminant mould respiration, insects pests, rodents, the key environmental factors (temperature, water availability and intergranular gas composition) and preservatives which are added to conserve moist grain for animal feed (Magan and Aldred, 2007).

During post-harvest, spoilage fungi colonising grain use different primary and secondary strategies to occupy the niche. Primary resource capture of grain is influenced by the germination rate, growth rate, enzyme production and the capacity for sporulation. Subsequent interactions between spoilage fungi result in combat, antagonism and niche overlap (Magan et al., 2003).

Stored maize and its microbial contaminants generally respire slowly when stored dry. However, when moisture content of maize is around 15-19%, *Aspergillus* species can grow and produce a significant increase in respiratory activities and then also in temperature and spontaneous heating. This results in CO_2 production derived from complete respiration of carbohydrates (dry matter loss). As a consequence, the greater the CO_2 production, the shorter the safe storage period without dry matter and nutritional quality losses (Magan and Aldred, 2007).

The environmental conditions under which *A. flavus* and maize interact are critical in determining whether aflatoxin contamination will occur. In

particular, interactions between these factors can determine if mycotoxins are produced (Wallace and Sinha, 1981; Magan et al., 2003; Magan et al., 2004).

Moisture content control can be considered essential to avoid *A. flavus* growth and aflatoxin production during storage. For example, blending wet corn with dry to achieve an average moisture content of ±15.5%, which is allowed in the USA, is considered to be a risk factor. There is concern that the wet pockets may remain at a high moisture content long enough to support growth of fungi, particularly *A. flavus*, with the subsequent production of aflatoxin (Sauer and Burroughs, 1980; Magan et al., 2004).

Lopez and Christensen (1967) found no evidence that *A. flavus* invaded any samples of inoculated corn when the moisture content was below 17%. They concluded that *A. flavus* would not grow appreciably, even at 35°C, in corn below about 17.5% moisture content. Trenk and Hartman (1970) reported that 18% moisture content was the lower practical limit for aflatoxin formation in naturally contaminated, artificially dried corn.

Lillehoj et al. (1976) in an experiment with dry and high-moisture corn blends, inoculated with *A. flavus*, found that the percentage of kernel invasion was high and aflatoxin was produced in dry corn fractions with moisture content that did not exceed 13%. Hunter (1969) established that *A. flavus* does not grow on corn held in storage in temperate climates if the moisture content is below ca. 17.5% (= 0.79 a_w). The growth and ability to produce aflatoxin was also dependent on the interaction between a_w and temperature.

Winn and Lane (1978) observed that growth of *A. flavus* and the production of aflatoxin required a minimum equilibrium relative humidity (ERH)

of 85%, which corresponds to about 18-18.5% moisture content in maize. A slightly higher ERH of 86-87% may induce rapid fungal growth and aflatoxin accumulation. Thus infected maize stored at an ERH of 90% may result in significant levels of aflatoxin within 48 h.

Maize is often treated with commercial preservatives mainly based on salts of propionic and sorbic acids to reduce spoilage. It is important that such treatments reach all grains surfaces to avoid fungal development because under-treated pockets can lead to growth and mycotoxin production (Magan and Aldred, 2007). Moreover, low dosages of preservatives based on aliphatic acids can result in stimulation of growth and mycotoxin production (Sanchis and Magan, 2004).

Insect pets are also a common problem in the stored grain ecosystem. They can grow at a_w lower than those indicated for fungi and can generate water *via* condensation on surfaces due to temperature differentials and develop classic hot spots which can quickly result in heating and complete spoilage (Magan et al., 2003).

1.9 PREVENTIVE MEASURES: GOOD CULTURAL PRACTICES

Preventive measures are of paramount importance in reducing the risk of mycotoxin contamination of grain. These can be summarized as good cultural practices.

A. flavus is adapted to extreme conditions, especially those associated with drought in tropical agricultural crops. The rate of fungal infection may also increase because of drought stress that compromises kernel integrity and health. Irrigation, that is able to reduce water stress, may be an effective method to reduce aflatoxin contamination but it is not always available or cost-effective for growers (Payne et al., 1986).

Drought stress may also affect the constituents of maize kernels, providing a better substrate for the establishment of *A. flavus* and for the biosynthesis of aflatoxin. Irrigation programmes on light soils may partially alleviate drought stress (Jones et al., 1981; Fortnum and Manwiller, 1985; Payne et al., 1985), but they can exacerbate the leaching problem of nitrogen derived by reduced water-holding capacity. This can occur as a result of dense plant or excessive weed populations (Anderson et al., 1975) which compete for soil nutrients (Cobb, 1977). In these cases a regular application of a well-balanced fertilizer is necessary to maintain a crop with a low inoculum level. In the USA, the excessive use of herbicides to control weeds resulted in an increase of susceptibility of maize to *A. flavus* infection and to aflatoxin contamination as well as increasing the population of undesirable insect and fungal pathogens (Oka and Pimentel, 1970).

Tillage systems and crop rotation can affect soil inoculum availability and root/soil interface and prevent inoculum build up (Jones, 1987).

Also harvesting practices can influence some aflatoxin contamination because it can have an effect on the level of kernel damage. A study conducted to evaluate the effect of harvest and de-husk machinery on the physical qualities of maize seeds underlined that those harvested manually presented less damage than those harvested mechanically. In addition, seeds harvested as maize ears yielded better physiological quality than those harvested as a maize grain (Oliveira et al., 1997). In addition, grain moisture at harvest seems to be closely related with the percentage of grain cracking (Plett, 1994). Damaged kernels can create an optimal way for fungal penetration in seed and, consequently, for aflatoxin production. In fact, the highest levels of aflatoxin are produced when the fungus invades the seed embryo, where simple sugars are present in high quantities compared to other parts of the seed where complex carbohydrates predominate (Bhatnagar et al., 2006).

Aflatoxin build up in the field occurs late in crop development. For this reason growers may harvest corn early at high moisture content (26-28%) and dry corn artificially to < 13% moisture content (Brown et al., 1999). Early harvesting and rapid drying are effective methods to limit aflatoxin accumulation (Jones, 1987; Payne et al., 1988); however the risk of further aflatoxin contamination has to exceed the expense involved in early harvesting and artificial drying for this practice to be employed (Jones, 1987). It is necessary to take into consideration that in regions with little late-season rainfall or where maturation occurs during hot periods of the year early harvesting is of limited usefulness (Brown et al., 1999).

Harvesting at the optimum stage of maturity and rapid drying after harvesting can represent good strategies for *A. flavus* and aflatoxin control (Brown et al., 1999).

Today the principal strategy to eliminate aflatoxin is to develop preharvest host resistance to aflatoxin accumulation. This strategy has gained even greater prominence due to recent discoveries of natural resistance in corn that can be exploited in plant-breeding strategies (Brown et al.,1999). For example, several studies show that AFB_1 contamination of grain was generally reduced in maize hybrids resistant to *Aspergillus* ear rot (Brown et al., 1995; Campbell and White, 1995). Current research is focused primarily on kernel pericarp resistance (morphologic and chemical) and kernel subpericarp biochemical resistance (antifungal proteins) to fungal infection. The resistant genotypes investigated generally seem to inhibit aflatoxin production indirectly through inhibition of fungal growth (Brown et al., 1995; Guo et al., 1996).

From results obtained by Norton (1997) carotenoids can markedly decrease aflatoxin level and those containing the α-ionone ring are most effective. Little information, however, is available on carotenoid formation in corn as a function of ripening. Zsolt et al. (1963) established that levels of total carotenoids at the waxy stage are approximately one-tenth of the levels of mature corn.

Volatiles generated from corn silks of individual genotypes of maize were found to have a profound effect on the growth of *A. flavus* and, consequently,

aflatoxin production. In particular, aflatoxin field resistant maize genotypes exhibited a larger relative concentration of the antifungal aldehyde, furfural (2-furancarboxaldehyde) when compared to the relative concentration of the field-susceptible varieties tested. The presence of furfural appears to contribute to a defence mechanism for protecting the developing maize kernel from fungal attack (Zeringue Jr., 2000).

Several phenols and related compounds in maize kernels have shown antibiotic activity against fungi. The relationship between phenolic content in kernels and resistance to infection by *A. flavus* was investigated. A significant negative correlation was found between the *A. flavus* incidence and the amount of phenolic content in kernels. Conventional breeding programmes should incorporate genotypes containing high concentrations of kernel phenolic content aiming for developing resistance to *A. flavus* (Kumar et al., 2001).

A. flavus strains appear to be significantly more sensitive to β-carotene than *A. parasiticus* strains (Norton, 1997). These studies could lead, in the near future, to commercially available, agronomically acceptable corn lines with multiple pre-harvest resistances to aflatoxin contamination.

1.10 REMEDIAL MEASURES

1.10.1 Use of biocontrol agents in field

The development of biocontrol agents (BCA) based on fungi able to control pests and plant disease is of great interest; this is evidenced by the number of commercial products available and also by the demand that these kind of products have especially from final consumers (Butt et al., 2001).

BCAs are microorganisms able to reduce the development of the mycotoxigenic fungi thanks to competition, niche overlap, parasitism or production of toxins. Usually, it is normal to chose fungi for biocontrol from field and crops where also mycotoxigenic fungi can be isolated. This could be interesting for maize and its aflatoxin control in the field since BCAs offer environmentally friendly alternatives to chemical pesticides.

Isolation of BCAs from the environment is the first step and then a great amount of experiments are necessary to obtain the necessary knowledge about their ecology, physiology and taxonomy (Butt et al., 2001). It Is of primary importance to demonstrate that their introduction in the environment at a high level will not create damage.

Interactions between aflatoxigenic fungi and other microorganisms is a common phenomenon in nature. This interaction results in continuous changes in the availability of nutrients, and production of metabolite by-products that can influence mould growth and aflatoxin production (Gourama and Bullerman, 1997). For example, *A. niger* was found to be a good competitor of *A. flavus*

strains (Mann and Rehm, 1977). Chaudhary et al. (2001) have observed that in vitro the highest reductions in aflatoxin B_1 and G_1 were present only when *A. niger* was inoculated prior to *A. flavus* and *A. parasiticus*. When *A. flavus* and *A. niger* were inoculated simultaneously, 100% degradation of aflatoxin B1 and 95% degradation of aflatoxin G1 were observed. Also in this case, the ability to reduce aflatoxin presence varied from strain to strain both of *A. niger* and of *A. flavus* and *A. parasiticus*. However, these studies were carried out prior to the knowledge that some species in the *A. niger* Section *Nigri* group produced ochratoxins. Aflatoxin can also be degraded by the same species that produce it (Doyle and Marth, 1978), so reduction is possible also with competitive non-aflatoxigenic strains of *A. flavus* and *A. parasiticus*. In field trials, Dorner et al. (1999) established that application of non-aflatoxigenic strains of *A. flavus* and *A. parasiticus* greatly altered the overall populations of those species in soil. A 87% aflatoxin reduction was seen during the first year of treatment and a 66% reduction during the second year. It appears that application of non-toxigenic *A. parasiticus* to soil may not be important in controlling aflatoxin in corn but the strain of *A. flavus* that is used as a biocompetitive agent is very important.

Some *in vitro* trials established that 9-day-old mycelia of *A. parasiticus* are able to degrade aflatoxin to a varying extent, depending only on the substrate used to grow the fungus. It has been established that aspergilli able to produce greater amounts of aflatoxin are also able to degrade aflatoxins more rapidly while those that produce minimal amounts of aflatoxin generally degraded aflatoxins less effectively (Doyle and Marth, 1978).

In laboratory cultures the amounts of aflatoxins synthesized by toxigenic strains decreases gradually after successive subculturing and morphological changes occur in these organisms. Torres et al. (1980) underlined how, as the number of successive subcultures of a strain increased, there was a progressive reduction in its capacity to synthesise aflatoxins. Not all strains were equally affected by the successive subculturing but *A. flavus* seemed to be more sensitive than *A. parasiticus*.

In the United States much of the early work on biocompetitive exclusion for aflatoxin management was performed on cotton and aflatoxin contaminated cottonseed was the target for the first atoxigenic strain biopesticide registration (Cole and Cotty, 1990; Cotty, 1990 and 1994). The species most frequently implicated in contamination of cotton is *A. flavus*. Atoxigenic individuals of this species are frequently isolated from infected crop tissue and the discovery that both ability to infect crops and virulence to crops were not correlated with aflatoxin-producing ability led to the suggestion that atoxigenic strains might be used as BCAs to competitively exclude aflatoxin producers and in so doing reduce the aflatoxin content of treated crops (Cotty, 1989 and 1992). *A. flavus* communities differed among agricultural fields in afaltoxin-producing potential, application of atoxigenic strains might reduce both the average aflatoxin-producing potential and the vulnerability of all crops planted in those fields to contamination (Cotty, 2006). It has been established that the use of atoxigenic strains of *A. flavus* was able to reduce the average aflatoxin-producing potential

of *A. flavus* communities in treated and nearby fields and that these changes to the fungal community persisted for multiple years (Cotty, 1994 and 2000).

In commercial practice, atoxigenic strains are applied on a nutrient source (i.e. wheat seed, barley, sorghum) on which the fungus grows, sporulates and disperses to developing plants and other nutrients in the crop environment (Antilla and Cotty, 2004). Solid formulations allow both residence in treated fields and spore production for relatively long periods and, as a result, provide a window of influence that extents considerably beyond application date (Cotty, 2006).

BCAs for *A. flavus* and *A. parasiticus* in peanuts has been developed in Australia and the United States and seem to have promising results. They are based on competitive exclusion since a large population of nonaflatoxigenic strain of *A. flavus* and/ora *A. parasiticus* is normally established in the soil where they compete with aflatoxigenic strains that are naturally present. In the U.S., good control of aflatoxin in peanuts was achieved with almost 90% of reduction but only at the second year of usage of a biocontrol product based on a non-aflatoxigenic strain of *A. flavus* (Dorner and Horn, 2007). While in Australia the biocontrol agent used was based on a strain of *A. parasiticus.* In this case they obtained a great reduction in soil of aflatoxigenic strain of A. flavus (95% of strains isolated from soil resulted nonaflatoxigenic) (Pitt, oral communication, 2006).

However, the economic costs of these kinds of products are still being evaluated in Australia and in the USA (Pitt and Hocking, 2006). A first

estimation is around 11$ per hectare but costs associated with the application of BCA were not considered (Cotty, 2006). Government incentives could be a good solution to increase their use.

1.10.2 Use of modified atmosphere in post-harvest

Only few experiments have been conducted on effects of elevated CO_2 on growth and mycotoxin production of *A. flavus*. Different CO_2 levels balanced with O_2 and N_2, showed that *A. flavus* grew on wheat and rye bread with up to 75% CO_2 (Suhr and Nielsen, 2005). Previously, Wilson and Jay (1975) tested a high CO_2 treatment (61.7% CO_2 balanced with O_2 and N_2) on moist maize and found that *A. flavus* growth was visible after 4 weeks at 27°C and that contamination with aflatoxin was lower than exposure in normal atmospheric conditions.

Similar experiments where conducted on *A. carbonarius* and ochratoxin production (Pateraki et al., 2007). These studies suggested that up to 50% CO_2 had only a slight impact on ochratoxin production by *A. carbonarius* over a wide range of a_w conditions (0.93-0.99).
Other studies found that fumonisin B_1 production by *F. verticillioides* was inhibited with 30% CO_2 at 0.984 a_w (Samapundo et al., 2007).

However, more information is required on the behaviour of *A. flavus* in response to modified atmospheres (increasing levels of CO_2) to examine

whether potential exists for it to be integrated into a prevention strategy post-harvest.

1.10.3 Decontamination

Ammoniation results in effective reduction in the level of aflatoxin B_1 in corn. The ammoniation process using either ammonium hydroxide or gaseous ammonia has been shown to reduce aflatoxin levels in corn by > 99% (Park et al., 1988) but the toxicology and carcinogenic potential of ammonia reaction products have to be considered.

Decontamination of corn using ozone gas (O_3) may be a possible alternative to ammoniation obtaining mycotoxin degradation in contaminated grains with minimal destruction of nutrients (Mckenzie et al., 1997). Results indicate that AFB_1 and AFG_1 are rapidly degraded using 2% O_3, while AFB_2 and AFG_2 are more resistant to oxidation and require higher levels of O_3 (20%) for rapid degradation. The difference in degradation rates for different aflatoxins suggests a propensity for oxidation by O_3 at the C8-C9 double bound which is present in AFB_1 and AFG_1 but not in AFB_2 and AFG_2 (Mckenzie et al., 1997). The degradation products of the aflatoxins were not identified, probably further reaction with O_3 should lead to the formation of CO_2 and H_2O as by-products (Mckenzie et al., 1997). Generally, practical methods to degrade mycotoxins using O_3 have been limited due to low O_3 production capabilities of conventional systems and their associated costs.

Also treatments with aqueous solutions of acids seems to be efficient in aflatoxin B_1 and G_1 reduction, but they are not able to have an effect on aflatoxin B_2 and G_2 and the possibility to use them practically seems difficult (Avantaggiato et al., 2002).

1.11 SPECIFIC OBJECTIVE OF THIS RESEARCH PROJECT

In 2003 in Northern Italy there was significant contamination of maize used as animal feed and, consequently, of milk, by aflatoxins. This occurred for the first time perhaps because of the unusually hot and dry conditions resulting in water stressed maize feed crops.

There was thus a requirement to understand the reasons why this occurred; in particular, the characteristics of Italian *Aspergillus* section *Flavi* populations and the ecological conditions favourable for their growth and aflatoxin production were needed in order to understand the reasons why they became dominant and to utilise such information to predict potential risk of contamination with aflatoxins in the future.

The main objective of this research project was to study the ecological needs of *A. flavus* and its behaviour on maize. To do this, the following studies were conducted:

1) A collection of *A. flavus* strains from Italian maize-growing areas was made and some comparisons made with other populations from other continents;
2) A preliminary characterization of these strains was carried out, based on their growth and toxin production at different levels of temperature and a_w;
3) Some representative strains of the Italian grouping were chosen, based on statistical analysis of the data, and more detailed studies were conducted. In particular:

- A range of ecological conditions (water availability and temperature) able to influence the growth and the aflatoxin production were examined;
- The effect of modified atmospheres and water stress in *in vitro* and *in situ* experiments were determined;
- The effect of solute and matric stress and temperature on growth, sporulation and aflatoxin production was investigated.

4) Artificial inoculation of maize kernels was done to verify the effect of growth stage on fungal development and aflatoxin content;
5) The effect of interaction between *A. flavus* and *F. verticilloides* by using the Niche Overlap Index (NOI) approach was studied to understand competitiveness under different environmental conditions;
6) Field trials were carried out to evaluate the behaviour of fungi on different maize hybrids in their natural environment.

The accumulated data and knowledge from this research project will be used to develop mathematical functions of the relationship between key environmental factors pre- and post-harvest to develop predictive models as part of Decision Support System (DSS) in the maize production chain to minimise contamination with aflatoxins.

The detailed components of the work programme and the links between them are shown in the Flow Diagram (Figure 1.7). The Thesis is organised as a series of linked Chapters which covers the different complimentary components of the work.

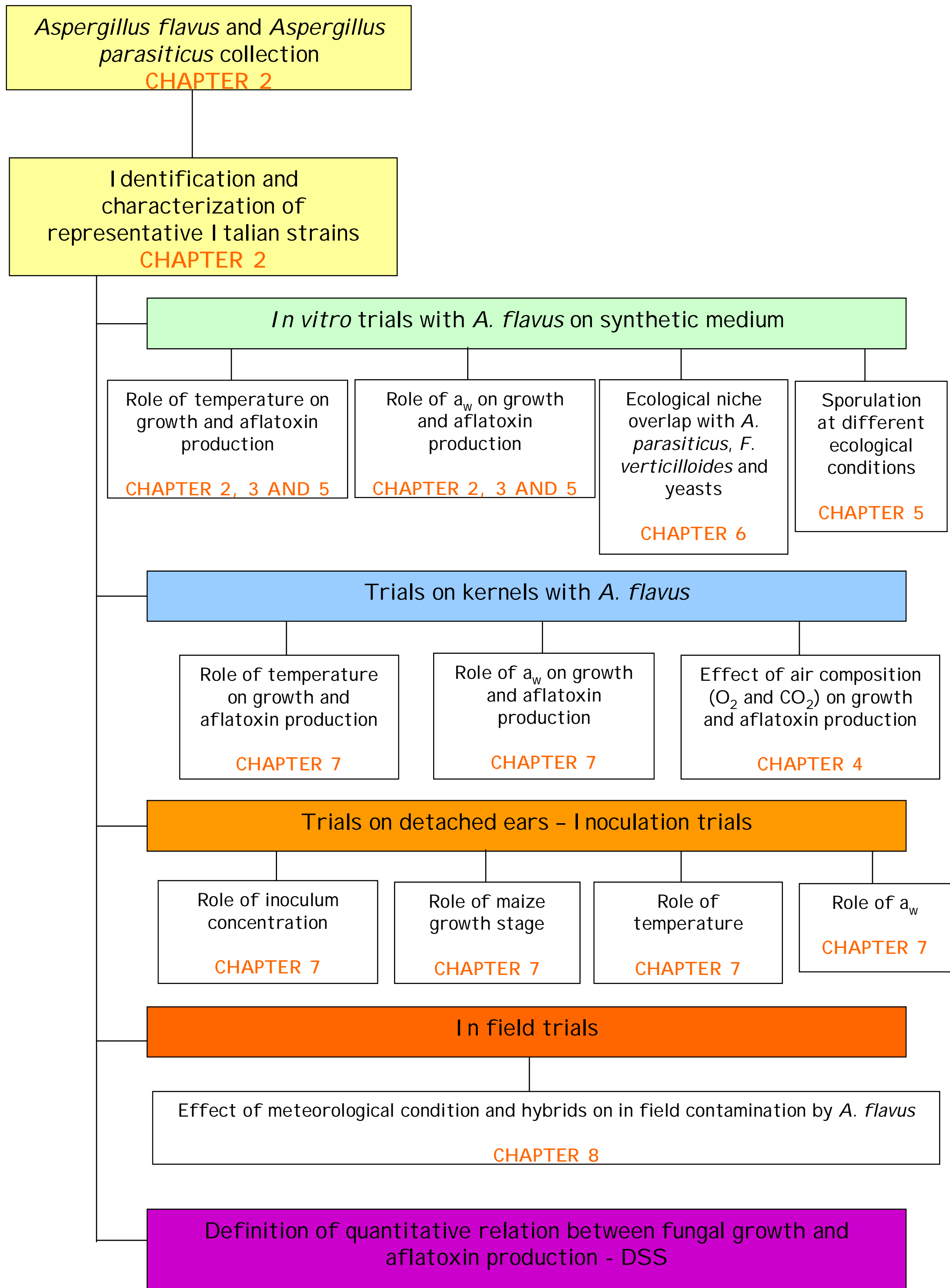


Figure 1.7 - Flow chart of different components of studies that will be considered in this research

CHAPTER 2

Studies on *Aspergillus* section *Flavi* isolated from maize in northern Italy

2.1. INTRODUCTION

Maize is a commodity considered to be one of the most susceptible crops to mycotoxins world-wide (Barug et al., 2004). Maize is colonized and contaminated by a range of different fusaria, including *F. graminearum, F. verticillioides, F. proliferatum, F. subglutinans,* causing maize ear rot, as well as by *Aspergillus* section *Flavi.* The dominant mycotoxigenic species present is strictly related to meteorological conditions in the regions of cultivation.

The optimal ecological conditions for growth and mycotoxin production differ for these important genera. *Fusarium* strains have optimum temperature for growth in the range 25-30°C, at which higher levels of toxins are produced, e.g. fumonisins (Marin et al., 1995). *Aspergillus* strains grow over a wider temperature range. Optimal growth of *A. flavus* occurs over the range 19-35°C (Northolt and van Egmond, 1981), with 28°C being optimum for aflatoxin production (Scott et al.,1970; Sanchis and Magan, 2004). Water availability (water activity, a_w) also has a significant impact and *Aspergillus* strains are able to grow and produce mycotoxins down to conditions of 0.73 and 0.85 a_w, respectively. These are extremely different from *Fusarium* species, which cannot often grow below 0.90 a_w and produce trichothecenes or fumonisins at > 0.93 a_w (Trucksess et al., 1988; Sanchis and Magan, 2004).

In Italy, maize is widely grown in the northern regions, where the main concern is contamination with fumonisins, produced by *F. verticillioides*, with a high incidence in most years. Deoxynivalenol is detected only sporadically,

especially in rainy years with temperature levels lower than usual for these regions, when *F. graminearum* becomes dominant (Pietri et al., 2004). In 2003, for the first time, significant problems arose due to aflatoxin contamination of maize. The summer was particularly dry and hot, with maize crops water-stressed and consequently maize grain was highly contaminated, resulting in problems with aflatoxin M_1 (AFM_1) in milk and derived products (Battilani et al., 2005; Pinelli et al., 2005). The problems were worsened by the lack of experience of local farmers and extension staff, with this new problem.

The main members of *Aspergillus* section *Flavi* able to produce aflatoxins (AFs) are *A. flavus* and *A. parasiticus* (Kurtzman et al., 1987). These are closely related fungi and difficult to distinguish from each other. It is now generally accepted that *A. flavus* produces only aflatoxin B_1 and B_2, while *A. parasiticus* produces all the four principal AFs (AFB_1, AFB_2, AFG_1 and AFG_2) (Diener et al., 1987; D'Mello and McDonald, 1997). However, Gabal et al. (1994) reported a high percentage of *A. flavus* strains producing AFG_1 and a minor group also producing AFG_2. The International Agency for Research on Cancer classified AFB_1 as a class 1 toxin because of its demonstrated carcinogenicity to humans, while AFM_1 is possibly carcinogenic and has been classified as 2B (Castegnaro and Wild, 1995). All aflatoxins are regulated in most countries throughout the world, Europe included, in different products as well as maize and milk (CE, 2001).

Some *A. flavus* strains are also reported to produce cyclopiazonic acid (CPA), a mycotoxin typical of several species of *Penicillium.* Contradictory results exist on the mutagenic effect of CPA; however, there is evidence of its inhibitory effect on the mutagenicity of AFB_1 (Kuilman-Wahls et al., 2002).

The main objective of this study was to obtain detailed information on the characteristics of Italian *Aspergillus* section *Flavi* populations in the key milk-producing regions of northern Italy. The diversity of *A. flavus* and *A. parasiticus* was examined in a detailed survey supported by ecological trials; grouping of strains was determined using cluster analysis and *in vitro* AFB_1 production. This was essential for a better understanding of the key role of the relevant strains in AF contamination of maize.

2.2. MATERIALS AND METHODS

A total of 70 isolates of *Aspergillus* section *Flavi* were examined in this study. These strains came from an Italian maize survey carried out in field in 2003 in Friuli Venezia Giulia, Lombardy, Piedmont, Tuscany and Veneto and in 2004 in Emilia Romagna; 33, 24, 12, 10, 17 and 90 samples were collected in the cited regions (Battilani et al., 2005). Twenty ears were harvested from each field and, after husk elimination, ears were dried at 40°C and shelled. Fifty grains of each sample were plated in Petri dishes with Peptone PCNB Agar (PPA) (Peptone 15g; KH_2PO_4 1g; $MgSO_4 \cdot 7H_2O$ 0.5g; PCNB 75% 1g; agar 8g; H_2O to 1L) and incubated at 25°C for 7 days. Moulds developed from grains

were purified, transferring them to Petri dishes with Potato Dextrose Agar (PDA) (infusion from potatoes 200g; dextrose 15g; agar 20g; H_2O to 1L) and after incubation at 25°C for 7 days fungi were identified to section level. Only 1 strain of *Aspergillus* section *Flavi* for each sampled field was stored, independently of the positive grains found. These strains are part of the culture collection of the Institute of Entomology and Plant Pathology of the Università Cattolica del Sacro Cuore of Piacenza (Italy, code MPVP).

2.2.1. Characterization of isolates

Colony morphology. Strains were inoculated at the central point on Petri dishes (Ø 6 cm) with Czapek Agar (CZ) (sucrose 30 g; $NaNO_3$ 2 g; KCl 0.5 g; $MgSO_4 \cdot 7H_2O$ 0.5 g; $FeSO_4 \cdot 7H_2O$ 0.01; K_2HPO_4 1 g; $ZnSO_4 \cdot 7H_2O$ 0.001 g; $CuSO_4 \cdot 7H_2O$ 0.005 g; agar 15 g; H_2O to 1 L) as medium and incubated at 30°C for 14 days in the dark. After incubation, dishes were observed for colony colour, sclerotial production and conidiophores, morphology and size. The characteristic colour of colonies for *A. flavus* is ivy green and for *A. parasiticus* cress green, according to Raper and Fennell (1965).

For microscopic observation, strains were prepared on glass slides after staining with lactic acid and lacto-phenol blue. The two relevant species, *A. flavus* and *A. parasiticus,* can be differentiated by relative conidiophore lengths (500 µm for *A. flavus* and from 200 µm to rarely more than 1 mm for *A. parasiticus*), conidiophore characteristics (*A. flavus* has thinner walled and less

roughened conidiophores than those of *A. parasiticus*), but primarily by the character of their sterigmata: *A. flavus* has primary and secondary sterigmata, while *A. parasiticus* has only primary sterigmata and they are respectively termed biseriate and uniseriate (Raper and Fennell, 1965). One type strain of *A. flavus* (IMI 348543) and one of *A. parasiticus* (IMI 283883) from the official collection of CABI Bioscience (Egham, UK) were used as reference strains. Observations were carried out with a magnification of between 100 and 400X.

Sclerotia. Kozakiewicz (1989) reported that production of sclerotia is a rare characteristic of *A. flavus* strains only. Petri dishes were observed macroscopically to verify the presence of sclerotia, structures easily identifiable. Sclerotial size is a phenotypic character within *A. flavus* strains (Abbas et al., 2005), that can be used to create two different groups: the large strains (L) having sclerotia > 400 μm in diameter and the small strains (S) with sclerotia < 400 μm (Horn, 2003); differences in strain ability to produce AFs can be linked to sclerotial size (Cotty, 1989; Chang et al., 2001).
Strains were transferred on Petri dishes with 5/2-Agar (5% V8-juice; 2% agar; pH 5.2) and incubated at 31°C for 5 to 7 days in darkness (Probst et al., 2005). Sclerotial size was evaluated by a measuring reticule with a Nikon Microscope (Nikon Inc., Garden City, NY, USA). Observations were carried out at 40X magnification.

Production and analysis of aflatoxins. Two approaches were followed to verify aflatoxin production: fluorescence and HPLC analysis. Strains were inoculated

at a central single point on Petri dishes (Ø 6 cm) containing Coconut Extract Agar (CEA) (20% desiccated coconut; 1.5% agar) and incubated at 25°C for 14 days in the dark. This medium was chosen because, due to the reaction of coconut fats, strains positive for aflatoxin production can be identified by fluorescence in the reverse side of the culture (Davis et al., 1987; Pitt, 1992); furthermore, coconut-based media are optimal for AFs production (Dyer and McCammon, 1994). After incubation, colonies of all the strains were observed for fluorescence and scored as positive or negative. Then, 3 plugs were cut from each Petri dish and 1 mL of methanol added (Bragulat et al., 2001). After 1 hour in methanol, the solution was filtered with a Millipore® filter (Ø 0.45 mm) (Bedford, MA, USA). The solution was analysed by reversed phase HPLC (Jasco, Easton, MD, USA) using post-column derivatization with pyridinium hydrobromide perbromide and fluorescence detection. The column was a superspher 100 RP-18 (Merck, Darmstadt, Germany) and the mobile phase was H_2O-CH_3CN-CH_3OH (64+13+23) at a flow rate of 1 mL min^{-1} (Stroka et al., 2003). Aflatoxin production was measured in ng g^{-1} of culture medium. The limit of detection was 0.5 ng g^{-1}.

Production and analysis of cyclopiazonic acid (CPA). All the strains were inoculated at a central single point on Petri dishes (Ø 6 cm) containing CZ and incubated at 30°C for 14 days in the dark. Then the methodology of Bragulat et al. (2001), previously applied for AFs analysis, was used for CPA extraction. The 1methanolic extract was analysed by reversed phase HPLC and UV detection. The column was a LiChrosorb NH_2 (Merck, Darmstadt, Germany)

and the mobile phase was CH_3CN-CH_3COONH_4 50 mM in water (80+20) at a flow rate of 1 mL min^{-1}. CPA was measured in ng g^{-1} of culture medium. The limit of detection was 50 ng g^{-1}.

Identification at species level. The identification of *Aspergillus* section *Flavi* was completed by taking into account a combination of all the observed criteria, including morphological observations, sclerotial production, colour of colony and AFs and CPA profiles.

2.2.2. Ecology of *A.* section *Flavi*

The effect of temperature and a_w level on fungal growth and AFs production was studied for 38 isolates of *A.* section *Flavi* selected among the 70 collected; strains were chosen on the basis of the place of isolation and AFs production. One strain isolated from pistachio nuts and one from peanuts were also included in the trial for comparison.

All the isolates were inoculated on CZ and incubated at 25°C for 7 days in the dark to provide inoculum. To prevent the formation of colonies from stray spores, inoculation was made from a semisolid suspension. Small vials were prepared with a solution of 1% water-agar; a needle point of conidia of each strain was added to each vial, mixed and used later as inoculum (Pitt, 1979). Petri dishes (Ø 9 cm) with CZ were inoculated centrally with the suspension. The a_w level of the unmodified medium was 0.995. Three different temperatures were considered: 15, 25 and 30°C, and 3 levels of a_w: 0.83, 0.94 and 0.995 (the

unmodified medium), obtained by adding respectively 800, 250 and 0 mL of glycerol to 1 L of CZ medium. The experiments were conducted with four replicates.

After incubation, the diameter of colonies was measured along two perpendicular diagonals crossing the inoculation point. Aflatoxin production was quantified following the method previously described.

2.2.3. Data analysis

Boxplot analysis, useful to highlight outliers, was performed to compare the distribution of values at the temperatures and a_w levels taken into account. This analysis was run using the statistical package SPSS (Statistical Package for Social Science ver. 11.5.1, 2002. SPSS Inc., Chicago, IL USA).

Data on aflatoxin production in ecological trials were logarithmically transformed before statistical analysis. Log transformation is always required for data that covers a wide range from single-digit numbers to numbers in hundreds or thousands (Clewer and Scarisbrick, 2001), as a wide range of values can be obtained for AF production. The analysis of variance (ANOVA) was carried out using the statistical package MSTAT-C (Michigan State University, ver. 1, 1991, East Lansing, MI, USA), experimental design number 2: completely randomised design for factor A (temperature or a_w), factor B (strains) is a split plot. Means were compared using the Tukeys test to indicate significant differences.

Cluster analysis was performed using SPSS to create homogenous groups of strains based on logarithm transformed data of AF production in ecological trials. This analysis is based on distances which are a measure of how far apart two objects are. Selection of a distance measure should be based both on the properties of the measure and on the algorithm chosen for cluster formation. The square Euclidean distance, which is the sum of squared differences over all the variables, was used as the distance index. The average linkage between groups, often called UPGMA (unweighted pair-group method using arithmetic averages) was the clustering method followed. It defines the distance between two clusters as the average of the distances between all pairs of cases in which one member of the pair is from each of the clusters.

2.3. RESULTS

2.3.1. Characterization of isolates

Colony morphology. All the information regarding the strains of *Aspergillus* section *Flavi* collected from the different maize growing regions, including colour of colonies, are shown in Table 2.4. All isolates were identified to species level; 65 out of the 70 strains of *Aspergillus* section *Flavi* collected from maize were identified as *A. flavus* and 5 as *A. parasiticus*. *A. flavus* represented almost all the strains collected in the regions sampled; only Emilia Romagna, Lombardy and Piedmont differed, with 3, 1 and 1 isolates of *A. parasiticus*, respectively.

Sclerotia. Forty-four strains (63% of total strains) were able to produce sclerotia at 30°C on CZ, 4 of these were identified as *A. parasiticus*.

Using the approach based on sclerotial size (Cotty, 1989; Chang et al., 2001), only 20 strains (29% of total strains) were able to produce sclerotia and among these only 1 produced the characteristic small sclerotia (S). Distribution of strains based on sclerotial diameter is shown in Figure 2.1.

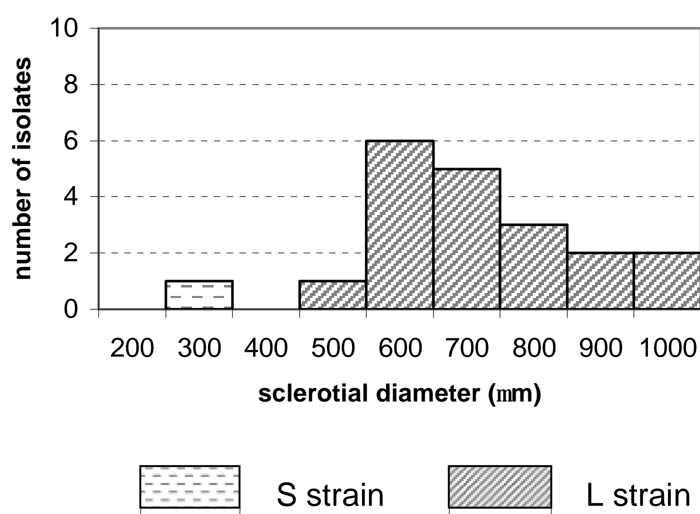


Figure 2.1 - Distribution of strains based on sclerotia diameter (S= sclerotia diameter < 400 µm; L= sclerotia diameter > 400 µm).

Production of aflatoxin. Seventy-three percent of *Aspergillus* section *Flavi* strains showed fluorescence when inoculated on CEA and 70% of strains were positive when tested by HPLC; 6 strains which showed fluorescence on CEA were not confirmed as AF producers using HPLC analysis and 4 strains without fluorescence on CEA were positive when tested with HPLC. Results of AF production can be summarized in classes of production from 1 (without AF production) to 5 (production higher than 1000 ng g^{-1}). In the population studied,

many strains (approx. 23%) produced < 10 ng g^{-1} of medium; however, approx. 25% of strains were able to produce > 1000 ng g^{-1} in 14 days in the *in vitro* conditions used (Table 2.1).

Production of cyclopiazonic acid. Forty-three strains (61% of tested strains) were able to produce CPA; results of CPA production can be summarized in classes of production from 1 (without CPA production) to 4 (production higher than 2000 ng g^{-1}). Around 20% of strains were able to produce > 2000 ng g^{-1} of medium and among these none was identified as *A. parasiticus* (Table 2.2).

Table 2.1. Distribution of *A*spergillus section *Flavi* strains, isolated from maize in 6 Italian regions, in classes of aflatoxin B1 production after incubation at 25°C for 14 days on CZ in the dark.

Class	AF (ng g^{-1})	Number of strains	% of strains
1	none	21	30
2	< 10	16	22.8
3	10 -100	7	10
4	100 - 1000	9	12.8
5	> 1000	17	24.3

Table 2.2. Distribution of *Aspergillus* section *Flavi* strains, isolated from maize in 6 Italian regions, in classes of cyclopiazonic acid production after incubation at 25°C for 14 days on CZ in the dark.

Class	CPA (ng g^{-1})	Number of strains	% of strains
1	none	27	39
2	< 1000	11	16
3	1000 - 2000	17	24
4	> 2000	15	21

Identification of chemotypes. The strains were classified into seven chemotypes based on AFs and CPA production patterns (Table 2.3). This classification was elaborated similarly to that obtained from a survey conducted in Iran (Razzagi-Abyaneh et al., 2006). Isolates able to produce both AFB and CPA represented the most represented chemotype (around 39% of total strains). No strains were found able to produce more AFB_2 than AFB_1. Isolates able to produce both AFB and AFG were classified as two different chemotypes: one with strains able to produce also CPA (around 11% of total strains) and one with strains not able to produce CPA (around 1% of total strains). Around 19% of total strains were of the chemotype representing isolates without ability to produce any toxin. Some other strains were able to produce either AFB or CPA and were included in two different chemotypes.

Table 2.3. Chemotype patterns of *Aspergillus* section *Flavi* strains based on aflatoxins and CPA production

Chemotype	Mycotoxins			
	AFB	**AFG**	**CPA**	**N. OF ISOLATES**
I (B1>B2)	+	-	+	27
II (B1<B2)	+	-	+	0
III	+	-	-	13
IV	-	-	+	8
V	-	-	-	13
VI	+	+	+	8
VII	+	+	-	1

2.3.2. Ecology of *A.* section *Flavi*

The strains used for ecological studies are detailed in Table 2.4.

Table 2.4. Characterization of *Aspergillus* section *Flavi* strains collected in 2003 and 2004 from 6 Italian regions.

CHARACTERIZATION									**ECOLOGICAL TRIALS**						
									AFB1[3]						
Code	**Region of maize origin[1]**	**Sclerotia (30°C)**	**Fluorescence**	**AFB1[2]**	**CPA[3]**	**Sclerotia Size[4]**	**Colour**	**Possible identification[5]**	**Temperature °C**			a_w			**Cluster analysis**
									15	25	30	0.83	0.94	0.99	
A 2087	ER	no	no	2	1		Ivy	AF							
A 2089	ER	no	no	1	1		Ivy	AF							
A 2090	ER	no	no	1	1	L	Ivy	AF							
A 2093	ER	yes	no	1	4		Ivy	AF							
A 2097	ER	yes	no	1	3	L	Ivy	AF	1	1	1	1	1	1	1
A 2098	ER	yes	no	1	1	L	Ivy	AF							
A 2102	ER	yes	no	1	3		Ivy	AF							
A 2103	ER	yes	no	1	1		Ivy	AF							

CHARACTERIZATION									ECOLOGICAL TRIALS						
									AFB1[3]						
Code	Region of maize origin[1]	Sclerotia (30°C)	Fluorescence	AFB1[2]	CPA[3]	Sclerotia Size [4]	Colour	Possible identification[5]	**Temperature °C**			a_w			Cluster analysis
									15	25	30	0.83	0.94	0.99	
A 2105	ER	yes	no	1	1		Ivy	AF							
A 2107	ER	no	no	4	4		Ivy	AF							
A 2109	ER	yes	no	1	3	L	Ivy	AF							
A 2045	FVG	yes	no	4	3		Ivy	AF							
A 2050	FVG	yes	no	1	3		Ivy	AF							
A 2061	V	no	no	3	3	L	Ivy	AF	2	3	2	1	2	4	3
A 2100	ER	yes	no	1	3		Ivy	AF							
A 2049	V	no	no	1	2		Ivy	AF	1	1	1	1	1	1	1
A 2086	ER	yes	yes	5	3		Ivy	AF	4	3	1	1	2	4	3
A 2091	ER	yes	yes	5	4		Ivy	AF	3	5	2	2	3	5	2
A 2092	ER	yes	yes	5	4		Ivy	AF	2	5	4	2	4	4	2
A 2094	ER	yes	yes	3	4		Ivy	AF	1	1	1	1	1	4	3
A 2095	ER	yes	yes	5	4		Ivy	AF	2	5	1	2	5	5	2
A 2099	ER	yes	yes	3	1		Ivy	AF	1	1	1	1	1	1	1
A 2101	ER	yes	yes	5	2		Ivy	AF	2	4	1	1	1	3	3
A 2104	ER	yes	yes	5	1	L	Ivy	AF	4	3	3	1	1	4	3
A 2106	ER	no	yes	4	2		Ivy	AF	1	2	1	1	1	1	1
A 2041	FVG	yes	yes	3	3		Ivy	AF	1	2	1	2	4	1	1
A 2044	FVG	yes	yes	2	1	L	Ivy	AF							
A 2046	FVG	no	yes	5	2		Ivy	AF	3	3	2	2	3	4	3
A 2056	FVG	no	yes	1	3		Ivy	AF	1	1	1	1	1	1	1
A 2067	FVG	yes	yes	5	4	L	Ivy	AF	2	2	1	1	2	3	3
A 2068	FVG	yes	yes	5	4	L	Ivy	AF	1	4	3	1	3	5	2
A 2071	FVG	no	yes	5	3		Ivy	AF	2	4	2	1	3	4	3
A 2074	FVG	yes	yes	2	2		Ivy	AF							
A 2075	FVG	yes	yes	2	2		Ivy	AF							
A 2079	FVG	no	yes	3	2		Ivy	AF	1	1	1	1	1	1	1
A 2080	FVG	yes	yes	2	1		Ivy	AF							
A 2081	FVG	no	yes	2	2		Ivy	AF							
A 2082	FVG	yes	yes	1	1		Ivy	AF	1	1	1	1	1	1	1
A 2047	L	yes	yes	2	2	L	Ivy	AF							
A 2052	L	yes	yes	3	4		Ivy	AF	2	4	1	1	3	4	3
A 2053	L	yes	yes	5	4		Ivy	AF	1	4	1	1	2	3	3
A 2063	L	yes	yes	4	4	L	Ivy	AF	3	4	3	1	4	4	2
A 2078	L	no	yes	5	3		Ivy	AF	1	3	3	1	1	4	3
A 2042	P	yes	yes	1	1		Ivy	AF	1	1	1	1	1	1	1
A 2059	P	yes	yes	5	3		Ivy	AF	2	5	2	1	2	5	2
A 2070	P	yes	yes	4	4	L	Ivy	AF	3	4	3	1	4	4	2
A 2073	P	yes	yes	4	4	L	Ivy	AF	1	3	1	1	1	4	3
A 2072	T	no	yes	2	2		Ivy	AF							
A 2040	V	yes	yes	4	1	S	Ivy	AF	3	5	4	1	1	5	2
A 2043	V	no	yes	2	1		Ivy	AF							
A 2055	V	yes	yes	4	1		Ivy	AF	2	3	1	1	1	4	3

CHARACTERIZATION									ECOLOGICAL TRIALS						
									AFB1[3]						
Code	Region of maize origin[1]	Sclerotia (30°C)	Fluorescence	AFB1[2]	CPA[3]	Sclerotia Size [4]	Colour	Possible identification[5]	Temperature °C			a_w			Cluster analysis
									15	25	30	0.83	0.94	0.99	
A 2060	V	no	yes	5	3		Ivy	AF	1	2	1	1	1	1	1
A 2064	V	yes	yes	2	1	L	Ivy	AF							
A 2076	V	yes	yes	2	1		Ivy	AF							
A 2077	V	no	yes	2	4	L	Ivy	AF	1	1	1	1	1	1	1
A 2069	ER	no	yes	5	3		Ivy	AF	1	4	3	1	1	4	3
A 2084	ER	yes	yes	2	1		Ivy	AF							
A 2085	ER	yes	yes	1	1		Ivy	AF							
A 2051	FVG	no	yes	1	1		Ivy	AF							
A 2058	FVG	no	yes	2	1		Ivy	AF							
A 2039	L	no	yes	5	2		Ivy	AF	3	3	3	1	4	3	3
A 2048	L	no	yes	1	1		Ivy	AF	1	1	1	1	1	1	1
A 2054	L	no	yes	4	3		Ivy	AF	1	1	1	1	1	1	1
A 2065	L	no	yes	3	3		Ivy	AF	1	3	2	1	2	3	3
A 2062	V	no	yes	5	4	L	Ivy	AF	3	4	2	1	1	5	3
A 2088	ER	yes	no	1	1	L	Cress	AP							
A 2096	ER	yes	no	1	1	L	Cress	AP							
A 2108	ER	no	no	1	1		Cress	AP							
A 2110	I - pe	no	yes	2	2		Ivy	AF	1	3	1	2	1	2	1
A 2111	I – pn	no	yes	2	4		Ivy	AF	2	2	1	1	1	2	1
A 2066	L	yes	yes	2	1	L	Cress	AP							
A 2057	P	yes	yes	2	1		Cress	AP							
IMI 283883	Unknown	yes	No	2	1		Cress	AP							
IMI 348543	USA	yes	no	1	1		Ivy	AF							

[1] ER=Emilia Romagna; FVG=Friuli Venezia Giulia; V=Veneto; L=Lombardy; P=Piedmont; T= Tuscany; I=Iran; pe= peanuts; pn=pistacho nuts

[2] Class of production of AFB1 as reported in Table 2.1
[3] Class of production of CPA as reported in Table 2.2
[4] Sclerotia size: L: sclerotia diameter > 400 μm; S: sclerotia diameter < 400 μm
[5] AF=*A. flavus*; AP=*A. parasiticus*

<u>Temperature</u>. Fungal growth was markedly affected by temperature (Figure 2.2). At 15°C the growth was the slowest, while at 25 and 30°C it was very similar and significantly higher, as shown by box-plot analysis and confirmed by ANOVA ($P \leq 0.01$).

As regards AFB_1 production (Figure 2.2), most strains produced the highest quantities at 25°C, while at 15°C and 30°C fewer strains produced aflatoxins and at lower levels. ANOVA showed significant differences between strains ($P \leq 0.01$). In particular, A 2092 and A 2040 were the best producers with 742 ng AFB_1 per g of medium, as mean of all temperatures.

Aflatoxin B_1 was produced by 29 of the tested strains (73%) at 25°C; 11 strains never produced AFs under any of the temperatures tested. The range of AF production was between 0 - 423 ng g^{-1} at 15 °C; between 0 - 2406 ng g^{-1} at 25 °C and between 0 - 505 ng g^{-1} at 30°C. Four strains of *A. flavus* were able to produce AFG_1 and AFG_2 at 15°C. Six strains of *A. flavus* produced G_1 only at 25°C. At 30°C none of the examined strains was able to produce AFG_1 or AFG_2. AFB_2 was synthesized at 15, 25 and 30°C, respectively by 21, 77 and 31% of strains able to produce also AFB_1. The behaviour of strains isolated from peanuts and pistachio nuts was in the range of variation of maize strains from northern Italian regions.

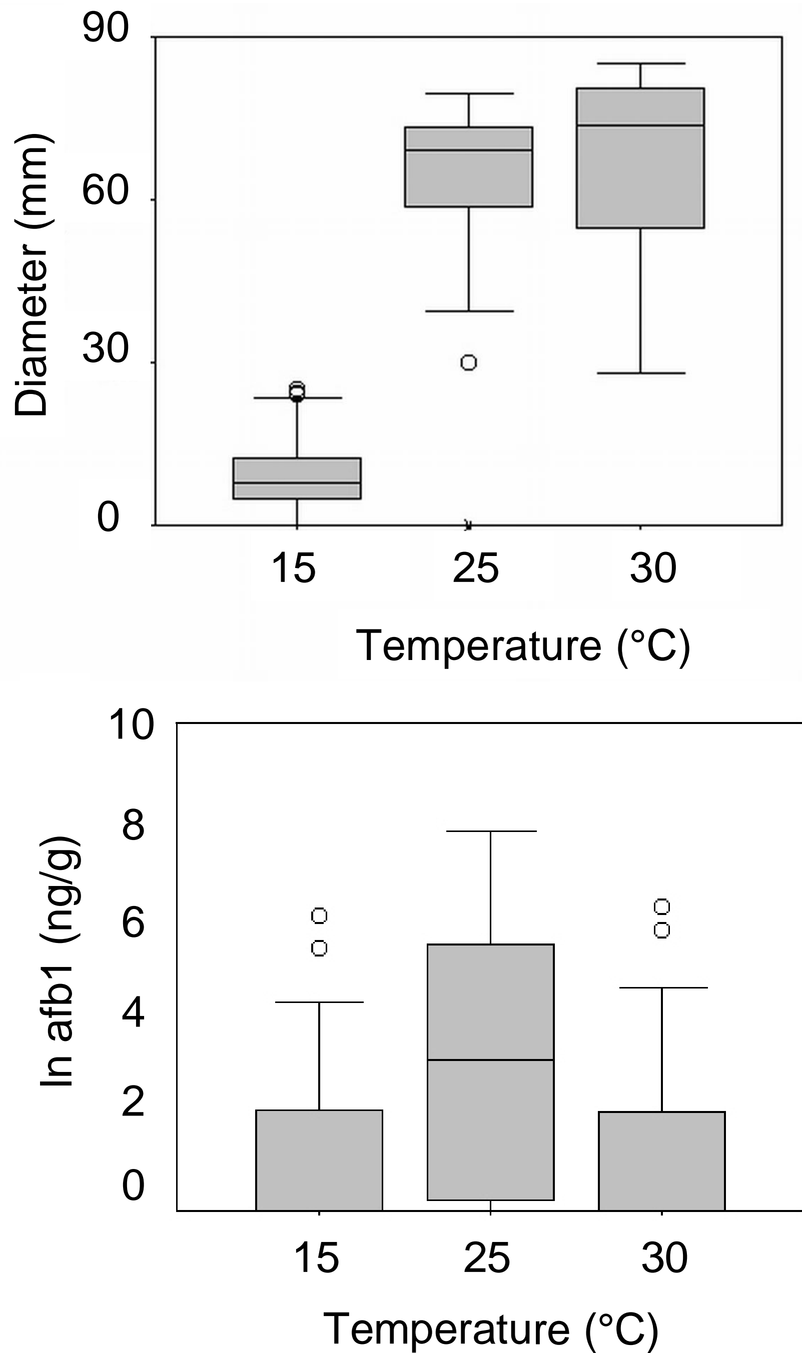


Figure 2.2 - Boxplot analysis for fungal growth (A) and aflatoxin B1 production (B) of 40 strains of *Aspergillus* section *Flavi* inoculated on CZ and incubated at 3 different T (15, 25 and 30°C) for 14 days in the dark. The box-plot analysis shows the inter-quartile range of each examined temperature (box), the median (line inside the box), minimum and maximum values (whiskers); circles represents values 1.5-3 times outside the interquartile range.

Water activity. Fungal growth was significantly influenced by a_w level as shown by the boxplot analysis (Figure 2.3). In particular, at 0.83 a_w growth values were very different and lower than those obtained at the other two a_w levels; significant differences were confirmed by ANOVA among all the a_w levels tested ($P \leq 0.01$). Significant differences among strains were also observed ($P \leq 0.01$); in particular, A 2046 showed the fastest and A 2095 the slowest colonisation rate (62.3 vs. 18.8 mm colony diameter, respectively).

Regarding AFs production, the boxplot analysis (Figure 2.3) shows that 0.99 a_w was the best condition, while only traces of AFs were detected in the driest condition tested (0.83 a_w). ANOVA demonstrated significant differences in aflatoxin production among all the tested a_w levels, confirming that 0.99 a_w is the optimal condition for *Aspergillus* section *Flavi* strains. Significant differences were also observed among strains ($P \leq 0.01$); AFB_1 production by strain A 2095 resulted significantly higher when compared to that of all the other strains.

Twelve strains never produced AFB_1 under any of the a_w levels considered. The range of AFs production was between 0 - 5 ng g^{-1} of medium at 0.83 a_w, between 0 - 1423 ng g^{-1} at 0.94 a_w and between 0 - 11039 ng g^{-1} at 0.99 a_w.

Aflatoxin B_1 alone was only produced by 6 strains of *A. flavus* at the lowest a_w (0.83) level tested. At 0.94 a_w, 43% of tested strains were positive, while at the highest a_w level the percentage of strains able to produce AFs increased to 68%. No strain was able to produce AFB_2 at 0.83 a_w, while 61% of strains were able to produce this mycotoxin at 0.94 and 0.99 a_w and 89% of strains were

able to produce also AFB_1. AFG_1 and AFG_2 were never detected in this experiment.

Cluster analysis. Cluster analysis run on AFB_1 produced in ecological trials (logarithm transformed) resulted in 3 groups of strains (Table 2.1). Group 1 included 14 strains, not producers or very weak producers; there were 8 strains in group 2 and they were all mean producers, while the 17 strains in group 3 were markedly influenced by ecological conditions and did not produce in marginal conditions. No geographic relation was found in strains included in the same cluster.

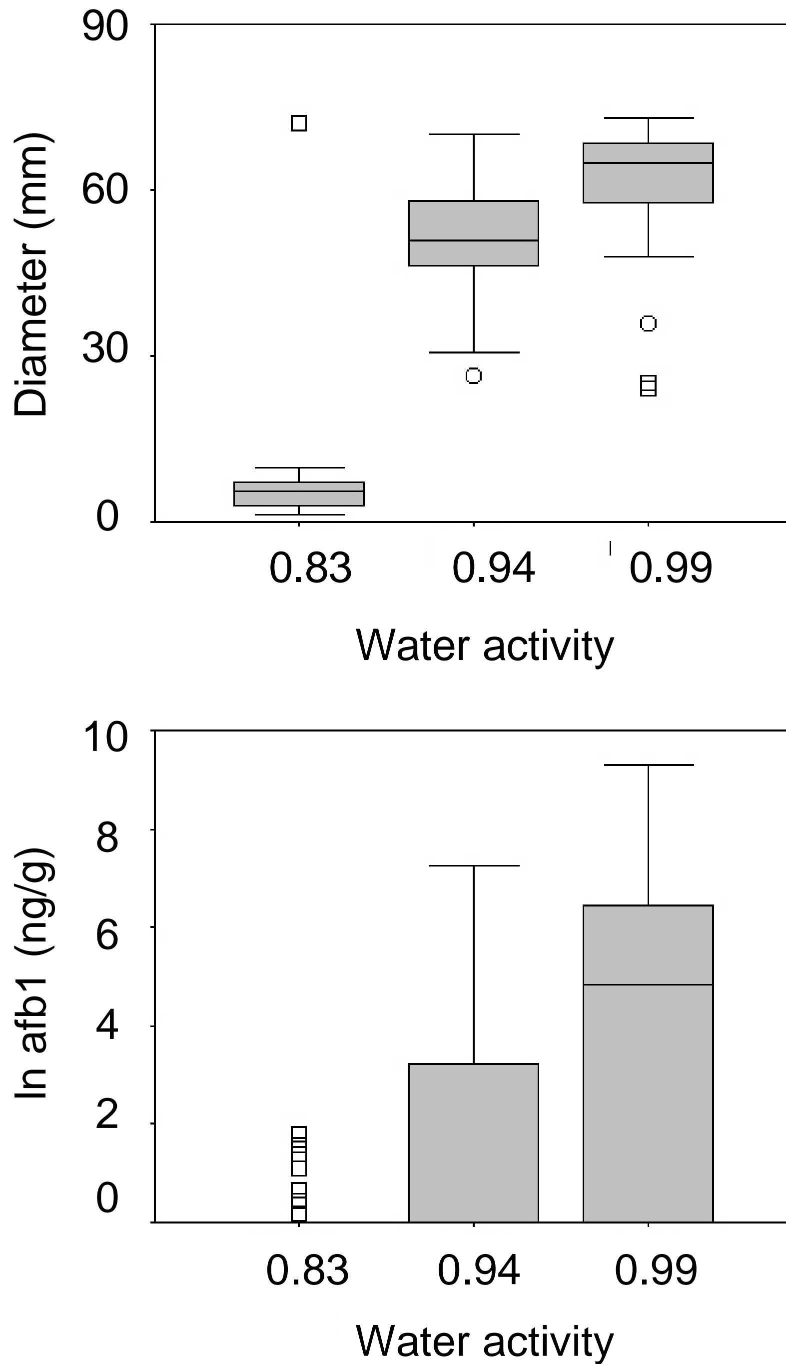


Figure 2.3 - Boxplot analysis for fungal growth (A) and aflatoxin B1 (B) production of 40 strains of *Aspergillus* section *Flavi* inoculated on CZ with 3 levels of a_w (0.83, 0.94 and 0.99) incubated at 25°C for 14 days in the dark. The box-plot analysis shows the inter-quartile range of each examined temperature (box), the median (line inside the box), minimum and maximum values (whiskers); circles represents values 1.5-3 times outside the interquartile range; squares represents values more than 3 time outside the interquartile range.

2.4. DISCUSSION

The results obtained in this study have provided, for the first time, important information about the presence, characteristics and distribution of *Aspergillus* section *Flavi* in maize in northern Italy. Ninety-three percent of the 70 strains studied belonged to *A. flavus* and only 7% to *A. parasiticus*. Distribution of strains between the two main species was quite different to other studies conducted in the United States (US). In fact, during a similar trial in Illinois, Wicklow et al. (1998) found that the percentage of *A. flavus* strains was 72% and that of *A. parasiticus* was 28%. However, the percentage of strains positive for aflatoxin production differed markedly, with 70% in the Italian population and only 53% in the US.

Sixty-two percent of Italian *A. flavus* strains and 80% of *A. parasiticus* were able to produce sclerotia at 30°C, 28-30°C being the optimal temperatures reported (Domsch et al., 1980). Our results are quite different from those obtained in a study conducted in Illinois, where 98% of *A. flavus* strains isolated from field produced sclerotia at 25°C (Wicklow et al., 1998) even if in the cited study the ability to produce sclerotia was additionally checked on PDA. Shearer et al. (1992) demonstrated, during a monitoring trial in the US, that the percentage of toxigenic strains changes consistently from one year to the next, as does sclerotia development. These aspects cannot be checked for the Italian strains because they were collected in the same year.

Regarding sclerotial size, determined according to Orum et al. (1997), only 1 strain developed S sclerotia and the other 19 L sclerotia. The distribution of these two different sizes of sclerotia seems related to environmental factors. In fact, in Kenya the majority belong to S strains (73% of tested strains) (Probst et al., 2005), while in the US, in a limited area of Texas, Louisiana and in Mississippi, S strains were more abundant; on the contrary, L strains were dominant in Virginia (Horn and Dorner, 1998). Bennett et al. (1979) found no correlation between aflatoxin and sclerotial production, but recently some tentative attempts to correlate high or low AF production to the size of sclerotia have given contrasting results. Probst et al. (2005) found S strains were high aflatoxin producers (665 $\mu g\ g^{-1}$ versus 40 $\mu g\ g^{-1}$ for L strains), while Abbas et al. (2005) found the opposite with L isolates producing the highest levels of AF (10000 $\mu g\ g^{-1}$). In our study no comments are possible on this aspect.

Sixty-one percent of the total strains were able to produce CPA; among these, none was *A. parasiticus*. Thirty-five strains of *A. flavus* were able to produce both CPA and AFs. Co-occurrence of both mycotoxins has previously been reported on maize and peanuts by Urano et al. (1992) and Fernandez-Pinto et al. (2001). This is interesting and relevant, but more detailed studies are required on CPA to understand its possible role in inhibiting the mutagenic action of AFB_1 (Kuilman-Wahls et al., 2002).

The chemotypes found in this study differ from those found in Iran (Razzaghi-Abyaneh et al., 2006). We never found strains able to produce more

AFB_2 than AFB_1; further, the group able to produce both AFB and CPA was the most represented in our study, while the non toxigenic group was dominant in Iran.

Ecological trials showed the range 25-30°C as optimal for *Aspergillus* section *Flavi* growth and 25°C for AFB_1 production. This suggests that Italian strains could be less thermophilic than those isolated in other geographic areas. In fact, previous studies by Scott et al. (1970) and Kheiralla et al. (1992), considered 28° and 30°C the optimal temperatures for toxin production. Another interesting point is that AFG_1 and AFG_2 were produced only at 15°C by 1 strain and at 25°C by only 3 strains. Regarding a_w, 0.99 was the optimal condition both for growth and AFs production. According to Hill et al. (1985), a_w profiles for growth and AFs production are different, as are marginal conditions for growth and AFs production, being 0.77 and 0.83 a_w, respectively (Sanchis and Magan, 2004). In this study, the only AF produced at the marginal condition of 0.83 a_w was AFB_1, detected only in 6 strains. At present it is possible to establish that 15°C and 0.83 a_w are the limit conditions for growth and AFs production by some strains of *Aspergillus* section *Flavi*, substantially in agreement with other studies (Sanchis and Magan, 2004); further trials are necessary to improve knowledge on conducive and inhibitory conditions for toxin synthesis .

In conclusion, this study has provided for the first time a significant body of relevant information on the key species responsible for AFs contamination of

maize used for human food and animal feed in the important milk producing northern regions of Italy. The information will be useful in identifying risk regions by linking regional climatic information to the levels of contamination present and the potential for AFs production.

CHAPTER 3

How Italian strains of Aspergillus flavus differ from other

3.1 INTRODUCTION

It is well known that in years characterized by having above-average temperatures and below-average rainfall the problem of *Aspergillus flavus* presence in maize fields could arise (Payne, 1998).

The exceptional hot weather conditions registered in Italy during the summer of 2003 created optimal environment for the development of *Aspergillus flavus* in maize and, consequently, also for aflatoxins production (AFs). This resulted in contamination, over the legal limits, of maize designated to become animal feed (EC Regulation 2003/100). This situation was unusual for Italy and increased the interest in *A. flavus*, in particular in the ecological needs of this fungus.

A. flavus isolates can be found in most of the world since it is ubiquitous. However they could have different behaviour depending on the area where they are adapted to live and survive. Their development is possible only when ecological conditions favour their proliferation and several factors may influence mycotoxins synthesis. In particular, temperature, humidity/water activity and chemical composition of the substrate seem to have a great impact on their presence (D'Mello and Macdonald, 1997, Sanchis and Magan, 2004).

Several *in vitro* studies were conducted on the role of ecological parameters on *A. flavus* strains collected in other areas of the world different from Italy. It is accepted that temperature range for the growth of *A. flavus* is 19-35°C (Northolt and van Egmond, 1981), with 28°C being optimum for aflatoxin production (Scott et al.,1970; Sanchis and Magan, 2004). This fungus is able to

grow and produce mycotoxins at low water activity (a_w) conditions of 0.73 and 0.85 a_w, respectively. Recent studies on strains of *A. flavus* isolated from maize in northern Italy show that these ecological parameters seem to differ slightly for these Italian regions (Giorni et al., 2007).

Composition of the substrate can also be important. In fact different nutritional substrates support fungal growth and aflatoxins production to different extents. Chemical composition of maize grain varies during several development stages and modifications of its compounds may influence *A. flavus* presence. In particular, proteins, degraded to amino acids by mould proteases, can be used as a nitrogen source or as a carbon source. When amino acids are used as a carbon source, large amounts of ammonia may be liberated which may affect aflatoxin production (Park and Bullerman, 1983).

The main objective of this study was to define ranges for growth and aflatoxin production for *A. flavus* strains isolated from maize in Northern Italy, regarding the main cardinal conditions of temperature, a_w and substrate composition. This will improve the knowledge on the behaviour of this species and create a firm base of information to be used for defining a predictive model to predict and monitor aflatoxin contamination in the field.

3.2 MATERIALS AND METHODS

3.2.1 Fungal strains and trials description

One strain of *A. flavus* (MPVP A 2092) isolated from maize in North Italy was used for *in vitro* experiments. The strain, stored in the fungal collection of the Institute of Entomology and Plant Pathology of the Università Cattolica del Sacro Cuore of Piacenza, was previously confirmed as potent aflatoxin producers (Giorni et al., 2007).

These strains were chosen because of their ability to grow well and produce high amounts of aflatoxin B_1 in *in vitro* experiments conducted at different temperatures and water activity (a_w) conditions (Chapter 2; Giorni et al, 2007). Aflatoxin B_2 was only produced in traces by both strains and only between 25 and 30°C and with a_w higher than 0.94; AFG_1 and AFG_2 were never detected.

Selected strains were grown on Water Agar (WA; 1.5 % of agar) and mycelial plugs were picked from these colonies and transferred to Petri dishes (Ø 60 mm) containing Czapek agar (CZ) (sucrose 30 g; $NaNO_3$ 2 g; KCl 0.5 g; $MgSO_4 \cdot 7H_2O$ 0.5 g; $FeSO_4 \cdot 7H_2O$ 0.01; K_2HPO_4 1 g; $ZnSO_4 \cdot 7H_2O$ 0.001 g; $CuSO_4 \cdot 7H_2O$ 0.005 g; agar 15 g; H_2O to 1 L); they were incubated for 7 days at 25°C and used as inoculum source.

Fungal spores were collected with a sterile loop, added to a water-agar solution (1% of agar) and then used to centrally inoculate Petri dishes (Ø 80 mm) filled with appropiate media for all trials (Pitt, 1979). A range of different factors were considered in the series of experiments detailed below, in particular temperature (T), a_w and maize kernels composition, related to growth stage and combinations.

The colonies were incubated in growth chambers with thermal regulation; fixed temperatures ±1°C were maintained. Different levels of a_w were obtained by adding increasing quantities of the non-ionic solute glycerol or the ionic solute sodium chloride (NaCl) to modify a_w. The a_w levels tested were obtained by adding, for each 100 mL of medium, the amount of glycerol or salt reported in Table 3.1. The a_w of all media was confirmed with AquaLab lite (version 1.3 © Decagon Devices Inc.) that uses a dielectric sensor to measure the prevailing a_w. All the trials were carried out in quadruplicate.

Preliminary trials were carried out in order to define the best incubation time to study growth and AFs production. Mycelial growth was measured along two orthogonal diagonals, every two days, until the maximum colony size (80 mm) was reached in at least one experimental treatment; incubation continued to study the dynamic of AFs production and chose the best time for analysis (see section "Aflatoxins analysis"). When border conditions were considered, growth was measured with a 15 day step to 60 days of incubation.

Table 3.1. Grams of glycerol or salt (NaCl) added to 100 grams of medium to obtain different levels of available water (Magan, personal communication).

	Available water						
	0.77	**0.80**	**0.83**	**0.87**	**0.90**	**0.93**	**0.95**
glycerol	124.0	101.0	92.0	69.0	50.6	36.8	23.0
salt					16.6	12.0	9.3

Four different trials were conducted as described below.

Trial 1. Marginal boundaries for growth

Levels of a_w and temperature parameters that limit growth of *A. flavus* were studied. Boundary conditions for *A. flavus* growth were studied. Four different levels of extremely low a_w were considered, 0.77, 0.80, 0.83 and 0.85 a_w, obtained with the addition of glycerol to CZ medium (see Table 3.1); Petri dishes were centrally inoculated with *A. flavus* and incubated at 25°C for 60 days. Low temperatures, 5 and 10 °C, were also considered and dishes with unmodified CZ medium (aw=0.99) were incubated for 60 days.

Trial 2. Role of temperature

Petri dishes with CZ (a_w=0.99) were centrally inoculated with *A. flavus* and incubated at 9 different temperatures (5-45 °C, step 5°C).

Trial 3. Role of available water

CZ agar medium was modified by adding different quantities of glycerol and salt; the a_w levels tested, with both solute, were 0.90, 0.93, 0.95. The unmodified medium was also included in the experiment. Petri dishes were centrally inoculated with *A. flavus* and incubated at three different temperatures: 20, 25 and 30°C.

Trial 4. Role of maize kernels composition

A minimal medium was prepared using maize flour obtained by milling maize ears harvested at different days after pollination (DAP) between 3 and 52, at a 7 days step (3% maize flour, 2% agar and double-distilled water to 1 L; Marin et al., 1998b). For 3 and 10 DAP the entire ears were milled while from 17 to 52 DAP only kernels were used to obtain flour. Petri dishes were centrally inoculated with *A. flavus* and incubated at 7 different temperatures (10-40°C, step 5 °C, 0.99 a_w) and at 4 different aw levels (0.83, 0.90, 0.94, 0.99 a_w, 25 °C).

3.2.2 Aflatoxins analysis

Three plugs (Ø 4.6 mm) were sampled from the colonies; they were put in a vial and 1 mL of methanol added (Bragulat et al., 2001). After 1 hour, the solution was filtered with a Millipore® filter (Ø 0.45 mm) (Bedford, MA, USA). Filtered solutions were analysed by reversed phase HPLC (Jasco, Easton, MD, USA) using post-column derivatization with pyridium hydrobromide perbromide and fluorescence detection. The column was a superspher 100 RP-18 (Merck,

Darmstadt, Germany) and the mobile phase was H_2O-CH_3CN-CH_3OH (64+13+23) at a flow rate of 1 mL min^{-1} (Stroka et al., 2003). Aflatoxins production was quantified in ng g^{-1} of culture medium. The limit of detection was 0.5 ng g^{-1}.

3.2.3 Statistical analysis of data

The diameter of the colonies (D) was used to compute daily radial growth rate (GR; mm day^{-1}) computed as

$$GR= (D_t/2)/t$$

where t is the incubation time in days and D_t is the diameter of the colony (mm) at time t.

Growth data were also transformed to a 0-1 scale rating the mean diameter at time t in each condition relative to the maximum reached diameter in the trial (80 mm). This was necessary to compare data obtained in different experimental conditions and to build general growth functions.

Data on aflatoxins production were logarithmically transformed before statistical analysis since a wide range of values were obtained; this is necessary to reduce the variance of data (Clewer and Scarisbrick, 2001). Analysis of variance (ANOVA) was performed for trials 1-3 considering all the factors involved. The generalized linear model of the statistical package SPSS was used and means were compared using LSD test to point out significant differences (P=0.05).

3.3 RESULTS

Preliminary trials showed that 10 days is the preferable incubation time for growth studies; in fact, at optimal conditions the colony covered all the available area and growth was clearly visible in all conducive conditions for the fungus. A longer incubation time of 21 days was suggested for AFs analysis because their production increased significantly until this time and remained practically similar thereafter (Figure 3.1). Only AFB_1 was considered in data analysis because AFB_2 was sometimes produced only in traces and AFG_1 and AFG_2 were never detected; these data confirm results of previous characterizations (Chapter 2; Giorni et al., 2007).

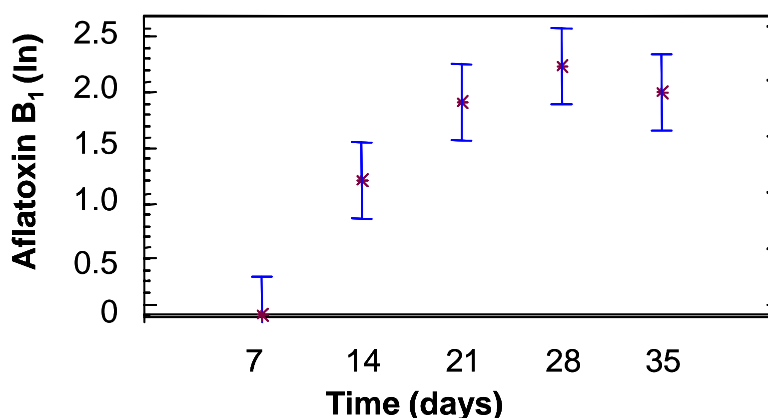


Figure 3.1. Dynamic of aflatoxin B_1 production by *Aspergillus flavus* (MPVP 2092) grown on Czapek medium and incubated at 25°C. Mean data are based on four replicates.

3.3.1 Effect of temperature, a_w level and maize growth stage on fungal growth and aflatoxins production *in vitro*

Trial 1. Marginal boundaries for growth

Colonies of *A. flavus* did not grow at 5 and 10 °C, and at 0.77 and 0.80 a_w even when incubation lasted 60 days. Fungal growth started at 15°C (0.99 a_w) and at 0.83 a_w and 0.85 a_w (25°C) after 20 and 10 days respectively (Figure 3.2).

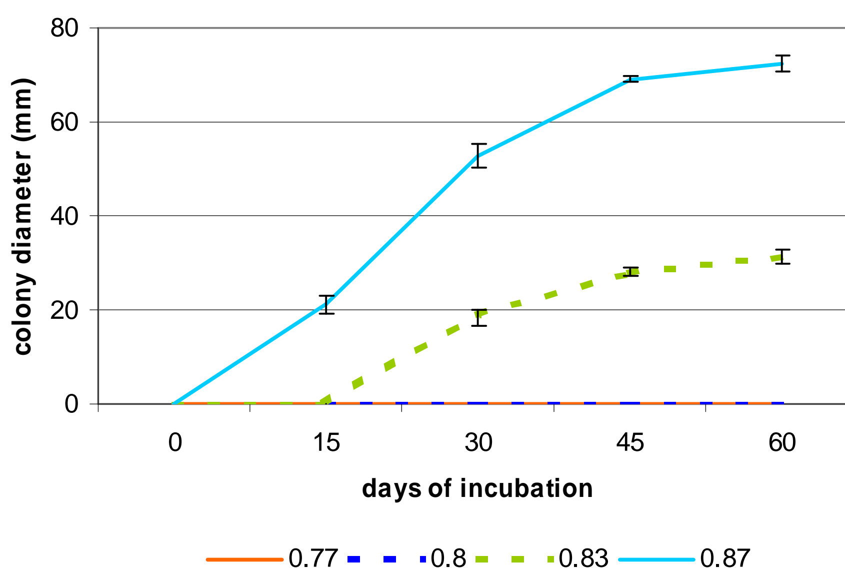


Figure 3.2. Mean colony diameter of *A. flavus* (MPVP 2092) inoculated *in vitro* on Czapek agar media, added with glycerol to obtain different a_w levels and incubated at 25 °C to 60 days. Error bars represent the standard error of mean data.

Trial 2. Role of temperature

Temperature had a significant effect on fungal growth ($P<0.01$). Fungal growth started at 15°C; significant differences were found among all the

temperatures tested ($P \leq 0.01$), except 30 and 35 °C at which maximum growth was reached, followed by 25, 40, 20 and 45 °C (Figure 3.3).

Regarding AFs, there was a narrower temperature range for their production, between 15 and 30°C, with significantly higher contamination at 20-25°C (Figure 3.3).

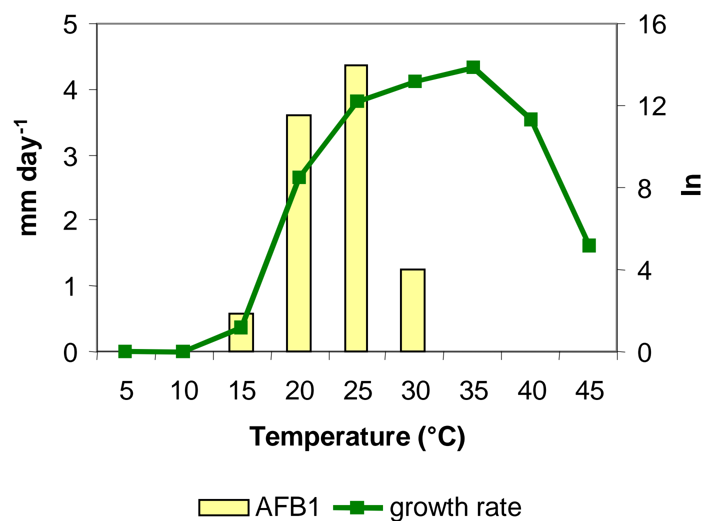


Figure 3.3 – Means of growth rate (mm day^{-1}) and aflatoxin B_1 production of *A. flavus* (MPVP A 2092) inoculated *in vitro* on Czapek agar media at different conditions of temperature.

Trial 3. Role of available water

The impact of non-ionic and ionic solute water stress on growth resulted significant ($P>0.01$) and higher than temperature effect; in fact, they explained respectively 74% and 17% of the variability.

Growth rates on glycerol were higher than on salt-amended media. The decrease in growth with 0.95 a_w was significant only when salt was added, and

it caused 74% and 94% decrease at 0.93 and 0.90 a_w respectively. In the same a_w conditions, obtained with glycerol addition, growth reduction was 15% and 65%, respectively (Table 3.2). No significant difference was found between growth rates at 0.90 a_w in glycerol amended and 0.95 a_w in NaCl amended media. The highest growth rate was observed at 30 °C, with significant decrease at 25 and 20 °C (Table 3.2).

Aflatoxin B_1 production followed a different trend; the highest amount was detected on glycerol modified medium at 0.90 a_w, but not significantly different from that in unmodified medium. The amount of AFB_1 produced increased with the decrease of a_w, with the addition of glycerol, while it was always significantly lower and minimum at 0.90 with salt addition (Table 3.2). The highest aflatoxin production was obtained at 25-30°C, with a significant lower amount at 20°C.

Table 3.2. Summary table based on analysis of variance of growth rate (mm day^{-1}) and aflatoxin B_1 production (ln value+1) by the *A. flavus* strain inoculated on Czapek medium modified for available water with glycerol (italics) or salt and incubated at three different temperatures. Different letters mean statistically significant differences among treatments (P≤0.01) and refers to the single main factor.

Factors		**Growth rate** (mm day^{-1})	**Aflatoxin B_1** [ln (value+1)]
Water activity (a_w)	0.99	3.8 a	7.7 a
glycerol	*0.95*	*3.6 ab*	*4.8 cd*
	0.93	*3.2 b*	*6.1 bc*
	0.90	*1.7 c*	*7.0 ab*
salt	0.95	2.0 c	3.1 e
	0.93	1.0 d	3.4 de
	0.90	0.2 e	1.2 f
Temperature (°C)	20	1.5 c	3.4 b
	25	2.2 b	5.4 a
	30	3.0 a	5.4 a

Trial 4. Role of maize kernels composition

Fungal growth was slightly influenced by growth stages of maize and 98% and 99% of explained variability was due to T and a_w, for the two experiments respectively. The highest development was obtained at 52 DAP and the growth rate decreased in media prepared with younger ears, not always significantly. The fungal growth was significantly lower than all the other growth stages at 3 DAP, but only in the trial with different temperature regimes. In the experiment with different a_w levels, growth resulted in the opposite effect, with higher fungal development at the lowest DAP (3, 10 and 17 DAP) (Table 3.3).

Regarding temperatures, the highest growth was registered at 35°C, followed by 25 and 30°C; the trend observed in the previous trial (trial 2) was confirmed (Table 3.3). Fungal growth, instead, was highly influenced by the a_w level. The mean value of fungal growth was highest at 0.99 a_w and lowest at 0.90 a_w. At 0.83 a_w, no growth occurred. No aflatoxins were found in any conditions studied.

Table 3.3. Summary of analysis of variance of *A. flavus* growth on flour-based media prepared with maize cobs collected at different days after pollination (DAP) incubated at seven different temperatures. Different letters mean statistically significant differences among treatments (P≤0.01) and refer to the single main factor. Experiment 1 refer to results obtained testing different growth stages in relation with temperature while Experiment 2 refer to results obtained testing different growth stages in relation with water availability.

Factors		**Growth rate** (mm day^{-1})	
		Experiment 1	**Experiment 2**
Growth stage (DAP)	3	2.0 d	1.4 ab
	10	2.3 c	1.6 a
	17	2.5 c	1.4 ab
	24	2.8 ab	1.4 b
	31	2.6 bc	1.3 bc
	38	2.8 ab	1.1 d
	45	2.8 ab	1.3 bc
	52	2.9 a	1.2 cd
Temperature (°C)	10	0 e	
	15	0.2 e	
	20	2.3 d	
	25	3.9 b	
	30	4.0 b	
	35	4.4 a	
	40	3.3 c	
Available water	0.83		0 d
	0.90		0.4 c
	0.94		1.8 b
	0.99		3.2 a

3.4 DISCUSSION

Usually the length of incubation time can influence the level of toxin produced by *A. flavus*. In particular, longer times than 14 days resulted in a decrease of aflatoxin levels probably due to degradation or re-adsorption by the fungus (Kheiralla et al., 1992). This was not confirmed by our study where only after 28 days was the maximum level of toxin reached and this was found not to be statistically significantly different from that obtained at 21 days.

The Italian strains of *A. flavus* were able to grow at 0.83 a_w while no growth occurred at lower a_w was impossible even after 60 days incubation at optimum temperature. This differed from what has been found in others studies in other parts of the world with strains which were able to grow down to 0.73 a_w (Trucksess et al., 1988; Sanchis and Magan, 2004). In previous *in vitro* trials on corn, the limiting a_w for *A. flavus* growth was 0.73 at 26 and 32°C (Trucksess et al., 1988).

High a_w levels are more conducive for faster growth in *A. flavus* strains. The problem of testing different levels of a_w is the way in which media has to be modified using different ingredients. These ingredients produce differences in the availability of nutritional compounds creating difficulties in the interpretation of results. Media modified with glycerol usually do not give problems in the growth response of fungus but produce similar aflatoxin B_1 in lower a_w levels similar to normal media (0.99 a_w), while with NaCl problems of toxicity reduce

fungal development and, consequently, also the analysis of aflatoxin results difficult.

Regarding optimal temperatures for *A. flavus* development and aflatoxins production, it is well accepted that on most substrates this occurs over a range of 19-35°C; with minimal and maximal temperatures of 12 and 43°C respectively (Northolt and van Egmond, 1981). However, the Italian strains were able to grow also at 45°C even if very slowly.

Generally 28°C is considered as the optimum for production of aflatoxin B_1 (Scott et al., 1970; Sanchis and Magan, 2004). For Italian strains the optimum appeared to be lower (25°C) even through the range of toxin production resulted from 15 to 30°C.

Growth stages of maize influenced fungal growth. In particular, results obtained resulted different and strictly linked to temperature patterns. My study suggests that maybe at 25°C younger plants were more susceptible to fungal growth while, considering mean data from a wide range of temperatures, older plants (close to harvesting) were more contaminated.

CHAPTER 4

Effect of a_w and CO_2 level on *Aspergillus flavus* growth and aflatoxin production in high moisture maize post-harvest

4.1. INTRODUCTION

Maize is one of the most widely distributed food plants in the world and its infection by fungi can result in mycotoxin contamination during growth, harvest, storage, transport and process stages (Bradburn et al., 1993). The main fungal species and mycotoxins of concern are *Aspergillus flavus* and aflatoxins, *Fusarium verticillioides* and fumonisins, *F. graminearum* and trichothecenes and zearalenones.

A. flavus can infect maize pre- and post-harvest and can result in an increase in aflatoxin contamination if the drying and storage phases are poorly managed. There is information on the effect of some abiotic factors on growth and aflatoxin production by *A. flavus.* It grows well in the range 19-35°C (Northolt and van Egmond, 1981) with 28°C being optimum for aflatoxin production (Scott et al., 1970; Sanchis and Magan, 2004). *A. flavus* can grow and produce mycotoxins at as low as 0.73 and 0.85 water activity (a_w) respectively (Sanchis and Magan, 2004). This corresponds to 8-12% and 17-19% moisture content (MC) (Battilani et al., 2005). Usually maize is stored in silos at 14% MC. Inefficient drying or water ingress can cause pockets of wetter grain resulting in a higher MC (Magan and Aldred, 2007).

In stored grain ecosystems, the most important abiotic conditions which influence growth and mycotoxin production are a_w, temperature and, when grain is moist, gas composition (Guynot et al., 2003; Magan et al., 2004). In particular, interactions between these factors can determine whether

contamination increases and mycotoxins are produced. While a significant body of information is available on water and temperature relations of mycotoxigenic fungi, less is available on interactions with gas composition. Detailed studies have been conducted on effects of elevated CO_2 on growth of both *Aspergillus ochraceus* and *Penicillium verrucosum* and ochratoxin production (Paster et al., 1983; Cairns-Fuller et al., 2005). Recently, studies have suggested that up to 50%, CO_2 had only a slight impact on ochratoxin production by *Aspergillus carbonarius* over a range of a_w conditions (Pateraki et al., 2007). Samapundo et al. (2007) found that fumonisin B_1 production by species of *Fusarium* in section Liseola was inhibited with 30% CO_2 at 0.984 a_w. However, only a few studies have examined *A. flavus.* Studies of several modified atmospheres with different CO_2 levels balanced with O_2 and N_2, showed that *A. flavus* grew on wheat and rye bread with up to 75% CO_2 (Suhr and Nielsen, 2005). Previously, Wilson and Jay (1975) tested a high CO_2 treatment (61.7% CO_2 balanced with O_2 and N_2) on moist maize and found that *A. flavus* growth was visible after 4 weeks at 27°C. The contamination with aflatoxin was lower than that in air.

The objectives of this study were to determine (a) the impact of interacting conditions of CO_2 (up to 75%) and a_w (0.92, 0.95) on growth and aflatoxin production on a Potato Dextrose Agar (PDA) medium and (b) its inhibitory effect on populations and aflatoxin B_1 (AFB_1) in stored maize grain inoculated with *A. flavus* spores.

4. 2. MATERIALS AND METHODS

An aflatoxin producing strain of *A. flavus* (MPVP A 2092; Giorni et al., 2007) was inoculated on Petri dishes containing PDA (Amersham), incubated at 25°C for 7 days and then used to produce the inoculum adjusted to 10^6 spores mL^{-1}.

4.2.1 Fungal growth

In vitro studies: Petri dishes (Ø 9 cm), containing PDA (Amersham Pharmacia Biotech, Inc., Uppsala, Sweden) adjusted with glycerol-water solutions at 0.92 and 0.95 a_w, were centrally inoculated with a drop of the *A. flavus* suspension (10^6 spores mL^{-1}). The diameter of the fungal colonies was measured, after 7 and 14 days, along two perpendicular diagonals crossing the inoculation point. All the trials were conducted in quadruplicate.

Maize grain studies: Maize grain, hybrid Lolita (FAO class 500) grown in Cremona province (northern Italy) in 2005, was used in this study. This maize was previously tested for fungal population and mycotoxins content. It was shown to have 13% of kernels infected by *Fusarium verticillioides* and, from a mass-mass HPLC analysis, a fumonisin B_1 level of 5.3 µg g^{-1}. No aflatoxin was detected.

A moisture adsorption curve was prepared for the maize in order to accurately determine the amounts of water required to add to 960 g maize to obtain the target a_w levels of 0.95 and 0.92. This curve was obtained by adding

different quantities of water to maize grains and calculating their moisture content (as difference in weight before and after 1 night at 130°C) and comparing it with their a_w level measured with Novasina a_w sprint (Novatron Ltd, Horsham, West Sussex, UK).

The required amounts of water were added to the maize and this was stored at 4ºC overnight to equilibrate the treatments. Then, maize was inoculated at room temperature in order to obtain a final concentration of 10^4 spores g^{-1}, by mixing thoroughly and then decanting the maize (20 g) into solid culture vessels (Magenta, Sigma Ltd, U.K.) closed with plastic lids containing a permeable membrane and placed in the chambers. A total of 20 kernels at each a_w were also plated on PDA and incubated for 5 days at 25°C and this showed that 100% of the plated kernels were contaminated with *A. flavus*.

Petri dishes and storage containers were put in plastic chambers (36 L volume) with inlet and outlet tubes to allow gas mixture to pass through them. The inlet was connected inside the chamber to a sparger, which was placed in a flask containing glycerol-water solutions appropriate to maintain the equilibrium relative humidity of the gas mixtures and the atmosphere in each chamber at the target a_w level. A computerised gas blender (Signal Series 850 Gas blender, Camberley, UK) was used to provide the four treatments: (1) normal air (21% O_2, 0.03% CO_2, 79% N_2); (2) 25% CO_2; (3) 50% CO_2; (4) 75% CO_2. The modified levels of CO_2 were obtained by reducing O_2 to <1% and increasing N_2 to 74, 49 and 24% respectively. Gas composition was also periodically checked with a gas chromatograph (GC; Carlo Erba model GC-8340, Carlo Erba

Instruments, Hemel Hempstead, UK) to ensure the maintenance of the correct gaseous proportions. All the chambers were maintained in a 25°C constant temperature room. The exhaust gases were channelled outside the room to avoid CO_2 build up.

Maize grain samples were destructively sampled after 7, 14 and 21 days and the *A. flavus* populations (CFUs) g^{-1} of grain determined by successively decimal dilutions in water-peptide (1%). In all cases three replicates were used per treatment condition.

4.2.2 Aflatoxin extraction and analysis

Three plugs (4.6 mm, diameter) of agar were sampled from the colonies grown on Petri dishes after 14 days incubation; they were put in a vial and 1 mL of methanol was added (Bragulat et al., 2001). After 1 hour, the solution was filtered through a Millipore® filter (Millex SLHV 013 NL, 0.45 μm) (Bedford, MA, USA).

For maize grain samples, 10 g sub-samples were milled into flour from each sample used for the analysis. Flour was extracted with 100 mL of CH_3OH-H_2O (80+20), stirring for 45 minutes and then the extract was filtered with a Whatman 595 ½ (Dassel, Germany) paper filter, 5 mL of the solution was passed, after dilution with 45 mL of H_2O, into an Easy Extract Aflatoxin immuno-affinity column (r-Biopharm Rhône Ltd, Glasgow, UK), then the column was washed with 5 mL of H_2O. Aflatoxins were eluted with 2.5 mL of CH_3OH and the

solution was concentrated to 1 mL with a stream of nitrogen. Then, 1 mL of CH_3CN-H_2O (25+75) was added and the solution was filtered through a Millipore® filter.

Filtered solutions, extracted from fungal colonies and maize grain, were analysed by reversed phase HPLC (Jasco, Easton, MD, USA) using post-column derivatization with a UVE instrument (LCTech GMBH, Postfach-Dorfen, Germany) set at 254 nm and fluorescence detection. The column was a Superspher 100 RP-18 (Merck, Darmstadt, Germany) and the mobile phase was H_2O-CH_3CN-CH_3OH (64+13+23) at a flow rate of 1 mL min^{-1} (Stroka et al., 2003). Aflatoxin production was quantified in ng g^{-1} of kernels or culture medium. The limit of detection was 0.1 ng g^{-1}**.** Average recovery values were: 97.8±1.6% for AFB_1 and 93.5 ± 2.3 % for AFB_2.

4.2.3 Statistical analysis of data

Data on CFU and AFB_1 production (values+1) were logarithmically transformed before statistical analysis. This was required because of the wide range of variability (from single-digit numbers to numbers in hundreds or thousands) (Clewer and Scarisbrick, 2001). Total AFB_1 produced in each fungal colony grown *in vitro* was computed taking into account the weight of the colony and the amount of AFB_1 produced per g. Mean values of AFB_1 content obtained at the four CO_2 conditions, both from the experiment *in vitro* and that with maize grains, were converted to a 0-1 scale before analysis. This conversion was performed by relating mean values to the maximum value obtained in the experiment; the results represent the rate of toxin production (0:

no aflatoxin production; 1: maximum aflatoxin production). This conversion was necessary to compare results of toxin production obtained in different experiments.

Analysis of variance was performed considering all factors (a_w, air composition and time, when appropriate); a randomized complete block design of the statistical package SPSS was used (Statistical Package for Social Science ver. 11.5.1, 2002. SPSS Inc., Chicago, IL USA). Means were compared using the LSD test to indicate significant differences.

4.3 RESULTS

4.3.1 Fungal growth on agar and maize grain

Fungal growth on artificial media was highly influenced by both CO_2 and a_w level. Mycelial extension of *A. flavus* was slower at 0.92 a_w than at 0.95 a_w (25 mm vs 41 mm). A significantly slower growth was observed at each increment in CO_2 level with a reduction of 40%, 70% and 90% respectively (Table 4.1). Statistically, all the factors considered (atmospheric gas composition and a_w) significantly influenced fungal growth ($P<0.01$) (see Table 4.2).

The populations of *A. flavus* (CFU g^{-1}) on stored maize grain were significantly lower with 25 and 75% CO_2 in the atmosphere. However, at 0.95 a_w the populations were about ten times higher with respect to 0.92 a_w and they significantly increased only after 21 days incubation (Table 4.1). Fungal growth

was significantly influenced by all three factors considered (atmospheric gas composition, a_w and incubation time) with CO_2 and its interaction with incubation time explaining 35% and 34% of variance, respectively (see Table 4.2).

Table 4.1. Effect of modified atmosphere and a_w on (a) *in vitro* growth (colony diameter, 7 days of incubation) and aflatoxin B_1 production at 25ºC (14 days incubation) (b) populations of *A. flavus*, and aflatoxin B_1 production at 25ºC (0, 7, 14 and 21 days incubation) in maize grain. Separation of means for AFB_1 was elaborated using logarithmic transformed values but in table real data are reported. Treatments with different letters mean differences statistically significant ($P \leq 0.01$) and refers only to main parameter considered (% CO_2, a_w or time).

	(a) Synthetic medium				**(b) Maize grain**			
	Growth (mm)		**AFB_1 (ng/g)**		**CFU/g (log 10)**		**AFB_1 (ng/g)**	
% CO_2 in air								
0	67	a	713	b	7	a	300	a
25	40	b	1237	a	6	b	79	bc
50	19	c	62	c	8	a	5	c
75	7	d	9	d	6	b	128	b
a_w								
0.92	25	b	541	a	6	b	40	b
0.95	41	a	470	b	8	a	216	a
Time (days)								
0	ND		ND		4	b	0	c
7	ND		ND		6	b	242	a
14	ND		ND		7	b	81	b
21	ND		ND		8	a	60	b

ND= not determined

Table 4.2. Analysis of variance of fungal growth and aflatoxin B_1 content for *in vitro* agar studies and on maize grain. Significant (S; P≤0.01) and non significant (NS) differences were indicated. Data were log transformed before statistical analyses.

Synthetic medium

	Fungal growth (Mean Diameter)		**AFB_1 production (ng/g)**	
	Explained variance (%)		**Explained variance (%)**	
A) CO_2 level	89	S	19	S
B) a_w	9	S	21	S
A x B	2	S	60	S

Maize grain

	Population (CFU/g)		**AFB_1 production (ng/g)**	
Factors	**% Explained variance**		**% Explained variance**	
A) CO_2 level	35	S	31	S
B) a_w	13	S	10	S
C) time	13	S	35	S
A x B	2	NS	7	NS
A x C	34	S	6	NS
B x C	1	NS	5	NS
A x B x C	2	NS	6	NS

4.3.2 Aflatoxins production

Aflatoxins were detected in both *in vitro* agar and stored maize samples analysed. Overall, AFB_1 was the predominant aflatoxin found, with AFB_2 being 0.5% and 5% of AFB_1 respectively on agar and on maize grain. In general, the mean production of AFB_1 after 14 days was 19.9 ng g^{-1} and 242.3 ng g^{-1} in the

in vitro trial and on kernels respectively, while AFB_2 was 0.1 ng g^{-1} and 6.7 ng g^{-1} in the same experiments (data not shown). Aflatoxins G_1 and G_2 were never detected.

The production of AFB_1 by *A. flavus* on synthetic medium almost doubled in the 25% CO_2 treatment, while incubation with 50% and 75% CO_2 reduced the toxin level by 91 and 99% relative to the untreated controls (see Table 4.1). Regarding a_w, significantly higher AFB_1 production was observed at 0.92 when compared to 0.95. From a statistical point of view, all the factors considered (atmospheric gas composition and a_w) significantly influenced the toxin production ($P<0.01$) (Table 4.2).

On stored maize, all the treatments with CO_2 could be considered efficient in reducing toxin production. Overall, 25%, 50% and 75% CO_2 were able to decrease AFB_1 by 74%, 98% and 57% respectively (Table 4.1). Significant differences in aflatoxin production were also found between the two a_w levels with AFB_1 content 81% lower at 0.92 a_w with respect to 0.95 a_w. There was also a temporal effect on AFB_1 production. The AFB_1 amounts were highest after 7 days and then decreased over the subsequent period up to the end of the experiment (21 days). ANOVA highlighted significant influences of all the principal factors involved (atmospheric gas composition, a_w and time) ($P\leq0.01$) (Table 4.2).

Interactions between CO_2 and a_w for both *in vitro* and in grain experiments are shown in Figure 4.1. On synthetic medium there was a stimulation of AFB_1 production by *A. flavus* colonies in air at 0.92 a_w and by 25% CO_2 at 0.95 a_w. In maize grain, AFB_1 content was highest in air while with increasing CO_2 levels the toxin production was significantly reduced.

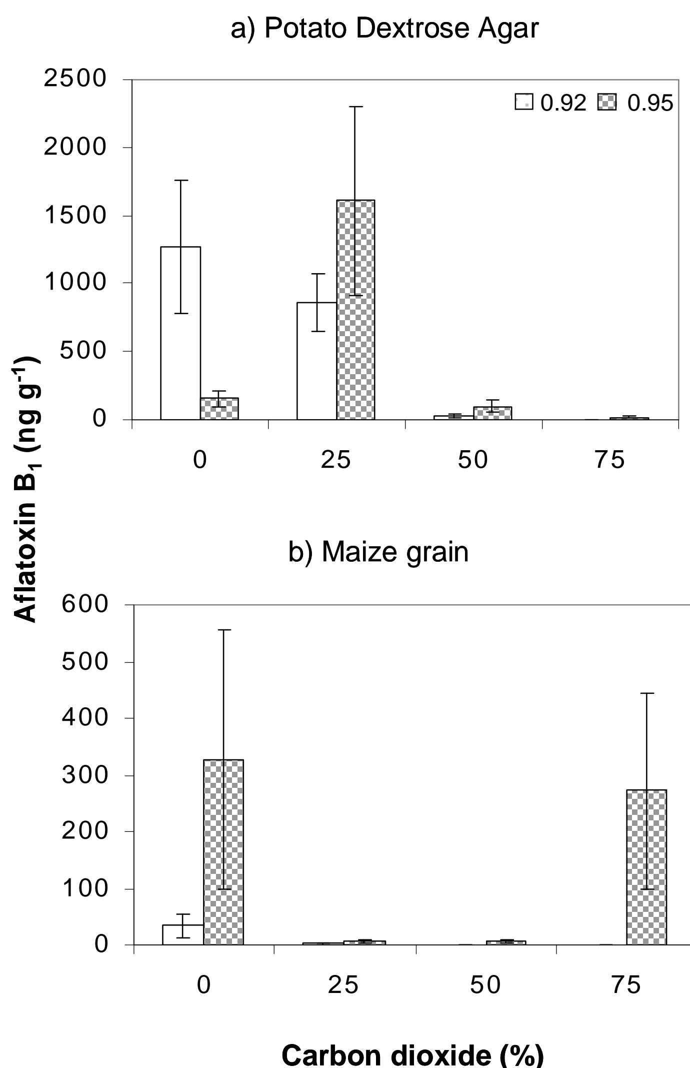


Figure 4.1 - Mean aflatoxin B_1 production by *A. flavus* on (a) Potato Dextrose Agar (b) on stored maize grain in relation to the different modified atmosphere conditions used at 25ºC (note that different scales are used in *in vitro* and maize grain plots). Error bars represent the standard error of mean data.

The overall evaluation of both trials are summarised in Figure 4.2. This shows the impact of elevated CO_2 and suggests that, while exposure to increased CO_2 does decrease AFB_1 production, at least 50% CO_2 is necessary to obtain a significant ($P<0.05$) reduction when compared to unmodified atmosphere.

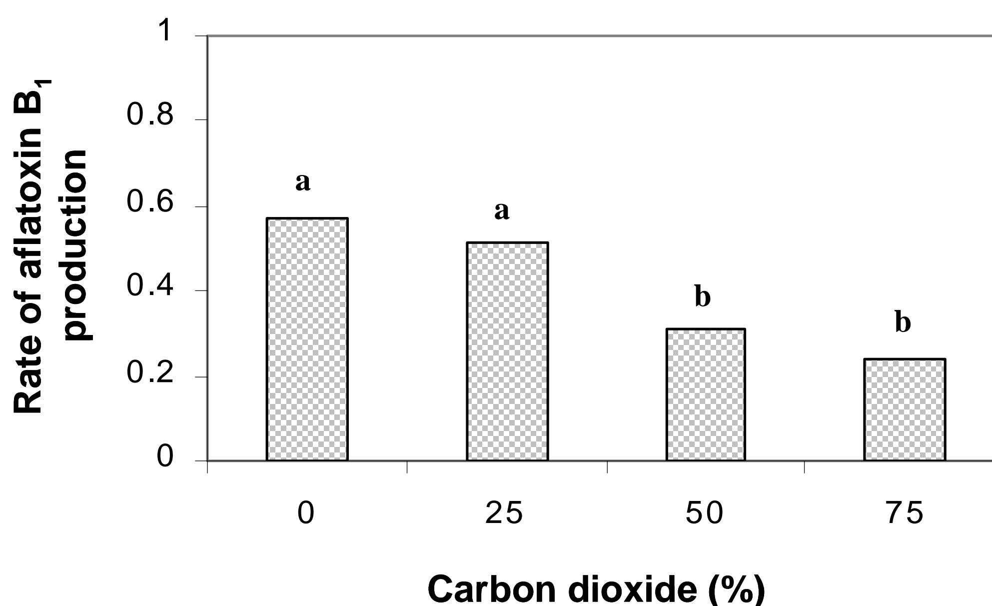


Figure 4.2 - Relative impact of different CO_2 concentrations on aflatoxin B_1 production by *A. flavus*. Data are shown in a 0-1 scale that represents a rate of toxin production (0: no aflatoxin; 1: maximum aflatoxin production) and include both data sets from *in vitro* and on maize grain after 14 days of incubation at 25°C (see materials and methods for details).Treatments followed by different letters are significantly different.

4.4 DISCUSSION

4.4.1 Effects of modified atmosphere on growth

This study considered the effect of interactions between a_w and CO_2 concentrations on mycelial extension of *A. flavus* and the ability to colonise maize grain from an initial spore-based inoculum. The study showed that the effect on growth and AFB_1 production varied significantly. Considering the experiments *in vitro* and *in situ* together, growth was more rapid at 0.95 than 0.92 a_w ($P<0.01$), while interaction with CO_2 significantly decreased the ability to grow and colonise maize grain. The use of modified atmospheres at 25 and 50% CO_2 resulted in about 30-35% inhibition of growth/CFUs/g grain. Exposure to 75% CO_2 resulted in >50% inhibition of growth regardless of a_w level (data not shown). However, this CO_2 percentage would be difficult to obtain and maintain post-harvest.

Previous studies, where exposure to 50% CO_2 at different a_w levels were carried out, showed that growth of ochratoxigenic species such as *P. verrucosum*, *A. ochraceus* and *A. carbonarius* were inhibited by 50-75%, depending on a_w levels, when compared to that in normal atmospheric conditions (Cairns-Fuller, 2004; Cairns-Fuller et al., 2005; Pateraki et al., 2007). Studies on bakery products showed that spoilage could be prevented with exposure to 70% CO_2 when the a_w level was 0.80, but it was only delayed when the a_w levels were 0.85 to 0.90 (Guynot et al., 2003). Recent studies with *Fusarium verticillioides* and *F. proliferatum* examined initial elevated CO_2 concentrations on growth rates at 0.984-0.93 a_w (Samapundo et al., 2007) and

they showed a reduction from 10-12 mm day^{-1} at 0.98 a_w and air to 2 mm day^{-1} at 0.93 a_w and >20% CO_2. However, these studies were conducted in static sealed systems, not with continuous slow flushing as in the present study; therefore results are not strictly comparable.

4.2 Efficacy on aflatoxin production

Aflatoxin production was influenced by both CO_2 concentration and a_w levels tested. Considering the experiments *in vitro* and *in situ* together, at 0.95 a_w, 48% more aflatoxin was produced than at 0.92 a_w ($P<0.05$). It appears that 25% CO_2 does not offer any significant control of aflatoxin content under the a_w treatments examined in this study. Only partial inhibition of growth occurred, resulting in the fungus being under stress and in aflatoxin levels similar to the untreated controls. For inhibition of aflatoxin production, 50% and 75% CO_2 were effective in reducing production levels by 46% and 58%, respectively. Overall, taking into account both *in vitro* and *in situ* trials, at least 50% CO_2 is required to inhibit aflatoxin production to any extent.

Previous studies of aflatoxin production in peanuts showed that a 25% reduction occurred with 20% CO_2 and that this modified atmosphere was insufficient to inhibit growth and sporulation of *A. flavus*. Growth and sporulation was inhibited to some extent by 25% CO_2 but, in this case, aflatoxin production increased (Diener and Davis, 1977). So, it is clear that to obtain a substantial reduction in aflatoxin production it is necessary to use high levels of CO_2. Studies with other mycotoxigenic fungi such as *A. ochraceus* showed that ochratoxin was produced in 30% CO_2, and inhibition of growth only occurred

with >60% CO_2 (Paster et al., 1983). Similar results were obtained with *Penicillium verrucosum*, which exhibited a decrease in growth and ochratoxin production only with 50% CO_2 (Cairns-Fuller et al., 2005). The latter study suggested that a_w had a greater influence than CO_2. In studies on *A. carbonarius*, 50% CO_2 significantly decreased ochratoxin production *in vitro* over a range of a_w levels, but again not completely (Pateraki et al., 2007). Samapundo et al. (2007) showed that fumonisin production by both *F. verticillioides* and *F. proliferatum* was inhibited by 30% CO_2 at 0.985 a_w, by about 10-20% at 0.951 a_w and by 10% at 0.93 a_w. However, these were initial concentrations in sealed systems, not active continuous flow through systems at the target a_w levels as used in the present study.

This study shows the potential target CO_2 concentrations required for inhibition of growth and aflatoxin synthesis. Further larger pilot scale studies are necessary to determine the feasibility of using controlled atmospheres, specifically for controlling *A. flavus* in stored maize grain destined for animal feed, where physical methods are required for safe storage.

CHAPTER 5

Effect of solute, matric potential and temperature on *in vitro* development of *Aspergillus flavus* strains from Italy

5.1 INTRODUCTION

Aspergillus section *Flavi* is the major group of fungi associated with aflatoxin contamination in several agricultural commodities. Three species of this section can produce aflatoxins (AFs): *A. flavus*, *A. parasiticus* and *A. nomius*, with the first two important in the colonisation of crops like maize, peanuts and nuts (Payne, 1998). They are wide spread in hot and dry geographic areas where they are often able to colonise and contaminate crops rapidly.

In 2003, for the first time, high aflatoxin levels in maize production, with levels above the European legal limits both in kernels (20 μg kg^{-1} and 2 μg kg^{-1} for feed and food, respectively) and in milk (0.05-2 μg kg^{-1}), were found in northern Italy. *Aspergillus flavus* overwinters in soil on crop debris and this represents the main source of primary inoculum for maize plant infection. Conidia are dispersed aerially and infected corn kernels soon after silking (Payne, 1992). The key abiotic factors influencing the development of such spoilage fungi in the plant are water availability (a_w) and temperature. Tolerance of both solute and matric potential stress are important for survival and to enable growth to occur in crop debris and in soil (Magan, 1988). Solute stress is imposed by ionic changes due to salt, and non-ionically due to water binding by components on crop residue or plant parts. Matric stress is due to water adsorption and surface tension phenomena in soil; it causes restricted solute transport and it limits growth responses.

Water potential is the potential energy of water compared to pure water in reference conditions. It quantifies the tendency of water to move from one area to another due to osmosis, gravity, mechanical pressure, or matrix effects including surface tension. Pure water at standard temperature and pressure is defined as having a water potential of 0. Water potential is measured in units of pressure (MPa) and values are negative; it represents the reduction of energy due to the addition of solutes to water (more solutes determines more negative values).

Growth variations in solute or matric stress conditions can also be due to nutritional imbalances, specific ion effects or to the decreased water content that restrict solute transport (Adebayo and Harris, 1971). Interactions between water stress and temperature are fundamental because they represent the two-dimensional niche in which fungi may be able to effectively germinate, grow and actively compete for available resources (Marin et al., 1998a).

Some studies have been conducted on the biology of *A. flavus* to determine favourable ecological parameters able to promote growth and aflatoxin production, especially in the USA (Trucksess et al., 1988; Kheiralla et al., 1992). These studies showed that 25-30°C were optimal for growth of *A.* Section *flavi* strains and 25°C for aflatoxin B1 (AFB_1) production (Giorni et al., 2007). However, very few studies have compared the effect of osmotic and matric stress on growth of *A. flavus* strains (Nesci et al., 2004) and none on sporulation, which is particularly important because spores produced on crop debris are the primary source of inoculum for maize ears infection.

The objective of this study was to obtain information on the capacity of mycotoxigenic *Aspergillus* section *flavi* strains collected from northern Italy to grow and sporulate under different interacting solute/matric stress and temperature combinations.

5.2. MATERIALS AND METHODS

5.2.1 Fungal strains and media preparation

Three *A. flavus* strains (MPVP A 2052, A 2073 and A 2092) stored in the fungal collection of the Università Cattolica del Sacro Cuore, Institute of Entomology and Plant Pathology, isolated from maize grown in Italy, previously characterised as able to produce AFB_1 and AFB_2 were used in this study (Giorni et al., 2007).

The medium used was a maize-based agar with 3% maize flour and 2% agar with a_w approx. -1.4 MPa (=0.99 a_w), measured with a Hygroskop-BT (Rotronic Instrument Corp.).The medium was modified osmotically by the addition of the ionic solute NaCl (Lang, 1967) and the non-ionic solute glycerol (Dallyn and Fox, 1980) to -2.8, -7.0, -14.0 and -21.0 MPa (=0.98, 0.95, 0.90 and 0.85 a_w).

Matric potential of the media was also modified using Polyethylene glycol 8000 (PEG 8000) instead of agar and obtaining a semi-solid media. Known

amounts of PEG 8000 were added according to the equation of Michel and Kaufmann (1973), as detailed by Magan (1988), to get matric potentials of -2.8, -7.0 and -9.8 MPa (=0.98, 0.95 and 0.93 a_w). Sterile circular discs (⌀ 8.5 cm) of capillary matting were placed in sterile 9 cm Petri dishes containing approx. 15 mL of cooled medium. The matting was overlayed with sterile discs of polyester fibre and cellophane (P400, Cannings Ltd., Bristol, U.K.).

5.2.2 Fungal growth and sporulation

Spores of the 3 strains of *A. flavus*, obtained from a 7 day old Czapek dox agar culture, were suspended in 1% peptone-water, shaken vigorously and spread onto plates of the basic medium. Plates were incubated overnight at 25°C to allow spore germination. The different osmotic and matric media were inoculated centrally with an agar plug obtained using a 4 mm surface-sterilised cork borer. Four replicates were prepared for each treatment. Plates of the same osmotic/matric potential were sealed in polyethylene bags and incubated at 25 and 30°C (12 hours day light).

The diameters of all colonies were measured in two orthogonal directions and carried out for a maximum of 14 days. These data were used to determine the growth rates (mm day^{-1}) for each growth medium and treatment. The growth rate for each strain was computed at the incubation time when the maximum growth was reached by at least one strain.

Data on sporulation were obtained in relation to solute stress. Petri dishes were inoculated as previously described and incubated for 7 days; colonies were washed with 5 ml of sterile water added with 0.05% Tween 80 and the spore production determined with a haemocytometer as detailed by Parra et al. (2004). The experiment was carried out with three replicates per treatment.

5.2.3 Data analysis

Two dimensional profiles were drawn using Excel (Microsoft Office 2000) to show the effect of time and water potential on fungal growth. Radial growth in the different environmental treatments and for all the fungi were rescaled to the range 0 - 1 considering 85 mm (diameter of Petri dishes used) as the maximum possible development area for the tested strains.

The analysis of variance (ANOVA) was carried out using the statistical package MSTAT-C (Michigan State University, ver. 1, 1991, East Lansing, MI, USA) and means were compared using the Tuckey test to determine significance of differences. Experimental design number 10: three factors (strain, temperature and water stress) in a randomised complete block design was used for growth data and for logarithmically transformed sporulation data [ln (value+1)].

5.3 RESULTS

5.3.1 Solute and matric stress effects on growth

The number of days necessary to reach the maximum growth (colony diameter 85 mm) by at least one strain in relation to solute stress and matric stress was 7 and 13 days, respectively. ANOVA of mean growth rate, respectively at 7 and 13 days for solute and matric stress, showed a significant effect ($P<0.01$) of all the main factors considered (strain, temperature and water potential) on fungal growth (Table 5.1) while no significant differences were found between replicates. The differences between strains were considered not relevant in practice and mean values of strains were considered for further analysis.

The growth rate at 25°C and 30°C was similar under optimal solute stress (< 7.0 MPa water stress), with both ionic and non-ionic solutes being used (Figure 5.1) . No growth was observed at -21.0 MPa regardless of the solute used. The limited difference due to temperature was considered not relevant in practice and the mean growth rate was used for further comparisons. In matric stress conditions, growth rate was generally about 50% of that measured with solute stress (Figure 5.1). The optimum temperature was 30°C, with no differences found between -1.4 and -2.8 MPa, while limits for growth were about -14.0 MPa to -17.0 MPa and -9.8 MPa, respectively for solute and matric stress.

Table 5.1 – Summary table based on results of ANOVA run with mean radial growth rate (mm day^{-1}) of the 3 strains grown on maize flour agar at 25 and 30°C with different solute (salt or glycerol) and matric potential (polyethylene glycol 8000) modifications. Different letters refer to the main factor considered (strain, temperature or water potential) and indicate statistically significant differences among treatments ($P \leq 0.01$).

		Radial growth (mm day^{-1})	
Factors		**Solute stress (1)**	**Matric stress (2)**
Strain	A 2092	3.30 c	1.97 a
	A 2052	3.33 b	1.45 b
	A 2073	3.37 a	1.93 a
Temperature (°C)	25	3.43 a	1.59 b
	30	3.24 b	1.97 a
Water potential (MPa)			
	glycerol		**matric**
	-21.0	0.00 e	
	-14.0*	1.02 d	0.00 c
	- 7.0	5.24 bc	1.04 b
	- 2.8	5.76 ab	3.09 a
	salt		
	-21.0	0.00 e	
	-14.0	1.14 d	
	- 7.0	5.05 c	
	- 2.8	6.07 a	
	control		
	-1.4	5.74 ab	2.99 a

(1) measured at 7 days of incubation

(2) measured at 13 days of incubation

*: -9.8 MPa (0.93 a_w) instead of –14.0 MPa (0.90 a_w) for matric potential treatment

Solute and matric stress were analysed separately.

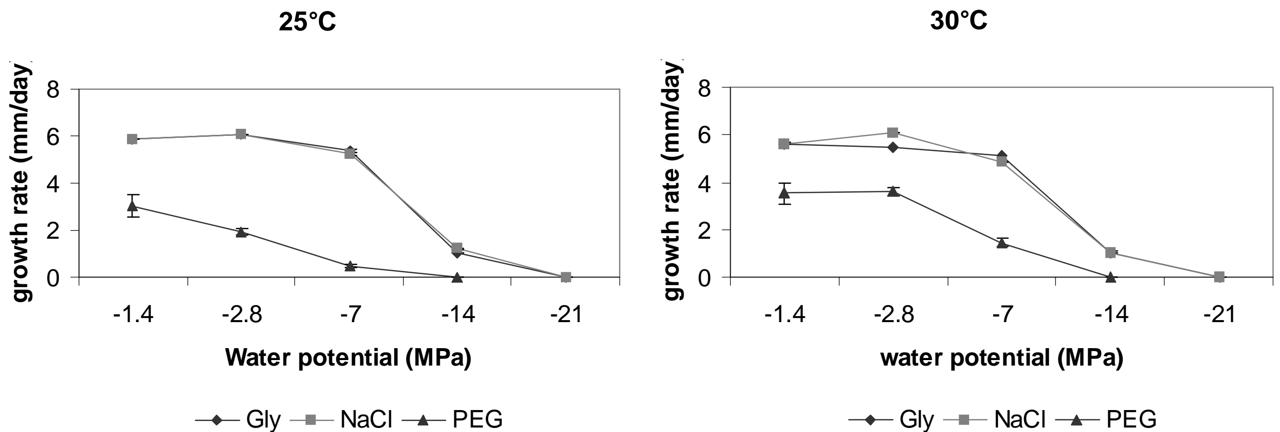


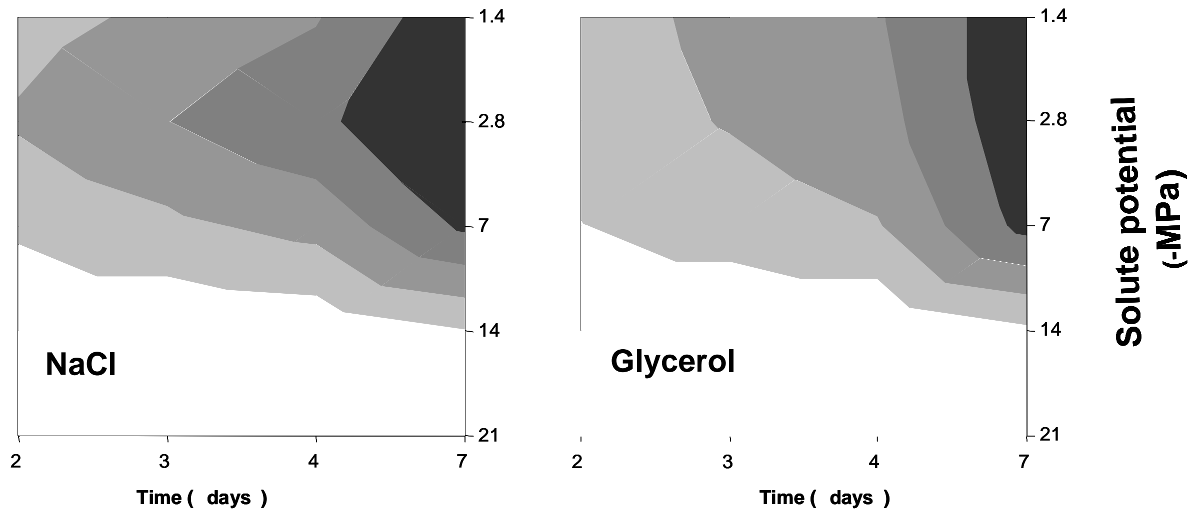
Figure 5.1 - Comparison of growth rates obtained in media modified with ionic solute (NaCl), non-ionic solute (glycerol) and PEG 8000 at all the tested water potentials at both 25 and 30°C after 7 days of incubation. Values refer to the mean growth rate of the 3 strains used for the experiment. Error bars represent the standard error of mean data, in some cases the value is very low and it is impossible to show it using this scale.

Two dimensional profiles were drawn based on solute or matric potential x time interactions (Figure 5.2) and differences between optimum and marginal solute potential x time conditions were observed. As an example (Figure 5.2a), at marginal time periods (2 days) and solute stress (-21 MPa) no growth was observed in both modified media; with higher levels of water potential, growth was influenced by solute type, with an optimum at 5 days and -2.8 MPa with salt modified media and at 6 days and -1.4 to -2.8 MPa when glycerol was added.

With regard to matric potential stress (Figure 5.2b), at 30°C the maximum growth was at 11 days and -2.8 MPa while at 25°C this was at 12 days and -1.4

to -2.8 MPa. At marginal matric potential and incubation time (2 days) no growth was observed at both temperatures, but differences were evident subsequently.

a)



b)

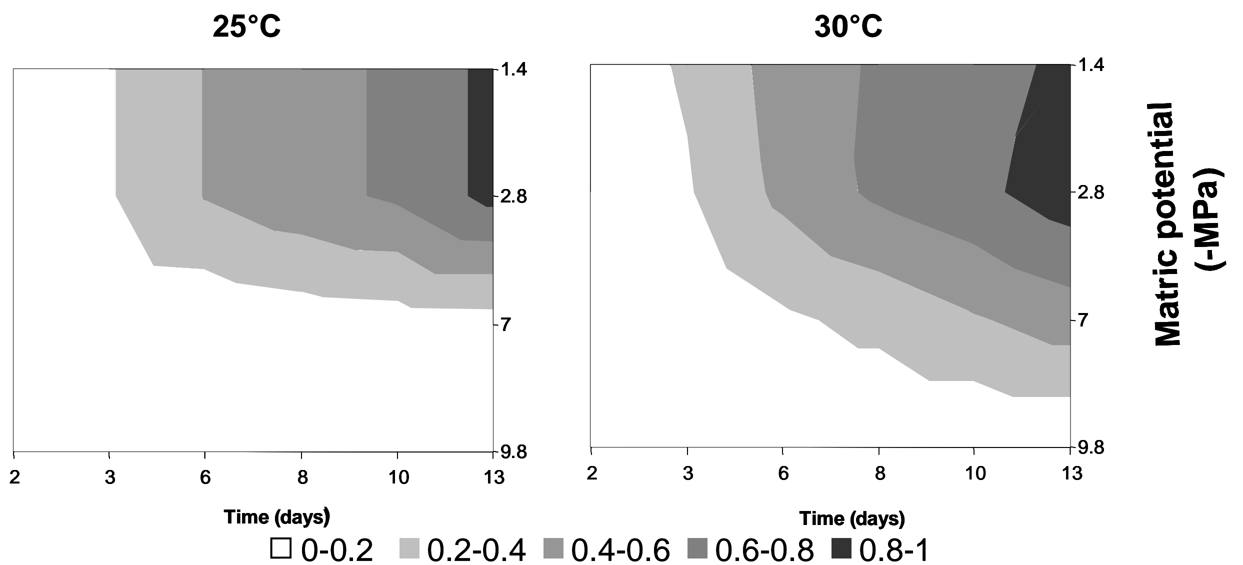


Figure 5.2 - Comparison of two dimensional profiles of mean growth of three *A. flavus* strains on media (a) modified with ionic and non-ionic solutes (NaCl, glycerol) in relation to time and water potential and (b) in relation to matric stress (modified with PEG 8000) at both 25 and 30°C. Different shading represents different growth rates. The scale represents a percentage of growth from 0 to 100%.

5.3.2 Solute stress effects on sporulation

A significant effect (P<0.01) of all the main factors considered (strain, temperature and water potential) on *A. flavus* sporulation was confirmed by ANOVA (Table 5.2), with no difference between replicates. Two strains were similar while A 2092 produced a significant higher amount of spores and sporulation was significantly higher at 25 than at 30°C.

The maximum number of spores was produced at -2.8 MPa in both modified media; significantly lower than that on the unmodified media. Spore production stopped or slightly reduced from -14.0 MPa, respectively with salt and glycerol addition.

Table 5.2 - Comparison of sporulation of *A. flavus* strains on maize flour agar (-1.4 MPa, 0.99 a_w) and on media modified with NaCl or glycerol to -2.8, -7.0 and -14.0 MPa (=0.98, 0.95, 0.90 a_w). Data were logarithmically transformed [ln (value+1)]. Different letters refer to the main factor considered (strain, temperature or water potential) and indicate statistically significant differences (P<0.01).

Factors		**Sporulation** [ln (value+1)]
Strain	A 2073	13.3 b
	A 2052	13.4 b
	A 2092	14.4 a
Temperature (°C)	25	14.1 a
	30	13.4 b
Water potential (MPa)		
	Glycerol	
	-21.0	10.4 f
	-14.0	17.2 e
	- 7.0	18.7 c
	- 2.8	19.4 b
	Salt	
	-21.0	0.0 g
	-14.0	0.0 g
	- 7.0	18.2 d
	- 2.8	19.6 b
	Control	
	-1.4	20.1 a

5.4 DISCUSSION

The present study compared the effects of solute and matric potential stress and temperature on mycelial growth of mycotoxigenic *A. flavus* strains from maize in northern Italy for the first time. The temperatures of 25°C and 30°C were chosen for the trials because they define the optimal range for growth of this fungus (Kheiralla et al., 1992; Nesci et al., 2004; Sanchis and Magan, 2004; Giorni et al., 2007).

A. flavus was more sensitive to matric than to solute stress and its growth was faster at 25 or 30°C respectively in the 2 conditions. In contrast, 30°C was the optimal temperature in both stress conditions in a similar study conducted in Argentina, but only 20 and 30°C were considered (Nesci et al., 2004).

Comparing the effects of solute and matric stress on mycelial growth, -2.8 MPa was optimal under solute stress and matrically modified media, not different from the unmodified media (-1.4 MPa). The Italian strains showed the ability to grow down to -14.0 MPa in a medium modified with NaCl, while under matric stress this was limited to -9.8 MPa. They seem more tolerant to both matric and solute imposed water stress than those from Argentina previously examined. In fact, Nesci et al. (2004) reported no growth at a water potential lower than -14.0 in solute (NaCl) or matrically derived (PEG 8000) water stress.

Italian mycotoxigenic strains have an optimal growth rate profile similar to that found in the USA for isolates from groundnuts and maize where germination/growth has been reported at down to -32.2 MPa, but after more

than 40 days incubation (Sanchis and Magan, 2004). Our interest was in how rapidly growth could occur and the Italian strains were unable to do this at -21 MPa after 7 days incubation.

Significant differences in tolerance of solute or matric potential stress were noticed. Growth rate on matrically-modified media was often about 50% with respect to similar conditions of solute stress, indicating higher sensitivity to this factor. This was also supported by the time required to reach the maximum growth which was equal to 7 days with solute stress and 13 days on matrically-modified media. The lower tolerance to matric stress confirms the greater difficulty involved in extracting water from soil pores and the consequent limited solute transport (Adebayo and Harris, 1971); as a consequence soil colonisation is expected over a narrower range of water availability respect to ear colonisation. This difference in sensitivity has been previously observed for Argentinean strains of *A. flavus* (Payne, 1992) and also with other species such as *Alternaria alternata* and some basidiomycetes (Adebayo and Harris, 1971; Whipps and Magan, 1987; Magan et al., 1995). In contrast, limited differences were observed in tolerance to solute and matric stress for ochratoxigenic strains of *A. ochraceus* (Lee and Magan, 1999; Ramos et al., 1999).

Regarding sporulation, very few studies have tried to quantify the efficacy of changing solute stress conditions (Battilani et al., 2003; Gervais and Molin, 2003; Parra et al., 2004). The present study suggests that temperature and

ionic/non-ionic solute stress significantly influence spore production by *A. flavus* on maize-based media, with the maximum at -1.4 MPa at both 25°C and 30°C.

It has been suggested by Calvo et al. (2002) that sporulation capacity and secondary metabolite production by *A. flavus* and *A. nidulans* are linked by the same induction pathways and influenced by environmental factors. They have provided information on this with regard to pH, temperature and carbon/nitrogen sources, but no studies have been conducted considering solute or matric stress.

Gervais and Molin (2003) found differences between optimal a_w conditions for growth and sporulation for *Penicillium roqueforti* being 0.97-0.98 a_w (corresponding to -2.8 - -4.2 MPa) and 0.96 a_w (-5.6 MPa) respectively.

The strains used for testing sporulation in this study were previously tested both for growth and AFB_1 production in different temperature and a_w conditions (Giorni et al., 2007). The results suggest that differences of 5°C and -0.7 MPa (=0.05 a_w) from the optimal conditions (25°C; -1.4 MPa; 0.99 a_w) can produce a 10-15% reduction in fungal growth and a higher reduction in AFB_1 production and sporulation (65-80% and 55% respectively) (data not shown). This could be explained by results reported by Brodhagen and Keller (2006) regarding the regulation of both sporulation and mycotoxin production in *A. flavus* by G protein signalling pathways. The relationship between mycotoxin production and sporulation were also found by Mostafa et al. (2005) who demonstrated that most of the toxins were produced after the fungus has

completed its initial growth phase and began the development stage, represented by sporulation and sclerotia formation.

Data obtained in this study is critical in building up a picture of the key factors which influence growth and sporulation of strains of this important mycotoxigenic species from northern Italy. They will contribute to the development of a Decision Support System aimed to predict the risk of aflatoxin contamination in maize and to optimise cropping systems and minimise aflatoxin contamination.

CHAPTER 6

Influence of environmental factors on niche overlap of common fungi present on maize

6.1 INTRODUCTION

Several *Aspergillus* and *Fusarium* species colonise maize during the growing season. The ability for both of these species to colonize plants, in particular the ears, and produce mycotoxins suggests that they may have strong competitive capabilities (Marin et al., 1998a).

In field, the dominance of one fungus over another depends on several factors. Environmental factors, such as water availability (a_w) and temperature have been demonstrated to affect the interactions and competitiveness of spoilage fungi (Marin et al., 1998a and b; Lee and Magan, 1999; Magan et al., 2003). Moreover, co-existence of microorganisms on plant surfaces may be mediated by nutritional resource partitioning (Wilson and Lindow, 1994). In particular, carbon source availability in the plant can determine fungal diversity and dominance.

Several studies have demonstrated that utilisation patterns of carbon sources relevant to maize grain could be used to examine and understand the dynamics of interaction and dominance of certain species, and eventually their competitiveness, in relation to both temperature and water availability (Marin et al., 1998 a and b; Lee and Magan, 1999; Magan et al., 2003). Recently, these patterns have also been shown to be influenced by interactions with preservatives which can influence nutritional partitioning and niche overlap indices (Arroyo et al., 2008, in press).

In vitro carbon source utilisation patterns could be used to determine niche overlap indices (NOI) and the level of ecological similarity. Based on the range of C-sources utilized and those unique to an individual species, Wilson and Lindow (1994) suggested that NOI values >0.90 were indicative of coexistence between species in an ecological niche, while scores of <0.90 represented occupation of separate niches.

6.2 MATERIALS AND METHODS

6.2.1 Fungal strains

Experiments were conducted using 5 fungal strains collected from maize kernels in North Italy. Four strains were *A. flavus*: MPVP A 2092 and MPVP A 2057, able to produce high amounts and low amounts of AFB_1 respectively, and MPVP A 2097 and MPVP A 2082, non-aflatoxin producers (Chapter 2; Giorni *et al.*, 2007); and a fumonisin producing strain of *Fusarium verticilloides* (ITEM 1744) isolated from maize kernels (Moretti et al., 1995). *Aspergillus* strains used belong to the fungal collection of the Institute of Entomology and Plant Pathology of the Università Cattolica del Sacro Cuore of Piacenza (Italy) and were officially identified by the Food Science Australia (CSIRO, Sydney, Australia). Identification was characterized by colony appearance typical of *A. flavus* for all the 4 strains but conidia and heads were slightly different from standard strains of this fungal species.

6.2.2 Microtitre plate preparation

Sterile microtitre plates (24 wells, IWAKI, Japan) provided with a lid and with a well capacity of 1 mL were used. A minimal medium was prepared with $NaNO_3$ (0.23%), $MgSO_4 \cdot 7H_2O$ (0.06%), K_2HPO_4 (0.17%) and KH_2PO_4 (0.13%). Carbon sources (CS) were incorporated into the media at a final concentration of $9.1x10^{-3}$ g C ml^{-1} $well^{-1}$ (carbon equivalent to 2% (w/v) glucose). Carbon sources tested represent the principal nutritional compounds of maize kernel and they are listed in Table 6.1.

The available water level of media was modified to four levels: 0.90, 0.93, 0.95 and 0.98 a_w by adding different amounts of NaCl (Dallyn and Fox, 1980). The pH of media was buffered at 6 with phosphate buffer (10nM, Sigma). Both NaCl and phosphate buffer were used because they did not represent an additional source of carbon. Each well of the sterile microtitre plate was filled with 700 µL of one CS solution; the trials were all conducted in triplicate and repeated.

Table 6.1 – Carbon sources and concentration used in niche overlap experiments. All compounds were from Sigma (Saint Louis, MO, USA).

CARBON SOURCE	% compound (w/v) (equivalent to 9.1 mgC/mL)
Aminoacids	
L-Leucine	1.65
L-Alanine	2.25
D-Alanine	2.25
D-L-Threonine	2.25
L-Serine	2.68
D-Serine	2.68
L-Histidine	1.96
L-Proline	1.74
L-Phenylalanine	2
L-Aspartic acid	2
L-Glutamic acid	2
Carbohydrates	
D-Galactose	2.28
D-Raffinose	2.50
D-Glucose	2.28
D-Maltose	2.28
D-Fructose	2.28
Sucrose	2.16
D-Melibiose	2.28
Dextrin	2
Amylopectin	2
Amylose	2
Fatty acids	
Oleic acid	2
Linoleic acid	2
Palmitic acid	2

6.2.3 Spore suspension preparation and inoculation

Spores of the different fungal species from 7 day old cultures on Czapek Agar (CZ; sucrose 30 g; $NaNO_3$ 2 g; KCl 0.5 g; $MgSO_4 \cdot 7H_2O$ 0.5 g; $FeSO_4 \cdot 7H_2O$ 0.01; K_2HPO_4 1 g; $ZnSO_4 \cdot 7H_2O$ 0.001 g; $CuSO_4 \cdot 7H_2O$ 0.005 g; agar 15 g; H_2O to 1 L) for *A. flavus* and Potato Dextrose Agar (PDA; infusion from potatoes 200g; dextrose 15g; agar 20g; H_2O to 1L) for *F. verticilloides* were harvested (with sterile water) and individually placed into sterile Universal bottles containing 20 mL of distilled water. Bottles were shaken vigorously for 3 minutes and centrifuged in a bench top microfuge for 15 minutes at 3000 rpm. After discarding the supernatant, a further 20 mL of sterile water were added and the washing process was repeated 3 times. After the third wash, spores were resuspended with the adequate buffer-NaCl sterile solution and their concentration was adjusted to 10^6 spores mL^{-1}.

For each considered fungus, wells were inoculated with 100 µL of spore solution. Microtitre plates were closed with parafilm® and incubated at 20 and 25°C. The presence or absence of growth was checked at 12 hour intervals, over a period of 60 hours. Similar microtitre plates were prepared and incubated without inoculum to verify the absence of fungal growth at all the conditions tested.

6.2.4 Calculation of niche overlap index (NOI)

Results of carbon sources utilisation were used to calculate a Niche Overlap Index (NOI) (Wilson and Lindow, 1994). The index is defined as follows:

$NOI_{A/B}$=number of CS utilised in common by species A and B/total number of CS used by species A

The index represents the coexistence or competition of different species in a nutritional niche: NOI values > 0.9 indicate occupation of the same nutritional niche and values < 0.9 indicate the occupation of different niches (Wilson and Lindow, 1994). NOI values are commonly presented in pairs as $NOI_{A/B}/NOI_{B/A}$; coexistence will appear when both NOI values are > 0.9 while values < 0.9 will indicate the occupation of separate nutritional niches.

6.3 RESULTS

Results shown refer to situation after 36 hours of incubation since many carbon sources were already used and because with longer times no significant differences were found in terms of carbon source utilization.

The use of carbon sources was influenced by both temperature and a_w. No sources were used by the tested fungi at 0.87 a_w and 20°C. Fatty acids seem the most difficult sources to be used by the test fungi; in fact, they were used initially at 0.93 a_w by *F. verticillioides* and from 0.93 a_w (A 2092), 0.95 a_w

(A 2082) or 0.98 a_w by different *A. flavus* strains. Fungal growth was very similar in media with sugars and amino acids; at 25 and 30°C and a_w levels from 0.90. Compounds from these 2 groups were useful for all fungi tested, with a few exceptions among *A. flavus* strains. *F. verticillioides* growth was also observed at 20°C (Figure 6.1).

	0.87			**0.9**			**0.93**			**0.95**			**0.98**		
A. flavus (**A 2057**)	**S**	**A**	**FA**	**S**	**A**	**FA**	**S**	**A**	**FA**	**S**	**A**	**FA**	**S**	**A**	**FA**
20															
25															
30															
A. flavus (**A 2092**)															
20															
25															
30															
A. flavus (**A 2097**)															
20															
25															
30															
A. flavus (**A 2082**)															
20															
25															
30															
F. verticillioides **(1744)**															
20															
25															
30															

Figure 6.1 – Type of carbon sources (S=sugars; A=amino acids; FA=fatty acids) used by *A. flavus* and *F. verticillioides* strains at the different conditions tested. The presence of a different colour represents that at least one carbon source belonging to sugars (blue), amino acids (green) or fatty acids (orange) was used by the fungus.

The number of carbon sources utilized by the 5 strains differed at all the treatment conditions. At 0.98 a_w and 30°C the number of C sources used was

the highest for all the strains of *A. flavus* with the exception of A 2092 that grew better at lower temperatures (20 and 25°C). The strain of *F. verticillioides*, instead, used more carbon sources at the lowest temperature tested (20°C) and with a_w from 0.93 to 0.98 (Figure 6.2).

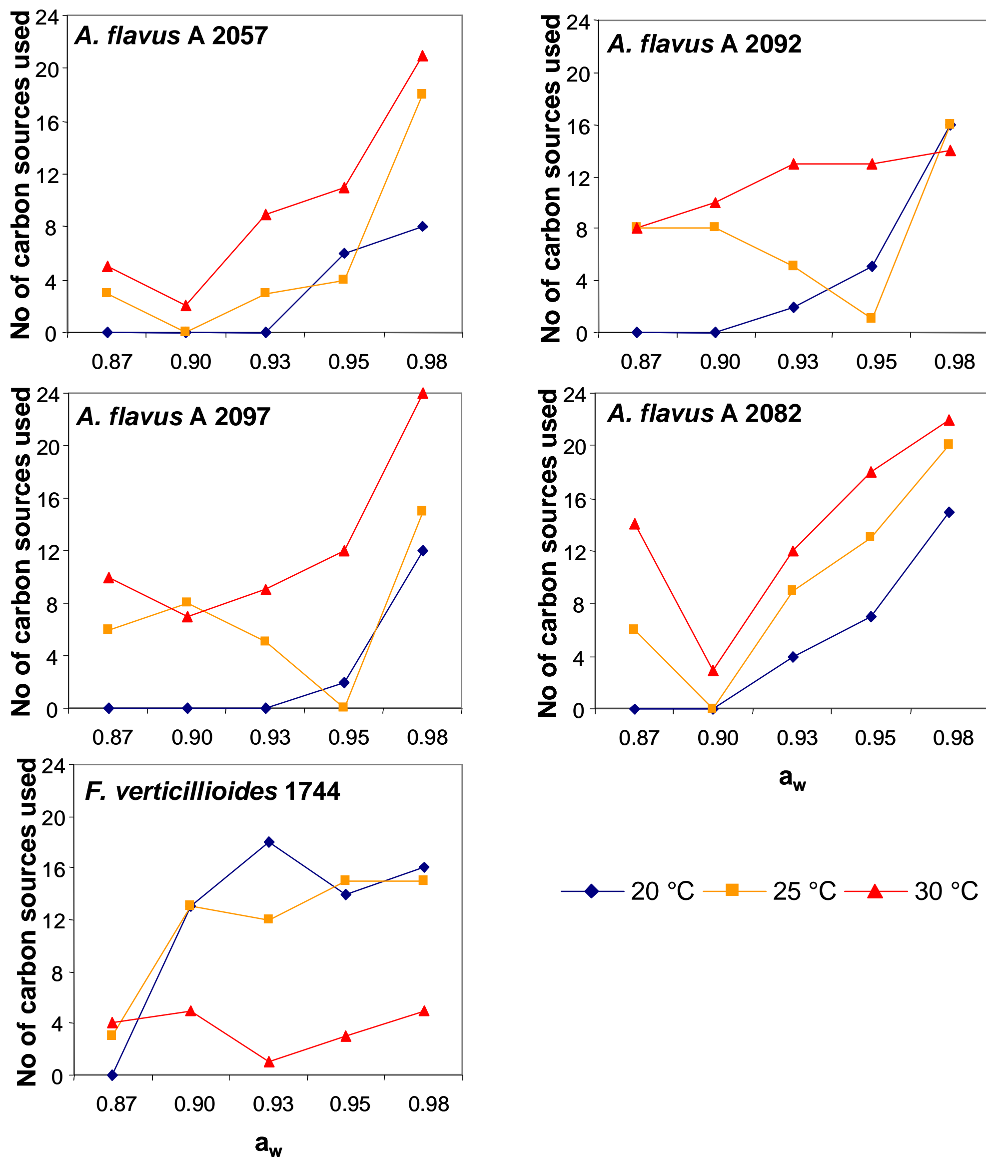


Figure 6.2 - Carbon sources used by the 5 species and strains considered at the different conditions tested.

Fusarium verticillioides never used amylose as a carbon source. D-serine, L-leucine and L-phenilalanine were never used by *A. flavus* 2092 and only at 30°C and 0.98 a_w by the others strains. Aspartic acid was never used by *A. flavus* 2082 and 2057, and only at 30°C and 0.98 a_w by the others strains.

Considering the NOI, co-existence or competition of different species in a nutritional niche was determined (Figure 6.3). At high temperatures (25 and 30°C) and low a_w level (0.87 a_w), *A. flavus* was always dominant with respect to *F. verticillioides* while at lower temperature (20°C) and more available water (0.95 and 0.98 a_w) *F. verticillioides* was dominant over *A. flavus*. In all the other conditions tested, the two species occupied separate niches.

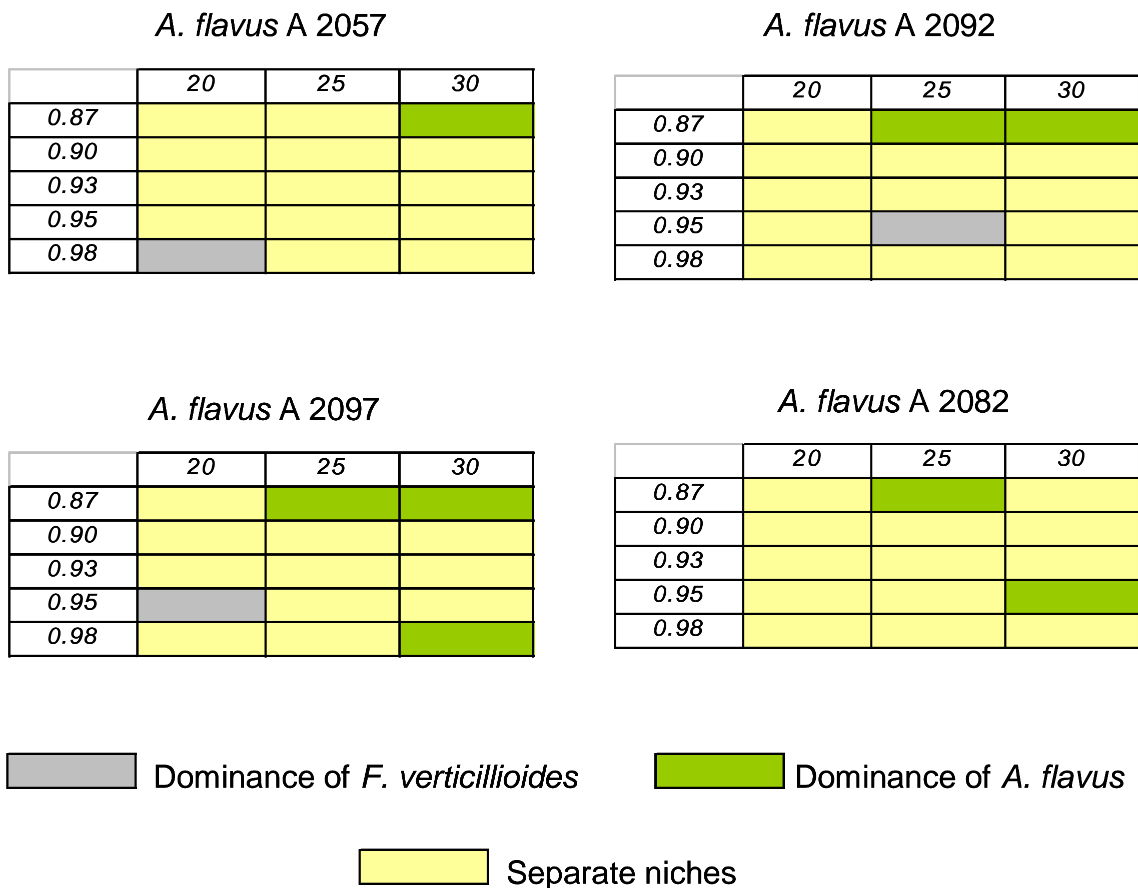


Figure 6.3 – Schematic representation of NOI for the different conditions of the strains of *A. flavus* used in the experiment respect to *F. verticillioides*.

6.4 Discussion

The behaviour of *A. flavus* strains at the different conditions considered was different. In particular, at 20°C both A 2057 and A 2097 started to use carbon sources only at a_w levels higher than 0.93, while for A 2092 and A 2082 at the same temperature less available water (0.90 a_w) was necessary. Even if strains with different abilities to produce aflatoxin were chosen for the experiment, no particular differences in general were noted regarding the

utilization of carbon sources. Some carbon sources were used earlier by one strain and only later and at optimal conditions by the others but this was not linked to high, low or inability to produce aflatoxin.

Strains A 2097 and A 2092 showed a modest decrease in the number of carbon sources utilised at 25°C and 0.95 a_w while for strains A 2057 and A 2082 this occurred at 0.90 a_w and both temperatures (25 and 30°C). This confirmed that the total and common carbon sources utilized by each fungus and the conditions of niche overlap modifications were markedly influenced by both water availability and temperature and can change with environmental conditions (Marin et al., 1998b). This may be further changed by interactions between environmental factors and interaction with potential control chemicals, including preservatives (Arroyo et al., 2008, in press).

Interestingly, the patterns of utilisation were sequential, with carbon sources such as sugars, which are easier to degrade, were used first, this was followed by amino acids. This could explain how their increase in kernels can help fungal development especially close to plant maturity. Fatty acids, instead, seemed to be used only when water was practically freely available.

The different optimal condition for the growth of *A. flavus* and *F. verticillioides* were confirmed also by analysis of NOI. *Fusarium verticillioides* was dominant at lower temperatures (20°C) and relatively higher a_w levels (> 0.95 a_w) while *A. flavus* was dominant only at high temperatures (25-30°C) and

drier conditions (0.87a_w) at the interface between growth boundaries for Fusarium species (Sanchis and Magan, 2004) These results confirms other reports in the literature (Marin et al., 1995; Sanchis and Magan, 2004). The capacity for assimilation of different carbon sources by the two genera reflect their competitiveness at certain environmental conditions. However, only extreme conditions were linked to dominance of one of the two tested Genera while in almost all cases both *A. flavus* and *F. verticillioides* appeared to occupy different niches. This could result in the possibility to find more than one fungal population on maize and thus could influence the role of mycotoxins in enabling these populations to competitively exploit the maize ecological niche to their advantage. This approach may also be very useful background information in screening and evaluating potential biocontrol agents to control these important mycotoxigenic species on maize. Thus, biocontrol agents will need to be able to effectively compete for these ranges of carbon sources to be able to competitively exclude these mycotoxigenic species over a wide environmental window.

CHAPTER 7

Efficiency of *Aspergillus flavus* in silk inoculation and role of maize ripening stage on fungal growth

7.1 INTRODUCTION

In this study the following experiments were carried out: (a) preliminary experiments to evaluate the possible role of growth stage and inoculum concentration on fungal development and final aflatoxin content in maize cobs; (b) the effect of different temperatures and relative humidities in maize on aflatoxin production and (c) the evolution of field infection using ripening maize cobs to recreate the natural nutritional matrices for infection by *A. flavus*.

7.2 MATERIALS AND METHODS

7.2.1 Inoculum preparation

One strain of *A. flavus* (A 2092; Giorni et al., 2007), belonging to the fungal collection of the Institute of Entomology and Plant Pathology of the Università Cattolica del Sacro Cuore of Piacenza, was used to produce the inoculum. The fungus was transferred from Water-Agar (WA) (1.5% agar) medium and inoculated in the central point on Petri dishes (Ø 6 cm) with Czapek Agar (CZ) as medium (sucrose 30 g; $NaNO_3$ 2 g; KCl 0.5 g; $MgSO_4 \cdot 7H_2O$ 0.5 g; $FeSO_4 \cdot 7H_2O$ 0.01; K_2HPO_4 1 g; $ZnSO_4 \cdot 7H_2O$ 0.001 g; $CuSO_4 \cdot 7H_2O$ 0.005 g; agar 15 g; H_2O to 1 L) and incubated at 25°C for 7 days in the dark.

At the end of the incubation period, the surface of media in one Petri dish was washed with sterile water and spores collected. These were used as an inoculum source for the different experiments.

7.2.2 Inoculation of ears, ears preparation and infection

Two different experiments on ears were conducted to verify both efficiency of the inoculum and how growth stage and temperature could influence fungal infection.

Ears of the maize hybrid PR33J24 of Pioneer (FAO class 600) at 3, 10 and 17 days after pollination (DAP) were collected and put under UV radiations (280nm wavelenght, Polylux XL) for 1 day to reduce microbial presence. Each ear was then inoculated by spraying 1.5 mL of the inoculum to the outside of the silks. Ears were then put in plastic bottles with 50 mL of Hoagland Solution (Hoagland and Arnon, 1950; Epstein, 1972) to avoid senescence and then incubated at different treatment conditions (Figure 7.1).

Figure 7.1 – Example of inoculated ears incubated in plastic bottles.

7.2.2.1 Efficiency of the inoculum

The spore suspension was adjusted to 7 different concentrations from 10^1 to 10^7 spores mL^{-1} and used to inoculate ears harvested at 17 days after pollination (DAP).

Ears were put into bottles as previously described and incubated at 30 °C. After 7 days of incubation, cob husks were removed and ears cut to a thickness of about 1 cm sections. The different sections were labelled and kept separate relative to their original position on the ear (upper, central or lower), and then cut into 4 smaller pieces as shown in Figure 7.2.

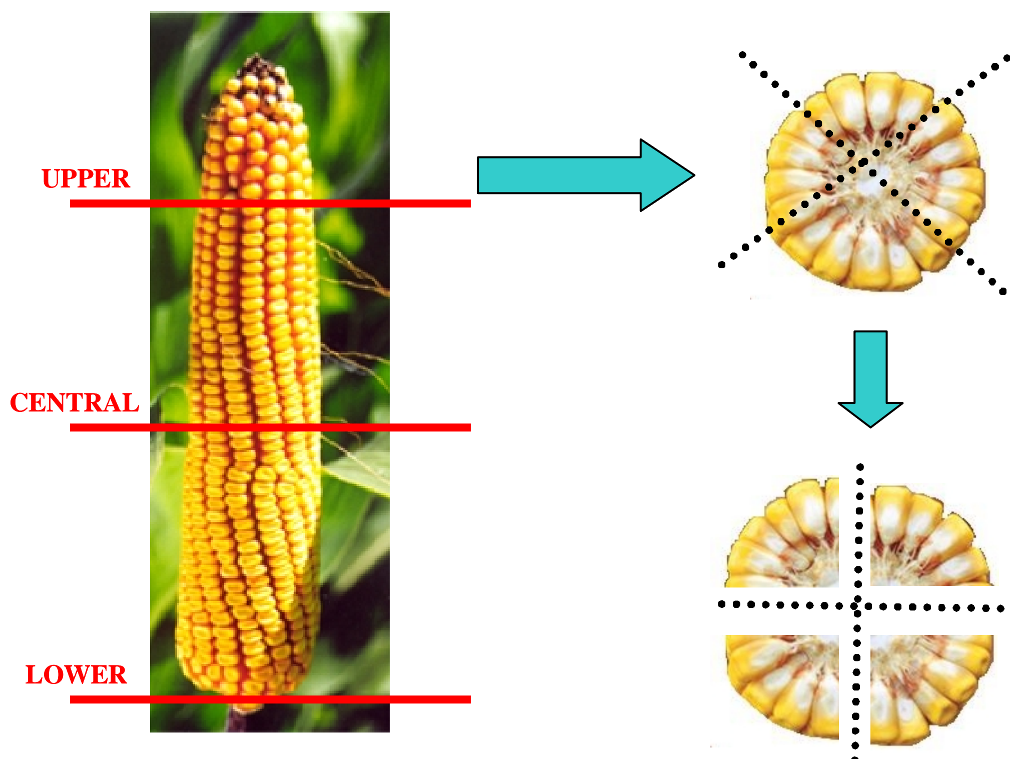


Figure 7.2 – Description of the preparation of the small portions of ears

Fifty small pieces of each ear were transferred to Petri dishes (5 pieces in each Petri dish) containing CZ medium and incubated for 1 week at 25°C (12 hours light photoperiod). The fifty pieces were chosen from all the ear levels considered in the study as follows: 10 pieces from the upper position, 20 from the middle position and 20 from the lower position. After incubation, dishes were checked for fungal growth and the number of ear pieces from which *A. flavus* grew was determined. The experiment was conducted in triplicate.

7.2.2.2 Role of growth stage and temperature on fungal growth

The spore suspension with the concentration of 10^2 spores mL^{-1} was used to inoculate ears at 3 different growth stages: 3, 10 and 17 DAP. Ears were put into bottles as previously described and incubated at 5 different temperatures from 15 to 35 °C (5 °C step) with 12 hours light photoperiod. The trial was carried out with 6 replicates.

After 21 days, 3 ears were used to produce small pieces from the upper, middle and lower parts to verify infection while the other 3 ears were dried at 45°C for 6 days and then milled. The flour obtained was used to determine the fungal populations (CFU g^{-1}) and contamination with aflatoxins.

7.2.3 Inoculation of maize grains

A concentration of 10^2 spores mL^{-1} was also used to inoculate kernels of 3 different maize Pioneer hybrids: PR34F02 (FAO 500), PR34N43 (FAO 500),

and PR32B14 (FAO 700), harvested every week from 21st July (14 days after pollination) to 29th September (10 times in total).

For each hybrid and each sampling time, grains were used to fill 3 Petri dishes (∅ 6 cm) and inoculated by spraying them with 1.5 mL of the inoculum suspension. Petri dishes were then put into bigger containers with or without sterile water on the bottom to maintain humidity at 100% or allowing a natural decrease respectively. Containers were closed and incubated.

A non-contaminated sample was considered for each experiment; this was inoculated only with sterile water.

After incubation at 25°C for 21 days, grains were dried at 45°C for 6 days and and aflatoxins were quantified in the flour.

7.2.4 Aflatoxin analysis

Flour was extracted with 100 mL of CH_3OH-H_2O (80+20), stirring for 45 minutes and then the extract was filtered with a Whatman 595 ½ (Dassel, Germany) paper filter and 5 mL of the solution was diluted with 45 mL of H_2O into an Easy Extract Aflatoxin immuno-affinity column (Biopharm, Rhone, Glasgow, UK) then the column was washed with 5 mL of H_2O. Aflatoxins were eluted with 2.5 mL of CH_3OH and the solution was concentrated to 1 mL with a stream of nitrogen. Then, 1 mL of CH_3CN-H_2O (25+75) was added and the solution was filtered through a Millipore® filter (0.45 µm) (Bedford, MA, USA).

Filtered solutions were analysed by reverse phase HPLC (Jasco, Easton, MD, USA) using post-column derivatization with a UVE instrument (LCTech GMBH, Postfach-Dorfen, Germany) set at 254 nm and fluorescence detection. The column was a superspher 100 RP-18 (Merck, Darmstadt, Germany) and the mobile phase was H_2O-CH_3CN-CH_3OH (64+13+23) at a flow rate of 1 mL min^{-1} (Stroka et al., 2003). Aflatoxin production was quantified in ng g^{-1} of ears. The limit of detection was 0.1 ng g^{-1}**.**

7.2.5 Data analysis

Data on CFU and aflatoxins production (values+1) were logarithmically transformed before statistical analysis. Log transformation is always required for data that covers a wide range of variability (from single-digit numbers to numbers in hundreds or thousands) (Clewer and Scarisbrick, 2001).

Analysis of variance was performed considering all factors (growth stage, temperature and hybrid, when appropriate); a generalized linear model of the statistical package SPSS was used (Statistical Package for Social Science ver. 11.5.1, 2002. SPSS Inc., Chicago, IL USA). Means were compared using the LSD test to indicate significant differences.

7.3 RESULTS

7.3.1 Inoculation of ears

7.3.1.1 Efficiency of the inoculum

Analysis of variance showed significant differences between treatments ($P \leq 0.01$), with higher concentration of inoculum resulting in a more efficient infection; in particular, 10^6-10^7 spores mL^{-1}caused almost 100% infection (Figure 7.3). The position in the ear did not have a significant effect on infection (data not shown).

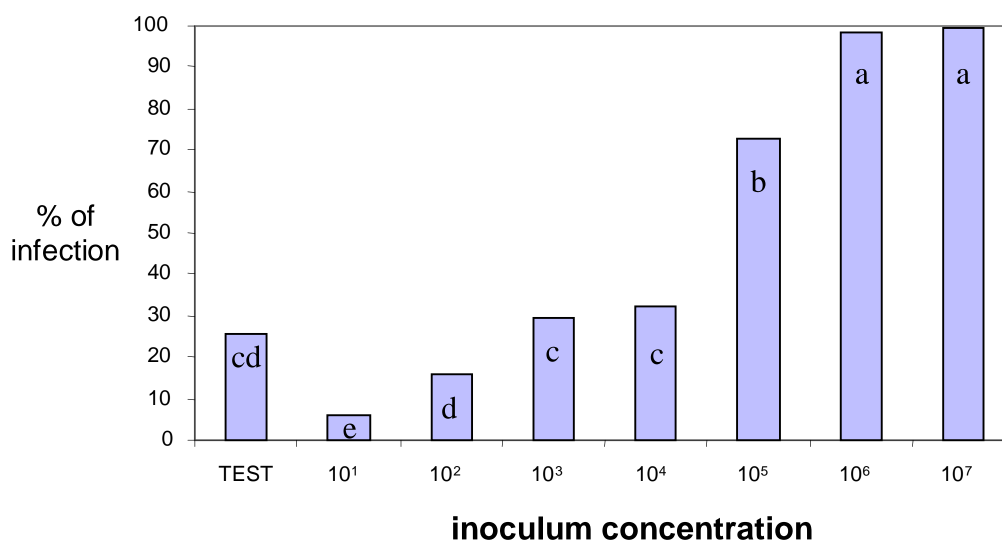


Figure 7.3 – Percentage of infection in the different parts of ears checked after 7 days of incubation at 30°C. Different letters represents statistically significant differences among treatments ($P \leq 0.01$).

7.3.1.2 Role of growth stage and temperature on fungal growth

Statistical analysis underlined significant differences between infections obtained at the different incubation temperatures ($P \leq 0.01$); contamination was higher at temperatures between 15 and 30°C, while at 35°C it was practically absent (Figure 7.4). The maize growth stage at inoculation and the portion of the ear considered, instead, were not statistically significant (data not shown).

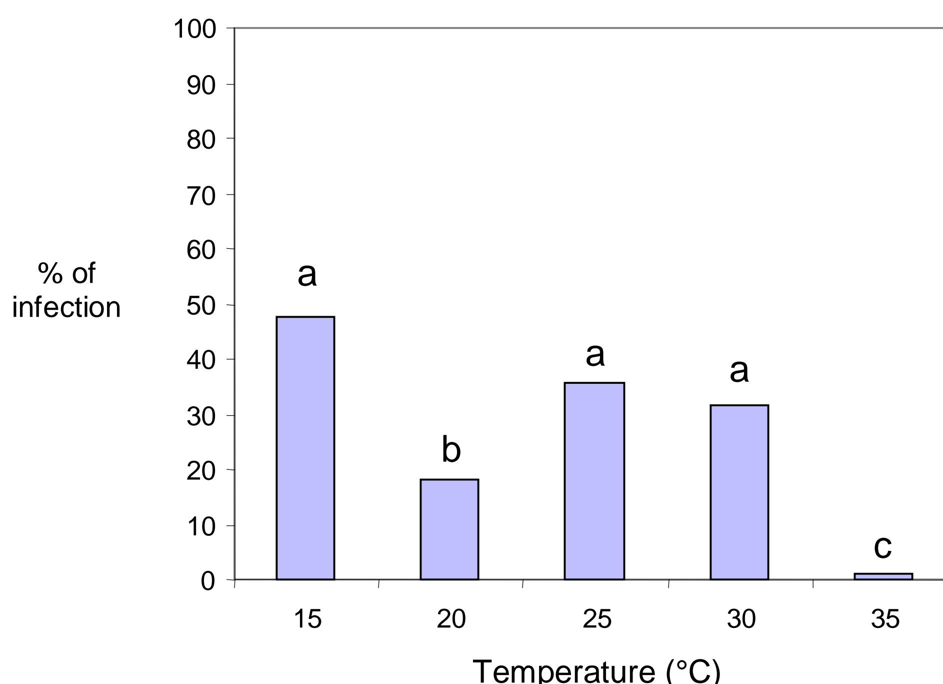


Figure 7.4 – Percentage of fungal infection in ears harvested at different DAP and incubated between 15 and 35°C (step 5°C). Different letters represent statistically significant differences among treatments ($P \leq 0.01$).

Fungal populations (CFU g^{-1}) were higher at 30°C followed by 35 and 25°C while at 15 and 20°C no fungal colonies were found. In relation to growth stage, the mean values indicated that earliest stages were more susceptible to *A.*

flavus infection. However, statistically, no significant differences were found in fungal development between both temperatures and growth stages.

Aflatoxin B_1 contamination of maize flour was shown to be linked to temperature; in fact, AFB_1 was detected with temperatures between 25 and 35°C, with a significantly higher amount at 25°C ($P \leq 0.05$; Figure 7.5).

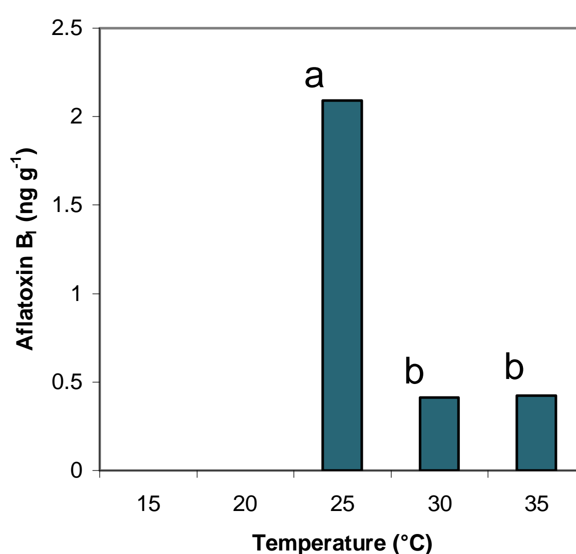


Figure 7.5. – Aflatoxin B_1 content in maize flour obtained from ears harvested at different DAP, artificially inoculated and incubated at several temperatures. Different letters represent statistically significant differences ($P \leq 0.01$).

7.3.2 Inoculation of maize grains

Aflatoxin B_1 content in maize grain of 3 hybrids collected at different growth stages, artificially inoculated and incubated with or without humidity control, was significantly influenced both by hybrids and growth stages. Regarding sampling time, samples collected on 28th July had more contaminated than all the others ($P \leq 0.01$) (Figure 7.6).

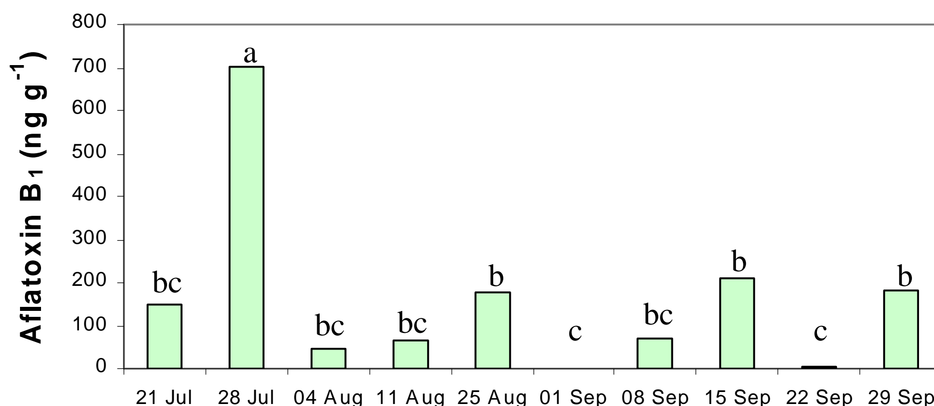


Figure 7.6 – Mean aflatoxin B_1content in kernels collected in different sampling dates and artificially inoculated. Different letters represent statistically significant differences among conditions (P≤0.01).

Hybrid B14 had higher AFB_1 content with respect to the other two hybrids considered ($P \leq 0.01$) (Figure 7.7).

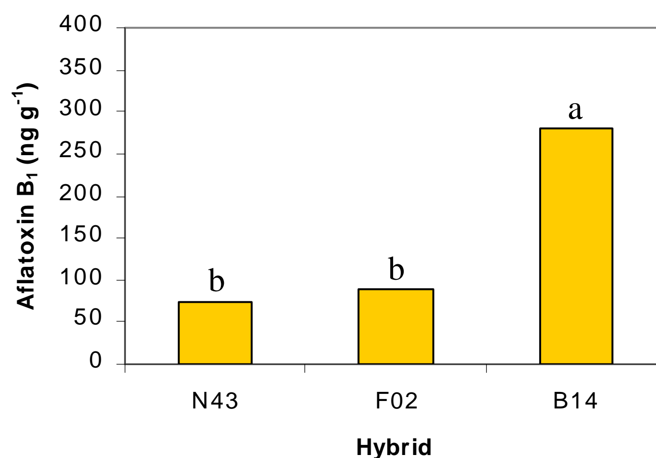


Figure 7.7 – Aflatoxin B_1 contamination of 3 maize hybrids artificially inoculated. Different letters indicate statistically significant differences among hybrids (P≤0.01).

7.4 DISCUSSION

The *in vitro* trials carried out in this study gave interesting results on the role of all parameters considered.

The inoculum concentration required to achieve high levels of ear infection were >10^5 spores mL^{-1}; lower inoculum caused less than 20% infection. Inoculum concentration in fields in Italy are normally very limited and it could explain the spot infection of ears, with few kernels highly contaminated with AFB_1.

Overall, the results obtained do underline the potential influence of growth stage on fungal development. Earlier growth stages are more susceptible to fungal infection and a significantly higher contamination resulted in kernels collected at 21 days after pollination. The reason why different ages were examined was because the natural resistance of the ripening maize kernels may vary with ripening stage. There may be natural inhibitors which become less active as maturity is reached. Previous studies also determined that *A. flavus* sclerotia placed on the soil surface are able to germinate 8 days prior to the maize silking date (Wicklow and Wilson, 1986) and then earlier stages have an higher inoculum concentration.

Also temperature appeared to have an important influence on both fungal development and AFB_1 production. Indeed, fungal populations were high between 15 and 30°C, with maximum AFB_1 accumulation at 25°C. Inoculum potential and infection capacity of *A. flavus* was found to be better at higher temperatures (Northolt and van Egmond, 1981).

With regard to aflatoxin production, 25°C was found to be optimal for production. This is further *in situ* evidence which supports previous *in vitro* trials (Giorni et al., 2007) that confirms the behaviour of Italian strains as being less thermophilic than others cited in the literature (Kheiralla et al. ,1992).

Interestingly, the trend of aflatoxin content obtained in the experiment in the present study are contradictory to that for fungal populations. Probably, this is a consequence of stress and of nutritional competition among *A. flavus* strains. On maize grains results can be very variable because of the high natural variation among ears in the field. Few information is available about the global factors that regulate aflatoxin biosynthesis, but there is a clear link between development and aflatoxin biosynthesis. A lot of physiological factors can influence mycotoxin production, such as pH level and chemical composition of kernels, but it is difficult to understand their role in the regulation of this pathway (Payne and Brown, 1998). A better method to analyse kernels directly coming from the field needs to be developed. Surely, the choice of hybrids is a key determinant to prevent *A. flavus* contamination.

These preliminary results do provide evidence that a specific relative humidity window exists which is conducive for fungal development and aflatoxin production in ripening maize ears.

CHAPTER 8

Field trials to evaluate maize hybrids resistance to *A. flavus*

A study conducted in collaboration with CRA - U09 Unità di ricerca per la maiscoltura - Bergamo (Research Unit on Maize of Bergamo), ITALY.

8.1 INTRODUCTION

In Italy, because of the high aflatoxin contamination problems in maize production in 2003, significant attention was given to potential hybrids which could be less susceptible to *A. flavus* infection and aflatoxin contamination. Although breeding selection eliminates genotypes particularly susceptible to diseases, cultivated hybrids frequently show serious fungal infection (Munkvold, 2003). There are no commercial hybrids completely resistant to *A. flavus* and, additionally, information about the comparative aflatoxin accumulation of commercial hybrids under field conditions is limited (Betrán and Isakeit, 2004). Current efforts are to map and characterize the genetic factors involved in resistance and to transfer them through marker-assisted selection to more suitable elite genotypes (Rocheford and White, 2002).

Beneficial secondary traits such as husk covering and tightness, physical properties of the pericarp, and drought or heat stress tolerance are factors which may contribute to *A. flavus* resistance. In general, the hybrids with good husk cover show a greater resistance to insect damage and accumulate lower levels of aflatoxins (Betrán et al., 2002). The incidence and severity of *A. flavus* infection and aflatoxin accumulation are also highly dependent on genotype, cultural practices and environmental conditions (Brown et al., 1998).

As a result of all these factors, hybrids of different maturities can influence alfatoxin accumulation. In Texas (USA) short-season maize could escape growth-limiting conditions of a hot, dry summer and associated aflatoxin contamination in contrast to the full-season maize; late maize hybrids can have

greater exposure to higher temperatures at flowering and post-flowering stages, greater *A. flavus* inoculum and increased insect activity compared with early hybrids; nevertheless, data reported by Betrán and Isakeit (2004) indicated that early maturation in hybrids was insufficient by itself to reduce aflatoxin contamination. In other parts of the world, including Italy, the situation seems to be the opposite since short-season maize seems to be more contaminated than late maize because of their growing in marginal areas for the crop where normally they are not irrigated and harvest takes place in hot periods (Bruns and Abbas, 2005).

The objective of this study conducted in 2005 and 2006 was to evaluate 34 commercial hybrids for resistance to *A. flavus* attack and aflatoxin accumulation.

8.2 MATERIALS AND METHODS

Thirty-four commercial hybrids with different days relative to maturity (DRM) (FAO 300: 96-105 DRM; FAO 400: 106-115 DRM; FAO 500: 116-120 DRM; FAO 600: 121-130 DRM; FAO 700: 131-140 DRM) were grown in the experimental fields at the CRA - U09 Unità di ricerca per la maiscoltura - Bergamo (Research Unit on maize-Bergamo), in a randomized block design of divided plots and replicated in two seasons (2005 and 2006). Plots were 4 m long and 0,75 m apart, with a plant density of 15 plants/row. In each plot, plants were shaken to encourage self pollination (self pollination breeding, SIB) and ears were covered with a paper bag to avoid cross pollination among plants and

contamination by other fungi. Environmental conditions, such as temperature and rainfall, which can influence hybrids response were recorded at the Weather-Station CRA - U09 Unità di ricerca per la maiscoltura - Bergamo (Research Unit on maize - Bergamo).

Seven days after pollination (DAP), 10 primary ears of each genotype in each elementary plot were artificially inoculated following the non-wounding SCIA (Silk Channel Inoculation Assay) methodology proposed by Zummo and Scott (1989). Silks of each ear were sprayed with 1,5 mL of a spore suspension of 5 *A. flavus* strains (MPVP A 2052, A 2055, A 2059, A 2082, A 2092) isolated from maize in the field during previous trials (Giorni et al., 2007). The suspension was obtained by transferring the strains for growth on Petri dishes (Ø 6 cm) containing Potato Dextrose Agar (PDA) and incubating them at 25°C for 7 days in the dark. After incubation, Petri dishes were washed with sterile water and spores of the 5 strains mixed. The spore suspension was then adjusted to a concentration of 10^8 colonies mL^{-1}.

The trial included:

1) *A. flavus* inoculated ears;
2) non-inoculated ears (SIB);
3) sterile water inoculated ears.

At ripening, ears were manually harvested, hand de-husked and evaluated for *A. flavus* infection using a visual rating scale (% of kernels with visible symptoms of infection such as rot and mycelium growth, reported as Disease

Severity Rating (DSR), ranging from 1 to 7), as proposed by Reid et al. (1996; Figure 8.1).

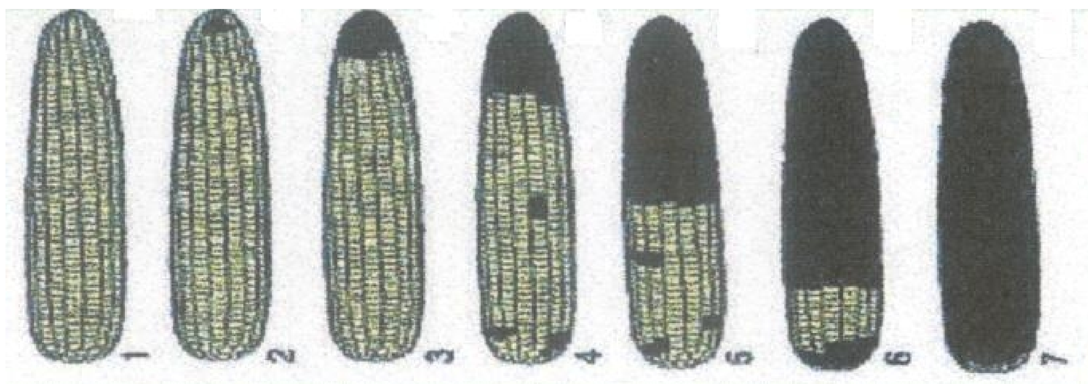


Figure 8.1 – Visual rating scale of fungal attack (Reid et al., 1996).

After visual infection, ears of each plot were dried, shelled, and the kernels bulked. To evaluate internal infection, 50 kernels, randomly chosen from each sample, were surface disinfected and plated on DRBC agar (King et al., 1979). Seven days after incubation at 25°C, the number of kernels showing visible *A. flavus* mycelium was counted.

Kernels derived from the inoculated ears and also from the controls were used to evaluate aflatoxin B_1 content. Kernels were milled and flour obtained analyzed using enzyme-immunoassay-ELISA kit (Kit Ridascreen-Aflatoxin B_1 30/15-R-Biopharm).

8.3 RESULTS

Data was obtained for: (A) *Aspergillus flavus* ear infection visual rating; (B) Percentage of *Aspergillus flavus* internal contaminated kernels; and (C) Ground

grain AFB_1 content (µg/kg), in the materials tested during 2005 and 2006 seasons. These are shown in Table 8.1 as an average of the 34 hybrids.

To test hybrids resistance to A. flavus, ears were rated individually following the visual scale reported in Figure 8.1. Results obtained are reported as mean of the 34 hybrids screened; variability in the hybrid response was observed in 2005 (DSR: 2,45 ± 0,96, range 1-5,05); in contrast, during 2006, DSR were lower than that observed in 2005 (see Table 8.1, A). From the ear visual inspection at maturity of the non-inoculated (SIB) and sterile water-inoculated ears, as control, no or very low disease symptoms were observed during both 2005 and 2006.

For internal kernel contamination, variability among hybrids was found with the percentage contaminated kernels ranging from 0 to 88 (2005) and from 0 to 76% (2006). In contrast, controls showed a value lower than that obtained in the corresponding inoculated hybrids both in 2005 and 2006 (see Table 8.1, B).

The analysis of AFB_1 content in grain samples of the hybrids under study showed that in inoculated samples the levels ranged from 0 to 180 µg/kg (2005) and from 0 to 570 (2006) with variability among hybrids, while in the controls AFB_1 was not found or present only in traces (see Table 8.1, C).

Ears and kernels were more infected in 2005 with respect to 2006, while aflatoxin contamination was higher in 2006. Differences between the two years

was predominantly due to the different meteorological conditions in the area (Figure 8.2).

Table 8.1 – Results, as average of the 34 hybrids, during 2005 and 2006 seasons.

A) *Aspergillus flavus* ear Infection visual rating **(visual rating 1-7)**									
	SIB			**H_2O**			**INOCULATED**		
	Mean value	**Error Standard**	**Range**	**Mean value**	**Error Standard**	**Range**	**Mean value**	**Error Standard**	**Range**
2005	1.02	0.06	1-1.3	1.01	0.03	1-1.4	2.45	0.96	1-5.1
2006	1.09	0.13	1-1.4	1.09	0.12	1-1.3	1.30	0.21	1-1.8
B) **Percentage of** *Aspergillus flavus* internal contaminated kernels									
2005	0.94	1.81	0-6	0.6	1.03	0-10	16.5	15.25	0-88
2006	1.88	6.57	0-10	1.4	4.73	0-14	9.3	8.38	0-76
C) Ground grain **AFB_1 content (µg/kg)** in the materials tested									
2005	2.0	2.89	0-18	2.0	5.09	0-21	27.0	48.16	0-180
2006	7.4	11.28	0.2-46	1.7	6.76	0-38	83.5	149.03	0-570

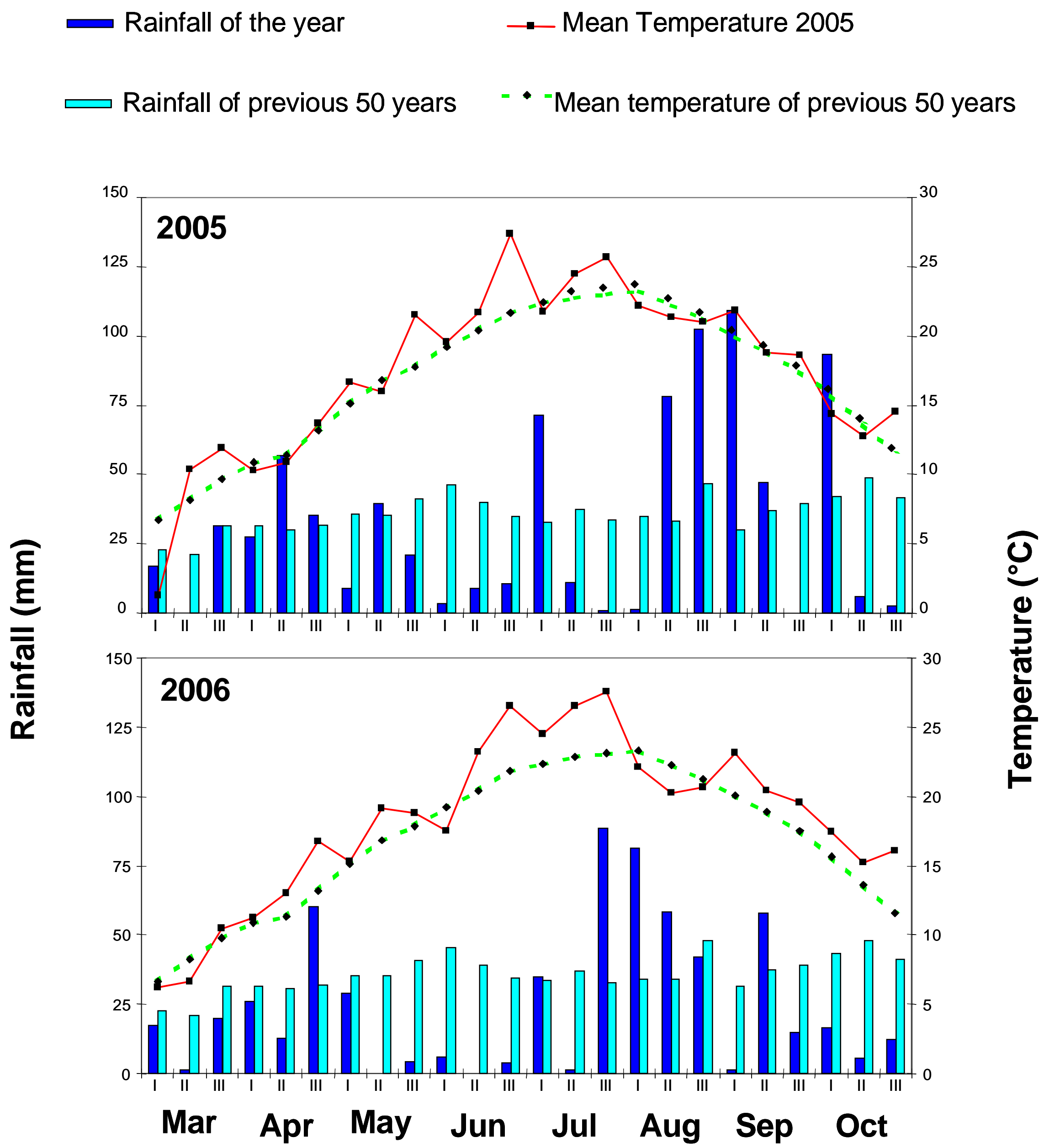


Figure 8.2 – Meteorological data recorded in 2005 and 2006 at the Weather-Station CRA - U09 Unità di ricerca per la maiscoltura - Bergamo (Research Unit on maize-Bergamo).

The capacity of hybrids to produce aflatoxin was classified into 3 groups: low (from 0 to 10 µg/kg), medium (from 10 to 100 µg/kg) and high (higher than 100 µg/kg) as reported in Figure 8.3. The most abundant class was that with low aflatoxin production, including around 60% of tested hybrids both in 2005 and 2006; the remaining 40% of hybrids under study, were shared in 2005, between medium (25%) and high class (12,6%); on the other hand in 2006 the 16.7% was in the medium and 25% in the high aflatoxin production class (Figure 8.3).

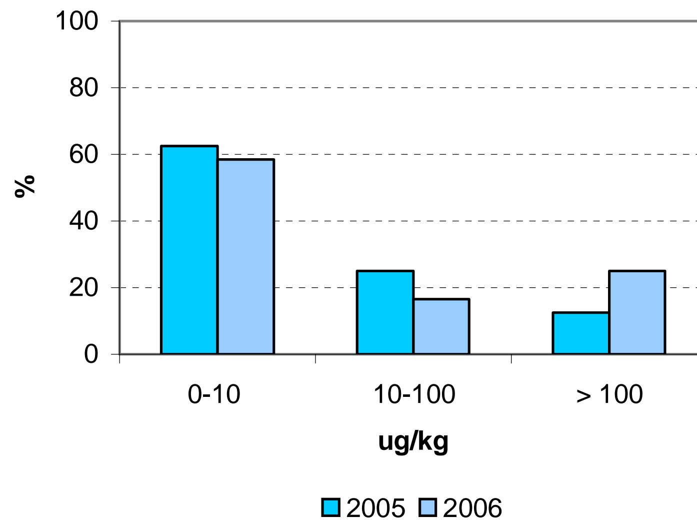


Figure 8.3 – Percentage of tested hybrids belonging to different production classes of AFB_1.

8.4 DISCUSSION

A. flavus ear infection visual rating and internal kernel contamination was lower while aflatoxin, contamination was higher in 2006, probably as a consequence of the conducive meteorological parameters.

In 2005 mean temperatures were lower than in 2006 over the whole growing period while rainfall was abundant, especially during August. The sum of rainfall registered was 374 mm against 307 mm in 2006. These hotter and drier conditions probably caused stress to the fungal populations increasing aflatoxin production, but limiting their growth (Magan and Aldred, 2007). This could explain why, even if both ear contamination, and kernel internal contamination by *A. flavus* were lower in 2006 than in 2005. In contrast, aflatoxin presence was higher than in the previous year.

However, in general, hybrids artificially inoculated with *A. flavus* resulted in a lower contamination with AFB_1 and some of them had no toxin content; this was independent of high or low visual presence of the fungus. Almost 40% of hybrids were contaminated with levels above the legal limit for humans in both years and, among these, 50% had a very high contamination (more than 100 µg/kg). This means that 60% of hybrids tested showed some resistance to fungal invasion and AFB_1 accumulation.

It is important to note that low levels of contamination in ears and kernels used as controls, indicate that the non-wounding silk channel inoculation technique applied in this study was effective in inducing *A. flavus* attack and in discriminating hybrids for resistance.

CHAPTER 9

Final discussion and conclusions

The Italian population of *Aspergillus* section *Flavi* associated with maize is mainly constituted by *A. flavus*, even if some strains have atypical morphological characters (Hocking, personal communication) and some show morphological characters similar to *A. parasiticus*. Among the studied strains, around 25% were not able to produce aflatoxins, and thus may have potential as biocontrol agents (Pitt and Hocking, 2006).

Ecological studies showed that the Italian strains of *A. flavus* were able to grow from 0.83 a_w and 15 °C, with an upper limit of 45°C with an optimum of 30°C, while for aflatoxins production this optimum was 25°C. They seem to be less xerophilic and thermophilic than strains collected from different geographic regions of the world reported in the literature. This could represent an adaptation to this region, where conditions are less hot and dry with respect to those conditions commonly associated with AFBs problems.

Sporulation of *A. flavus* strains studied, as well as fungal growth and AFB_1 production, was significantly influenced by temperature and ionic/non-ionic solute stress. Differences of 5°C and -0.7 MPa (=0.05 a_w) from the optimal conditions (25°C; -1.4 MPa; 0.99 a_w) can produce a 10-15% reduction in fungal growth and a greater reduction in AFB_1 production and sporulation (65-80% and 55% respectively).

A. flavus was more sensitive to matric than to solute stress and its growth was faster at 25 or 30°C, respectively in these two water stress conditions. The Italian strains showed the ability to grow down to -14.0 MPa in a medium modified with NaCl, while under matric stress this was limited to -9.8 MPa. Significant differences in tolerance of solute or matric potential stress were observed; growth rate on matrically-modified media was often about 50% lower with respect to similar conditions of solute stress, indicating a higher sensitivity to this factor. This was also supported by the time required to reach the maximum growth

which was equal to 7 days under solute stress and 13 days in matrically-modified media. As a consequence soil colonisation will only occur over a narrower range of water availability with respect to ear colonisation.

A. flavus overwinters in soil or on maize debris; these results suggests that limiting factors can frequently be encountered and sporulation can produce inoculum of a limited concentration. In addition, spores are air-borne and they are not detected in air on rainy days. As a consequence, there is probably a limited concentration of inoculum on maize ears. The inoculum concentration required to achieve high levels of ear infection were $>10^5$ spores mL^{-1}; with lower inoculum size causing <20% infection. Inoculum concentration in fields in Italy are normally very limited and it could explain the spot infection of ears, with only a few kernels highly contaminated with AFB_1.

A. flavus inoculum had a variable infection efficiency which appeared to be related to maize growth stage at inoculation. Earlier growth stages were more susceptible to fungal infection and a significantly higher contamination resulted in kernels infected 21 days after pollination with respect to all the other growth stages considered, between 3 and 52 DAP.

Aspergillus flavus in the field is frequently co-existing with *Fusarium verticillioides*. These fungi are known to have different optimal ecological conditions (see Sanchis and Magan, 2004; Marin et al., 2004). In a specific trial managed to define the usage of carbon sources by these two species in different environmental conditions, the number of carbon sources utilized by *A. flavus* and *F. verticillioides* differed in all the conditions examined. At 0.98 a_w and 30°C the number of C-sources used was highest for *A. flavus;* in contrast *F. verticillioides* used more carbon sources at the lowest temperature tested (20°C) and with

a_w levels from 0.93 to 0.98. *Fusarium verticillioides* was dominant at lower temperatures (20°C) and when a certain level of water was available (> 0.95 a_w). In contrast, *A. flavus* was dominant only at high temperature (25-30°C) and under dry conditions (0.87a_w). The ability to assimilate different carbon sources by the two genera reflect their competitiveness in certain environmental conditions. However, only extreme conditions were linked to dominance of one of the two species tested while in almost all cases both *A. flavus* and *F. verticillioides* appeared to occupy different niches. This type of niche exclusion might partially be aided by the production of the mycotoxins by these two species which enables them to occupy separate niches (Magan and Aldred, 2007).

In field trials with different (34) maize hybrids artificially inoculated, *A. flavus* ear infection gave different visual infection ratings and internal kernel contamination. Overall in 2006 this was lower than in 2005, although aflatoxin B_1 contamination was higher in this season. This suggests that in 2006, more conducive meteorological parameters occurred. In 2005 mean temperatures were lower than in 2006 over the whole growing period while rainfall was abundant, especially during August. The hotter and drier conditions in 2006 probably caused more abiotic stress on the fungal populations, increasing aflatoxin production, although limiting growth of *A. flavus*.

Hybrids artificially inoculated with *A. flavus* contained variable contamination levels with AFB_1 and some of them had no toxin content at all; this was independent of high or low visual presence of the fungus. Almost 40% of hybrids were contaminated with levels above the legal limit for humans in both years, but 60% of the hybrids tested showed some resistance to fungal invasion and AFB_1 accumulation.

The risk of AFB_1 contamination in maize is predominantly determined by field conditions, but during storage the toxin can increase if grain management during drying

and storage is not efficient or is too long (Magan and Aldred, 2007). Storage in modified atmospheres can contribute to safe storage. It appears that 25% CO_2 does not offer any significant control of aflatoxin content under the a_w treatments examined in this study. Only partial inhibition of growth occurred, resulting in the fungus being placed under stress and aflatoxin levels similar to the untreated controls being produced. For inhibition of aflatoxin production, 50% and 75% CO_2 were effective in reducing production by 46% and 58%, respectively.

The full range of ecological data gathered in this research project should be sufficient to define quantitative relationships between fungal growth, sporulation and aflatoxin production and, as a consequence, develop a Decision Support System to enable more effective control of aflatoxin contamination of maize production in northern Italy.

CHAPTER 10

Suggestions for future work

Many interesting results were obtained on the characteristics and ecology of *Aspergillus* section *Flavi* strains isolated from northern Italy. However, it could be interesting to improve knowledge on other aspects of these fungi.

In particular, it would be important to define:

- Molecular characterization of strains collected and verify their expression in different conditions;
- The possibility to use non-aflatoxigenic isolates of *A. flavus* as biocontrol agents in the field. Molecular studies to check the presence of genes involved in AFs production and vegetative compatibility group (VCG) have to be evaluated to ensure proper use of such strains in the field;
- The dynamics of AFB_1 in post-harvest to understand possible critical control points.
- A predictive model based on systems analysis to be able to predict the risk of the presence of *A. flavus* and the production of AFBs during the growing season;
- The development of a Decision Support System that could help farmers in the management of maize crop;
- Risk areas in Italy where fungal contamination is more likely to occur and where AFBs content could be predicted and then effectively managed with proper control strategies.

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APPENDIX 1

Published papers and accepted manuscripts

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Studies on *Aspergillus* section *Flavi* isolated from maize in northern Italy

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Abstract

In 2003, for the first time in Italy, significant problems arose with colonization and contamination of maize destined for animal feed with *Aspergillus* section *Flavi* and aflatoxins (AFs). This resulted in milk and derived products being contaminated with AFM_1 at levels above the legislative limit. There was little knowledge and experience of this problem in Italy. The objectives of this research were thus to study the populations of *Aspergillus* section *Flavi* in six northern Italian regions and obtain information on the relative role of the key species, ability to produce sclerotia, production of the main toxic secondary metabolites, aflatoxins and cyclopiazonic acid, and tolerance of key environmental parameters. A total of 70 strains were isolated and they included the toxigenic species *A. flavus* and *A. parasiticus*. *A. flavus* was dominant in the populations studied, representing 93% of the strains. Seventy percent of strains of *Aspergillus* section *Flavi* produced AFs, with 50% of strains also producing cyclopiazonic acid. Sixty-two percent of *A. flavus* strains and 80% of *A. parasiticus* were able to produce sclerotia at 30 °C. Using 5/2 agar, only 1 strain developed S sclerotia and 19 L sclerotia. With regard to ecological studies, growth of *Aspergillus* section *Flavi* was optimal at between 25 and 30 °C, while AFB_1 production was optimal at 25 °C. Regarding water availability (water activity, a_w), 0.99 a_w was optimal for both growth and AFs production, while the only aflatoxin produced in the driest condition tested (0.83 a_w) was AFB_1. This information will be very useful in identifying regions at risk in northern Italy by linking climatic regional information to levels of fungal contamination present and potential for aflatoxin production in maize destined for animal feed. This would be beneficial as part of a prevention strategy for minimising AFs in this product.
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Keywords: *Aspergillus* section *Flavi*; Aflatoxin; Cyclopiazonic acid; Temperature; Water activity

1. Introduction

MAIZE is a commodity considered to be one of the most susceptible to mycotoxins world-wide (Barug et al., 2004). Maize is colonized and contaminated by a range of different fusaria, including *F. graminearum*, *F. verticillioides*, *F. proliferatum*, *F. subglutinans*, causing maize ear rot, as well as by *Aspergillus* section *Flavi*. The dominant mycotoxigenic species is strictly related to meteorological conditions in the regions of cultivation.

The optimal ecological conditions for growth and mycotoxin production differ for these important genera. *Fusarium* strains have optimum temperature for growth in the range 25–30 °C, at which higher levels of toxins are produced, e.g. fumonisins (Marin et al., 1995). *Aspergillus* strains grow over a wider temperature range. Optimal growth of *A. flavus* occurs over the range 19–35 °C (Northolt and van Egmond, 1981), with 28 °C being optimum for aflatoxin production (Scott et al., 1970; Sanchis and Magan, 2004). Water availability (water activity, a_w) also has a significant impact and *Aspergillus* strains are able to grow and produce mycotoxins down to conditions of 0.73 and 0.85 a_w, respectively. These are extremely different from *Fusarium* species, which cannot often grow below 0.90 a_w and produce trichothecenes or fumonisins at >0.93 a_w (Trucksess et al., 1988; Sanchis and Magan, 2004).

In Italy, maize is widely grown in the northern regions, where the main concern is contamination with fumonisins, produced by *F. verticillioides*, with a high incidence in most years. Deoxynivalenol is detected only sporadically, especially

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in rainy years with temperature levels lower than usual for these regions, when *F. graminearum* becomes dominant (Pietri et al., 2004). In 2003, for the first time, significant problems arose due to aflatoxin contamination of maize. The summer was particularly dry and hot, with maize crops water-stressed and consequently maize grain was highly contaminated, resulting in problems with aflatoxin M_1 (AFM_1) in milk and derived products (Battilani et al., 2005; Pinelli et al., 2005). The problems were worsened by the lack of experience of local farmers and extension staff with this new problem.

The main members of *Aspergillus* section *Flavi* able to produce aflatoxins (AFs) are *A. flavus* and *A. parasiticus* (Kurtzman et al., 1987). These are closely related fungi and difficult to distinguish from each other. It is now generally accepted that *A. flavus* produces only aflatoxin B_1 and B_2, while *A. parasiticus* produces all the four principal AFs (AFB_1, AFB_2, AFG_1 and AFG_2) (Diener et al., 1987; D'Mello and MacDonald, 1997). However, Gabal et al. (1994) reported a high percentage of *A. flavus* strains producing AFG_1 and a minor group also producing AFG_2. The International Agency for Research on Cancer classified AFB_1 as a class 1 toxin because of its demonstrated carcinogenicity to humans, while AFM_1 is possibly carcinogenic and has been classified as 2B (Castegnaro and Wild, 1995). All aflatoxins are regulated in most countries throughout the world, Europe included, in different products as well as maize and milk (CE, 2001).

Some *A. flavus* strains are also reported to produce cyclopiazonic acid (CPA), a mycotoxin typical of several species of *Penicillium*. Contradictory results exist on the mutagenic effect of CPA; however, there is evidence of its inhibitory effect on the mutagenicity of AFB_1 (Kuilman-Wahls et al., 2002).

The main objective of this study was to obtain detailed information on the characteristics of Italian *Aspergillus* section *Flavi* populations in the key milk-producing regions of northern Italy. The diversity of *A. flavus* and *A. parasiticus* was examined in a detailed survey supported by ecological trials; grouping of strains was determined using cluster analysis and *in vitro* AFB_1 production. This was essential for a better understanding of the key role of the relevant strains in AF contamination of maize.

2. Materials and methods

A total of 70 isolates of *Aspergillus* section *Flavi* were examined in this study. These strains came from an Italian maize survey carried out in field in 2003 in Friuli Venezia Giulia, Lombardy, Piedmont, Tuscany and Veneto and in 2004 in Emilia Romagna; 33, 24, 12, 10, 17 and 90 samples were collected in the cited regions (Battilani et al., 2005). Twenty ears were harvested from each field and, after husk elimination, ears were dried at 40 °C and shelled. Fifty grains of each sample were plated in Petri dishes with Peptone PCNB Agar (PPA) (Peptone 15 g; KH_2PO_4 1 g; $MgSO_4 \cdot 7H_2O$ 0.5 g; PCNB 75% 1 g; agar 8 g; H_2O to 1 L) and incubated at 25 °C for 7 days. Moulds developed from grains were purified, transferring them to Petri dishes with Potato Dextrose Agar (PDA) (infusion from potatoes 200 g; dextrose 15 g; agar 20 g; H_2O to 1 L) and after incubation at 25 °C for 7 days fungi were identified to section level. Only 1 strain of *Aspergillus* section *Flavi* for each sampled field was stored, independently of the positive grains found. These strains are part of the culture collection of the Institute of Entomology and Plant Pathology of the Università Cattolica del Sacro Cuore of Piacenza (Italy, code MPVP).

2.1. Characterization of isolates

2.1.1. Colony morphology

Strains were inoculated at the central point on Petri dishes (∅ 6 cm) with Czapek Agar (CZ) (sucrose 30 g; $NaNO_3$ 2 g; KCl 0.5 g; $MgSO_4 \cdot 7H_2O$ 0.5 g; $FeSO_4 \cdot 7H_2O$ 0.01 g; K_2HPO_4 1 g; $ZnSO_4 \cdot 7H_2O$ 0.001 g; $CuSO_4 \cdot 7H_2O$ 0.005 g; agar 15 g; H_2O to 1 L) as medium and incubated at 30 °C for 14 days in the dark. After incubation, dishes were observed for colony colour, sclerotial production and conidiophores, morphology and size. The characteristic colour of colonies for *A. flavus* is ivy green and for *A. parasiticus* cress green, according to Raper and Fennell (1965).

For microscopic observation, strains were prepared on glass slides after staining with lactic acid and lacto-phenol blue. The two relevant species, *A. flavus* and *A. parasiticus*, can be differentiated by relative conidiophore lengths (500 µm for *A. flavus* and from 200 µm to rarely more than 1 mm for *A. parasiticus*), conidiophore characteristics (*A. flavus* has thinner walled and less roughened conidiophores than those of *A. parasiticus*), but primarily by the character of their sterigmata: *A. flavus* has primary and secondary sterigmata, while *A. parasiticus* has only primary sterigmata and they are respectively termed biseriate and uniseriate (Raper and Fennell, 1965). One type strain of *A. flavus* (IMI 348543) and one of *A. parasiticus* (IMI 283883) from the official collection of CABI Bioscience (Engham, UK) were used as reference strains. Observations were carried out with a magnification of between 100 and 400×.

2.1.2. Sclerotia

Kozakiewicz (1989) reported that production of sclerotia is a rare characteristic of *A. flavus* strains only. Petri dishes were observed macroscopically to verify the presence of sclerotia, structures easily identifiable.

Sclerotial size is a phenotypic character within *A. flavus* strains (Abbas et al., 2005), that can be used to create two different groups: the large strains (L) having sclerotia >400 µm in diameter and the small strains (S) with sclerotia <400 µm (Horn, 2003); differences in strain ability to produce AFs can be linked to sclerotial size (Cotty, 1989; Chang et al., 2001).

Strains were transferred on Petri dishes with 5/2 agar (5% V8-juice; 2% agar; pH 5.2) and incubated at 31 °C for 5 to 7 days in darkness (Probst et al., 2005). Sclerotial size was evaluated by a measuring reticule with a Nikon Microscope (Nikon Inc., Garden City, NY, USA). Observations were carried out at 40× magnification.

2.1.3. Production and analysis of aflatoxins

Two approaches were followed to verify aflatoxin production: fluorescence and HPLC analysis. Strains were inoculated

at a central single point on Petri dishes (∅ 6 cm) containing Coconut Extract Agar (CEA) (20% desiccated coconut; 1.5% agar) and incubated at 25 °C for 14 days in the dark. This medium was chosen because, due to the reaction of coconut fats, strains positive for aflatoxin production can be identified by fluorescence in the reverse side of the culture (Davis et al., 1987; Pitt, 1994); furthermore, coconut-based media are optimal for AFs production (Dyer and McCammon, 1994). After incubation, colonies of all the strains were observed for fluorescence and scored as positive or negative. Then, 3 plugs were cut from each Petri dish and 1 mL of methanol added (Bragulat et al., 2001). After 1 h in methanol, the solution was filtered with a Millipore® filter (∅ 0.45 mm) (Bedford, MA, USA). The solution was analysed by reversed phase HPLC (Jasco, Easton, MD, USA) using post-column derivatization with pyridinium hydrobromide perbromide and fluorescence detection. The column was a superspher 100 RP-18 (Merck, Darmstadt, Germany) and the mobile phase was H_2O–CH_3CN–CH_3OH (64+13+23) at a flow rate of 1 mL min^{-1} (Stroka et al., 2003). Aflatoxin production was measured in ng g^{-1} of culture medium. The limit of detection was 0.5 ng g^{-1}.

2.1.4. Production and analysis of cyclopiazonic acid

All the strains were inoculated at a central single point on Petri dishes (∅ 6 cm) containing CZ and incubated at 30 °C for 14 days in the dark. Then the methodology of Bragulat et al. (2001), previously applied for AFs analysis, was used for CPA extraction. The methanolic extract was analysed by reversed phase HPLC and UV detection. The column was a LiChrosorb NH_2 (Merck, Darmstadt, Germany) and the mobile phase was CH_3CN–CH_3COONH_4 50 mM in water (80+20) at a flow rate of 1 mL min^{-1}. CPA was measured in ng g^{-1} of culture medium. The limit of detection was 50 ng g^{-1}.

2.1.5. Identification at species level

The identification of *Aspergillus* section *Flavi* was completed by taking into account a combination of all the observed criteria, including morphological observations, sclerotial production, colour of colony and AFs and CPA profiles.

2.2. Ecology of A. section Flavi

The effect of temperature and a_w level on fungal growth and AFs production was studied for 38 isolates of *A.* section *Flavi* selected among the 70 collected; strains were chosen on the basis of the place of isolation and AFs production. One strain isolated from pistachio nuts and one from peanuts were also included in the trial for comparison.

All the isolates were inoculated on CZ and incubated at 25 °C for 7 days in the dark to provide inoculum. To prevent the formation of colonies from stray spores, inoculation was made from a semisolid suspension. Small vials were prepared with a solution of 1% water–agar; a needle point of conidia of each strain was added to each vial, mixed and used later as inoculum (Pitt, 1979). Petri dishes (∅ 9 cm) with CZ were inoculated centrally with the suspension. The a_w level of the medium was 0.995. Three different temperatures were considered: 15, 25 and 30 °C, and 3 levels of a_w: 0.83, 0.94 and 0.995 (the unmodified medium), obtained by adding respectively 800, 250 and 0 mL of glycerol to 1 L of CZ medium. All plates with different a_w were incubated at 25 °C; the experiments were conducted with four replicates.

After incubation, the diameter of colonies was measured along two perpendicular diagonals crossing the inoculation point. Aflatoxin production was quantified following the method previously described.

2.3. Data analysis

Boxplot analysis, useful to highlight outliers, was performed to compare the distribution of values at the temperatures and a_w levels taken into account. This analysis was run using the statistical package SPSS (Statistical Package for Social Science ver. 11.5.1, 2002. SPSS Inc., Chicago, IL, USA).

Data on aflatoxin production in ecological trials were logarithmically transformed before statistical analysis. Log transformation is always required for data that covers a wide range from single-digit numbers to numbers in hundreds or thousands (Clewer and Scarisbrick, 2001), as a wide range of values can be obtained for AF production. The analysis of variance (ANOVA) was carried out using the statistical package MSTAT-C (Michigan State University, ver. 1, 1991, East Lansing, MI, USA), experimental design number 2: completely randomised design for factor A (temperature or a_w), factor B (strains) is a split plot. Means were compared using the Tukeys test to indicate significant differences.

Cluster analysis was performed using SPSS to create homogenous groups of strains based on logarithm transformed data of AF production in ecological trials. This analysis is based on distances which are a measure of how far apart two objects are. Selection of a distance measure should be based both on the properties of the measure and on the algorithm chosen for cluster formation. The square Euclidean distance, which is the sum of squared differences over all the variables, was used as the distance index. The average linkage between groups, often called UPGMA (unweighted pair-group method using arithmetic averages) was the clustering method followed. It defines the distance between two clusters as the average of the distances between all pairs of cases in which one member of the pair is from each of the clusters.

3. Results

3.1. Characterization of isolates

3.1.1. Colony morphology

All the information regarding the strains of *Aspergillus* section *Flavi* collected from the different maize growing regions, including colour of colonies, are shown in Table 1. All isolates were identified to species level; 65 out of the 70 strains of *Aspergillus* section *Flavi* collected from maize were identified as *A. flavus* and 5 as *A. parasiticus*. *A. flavus* represented almost all the strains collected in the regions sampled; only Emilia Romagna, Lombardy and Piedmont differed, with 3, 1 and 1 isolates of *A. parasiticus*, respectively.

Table 1
Characterization of *Aspergillus* section *Flavi* strains collected in 2003 and 2004 from 6 Italian regions

Characterization									Ecological trials							
Code	Region of maize origin[a]	Sclerotia (30°C)	Fluorescence	AFB1[b]	CPA[c]	Sclerotia size[d]	Colour	Possible identification[e]	Selected stains	AFB1[f]						Cluster analysis
										Temperature (°C)			a_w			
										15	25	30	0.83	0.94	0.99	
A 2087	ER	No	No	2	1		Ivy	AF								
A 2089	ER	No	No	1	1		Ivy	AF								
A 2090	ER	No	No	1	1	L	Ivy	AF								
A 2093	ER	Yes	No	1	4		Ivy	AF								
A 2097	ER	Yes	No	1	3	L	Ivy	AF	11	1	1	1	1	1	1	1
A 2098	ER	Yes	No	1	1	L	Ivy	AF								
A 2102	ER	Yes	No	1	3		Ivy	AF								
A 2103	ER	Yes	No	1	1		Ivy	AF								
A 2105	ER	Yes	No	1	1		Ivy	AF								
A 2107	ER	No	No	4	4		Ivy	AF								
A 2109	ER	Yes	No	1	3	L	Ivy	AF								
A 2045	FVG	Yes	No	4	3		Ivy	AF								
A 2050	FVG	Yes	No	1	3		Ivy	AF								
A 2061	V	No	No	3	3	L	Ivy	AF	10	2	3	2	1	2	4	3
A 2100	ER	Yes	No	1	3		Ivy	AF								
A 2049	V	No	No	1	2		Ivy	AF	32	1	1	1	1	1	1	1
A 2086	ER	Yes	Yes	5	3		Ivy	AF	8	4	3	1	1	2	4	3
A 2091	ER	Yes	Yes	5	4		Ivy	AF	36	3	5	2	2	3	5	2
A 2092	ER	Yes	Yes	5	4		Ivy	AF	18	2	5	4	2	4	4	2
A 2094	ER	Yes	Yes	3	4		Ivy	AF	22	1	1	1	1	1	4	3
A 2095	ER	Yes	Yes	5	4		Ivy	AF	27	2	5	1	2	5	5	2
A 2099	ER	Yes	Yes	3	1		Ivy	AF	13	1	1	1	1	1	1	1
A 2101	ER	Yes	Yes	5	2		Ivy	AF	29	2	4	1	1	1	3	3
A 2104	ER	Yes	Yes	5	1	L	Ivy	AF	12	4	3	3	1	1	4	3
A 2106	ER	No	Yes	4	2		Ivy	AF	37	1	2	1	1	1	1	1
A 2041	FVG	Yes	Yes	3	3		Ivy	AF	16	1	2	1	2	4	1	1
A 2044	FVG	Yes	Yes	2	1	L	Ivy	AF								
A 2046	FVG	No	Yes	5	2		Ivy	AF	17	3	3	2	2	3	4	3
A 2056	FVG	No	Yes	1	3		Ivy	AF	39	1	1	1	1	1	1	1
A 2067	FVG	Yes	Yes	5	4	L	Ivy	AF	9	2	2	1	1	2	3	3
A 2068	FVG	Yes	Yes	5	4	L	Ivy	AF	21	1	4	3	1	3	5	2
A 2071	FVG	No	Yes	5	3		Ivy	AF	5	2	4	2	1	3	4	3
A 2074	FVG	Yes	Yes	2	2		Ivy	AF								
A 2075	FVG	Yes	Yes	2	2		Ivy	AF								
A 2079	FVG	No	Yes	3	2		Ivy	AF	31	1	1	1	1	1	1	1
A 2080	FVG	Yes	Yes	2	1		Ivy	AF								
A 2081	FVG	No	Yes	2	2		Ivy	AF								
A 2082	FVG	Yes	Yes	1	1		Ivy	AF	34	1	1	1	1	1	1	1
A 2047	L	Yes	Yes	2	2	L	Ivy	AF								
A 2052	L	Yes	Yes	3	4		Ivy	AF	38	2	4	1	1	3	4	3
A 2053	L	Yes	Yes	5	4		Ivy	AF	20	1	4	1	1	2	3	3
A 2063	L	Yes	Yes	4	4	L	Ivy	AF	14	3	4	3	1	4	4	2
A 2078	L	No	Yes	5	3		Ivy	AF	33	1	3	3	1	1	4	3
A 2042	P	Yes	Yes	1	1		Ivy	AF	28	1	1	1	1	1	1	1
A 2059	P	Yes	Yes	5	3		Ivy	AF	23	2	5	2	1	2	5	2
A 2070	P	Yes	Yes	4	4	L	Ivy	AF	15	3	4	3	1	4	4	2
A 2073	P	Yes	Yes	4	4	L	Ivy	AF	30	1	3	1	1	1	4	3
A 2072	T	No	Yes	2	2		Ivy	AF								
A 2040	V	Yes	Yes	4	1	S	Ivy	AF	26	3	5	4	1	1	5	2
A 2043	V	No	Yes	2	1		Ivy	AF								
A 2055	V	Yes	Yes	4	1		Ivy	AF	19	2	3	1	1	1	4	3
A 2060	V	No	Yes	5	3		Ivy	AF	25	1	2	1	1	1	1	1
A 2064	V	Yes	Yes	2	1	L	Ivy	AF								
A 2076	V	Yes	Yes	2	1		Ivy	AF								
A 2077	V	No	Yes	2	4	L	Ivy	AF	6	1	1	1	1	1	1	1
A 2069	ER	No	Yes	5	3		Ivy	AF	3	1	4	3	1	1	4	3
A 2084	ER	Yes	Yes	2	1		Ivy	AF								

(continued on next page)

Table 1 (*continued*)

Characterization									Ecological trials							
Code	Region of maize origin[a]	Sclerotia (30°C)	Fluorescence	AFB1[b]	CPA[c]	Sclerotia size[d]	Colour	Possible identification[e]	Selected stains	AFB1[f]						Cluster analysis
										Temperature (°C)			a_w			
										15	25	30	0.83	0.94	0.99	
A 2085	ER	Yes	Yes	1	1		Ivy	AF								
A 2051	FVG	No	Yes	1	1		Ivy	AF								
A 2058	FVG	No	Yes	2	1		Ivy	AF								
A 2039	L	No	Yes	5	2		Ivy	AF	24	3	3	3	1	4	3	3
A 2048	L	No	Yes	1	1		Ivy	AF	35	1	1	1	1	1	1	1
A 2054	L	No	Yes	4	3		Ivy	AF	1	1	1	1	1	1	1	1
A 2065	L	No	Yes	3	3		Ivy	AF	7	1	3	2	1	2	3	3
A 2062	V	No	Yes	5	4	L	Ivy	AF	40	3	4	2	1	1	5	3
A 2088	ER	Yes	No	1	1	L	Cress	AP								
A 2096	ER	Yes	No	1	1	L	Cress	AP								
A 2108	ER	No	No	1	1		Cress	AP								
A 2110	I — pe	No	Yes	2	2		Ivy	AF	2	1	3	1	2	1	2	1
A 2111	I — pn	No	Yes	2	4		Ivy	AF	4	2	2	1	1	1	2	1
A 2066	L	Yes	Yes	2	1	L	Cress	AP								
A 2057	P	Yes	Yes	2	1		Cress	AP								
IMI 283883	Unknown	Yes	No	2	1		Cress	AP								
IMI 348543	USA	Yes	No	1	1		Ivy	AF								

[a] ER = Emilia Romagna; FVG = Friuli Venezia Giulia; V = Veneto; L = Lombardy; P = Piedmont; T = Tuscany; I = Iran; pe = peanuts; pn = pistachio nuts.
[b] Class of production of AFB1 as reported in Table 2.
[c] Class of production of CPA as reported in Table 3.
[d] Sclerotia size: L: sclerotia diameter > 400 μm; S: sclerotia diameter < 400 μm.
[e] AF = *A. flavus*; AP = *A. parasiticus*.
[f] Data from ecological trials.

3.1.2. Sclerotia

Forty-four strains (63% of total strains) were able to produce sclerotia at 30 °C on CZ, 4 of these were identified as *A. parasiticus*.

Using the approach based on sclerotial size (Cotty, 1989; Chang et al., 2001), only 20 strains (29% of total strains) were able to produce sclerotia and among these only 1 produced the characteristic small sclerotia (S). Distribution of strains based on sclerotial diameter is shown in Fig. 1.

3.1.3. Production of aflatoxin

Seventy-three percent of *Aspergillus* section *Flavi* strains showed fluorescence when inoculated on CEA and 70% of strains were positive when tested by HPLC; 6 strains which showed fluorescence on CEA were not confirmed as AF producers using HPLC analysis and 4 strains without fluorescence on CEA were positive when tested with HPLC. In the population studied, many strains (approx. 23%) produced <10 ng g^{-1} of medium; however, approx. 25% of strains were able to produce >1000 ng g^{-1} in 14 days in the *in vitro* conditions used (Table 2).

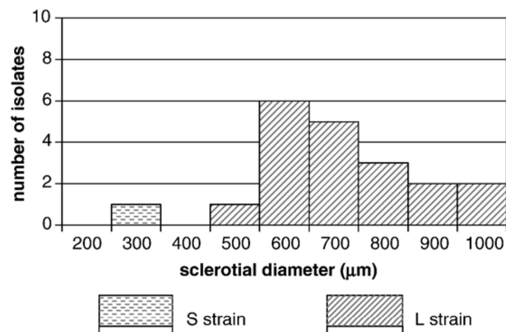

Fig. 1. Distribution of strains based on sclerotia diameter (S = sclerotia diameter <400 μm; L = sclerotia diameter >400 μm).

3.1.4. Production of cyclopiazonic acid

Forty-three strains (61% of tested strains) were able to produce CPA; around 20% of strains were able to produce >2000 ng g^{-1} of medium and among these none was identified as *A. parasiticus* (Table 3).

3.1.5. Identification of chemotypes

The strains were classified into seven chemotypes based on AFs and CPA production patterns (Table 4). This classification was elaborated similarly to that obtained from a survey

Table 2
Distribution of *Aspergillus* section *Flavi* strains, isolated from maize in 6 Italian regions, in classes of aflatoxin B1 production after incubation at 25 °C for 14 days on CZ in the dark

Class	AF (ng g^{-1})	Number of strains	% of strains
1	None	21	30
2	<10	16	22.8
3	10–100	7	10
4	100–1000	9	12.8
5	>1000	17	24.3

Table 3
Distribution of *Aspergillus* section *Flavi* strains, isolated from maize in 6 Italian regions, in classes of cyclopiazonic acid production after incubation at 25 °C for 14 days on CZ in the dark

Class	CPA (ng g^{-1})	Number of strains	% of strains
1	None	27	39
2	<1000	11	16
3	1000–2000	17	24
4	>2000	15	21

conducted in Iran (Razzaghi-Abyaneh et al., 2006). Isolates able to produce both AFB and CPA represented the most represented chemotype (around 39% of total strains). No strains were found able to produce more AFB_2 than AFB_1. Isolates able to produce both AFB and AFG were classified as two different chemotypes: one with strains able to produce also CPA (around 11% of total strains) and one with strains not able to produce CPA (around 1% of total strains). Around 19% of total strains were of the chemotype representing isolates without ability to produce any toxin. Some other strains were able to produce either AFB or CPA and were included in two different chemotypes.

3.2. Ecology of Aspergillus section Flavi

The strains used for ecological studies are detailed in Table 1.

3.2.1. Temperature

Fungal growth was markedly affected by temperature (Fig. 2). At 15 °C the growth was the slowest, while at 25 and 30 °C it was very similar and significantly higher, as shown by boxplot analysis and confirmed by ANOVA ($P \leq 0.01$).

As regards AFB_1 production (Fig. 2), most strains produced the highest quantities at 25 °C, while at 15 °C and 30 °C the number of positive strains decreased as did the amount produced. ANOVA showed significant differences between strains ($P \leq 0.01$). In particular, A 2092 and A 2040 were the best producers with 742 ng AFB_1 per g of medium, as mean of all temperatures.

Aflatoxin B_1 was produced by 29 of the tested strains (73%) at 25 °C; 11 strains never produced AFs under any of the temperatures tested. The range of AF production was between 0 and 423 ng g^{-1} at 15 °C; between 0 and 2406 ng g^{-1} at 25 °C and between 0 and 505 ng g^{-1} at 30 °C. Four strains of *A. flavus* were able to produce AFG_1 and AFG_2 at 15 °C. Six

Table 4
Chemotype patterns of *Aspergillus* section *Flavi* strains based on aflatoxins and CPA production

Chemotype	Mycotoxins			
	AFB	AFG	CPA	No. of isolates
I (B1>B2)	+	−	+	27
II (B1<B2)	+	−	+	0
III	+	−	−	13
IV	−	−	+	8
V	−	−	−	13
VI	+	+	+	8
VII	+	+	−	1

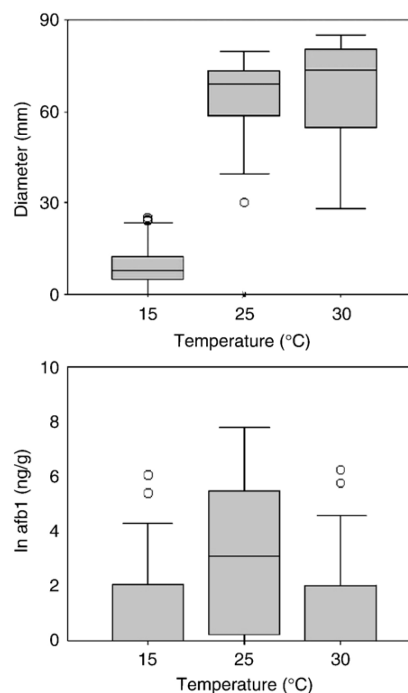

Fig. 2. Boxplot analysis for fungal growth (Diameter) and aflatoxin B1 production (ln afb1) of 40 strains of *Aspergillus* section *Flavi* inoculated on CZ and incubated at 3 different *T* (15, 25 and 30 °C) for 14 days in the dark. The boxplot analysis shows the interquartile range of each examined temperature (box), the median (line inside the box), minimum and maximum values (whiskers); circles represent values 1.5–3 times outside the interquartile range; squares represent values more than 3 times outside the interquartile range.

strains of *A. flavus* produced G_1 only at 25 °C. At 30 °C none of the examined strains was able to produce AFG_1 or AFG_2. AFB_2 was synthesized at 15, 25 and 30 °C, respectively by 21, 77 and 31% of strains able to produce also AFB_1. The behaviour of strains isolated from peanuts and pistachio nuts was in the range of variation of maize strains from northern Italian regions.

3.2.2. Water activity

Fungal growth was significantly influenced by a_w level as shown by the boxplot analysis (Fig. 3). In particular, at 0.83 a_w growth values were very different and lower than those obtained at the other two a_w levels; significant differences were confirmed by ANOVA among all the a_w levels tested ($P \leq 0.01$). Significant differences among strains were also observed ($P \leq 0.01$); in particular, A 2046 showed the fastest and A 2095 the slowest colonization rate (62.3 vs. 18.8 mm colony diameter, respectively).

Regarding AFs production, the boxplot analysis (Fig. 3) shows that 0.99 a_w was the best condition, while only traces of AFs were detected in the driest condition tested (0.83 a_w). ANOVA demonstrated significant differences in aflatoxin production among all the tested a_w levels, confirming that 0.99 a_w is the optimal condition for *Aspergillus* section *Flavi* strains. Significant differences were also observed among strains ($P \leq 0.01$); AFB_1 production by strain A 2095 resulted

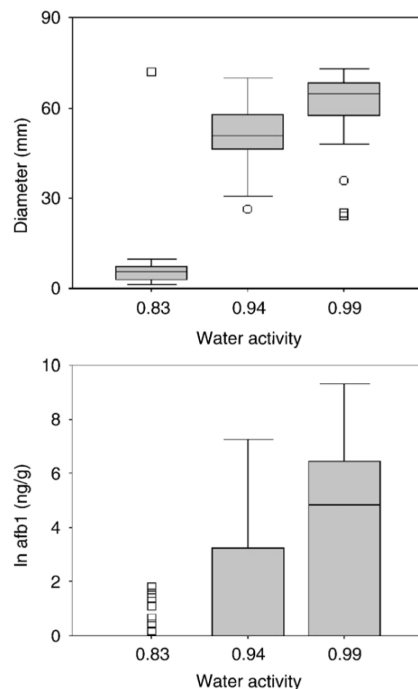

Fig. 3. Boxplot analysis for fungal growth (Diameter) and aflatoxin B1 (ln afb1) production of 40 strains of *Aspergillus* section *Flavi* inoculated on CZ with 3 levels of a_w (0.83, 0.94 and 0.99) incubated at 25 °C for 14 days in the dark.

significantly higher when compared to that of all the other strains.

Twelve strains never produced AFB_1 under any of the a_w levels considered. The range of AFB_1 production was between 0 and 5 ng g^{-1} of medium at 0.83 a_w, between 0 and 1423 ng g^{-1} at 0.94 a_w and between 0 and 11039 ng g^{-1} at 0.99 a_w.

Six, 17 and 25 of the tested strains were able to produce AFB_1 at 0.83, 0.94 and 0.99 a_w, respectively. No strain was able to produce AFB_2 at 0.83 a_w, while 11 and 24 strains were able to produce this aflatoxin at 0.94 and 0.99 a_w respectively. AFG_1 and AFG_2 were never detected in this experiment.

3.2.3. Cluster analysis

Cluster analysis ran on AFB_1 produced in ecological trials (logarithm transformed) resulted in 3 groups of strains (Table 1). Group 1 included 14 strains, not producers or very weak producers; there were 8 strains in group 2 and they were all mean producers, while the 17 strains in group 3 were markedly influenced by ecological conditions and did not produce in marginal conditions. No geographic relation was found in strains included in the same cluster.

4. Discussion

The results obtained in this study have provided, for the first time, important information about the presence, characteristics and distribution of *Aspergillus* section *Flavi* in maize in northern Italy. Ninety-three percent of the 70 strains studied belonged to *A. flavus* and only 7% to *A. parasiticus*. Distribution of strains between the two main species was quite different to other studies conducted in the United States (US). In fact, during a similar trial in Illinois, Wicklow et al. (1998) found that the percentage of *A. flavus* strains was 72% and that of *A. parasiticus* was 28%. However, the percentage of strains positive for aflatoxin production differed markedly, with 70% in the Italian population and only 53% in the US.

Sixty-two percent of Italian *A. flavus* strains and 80% of *A. parasiticus* were able to produce sclerotia at 30 °C, 28–30 °C being the optimal temperatures reported (Domsch et al., 1980). Our results are quite different from those obtained in a study conducted in Illinois, where 98% of *A. flavus* strains isolated from field produced sclerotia at 25 °C (Wicklow et al., 1998) even if in the cited study the ability to produce sclerotia was additionally checked on PDA. Shearer et al. (1992) demonstrated, during a monitoring trial in the US, that the percentage of toxigenic strains changes consistently from one year to the next, as does sclerotia development. These aspects cannot be checked for the Italian strains because they were collected in the same year.

Regarding sclerotial size, determined according to Orum et al. (1997), only 1 strain developed S sclerotia and the other 19 L sclerotia. The distribution of these two different sizes of sclerotia seems related to environmental factors. In fact, in Kenya the majority belong to S strains (73% of tested strains) (Probst et al., 2005), while in the US, in a limited area of Texas, Louisiana and in Mississippi, S strains were more abundant; on the contrary, L strains were dominant in Virginia (Horn and Dorner, 1998). Bennett et al. (1979) found no correlation between aflatoxin and sclerotial production, but recently some tentative attempts to correlate high or low AF production to the size of sclerotia have given contrasting results. Probst et al. (2005) found S strains were high aflatoxin producers (665 μg g^{-1} versus 40 μg g^{-1} for L strains), while Abbas et al. (2005) found the opposite with L isolates producing the highest levels of AF (10,000 μg g^{-1}). In our study no comments are possible on this aspect.

Sixty-one percent of the total strains were able to produce CPA; among these, none was *A. parasiticus*. Thirty-five strains of *A. flavus* were able to produce both CPA and AFs. Co-occurrence of both mycotoxins has previously been reported on maize and peanuts by Urano et al. (1992) and Fernandez-Pinto et al. (2001). This is interesting and relevant, but more detailed studies are required on CPA to understand its possible role in inhibiting the mutagenic action of AFB_1 (Kuilman-Wahls et al., 2002).

The chemotypes found in this study differ from those found in Iran (Razzaghi-Abyaneh et al., 2006). We never found strains able to produce more AFB_2 than AFB_1; further, the group able to produce both AFB and CPA was the most represented in our study, while the non-toxigenic group was dominant in Iran.

Ecological trials showed the range 25–30 °C as optimal for *Aspergillus* section *Flavi* growth and 25 °C for AFB_1 production. This suggests that Italian strains could be less thermophilic than those isolated in other geographic areas. In fact, previous studies by Scott et al. (1970) and Kheiralla et al. (1992), considered 28 and 30 °C the optimal temperatures for

toxin production. Another interesting point is that AFG_1 and AFG_2 were produced only at 15 °C by 1 strain and at 25 °C by only 3 strains. Regarding a_w, 0.99 was the optimal condition both for growth and AFs production. According to Hill et al. (1985), a_w profiles for growth and AFs production are different, as are marginal conditions for growth and AFs production, being 0.77 and 0.83 a_w, respectively (Sanchis and Magan, 2004). In this study, the only AF produced at the marginal condition of 0.83 a_w was AFB_1, detected only in 6 strains. At present it is possible to establish that 15 °C and 0.83 a_w are the limit conditions for growth and AFs production by some strains of *Aspergillus* section *Flavi*, substantially in agreement with other studies (Sanchis and Magan, 2004); further trials are necessary to improve knowledge on conducive and inhibitory conditions for toxin synthesis.

In conclusion, this study has provided for the first time a significant body of relevant information on the key species responsible for AFs contamination of maize used for human food and animal feed in the important milk-producing northern regions of Italy. The information will be useful in identifying risk regions by linking regional climatic information to the levels of contamination present and the potential for AFs production.

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Effect of a_w and CO_2 level on *Aspergillus flavus* growth and aflatoxin production in high moisture maize post-harvest

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Abstract

The potential for using modified atmospheres of 25–75% CO_2 (balanced with N_2) and water activity (a_w, 0.95, 0.92) to control *Aspergillus flavus* development and aflatoxin B_1 production has been evaluated (a) on synthetic medium and (b) on maize grain during storage for up to 21 days at 25 °C. On agar medium up to 75% CO_2 at both 0.95 and 0.92 a_w significant inhibition of growth was obtained ($P<0.05$). In stored grain inoculated with spores of *A. flavus* there was significantly higher populations of the species at 0.95 a_w than 0.92 a_w. Up to 75% CO_2 resulted in an inhibition of the populations of *A. flavus* isolated from the grain. Contrasting aflatoxin B_1 production was obtained on agar and in stored maize grain. On agar, greatest amounts were produced at 0.92 a_w, while more was produced at 0.95 a_w on maize grain. Overall, the efficacy of controlled atmospheres × a_w showed that treatment with 25% CO_2 could be sufficient to efficiently reduce *A. flavus* development but at least 50% CO_2 was required to obtain a significant reduction of aflatoxin synthesis.
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Keywords: *Aspergillus flavus*; CO_2; Water activity; Post-harvest; Aflatoxin

1. Introduction

Maize is one of the most widely distributed food plants in the world and its infection by fungi can result in mycotoxin contamination during the growing, harvesting, storage, transporting and processing stages (Bradburn et al., 1993). The main fungal species and mycotoxins of concern are *Aspergillus flavus* and aflatoxins, *Fusarium verticillioides* and fumonisins, *F. graminearum* and trichothecenes and zearalenones.

A. flavus can infect maize pre-and post-harvest and can result in an increase in aflatoxin contamination if the drying and storage phases are poorly managed. There is information on the effect of some abiotic factors on growth and aflatoxin production by *A. flavus*. It grows well in the range 19–35 °C (Northolt and van Egmond, 1981) with 28 °C being optimum for aflatoxin production (Scott et al., 1970; Sanchis and Magan, 2004). *A. flavus* can grow and produce mycotoxins down to 0.73 and 0.85 water activity (a_w) respectively (Sanchis and Magan, 2004). This corresponds to 8–12% and 17–19% moisture content (MC) (Battilani et al., 2007). Usually maize is stored in silos at 14% MC. Inefficient drying or water ingress can cause pockets of wetter grain resulting in a higher MC (Magan and Aldred, 2007).

In stored grain ecosystems, the most important abiotic conditions identified which influence growth and mycotoxin production are a_w, temperature and, when grain is moist, gas composition (Guynot et al., 2003; Magan et al., 2004). In particular, interactions between these factors can determine whether contamination increases and mycotoxins are produced. While a significant body of information is available on water and temperature relations of mycotoxigenic fungi, less is available on interactions with gas composition. Detailed studies have been conducted on effects of elevated CO_2 on growth of both *Aspergillus ochraceus* and *Penicillium verrucosum* and ochratoxin production (Paster et al., 1983; Cairns-Fuller et al., 2005). Recently, studies have suggested that up to 50%, CO_2 had only a slight impact on ochratoxin production by *Aspergillus carbonarius* over a range of a_w conditions (Pateraki et al., 2007). Samapundo et al. (2007) found that fumonisin B_1

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production by *Fusarium* section Liseola species was inhibited with 30% CO_2 at 0.984 a_w. However, only a few experiments have examined *A. flavus*. Studies of several modified atmospheres with different CO_2 levels balanced with O_2 and N_2, showed that *A. flavus* grew on wheat and rye bread with up to 75% CO_2 (Suhr and Nielsen, 2005). Previously, Wilson and Jay (1975) tested a high CO_2 treatment (61.7% CO_2 balanced with O_2 and N_2) on moist maize and found that *A. flavus* growth was visible after 4 weeks at 27 °C. The contamination with aflatoxin was lower than that in air.

The objectives of this study were to determine (a) the impact of interacting conditions of CO_2 (up to 75%) and a_w (0.92, 0.95) on growth and aflatoxin production on a Potato Dextrose Agar (PDA) medium and (b) its inhibitory effect on populations and aflatoxin B_1 (AFB_1) in stored maize grain inoculated with *A. flavus* spores.

2. Materials and methods

An aflatoxin producer strain of *A. flavus* (MPVP A 2092; Giorni et al., 2007) was inoculated on Petri dishes containing PDA (Amersham), incubated at 25 °C for 7 days and then used to produce the inoculum adjusted to 10^6 spores mL^{-1}.

2.1. Fungal growth

2.1.1. In vitro studies

Petri dishes (Ø 9 cm), containing PDA (Amersham Pharmacia Biotech, Inc., Uppsala, Sweden) adjusted with glycerol–water solutions at 0.92 and 0.95 a_w, were centrally inoculated with a drop of the *A. flavus* suspension (10^6 spores mL^{-1}). The diameter of the fungal colonies was measured, after 7 and 14 days, along two perpendicular diagonals crossing the inoculation point. All the trials were conducted in quadruplicate.

2.1.2. Maize grain studies

Maize grain, hybrid Lolita (FAO class 500) grown in Cremona province (northern Italy) in 2005, was used in this study. This maize was previously tested for fungal population and mycotoxins content. It resulted to have 13% of kernels infected by *Fusarium verticillioides* and, from a mass–mass HPLC analysis, a fumonisin B_1 level of 5.3 µg g^{-1}. No aflatoxin was detected.

A moisture adsorption curve was prepared for the maize in order to accurately determine the amounts of water required to add to 960 g maize to obtain the target a_w levels of 0.95 and 0.92. This curve was obtained adding different quantities of water to maize grains and calculating their moisture content (as difference in weight before and after 1 night at 130 °C) and comparing it with their a_w level measured with Novasina a_w sprint (Novatron Ltd, Horsham, West Sussex, UK).

The required amounts of water were added to the maize and this was stored at 4 °C overnight to equilibrate the treatments. Then, maize was inoculated at room temperature in order to obtain a final concentration of 10^4 spores g^{-1}, by mixing thoroughly and then decanting the maize (20 g) into solid culture vessels (Magenta, Sigma Ltd, U.K.) closed with plastic lids containing a permeable membrane and placed in the chambers. A total of 20 kernels at each a_w were also plated on PDA and incubated for 5 days at 25 °C and this showed that 100% of the plated kernels were contaminated with *A. flavus*.

Petri dishes and storage containers were put in plastic chambers (36 L volume) with inlet and outlet tubes to allow gas mixture to pass through them. The inlet was connected inside the chamber to a sparger, which was placed in a flask containing glycerol–water solutions appropriate to maintain the equilibrium relative humidity of the gas mixtures and the atmosphere in each chamber at the target a_w level. A computerised gas blender (Signal Series 850 Gas blender, Camberley, UK) was used to provide the four treatments: (1) normal air (21% O_2, 0.03% CO_2, 79% N_2); (2) 25% CO_2; (3) 50% CO_2; (4) 75% CO_2. The modified levels of CO_2 were obtained by reducing O_2 to$<$1% and increasing N_2 to 74, 49 and 24% respectively. Gas composition was also periodically checked with a gas chromatograph (GC; Carlo Erba model GC-8340, Carlo Erba Instruments, Hemel Hempstead, UK) to ensure the maintenance of the correct gases proportions. All the chambers were maintained in a 25 °C constant temperature room. The exhaust gases were channelled outside the room to avoid CO_2 build up.

Maize grain samples were destructively sampled after 7, 14 and 21 days and the *A. flavus* populations (CFUs) g^{-1} of grain determined. In all cases three replicate Petri dishes were used per treatment condition.

2.2. Aflatoxin extraction and analysis

Three plugs (4.6 mm, diameter) of agar were sampled from the colonies grown on Petri dishes after 14 days incubation; they were put in a vial and 1 mL of methanol was added (Bragulat et al., 2001). After 1 h, the solution was filtered through a Millipore® filter (Millex SLHV 013 NL, 0.45 µm) (Bedford, MA, USA).

For maize grain samples, 10 g sub-samples were milled into flour from each sample used for the analysis. Flour was extracted with 100 mL of CH_3OH-H_2O (80+20), stirring for 45 min and then the extract was filtered with a Whatman 595 1/2 (Dassel, Germany) paper filter, 5 mL of the solution was passed, after dilution with 45 mL of H_2O, into an Easy Extract Aflatoxin immuno-affinity column (*r-Biopharm Rhône Ltd*, Glasgow, UK), then the column was washed with 5 mL of H_2O. Aflatoxins were eluted with 2.5 mL of CH_3OH and the solution was concentrated to 1 mL with a stream of nitrogen. Then, 1 mL of CH_3CN-H_2O (25+75) was added and the solution was filtered through a Millipore® filter.

Filtered solutions, extracted from fungal colonies and maize grain, were analysed by reversed phase HPLC (Jasco, Easton, MD, USA) using post-column derivatization with a UVE instrument (LCTech GMBH, Postfach-Dorfen, Germany) set at 254 nm and fluorescence detection. The column was a Superspher 100 RP-18 (Merck, Darmstadt, Germany) and the mobile phase was H_2O-CH_3CN-CH_3OH (64+13+23) at a flow rate of 1 mL min^{-1} (Stroka et al., 2003). Aflatoxin production was quantified in ng g^{-1} of kernels or culture medium. The limit of

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Table 1
Effect of modified atmosphere and a_w on (a) in vitro growth (colony diameter, 7 days of incubation) and aflatoxin B_1 production at 25 °C (14 days incubation) (b) populations of *A. flavus*, and aflatoxin B_1 production at 25 °C (0, 7, 14 and 21 days incubation)

	(a) Synthetic medium				(b) Maize grain			
	Growth (mm)		AFB_1 (ng/g)		CFU/g (log 10)		AFB_1 (ng/g)	
% CO_2 in air								
0	67	a	713	b	7	a	300	a
25	40	b	1237	a	6	b	79	bc
50	19	c	62	c	8	a	5	c
75	7	d	9	d	6	b	128	b
a_w								
0.92	25	b	541	a	6	b	40	b
0.95	41	a	470	b	8	a	216	a
Time (days)								
0	ND		ND		4	b	0	c
7	ND		ND		6	b	242	a
14	ND		ND		7	b	81	b
21	ND		ND		8	a	60	b

ND = not detected.
Treatments with different letters mean differences statistically significant.

detection was 0.1 ng g^{-1}. Average recovery values were: 97.8± 1.6% for AFB_1 and 93.5±2.3% for AFB_2.

2.3. Statistical analysis

Data on CFU and AFB_1 production (values + 1) were logarithmically transformed before statistical analysis. This was required because of the wide range of variability (from single-digit numbers to numbers in hundreds or thousands) (Clewer and Scarisbrick, 2001). Total AFB_1 produced in each fungal colony grown *in vitro* was computed taking into account the weight of the colony and the amount of AFB_1 produced per g. Mean values of AFB_1 content obtained at the four CO_2 conditions, both from the experiment *in vitro* and that with maize grains, were converted to a 0–1 scale before analysis. This conversion was performed by relating mean values to the maximum value obtained in the experiment; the results represent the rate of toxin production (0: no aflatoxin production; 1: maximum aflatoxin production). This conversion was necessary to compare results of toxin production obtained in different experiments.

Analysis of variance was performed considering all factors (a_w, air composition and time, when appropriate); a randomized complete block design of the statistical package SPSS was used (Statistical Package for Social Science ver. 11.5.1, 2002. SPSS Inc., Chicago, IL USA). Means were compared using the LSD test to indicate significant differences.

3. Results

3.1. Fungal growth on agar and maize grain

Fungal growth on artificial media was highly influenced by both CO_2 and a_w level. Mycelial extension of *A. flavus* was slower at 0.92 a_w than at 0.95 a_w (25 mm vs 41 mm). A significantly slower growth was observed at each increment in CO_2 level with a reduction of 40%, 70% and 90% respectively (Table 1). Statistically, all the factors considered (atmospheric gas composition and a_w) significantly influenced fungal growth ($P<0.01$) (see Table 2).

The populations of *A. flavus* (CFU g^{-1}) on stored maize grain were significantly lower with 25 and 75% CO_2 in the atmosphere. However, at 0.95 a_w the populations were about ten times higher with respect to 0.92 a_w and they significantly increased only after 21 days incubation (Table 1). Fungal growth was significantly influenced by all three factors considered (atmospheric gas composition, a_w and incubation time) with CO_2 and its interaction with incubation time explaining 35% and 34% of variance respectively (see Table 2).

3.2. Aflatoxins production

Aflatoxins were detected in both *in vitro* agar and stored maize samples analysed. Overall, AFB_1 was the predominant aflatoxin found, with AFB_2 being 0.5% and 5% of AFB_1 respectively on agar and on maize grain. In general, the mean production of AFB_1 was 19.9 ng g^{-1} and 242.3 ng g^{-1} in the *in vitro* trial and on kernels respectively, while AFB_2 was 0.1 ng g^{-1} and 6.7 ng g^{-1} in the same experiments. Aflatoxins G_1 and G_2 were never detected.

The production of AFB_1 by *A. flavus* on synthetic medium almost doubled in the 25% CO_2 treatment, while incubation with 50% and 75% CO_2 reduced the toxin level by 91 and 99% relative to the untreated controls (see Table 1). Regarding a_w, significantly higher AFB_1 production was

Table 2
Analysis of variance of fungal growth and aflatoxin B_1 content for in vitro agar studies and on maize grain

	Synthetic medium			
	Fungal growth (mean diameter)		AFB_1 production (ng/g)	
	% Explained variance		% Explained variance	
A) CO_2 level	89	S	19	S
B) a_w	9	S	21	S
A×B	2	S	60	S
	Maize grain			
	Population (CFU/g)		AFB_1 production (ng/g)	
Factors	% Explained variance		% Explained variance	
A) CO_2 level	35	S	31	S
B) a_w	13	S	10	S
C) time	13	S	35	S
A×B	2	NS	7	NS
A×C	34	S	6	NS
B×C	1	NS	5	NS
A×B×C	2	NS	6	NS

Significant (*S*; $P \leq 0.01$) and non significant (NS) differences were indicated. Data were log transformed before statistical analyses.

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observed at 0.92 when compared to 0.95. From a statistical point of view, all the factors considered (atmospheric gas composition and a_w) significantly influenced the toxin production ($P<0.01$) (Table 2).

On stored maize, all the treatments with CO_2 could be considered efficient in reducing toxin production. Overall, 25%, 50% and 75% CO_2 were able to decrease AFB_1 by 74%, 98% and 57% respectively (Table 1). Significant differences in aflatoxin production were also found between the two a_w levels with AFB_1 content 81% lower at 0.92 a_w with respect to 0.95 a_w. There was also a temporal effect on AFB_1 production. The AFB_1 amounts were highest after 7 days and then decreased over the subsequent period up to the end of the experiment (21 days). ANOVA highlighted significant influences of all the principal factors involved (atmospheric gas composition, a_w and time) ($P\leq 0.01$) (Table 2).

Interactions between CO_2 and a_w for both *in vitro* and in grain experiments are shown in Fig. 1. On synthetic medium there was a stimulation of AFB_1 production by *A. flavus* colonies in air at 0.92 a_w and by 25% CO_2 at 0.95 a_w. In maize grain, AFB_1 content was highest in air while with increasing CO_2 levels the toxin production was significantly reduced.

The overall evaluation of both trials are summarised in Fig. 2. This shows the impact of elevated CO_2 and suggests that, while exposure to increased CO_2 does decrease AFB_1 production, at least 50% CO_2 is necessary to obtain a significant ($P<0.05$) reduction when compared to unmodified atmosphere.

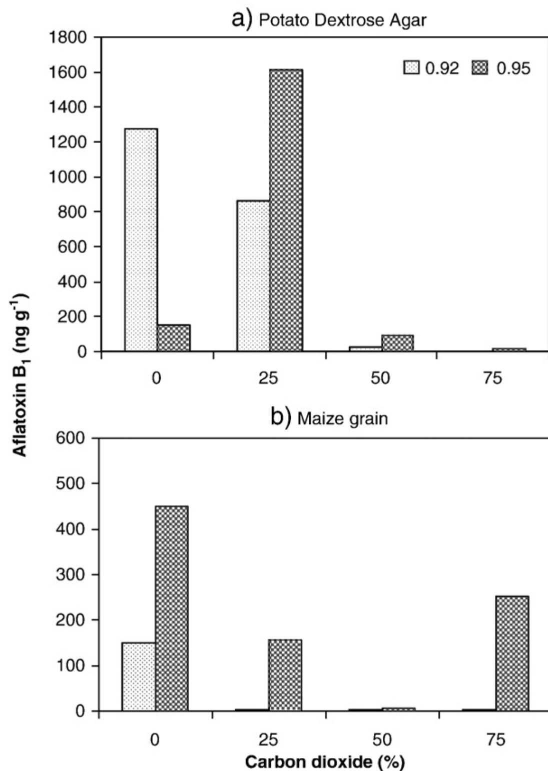

Fig. 1. Mean aflatoxin B_1 production by *A. flavus* on (a) potato dextrose agar (b) on stored maize grain in relation to the different modified atmosphere conditions used at 25 °C (note that different scales are used in *in vitro* and maize grain plots).

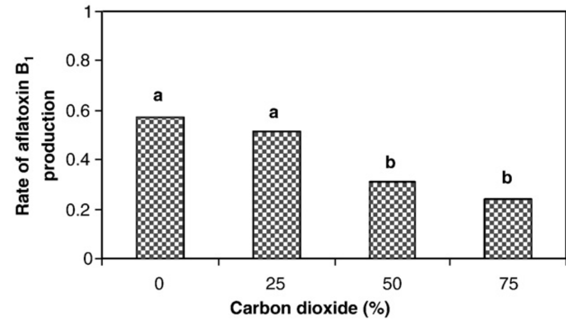

Fig. 2. Relative impact of different CO_2 concentrations on aflatoxin B_1 production by A. flavus. Data are shown in a 0–1 scale that represents a rate of toxin production (0: no aflatoxin; 1: maximum aflatoxin production) and include both data sets from in vitro and on maize grain after 14 days of incubation at 25 °C (see Materials and methods for details).Treatments followed by different letters are significantly different.

4. Discussion

4.1. Effects of modified atmosphere on growth

This study considered the effect of interactions between a_w and CO_2 concentrations on mycelial extension of *A. flavus* and the ability to colonise maize grain from an initial spore-based inoculum. The study showed that the effect on growth and AFB_1 production varied significantly. Considering the experiments *in vitro* and *in situ* together, growth was more rapid at 0.95 than 0.92 a_w ($P<0.01$), while interaction with CO_2 significantly decreased the ability to grow and colonise maize grain. The use of modified atmospheres at 25 and 50% CO_2 resulted in about 30–35% inhibition of growth/CFUs/g grain. Exposure to 75% CO_2 resulted in > 50% inhibition of growth regardless of a_w level (data not shown). However, this CO_2 percentage would be difficult to obtain and maintain post-harvest.

Previous studies, where exposure to 50% CO_2 at different a_w levels were carried out, showed that growth of ochratoxigenic species such as *P. verrucosum*, *A. ochraceus* and *A. carbonarius* was inhibited by 50–75%, depending on a_w levels, when compared to that in normal atmospheric conditions (Cairns-Fuller, 2004; Cairns-Fuller et al., 2005; Pateraki et al., 2007). Studies on bakery products showed that spoilage could be prevented with exposure to 70% CO_2 when the a_w level was 0.80, but it was only delayed when the a_w levels were 0.85 to 0.90 (Guynot et al., 2003). Recent studies with *F. verticillioides* and *F. proliferatum* examined initial elevated CO_2 concentrations on growth rates at 0.984–0.93 a_w (Samapundo et al., 2007) and they showed a reduction from 10–12 mm day^{-1} at 0.98 a_w and air to 2 mm day^{-1} at 0.93 a_w and > 20% CO_2. However, these studies were conducted in static sealed systems, not with continuous slow flushing as in the present study; therefore results are not strictly comparable.

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4.2. Efficacy on aflatoxin production

Aflatoxin production was influenced by both CO_2 concentration and a_w levels tested. Considering the experiments *in vitro* and *in situ* together, at 0.95 a_w, 48% more aflatoxin was produced than at 0.92 a_w ($P<0.05$). It appears that 25% CO_2 does not offer any significant control of aflatoxin content under the a_w treatments examined in this study. Only partial inhibition of growth occurred, resulting in the fungus being under stress and in aflatoxin levels similar to the untreated controls. For inhibition of aflatoxin production, 50% and 75% CO_2 were effective in reducing production levels by 46% and 58%, respectively. Overall, taking into account both *in vitro* and *in situ* trials, at least 50% CO_2 is required to inhibit aflatoxin production to any extent.

Previous studies of aflatoxin production in peanuts showed that a 25% reduction occurred with 20% CO_2 and that this modified atmosphere was insufficient to inhibit growth and sporulation of *A. flavus*. Growth and sporulation was inhibited to some extent by 25% CO_2 but, in this case, aflatoxin production increased (Diener and Davis, 1977). So, it is clear that to obtain a substantial reduction in aflatoxin production it is necessary to use high levels of CO_2. Studies with other mycotoxigenic fungi such as *A. ochraceus* showed that ochratoxin was produced in 30% CO_2, but inhibition of growth only occurred with>60% CO_2 (Paster et al., 1983). Similar results were obtained with *P. verrucosum*, that exhibited a decrease in growth and ochratoxin production only with 50% CO_2 (Cairns-Fuller et al., 2005). The latter study suggested that a_w had a greater influence than CO_2. In studies on *A. carbonarius*, 50% CO_2 significantly decreased ochratoxin production *in vitro* over a range of a_w levels, but again not completely (Pateraki et al., 2007). Samapundo et al. (2007) showed that fumonisin production by both *F. verticillioides* and *F. proliferatum* was inhibited by 30% CO_2 at 0.985 a_w, by about 10–20% at 0.951 a_w and by 10% at 0.93 a_w. However, these were initial concentrations in sealed systems, not active continuous flow through systems at the target a_w levels as used in the present study.

This study shows the potential target CO_2 concentrations required for inhibition of growth and aflatoxin synthesis. Further larger pilot scale studies are necessary to determine the feasibility of using controlled atmospheres, specifically for controlling *A. flavus* in stored maize grain destined for animal feed, where physical methods are required for safe storage.

Acknowledgements

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APPENDIX 2

Statistical Elaborations

CHAPTER 1

TEMPERATURE

		N	Media	Deviazione std.	Errore std.	Intervallo di confidenza 95% per la media		Minimo	Massimo
						Limite inferiore	Limite superiore		
MEDIA	1	120	9.254	6.3985	.5841	8.098	10.411	.0	25.0
	2	120	64.013	14.5075	1.3243	61.390	66.635	.0	79.5
	3	120	67.392	15.9960	1.4602	64.500	70.283	28.0	85.0
	Totale	360	46.886	29.6667	1.5636	43.811	49.961	.0	85.0
tasso di crescita	1	120	.66101190476191	.457037705337236	.041721643473934	.57839888367361	.74362492585020	.000000000000	1.78571428571
	2	120	4.57232142857143	1.036251904387440	.094596423881781	4.38501106132989	4.75963179581297	.000000000000	5.67857142857
	3	120	4.81369047619048	1.142568084443189	.104301718889167	4.60716265550384	5.02021829687712	2.00000000000	6.07142857143
	Totale	360	3.34900793650794	2.119050776782337	.111683782203021	3.12937128748050	3.56864458553537	.000000000000	6.07142857143

ANOVA univariata

		Somma dei quadrati	df	Media dei quadrati	F	Sig.
MEDIA	Fra gruppi	255594.510	2	127797.255	755.779	.000
	Entro gruppi	60366.321	357	169.093		
	Totale	315960.831	359			
tasso di crescita	Fra gruppi	1304.054	2	652.027	755.779	.000
	Entro gruppi	307.991	357	.863		
	Totale	1612.045	359			

Test post hoc

Confronti multipli

LSD

Variabile dipendente	(I) T	(J) T	Differenza fra medie (I-J)	Errore std.	Sig.	Intervallo di confidenza 95%	
MEDIA	1	2	-54.758(*)	1.6788	.000	-58.060	-51.457
		3	-58.138(*)	1.6788	.000	-61.439	-54.836
	2	1	54.758(*)	1.6788	.000	51.457	58.060
		3	-3.379(*)	1.6788	.045	-6.681	-.078
	3	1	58.138(*)	1.6788	.000	54.836	61.439
		2	3.379(*)	1.6788	.045	.078	6.681
tasso di crescita	1	2	-3.91130952380953(*)	.119911154634372	.000	-4.14713054226495	-3.67548850535409
		3	-4.15267857142858(*)	.119911154634372	.000	-4.38849958988400	-3.91685755297314
	2	1	3.91130952380952(*)	.119911154634372	.000	3.67548850535409	4.14713054226495

	3	-.24136904761905(*)	.119911154634372	.045	-.47719006607448	-.00554802916362
3	1	4.15267857142857(*)	.119911154634372	.000	3.91685755297314	4.38849958988400
	2	.24136904761905(*)	.119911154634372	.045	.00554802916362	.47719006607448

* La differenza tra le medie è significativa al livello .05.

AW

ANOVA univariata

DIAMETER

	N	Media	Deviazione std.	Errore std.	Intervallo di confidenza 95% per la media		Minimo	Massimo
					Limite inferiore	Limite superiore		
.83	40	6.913	10.8301	1.7124	3.449	10.376	1.3	72.0
.94	40	50.592	9.6604	1.5274	47.502	53.681	26.3	70.0
.99	40	61.346	11.7296	1.8546	57.595	65.097	24.0	73.2
Totale	120	39.617	25.9389	2.3679	34.928	44.305	1.3	73.2

ANOVA univariata

DIAMETER

	Somma dei quadrati	df	Media dei quadrati	F	Sig.
Fra gruppi	66486.793	2	33243.397	286.417	.000
Entro gruppi	13579.782	117	116.067		
Totale	80066.575	119			

Test post hoc

Confronti multipli

Variabile dipendente: DM
LSD

(I) AW	(J) AW	Differenza fra medie (I-J)	Errore std.	Sig.	Intervallo di confidenza 95%	
					Limite inferiore	Limite superiore
.83	.94	-43.679(*)	2.4090	.000	-48.450	-38.908
	.99	-54.433(*)	2.4090	.000	-59.204	-49.662
.94	.83	43.679(*)	2.4090	.000	38.908	48.450
	.99	-10.754(*)	2.4090	.000	-15.525	-5.983
.99	.83	54.433(*)	2.4090	.000	49.662	59.204
	.94	10.754(*)	2.4090	.000	5.983	15.525

* La differenza tra le medie è significativa al livello .05.

Cluster con gli zeri

Riepilogo dei casi(a,b)

Casi					
Validi		Mancanti		Totale	
N	Percentuale	N	Percentuale	N	Percentuale
40	100.0	0	.0	40	100.0

a Distanza euclidea quadratica utilizzata
b Legame medio (fra gruppi)

Legame medio (fra gruppi)

Programma di agglomerazione

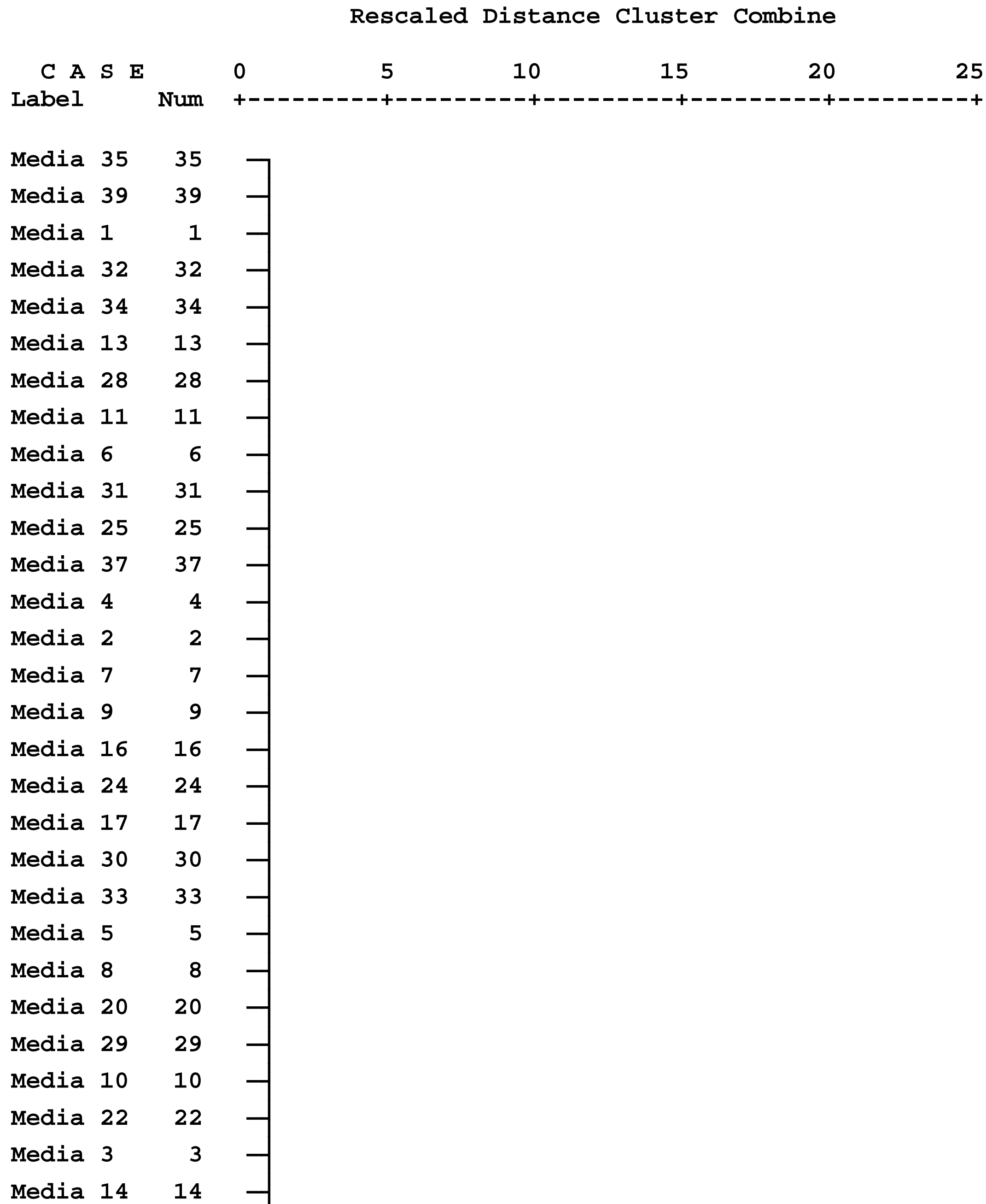
Stadio	Cluster accorpati		Coefficienti	Stadio di formazione del cluster		Stadio successivo
	Cluster 1	Cluster 2		Cluster 1	Cluster 2	
1	35	39	.000	0	0	2
2	1	35	.000	0	1	4
3	32	34	.000	0	0	4
4	1	32	.000	2	3	6
5	13	28	.000	0	0	6
6	1	13	.000	4	5	7
7	1	11	.000	6	0	8
8	1	6	.309	7	0	9
9	1	31	1.715	8	0	12
10	25	37	15.123	0	0	11
11	4	25	17.747	0	10	12
12	1	4	64.170	9	11	13
13	1	2	288.021	12	0	16
14	7	9	724.623	0	0	16
15	17	30	2983.539	0	0	18
16	1	7	3842.781	13	14	17
17	1	16	11262.056	16	0	22
18	17	33	11648.971	15	0	20
19	20	29	13833.230	0	0	27
20	5	17	29393.427	0	18	23
21	10	22	39724.897	0	0	24
22	1	24	50197.884	17	0	26
23	5	8	54484.525	20	0	26
24	3	10	66458.196	0	21	28
25	14	19	70266.084	0	0	28
26	1	5	70631.861	22	23	27
27	1	20	81306.514	26	19	32
28	3	14	176158.522	24	25	31
29	15	38	194704.595	0	0	33
30	21	26	206470.713	0	0	34
31	3	12	250188.896	28	0	32
32	1	3	451471.084	27	31	33
33	1	15	536260.240	32	29	35
34	21	40	999790.972	30	0	35
35	1	21	2129012.339	33	34	37

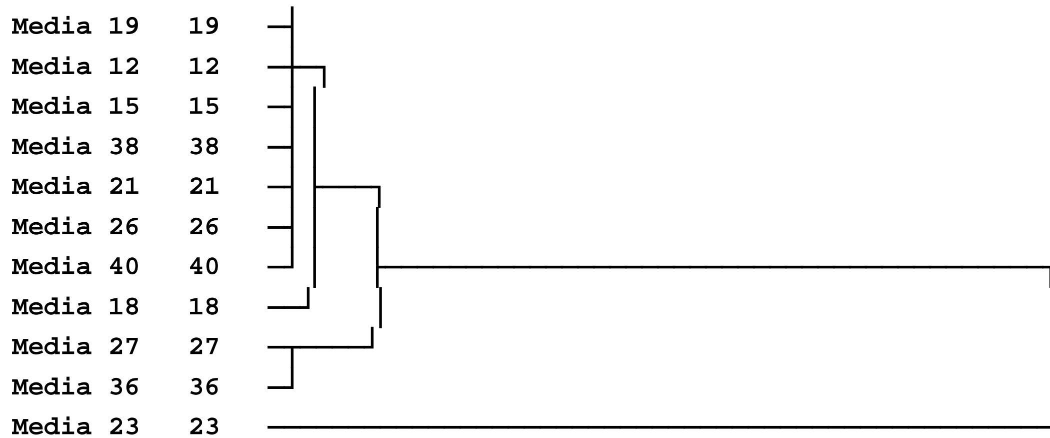
36	27	36	2163977.538	0	0	38
37	1	18	5813964.730	35	0	38
38	1	27	15127178.379	37	36	39
39	1	23	114300667.302	38	0	0

Dendrogramma

*** * * * * * H I E R A R C H I C A L C L U S T E R A N A L Y S I S * * * * * ***

Dendrogram using Average Linkage (Between Groups)

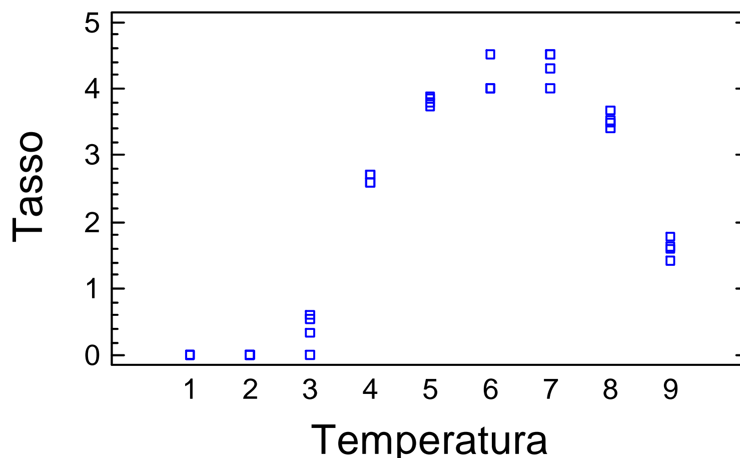




CHAPTER 3

One-Way ANOVA – Growth at different Temperatures

```
Analysis Summary

Dependent variable: Tasso
Factor: Temperatura

Number of observations: 36
Number of levels: 9


The StatAdvisor
---------------
   This procedure performs a one-way analysis of variance for Tasso. It constructs various tests
and graphs to compare the mean values of Tasso for the 9 different levels of Temperatura. The F-
test in the
ANOVA table will test whether there are any significant differences amongst the means.  If there
are, the Multiple Range Tests will tell you which means are significantly different from which
others.  If you
are worried about the presence of outliers, choose the Kruskal-Wallis Test which compares medians
instead of means.  The various plots will help you judge the practical significance of the
results, as well as
allow you to look for possible violations of the assumptions underlying the analysis of variance.
```



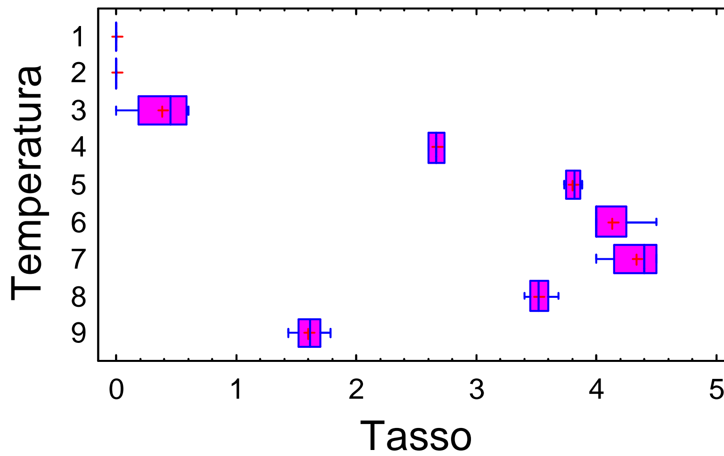
ANOVA Table for Tasso by Temperatura

Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
Between groups	104.362	8	13.0453	497.62	0.0000
Within groups	0.707812	27	0.0262153		
Total (Corr.)	105.07	35			

The StatAdvisor

The ANOVA table decomposes the variance of Tasso into two components: a between-group component and a within-group component. The F-ratio, which in this case equals 497.622, is a ratio of the between-group estimate to the within-group estimate. Since the P-value of the F-test is less than 0.05, there is a statistically significant difference between the mean Tasso from one level of Temperatura to another at the 95.0% confidence level. To determine which means are significantly different from which others, select Multiple Range Tests from the list of Tabular Options.



Multiple Range Tests for Tasso by Temperatura

Method: 95.0 percent LSD

Temperatura	Count	Mean	Homogeneous Groups
1	4	0.0	X
2	4	0.0	X
3	4	0.375	X
9	4	1.60625	X
4	4	2.6625	X
8	4	3.525	X
5	4	3.80625	X
6	4	4.125	X
7	4	4.325	X

Contrast	Difference	+/- Limits
1 - 2	0.0	0.234912
1 - 3	*-0.375	0.234912
1 - 4	*-2.6625	0.234912
1 - 5	*-3.80625	0.234912
1 - 6	*-4.125	0.234912
1 - 7	*-4.325	0.234912
1 - 8	*-3.525	0.234912
1 - 9	*-1.60625	0.234912
2 - 3	*-0.375	0.234912
2 - 4	*-2.6625	0.234912
2 - 5	*-3.80625	0.234912
2 - 6	*-4.125	0.234912
2 - 7	*-4.325	0.234912

2 - 8	*-3.525	0.234912
2 - 9	*-1.60625	0.234912
3 - 4	*-2.2875	0.234912
3 - 5	*-3.43125	0.234912
3 - 6	*-3.75	0.234912
3 - 7	*-3.95	0.234912
3 - 8	*-3.15	0.234912
3 - 9	*-1.23125	0.234912
4 - 5	*-1.14375	0.234912
4 - 6	*-1.4625	0.234912
4 - 7	*-1.6625	0.234912
4 - 8	*-0.8625	0.234912
4 - 9	*1.05625	0.234912
5 - 6	*-0.31875	0.234912
5 - 7	*-0.51875	0.234912
5 - 8	*0.28125	0.234912
5 - 9	*2.2	0.234912
6 - 7	-0.2	0.234912
6 - 8	*0.6	0.234912
6 - 9	*2.51875	0.234912
7 - 8	*0.8	0.234912
7 - 9	*2.71875	0.234912
8 - 9	*1.91875	0.234912

* denotes a statistically significant difference.

The StatAdvisor

This table applies a multiple comparison procedure to determine which means are significantly different from which others. The bottom half of the output shows the estimated difference between each pair of means. An asterisk has been placed next to 34 pairs, indicating that these pairs show statistically significant differences at the 95.0% confidence level. At the top of the page, 7 homogenous groups are identified using columns of X's. Within each column, the levels containing X's form a group of means within which there are no statistically significant differences. The method currently being used to discriminate among the means is Fisher's least significant difference (LSD) procedure. With this method, there is a 5.0% risk of calling each pair of means significantly different when the actual difference equals 0.

Multifactor ANOVA – Growth rate at different temperature and a_ws

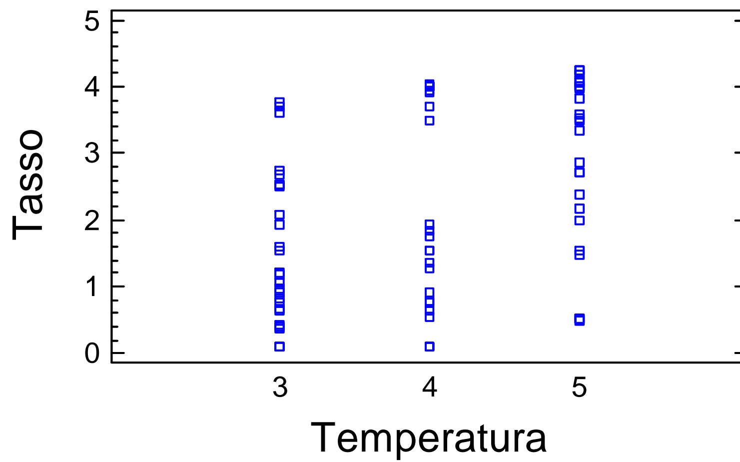
Analysis Summary

Dependent variable: Tasso
Factors:
Temperatura
aw

Number of complete cases: 84

The StatAdvisor

This procedure performs a multifactor analysis of variance for Tasso. It constructs various tests and graphs to determine which factors have a statistically significant effect on Tasso. It also tests for significant interactions amongst the factors, given sufficient data. The F-tests in the ANOVA table will allow you to identify the significant factors. For each significant factor, the Multiple Range Tests will tell you which means are significantly different from which others. The Means Plot and Interaction Plot will help you interpret the significant effects. The Residual Plots will help you judge whether the assumptions underlying the analysis of variance are violated by the data.



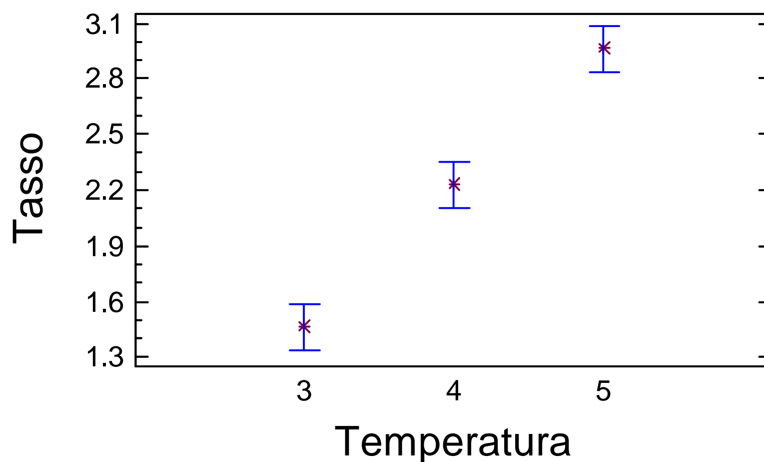
Analysis of Variance for Tasso - Type III Sums of Squares

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
MAIN EFFECTS					
A:Temperatura	31.4666	2	15.7333	71.94	0.0000
B:aw	133.385	6	22.2308	101.65	0.0000
RESIDUAL	16.4026	75	0.218701		
TOTAL (CORRECTED)	181.254	83			

All F-ratios are based on the residual mean square error.

The StatAdvisor

The ANOVA table decomposes the variability of Tasso into contributions due to various factors. Since Type III sums of squares (the default) have been chosen, the contribution of each factor is measured having removed the effects of all other factors. The P-values test the statistical significance of each of the factors. Since 2 P-values are less than 0.05, these factors have a statistically significant effect on Tasso at the 95.0% confidence level.



Multiple Range Tests for Tasso by Temperatura

Method: 95.0 percent LSD

Temperatura	Count	LS Mean	LS Sigma	Homogeneous Groups
3	28	1.46518	0.0883785	X
4	28	2.22946	0.0883785	X
5	28	2.96429	0.0883785	X

Contrast	Difference	+/- Limits
3 - 4	*-0.764286	0.248986
3 - 5	*-1.49911	0.248986
4 - 5	*-0.734821	0.248986

* denotes a statistically significant difference.

The StatAdvisor

This table applies a multiple comparison procedure to determine which means are significantly different from which others. The bottom half of the output shows the estimated difference between each pair of means. An asterisk has been placed next to 3 pairs, indicating that these pairs show statistically significant differences at the 95.0% confidence level. At the top of the page, 3 homogenous groups are identified using columns of X's. Within each column, the levels containing X's form a group of means within which there are no statistically significant differences. The method currently being used to discriminate among the means is Fisher's least significant difference (LSD) procedure. With this method, there is a 5.0% risk of calling each pair of means significantly different when the actual difference equals 0.

Multifactor ANOVA - Tasso

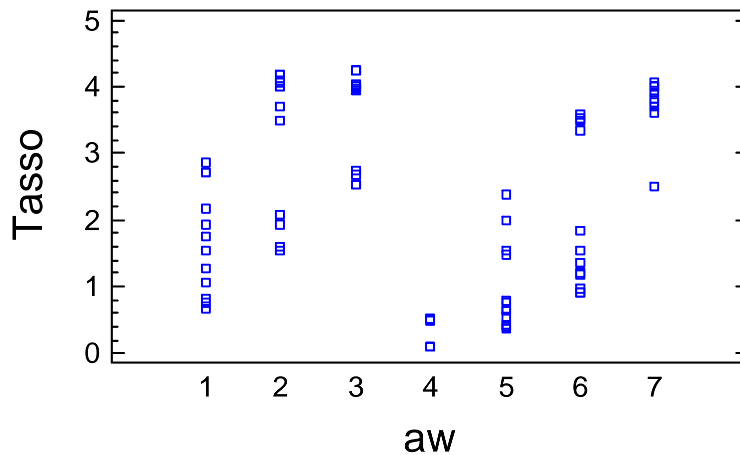
Analysis Summary

Dependent variable: Tasso
Factors:
aw
Temperatura

Number of complete cases: 84

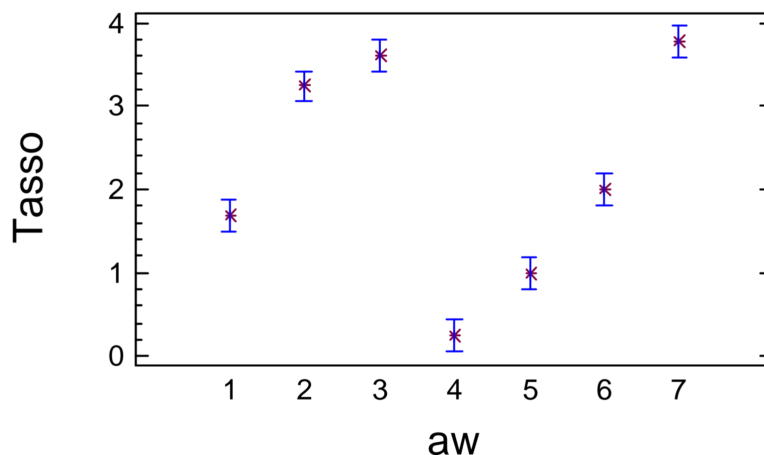
The StatAdvisor

This procedure performs a multifactor analysis of variance for Tasso. It constructs various tests and graphs to determine which factors have a statistically significant effect on Tasso. It also tests for significant interactions amongst the factors, given sufficient data. The F-tests in the ANOVA table will allow you to identify the significant factors. For each significant factor, the Multiple Range Tests will tell you which means are significantly different from which others. The Means Plot and Interaction Plot will help you interpret the significant effects. The Residual Plots will help you judge whether the assumptions underlying the analysis of variance are violated by the data.




```
Analysis of Variance for Tasso - Type III Sums of Squares
--------------------------------------------------------------------------------
Source                    Sum of Squares     Df    Mean Square    F-Ratio     P-Value
--------------------------------------------------------------------------------
MAIN EFFECTS
 A:aw                            133.385      6        22.2308     101.65      0.0000
 B:Temperatura                   31.4666      2        15.7333      71.94      0.0000

RESIDUAL                         16.4026     75       0.218701
--------------------------------------------------------------------------------
TOTAL (CORRECTED)                181.254     83
--------------------------------------------------------------------------------
All F-ratios are based on the residual mean square error.
```

```
The StatAdvisor
---------------
   The ANOVA table decomposes the variability of Tasso into contributions due to various factors.
Since Type III sums of squares (the default) have been chosen, the contribution of each factor is
measured having removed the effects of all other factors. The P-values test the statistical
significance of each of the factors. Since 2 P-values are less than 0.05, these factors have a
statistically significant effect on Tasso at the 95.0% confidence level.
```




```
Multiple Range Tests for Tasso by aw

--------------------------------------------------------------------------------
Method: 95.0 percent LSD
```

```
aw            Count     LS Mean       LS Sigma      Homogeneous Groups
--------------------------------------------------------------------------------
4             12        0.239583      0.135         X
5             12        1.0           0.135          X
1             12        1.68958       0.135           X
6             12        1.99167       0.135           X
2             12        3.2375        0.135            X
3             12        3.59792       0.135            XX
7             12        3.78125       0.135             X
--------------------------------------------------------------------------------
Contrast                                 Difference          +/-  Limits
--------------------------------------------------------------------------------
1 - 2                                   *-1.54792           0.380332
1 - 3                                   *-1.90833           0.380332
1 - 4                                   *1.45               0.380332
1 - 5                                   *0.689583           0.380332
1 - 6                                    -0.302083          0.380332
1 - 7                                   *-2.09167           0.380332
2 - 3                                    -0.360417          0.380332
2 - 4                                   *2.99792            0.380332
2 - 5                                   *2.2375             0.380332
2 - 6                                   *1.24583            0.380332
2 - 7                                   *-0.54375           0.380332
3 - 4                                   *3.35833            0.380332
3 - 5                                   *2.59792            0.380332
3 - 6                                   *1.60625            0.380332
3 - 7                                    -0.183333          0.380332
4 - 5                                   *-0.760417          0.380332
4 - 6                                   *-1.75208           0.380332
4 - 7                                   *-3.54167           0.380332
5 - 6                                   *-0.991667          0.380332
5 - 7                                   *-2.78125           0.380332
6 - 7                                   *-1.78958           0.380332
--------------------------------------------------------------------------------
* denotes a statistically significant difference.
```

The StatAdvisor

This table applies a multiple comparison procedure to determine which means are significantly different from which others. The bottom half of the output shows the estimated difference between each pair of means. An asterisk has been placed next to 18 pairs, indicating that these pairs show statistically significant differences at the 95.0% confidence level. At the top of the page, 5 homogenous groups are identified using columns of X's. Within each column, the levels containing X's form a group of means within which there are no statistically significant differences. The method currently being
used to discriminate among the means is Fisher's least significant difference (LSD) procedure. With this method, there is a 5.0% risk of calling each pair of means significantly different when the actual difference equals 0.

Multifactor ANOVA - AFB_1 production at different temperatures and a_ws

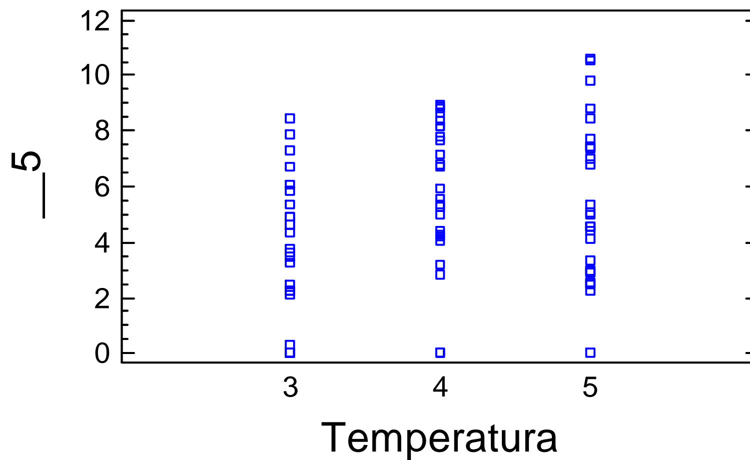
Analysis Summary

Dependent variable: afb1
Factors:
        Temperatura
        aw

Number of complete cases: 84

The StatAdvisor

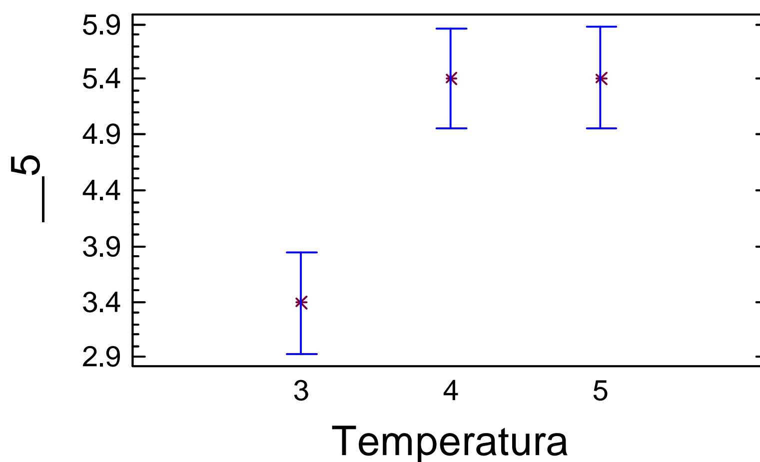
This procedure performs a multifactor analysis of variance for afb1. It constructs various tests and graphs to determine which factors have a statistically significant effect on afb1. It also tests for
significant interactions amongst the factors, given sufficient data. The F-tests in the ANOVA table will allow you to identify the significant factors. For each significant factor, the Multiple Range Tests will tell you which means are significantly different from which others. The Means Plot and Interaction Plot will help you interpret the significant effects. The Residual Plots will help you judge whether the assumptions underlying the analysis of variance are violated by the data.




```
Analysis of Variance for __5 - Type III Sums of Squares
--------------------------------------------------------------------------------
Source                    Sum of Squares     Df      Mean Square     F-Ratio     P-Value
--------------------------------------------------------------------------------
MAIN EFFECTS
 A:Temperatura                 76.4234        2          38.2117       12.90      0.0000
 B:aw                          399.428        6          66.5713       22.47      0.0000

RESIDUAL                       222.164       75          2.96219
--------------------------------------------------------------------------------
TOTAL (CORRECTED)              698.015       83
--------------------------------------------------------------------------------
All F-ratios are based on the residual mean square error.
```

The StatAdvisor

   The ANOVA table decomposes the variability of afb1 into contributions due to various factors. Since Type III sums of squares (the default) have been chosen, the contribution of each factor is measured having removed the effects of all other factors. The P-values test the statistical significance of each of the factors. Since 2 P-values are less than 0.05, these factors have a statistically significant effect on afb1 at the 95.0% confidence level.



Multiple Range Tests for __5 by Temperatura

Method: 95.0 percent LSD

Temperatura	Count	LS Mean	LS Sigma	Homogeneous Groups
3	28	3.38706	0.325257	X
4	28	5.40857	0.325257	X
5	28	5.41232	0.325257	X

Contrast	Difference	+/- Limits
3 - 4	*-2.02151	0.916336
3 - 5	*-2.02527	0.916336
4 - 5	-0.00375522	0.916336

* denotes a statistically significant difference.

The StatAdvisor

This table applies a multiple comparison procedure to determine which means are significantly different from which others. The bottom half of the output shows the estimated difference between each pair of means. An asterisk has been placed next to 2 pairs, indicating that these pairs show statistically significant differences at the 95.0% confidence level. At the top of the page, 2 homogenous groups are identified using columns of X's. Within each column, the levels containing X's form a group of means within which there are no statistically significant differences. The method currently being
used to discriminate among the means is Fisher's least significant difference (LSD) procedure. With this method, there is a 5.0% risk of calling each pair of means significantly different when the actual
difference equals 0.

Multifactor ANOVA - afb1

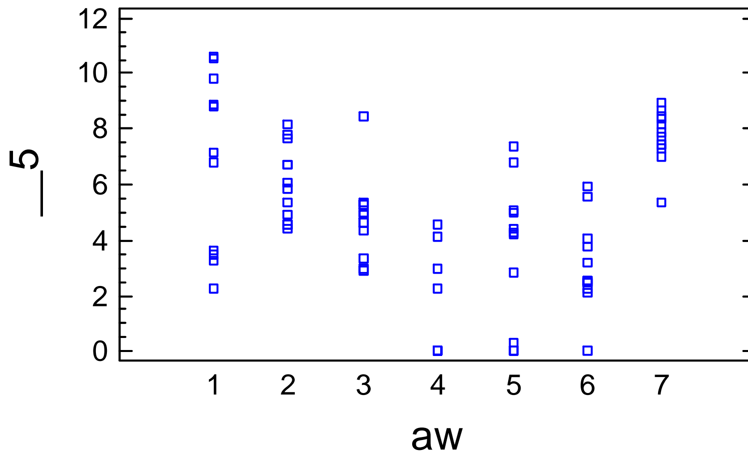
Analysis Summary

Dependent variable: afb1
Factors:
aw
Temperatura

Number of complete cases: 84

The StatAdvisor

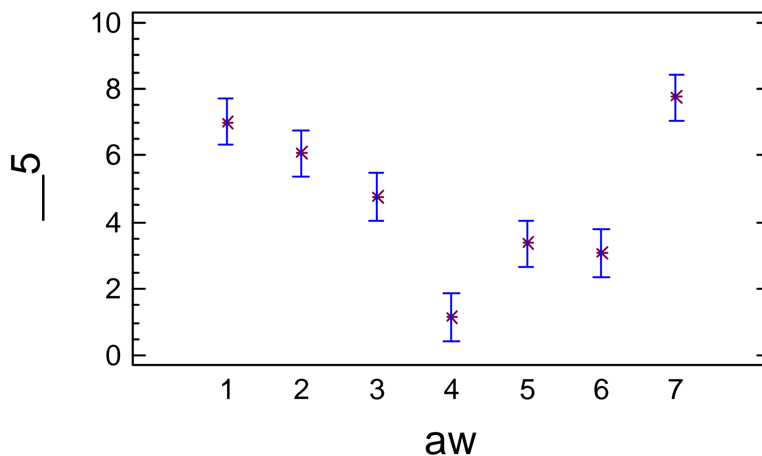
This procedure performs a multifactor analysis of variance for afb1. It constructs various tests and graphs to determine which factors have a statistically significant effect on afb1. It also tests for significant interactions amongst the factors, given sufficient data. The F-tests in the ANOVA table will allow you to identify the significant factors. For each significant factor, the Multiple Range Tests will tell you which means are significantly different from which others. The Means Plot and Interaction Plot will help you interpret the significant effects. The Residual Plots will help you judge whether the assumptions underlying the analysis of variance are violated by the data.




```
Analysis of Variance for __5 - Type III Sums of Squares
--------------------------------------------------------------------------------
Source                   Sum of Squares     Df    Mean Square    F-Ratio     P-Value
--------------------------------------------------------------------------------
MAIN EFFECTS
 A:aw                           399.428      6        66.5713      22.47      0.0000
 B:Temperatura                  76.4234      2        38.2117      12.90      0.0000

RESIDUAL                        222.164     75        2.96219
--------------------------------------------------------------------------------
TOTAL (CORRECTED)               698.015     83
--------------------------------------------------------------------------------
All F-ratios are based on the residual mean square error.
```

The StatAdvisor

The ANOVA table decomposes the variability of afb1 into contributions due to various factors. Since Type III sums of squares (the default) have been chosen, the contribution of each factor is measured having removed the effects of all other factors. The P-values test the statistical significance of each of the factors. Since 2 P-values are less than 0.05, these factors have a statistically significant effect on afb1 at the 95.0% confidence level.



Multiple Range Tests for __5 by aw

Method: 95.0 percent LSD

aw	Count	LS Mean	LS Sigma	Homogeneous Groups
4	12	1.16163	0.496839	X
6	12	3.08003	0.496839	X
5	12	3.36006	0.496839	XX
3	12	4.75015	0.496839	XX
2	12	6.06957	0.496839	XX
1	12	6.99783	0.496839	XX
7	12	7.7326	0.496839	X

Contrast	Difference	+/- Limits
1 - 2	0.928258	1.39973
1 - 3	*2.24767	1.39973
1 - 4	*5.8362	1.39973
1 - 5	*3.63776	1.39973
1 - 6	*3.9178	1.39973
1 - 7	-0.734768	1.39973
2 - 3	1.31942	1.39973
2 - 4	*4.90794	1.39973
2 - 5	*2.70951	1.39973
2 - 6	*2.98954	1.39973
2 - 7	*-1.66303	1.39973
3 - 4	*3.58853	1.39973
3 - 5	1.39009	1.39973
3 - 6	*1.67012	1.39973
3 - 7	*-2.98244	1.39973
4 - 5	*-2.19844	1.39973
4 - 6	*-1.91841	1.39973
4 - 7	*-6.57097	1.39973
5 - 6	0.280032	1.39973
5 - 7	*-4.37253	1.39973
6 - 7	*-4.65256	1.39973

* denotes a statistically significant difference.

The StatAdvisor

This table applies a multiple comparison procedure to determine which means are significantly different from which others. The bottom half of the output shows the estimated difference between each pair of means. An asterisk has been placed next to 16 pairs, indicating that these pairs show statistically significant differences at the 95.0% confidence level. At the top of the page, 6 homogenous groups are identified using columns of X's. Within each column, the levels containing X's form a group of means within which there are no statistically significant differences. The method currently being
used to discriminate among the means is Fisher's least significant difference (LSD) procedure. With this method, there is a 5.0% risk of calling each pair of means significantly different when the actual
difference equals 0.

Multifactor ANOVA – Growth at different temperatures and growth stages

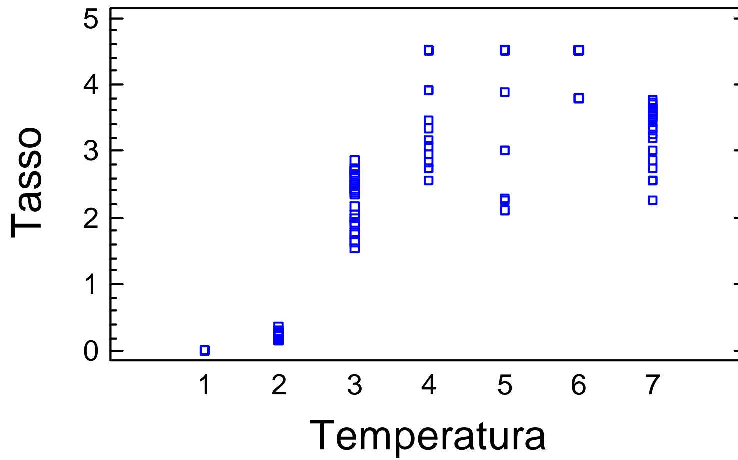
Analysis Summary

Dependent variable: Tasso
Factors:
Temperatura
DAP

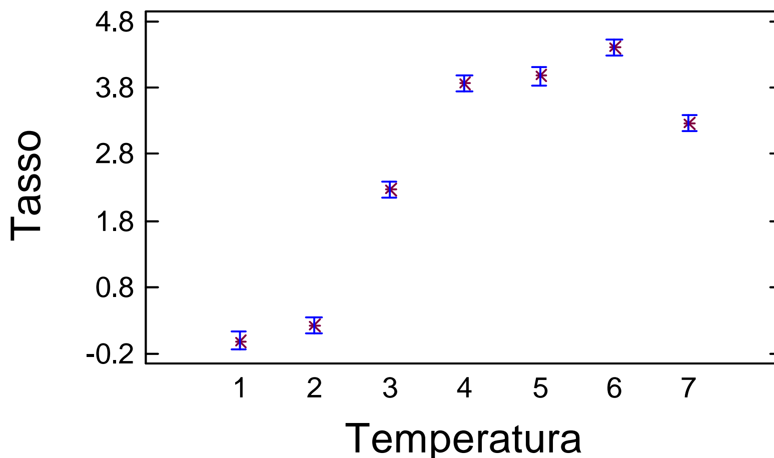
Number of complete cases: 168

The StatAdvisor

This procedure performs a multifactor analysis of variance for Tasso. It constructs various tests and graphs to determine which factors have a statistically significant effect on Tasso. It also tests for significant interactions amongst the factors, given sufficient data. The F-tests in the ANOVA table will allow you to identify the significant factors. For each significant factor, the Multiple Range Tests will tell you which means are significantly different from which others. The Means Plot and Interaction Plot will help you interpret the significant effects. The Residual Plots will help you judge whether the assumptions underlying the analysis of variance are violated by the data.




```
Analysis of Variance for Tasso - Type III Sums of Squares
--------------------------------------------------------------------------------
Source                     Sum of Squares     Df      Mean Square     F-Ratio     P-Value
--------------------------------------------------------------------------------
MAIN EFFECTS
 A:Temperatura                    470.619      6          78.4365      407.62      0.0000
 B:DAP                            12.0226      7          1.71752        8.93      0.0000

RESIDUAL                          29.6334    154         0.192425
--------------------------------------------------------------------------------
TOTAL (CORRECTED)                 512.275    167
--------------------------------------------------------------------------------
All F-ratios are based on the residual mean square error.


The StatAdvisor
---------------
   The ANOVA table decomposes the variability of Tasso into contributions due to various factors.
Since Type III sums of squares (the default) have been chosen, the contribution of each factor is
measured having removed the effects of all other factors. The P-values test the statistical
significance of each of the factors. Since 2 P-values are less than 0.05, these factors have a
statistically significant effect on Tasso at the 95.0% confidence level.
```




```
Multiple Range Tests for Tasso by Temperatura

--------------------------------------------------------------------------------
Method: 95.0 percent LSD
```

Temperatura	Count	LS Mean	LS Sigma	Homogeneous Groups				
1	24	0.0	0.0895416	X				
2	24	0.2375	0.0895416	X				
3	24	2.26771	0.0895416		X			
7	24	3.26771	0.0895416			X		
4	24	3.85625	0.0895416				X	
5	24	3.96562	0.0895416				X	
6	24	4.40937	0.0895416					X

Contrast	Difference	+/- Limits
1 - 2	-0.2375	0.250158
1 - 3	*-2.26771	0.250158
1 - 4	*-3.85625	0.250158
1 - 5	*-3.96562	0.250158
1 - 6	*-4.40937	0.250158
1 - 7	*-3.26771	0.250158
2 - 3	*-2.03021	0.250158
2 - 4	*-3.61875	0.250158
2 - 5	*-3.72812	0.250158
2 - 6	*-4.17187	0.250158
2 - 7	*-3.03021	0.250158
3 - 4	*-1.58854	0.250158
3 - 5	*-1.69792	0.250158
3 - 6	*-2.14167	0.250158
3 - 7	*-1.0	0.250158
4 - 5	-0.109375	0.250158
4 - 6	*-0.553125	0.250158
4 - 7	*0.588542	0.250158
5 - 6	*-0.44375	0.250158
5 - 7	*0.697917	0.250158
6 - 7	*1.14167	0.250158

* denotes a statistically significant difference.

The StatAdvisor

This table applies a multiple comparison procedure to determine which means are significantly different from which others. The bottom half of the output shows the estimated difference between each pair of means. An asterisk has been placed next to 19 pairs, indicating that these pairs show statistically significant differences at the 95.0% confidence level. At the top of the page, 5 homogenous groups are identified using columns of X's. Within each column, the levels containing X's form a group of means within which there are no statistically significant differences. The method currently being used to discriminate among the means is Fisher's least significant difference (LSD) procedure. With this method, there is a 5.0% risk of calling each pair of means significantly different when the actual difference equals 0.

Multifactor ANOVA - Tasso

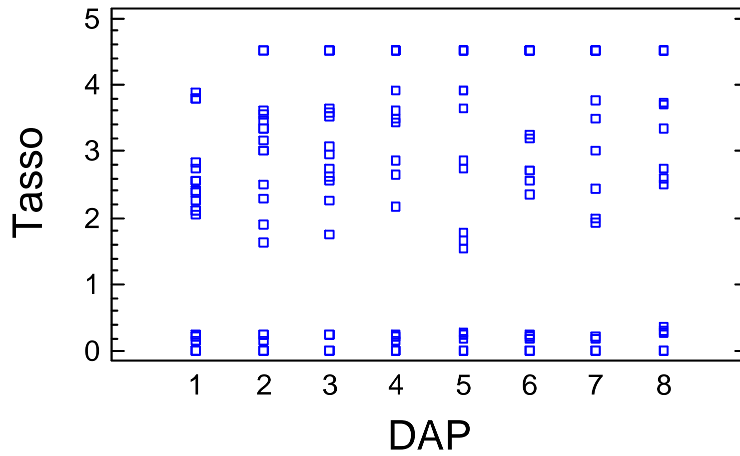
Analysis Summary

Dependent variable: Tasso
Factors:
DAP
Temperatura

Number of complete cases: 168

The StatAdvisor

This procedure performs a multifactor analysis of variance for Tasso. It constructs various tests and graphs to determine which factors have a statistically significant effect on Tasso. It also tests for significant interactions amongst the factors, given sufficient data. The F-tests in the ANOVA table will allow you to identify the significant factors. For each significant factor, the Multiple Range Tests will tell you which means are significantly different from which others. The Means Plot and Interaction Plot will help you interpret the significant effects. The Residual Plots will help you judge whether the assumptions underlying the analysis of variance are violated by the data.



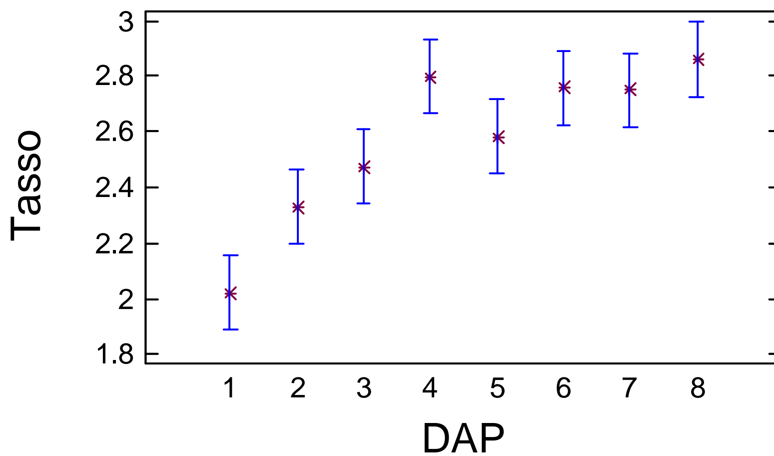
Analysis of Variance for Tasso - Type III Sums of Squares

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
MAIN EFFECTS					
A:DAP	12.0226	7	1.71752	8.93	0.0000
B:Temperatura	470.619	6	78.4365	407.62	0.0000
RESIDUAL	29.6334	154	0.192425		
TOTAL (CORRECTED)	512.275	167			

All F-ratios are based on the residual mean square error.

The StatAdvisor

The ANOVA table decomposes the variability of Tasso into contributions due to various factors. Since Type III sums of squares (the default) have been chosen, the contribution of each factor is measured having removed the effects of all other factors. The P-values test the statistical significance of each of the factors. Since 2 P-values are less than 0.05, these factors have a statistically significant effect on Tasso at the 95.0% confidence level.



Multiple Range Tests for Tasso by DAP

Method: 95.0 percent LSD

DAP	Count	LS Mean	LS Sigma	Homogeneous Groups
1	21	2.02143	0.095724	X
2	21	2.32976	0.095724	X
3	21	2.475	0.095724	X
5	21	2.58214	0.095724	XX
7	21	2.75119	0.095724	XX
6	21	2.75714	0.095724	XX
4	21	2.79881	0.095724	XX
8	21	2.86071	0.095724	X

Contrast	Difference	+/- Limits
1 - 2	*-0.308333	0.267431
1 - 3	*-0.453571	0.267431
1 - 4	*-0.777381	0.267431
1 - 5	*-0.560714	0.267431
1 - 6	*-0.735714	0.267431
1 - 7	*-0.729762	0.267431
1 - 8	*-0.839286	0.267431
2 - 3	-0.145238	0.267431
2 - 4	*-0.469048	0.267431
2 - 5	-0.252381	0.267431
2 - 6	*-0.427381	0.267431
2 - 7	*-0.421429	0.267431
2 - 8	*-0.530952	0.267431
3 - 4	*-0.32381	0.267431
3 - 5	-0.107143	0.267431
3 - 6	*-0.282143	0.267431
3 - 7	*-0.27619	0.267431
3 - 8	*-0.385714	0.267431
4 - 5	0.216667	0.267431
4 - 6	0.0416667	0.267431
4 - 7	0.047619	0.267431
4 - 8	-0.0619048	0.267431
5 - 6	-0.175	0.267431
5 - 7	-0.169048	0.267431
5 - 8	*-0.278571	0.267431
6 - 7	0.00595238	0.267431
6 - 8	-0.103571	0.267431
7 - 8	-0.109524	0.267431

* denotes a statistically significant difference.

The StatAdvisor

This table applies a multiple comparison procedure to determine which means are significantly different from which others. The bottom half of the output shows the estimated difference between each pair of means. An asterisk has been placed next to 16 pairs, indicating that these pairs show statistically significant differences at the 95.0% confidence level. At the top of the page, 4 homogenous groups are identified using columns of X's. Within each column, the levels containing X's form a group of means within which there are no statistically significant differences. The method currently being used to discriminate among the means is Fisher's least significant difference (LSD) procedure. With this method, there is a 5.0% risk of calling each pair of means significantly different when the actual difference equals 0.

Multifactor ANOVA – Growth at different a_w and growth stage

Analysis Summary

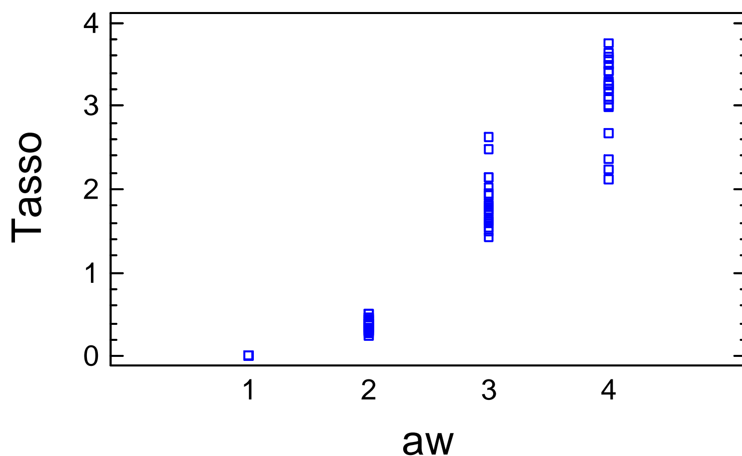
Dependent variable: Tasso
Factors:
aw
DAP

Number of complete cases: 96

The StatAdvisor

This procedure performs a multifactor analysis of variance for Tasso. It constructs various tests and graphs to determine which factors have a statistically significant effect on Tasso. It also tests for significant interactions amongst the factors, given sufficient data. The F-tests in the ANOVA table will allow you to identify the significant factors. For each significant factor, the Multiple Range Tests will tell you which means are significantly different from which

others. The Means Plot and Interaction Plot will help you interpret the significant effects. The Residual Plots will help you judge whether the assumptions underlying the analysis of variance are violated by the data.



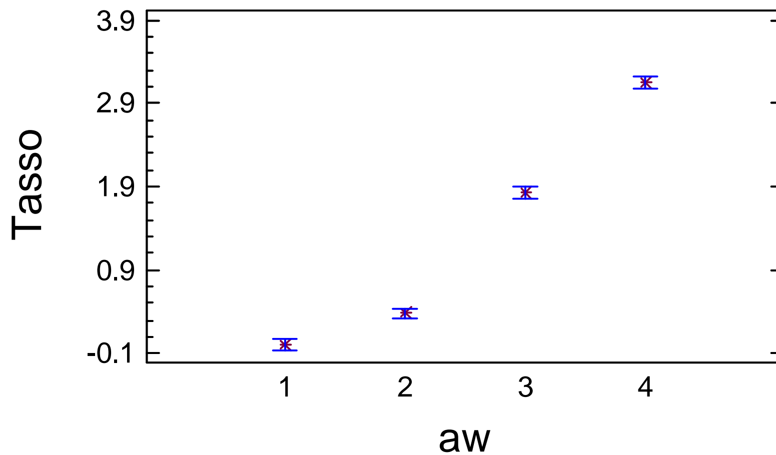
Analysis of Variance for Tasso - Type III Sums of Squares

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
MAIN EFFECTS					
A:aw	149.639	3	49.8798	1000.43	0.0000
B:DAP	1.99917	7	0.285595	5.73	0.0000
RESIDUAL	4.23797	85	0.0498585		
TOTAL (CORRECTED)	155.877	95			

All F-ratios are based on the residual mean square error.

The StatAdvisor

The ANOVA table decomposes the variability of Tasso into contributions due to various factors. Since Type III sums of squares (the default) have been chosen, the contribution of each factor is measured having removed the effects of all other factors. The P-values test the statistical significance of each of the factors. Since 2 P-values are less than 0.05, these factors have a statistically significant effect on Tasso at the 95.0% confidence level.



Multiple Range Tests for Tasso by aw

Method: 95.0 percent LSD

aw	Count	LS Mean	LS Sigma	Homogeneous Groups
1	24	0.0	0.0455789	X
2	24	0.38125	0.0455789	X
3	24	1.83021	0.0455789	X
4	24	3.15104	0.0455789	X

Contrast	Difference	+/- Limits
1 - 2	*-0.38125	0.128161
1 - 3	*-1.83021	0.128161
1 - 4	*-3.15104	0.128161
2 - 3	*-1.44896	0.128161
2 - 4	*-2.76979	0.128161
3 - 4	*-1.32083	0.128161

* denotes a statistically significant difference.

The StatAdvisor

This table applies a multiple comparison procedure to determine which means are significantly different from which others. The bottom half of the output shows the estimated difference between each pair of means. An asterisk has been placed next to 6 pairs, indicating that these pairs show statistically significant differences at the 95.0% confidence level. At the top of the page, 4 homogenous groups are identified using columns of X's. Within each column, the levels containing X's form a group of means within which there are no statistically significant differences. The method currently being
used to discriminate among the means is Fisher's least significant difference (LSD) procedure. With this method, there is a 5.0% risk of calling each pair of means significantly different when the actual
difference equals 0.

Multifactor ANOVA - Tasso

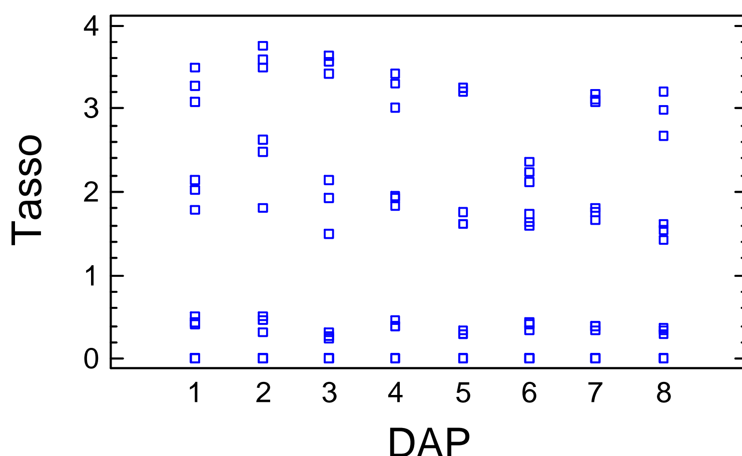
Analysis Summary

Dependent variable: Tasso
Factors:
DAP
aw

Number of complete cases: 96

The StatAdvisor

This procedure performs a multifactor analysis of variance for Tasso. It constructs various tests and graphs to determine which factors have a statistically significant effect on Tasso. It also tests for significant interactions amongst the factors, given sufficient data. The F-tests in the ANOVA table will allow you to identify the significant factors. For each significant factor, the Multiple Range Tests will tell you which means are significantly different from which others. The Means Plot and Interaction Plot will help you interpret the significant effects. The Residual Plots will help you judge whether the assumptions underlying the analysis of variance are violated by the data.



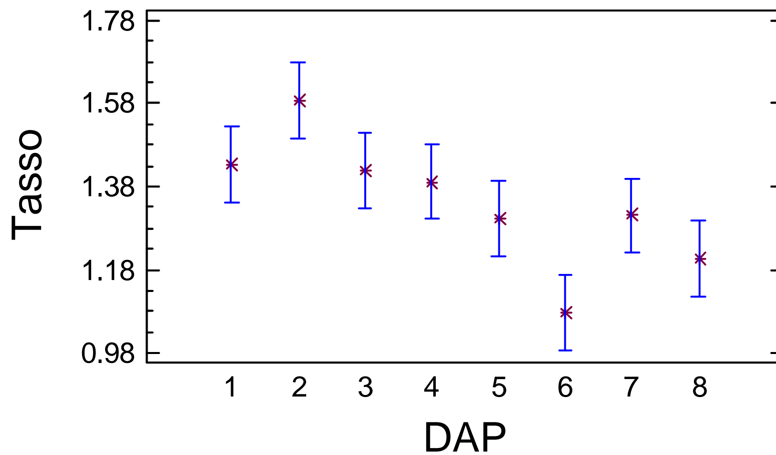
Analysis of Variance for Tasso - Type III Sums of Squares

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
MAIN EFFECTS					
A:DAP	1.99917	7	0.285595	5.73	0.0000
B:aw	149.639	3	49.8798	1000.43	0.0000
RESIDUAL	4.23797	85	0.0498585		
TOTAL (CORRECTED)	155.877	95			

All F-ratios are based on the residual mean square error.

The StatAdvisor

The ANOVA table decomposes the variability of Tasso into contributions due to various factors. Since Type III sums of squares (the default) have been chosen, the contribution of each factor is measured having removed the effects of all other factors. The P-values test the statistical significance of each of the factors. Since 2 P-values are less than 0.05, these factors have a statistically significant effect on Tasso at the 95.0% confidence level.



Multiple Range Tests for Tasso by DAP

Method: 95.0 percent LSD

DAP	Count	LS Mean	LS Sigma	Homogeneous Groups
6	12	1.07708	0.0644583	X
8	12	1.20625	0.0644583	XX
5	12	1.30417	0.0644583	XX
7	12	1.31042	0.0644583	XX
4	12	1.39167	0.0644583	X
3	12	1.41875	0.0644583	XX
1	12	1.43125	0.0644583	XX
2	12	1.58542	0.0644583	X

Contrast	Difference	+/- Limits
1 - 2	-0.154167	0.181246
1 - 3	0.0125	0.181246
1 - 4	0.0395833	0.181246
1 - 5	0.127083	0.181246
1 - 6	*0.354167	0.181246
1 - 7	0.120833	0.181246
1 - 8	*0.225	0.181246
2 - 3	0.166667	0.181246
2 - 4	*0.19375	0.181246
2 - 5	*0.28125	0.181246
2 - 6	*0.508333	0.181246
2 - 7	*0.275	0.181246
2 - 8	*0.379167	0.181246
3 - 4	0.0270833	0.181246
3 - 5	0.114583	0.181246
3 - 6	*0.341667	0.181246
3 - 7	0.108333	0.181246
3 - 8	*0.2125	0.181246
4 - 5	0.0875	0.181246
4 - 6	*0.314583	0.181246
4 - 7	0.08125	0.181246
4 - 8	*0.185417	0.181246
5 - 6	*0.227083	0.181246
5 - 7	-0.00625	0.181246
5 - 8	0.0979167	0.181246
6 - 7	*-0.233333	0.181246
6 - 8	-0.129167	0.181246
7 - 8	0.104167	0.181246

* denotes a statistically significant difference.

The StatAdvisor

This table applies a multiple comparison procedure to determine which means are significantly different from which others. The bottom half of the output shows the estimated difference between

each pair of means. An asterisk has been placed next to 13 pairs, indicating that these pairs show statistically significant differences at the 95.0% confidence level. At the top of the page, 4 homogenous groups are identified using columns of X's. Within each column, the levels containing X's form a group of means within which there are no statistically significant differences. The method currently being used to discriminate among the means is Fisher's least significant difference (LSD) procedure. With this method, there is a 5.0% risk of calling each pair of means significantly different when the actual difference equals 0.

CHAPTER 4

Multifactor ANOVA – growth on synthetic medium

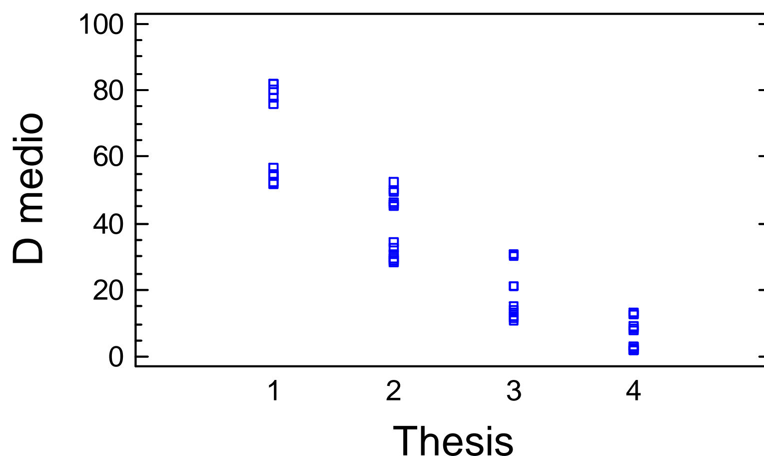
Analysis Summary

Dependent variable: D medio
Factors:
  Thesis
  aw
  Replicate

Number of complete cases: 48

The StatAdvisor

This procedure performs a multifactor analysis of variance for D medio. It constructs various tests and graphs to determine which factors have a statistically significant effect on D medio. It also tests for significant interactions amongst the factors, given sufficient data. The F-tests in the ANOVA table will allow you to identify the significant factors. For each significant factor, the Multiple Range Tests will tell you which means are significantly different from which others. The Means Plot and Interaction Plot will help you interpret the significant effects. The Residual Plots will help you judge whether the assumptions underlying the analysis of variance are violated by the data.



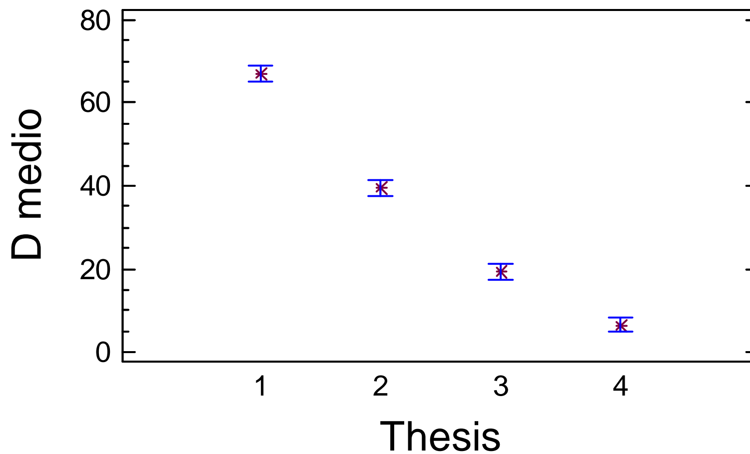
Analysis of Variance for D medio - Type III Sums of Squares

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
MAIN EFFECTS					
A:Thesis	24791,1	3	8263,69	442,78	0,0000
B:aw	3104,08	1	3104,08	166,32	0,0000
C:Replicate	36,2917	5	7,25833	0,39	0,8533
RESIDUAL	709,208	38	18,6634		
TOTAL (CORRECTED)	28640,7	47			

All F-ratios are based on the residual mean square error.

The StatAdvisor

The ANOVA table decomposes the variability of D medio into contributions due to various factors. Since Type III sums of squares (the default) have been chosen, the contribution of each factor is
measured having removed the effects of all other factors. The P-values test the statistical significance of each of the factors. Since 2 P-values are less than 0,05, these factors have a statistically significant effect on D medio at the 95,0% confidence level.



Multiple Range Tests for D medio by Thesis

Method: 95,0 percent LSD

Thesis	Count	LS Mean	LS Sigma	Homogeneous Groups
4	12	6,625	1,24711	X
3	12	19,375	1,24711	X
2	12	39,5417	1,24711	X
1	12	66,7917	1,24711	X

Contrast	Difference	+/- Limits
1 - 2	*27,25	3,57039
1 - 3	*47,4167	3,57039
1 - 4	*60,1667	3,57039
2 - 3	*20,1667	3,57039
2 - 4	*32,9167	3,57039
3 - 4	*12,75	3,57039

* denotes a statistically significant difference.

The StatAdvisor

This table applies a multiple comparison procedure to determine which means are significantly different from which others. The bottom half of the output shows the estimated difference between each pair of means. An asterisk has been placed next to 6 pairs, indicating that these pairs show statistically significant differences at the 95,0% confidence level. At the top of the page, 4 homogenous groups are identified using columns of X's. Within each column, the levels containing X's form a group of means within which there are no statistically significant differences. The method currently being used to discriminate among the means is Fisher's least significant difference (LSD) procedure. With this method, there is a 5,0% risk of calling each pair of means significantly different when the actual difference equals 0.

Multifactor ANOVA - AFB_1 on synthetic medium

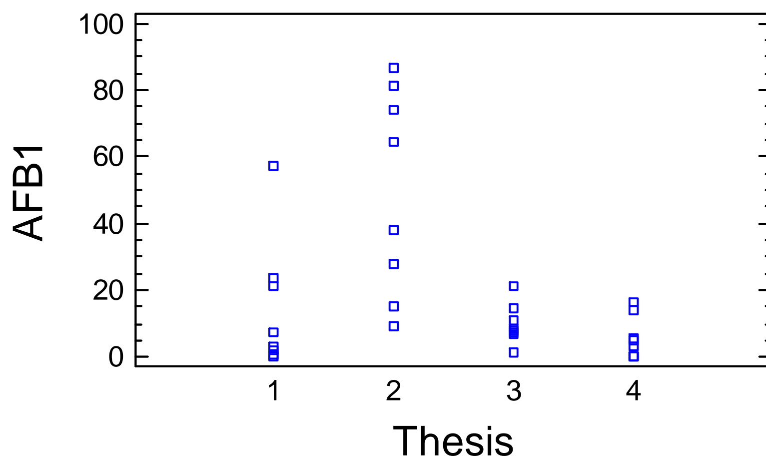
Analysis Summary

Dependent variable: AFB1

```
Factors:
        Thesis
        aw
        Replicate

Number of complete cases: 32
```

The StatAdvisor

This procedure performs a multifactor analysis of variance for AFB1. It constructs various tests and graphs to determine which factors have a statistically significant effect on AFB1. It also tests for significant interactions amongst the factors, given sufficient data. The F-tests in the ANOVA table will allow you to identify the significant factors. For each significant factor, the Multiple Range Tests will tell you which means are significantly different from which others. The Means Plot and Interaction Plot will help you interpret the significant effects. The Residual Plots will help you judge whether the assumptions underlying the analysis of variance are violated by the data.



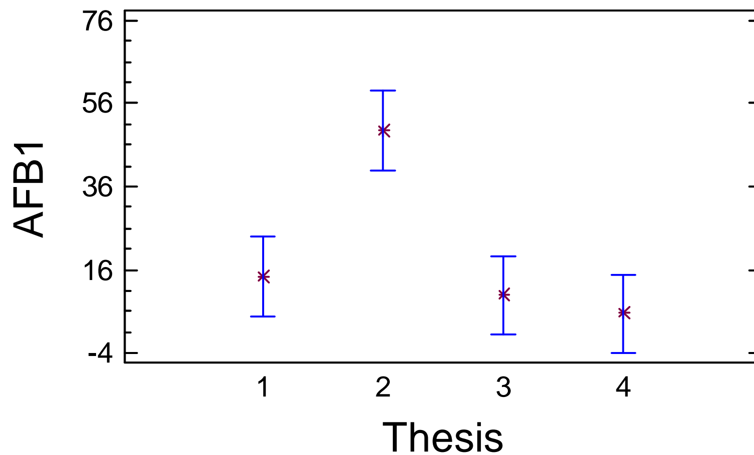
Analysis of Variance for AFB1 - Type III Sums of Squares

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
MAIN EFFECTS					
A:Thesis	9694,76	3	3231,59	9,59	0,0002
B:aw	419,051	1	419,051	1,24	0,2759
C:Replicate	1317,16	3	439,052	1,30	0,2966
RESIDUAL	8090,8	24	337,117		
TOTAL (CORRECTED)	19521,8	31			

All F-ratios are based on the residual mean square error.

The StatAdvisor

The ANOVA table decomposes the variability of AFB1 into contributions due to various factors. Since Type III sums of squares (the default) have been chosen, the contribution of each factor is measured having removed the effects of all other factors. The P-values test the statistical significance of each of the factors. Since one P-value is less than 0,05, this factor has a statistically significant effect on AFB1 at the 95,0% confidence level.



Multiple Range Tests for AFB1 by Thesis

Method: 95,0 percent LSD

Thesis	Count	LS Mean	LS Sigma	Homogeneous Groups
4	8	5,625	6,4915	X
3	8	9,85	6,4915	X
1	8	14,475	6,4915	X
2	8	49,525	6,4915	X

Contrast	Difference	+/- Limits
1 - 2	*-35,05	18,9474
1 - 3	4,625	18,9474
1 - 4	8,85	18,9474
2 - 3	*39,675	18,9474
2 - 4	*43,9	18,9474
3 - 4	4,225	18,9474

* denotes a statistically significant difference.

The StatAdvisor

This table applies a multiple comparison procedure to determine which means are significantly different from which others. The bottom half of the output shows the estimated difference between each pair of means. An asterisk has been placed next to 3 pairs, indicating that these pairs show statistically significant differences at the 95,0% confidence level. At the top of the page, 2 homogenous groups are identified using columns of X's. Within each column, the levels containing X's form a group of means within which there are no statistically significant differences. The method currently being used to discriminate among the means is Fisher's least significant difference (LSD) procedure. With this method, there is a 5,0% risk of calling each pair of means significantly different when the actual difference equals 0.

Multifactor ANOVA - AFB2

Analysis Summary

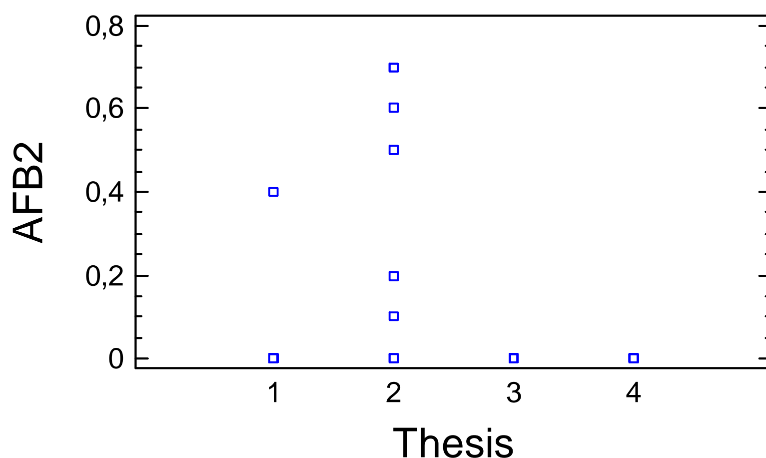
Dependent variable: AFB2
Factors:
Thesis
aw
Replicate

Number of complete cases: 32

The StatAdvisor

This procedure performs a multifactor analysis of variance for AFB2. It constructs various

tests and graphs to determine which factors have a statistically significant effect on AFB2. It also tests for significant interactions amongst the factors, given sufficient data. The F-tests in the ANOVA table will allow you to identify the significant factors. For each significant factor, the Multiple Range Tests will tell you which means are significantly different from which others. The Means Plot and Interaction Plot will help you interpret the significant effects. The Residual Plots will help you judge whether the assumptions underlying the analysis of variance are violated by the data.



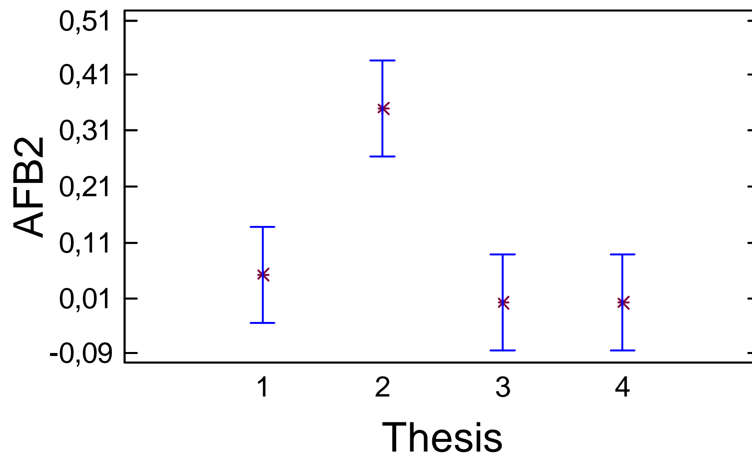
Analysis of Variance for AFB2 - Type III Sums of Squares

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
MAIN EFFECTS					
A:Thesis	0,68	3	0,226667	8,09	0,0007
B:aw	0,02	1	0,02	0,71	0,4065
C:Replicate	0,1075	3	0,0358333	1,28	0,3042
RESIDUAL	0,6725	24	0,0280208		
TOTAL (CORRECTED)	1,48	31			

All F-ratios are based on the residual mean square error.

The StatAdvisor

The ANOVA table decomposes the variability of AFB2 into contributions due to various factors. Since Type III sums of squares (the default) have been chosen, the contribution of each factor is measured having removed the effects of all other factors. The P-values test the statistical significance of each of the factors. Since one P-value is less than 0,05, this factor has a statistically significant effect on AFB2 at the 95,0% confidence level.



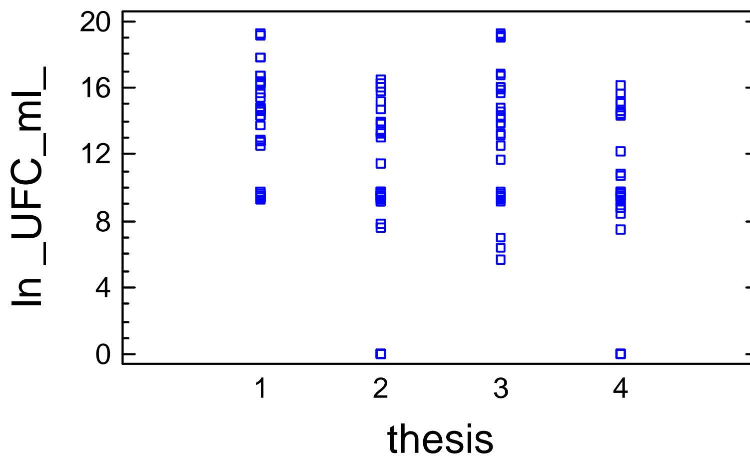

```
Multiple Range Tests for AFB2 by Thesis

--------------------------------------------------------------------------------
Method: 95,0 percent LSD
Thesis          Count     LS Mean       LS Sigma      Homogeneous Groups
--------------------------------------------------------------------------------
4               8         0,0           0,0591828     X
3               8         0,0           0,0591828     X
1               8         0,05          0,0591828     X
2               8         0,35          0,0591828      X
--------------------------------------------------------------------------------
Contrast                                 Difference           +/-  Limits
--------------------------------------------------------------------------------
1 - 2                                    *-0,3                0,172743
1 - 3                                     0,05                0,172743
1 - 4                                     0,05                0,172743
2 - 3                                    *0,35                0,172743
2 - 4                                    *0,35                0,172743
3 - 4                                     0,0                 0,172743
--------------------------------------------------------------------------------
* denotes a statistically significant difference.


The StatAdvisor
---------------
   This table applies a multiple comparison procedure to determine which means are significantly
different from which others. The bottom half of the output shows the estimated difference between
each pair of means. An asterisk has been placed next to 3 pairs, indicating that these pairs show
statistically significant differences at the 95,0% confidence level. At the top of the page, 2
homogenous groups are identified using columns of X's. Within each column, the levels containing
X's form a group of means within which there are no statistically significant differences. The
method currently being
used to discriminate among the means is Fisher's least significant difference (LSD) procedure.
With this method, there is a 5,0% risk of calling each pair of means significantly different when
the actual
difference equals 0.
```

Multifactor ANOVA – growth (UFC) on maize grains

```
Analysis Summary

Dependent variable: ln _UFC_ml_
Factors:
        thesis
        aw
        time
        replicate

Number of complete cases: 120
```

The StatAdvisor

This procedure performs a multifactor analysis of variance for ln_UFC_ml_. It constructs various tests and graphs to determine which factors have a statistically significant effect on ln _UFC_ml_. It also tests for significant interactions amongst the factors, given sufficient data. The F-tests in the ANOVA table will allow you to identify the significant factors. For each significant factor, the Multiple Range Tests will tell you which means are significantly different from which others. The Means Plot and Interaction Plot will help you interpret the significant effects. The Residual Plots will help you judge whether the assumptions underlying the analysis of variance are violated by the data.



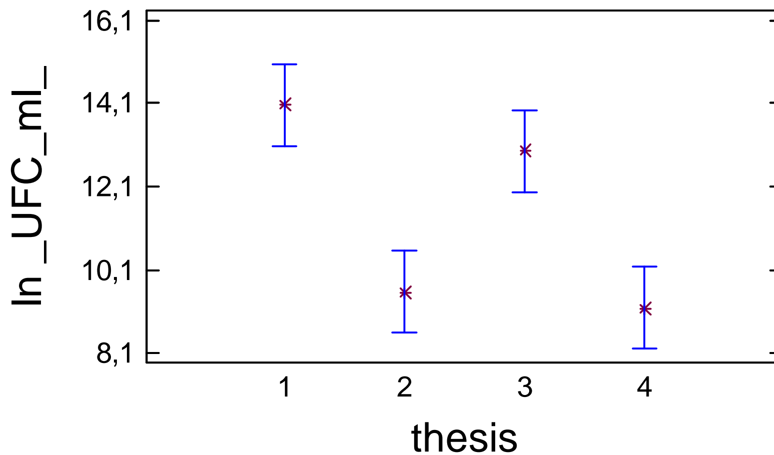
Analysis of Variance for ln _UFC_ml_ - Type III Sums of Squares

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
MAIN EFFECTS					
A:thesis	530,648	3	176,883	12,01	0,0000
B:aw	276,16	1	276,16	18,75	0,0000
C:time	234,533	3	78,1778	5,31	0,0019
D:replicate	81,6787	3	27,2262	1,85	0,1426
RESIDUAL	1605,25	109	14,727		
TOTAL (CORRECTED)	2707,79	119			

All F-ratios are based on the residual mean square error.

The StatAdvisor

The ANOVA table decomposes the variability of ln _UFC_ml_ into contributions due to various factors. Since Type III sums of squares (the default) have been chosen, the contribution of each factor is
measured having removed the effects of all other factors. The P-values test the statistical significance of each of the factors. Since 3 P-values are less than 0,05, these factors have a statistically significant effect on ln _UFC_ml_ at the 95,0% confidence level.



Multiple Range Tests for ln _UFC_ml_ by thesis

Method: 95,0 percent LSD

thesis	Count	LS Mean	LS Sigma	Homogeneous Groups
4	30	9,18562	0,704283	X
2	30	9,5642	0,704283	X
3	30	12,9537	0,704283	X
1	30	14,0469	0,704283	X

Contrast	Difference	+/- Limits
1 - 2	*4,48273	1,96385
1 - 3	1,09321	1,96385
1 - 4	*4,86131	1,96385
2 - 3	*-3,38952	1,96385
2 - 4	0,378582	1,96385
3 - 4	*3,7681	1,96385

* denotes a statistically significant difference.

The StatAdvisor

This table applies a multiple comparison procedure to determine which means are significantly different from which others. The bottom half of the output shows the estimated difference between each pair of means. An asterisk has been placed next to 4 pairs, indicating that these pairs show statistically significant differences at the 95,0% confidence level. At the top of the page, 2 homogenous groups are identified using columns of X's. Within each column, the levels containing X's form a group of means within which there are no statistically significant differences. The method currently being
used to discriminate among the means is Fisher's least significant difference (LSD) procedure. With this method, there is a 5,0% risk of calling each pair of means significantly different when the actual
difference equals 0.

Multifactor ANOVA - AFB_1 production on maize grains

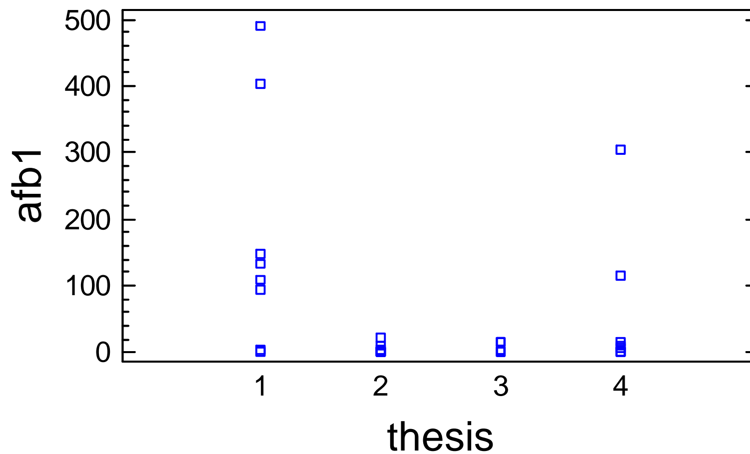
Analysis Summary

Dependent variable: afb1
Factors:
    thesis
    aw
    replicate

Number of complete cases: 32

The StatAdvisor

   This procedure performs a multifactor analysis of variance for afb1. It constructs various tests and graphs to determine which factors have a statistically significant effect on afb1. It also tests for significant interactions amongst the factors, given sufficient data. The F-tests in the ANOVA table will allow you to identify the significant factors. For each significant factor, the Multiple Range Tests will tell you which means are significantly different from which others. The Means Plot and Interaction Plot will help you interpret the significant effects. The Residual Plots will help you judge whether the assumptions underlying the analysis of variance are violated by the data.



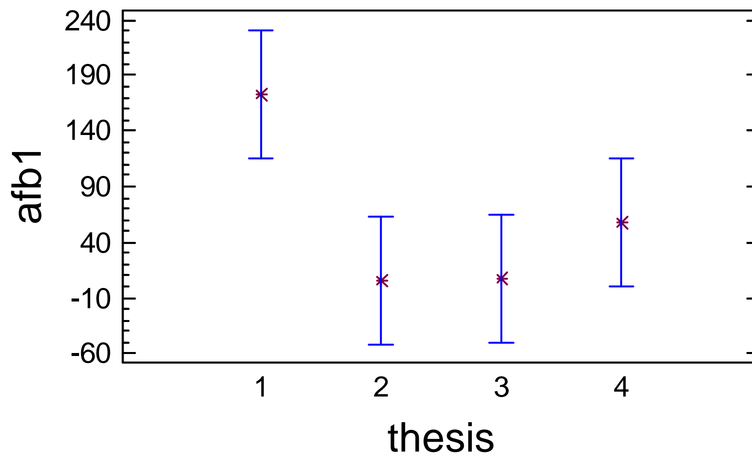
Analysis of Variance for afb1 - Type III Sums of Squares

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
MAIN EFFECTS					
A:thesis	148282,0	3	49427,4	3,97	0,0198
B:aw	4559,81	1	4559,81	0,37	0,5508
C:replicate	2878,66	3	959,552	0,08	0,9718
RESIDUAL	298933,0	24	12455,5		
TOTAL (CORRECTED)	454654,0	31			

All F-ratios are based on the residual mean square error.

The StatAdvisor

   The ANOVA table decomposes the variability of afb1 into contributions due to various factors. Since Type III sums of squares (the default) have been chosen, the contribution of each factor is measured having removed the effects of all other factors. The P-values test the statistical significance of each of the factors. Since one P-value is less than 0,05, this factor has a statistically significant effect on afb1 at the 95,0% confidence level.



Multiple Range Tests for afb1 by thesis

Method: 95,0 percent LSD

thesis	Count	LS Mean	LS Sigma	Homogeneous Groups
2	8	5,01967	39,4581	X
3	8	6,63852	39,4581	X
4	8	57,2567	39,4581	X
1	8	172,508	39,4581	X

Contrast	Difference	+/- Limits
1 - 2	*167,488	115,17
1 - 3	*165,869	115,17
1 - 4	*115,251	115,17
2 - 3	-1,61885	115,17
2 - 4	-52,237	115,17
3 - 4	-50,6182	115,17

* denotes a statistically significant difference.

The StatAdvisor

This table applies a multiple comparison procedure to determine which means are significantly different from which others. The bottom half of the output shows the estimated difference between each pair of means. An asterisk has been placed next to 3 pairs, indicating that these pairs show statistically significant differences at the 95,0% confidence level. At the top of the page, 2 homogenous groups are identified using columns of X's. Within each column, the levels containing X's form a group of means within which there are no statistically significant differences. The method currently being
used to discriminate among the means is Fisher's least significant difference (LSD) procedure. With this method, there is a 5,0% risk of calling each pair of means significantly different when the actual
difference equals 0.

Multifactor ANOVA - afb2

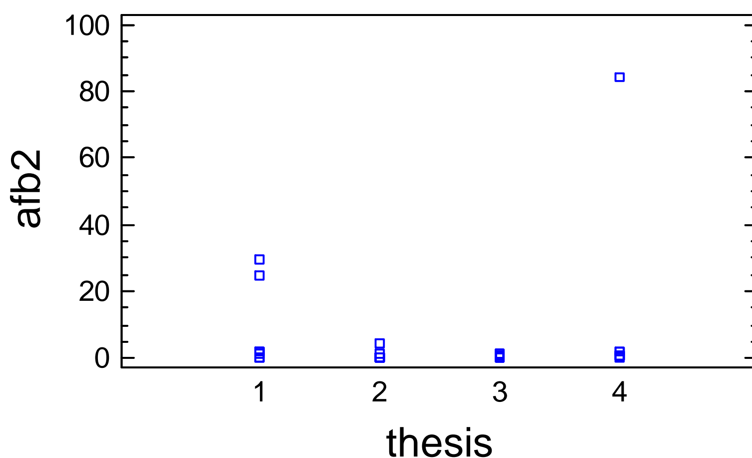
Analysis Summary

Dependent variable: afb2
Factors:
thesis
aw
replicate

Number of complete cases: 32

The StatAdvisor

This procedure performs a multifactor analysis of variance for afb2. It constructs various tests and graphs to determine which factors have a statistically significant effect on afb2. It also tests for significant interactions amongst the factors, given sufficient data. The F-tests in the ANOVA table will allow you to identify the significant factors. For each significant factor, the Multiple Range Tests will tell you which means are significantly different from which others. The Means Plot and Interaction Plot will help you interpret the significant effects. The Residual Plots will help you judge whether the assumptions underlying the analysis of variance are violated by the data.



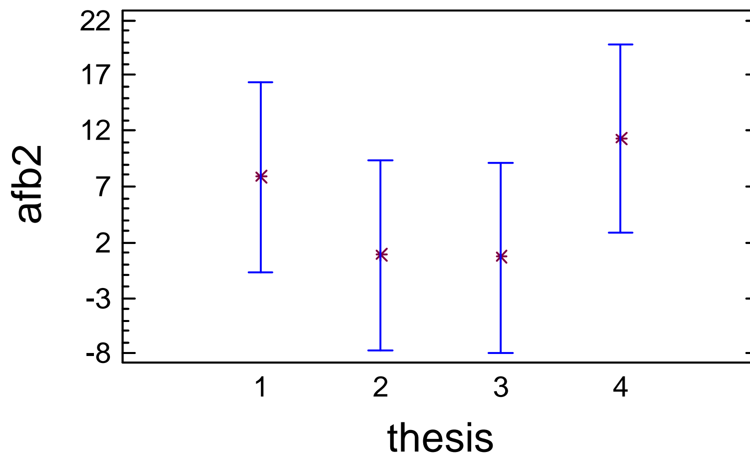
Analysis of Variance for afb2 - Type III Sums of Squares

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
MAIN EFFECTS					
A:thesis	674,861	3	224,954	0,82	0,4935
B:aw	42,6492	1	42,6492	0,16	0,6961
C:replicate	519,949	3	173,316	0,63	0,5998
RESIDUAL	6551,17	24	272,965		
TOTAL (CORRECTED)	7788,63	31			

All F-ratios are based on the residual mean square error.

The StatAdvisor

The ANOVA table decomposes the variability of afb2 into contributions due to various factors. Since Type III sums of squares (the default) have been chosen, the contribution of each factor is measured having removed the effects of all other factors. The P-values test the statistical significance of each of the factors. Since no P-values are less than 0,05, none of the factors have a statistically significant effect on afb2 at the 95,0% confidence level.



Multiple Range Tests for afb2 by thesis

Method: 95,0 percent LSD

thesis	Count	LS Mean	LS Sigma	Homogeneous Groups
3	8	0,669012	5,84129	X
2	8	0,849708	5,84129	X
1	8	7,87406	5,84129	X
4	8	11,3444	5,84129	X

Contrast	Difference	+/- Limits
1 - 2	7,02435	17,0496
1 - 3	7,20505	17,0496
1 - 4	-3,47029	17,0496
2 - 3	0,180696	17,0496
2 - 4	-10,4946	17,0496
3 - 4	-10,6753	17,0496

* denotes a statistically significant difference.

The StatAdvisor

This table applies a multiple comparison procedure to determine which means are significantly different from which others. The bottom half of the output shows the estimated difference between each pair of means. There are no statistically significant differences between any pair of means at the 95,0% confidence level. At the top of the page, one homogenous group is identified by a column of X's. Within each column, the levels containing X's form a group of means within which there are no statistically significant differences. The method currently being used to discriminate among the means is Fisher's least significant difference (LSD) procedure. With this method, there is a 5,0% risk of calling each pair of means significantly different when the actual difference equals 0.

Multifactor ANOVA – trials on grains and synthetic medium analysed together (0-1 scale)

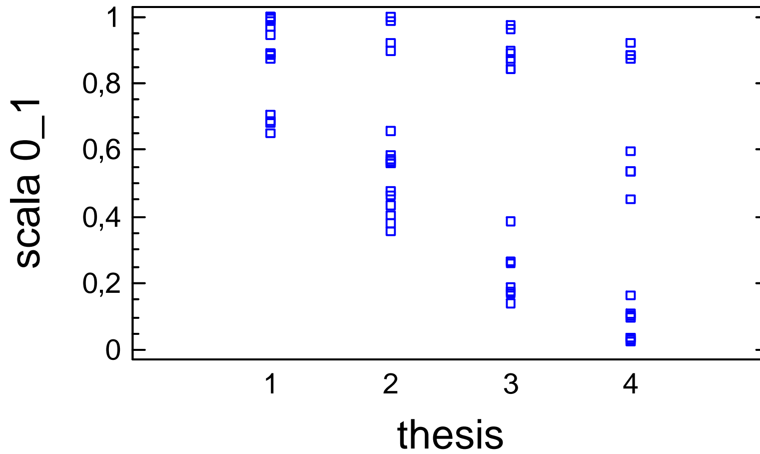
Analysis Summary

Dependent variable: scala 0_1
Factors:
- thesis
- aw
- replicate
- trial

Number of complete cases: 64

The StatAdvisor

This procedure performs a multifactor analysis of variance for scala 0_1. It constructs various tests and graphs to determine which factors have a statistically significant effect on scala 0_1. It also
tests for significant interactions amongst the factors, given sufficient data. The F-tests in the ANOVA table will allow you to identify the significant factors. For each significant factor, the Multiple Range Tests will tell you which means are significantly different from which others. The Means Plot and Interaction Plot will help you interpret the significant effects. The Residual Plots will help you judge whether the assumptions underlying the analysis of variance are violated by the data.



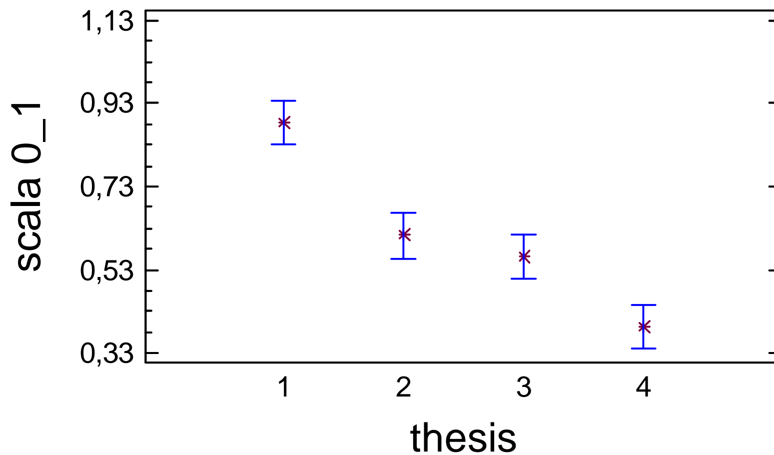
Analysis of Variance for scala 0_1 - Type III Sums of Squares

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
MAIN EFFECTS					
A:thesis	1,98682	3	0,662273	28,15	0,0000
B:aw	0,674307	1	0,674307	28,66	0,0000
C:replicate	0,00496888	3	0,00165629	0,07	0,9755
D:trial	2,69603	1	2,69603	114,61	0,0000
RESIDUAL	1,29382	55	0,023524		
TOTAL (CORRECTED)	6,65595	63			

All F-ratios are based on the residual mean square error.

The StatAdvisor

The ANOVA table decomposes the variability of scala 0_1 into contributions due to various factors. Since Type III sums of squares (the default) have been chosen, the contribution of each factor is
measured having removed the effects of all other factors. The P-values test the statistical significance of each of the factors. Since 3 P-values are less than 0,05, these factors have a statistically significant effect on scala 0_1 at the 95,0% confidence level.




```
Multiple Range Tests for scala 0_1 by thesis

--------------------------------------------------------------------------------
Method: 95,0 percent LSD
thesis         Count     LS Mean       LS Sigma      Homogeneous Groups
--------------------------------------------------------------------------------
4              16        0,393469      0,0383439     X
3              16        0,562513      0,0383439      X
2              16        0,613825      0,0383439      X
1              16        0,883986      0,0383439       X
--------------------------------------------------------------------------------
Contrast                                  Difference           +/-  Limits
--------------------------------------------------------------------------------
1 - 2                                    *0,270161             0,108672
1 - 3                                    *0,321473             0,108672
1 - 4                                    *0,490517             0,108672
2 - 3                                     0,051312             0,108672
2 - 4                                    *0,220356             0,108672
3 - 4                                    *0,169045             0,108672
--------------------------------------------------------------------------------
* denotes a statistically significant difference.
```

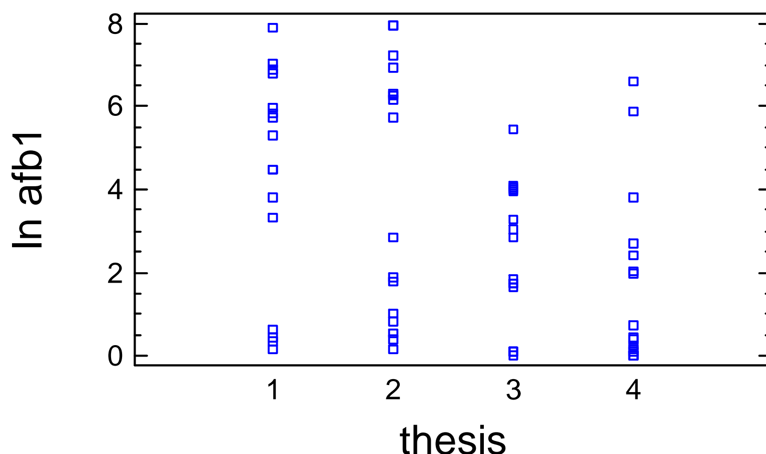
The StatAdvisor

This table applies a multiple comparison procedure to determine which means are significantly different from which others. The bottom half of the output shows the estimated difference between each pair of means. An asterisk has been placed next to 5 pairs, indicating that these pairs show statistically significant differences at the 95,0% confidence level. At the top of the page, 3 homogenous groups are identified using columns of X's. Within each column, the levels containing X's form a group of means within which there are no statistically significant differences. The method currently being used to discriminate among the means is Fisher's least significant difference (LSD) procedure. With this method, there is a 5,0% risk of calling each pair of means significantly different when the actual difference equals 0.

```
Multifactor ANOVA - ln afb1

Analysis Summary

Dependent variable: ln afb1
Factors:
        thesis
        aw
        replicate
        trial

Number of complete cases: 64
```

The StatAdvisor

This procedure performs a multifactor analysis of variance for ln afb1. It constructs various tests and graphs to determine which factors have a statistically significant effect on ln afb1. It also
tests for significant interactions amongst the factors, given sufficient data. The F-tests in the ANOVA table will allow you to identify the significant factors. For each significant factor, the Multiple Range Tests will tell you which means are significantly different from which others. The Means Plot and Interaction Plot will help you interpret the significant effects. The Residual Plots will help you judge whether the assumptions underlying the analysis of variance are violated by the data.



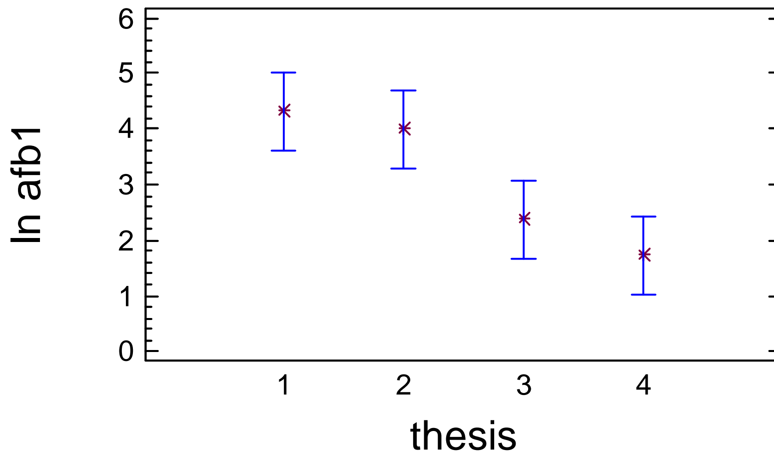
Analysis of Variance for ln afb1 - Type III Sums of Squares

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
MAIN EFFECTS					
A:thesis	75,5476	3	25,1825	6,41	0,0008
B:aw	19,4321	1	19,4321	4,95	0,0302
C:replicate	4,83994	3	1,61331	0,41	0,7458
D:trial	111,974	1	111,974	28,52	0,0000
RESIDUAL	215,974	55	3,92679		
TOTAL (CORRECTED)	427,767	63			

All F-ratios are based on the residual mean square error.

The StatAdvisor

The ANOVA table decomposes the variability of ln afb1 into contributions due to various factors. Since Type III sums of squares (the default) have been chosen, the contribution of each factor is
measured having removed the effects of all other factors. The P-values test the statistical significance of each of the factors. Since 3 P-values are less than 0,05, these factors have a statistically significant effect on ln afb1 at the 95,0% confidence level.




```
Multiple Range Tests for ln afb1 by thesis

--------------------------------------------------------------------------------
Method: 95,0 percent LSD
thesis          Count      LS Mean       LS Sigma      Homogeneous Groups
--------------------------------------------------------------------------------
4               16         1,72305       0,495403      X
3               16         2,37519       0,495403      X
2               16         3,99887       0,495403       X
1               16         4,32168       0,495403       X
--------------------------------------------------------------------------------
Contrast                                  Difference           +/-  Limits
--------------------------------------------------------------------------------
1 - 2                                      0,322818            1,40405
1 - 3                                     *1,9465              1,40405
1 - 4                                     *2,59863             1,40405
2 - 3                                     *1,62368             1,40405
2 - 4                                     *2,27581             1,40405
3 - 4                                      0,652133            1,40405
--------------------------------------------------------------------------------
* denotes a statistically significant difference.
```

The StatAdvisor

This table applies a multiple comparison procedure to determine which means are significantly different from which others. The bottom half of the output shows the estimated difference between each pair of means. An asterisk has been placed next to 4 pairs, indicating that these pairs show statistically significant differences at the 95,0% confidence level. At the top of the page, 2 homogenous groups are identified using columns of X's. Within each column, the levels containing X's form a group of means within which there are no statistically significant differences. The method currently being used to discriminate among the means is Fisher's least significant difference (LSD) procedure. With this method, there is a 5,0% risk of calling each pair of means significantly different when the actual difference equals 0.

CHAPTER 5

MEANS SEPARATIONS

SOLUTE STRESS

Experiment Model Number 10:
Three Factor Randomized Complete Block Design

Data case no. 1 to 216.

Factorial ANOVA for the factors:

```
    Replication (Var 4: repl) with values from 1 to 4
    Factor A (Var 1: strain) with values from 1 to 3
    Factor B (Var 2: temp) with values from 1 to 2
    Factor C (Var 3: tesi) with values from 1 to 9


Grand Mean = 23.348   Grand Sum = 5043.250   Total Count = 216


              T A B L E   O F   M E A N S

  4   1   2   3              5             Total
-------------------------------------------------------------
  1   *   *   *           23.491         1268.500
  2   *   *   *           23.315         1259.000
  3   *   *   *           23.319         1259.250
  4   *   *   *           23.269         1256.500
-------------------------------------------------------------
  *   1   *   *           23.080         1661.750
  *   2   *   *           23.337         1680.250
  *   3   *   *           23.628         1701.250
-------------------------------------------------------------
  *   *   1   *           24.002         2592.250
  *   *   2   *           22.694         2451.000
-------------------------------------------------------------
  *   1   1   *           24.090          867.250
  *   1   2   *           22.069          794.500
  *   2   1   *           23.597          849.500
  *   2   2   *           23.076          830.750
  *   3   1   *           24.319          875.500
  *   3   2   *           22.938          825.750
-------------------------------------------------------------
  *   *   *   1            0.000            0.000
  *   *   *   2            7.146          171.500
  *   *   *   3           36.698          880.750
  *   *   *   4           40.302          967.250
  *   *   *   5            0.000            0.000
  *   *   *   6            7.969          191.250
  *   *   *   7           35.323          847.750
  *   *   *   8           42.500         1020.000
  *   *   *   9           40.198          964.750
-------------------------------------------------------------
  *   1   *   1            0.000            0.000
  *   1   *   2            7.188           57.500
  *   1   *   3           36.688          293.500
  *   1   *   4           40.188          321.500
  *   1   *   5            0.000            0.000
  *   1   *   6            5.594           44.750
  *   1   *   7           36.156          289.250
  *   1   *   8           42.500          340.000
  *   1   *   9           39.406          315.250
  *   2   *   1            0.000            0.000
  *   2   *   2            7.531           60.250
  *   2   *   3           36.094          288.750
  *   2   *   4           40.156          321.250
  *   2   *   5            0.000            0.000
  *   2   *   6            8.219           65.750
  *   2   *   7           35.406          283.250
  *   2   *   8           42.500          340.000
  *   2   *   9           40.125          321.000
  *   3   *   1            0.000            0.000
  *   3   *   2            6.719           53.750
  *   3   *   3           37.313          298.500
```

*	3	*	4	40.563	324.500
*	3	*	5	0.000	0.000
*	3	*	6	10.094	80.750
*	3	*	7	34.406	275.250
*	3	*	8	42.500	340.000
*	3	*	9	41.063	328.500
*	*	1	1	0.000	0.000
*	*	1	2	7.250	87.000
*	*	1	3	37.667	452.000
*	*	1	4	42.500	510.000
*	*	1	5	0.000	0.000
*	*	1	6	8.542	102.500
*	*	1	7	36.646	439.750
*	*	1	8	42.500	510.000
*	*	1	9	40.917	491.000
*	*	2	1	0.000	0.000
*	*	2	2	7.042	84.500
*	*	2	3	35.729	428.750
*	*	2	4	38.104	457.250
*	*	2	5	0.000	0.000
*	*	2	6	7.396	88.750
*	*	2	7	34.000	408.000
*	*	2	8	42.500	510.000
*	*	2	9	39.479	473.750
*	1	1	1	0.000	0.000
*	1	1	2	7.188	28.750
*	1	1	3	37.500	150.000
*	1	1	4	42.500	170.000
*	1	1	5	0.000	0.000
*	1	1	6	5.688	22.750
*	1	1	7	38.938	155.750
*	1	1	8	42.500	170.000
*	1	1	9	42.500	170.000
*	1	2	1	0.000	0.000
*	1	2	2	7.188	28.750
*	1	2	3	35.875	143.500
*	1	2	4	37.875	151.500
*	1	2	5	0.000	0.000
*	1	2	6	5.500	22.000
*	1	2	7	33.375	133.500
*	1	2	8	42.500	170.000
*	1	2	9	36.313	145.250
*	2	1	1	0.000	0.000
*	2	1	2	7.750	31.000
*	2	1	3	36.625	146.500
*	2	1	4	42.500	170.000
*	2	1	5	0.000	0.000
*	2	1	6	8.688	34.750
*	2	1	7	36.563	146.250
*	2	1	8	42.500	170.000
*	2	1	9	37.750	151.000
*	2	2	1	0.000	0.000
*	2	2	2	7.313	29.250
*	2	2	3	35.563	142.250
*	2	2	4	37.813	151.250
*	2	2	5	0.000	0.000
*	2	2	6	7.750	31.000
*	2	2	7	34.250	137.000
*	2	2	8	42.500	170.000
*	2	2	9	42.500	170.000
*	3	1	1	0.000	0.000

```
  *   3   1   2              6.813            27.250
  *   3   1   3             38.875           155.500
  *   3   1   4             42.500           170.000
  *   3   1   5              0.000             0.000
  *   3   1   6             11.250            45.000
  *   3   1   7             34.438           137.750
  *   3   1   8             42.500           170.000
  *   3   1   9             42.500           170.000
  *   3   2   1              0.000             0.000
  *   3   2   2              6.625            26.500
  *   3   2   3             35.750           143.000
  *   3   2   4             38.625           154.500
  *   3   2   5              0.000             0.000
  *   3   2   6              8.938            35.750
  *   3   2   7             34.375           137.500
  *   3   2   8             42.500           170.000
  *   3   2   9             39.625           158.500
--------------------------------------------------------------
```

A N A L Y S I S   O F   V A R I A N C E   T A B L E

K Value	Source	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Prob
1	Replication	3	1.545	0.515	1.5783	0.1968
2	Factor A	2	10.850	5.425	16.6266	0.0000
4	Factor B	1	92.368	92.368	283.1035	0.0000
6	AB	2	20.398	10.199	31.2596	0.0000
8	Factor C	8	68377.641	8547.205	26196.6758	0.0000
10	AC	16	103.692	6.481	19.8631	0.0000
12	BC	8	108.634	13.579	41.6197	0.0000
14	ABC	16	146.216	9.139	28.0090	0.0000
-15	Error	159	51.877	0.326		
	Total	215	68913.222			

Coefficient of Variation: 2.45%

$s_{\bar{y}}$ for means group 1: 0.0777 Number of Observations: 54

$s_{\bar{y}}$ for means group 2: 0.0673 Number of Observations: 72

$s_{\bar{y}}$ for means group 4: 0.0550 Number of Observations: 108

$s_{\bar{y}}$ for means group 6: 0.0952 Number of Observations: 36

$s_{\bar{y}}$ for means group 8: 0.1166 Number of Observations: 24

$s_{\bar{y}}$ for means group 10: 0.2020 Number of Observations: 8

$s_{\bar{y}}$ for means group 12: 0.1649 Number of Observations: 12

```
     s_ for means group 14:     0.2856       Number of Observations: 4
      y
```

STRAINS

```
Error Mean Square = 0.3260
Error Degrees of Freedom = 159
No. of observations to calculate a mean = 72

Least Significant Difference Test
LSD value = 0.1879     at alpha = 0.050

      Original Order               Ranked Order

 Mean    1 =    23.08    C    Mean    3 =    23.63  A
 Mean    2 =    23.34   B     Mean    2 =    23.34   B
 Mean    3 =    23.63  A      Mean    1 =    23.08    C
```

WATER POTENTIAL

```
Error Mean Square = 0.007000
Error Degrees of Freedom = 159
No. of observations to calculate a mean = 24

Tukey's Honestly Significant Difference Test
s_ = 0.01708    at alpha = 0.050
 x

        Original Order                  Ranked Order

 Mean    1 =   0.0000      E      Mean    8 =    6.070  A
 Mean    2 =    1.021     D       Mean    4 =    5.758  AB
 Mean    3 =    5.242   BC        Mean    9 =    5.741  AB
 Mean    4 =    5.758  AB         Mean    3 =    5.242   BC
 Mean    5 =   0.0000      E      Mean    7 =    5.045    C
 Mean    6 =    1.138     D       Mean    6 =    1.138     D
 Mean    7 =    5.045    C        Mean    2 =    1.021     D
 Mean    8 =    6.070  A          Mean    1 =   0.0000      E
 Mean    9 =    5.741  AB         Mean    5 =   0.0000      E
```

MATRIC STRESS

```
Experiment Model Number 10:
Three Factor Randomized Complete Block Design

     Data case no. 1 to 72.

     Factorial ANOVA for the factors:
          Replication (Var 4: repl) with values from 1 to 3
          Factor A (Var 1: strains) with values from 1 to 3
          Factor B (Var 2: temp) with values from 1 to 2
          Factor C (Var 3: aw) with values from 1 to 4


     Grand Mean = 23.167   Grand Sum = 1668.000   Total Count = 72


                   T A B L E   O F   M E A N S
```

4	1	2	3	5	Total
1	*	*	*	24.729	593.500
2	*	*	*	20.823	499.750
3	*	*	*	23.948	574.750
*	1	*	*	25.604	614.500
*	2	*	*	18.813	451.500
*	3	*	*	25.083	602.000
*	*	1	*	20.715	745.750
*	*	2	*	25.618	922.250
*	1	1	*	24.583	295.000
*	1	2	*	26.625	319.500
*	2	1	*	16.521	198.250
*	2	2	*	21.104	253.250
*	3	1	*	21.042	252.500
*	3	2	*	29.125	349.500
*	*	*	1	0.000	0.000
*	*	*	2	13.583	244.500
*	*	*	3	40.167	723.000
*	*	*	4	38.917	700.500
*	1	*	1	0.000	0.000
*	1	*	2	14.250	85.500
*	1	*	3	43.167	259.000
*	1	*	4	45.000	270.000
*	2	*	1	0.000	0.000
*	2	*	2	10.958	65.750
*	2	*	3	37.542	225.250
*	2	*	4	26.750	160.500
*	3	*	1	0.000	0.000
*	3	*	2	15.542	93.250
*	3	*	3	39.792	238.750
*	3	*	4	45.000	270.000
*	*	1	1	0.000	0.000
*	*	1	2	5.028	45.250
*	*	1	3	39.167	352.500
*	*	1	4	38.667	348.000
*	*	2	1	0.000	0.000
*	*	2	2	22.139	199.250
*	*	2	3	41.167	370.500
*	*	2	4	39.167	352.500
*	1	1	1	0.000	0.000
*	1	1	2	8.333	25.000
*	1	1	3	45.000	135.000
*	1	1	4	45.000	135.000
*	1	2	1	0.000	0.000
*	1	2	2	20.167	60.500
*	1	2	3	41.333	124.000
*	1	2	4	45.000	135.000
*	2	1	1	0.000	0.000
*	2	1	2	2.167	6.500
*	2	1	3	37.917	113.750
*	2	1	4	26.000	78.000
*	2	2	1	0.000	0.000
*	2	2	2	19.750	59.250
*	2	2	3	37.167	111.500
*	2	2	4	27.500	82.500

```
  *   3   1   1            0.000           0.000
  *   3   1   2            4.583          13.750
  *   3   1   3           34.583         103.750
  *   3   1   4           45.000         135.000
  *   3   2   1            0.000           0.000
  *   3   2   2           26.500          79.500
  *   3   2   3           45.000         135.000
  *   3   2   4           45.000         135.000
--------------------------------------------------------------
```

A N A L Y S I S   O F   V A R I A N C E   T A B L E

K Value	Source	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Prob
1	Replication	2	205.078	102.539	1.9480	0.1541
2	Factor A	2	685.771	342.885	6.5139	0.0032
4	Factor B	1	432.670	432.670	8.2196	0.0062
6	AB	2	110.424	55.212	1.0489	0.3586
8	Factor C	3	20980.750	6993.583	132.8601	0.0000
10	AC	6	809.688	134.948	2.5637	0.0317
12	BC	3	904.010	301.337	5.7246	0.0020
14	ABC	6	134.354	22.392	0.4254	
-15	Error	46	2421.380	52.639		
	Total	71	26684.125			

Coefficient of Variation: 31.32%

$s_{\bar{y}}$ for means group 1: 1.4810 Number of Observations: 24

$s_{\bar{y}}$ for means group 2: 1.4810 Number of Observations: 24

$s_{\bar{y}}$ for means group 4: 1.2092 Number of Observations: 36

$s_{\bar{y}}$ for means group 6: 2.0944 Number of Observations: 12

$s_{\bar{y}}$ for means group 8: 1.7101 Number of Observations: 18

$s_{\bar{y}}$ for means group 10: 2.9619 Number of Observations: 6

$s_{\bar{y}}$ for means group 12: 2.4184 Number of Observations: 9

$s_{\bar{y}}$ for means group 14: 4.1888 Number of Observations: 3

STRAIN

Error Mean Square = 52.64
Error Degrees of Freedom = 46

```
No. of observations to calculate a mean = 24

Tukey's Honestly Significant Difference Test
s_ = 1.481      at alpha = 0.050
 x

     Original Order                Ranked Order

 Mean    1 =    25.60  A     Mean    1 =    25.60  A
 Mean    2 =    18.81   B    Mean    3 =    25.08  A
 Mean    3 =    25.08  A     Mean    2 =    18.81   B
```

WATER POTENTIAL

```
Error Mean Square = 52.64
Error Degrees of Freedom = 46
No. of observations to calculate a mean = 18

Tukey's Honestly Significant Difference Test
s_ = 1.710      at alpha = 0.050
 x

      Original Order                Ranked Order

 Mean    1 =   0.0000    C    Mean    3 =    40.17  A
 Mean    2 =    13.58   B     Mean    4 =    38.92  A
 Mean    3 =    40.17  A      Mean    2 =    13.58   B
 Mean    4 =    38.92  A      Mean    1 =   0.0000    C
```

SPORULATION

ANOVA UNIVARIATE

Fattori tra soggetti

		N
aw	1	18
	2	18
	3	18
	4	18
	5	18
	6	18
	7	18
	8	18
	9	18
ceppo	1	54
	2	54
	3	54
temp	1	81
	2	81

Test degli effetti fra soggetti

Variabile dipendente: ln

Sorgente	Somma dei quadrati Tipo III	df	Media dei quadrati	F	Sig.
Modello corretto	10910.265[a]	53	205.854	3780.178	.000
Intercetta	30479.256	1	30479.256	559702.5	.000
aw	9905.372	8	1238.172	22737.028	.000
ceppo	36.396	2	18.198	334.179	.000
temp	18.013	1	18.013	330.786	.000
aw * ceppo	268.474	16	16.780	308.131	.000
aw * temp	431.704	8	53.963	990.944	.000
ceppo * temp	22.931	2	11.465	210.545	.000
aw * ceppo * temp	227.374	16	14.211	260.960	.000
Errore	5.881	108	.054		
Totale	41395.402	162			
Totale corretto	10916.146	161			

a. R quadrato = .999 (R quadrato corretto = .999)

Test post-hoc

aw

Confronti multipli

Variabile dipendente: ln

	(I) aw	(J) aw	Differenza fra medie (I-J)	Errore std.	Sig.	Intervallo di confidenza 95% Limite inferiore	Limite superiore
HSD di Tukey	1	2	-6.7494*	.07779	.000	-6.9957	-6.5032
		3	-8.2906*	.07779	.000	-8.5368	-8.0443
		4	-8.9511*	.07779	.000	-9.1974	-8.7049
		5	10.4089*	.07779	.000	10.1626	10.6551
		6	10.4089*	.07779	.000	10.1626	10.6551
		7	-7.7789*	.07779	.000	-8.0251	-7.5326
		8	-9.1650*	.07779	.000	-9.4112	-8.9188
		9	-9.6517*	.07779	.000	-9.8979	-9.4054
	2	1	6.7494*	.07779	.000	6.5032	6.9957
		3	-1.5411*	.07779	.000	-1.7874	-1.2949
		4	-2.2017*	.07779	.000	-2.4479	-1.9554
		5	17.1583*	.07779	.000	16.9121	17.4046
		6	17.1583*	.07779	.000	16.9121	17.4046
		7	-1.0294*	.07779	.000	-1.2757	-.7832
		8	-2.4156*	.07779	.000	-2.6618	-2.1693
		9	-2.9022*	.07779	.000	-3.1485	-2.6560
	3	1	8.2906*	.07779	.000	8.0443	8.5368
		2	1.5411*	.07779	.000	1.2949	1.7874
		4	-.6606*	.07779	.000	-.9068	-.4143
		5	18.6994*	.07779	.000	18.4532	18.9457
		6	18.6994*	.07779	.000	18.4532	18.9457
		7	.5117*	.07779	.000	.2654	.7579
		8	-.8744*	.07779	.000	-1.1207	-.6282
		9	-1.3611*	.07779	.000	-1.6074	-1.1149
	4	1	8.9511*	.07779	.000	8.7049	9.1974
		2	2.2017*	.07779	.000	1.9554	2.4479
		3	.6606*	.07779	.000	.4143	.9068
		5	19.3600*	.07779	.000	19.1138	19.6062
		6	19.3600*	.07779	.000	19.1138	19.6062
		7	1.1722*	.07779	.000	.9260	1.4185
		8	-.2139	.07779	.143	-.4601	.0324
		9	-.7006*	.07779	.000	-.9468	-.4543
	5	1	-10.4089*	.07779	.000	-10.6551	-10.1626
		2	-17.1583*	.07779	.000	-17.4046	-16.9121
		3	-18.6994*	.07779	.000	-18.9457	-18.4532
		4	-19.3600*	.07779	.000	-19.6062	-19.1138
		6	.0000	.07779	1.000	-.2462	.2462
		7	-18.1878*	.07779	.000	-18.4340	-17.9415
		8	-19.5739*	.07779	.000	-19.8201	-19.3276
		9	-20.0606*	.07779	.000	-20.3068	-19.8143
	6	1	-10.4089*	.07779	.000	-10.6551	-10.1626
		2	-17.1583*	.07779	.000	-17.4046	-16.9121
		3	-18.6994*	.07779	.000	-18.9457	-18.4532
		4	-19.3600*	.07779	.000	-19.6062	-19.1138
		5	.0000	.07779	1.000	-.2462	.2462
		7	-18.1878*	.07779	.000	-18.4340	-17.9415
		8	-19.5739*	.07779	.000	-19.8201	-19.3276
		9	-20.0606*	.07779	.000	-20.3068	-19.8143
	7	1	7.7789*	.07779	.000	7.5326	8.0251

Sottoinsiemi omogenei

ln

	aw	N	Sottoinsieme 1	2	3	4	5	6
Student-Newman-Keuls[a,b]	5	18	.0000					
	6	18	.0000					
	1	18		10.4089				
	2	18			17.1583			
	7	18				18.1878		
	3	18					18.6994	
	4	18						19.36
	8	18						
	9	18						
	Sig.		1.000	1.000	1.000	1.000	1.000	1.0
HSD di Tukey[a,b]	5	18	.0000					
	6	18	.0000					
	1	18		10.4089				
	2	18			17.1583			
	7	18				18.1878		
	3	18					18.6994	
	4	18						19.36
	8	18						19.57
	9	18						
	Sig.		1.000	1.000	1.000	1.000	1.000	.1

Sono visualizzate le medie per i gruppi di sottoinsiemi omogenei.
Basato sulla somma dei quadrati Tipo III
Il termine di errore è Media dei quadrati(Errore) = .054.

a. Utilizza dimensione campionaria media armonica = 18.000

b. Alfa = .05

strain

Confronti multipli

Variabile dipendente: ln

	(I) ceppo	(J) ceppo	Differenza fra medie (I-J)	Errore std.	Sig.	Intervallo di confidenza 95% Limite inferiore	Limite superiore
HSD di Tukey	1	2	-.0776	.04491	.200	-.1843	.0291
		3	-1.0420*	.04491	.000	-1.1488	-.9353
	2	1	.0776	.04491	.200	-.0291	.1843
		3	-.9644*	.04491	.000	-1.0712	-.8577
	3	1	1.0420*	.04491	.000	.9353	1.1488
		2	.9644*	.04491	.000	.8577	1.0712

Basato sulle medie osservate.

*. La differenza fra medie è significativa al livello .05.

Sottoinsiemi omogenei

ln

	ceppo	N	Sottoinsieme 1	Sottoinsieme 2
Student-Newman-Keuls[a,b]	1	54	13.3433	
	2	54	13.4209	
	3	54		14.3854
	Sig.		.087	1.000
HSD di Tukey[a,b]	1	54	13.3433	
	2	54	13.4209	
	3	54		14.3854
	Sig.		.200	1.000

Sono visualizzate le medie per i gruppi di sottoinsiemi omogenei.
Basato sulla somma dei quadrati Tipo III
Il termine di errore è Media dei quadrati(Errore) = .054.

a. Utilizza dimensione campionaria media armonica = 54.000

b. Alfa = .05

CHAPTER 7

Multifactor ANOVA – Concentration of the inoculum

```
Analysis Summary

Dependent variable: n° di pezzi infetti da A_ flavus
Factors:
        Tesi
        Zona
        Replica

Number of complete cases: 240


The StatAdvisor
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```
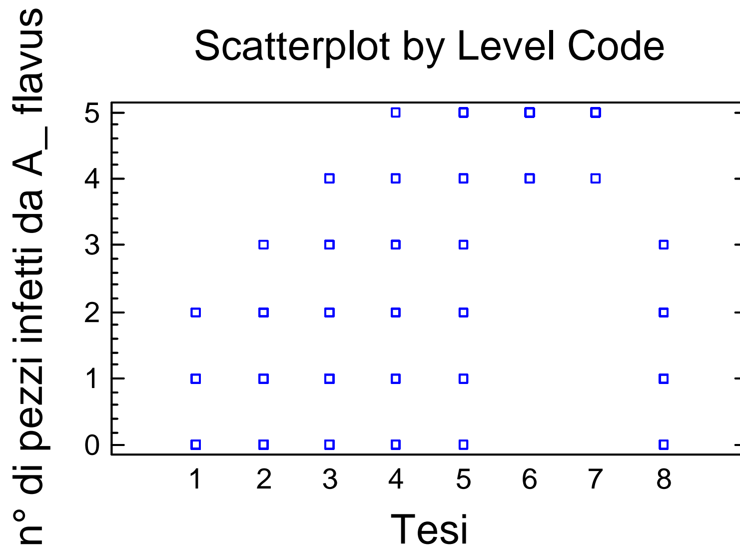

This procedure performs a multifactor analysis of variance for n° di pezzi infetti da A_ flavus. It constructs various tests and graphs to determine which factors have a statistically significant effect on
n° di pezzi infetti da A_ flavus. It also tests for significant interactions amongst the factors, given sufficient data. The F-tests in the ANOVA table will allow you to identify the significant factors.
For each significant factor, the Multiple Range Tests will tell you which means are significantly different from which others. The Means Plot and Interaction Plot will help you interpret the significant
effects. The Residual Plots will help you judge whether the assumptions underlying the analysis of variance are violated by the data.



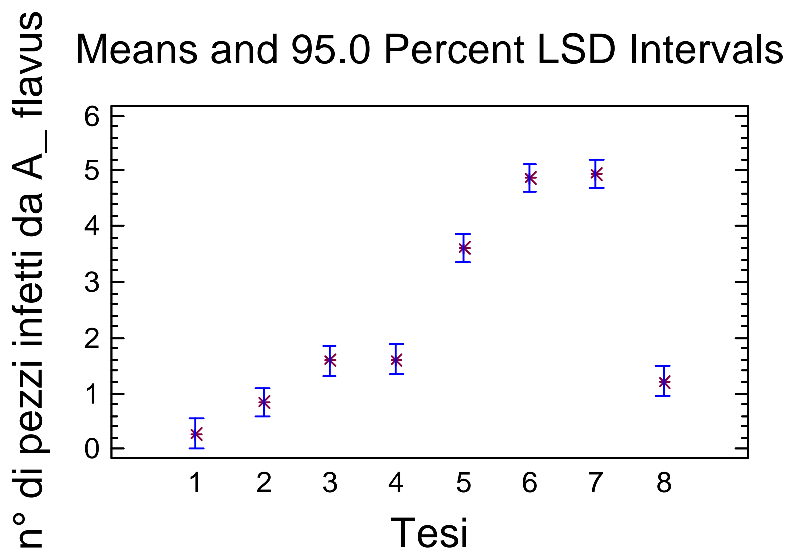
Analysis of Variance for n° di pezzi infetti da A_ flavus - Type III Sums of Squares

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
MAIN EFFECTS					
A:Tesi	711.25	7	101.607	98.14	0.0000
B:Zona	2.38125	2	1.19062	1.15	0.3185
C:Replica	11.5083	2	5.75417	5.56	0.0044
RESIDUAL	236.044	228	1.03528		
TOTAL (CORRECTED)	961.183	239			

All F-ratios are based on the residual mean square error.

The StatAdvisor

The ANOVA table decomposes the variability of n° di pezzi infetti da A_ flavus into contributions due to various factors. Since Type III sums of squares (the default) have been chosen, the contribution
of each factor is measured having removed the effects of all other factors. The P-values test the statistical significance of each of the factors. Since 2 P-values are less than 0.05, these factors have
a statistically significant effect on n° di pezzi infetti da A_ flavus at the 95.0% confidence level.



Multiple Range Tests for nº di pezzi infetti da A_ flavus by Tesi

Method: 95.0 percent LSD

Tesi	Count	LS Mean	LS Sigma	Homogeneous Groups				
1	30	0.279861	0.187052	X				
2	30	0.846528	0.187052		X			
8	30	1.21319	0.187052		X	X		
3	30	1.57986	0.187052			X		
4	30	1.61319	0.187052			X		
5	30	3.61319	0.187052				X	
6	30	4.87986	0.187052					X
7	30	4.94653	0.187052					X

Contrast	Difference	+/- Limits
1 - 2	*-0.566667	0.517659
1 - 3	*-1.3	0.517659
1 - 4	*-1.33333	0.517659
1 - 5	*-3.33333	0.517659
1 - 6	*-4.6	0.517659
1 - 7	*-4.66667	0.517659
1 - 8	*-0.933333	0.517659
2 - 3	*-0.733333	0.517659
2 - 4	*-0.766667	0.517659
2 - 5	*-2.76667	0.517659
2 - 6	*-4.03333	0.517659
2 - 7	*-4.1	0.517659
2 - 8	-0.366667	0.517659
3 - 4	-0.0333333	0.517659
3 - 5	*-2.03333	0.517659
3 - 6	*-3.3	0.517659
3 - 7	*-3.36667	0.517659
3 - 8	0.366667	0.517659
4 - 5	*-2.0	0.517659
4 - 6	*-3.26667	0.517659
4 - 7	*-3.33333	0.517659
4 - 8	0.4	0.517659
5 - 6	*-1.26667	0.517659
5 - 7	*-1.33333	0.517659
5 - 8	*2.4	0.517659
6 - 7	-0.0666667	0.517659
6 - 8	*3.66667	0.517659
7 - 8	*3.73333	0.517659

* denotes a statistically significant difference.

The StatAdvisor

This table applies a multiple comparison procedure to determine which means are significantly different from which others. The bottom half of the output shows the estimated difference between each pair of means. An asterisk has been placed next to 23 pairs, indicating that these pairs show statistically significant differences at the 95.0% confidence level. At the top of the page, 5 homogenous groups are identified using columns of X's. Within each column, the levels containing X's form a group of means within which there are no statistically significant differences. The method currently being used to discriminate among the means is Fisher's least significant difference (LSD) procedure. With this method, there is a 5.0% risk of calling each pair of means significantly different when the actual difference equals 0.

Multifactor ANOVA – infection at different temperatures

Analysis Summary

Dependent variable: nº di A_flavus su 5 pezzi di mai

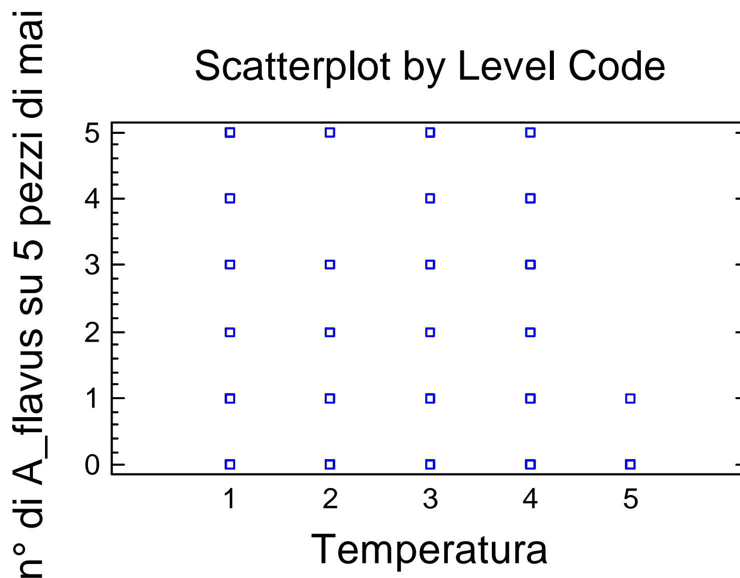
Factors:

- Temperatura
- DAP
- Zona

Number of complete cases: 150

The StatAdvisor

This procedure performs a multifactor analysis of variance for n° di A_flavus su 5 pezzi di mai. It constructs various tests and graphs to determine which factors have a statistically significant effect on
n° di A_flavus su 5 pezzi di mai. It also tests for significant interactions amongst the factors, given sufficient data. The F-tests in the ANOVA table will allow you to identify the significant factors.
For each significant factor, the Multiple Range Tests will tell you which means are significantly different from which others. The Means Plot and Interaction Plot will help you interpret the significant
effects. The Residual Plots will help you judge whether the assumptions underlying the analysis of variance are violated by the data.



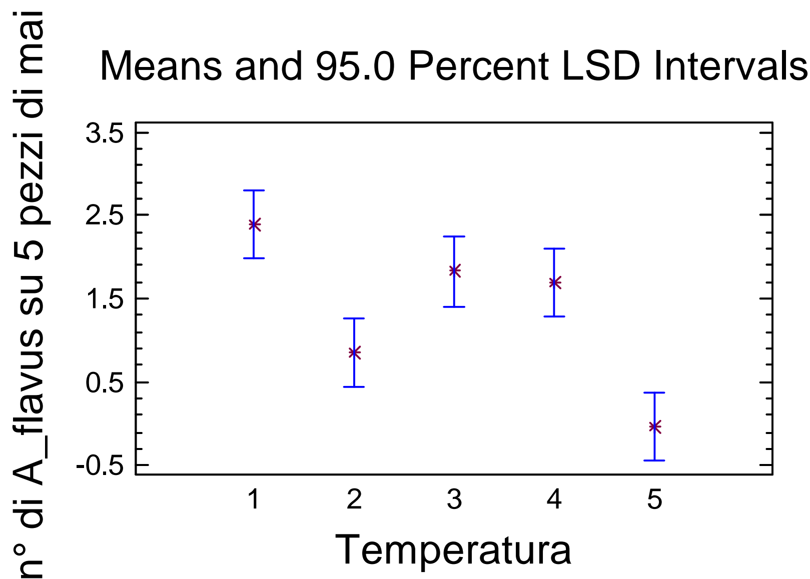
Analysis of Variance for n° di A_flavus su 5 pezzi di mai - Type III Sums of Squares

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
MAIN EFFECTS					
A:Temperatura	108.173	4	27.0433	10.61	0.0000
B:DAP	3.24	2	1.62	0.64	0.5311
C:Zona	11.74	2	5.87	2.30	0.1037
RESIDUAL	359.387	141	2.54884		
TOTAL (CORRECTED)	482.54	149			

All F-ratios are based on the residual mean square error.

The StatAdvisor

The ANOVA table decomposes the variability of n° di A_flavus su 5 pezzi di mai into contributions due to various factors. Since Type III sums of squares (the default) have been chosen, the contribution
of each factor is measured having removed the effects of all other factors. The P-values test the statistical significance of each of the factors. Since one P-value is less than 0.05, this factor has a
statistically significant effect on n° di A_flavus su 5 pezzi di mai at the 95.0% confidence level.




```
Multiple Range Tests for n° di A_flavus su 5 pezzi di mai by Temperatura

--------------------------------------------------------------------------------
Method: 95.0 percent LSD
Temperatura     Count      LS Mean        LS Sigma      Homogeneous Groups
--------------------------------------------------------------------------------
5               30         -0.0422222     0.294702      X
2               30         0.857778       0.294702       X
4               30         1.69111        0.294702        X
3               30         1.82444        0.294702        X
1               30         2.39111        0.294702        X
--------------------------------------------------------------------------------
Contrast                                   Difference           +/-  Limits
--------------------------------------------------------------------------------
1 - 2                                     *1.53333              0.814926
1 - 3                                      0.566667             0.814926
1 - 4                                      0.7                  0.814926
1 - 5                                     *2.43333              0.814926
2 - 3                                     *-0.966667            0.814926
2 - 4                                     *-0.833333            0.814926
2 - 5                                     *0.9                  0.814926
3 - 4                                      0.133333             0.814926
3 - 5                                     *1.86667              0.814926
4 - 5                                     *1.73333              0.814926
--------------------------------------------------------------------------------
* denotes a statistically significant difference.


The StatAdvisor
---------------
   This table applies a multiple comparison procedure to determine which means are significantly
different from which others. The bottom half of the output shows the estimated difference between
each pair of means. An asterisk has been placed next to 7 pairs, indicating that these pairs show
statistically significant differences at the 95.0% confidence level. At the top of the page, 3
homogenous groups are identified using columns of X's. Within each column, the levels containing
X's form a group of means within which there are no statistically significant differences. The
method currently being
used to discriminate among the means is Fisher's least significant difference (LSD) procedure.
With this method, there is a 5.0% risk of calling each pair of means significantly different when
the actual
difference equals 0.
```

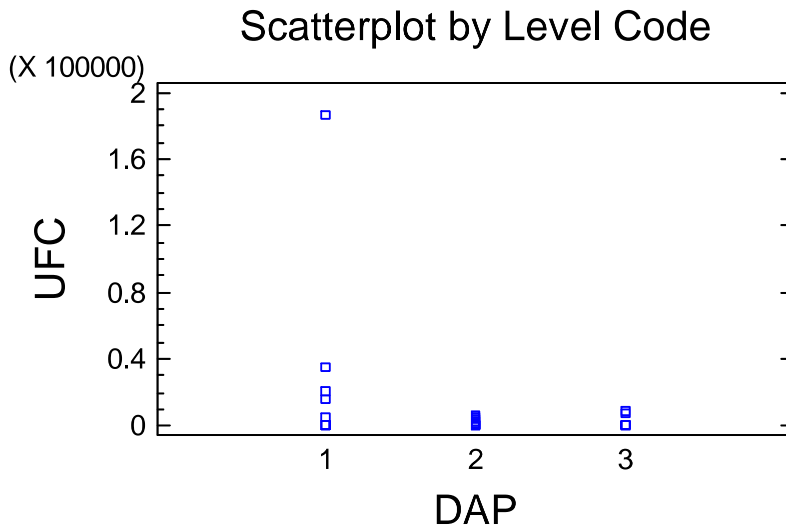
Multifactor ANOVA – growth (UFC) at different growth stages

```
Analysis Summary

Dependent variable: UFC
Factors:
        DAP
        Temperatura
        Replica
```

Number of complete cases: 45

The StatAdvisor

This procedure performs a multifactor analysis of variance for UFC. It constructs various tests and graphs to determine which factors have a statistically significant effect on UFC. It also tests for
significant interactions amongst the factors, given sufficient data. The F-tests in the ANOVA table will allow you to identify the significant factors. For each significant factor, the Multiple Range Tests will tell you which means are significantly different from which others. The Means Plot and Interaction Plot will help you interpret the significant effects. The Residual Plots will help you judge whether the assumptions underlying the analysis of variance are violated by the data.



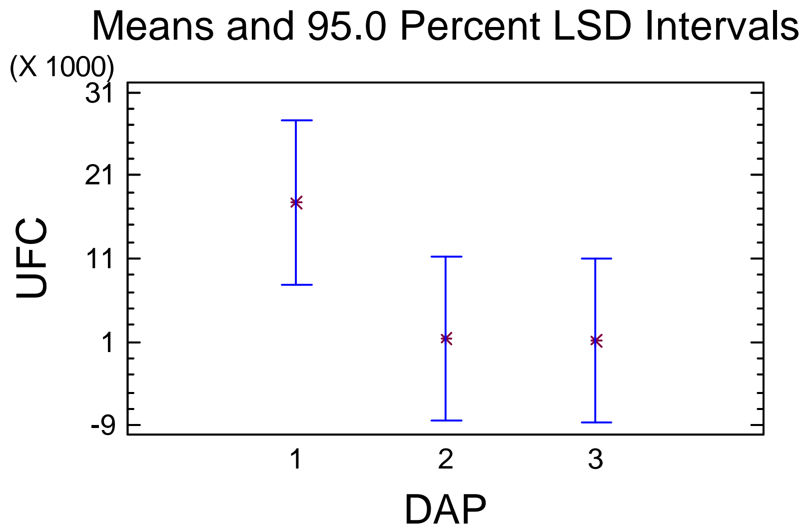
Analysis of Variance for UFC - Type III Sums of Squares

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
MAIN EFFECTS					
A:DAP	2.69355E9	2	1.34677E9	1.91	0.1634
B:Temperatura	5.49922E9	4	1.3748E9	1.95	0.1240
C:Replica	1.47097E9	2	7.35484E8	1.04	0.3636
RESIDUAL	2.54418E10	36	7.06716E8		
TOTAL (CORRECTED)	3.51055E10	44			

All F-ratios are based on the residual mean square error.

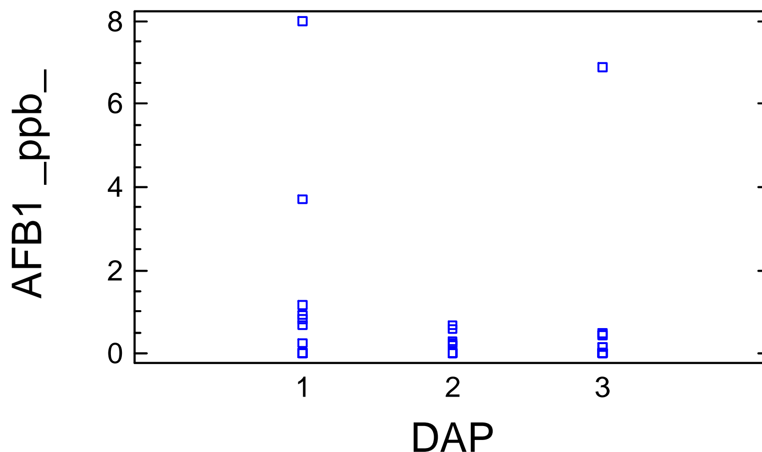
The StatAdvisor

The ANOVA table decomposes the variability of UFC into contributions due to various factors. Since Type III sums of squares (the default) have been chosen, the contribution of each factor is measured having removed the effects of all other factors. The P-values test the statistical significance of each of the factors. Since no P-values are less than 0.05, none of the factors have a statistically significant effect on UFC at the 95.0% confidence level.



Multifactor ANOVA – aflatoxin production at different growth stages

```
Analysis Summary

Dependent variable: AFB1 _ppb_
Factors:
        DAP
        Temperatura
        Replica

Number of complete cases: 45


The StatAdvisor
---------------
   This procedure performs a multifactor analysis of variance for AFB1 _ppb_. It constructs
various tests and graphs to determine which factors have a statistically significant effect on
AFB1 _ppb_. It also tests for significant interactions amongst the factors, given sufficient
data. The F-tests in the ANOVA table will allow you to identify the significant factors. For each
significant factor, the Multiple Range Tests will tell you which means are significantly
different from which others. The Means Plot and Interaction Plot will help you interpret the
significant effects. The Residual Plots will help you judge whether the assumptions underlying
the analysis of variance are violated by the data.
```



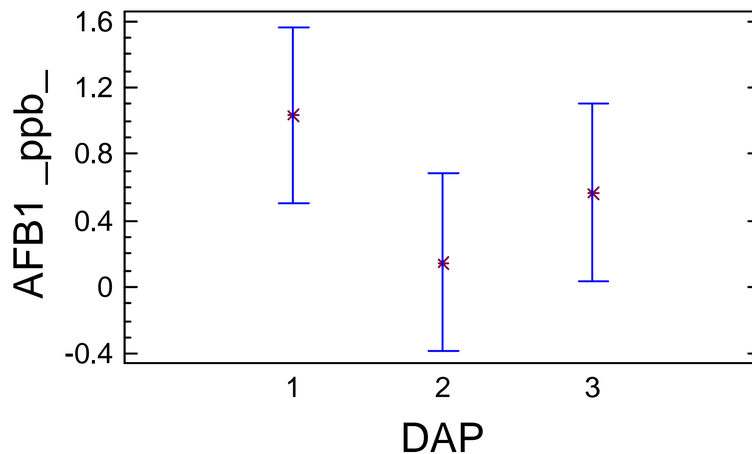

```
Analysis of Variance for AFB1 _ppb_ - Type III Sums of Squares
```

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
MAIN EFFECTS					
A:DAP	5.89276	2	2.94638	1.44	0.2495
B:Temperatura	27.0795	4	6.76988	3.32	0.0206
C:Replica	7.88585	2	3.94293	1.93	0.1597
RESIDUAL	73.4944	36	2.04151		
TOTAL (CORRECTED)	114.353	44			

All F-ratios are based on the residual mean square error.

The StatAdvisor

The ANOVA table decomposes the variability of AFB1 _ppb_ into contributions due to various factors. Since Type III sums of squares (the default) have been chosen, the contribution of each factor is
measured having removed the effects of all other factors. The P-values test the statistical significance of each of the factors. Since one P-value is less than 0.05, this factor has a statistically significant effect on AFB1 _ppb_ at the 95.0% confidence level.



Multifactor ANOVA – aflatoxin production at different temperatures

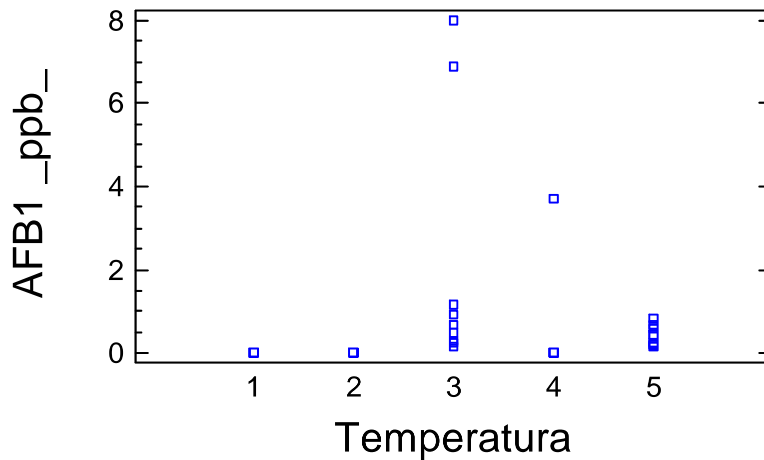
Analysis Summary

Dependent variable: AFB1 _ppb_
Factors:
Temperatura
DAP
Replica

Number of complete cases: 45

The StatAdvisor

This procedure performs a multifactor analysis of variance for AFB1 _ppb_. It constructs various tests and graphs to determine which factors have a statistically significant effect on AFB1 _ppb_. It also tests for significant interactions amongst the factors, given sufficient data. The F-tests in the ANOVA table will allow you to identify the significant factors. For each significant factor, the Multiple Range Tests will tell you which means are significantly different from which others. The Means Plot and Interaction Plot will help you interpret the significant effects. The Residual Plots will help you judge whether the assumptions underlying the analysis of variance are violated by the data.



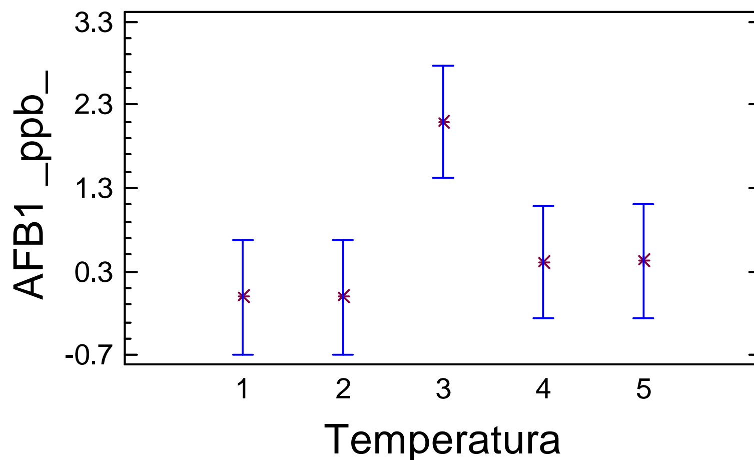
Analysis of Variance for AFB1 _ppb_ - Type III Sums of Squares

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
MAIN EFFECTS					
A:Temperatura	27.0795	4	6.76988	3.32	0.0206
B:DAP	5.89276	2	2.94638	1.44	0.2495
C:Replica	7.88585	2	3.94293	1.93	0.1597
RESIDUAL	73.4944	36	2.04151		
TOTAL (CORRECTED)	114.353	44			

All F-ratios are based on the residual mean square error.

The StatAdvisor

The ANOVA table decomposes the variability of AFB1 _ppb_ into contributions due to various factors. Since Type III sums of squares (the default) have been chosen, the contribution of each factor is
measured having removed the effects of all other factors. The P-values test the statistical significance of each of the factors. Since one P-value is less than 0.05, this factor has a statistically significant effect on AFB1 _ppb_ at the 95.0% confidence level.



Multiple Range Tests for AFB1 _ppb_ by Temperatura

```
--------------------------------------------------------------------------------
Method: 95.0 percent LSD
Temperatura    Count     LS Mean       LS Sigma      Homogeneous Groups
--------------------------------------------------------------------------------
1              9         0.0           0.476271      X
2              9         0.0           0.476271      X
4              9         0.411111      0.476271      X
5              9         0.424444      0.476271      X
3              9         2.09111       0.476271       X
--------------------------------------------------------------------------------
Contrast                                 Difference          +/-  Limits
--------------------------------------------------------------------------------
1 - 2                                    0.0                 1.36602
1 - 3                                   *-2.09111            1.36602
1 - 4                                    -0.411111           1.36602
1 - 5                                    -0.424444           1.36602
2 - 3                                   *-2.09111            1.36602
2 - 4                                    -0.411111           1.36602
2 - 5                                    -0.424444           1.36602
3 - 4                                   *1.68                1.36602
3 - 5                                   *1.66667             1.36602
4 - 5                                    -0.0133333          1.36602
--------------------------------------------------------------------------------
* denotes a statistically significant difference.
```

The StatAdvisor

This table applies a multiple comparison procedure to determine which means are significantly different from which others. The bottom half of the output shows the estimated difference between each pair of means. An asterisk has been placed next to 4 pairs, indicating that these pairs show statistically significant differences at the 95.0% confidence level. At the top of the page, 2 homogenous groups are identified using columns of X's. Within each column, the levels containing X's form a group of means within which there are no statistically significant differences. The method currently being
used to discriminate among the means is Fisher's least significant difference (LSD) procedure. With this method, there is a 5.0% risk of calling each pair of means significantly different when the actual difference equals 0.

Multifactor ANOVA - AFB_1 production for hybrids considered

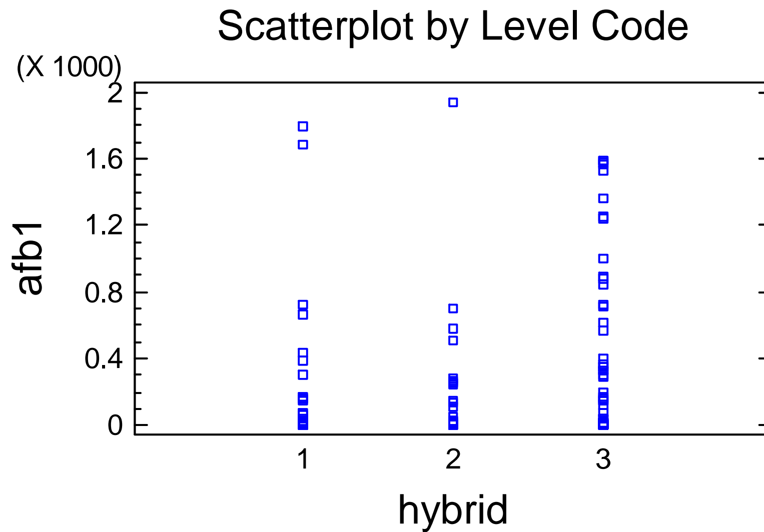
Analysis Summary

Dependent variable: afb1
Factors:
hybrid
thesis
time
repl

Number of complete cases: 234

The StatAdvisor

This procedure performs a multifactor analysis of variance for afb1. It constructs various tests and graphs to determine which factors have a statistically significant effect on afb1. It also tests for significant interactions amongst the factors, given sufficient data. The F-tests in the ANOVA table will allow you to identify the significant factors. For each significant factor, the Multiple Range Tests will tell you which means are significantly different from which others. The Means Plot and Interaction Plot will help you interpret the significant effects. The Residual Plots will help you judge whether the assumptions underlying the analysis of variance are violated by the data.



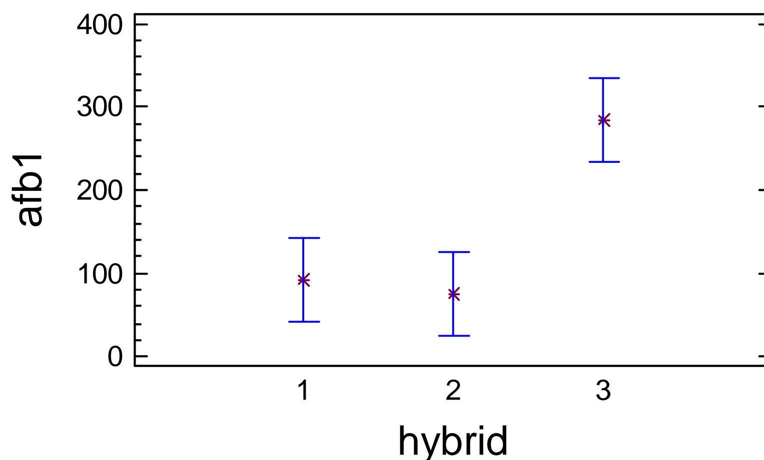
Analysis of Variance for afb1 - Type III Sums of Squares

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
MAIN EFFECTS					
A:hybrid	2.10213E6	2	1.05106E6	10.70	0.0000
B:thesis	196495.0	2	98247.3	1.00	0.3695
C:time	6.9014E6	9	766822.0	7.81	0.0000
D:repl	55942.9	2	27971.4	0.28	0.7525
RESIDUAL	2.14132E7	218	98225.7		
TOTAL (CORRECTED)	3.10462E7	233			

All F-ratios are based on the residual mean square error.

The StatAdvisor

The ANOVA table decomposes the variability of afb1 into contributions due to various factors. Since Type III sums of squares (the default) have been chosen, the contribution of each factor is measured having removed the effects of all other factors. The P-values test the statistical significance of each of the factors. Since 2 P-values are less than 0.05, these factors have a statistically significant effect on afb1 at the 95.0% confidence level.



Multiple Range Tests for afb1 by hybrid

Method: 95.0 percent LSD

hybrid	Count	LS Mean	LS Sigma	Homogeneous Groups
2	78	75.4532	36.3951	X
1	78	91.3829	36.3951	X
3	78	284.005	36.3951	X

Contrast	Difference	+/- Limits
1 - 2	15.9297	98.9115
1 - 3	*-192.622	98.9115
2 - 3	*-208.552	98.9115

* denotes a statistically significant difference.

The StatAdvisor

This table applies a multiple comparison procedure to determine which means are significantly different from which others. The bottom half of the output shows the estimated difference between each pair of means. An asterisk has been placed next to 2 pairs, indicating that these pairs show statistically significant differences at the 95.0% confidence level. At the top of the page, 2 homogenous groups are identified using columns of X's. Within each column, the levels containing X's form a group of means within which there are no statistically significant differences. The method currently being used to discriminate among the means is Fisher's least significant difference (LSD) procedure. With this method, there is a 5.0% risk of calling each pair of means significantly different when the actual difference equals 0.

Multifactor ANOVA - AFB_1 production at different periods

Analysis Summary

Dependent variable: afb1

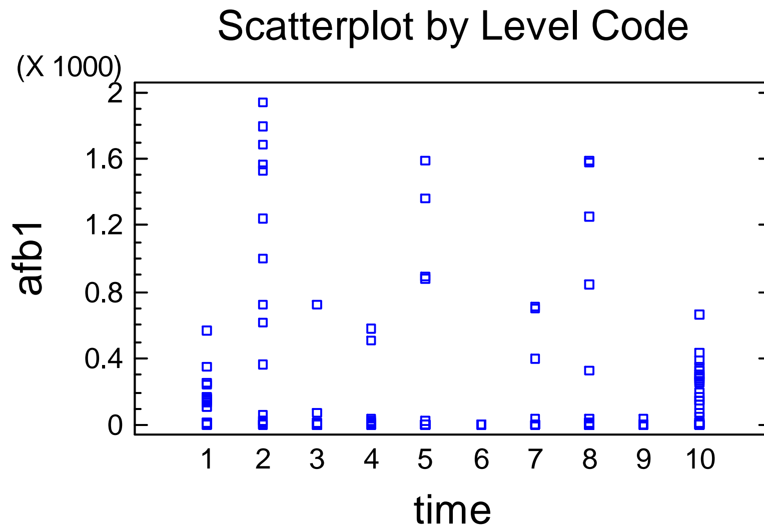
Factors:

time
hybrid
thesis
repl

Number of complete cases: 234

The StatAdvisor

This procedure performs a multifactor analysis of variance for afb1. It constructs various tests and graphs to determine which factors have a statistically significant effect on afb1. It also tests for significant interactions amongst the factors, given sufficient data. The F-tests in the ANOVA table will allow you to identify the significant factors. For each significant factor, the Multiple Range Tests will tell you which means are significantly different from which others. The Means Plot and Interaction Plot will help you interpret the significant effects. The Residual Plots will help you judge whether the assumptions underlying the analysis of variance are violated by the data.



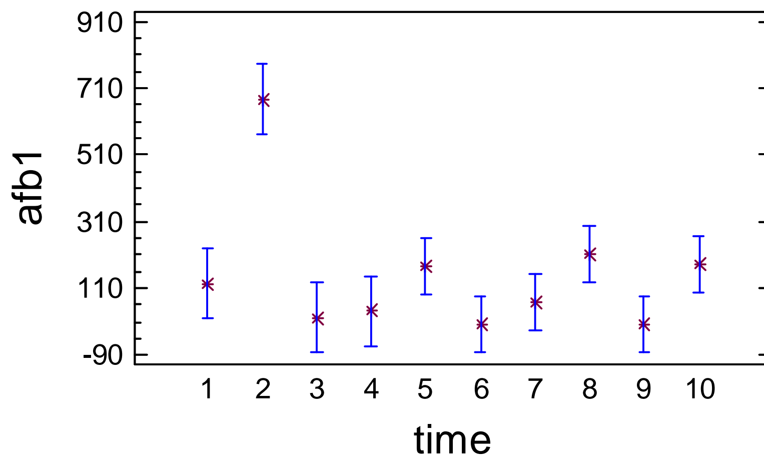
Analysis of Variance for afb1 - Type III Sums of Squares

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
MAIN EFFECTS					
A:time	6.9014E6	9	766822.0	7.81	0.0000
B:hybrid	2.10213E6	2	1.05106E6	10.70	0.0000
C:thesis	196495.0	2	98247.3	1.00	0.3695
D:repl	55942.9	2	27971.4	0.28	0.7525
RESIDUAL	2.14132E7	218	98225.7		
TOTAL (CORRECTED)	3.10462E7	233			

All F-ratios are based on the residual mean square error.

The StatAdvisor

The ANOVA table decomposes the variability of afb1 into contributions due to various factors. Since Type III sums of squares (the default) have been chosen, the contribution of each factor is measured having removed the effects of all other factors. The P-values test the statistical significance of each of the factors. Since 2 P-values are less than 0.05, these factors have a statistically significant effect on afb1 at the 95.0% confidence level.



Multiple Range Tests for afb1 by time

Method: 95.0 percent LSD

time	Count	LS Mean	LS Sigma	Homogeneous Groups
6	27	0.525444	60.3157	X
9	27	2.47037	60.3157	X
3	18	21.9802	75.8956	XX
4	18	42.1149	75.8956	XX
7	27	69.1143	60.3157	XX
1	18	124.046	75.8956	XX
5	27	176.767	60.3157	X
10	27	179.37	60.3157	X
8	27	210.53	60.3157	X
2	18	675.887	75.8956	X

Contrast	Difference	+/- Limits
1 - 2	*-551.841	205.901
1 - 3	102.065	205.901
1 - 4	81.9306	205.901
1 - 5	-52.7212	191.068
1 - 6	123.52	191.068
1 - 7	54.9312	191.068
1 - 8	-86.4841	191.068
1 - 9	121.575	191.068
1 - 10	-55.3248	191.068
2 - 3	*653.906	205.901
2 - 4	*633.772	205.901
2 - 5	*499.12	191.068
2 - 6	*675.361	191.068
2 - 7	*606.772	191.068
2 - 8	*465.357	191.068
2 - 9	*673.416	191.068
2 - 10	*496.516	191.068
3 - 4	-20.1347	205.901
3 - 5	-154.787	191.068
3 - 6	21.4548	191.068
3 - 7	-47.134	191.068
3 - 8	-188.549	191.068
3 - 9	19.5099	191.068
3 - 10	-157.39	191.068
4 - 5	-134.652	191.068
4 - 6	41.5895	191.068
4 - 7	-26.9993	191.068
4 - 8	-168.415	191.068
4 - 9	39.6446	191.068
4 - 10	-137.255	191.068
5 - 6	*176.241	168.117
5 - 7	107.652	168.117
5 - 8	-33.7629	168.117
5 - 9	*174.296	168.117
5 - 10	-2.6036	168.117
6 - 7	-68.5888	168.117
6 - 8	*-210.004	168.117
6 - 9	-1.94493	168.117
6 - 10	*-178.845	168.117
7 - 8	-141.415	168.117
7 - 9	66.6439	168.117
7 - 10	-110.256	168.117
8 - 9	*208.059	168.117
8 - 10	31.1593	168.117
9 - 10	*-176.9	168.117

* denotes a statistically significant difference.

The StatAdvisor

This table applies a multiple comparison procedure to determine which means are significantly different from which others. The bottom half of the output shows the estimated difference between each pair of means. An asterisk has been placed next to 15 pairs, indicating that these pairs show statistically significant differences at the 95.0% confidence level. At the top of the page, 3 homogenous groups are identified using columns of X's. Within each column, the levels containing X's form a group of means within which there are no statistically significant differences. The method currently being used to discriminate among the means is Fisher's least significant difference (LSD) procedure. With this method, there is a 5.0% risk of calling each pair of means significantly different when the actual difference equals 0.