EPIDEMIOLOGY AND CONTROL OF DISEASES CAUSED BY *ALTERNARIA* SPECIES ON PISTACHIO

By

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PREFACE

Pistachio (*Pistacia vera* L.) is a woody nut producing tree belonging to the family Anacardiaceae. Also known as the green almond, with reference to the bright green colour of the kernel, pistachio is the fifth most important commercial nut crop in the world. The species has been cultivated in Iran, Iraq, Turkey, Greece, Tunisia and Italy for centuries but more recently, it is being cultivated extensively in California, USA and Australia. During the last 15 years the crop has also been established near Prieska in South Africa by the Industrial Development Corporation (IDC) as part of a project aimed at providing labour for residents of the Northern Cape Province.

The major constraints to commercial production of pistachio in South Africa are rainfall and temperature. Although the tree does well in arid areas on shallow soils, timeous irrigation and fertilization are needed for optimal yields. Furthermore, in order to reduce losses due to disease, it is important to have a sound understanding of abiotic and biotic factors that contribute to disease development. Under conditions created by monoculture, numerous diseases have been recorded on all crops in the USA and elsewhere in the world. Special reference is made in this dissertation to *Alternaria* spp. as important opportunistic and quiescent fungal pathogens of pistachio in South Africa. The main purpose of the study was to determine the true identity of the causal agent, its biology and possible steps that can be taken to reduce its negative impact on yield. The dissertation is a compilation of four independent manuscripts which may result in some redundancy between chapters.

Chapter 1 is a literature review examining certain pertinent aspects pertaining to the intensive cultivation of pistachio worldwide. Emphasis is placed on environmental constraints to production in addition to management practices that affect the health and yield of pistachio orchards. Specific reference is made to the ultimate effect of these practices on the susceptibility of the crop to opportunistic fungal pathogens.

Blackening of the stylar end of developing nuts resulted in losses of up to 70% in local orchards during the 2003/2004 growing season by the Plant Pathology Division at the University of the Free State. During an intensive investigation by the author in the 2005/06 growing season, many culturally different *Alternaria* isolates were obtained from tissue aquired from asymptomatic flower buds, leaf buds, leaves and nuts, as well as from diseased leaves and nuts. The research carried out in Chapter 2 reported on whether differences

between quiescent and pathogenic isolates could be related to different *Alternaria* species, by means of cultural techniques and molecular (AFLP) analysis, as well as whether there was any niche specificity with regard to tissue types among species.

In Chapter 3 the evaluation of the use of eleven fungicides were described for their efficacy *in vitro* and *in vivo* in reducing the incidence of *Alternaria* spp. in asymptomatic nut tissue and their ultimate effect on yield. Special attention is given to the time and frequency of application in addition to the concentration of the most effective fungicides.

Microclimatic conditions created by increased free moisture and relative humidity create conditions that are ideal for germination of *Alternaria* spores and subsequent infection of pistachio tissue. In Chapter 4 it was investigated whether wetting of the tree canopy during the cooler autumn and spring months, in order to increase chilling units for optimal fruit set, has any effect on endophytic colonization of pistachio tissue by *Alternaria* spp. which will ultimately affect nut yield. The rationale was that quiescent infection of asymptomatic flower buds would be facilitated by means of this practice which would result in increased disease incidence in nut clusters during the growing season.

CHAPTER 1

Literature review

Constraints to Pistachio Production under Intensive Cultivation with Specific Reference to Opportunistic Diseases caused by *Alternaria* spp.

1. Introduction

The pistachio, *Pistacia vera* L. is a deciduous tree native to Western Asia and parts of the Middle East that belongs to the family Anacardiaceae (Woodroof, 1967; Tous & Ferguson, 1996). It is the only species of the genus *Pistacia* that is commercially cultivated (Labavitch *et al*., 1982; Kaska, 2005) and is mentioned in the Bible (Genesis 43:11) as the green almond, with reference to the bright green colour of the kernel. The nuts have always been highly prized and even before the advent of agriculture, the trees were nurtured and protected by stone age man and his successors (Caruso, 2005). Pistachio is the fifth most important commercially produced nut crop in the world, supplying 8% of total production after cashews (31%), almonds (27%), walnuts (22%) and hazelnuts (10%), and is followed by macadamia and pecan nuts at 1% each (FAO, 2004).

During the height of the Roman Empire, seeds were collected and distributed throughout the Mediterranean basin and seedlings were successfully grown in Iran, Iraq, Turkey, Greece, Tunisia and Italy (Fabbri & Valenti, 1998; Kaska, 2005). In the 20th century, the species was cultivated extensively in California and Arizona (Michailides, Morgan & Doster, 1995), with increased cultivation in Australia (Facelli *et al*., 2005a) and South Africa in the last 15 years (Jooste, 2000; 2005).

The tree was introduced into South Africa in 1990 by the Industrial Development Corporation (IDC) to Prieska in the Northern Cape Province. Pistachio is a high potential crop not only in terms of yield, but in its ability to play a role in work creation for disadvantaged South Africans while generating revenue as an export item. The major constraints to commercial production of pistachio in South Africa are rainfall and temperature (Kanber *et al*., 2004). Although the tree does well in arid areas on shallow soils, timeous irrigation and fertilization are needed for optimal yields.

The species is xerophytic (Woodroof, 1967; Tous & Ferguson, 1996) and drought tolerance is enhanced by a thick cuticle epidermis on both sides of the leaf (Caruso, 2005). In the centre of highest genetic variation of the genus *Pistacia*, the plant is found in rocky areas with sandy soils and an annual rainfall less than 500 mm. The cultivation area of this crop is

limited by the very specific cold requirements required for flower set, and heat required for ripening of the nuts. High summer temperatures and average winter temperatures below 13°C are required for the tree to go into dormancy (Woodroof, 1967).

Flowers are carried in clusters. Inflorescent buds start to develop during spring, become latent in summer and resume growth in autumn. Flower differentiation is completed during the following spring just before anthesis (Polito & Pinney, 1999; Beyers, Costa & Vizzotto, 2003; Caruso, 2005). The fruit is called a drupe and fruit set in pistachio is dependent on the number of chilling hours accumulated during winter. If the optimum number of hours is not reached, yield is adversely affected (Küden, *et al*., 1995a). After fertilization and fruit set, the hull and shell develop quickly while the kernel and cotyledons develop six to eight weeks later. Approximately six weeks before ripening, the shell splits along a prominent ventral suture in a process known as endocarp dehiscence (Shuraki & Sedgley, 1996; Polito & Pinney, 1999) and two weeks before ripening the hull changes from light green to red as chlorophyll is broken down (Labavitch *et al*., 1982; Ferguson, Polito & Kallsen, 2005). After harvest the hull is easily slipped off the shell, exposing the green kernel within the split shell (Labavitch *et al*., 1982; Tous & Ferguson, 1996; Kallsen, 2003; Caruso, 2005).

While the plant can tolerate arid conditions, drought stress will predispose pistachio to increased attack by opportunistic plant pathogens (Schoeneweiss, 1975; Ma, Morgan & Michailides, 2001). Numerous opportunistic pathogens of pistachio have been reported from around the world (Funk, 2005). Diseases previously unknown or unreported on pistachio elsewhere have been observed in South African orchards. The two main pathogens identified to date are *Botryosphaeria obtusa* Schwein. and *B. dothidea* Moug. Fr., the cause of basal stem canker (Swart & Blodgett, 1998). This disease is characterised by cambial lesions (cancers) on the lower stems near the graft which caused dieback of the trees (Swart & Botes, 1995; Swart & Blodgett, 1998). More recently, *Alternaria alternata* L. has become increasingly important in South Africa as the cause of black apical nut necrosis (Swart, 2006). This fungus caused brown to black lesions on the ripeing fruit which can spread to the nut meat during dihescense. At the end of the season, lesions appear on the leaves, in some cases totally covering these at leaf drop (Hong *et.al*., 2006).

Climatic conditions are the main constraints to production of pistachio. Most of the other abiotic and biotic factors in the environment can be modified through correct cultivation practices and management decisions. These include the use of fertilizers, soil cultivation

practices and the control of weeds and other pests in the orchards. Pistachio, like other orchard crops, is expensive to establish and maintain. Improved managerial practices, especially with regard to irrigation, with not only the amount of water applied, but also the method of application, have led to higher yields and bigger nuts (Michailides *et al.*, 1995).

This review examines some aspects pertaining to the intensive cultivation of pistachio worldwide. Emphasis will be placed on constraints to obtaining optimal yields by various factors associated with crop production. Cultivation practices in the nursery and orchard that ultimately affect the health of trees will also be reviewed with specific reference to how these practices influence the tree's susceptibility to opportunistic fungal pathogens.

2. Nursery Practices

Pistachio is a known outbreeder and difficult to grow from cuttings. Various pistachio species can cross-pollinate, resulting in the production of seed that differs genetically from the parents (Shuraki & Sedgley, 1996; Isfendiyaroglu, *et al*., 1999). In order to produce uniform nuts with respect to shape, size and taste, as well as the saturated fat content, no *de novo* production of pistachio planting material is presently generated from seed (Caruso, 2005). Rootstocks are produced from seed (Labavitch *et al*., 1982) and all scion materials are presently harvested from existing trees.

2.1 Choice of rootstock

The rootstock affects a variety of factors and the use of more than one species of rootstock in an orchard leads to multiple protection from environmental constraints. The choice of adapted rootstock is essential in the ability of the plant to adapt to adverse soil and other environmental conditions (Chao, Parfit, & Michailides, 2001; Atli & Arpaci, 2002; Ferguson *et al.*, 2002a; 2002b). Rootstocks are often chosen for their resistance to soil-borne diseases caused by *Fusarium* spp. and *Verticillium* spp. These fungi cause wilt and ultimate death of newly planted trees (Kaska & Nikpeyma, 1999; Epstein *et al*., 2004; Kaska, 2005). *Pistacia mutica* Fisher & Meyer is resistant to root-knot nematodes and *P. terebinthus* L. is resistant to *Phytophthora* spp. (Can *et al.*, 2006).

A variety of rootstocks from other pistachio species are used to improve the health and quality of nuts (Kaska & Nikpeyma, 1999; Kaska, 2005). Trunk diameter, height, and branching, as well as the weight of the root system and the whole tree, are affected by the choice of

rootstock (Vargas, Romero & Clavè, 2001). Rootstock does not contribute to the percentage of shell split, however, pollen from the male scions, 'Peters' and 'Ask', produces a higher percentage of split shells than 'Altantica' male trees, (Ferguson *et al*., 2005), making the choice of scion as important as that of the rootstock.

The Californian pistachio industry relies on *P. atlantica* Desf., *P. integerrima* Stewart as well as a hybrid *P. atlantica x P. integerrima* to supply most of the rootstock (Ferguson *et al*., 2002a; 2002b). Studies conducted in Spain, showed that rootstocks from *P. integerrima* (originally from the USA), *P. atlantica* (originally from Syria), *P. terebinthus* (originally from Spain) and *P. palaestine* Bois. hybrids (originally from Greece) are most promising in terms of plant health and yield (Vargas, Romero & Clavé, 1999). *P. atlantica*, *P. integerrima* and a hybrid *P. atlantica x P. integerrima* rootstocks were compared in relation to the type of new season growth exhibited. *P. integerrima* and the hybrid displayed better neoformed growth, greater vigour and a more acceptable canopy form, which plays an important role in mechanical harvesting (Spann *et al*., 2004).

2.2 Grafting

Woody tree seed often does not breed true due to uncontrolled pollination and favourable characteristics of the parent tree cannot be reliably transferred via seed,. By grafting a scion of known production quality onto a rootstock with known features, the total tree can be reproduced and the quality of its fruit ensured (Onay *et al*., 2003). A variety of grafting methods are used in commercial orchard nurseries. The most common grafting techniques are basic angle grafting, whip and tongue grafting and side veneer grafting. Bud grafting consists of using a piece of bark containing a bud from the scion and uniting it with the rootstock (Arizona Cooperative Extension, 1998).

Although grafting is essential for the production of a healthy, optimal fruit bearing tree, there are problems associated with the procedures. Failure of grafting may occur due to incompatibility of rootstock and scion which leads to rejection, browning of the cut surfaces due to oxidation, or the poor development of the root system. Micrografting may offer a solution to the problem of incompatibility between rootstock and scion. In this technique a shoot tip is aseptically placed into a decapitated rootstock (Onay *et al*., 2003; Can *et a*l., 2006). Apart from failure of the grafting to take, infection by opportunistic fungi may occur at the cut surfaces and cause weakening or death of the plant.

Air layering is a technique that is used to form a separate plant on an existing branch. A shallow cut is made into the bark of the branch and cushioned with a wrapping of wet moss. This is covered with dark plastic and kept damp until roots start to develop. This branch can then be severed from the parent and transplanted (Delgado, *et al.,* 2005). This technique has been successfully used in the propagation of *Mangifera indica* L. which also belongs to the Anacardiaceae family (Chhonkar & Singh, 1972) and is currently used on pistachio in South Africa (Snyman, 2005).

3. Orchard Practices

The exact spacing between trees, as well as the proportion of male to female trees is governed by the biology of the pistachio, as well as cultural practices such as fertilization, pruning and harvesting. Orchard establishment and upkeep is costly, and the original placement of transplants must be done with careful consideration to cultural activities and harvesting procedures as pistachio trees only start to produce commercially after seven to ten years, and can stay productive for up to 40 years (Tous & Ferguson, 1996; Caruso, 2005).

3.1 Choice of site

Pistachio trees will grow and survive in a wide range of conditions, but optimum yields and high quality nuts are only realized under specific climatic conditions. Pistachios require an annual rainfall of at least 300-450 mm. They do not tolerate high humidity or prolonged wet conditions during the growing season (Pillai, 1995), and spring frost can kill flowers and young leaves. Pistachios require cold winters and hot dry summers with 2200-2800 heat units (Ferguson *et al.,* 2005). Fruit set in pistachio is dependent on a minimum number of chilling hours, ranging from 500 to 11 500 hours depending on the cultivar. If the climate at the site of choice does not allow optimal accumulation of chilling hours, yields will be negatively affected (Küden *et al.,* 1995a).

Ideally, soils should be deep, friable and well-drained, but moisture-retaining (Labavitch *et al*., 1982). Pistachios can survive on poor, stony, calcareous, highly alkaline or slightly acid, or even saline soils, and are more tolerant of these conditions than most other commercial fruit and nut trees. Although the tree can survive a wide acidic range, a soil pH between 7.1-7.8 is optimal (Pillai, 1995).

3.2 Transplanting

Due to the cost and delicate nature of young pistachios, the process of producing a pistachio plant is performed in a nursery where all the production steps as well as the hardening-off can be controlled. The young plant is protected from the environment with its inherent pests until it is strong enough to be transplanted into the orchard (Robinson, 1997). The success of the orchard is related to the treatment of the young trees prior to transplanting. Arpaci & Ak (2001) found significant differences in the progress of experimental seedlings and budded plants after transplanting when seedlings of different ages were planted into the orchards at different times of the year. One-year-old potted pistachios planted in autumn showed a 100% transplant success, while the three-year-old seedlings only showed 80% success. Older plants not in pots were also less successful when transplanted. The success of the potted plants was related to the large number of capillary roots which were not disturbed during transplanting, and the fact that plants were transplanted with their own soil. These plants also showed the highest percentage of budding in the orchard (Arpaci & Ak, 2001).

3.3 Male: female ratio

In order to ensure pollination, the correct proportion of male to female trees in an orchard is essential (Ferguson *et al*., 2005). This usually varies from one in five to one in twenty depending on the area and the cultivar of the male. In order to co-ordinate release of pollen from the male and female, great care has to be taken with the choice of cultivars. As pollen is wind distributed, the placement of male trees in the orchard in relation to the predominant wind direction is also of great importance (Caruso, 2005).

3.4 Fertilization

Limited availability of any macro- or micro-elements imposes stress on plants, which predisposes the plants to disease and insect attack (Schoeneweiss, 1975; Michailides *et al*., 2002; Norris, Caswell-Chen & Kogan, 2003). Michailides *et al*. (2002) stated that the occurrence of both Botryosphaeria blight and Alternaria late blight can be alleviated by the correct use of fertilizers. Fertilization of pistachio has therefore been aimed at enhancing the production of nuts (Arzani, Hokmabadi & Dehghani-Shuraki 2002), as well as increasing plant health as a whole. It is critical to use the correct formulation and concentration, as certain chemical salts such as $CaCl_3$ at 2% and 4%, are phytotoxic. Excess nitrogen, that stimulates vegetative growth, can be detrimental to the plant as excessive foliage production can predispose the plant to drought stress (Michailides *et al*, 2002; Swart, Nieuwoudt &

Pretorius, 2003).

Potassium (K) increases the leaf K status as well as the nut yield and quality (Zeng, Brown & Rosecrance, 1998; Zeng, Brown & Holz, 1999) and when applied at the correct times during the growth season, improves the ability of the plant to tolerate stress. The alternate bearing nature of the pistachio tree of high production one year, followed by low production the next, creates unique nutritional needs. There is an increased uptake of K during high production years (on-years) to replace K that was removed in fruit and leaves abscised during summer, which in turn necessitates adequate and timeous application of K before spring (Rosecrance, Weinbaum & Brown, 2002). Michailides *et al*. (2002) found that the application of double the recommended rate of K (0.468 g/ℓ) reduced the severity of Botryosphaeria blight on leaves.

Phosphorus (P) fertilizer appears to be stored in the trees during the low production years (off-years) and redistributed during on-years for production, as the tree does not seem to be able to absorb enough P from fertilization during the on-years (Rosecrance *et al.*, 2002). For this storage to be adequate it is critical that P be available to the tree during the whole growing season. P is a necessary component of the fertilization program for improvement of general productivity, fruit yield, size of the nuts, quality of nutmeat and the percentage of dehisced fruits. Dihisced nuts attractively expose the green meat within the pale yellow shell, which is an important selling point for pistachio sold unshelled (Tekin, Guzel & Ibrikci, 1995).

Presenting the attractive green kernel within its cracked shell is an important visual characteristic of the pistachio nut. Un-split shells are expensive to treat, and shells that split too early can lead to infection of the nut. Brown, Ferguson & Picchioni (1995) recommend the use of foliar boron (B) during the growing season from summer, late dormancy, to spring when the leaf canopy is full, for optimal reduction of blanking and non-splits, thus increasing yield. Tsipouridis *et al*. (2005) found that timeous application of boric acid led to high shell split without any infection of the nuts by opportunistic fungi such as *Alternaria* spp. Ferguson *et al.* (2005) reported that a lack of B leads to high percentages of blank nuts being produced.

Nitrogen (N) and calcium (Ca) applied correctly can increase yield and the ability of pistachio to resist diseases such as Botryosphaeria and Alternaria disease (Daane *et al*., 1995;

Michailides *et al.,* 2002). Michailides *et al*. (2002) found that the application of $Ca(NO_3)_2$ decreased the level of infection by *Botryosphaeria* spp. by up to 33% compared to untreated controls. Application of $Ca(Cl)_2$ also exhibited a trend towards smaller numbers of latent infections caused by *Alternaria* spp. on pistachio leaves (Michailides *et al.,* 2002). Swart *et al.* (2003) found that increased N application to various pistachio rootstocks decreased lesions caused by B. *obtusa* and *B. dothidea* in the greenhouse, but warned that an excess of this nutrient can predispose the plants to drought and freeze stress due to excessive vegetative growth, leading to infection by opportunistic fungi such as *Alternaria* spp.

During fruiting there is competition for carbohydrates between vegetative growth and kernel development. Arzani *et al.* (2002) found that a mixture of 3% sucrose and 2% glucose was absorbed by the leaves after foliar application and increased nut quality. Zakinthinos & Touskas (1995) found that such mixtures improved shell splitting and lead to an increase in dry weight of the nuts.

3.5 Agro-chemicals

3.5.1 Fungicides

Chemical control is a fast and effective method of fungal control. Two disadvantages of this practice are increasing consumer resistance to chemical residues and the development of pathogen resistance to fungicides (Meena *et al.,* 2004). Alternaria late blight on pistachio in California was successfully controlled with cupric hydroxide (Kocide 101®) and cuprous oxide (Nordox®) (Michailides & Morgan, 1993). Michailides & Morgan (1993) reported a reduction in fruit staining caused by *Alternaria* spp. with the use of benomyl. Cupric hydroxide and benomyl were subsequently registered for use against Alternaria late blight on pistachio in the USA (Michailides *et al.,* 1995). Later Michailides, Morgan & Felts, (1999) reported excellent control of Alternaria late blight on pistachio with the strobilurin fungicide azoxystobin (Abound®). Ma & Michailides (2004), however, subsequently reported that certain *Alternaria* isolates have become resistant to this fungicide. This resistance was shown in orchards previously exposed to the fungicide, but no resistance was found in wild types of this fungus not previously exposed to the fungicide. Due to resistance, this fungicide is no longer used in California. Use of anti-fungal plant extracts and antagonistic micro-organisms in synergy with chemicals is being investigated (Ram & Ram, 1997; Meena *et al.,* 2004).

3.5.2 Insecticides and Nematicides

On pistachio, insects cause damage by actively feeding on the buds (Farivar-Mehin, 2002) and nuts (Bentley *et al.,* 2003b), creating entry wounds for opportunistic pathogens (Doster & Michailides, 1991), or by acting as vectors for disease (Michailides, Morgan & Felts; 1998; Michailides & Teviotdale, 2003b). One example of a feeder is *Megastigmus pistaciae* which does not act as a vector, but causes serious damage to nuts, especially where these nuts are produced as seed. Orchard sanitation in the form of removal of fallen nuts reduces their numbers (Bentley, *et al.,* 2003b). *Hylesinus vestilus* Mulsant & Rey bore into pistachio twigs through the leaf or flower buds, destroying the bud. During spring the beetles emerge and lay their eggs on pruned, dead or damaged branches in or outside the orchard and removal and destruction of such off-cuts are essential. The pistachio root beetle, *Canodis cariosa hauseri* Ob. larvae emerge from eggs deposited on the branches, often near the collar, and bore into the roots. This causes general weakness and eventually death of the trees. The pistachio weevil, *Polydrosus davatchii* Hoffman feeds on flower clusters and young fruit (Farivar-Mehin, 2002). Both of the above pests predispose pistachio to attack by opportunistic fungi such as *Alternaria* spp.

Daane *et al*. (2000) investigated the role of hemipteran insects in the spread of *B. dothidea,* and found that even if insect damage is extensive, there is no significant increase in the occurrence of disease caused by this pathogen, as the insects tested did not carry the fungal spores. Doster & Michailides (1991) found that a large percentage of *Aspergillus* spp. found within pistachio fruits were associated with insect bites.

Hemipterans such as the stinkbugs *Thyanta pallidovirens* Stal, *Chlorochroa uhleri* Stal and *C. ligata* Say, and a leaf footed bug *Leptoglossus clypealis* Heidemann feed on the pistachio fruit, infecting it with stigmatomycosis caused by *Nematospora coryli* Peglion or *Aureobasidium pullulans* Pullaria (Michailides *et al.*, 1998; Michailides & Teviotdale, 2003a). Insects are known to carry *Alternaria* spp. and transmit these to plants during feeding (Holtz, 2002; Swart & Swart, 2003). Swart *et al.* (2002) identified two nut feeding beetles, Lygaeidae of the family Heteroptera and Coreidae of the family Hemiptera as carriers and transmitters of *Alternaria* spp.

Rootstocks from *P. atlantica* and *P. terebinthus* produce pistachio trees that are resistant to root lesion as well as root knot nematodes (Daane *et al.,* 2000). *Paratylenchus hamatus* Thorne & Allen (pin nematode), *Pratylenchus neglectus* Rensch (root lesion nematode), *Xiphinema americanum* Cobb (dagger nematode) and *Meloidogyne* spp (root knot nematode)

have been isolated from pistachio orchards. Although nematodes are currently not regarded as a constraint to commercial production of pistachio locally, their presence must be monitored, as damage caused by their feeding can facilitate entrance of soil borne pathogens such as *Verticillium* spp. (Westerdahl, 2000). As pathogenic nematodes occur in most soils, it is critical that resistant rootstock is used.

Insect infestation in orchards is reduced by the removal of unharvested nuts and pruned branches. Chemical insecticides are applied when between 300 and 400 degree days are reached. Currently the following chemical insecticides are registered for use against the navel orangeworm in the USA: azinphosmethyl, carbaryl, permethrin, phosmet and tebufenozide, with the parasite *Goniozus legneri* Gordh**.** registered as a biological insecticide against the orangeworm in the USA (Bentley, Beede & Daane, 2003a). The pistachio twig borer, *Kermania pistaciella* Ams. is controlled with monocrotophos, azinphos-methyl and carbaryl applied one week after the adults emerge and again 10-15 days later (Mart, Yigit & Çelik, 1995).

Consumer resistance to the use of chemicals in agriculture is increasing and alternatives are being sought (Townsend, 2003). Most commercially available horticultural oils used as insecticides are refined from petroleum and are collectively known as mineral oils (Cranshaw & Baxendale 2006). Light oils are used during active growth of the tree for the control of active insects, and dormant oils are applied before bud break and target inactive insects (Townsend, 2003). In order to facilitate application, these oils are mixed with emulsifying agents. Impurities in the oils such as aromatic compounds and sulphur, nitrogen or oxygen containing compounds are associated with plant injury. Impurities are normally removed during refining. It is therefore important to obtain such oils from a reputable firm (Ferguson *et al*., 2005).

Plant pathogenic nematodes occur in all soils, and are known inhabitants in woody tree rhizospheres. They are currently not a problem in Californian pistachio orchards, but numbers must be monitored (Westerdahl, 2000). Aliramaji, Pourjam & Karegar (2006) isolated 13 nematode genera from several pistachio production regions in Iran, though it is not yet a commercial problem. *Heliotylenchus* was identified in Pakistan pistachio orchards, but again not in commercially important numbers (Maqbool & Qasim, 1988). No nematicide is presently registered for use on pistachio (Westerdahl, 2000).

3.5.3 Herbicides

Weeds, like cover crops, can harbour insects and act as alternative hosts to opportunistic fungi such as *Alternaria* spp. (Table 2). Weeds also compete with the orchard trees for resources such as water and nutrients (Norris *et al.*, 2003). Young trees are especially vulnerable as the presence of weeds can delay growth and thereby, maturity and optimal production. Weeds also interfere with harvesting. In new orchards the young trees can be sensitive to chemicals and care should be taken that any herbicides used are selective and target the problem weeds. Herbicide run-off and contamination of irrigation water must be prevented. Weeds should be monitored constantly and chemical weed control should be combined with mechanical control, such as mulching, subsurface irrigation and flaming of weeds (Hembree & Shrestha, 2004). An in depth study of weeds in pistachio orchard in South Africa is required to determine which managerial practice should be implemented.

Table 2. Common weeds found in pistachio orchards in California (Hembree & Shrestha, 2004).

Summer annuals	Winter annuals	Perennials
Barnyard grass	Annual bluegrass	Yellow nutsedge
Sprangletop	Filaree spp.	Bermuda grass
Large crabgrass	Cheeseweed	Johnsongrass
Lambsquarter	Mustard spp.	Filed bindweed
Pigweed spp.	Chickweed	Dallisgrass
Evening primrose spp.	Shepard's purse	Annual morning glory
Spotted spurge	Cudweed	
Sowthistle	Nettle	
Prickly lettuce		
Horseweed		
Mullein		
Flaxleaved fleabane		
Nightshade spp.		

Weeds and weed control are as old as agriculture itself, and a variety of methods have been developed to control weeds. Weeds may be comprehensively defined as unwanted and undesirable plants that interfere with the utilization of land and water resources, and so have an adverse effect on human welfare (Rao, 2000). From an agricultural perspective weeds compete with crop plants for nutrients, soil moisture and light, resulting in a reduction in yield and quality of the produce (Anderson, 1996; Rao, 2000). Weeds may also harbour a variety of insect pests and diseases that can have a negative effect on the crop, as well as a number of rodent species that cause physical damage to crop plants, particularly the bark of tree crops (Ashton & Monaco, 1991).

In orchard crops the very wide spacing required between trees to allow easy movement through the orchard allows far more weed growth than in agronomic crops, as the trees are only able to shade out weeds directly under their canopy (Rao, 2000). It is crucial that weeds be kept under control during the establishment phase of the orchard as this is the period of most intense competition, that decrease as the orchard gets older (Ashton & Monaco, 1991; Rao, 2000). The majority of weed problems are caused by persistent perennial weeds, although annual species normally predominate. The effect of weeds in pistachio orchards are

discussed in section 5.

Cover crops are normally established between the tree rows to assist in shading of weeds (Ashton & Monaco, 1991). The use of cover crops in pistachio orchards is discussed in section 3.6. Where weeds do occur they can be controlled mechanically, by hand, or through use of chemicals (Ashton & Monaco, 1991; Rao, 2000). At this stage no herbicides are registered for weed control in Pistachio orchards in South Africa (Directorate: Food Safety and Quality Assurance, 2004), but the herbicides that are registered in California are given in Table 1.

Table 1. Herbicides recommended for use in pistachio orchards in California (Hembree & Shrestha, 2004).

Use	Time of application	Active ingredient
Site preparation, established weeds	Active growing annual weeds and flowering perennials	Glyphosate®
	Seedling weeds	Sulfosate®, Paraquat®
After planting, before weed emergence	Non-bearing orchards only, not to be used 1 year before harvest	Isoxaben®, Pendimethalin®, Thiazopyr®
	Bearing or non-bearing orchard, incorporate with irrigation, ensure absence of debris	Napropamide®, Oryzalin®, Oxyfuorfen®
Established weeds	Non-bearing orchards, not to be used 1 year before harvest	Clethodim®, Diquat®, Fluazifop-p-butyl®, Sethoxydim®, Sulfosate®
	Bearing and non-bearing orchards, active growing annual weeds and flowering perennials	Glyphosate®
	Bearing and non-bearing orchards at least one year old, avoid contact with foliage and roots	Halosulfuron, 2,4-D amine®
	Dormant weeds, in-season	Oxyfuorfen®
	Active growing annual weeds and flowering perennials	Paraquat®

3.5.4 Bud break chemicals

Most commercial crops have been exported from areas where they occur naturally and are

often cultivated under less than ideal conditions. One recurrent problem in pistachio is the occurrence of bud break too early in the season. If temperatures drop sharply, budding leaves and flowers can be damaged or killed. In contrast, bud break can occur too late in the season, exposing ripening fruit to early frost, again causing yield and quality losses (Polito & Pinney, 1999).

Bud break is achieved by the application of horticultural mineral oil to break the winter rest period of a woody tree (Küden, *et al*., 1995b; Beede & Ferguson, 2002). Repeated spraying in mid-winter increases vegetative growth, flowering and the number of filled nuts, with a definite increase in fruit set. Citronella oil was successfully used by Rodriquez & Almaguer (1980) for bud break, while distillates of petroleum are commonly used (Baker & Powell, 1996; Townsend, 2003), as well as other chemicals such as thiourea, potassium nitrate, gibberellic acid, cytokinins, paclibutrazole and Armobreak®, an alkoxylated fatty alkylamine polymer (a fatty amine surfactant) (Küden *et al*., 1995b; Beyers *et al.*, 2003; Fahemi & Asghari, 2004).

3.6 Biological Control

The pistachio seed wasp larvae *Eurytoma plotnikovi* Nikolskaya in Tunisia is not controlled by chemicals containing dimethoate and deltanethrin. The use of spinosad, produced by an actinomycete *Saccharopolyspora spinosa* Mertz and Yao, at a concentration of 0.05% proved successful in combating this insect, if applied during spring (Mohamed, 2005). The use of Neem extract (antiecdysone) at a rate of 0.7%, and the antagonistic fungus *Beauvaria bassiana* Bals. used in conjunction with flufenoxuron and teflubenzuron, is showing promise against *Agonoscena targianii* Licht., a pistachio psyllid (Lababidi, 2002). The Navel Orangeworm, *Amyelois transitella* Walker is controlled by parasites including *Goniozus legneri* and *Copidosomopsis plethorica* Caltagirone (Bentley *et al*., 2003a). Bentley *et al*. (2003a) states that the application of these biocontrol agents must take place between 300 and 400 degree days after the third laying which is preceded by an increase in two consecutive egg trappings. Entomopathogenic nematodes are being cultivated for use against the flatheaded woodborer, *Capnodis* spp. which causes feeding damage to pistachio trees (Hazir *et al*., 2003). A variety of application methods are used to apply the nematodes during the summer including using irrigation and squeezing sponges containing the nematodes into holes eaten by the woodborers. The nematodes are applied when signs of activity by woodborers are noticed.

3.7 Irrigation

Though pistachio is a xerophyte, supplementary water in the form of irrigation ensures greater yields (Ferguson *et al*., 2005). The correct amounts of water supplied at bud break when the leaves emerge and when shell filling takes place is critical for shell splitting (dehiscence). Presentation of the nut within a split shell is one of the main selling points of pistachio, and plays a role in the determining of the price paid to the producer (Tous & Ferguson, 1996; Kallsen, 2003; Kanber *et al.,* 2004). Water stress also leads to more blank nuts being produced with concomitant loss of commercial yield (Ferguson *et al.*, 2005).

In arid and semi-arid regions, it is possible for water stress to occur even during irrigation due to the high evaporation demand of the atmosphere. This places the plant under stress, and places pathogens at an advantage (Blodgett, Kruger & Stanosz, 1997a; 1997b; Maxwell, Kruger & Stanosz, 1997; Stanosz *et al.*, 2001, McElrone, Sherald & Forseth, 2003). Various researchers have indicated that irrigation using spray heads elevated above the ground, leads to higher levels of *Alternaria alternata* infection than drip or sub-surface level irrigation (Michailides *et al.,* 1995; Goldhamer, 1996; Robinson, 1997; Goldhamer, Michailides & Morgan, 2002; Holtz, 2002). Michailides & Teviotdale (2003a) found that the use of sprinklers or flood irrigation increased the relative humidity in the orchard which allowed high levels of potential inoculum on fallen leaves to become airborne, thus favouring infection by *Alternaria* spp. Although flood irrigation is a cost effective way of irrigating orchards, this has to be balanced against the possible increase in infection by *Alternaria alternata* (Goldhamer, 1996). Goldhamer (1996) reported that irrigation at the sub-soil level reduces relative humidity in the orchard, leading to a four-fold reduction in the incidence of Alternaria late blight, and a 45% reduction in fruit infection. Goldhamer *et al.* (2002) expanded on this, stating that the cost of installing "buried drip irrigation" is amply repaid by the lessening of infection by *Alternaria* spp. both on the leaves and the developing fruit. Coupled with the high levels of potential inoculum in the fallen leaves that can become airborne during overhead or aerial irrigation, buried irrigation must be seriously considered (Goldhamer *et al.*, 2002). Michailides & Morgan (1991) found that skipping an irrigation cycle in late summer reduced the occurrence of *Alternaria* spp. by 60 %. Conditions of high humidity have also been found to occur in low laying areas in orchards, as well as under conditions of excessive dew formation (Michailides & Teviotdale, 2003a). The quality of irrigation water also plays a role in the health of the pistachio tree, since high salinity levels

can lead to reduced shell splitting (Ferguson *et al.*, 2005).

In order for fruit set to be successfull, pistachio trees require exposure to a minimum period of low temperatures. This is known as the chilling requirement (Beede, 2005). Chilling requirements differ between trees, location and even between cultivars (Küden *et al*., 1995a). The requirement for pistachio trees varies between 500 and 1000 cumulative hours below 7.2°C (Caruso, 2005; Beede, 2005; Küden *et al*., 1995a). Various chemicals can be used in areas where inadequate chilling is not naturally achieved. Use of cyanamide not only leads to uniform budbreak, but also to increases in fertility (Inglese, Gullo & Pace, 1998).

In South Africa, fruit-set is often not achieved naturally, and canopy wetting is thus carried out during autumn and spring in order to achieve optimum chilling hours. This process increases the number of chilling hours to above the minimum required by reducing the transpiration rate in the leaves (Plaut & Zieslin, 1977) and delaying blooming due to evaporative cooling (Uzun & Caslar, 1999). An irrigation system with a sprinkler head is lead up into a tree to a suitable height so as to totally wet the canopy, allowing for sufficient runoff to the roots. The selected trees are watered for specific time periods at different times of the year. Wetting of the canopy, however, could also inadvertently create conditions of high moisture which is ideal for opportunistic fungi, such as *Alternaria* spp.

3.8 Cover cropping and wind breaks

The functions of cover crops include soil binding to prevent airborne sand damage to the trees, soil building to ensure better control of irrigation run-off and soil enrichment with the use of annual legumes (Michailides & Morgan, 2004). Michailides & Morgan (2004) examined a variety of cover crops in California, and warned against using cover crops such as vetch or other leguminous plants that harbour insects that cause physical damage to nuts by feeding and may be vectors, that act as disease carriers. Cover crops that act as alternative hosts to fungal and viral pathogens of pistachio are not suitable for use (Daane *et al*., 2000). In South Africa, grass crops such as oats, are planted between rows of trees to reduce the danger of wind driven sand (Snyman, 2005).

Fruit trees are susceptible to wind cull due to blossoms and small branches being broken off by the wind (Holmes & Farrell, 1993; Goldhamer *et al*., 1995). Damage by sand blown onto trunks can also provide entry wounds and place stress on the tree in general, predisposing it to opportunistic pathogens. As pistachio is wind pollinated, wind in the wrong direction or at

the inappropriate time can inhibited pollenation of the flowers (Kallsen, 2003). Many types of trees and shrubs are therefore, used as windbreaks. In South Africa, *Casurina cunninghamiana* Miq. is planted as a wind break between the orchards (Swart, 2006).

3.9 Pruning and orchard sanitation

Pistachio trees need to be pruned extensively for at least the first five years to provide a canopy that will enable mechanical harvesting and to counteract apical dominance (Beede & Ferguson, 2005). Caruso (2005) reports that heavy pruning after the initial formative stage can cause yield losses, as flowers are borne along current and one-year old wood, auxiliary to the leaf (Caruso, 2005). The trees start bearing nuts commercially after seven to ten years (Tous & Ferguson, 1996) and correct pruning is therefore essential for maximum yields.

Timing of pruning effects subsequent growth. Pruning in autumn and winter causes decreased shoot growth in high production years, while autumn pruning causes an increase in shoot development and decreased fruit drop when carried out in low production years (Küden *et al*., 1998). Summer pruning is carried out on young trees to remove diseased branches such as those infected by Botryosphaeria blight (Beede & Ferguson, 2005). Recent research has indicated that rejuvenating pruning of older trees, which involves drastic removal of branches, may alleviate the severe alternate bearing of pistachio (Ferguson *et al.*, 2005).

Pruning practices and disease incidence are often inter-related. Michailides & Morgan (2004) report that branches infected with *Botryosphaeria* spp. can be pruned in order to stop the spread of the disease. All pruned and diseased debris should be removed from the orchard immediately and either burned or correctly composted so as to prevent inoculum spread. Mechanical damage to plants allows entry for potential pathogens and pruning is especially important in this regard. If a pruning cut is not treated with fungicides and tools are not sterilized between branches and trees, opportunistic pathogens will gain easy entrance to internal tree tissue (Norris *et al.*, 2003; Michailides & Morgan, 2004). The pistachio seed chalcid, *Megastigmus pistaciae* Walker is controlled through orchard sanitation with the removal and destruction of nuts left on the trees after harvest, as well as nuts that have fallen on the ground (Bentley, Beede & Daane, 2003b).

4. Diseases

Pistachio trees, like many other woody trees are subject to attack by pathogenic micro-organisms, which reduces the plant's resistance to infection by opportunistic fungi such as

Alternaria spp. (Lenti, 1990; Michailides, 1997). Hatcher & Paul (2000) reported that the presence of *Uromyces rumicis* Schum or *Ramularia rubella* Bonord. on *Romex obtusifolius* L. leaves, lead to higher rates of infection by *Venturia rumicis* Desm. Most pistachio pathogens are fungi, with viruses and bacteria not yet reported to cause commercial losses. However, *Xanthomonas translucens* Jones, Johnson & Reddy was identified as the causal agent of wilt of young nursery plants. *Verticillium* spp. often attack young plants in the nursery and rootstocks that are resistant to *Verticillium* spp. are critical where the orchard soil harbours the pathogen (Epstein, *et al*., 2004; Facelli *et al.,* 2005b). Botryosphaeria panicle and shoot blight causes between 40% and 100% of yield loss in some Californian orchards (Michailides *et al.*, 1998). Root and crown rot can quickly spread throughout a nursery (Eskalen *et al.*, 1999) leading to loss of planting material. Septoria leaf spot infects the leaves and nuts later in the season and also causes post-harvest decay (Call & Matherton, 1998).

Table 3. Diseases of pistachio in California, East Mediterranean and Southeast Anatolian regions. Compiled from Michailides *et al.* (1998), Eskalen *et al.* (1999) and Adaskaveg *et al.* (2006).

Disease	Organism
Verticillium wilt*	*Verticillium dahliae* Kleb.
Botrytis blossom and shoot blight*	*Botrytis cinerea* Pers.
Alternaria blight*	*Alternaria alternata*
Botryosphaeria panicle and shoot blight*	*Botryosphaeria dothidea* Moung.Fr.
Root and crown rot*	*Phytophthora* spp and *Fusarium equiseti* Corda
Armillaria root rot	*Armillaria mellea* Vahl. Fr.
Schizophyllum wood decay	*Schizophyllum commune* Fries.
Sclerotinia shoot blight	*Sclerotinia sclerotiorum* Lib.
Phomopsis shot blight	*Phomopsis spp*
Powdery mildew	*Phyllactinia angulata* Wallr. Fr. and an unknown powdery mildew fungus
Septoria leaf and fruit spot*	*Septoria pistaciae* Desmaz.
Stigmatomycosis	*Nematospora coryli* and *Aureobasidium pullulans*
Aspergillus blight	*Aspergillus niger* Tiegh. and other *Aspergillus* spp.

* Major diseases

4.1 Diseases caused by *Botryosphaeria* spp.

Botryosphaeria dothidea (Moug.:Fr.) Ces. & De Not. is a fungus that causes branch and stem canker in woody trees, and has been reported on English walnut (*Juglans regia* L.), willow trees (*Salix lasiolepis* Benth.) and almonds (*Prunus dulcis* (Mill) Webb) (Michailides *et al.,* 1995). It was first identified in 1984 in California as the cause of panicle and shoot blight in pistachio (Michailides *et al.*, 1998; Ma *et al.*, 2001; Michailides, & Morgan, 2004). The fungus infects young fruit clusters as well as shoots, often leading to secondary infection, and to panicle blight (Michailides *et al*., 1995), which can cause up to 100% yield loss.

Panicle and shoot blight is regarded as one of the most important threats to commercial production of pistachio in California. Presently pruning of infected material is more effective than the use of fungicides. Care must, however, be taken to remove and destroy all infected material (Holtz, Michailides & Hoffman, 2000). Ma *et al.* (2001) found that drought stress on pistachio led to a severe increase in the incidence of this disease. As no chemicals have yet successfully controlled this fungus, sufficient irrigation has to be applied at the correct times to prevent water stress in the trees. Nutrients deficiencies such as potassium and calcium increase the predisposition of pistachio to this disease (Michailides *et al*., 2002). Michailides

et al. (2002), however, found that whereas $Ca(NO_3)_2$ caused a significant decrease in Botryosphaeria panicle and shoot blight, $CaCl_2$ was phytotoxic to pistachio.

Botryosphaeria obtusa Schwein. was first reported on pistachio in South Africa in 1995 (Swart & Botes, 1995). Swart & Blodgett (1998) subsequently also reported the presence of *B. dothidea* as the causal agent of stem cankers of pistachio which leads to weakening of the trees, thus predisposing them to other opportunistic fungi such as *Alternaria* spp.

4.2. Diseases caused by *Alternaria* spp.

In 1985, Alternaria late blight on pistachio caused by *Alternaria alternata* Fr. was first reported in California (Michailides *et al*., 1995). The disease becomes severe late in the season and is characterized by angular brown spots on the fruit and lesions on leaves (Michailides & Teviotdale, 2003a). According to Michailides *et al.* (1995) these lesions originate from lenticels, causing deterioration of the endocarp that could lead to shell staining. Doster & Michailides (1999) suggested that shell staining could be used as a measure of infection and stained shells should be discarded. If the nuts split pre-maturely, the kernels can become infected. The fungus attacks many commercial crops and especially fruits and berries throughout the world (Belisario *et al.,* 1999; 2004; Wright *et al.,* 2004). During 1998, black discolouration at the stylar end of developing nuts in South African pistachio orchards was associated with *Alternaria alternata* (Swart & Blodgett, 1998).

4.2.1 Morphology

Members of the genus *Alternaria* are classified as Hyphomycetes and form small light green to black catenate dictyospores or conidia (Kendrick, 2000). The chains of conidia can be abundantly or sparsely branched. The spores are typically small, less than 30µm in length and characterised by transverse septa and the presence of a beak region (Simmons, 1996). The fungus is omnipresent and can be isolated from many surfaces, including plant material and the soil (Rotem, 1994). The most common *Alternaria* spp. associated with Alternaria late blight of pistachio are *A. alternata, A. arborescens, A. tenuissima* and *A. infectoria*. Although the causative organism of Alternaria late blight has been identified as *A. alternata,* there is considerable variation in morphology of these small spore catenulate taxa. The morphology of a culture can vary depending on the cultural condition such as medium, temperature, light and humidity. Simmons (1996) identifies the different species using the shape and size of the conidia, however, it has been found that conidial morphology can differ within an isolate

depending on the conidial age (Pryor & Michailides, 2002). Conidial morphology overlaps between the taxa, making it an unreliable source of identification of *Alternaria* species. In addition to this, host specific isolates are also morphologically distinct, necessitating reclassification of such isolates.

4.2.2 Molecular identification

Due to variation in the morphology of *Alternaria* spp. molecular techniques are needed to verify the identity of the species (Weir *et al.,* 1998). A variety of methods for determining genetic relationships using genomic DNA have been developed. Random Amplification of Polymorphic DNA Polymerase Chain Reaction (RAPD-PCR) is often used for the identification of *Alternaria* spp. (Weir *et al*., 1998; Cooke *et al*., 1998; Roberts, Reymond & Anderson, 2000; Pryor & Michailides, 2002). Sequence variation among species in the laternata specie-group are often so small that use of *formae specialis* was suggested within *A. alternata*. For example, the previously classified *A. citri*, was reclassified as *A. alternata* f. sp. *citri,* an intraspecific variant (Weir *et al*., 1998). Such differences within species might indicate host specificity (Weir *et al.,* 1998). Cooke *et al.* (1998) found a high genetic correlation between various *A. brassicae* isolates, but also found a close genetic grouping between *Alternaria* spp., *Embellisia* spp. and *Stemphylium* spp. In contrast, Bock, Thrall and Burdon (2005) found sizeable polymorphisms in *A. brassicicola* in Australia, and had difficulty grouping isolates. This was confirmed by Avenot *et al*. (2005). Roberts *et al.*, (2000) disagreed with the pathotype system for *Alternaria* spp. and claimed to have distinguished species morphologically distinct, confirming this with RAPD-PCR. Plasticity in morphology is blamed on non-standard growth conditions, including variation in temperature, media and light regimes (Roberts *et al.*, 2000). Pryor & Michailides (2002) identified three *Alternaria* clusters in pistachio orchards morphologically and confirmed these groupings using RAPD-PCR. This method found little variation between the alternata and tenuissima groups, but was able to differentiate between these two and the arborescens group

Aradhya, Arjmand & Chan (2000) made use of Restriction Length Polymorphism (RFLP) variation in the rDNA region of the genome to analyse *Alternaria alternata* pistachio pathotype populations, and found significant genetic diversity within this population. Aradhya, Chan & Parfitt (2001) used PCR-RFLP to study genetic variations of *A. alternata* causing pistachio late blight, and found 34 different rDNA haplotypes in the isolates. Using RFLP, Pryor and Michailides (2002) found comparable results to the RAPD-PCR method

with regard to the similarity between the alternata and tenuissima groups and the distinction between these and the arborescens group.

The above studies highlight genetic variation within the *Alternaria* spp. culminating in the fear that this fungus represents a great danger to pistachio due to its apparent adaptability (Schrader *et al*., 2001). Van der Waals, Korsten & Slippers (2004) used Random Amplified Microsatellite (RAMS) primers to study *A. solani* Ell. and Mart. isolated from potatoes. They also found very high diversity within this species that occurs randomly throughout South Africa, indicating a danger to the potato producing industry due to the fungus' high adaptability.

4.2.3. Other identification methods

Vegetative compatibility tests where non-reactive hyphal intermingling denotes similarity and an antagonistic zone denotes differences can be used instead of, or in addition to, morphological differences. These tests are then combined with DNA analysis for identification of pathogenic fungal species (Cilliers, Herselman & Pretorius, 2000; Cai & Schneider, 2005). Andersen, Kroger & Roberts (2002) made use of secondary metabolites to differentiate between the infectoria, arborescens and tenuissima groups. Using high performance liquid chromatography for separation of the metabolites and a diode array detector, Andersen *et al.* (2002) found that the infectoria group differed significantly from the other groups with regard to the secondary metabolites formed, and also found tentoxin in one arborescens cluster not previously reported. This led to subsequent studies which compiled a chemical profile for fungi, including the genus *Alternaria* (Hansen, Andersen & Smedsgaard, 2005). This information could be used in conjunction with previous classification methods to clarify the relationships between the different *Alternaria* spp.

4.3 Infection of pistachio by *Alternaria* spp.

4.3.1 Dissemination

Conidia of *Alternaria* spp. are commonly found in spore traps in pistachio orchards (Timmer *et al.,* 1998; Swart, 2003) indicating their presence throughout the year. Within as little as 24 hours after a conidium has settled on a host, the fungus can colonize the host and start producing secondary conidia (Rotem, 1994; Michailides *et al*., 1995; Simmons, 1996; Timmer et al., 2000). Conidia are easily released from the hyphal mass and are distributed mainly by wind (Baidya, Pasha & Sunirmal-Chandra, 1994; Rotem, 1994; Michailides *et al.*,

1995; Timmer *et al.,* 1998). Bilgrami & Ghaffar (1994) found *A. alternata* and *A. tenuissima* Fr. on pistachio trees throughout their studies in Pakistan. Similar reports have come from most areas where *Pistacia vera* is now commercially planted (Michailides *et al*., 1995; Michailides, 1997; Swart & Blodgett, 1998; Ash & Lanoiselet, 2001; Bharat, 2002). Pistachio leaves become blackened with conidia of *Alternaria* spp. towards the end of the growing season at leaf drop, and are also a likely source of primary infection of emerging buds in spring (Rotem, 1994). Due to pistachio's deciduous nature, leaf debris collects below the trees, and when humidity is artificially increased through the use of sprinkler and flood irrigation (Goldhamer, 1996), the conidia are released and can become airborne (Timmer *et al.,* 1998).

Low winter temperatures in the soil do not support vigorous saprophytic growth of opportunistic fungi. The conidia go into a dormant state in the soil, on fallen leaves, branches that have been pruned and left in the orchard, grasses planted between the rows as well as on organic material found in the soil (Michailides *et al.,* 1999; Van der Waals, 2002). Pryor, Davis & Gilbertson, (1998) recovered *Alternaria* spp. from dry soils that had been stored for four years, indicating that the dry spores can remain viable for lang periods of time. *Alternaria* spp. can also survive epiphytically on plant surfaces (Logrieco *et al.*, 2003; Simmons, 1996), or even within plant tissue without causing any disease symptoms (Kriel, Swart & Crous, 2000; Gennaro, Gonthier & Nicolotti, 2003), until the plant is stressed and it becomes pathogenic. This ubiquitous fungus survives well on plant debris and is favoured by high humidity (Rotem, 1994; Ferguson *et al.*, 2005). Conidia over-winter in the soil while the trees are in a dormant phase, forming a spore bank for reinfection of the orchards the following season (Michailides & Morgan, 2004).

Alternaria spp. are consistently isolated from asymptomatic flower buds and leaf buds that have been surface sterilized to exclude any external contamination. This fungus' presence within plant tissue has been proven (Devi, Garg & Dwivedi, 1995) both through stimulation of growth in the laboratory and by electron microscopy. Evans *et al.* (1999) state that flower and leaf buds are sources of *Alternaria* spp. inoculum. They found that the mean percentage of buds infected was 93.2%, but none of the flower buds exhibited any visual symptoms. As latency is broken by the release of sugars during the ripening of the fruit, lesions appear on the fruits and the conidia are wind borne to form secondary infections (Biles, Bruton, & Zhang, 1999). Sudden deterioration of walnuts, hazelnuts and pistachio at or just after harvest prompted Michailides *et al.* (1995) to sample asymptomatic plant tissue and they

found *Alternaria* spp. within the fruit tissue as early as spring, only causing brown spot in midsummer. *Alternaria* spp. have also been recovered from surface sterilized almonds Bilgrami & Ghaffar (1997).

Other opportunistic fungi also infect woody tree fruits. Washington, Hood & Stewart-Wade (1999) identified *Phomopsis castanea* Sacc. in asymptomatic chestnuts that led to post-harvest rotting of the nuts. Belisario *et al.* (2002) reported similar symptoms on walnuts which yielded the presence of *Fusarium* spp. and *Alternaria* spp. within the stylar end. Belisario *et al.* (2004) inoculated attached walnuts blossoms with a water drop containing *Alternaria* spp. Between 20 and 50% of the fruit subsequently exhibited brown apical necrosis, and the introduced pathogens were recovered from the diseased tissue. *Alternaria* spp. present in apparently healthy pistachio plant material can be regarded as living endophytically within the plant material until certain factors change in the plant itself, or in its environment (Schardl & Phillips, 1997; Michailides *et al*., 1995). Andrews (1996) used an intensified method of surface sterilization on seemingly healthy pistachio tissue and found that 70% of the organisms subsequently recovered were *Alternaria* spp. Although *Alternaria* spp. are not true endophytes (Kim *et al*., 2001; Christensen & Bennett, 2005), they spend part of their life cycle in an endophytic state within plant tissue.

The time between infection and symptom development can be as long as six weeks (Caruso, 2005) with symptoms sometimes only developing during post-harvest storage of the fruit (Prusky, 1996). The fungus can therefore be said to be in a state of quiescence or latency (Evans *et al.,* 1999; Chen, Brown & Cleveland, 2004). Although symptomless, these infections can be observed by transmission and scanning electron microscopy (Devi *et al.*, 1995, Kim, *et al.*, 2001). Once conditions for growth, such as release of sugars during fruit ripening are met, the fungus will start growing, leading to the development of symptoms (Prusky, 1996; Biles *et al.*, 1999). During active plant growth, large amounts of sugar are present in the leaves. Sugar inhibits fungal spore germination *in vitro*, and this is related to the active plant being able to resist fungal infection (Rotem, 1994). Leaf senescence leads to natural loss of chlorophyll and high concentrations of conidia on leaves infected by *A. solani,* Ell. and Mart., *A. macrospora* and *A. alternata* (Rotem, 1994). This is consistent with pistachio leaves becoming chlorotic and eventually necrotic towards the end of the growing season and later blackened with *Alternaria* conidia.

4.3.2 Infection

Hong & Pryor (2004) reported that *Alternaria* spp. opportunistically infect plants through mechanical injuries or injuries caused by insects. *Hylensinus vestitus,* a beetle that damages leaf and flower buds, lays its eggs on pruned, damaged and dead pistachio branches. This beetle does not transmit *Alternaria* spp., but causes injuries which can allow the fungus entrance to the plant. It is critical to maintain proper orchard sanitation in the form of removal and burning of debris which may contain eggs of this beetle (Farivar-Mehin, 2002). Strong wind can damage young trees, allowing entrance of opportunistic fungal spores into the plant tissue (Ferguson *et al.*, 2005) and pruning without sealing the wounds with appropriate fungicides will also cause potential entry wounds (Michailides & Morgan, 2004).

The apetulous flower of the Anacardiaceae family has nectaries, an adaptation for capturing wind born pollen (Hormanza & Polito, 1996). Spores of *Alternaria* spp. are also disseminated by wind (Rotem, 1994). Pistachio pollen varies from 20-24 µm in diameter (Davarynejad *et al.,* 1995) to 31 µm in diameter (Erdtman, 1966) and spores vary in diameter from 25-40 x 15-25 µm in *A. citri*, 30-50 x 8-13 µm in *A. alternata* to 60-75 x 12-16 µm in *A. tenuissima* (Simmons, 1996). It is thus conceivable that the same wind that carries the pollen can deposit *Alternaria* spp. spores on the flowers. Ngugi & Scherm (2006) reported that fungi specializing in infecting flowers include unspecialised opportunistic pathogens such as *Alternaria* spp.

4.3.3. Colonization

Managerial and environmental factors often combine to predispose pistachio to colonization by opportunistic fungi such as *Alternaria* spp. For example, elevations above 800 meters could result in insufficient summer heat units and may lead to incomplete kernel development which leaves the nuts susceptible to infection (Ferguson *et al.*, 2005). Ferguson *et al.* (2005) reported that weather conditions that lead to insufficient chilling hours may result in irregular and/or delayed blooming and leaf emergence and malformed leaves with subsequent yield losses and general loss of plant health, predisposing it to opportunistic infection.

Soils with inadequate drainage can lead to water logging which can predispose both rootstock and scion to opportunistic fungal infection (Schoeneweiss, 1975; Ferguson *et al.*, 2005). The number of continuous rain days in spring has been correlated with conditions conducive to fungal growth (Mila *et al*., 2005), requiring good fungicide management. If the chosen

rootstock does not allow for the correct branching of the radicular system, the new plant may either not survive transplanting or be so weakened that it becomes predisposed to opportunistic fungal attack (Vargas *et al.*, 1999; 2001). Arpaci & Ak (2001) determined that the timing of transplanting of seedlings affects the vigour of the tree, predisposing it to infection by opportunistic fungi if transplanting does not take place at the optimum time of the year.

Potassium, nitrogen, phosphate and calcium deficiencies predispose pistachio to *Alternaria* spp. Plant vigour improves after application of fertilizers and yields increase due to a reduction in fungal infection (Michailides *et al.,* 2002). Incorrect use of fungicides, especially concentrations lower than the recommended rate will not only be ineffective but may even stimulate the growth of the pathogen through a process known as hormesis (Watkins & Klemme, 1948; Kenyon, Dixon & Helfer, 1997; Calabrese & Baldwin, 1998).

Weeds compete with pistachio trees for water and nutrients (Hembree & Shrestha, 2004) leading to stress and predisposing them to opportunistic infection. Weeds can also harbour insects, fungi and viruses (Lopes *et al*., 2003) thus acting as an alternative host. If herbicide labels are not followed, damage can occur to pistachio trees, predisposing them to infection by *Alternaria* spp. (Mohassel, Nassiri & Poorkzem, 1999; Hembree & Shrestha, 2004). Sublethal concentrations of the herbicides may stimulate growth of the weeds, compounding the problem (Wiedman & Appleby, 1972).

Application of mineral oils for the purpose of bud break during cold weather can increase infection by *Botrytis* spp. (Beede & Ferguson, 2002). Late application of bud break oils such as dormex (hydrogen cyanamide) can lead to inadequate chilling resulting in severe reduction in pistachio yield (Rahemi & Asghari, 2004). Impurities such as aromatic compounds and sulphur, nitrogen or oxygen containing compounds in inadequately purified mineral oils are often associated with plant injury (Townsend, 2003). These are normally removed during refining, and it is therefore important to obtain such oils from a reputable firm (Baker & Powell, 1996; Polito & Pinney, 1999). Rodriquez & Almaguer (1980) found that application of citronella oil from mid- to late summer was effective, but application one week before bud break killed many buds. The heavier or dormant oils primarily used as insecticides must be applied before active growth, as these oils can cause injury to the trees (Townsend, 2003).

Various studies have indicated that the method of irrigation, e.g. drip or low level irrigation vs. high level irrigation, may affect the incidence of infection by *Alternaria* spp. (Michailides *et al*., 1995; Goldhamer, 1996; Robinson, 1997; Goldhamer *et al.*, 2002; Holtz, 2002). Flood irrigation is a cost effective way of irrigating orchards, however, this creates conditions of high humidity that favours *Alternaria alternata* causing large increases in disease incidence (Goldhamer, 1996). Goldhamer (1996) reports that irrigation on the sub-surface level reduces relative humidity in the orchard, leading to a four fold reduction of the incidence of Alternaria late blight, and a 45% reduction in fruit infection. Coupled with the high levels of potential inoculum in the fallen leaves that can become airborne during over-head or above-ground irrigation, buried irrigation should therefore be seriously considered (Goldhamer *et al.*, 2002). Frequent irrigation leads to higher root activity in the shallower soil layers which could lead to damage during mechanical weeding or the uptake of potentially damaging herbicides in run-offs (Kanber *et al*., 2004). Pistachio also does not tolerate excess water in its root zone, and this practice can lead to predisposition of the tree to infection by opportunistic fungi such as *Alternaria* spp. (Canihos, Peever, & Timmer, 1999; Kirnak, Ak & Acar, 1999).

Water stress can lead to ending the latent stage of fungal development, and often rapid disease development (Stanosz *et al.,* 2001; McElrone *et al.,* 2003). Ma *et al*. (2001) determined that drought stress in pistachios increased the incidence and severity of Botryosphaeria blight, and this would probably hold true for cases of Alternaria blight on pistachio as well. Skipping an irrigation during shell formation, or delaying harvesting, increases the incidence of unwanted early split and increases the incidence of Navel Orangeworm as well as opportunistic pathogenic fungi such as *Alternaria* spp. (Doster & Michailides, 1995; Washington, Allen & Doodley, 1997; Ferguson *et al.*, 2005). Mechanical harvesting may also damage the bark, leading to infection by *Botryosphaeria* spp. generally weakening the plant.

5. Conclusions

Alternaria spp. are omnipresent in commercial pistachio orchards, with inoculum present in sufficient concentrations to allow for repeated infection. Healthy tissue is not susceptible to this opportunistic fungus, and infection and colonization can therefore only take place in pistachios that have been predisposed to disease due to stress. Stress is caused by endogenous factors related to the genotype of the rootstock and scion, and unalterable exogenous factors related to the environment including the basic soil type, height above sea level, minimum and maximum temperatures, the time and amounts of rainfall. These factors play a major role in

the choice of young plants, their treatment and eventual transplantation and are the basis of a healthy and productive orchard.

Once seeding material and the site have been decided upon, pistachio trees will be exposed to a variety of changing and sometimes unpredictable stresses caused by managerial practices imposed on the orchard. In commercial production the emphasis is on maximum yield of high quality nuts. This is achieved by optimizing growing conditions through planting of cover crops and windbreaks, pruning and orchard sanitation, fertilization, irrigation, control of weeds, insects, other pests and diseases, and artificial forcing or retardation of bud break. If any of the methods used to increase production causes stress in pistachio, the trees will be predisposed to infection by opportunistic *Alternaria* spp. with concomitant loss in yield.

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CHAPTER 2

Characterization of Quiescent *Alternaria* Isolates from Asymptomatic Pistachio Tissue in South Africa

Introduction

Pistachio (*Pistachio vera* L.) is a relatively new crop in South Africa with considerable commercial potential, providing that constraints such as pests and infection by facultative parasites or opportunistic pathogens, as well as the pressure of environmental factors, can be overcome (Canihos, Peever, & Timmer, 1999; Kirnak, Ak & Acar, 1999). During 2003, observations in pistachio orchards near Prieska in the Northern Cape Province indicated blackening at the stylar end of developing nuts (Swart, 2006). During the 2004/2005 season nut yield losses of up to 70% occurred in some blocks of six to eight year-old trees. At the end of the growing season in April twig dieback was also noted on trees in certain blocks where senescent leaves and dead nuts had become covered by *Alternaria* spp. spores (Swart, 2006).

Pryor & Michailides (2002) and Belisario *et al*. (2004) reported that samples from diseased nuts and other symptomatic plant tissues such as twigs and leaves from pistachio and walnuts, revealed *Alternaria* spp. that differed significantly in terms of their culture morphology. *Alternaria* spp. were isolated from seemingly healthy pistachio leaves, bud tissue and flowers indicating a possible endophytic or quiescent phase in the development of the fungus as indicated by other researchers (Devi, Garg & Dwivendi, 1995; Evans *et al*., 1999; Pryor & Michailides, 2002). Pryor & Michailides (2002) remarked that a complex of several small-spored catenulate *Alternaria* spp. were associated with leaves of pistachio, as well as with those of pears, citrus and almonds, occupied similar ecological niches and represented a closely related monophyletic group.

Morphological characteristics have been used to differentiate between species in the genus *Alternaria* (Rotem, 1994; Simmons 1996). Roberts, Reymond & Andersen (2000) and Pryor & Michailides (2002) claimed to be able to use morphological differences for differentiation between isolates, but emphasized the importance of strictly standardized growing conditions. According to Rotem (1994) morphological variation seems to be typical for this fungus and should not be used exclusively in species determination, but employed as a guide towards possible species identification.

It is of vital importance to identify a phytopathogen in order to implement an integrated pest management programme (Pryor & Michailides, 2002; Hong *et al*., 2006). These strategies include chemical and biological control (Adaskaveg *et al*., 2007), planting of catch crops

(Norris, Caswell-Chen & Kogan, 2003), as well as orchard sanitation including removal and destruction of contaminated and pruned plant material (Doster & Michailides, 1995) and the timing and type of irrigation (Goldhamer, 1996).

The objectives of the present study were to determine a) whether differences in culture morphology observed between *Alternaria* isolates from pistachio tissue could be attributed to different species of *Alternaria* using molecular techniques; b) whether there was any indication of niche specificity with regard to *Alternaria* spp. that inhabit asymptomatic as opposed to diseased pistachio tissue and c) the dynamics of endophytic colonization and its influence on disease incidence and control strategies for nut diseases.

Materials and Methods

Preliminary characterization of Alternaria *isolates in culture*

In a preliminary study, ten *Alternaria* isolates obtained from asymptomatic pistachio leaves during previous research by Swart (unpublished) and stored in the culture collection of the Division of Plant Pathology in the Department of Plant Sciences at the University of the Free State, South Africa, were cultured on seven types of agar media to determine macroscopic differences between isolates. Media used were corn meal agar (CMA) (Biolab), malt extract agar (MEA) (Biolab), potato carrot agar (PCA) (Bioblab), water agar (WA) (Biolab), potato dextrose agar (PDA) (Biolab), as well as acidified PDA (APDA) and acidified weak PDA (AWPDA) as developed by Hong & Pryor (2004). Each isolate was plated in triplicate and the entire experiment was repeated in triplicate. Inoculated plates were incubated at 20°C, 25°C, 30°C and 35°C and differences in culture morphology were noted after 5 and 11 days. Characteristics noted included the colour and texture of the mycelial growth as well as discolouration of the agar and all isolates were classified into four morphotypes (Table 2.3). Branching of conidial chains was microscopically observed (x 60) under a dissection microscope after growing these isolates on WA.

Characterization of Alternaria *isolates from asymptomatic nuts*

During the fungicide (Chapter 3) and canopy wetting (Chapter 4) experiments more than 5 000 *Alternaria* isolates were collected. These were classified according to morphotypes distinguished during the preliminary characterization of *Alternaria* isolates. Thirteen isolates

were selected from morphotypes 1, 2 and 3 and 11 from morphotype 4. Ten type cultures of five *Alternaria* spp. were obtained from the Centraalbureau voor Schimmelcultures (CBS): *Alternaria arborescens* Simmons (102605), *A. arborescens* (109730), *A. alternata* Fr. (105.24), *A. alternata* (101.13), *A. tenuissima* Simmons (117.44), *A. tenuissima* var *godetiae* (878.95), *A. brassicae* Berk. (102.24), *A. brassicae* (106.36), *A. citri* Ellis & N. Pierce (107.27) and *A. citri* (106.27). These were used as taxonomic markers against which to compare the pistachio isolates and were also grouped into the morphotypes. *A. brassicae* and *A. citri* were used as outgroups. All 60 isolates were plated onto AWPD and incubated at 15°C, 22°C, 29°C and 35°C. Colony diameter was measured after 11 days. Cluster analysis was performed on the data using the Number Cruncher Statistical System (NCSS) 2000 (Hintze, 2001) in order to determine relationships between isolates at different temperatures.

Molecular characterization of Alternaria *isolates*

Diseased nuts, asymptomatic flower buds, leaves and twigs were sampled for the presence of *Alternaria* spp. Leaves and twigs were placed in a freezer overnight, then incubated at room temperature in a moist chamber. A small sample of the the resultant fungal growth was collected using a heat sterilized scaple blade and plated on AWPDA (ONFIT technique) (Emery & Michailides, 2000). The ensuing colonies were grouped according to the four morphotypes described in the preliminary characterization of *Alternaria* isolates. Twenty of the abovementioned isolates and 50 isolates from asymptomatic nuts were used in the molecular study. Ten type cultures representing five species (CBS cultures) were used as reference isolates (Table 2.1). With the exception of one *A. tenuissima* isolate which was discarded, all isolates were grown in a broth containing 2% malt extract (Bioblab) and 0.3% bacterial peptone (Biolab). DNA was extracted using the CTAB (hexa-decyl-trimethylammonium bromide) DNA isolation method (Saghai-Maroof *et al*., 1984). For molecular characterization, AFLP analysis method developed by Vos *et al*. (1995) and modified by Herselman (2003) was used. *Eco*RI and *Mse*I were used as restriction enzymes: Primers used for selective amplification were: *Eco*RI-AG/*Mse*I -AT, *Eco*RI -AG/*Mse*I -TT; *Eco*RI -AC/*Mse*I -AT and *Eco*RI -TT/*Mse*I -AG. (Hong *et al*., 2006) (Table 2.2).

PCR products were separated on 5% (w/v) denaturing polyacrylamide gel [19:1 acrylamide/bis-acrylamide; 7 M urea; 1 x TBE buffer (0.89 M Tris-HCl; 0.89 M boric acid; 2.0 mM EDTA)], polymerised using 0.04% ammonium persulfate and 0.09% TEMED (N,N,N',N'-

Tetramethylethylenediamine) added to 31 ml of double distilled water. In preparation for gel electrophoresis, the 20 µℓ of amplified sample was mixed with 20 µℓ of formamide dye consisting of 98% (v/v) de-ionized formamide, 10 mM EDTA at pH 8.0, 0.05% (w/v) bromophenol blue and 0.05% (w/v) xylene cyanol. Samples were incubated at 95°C for five minutes and immediately placed on ice prior to loading on the gel. Electrophoresis was performed at constant power of 80 W for two hours (Herselman, 2003).

The resulting gels were stained using the Silver Staining Sequence™ DNA Sequencing System manual following the instruction supplied by Promega (Madison, WI, USA) as revised in June 1998. A negative image, the same size as the gel was produced using Kodak Polymax II RC photographic paper underneath the gel exposed at dim light for about 20s. Visible fragments were scored as either present (1) or absent (0) for each sample generated by each primer combination. Only reliable (between 150 and 700 bp) and repeatable fragments were considered. Pair wise genetic distances were expressed as the complement of Dice's coefficient (Dice, 1945). Cluster analysis was performed using the unweighted pair group method using arithmetic averages (UPGMA) (Sokal & Michener, 1958). Statistical analysis was performed using the NTSys-pc version 2.02 (Exetor Software, NY, USA) software package (Rohlf, 1998). The placement of the ten reference isolates and the cultures isolated from pistachio tissue in the four morphotypes were compared to the results of the molecular analysis to see whether these distinctions held true to the genetic divisions.

Results

Characterization of Alternaria *isolates in culture*

All *Alternari*a isolates showed similar growth morphology on CMA, MEA, PCA, WA or PDA at all temperatures. At 15°C and 35°C the colonies on APDA and AWPDA were small and dark with little difference in appearance (results not shown). Growth on APDA and AWPDA showed distinct morphological differences in terms of colony appearance at both 25°C and 30°C (Table 2.3). These characteristics were most prominent on AWPDA rather than on APDA at 25°C. Using the colour and texture of the mycelial growth, as well as agar discolouration and conidial chain branching, isolates were placed into four morphotypes, which were used throughout the study (Figure 2.1). In morphotype 1 the conidial chains were non-branching, in

morphotype 2 few chains were observed, while morphotypes 3 and 4 were mostly branched in a similar fasion as shown in Figure 2.2.

Characterization of Alternaria *spp. isolated from asymptomatic nuts*

Average colony diameter of each of the isolates, including the type species, grown at different temperatures and measured at 11 days, did not show any distinctive groupings. Dendrograms were compiled of the relationships between the isolates at different temperatures using the NSCC statistical software programme. The type species did not group together according to species and neither the type species, nor the other isolates clustered according to the proposed morphotypes (data not shown).

Molecular characterization of Alternaria *isolates*

All isolates from pistachio, irrespective of the tissue type from which they were isolated, or whether the tissue was seemingly healthy or diseased, clustered together within the cluster containing the type cultures of *A. arborescens* and *A. alternata* (Cluster B, Figure 2.3). AFLP analysis revealed a total level of variation of 20.6% similarity between all isolates tested. Except for isolates 19 and 20, all isolates could be distinguished from each other using the four tested AFLP primer combinations. The highest level of variation was detected between the two *A. citri* isolates and the rest of the isolates. Except for isolates 17, 73 and 77, all *Alternaria* isolates isolated from pistachio clustered together in cluster b with a similarity of 56.9%. The type isolates of *A. arborescens* and *A. alternata,* as well as isolates 17, 73 and 77, clustered together in cluster with a similarity of 66.1%. The four isolates of *A. arborescens* and *A. alternata* were 92.6% similar. The type isolates from *A. brassicae* and *A. tenuissima* clustered together in cluster A with a similarity of 83.7%. It was interesting to note that the type isolate of *A. tenuissima* was 97.9% similar to one of the type isolates of *A. brassicae.* The level of variation between *Alternaria* isolates isolated from pistachio was much higher compared to the variation between isolates of the different type species.

With the exception of isolates 27, 48 and 17, all isolates from asymptomatic nuts (isolates 11-60) formed a separate cluster (cluster i, Figure 2.3) with a similarity coefficient of 70.6%. Isolates from infected twigs, diseased nuts, asymptomatic flower buds and asymptomatic leaves formed a secondary cluster (cluster ii) with a similarity coefficient of 66.2%. Isolates 27 (cluster iii) and isolate 48 (cluster iv) clustered separately from the other *Alternaria* isolates in

cluster i and ii. Isolates 73 (asymptomatic blossom) and 77 (asymptomatic twig) were the most dissimilar to the other isolates isolated from pistachio and cluster together with the type isolates of *A. arborescens* and *A. alternata*. These two isolates were 94.7% similar to each other and 72.6% similar to the type isolates of *A. arborescens* and *A. alternata.* Although some of the isolates clustered together based on morphotype (isolates 13-23, except isolate 17, of morphotype 1 clustered together as well as isolates 51-57 of morphotype 4), isolates generally did not cluster according to morphotype.

Discussion

Belisario *et al*. (2004) and Hong *et al*. (2006) emphasized the importance of identifying the causal organism of a plant disease before any control measures can be implemented. The niche specificity and timing of infection also play a role in the determination of control measures. All isolates used in this study were microscopically identified as *Alternaria* spp. based on conidia morphology and identification was confirmed through the molecular study.

The fact that isolates did not group according to morphotype in temperature studies confirmed previous findings in literature (Pryor & Michailides, 2002; Pryor, Matherton & Figuli, 2003; Hong *et al*., 2006). Growth rate of small spored catenate *Alternaria* spp. is not used as an identification criterium. However, conidial shape and branching patterns are used for identification, providing that growth conditions are strictly controlled and consistent for all observations (Pryor & Michailides, 2002; Pryor *et al*., 2003; Hong *et al*., 2006). The lack of grouping in this study based on culture morphology holds true for isolates from asymptomatic nuts as well as the type cultures where no clustering occurred within species or morphotypes. Although all non-branched isolates were in morphotypes 1 and 2 (Table 2.1, Figure 2.3) AFLP analysis did not differentiate them from branched isolates. AFLP analysis done during the present study, employing primers used by Hong *et al* (2006), did not correlate with results based on culture morphology that were used to group isolates into the four proposed morphotypes.

Based on molecular analysis, all *Alternari*a spp. isolated from different types of healthy and diseased pistachio tissues fell within the small spored catenate *Alternaria* spp. group which encompasses *A. alternata, A. arborescens* and *A. tenuissima*. There is therefore no evidence of specific niches being occupied by specific *Alternaria* spp. Although no niche specificity was

noted, isolations from flower buds suggested that infection of pistachio tissue occurred early on in the growth season (Evans et al., 1999). All three the abovementioned species are pathogenic on pistachio (Caruso, Fabbri & Giovannini, 1995; Roberts *et al*., 2000; Pryor & Michailides, 2002; Michailides *et al*., 2005; Hong *et al.*, 2006). Spores of these fungi are easily disseminated by wind in orchards and can be deposited on flowers where the microclimate is ideal for germination and could lead to infection. Ngugi & Scherm (2006) reported that fungi specializing in infecting flowers include unspecialized opportunistic pathogens of which *Alternaria* spp. are typical examples (Rotem, 1994). In the present study *Alternaria* spp. were frequently isolated from flower buds as found by Evans *et al*. (1999). Infection of the developing flower bud or flower became latent or quiescent (Evans *et al*., 1999) until the fruit started to ripen after which symptoms started to appear (Devi *et al.*, 1995; Pryor & Michailides, 2002). Infection of young developing fruit through stomata or the stylar end is also possible (Verhoeff, 1974) and the typical stylar rot symptom observed on mature pistachio fruits in the present study is consistent with this avenue for infection.

The AFLP typing results of *Alternaria* isolates are inconsistent with those of Belisario *et al*. (2004) who isolated *Alternaria* spp. from hazelnut and walnut fruit and was able to differentiate isolates from each host into an *A. alternata*/*A. tenuissima* group and an *A. arborescens* group. In the present study type cultures of *A. alternata* and *A. arborescens* obtained from CBS clustered together and were different from the single isolate of *A. tenuissima* which grouped with two isolates of *A. brassicae*. This is in direct contrast with findings of Hong *et al.* (2006) where *A. alternata* and *A. tenuissima* clustered together and were distinct from *A. arborescens*. Similarly, Pryor & Michailides (2002), using RAPDs and RFLPs, found that *A. arborescens* clustered separate from *A. alternata* and *A. tenuissima*. However, using internal transcribed spacer (ITS) regions, all three of these groups clustered into a single monophyletic clade (Pryor & Michailides, 2002).

The unexpected clustering of the A. *tenuissima* isolate with the *A. brassicae* isolates may have been caused by any of a variety of factors. The *A. tenuissima* isolate could have been contaminated by an *A. brassicae* isolate during production of hyphal material for molecular analysis. Based on the described morphotypes, these two isolates should have been morphologically different and contamination should have been noted. AFLP analysis indicated that isolates did not cluster according to described morphotypes, indicating that contamination

may not have been visible. Another possible cause for the unexpected clustering might be contamination of the material used during the DNA extraction process, leading to inconsistent enzyme restrictions and ligations. Lastly, as De Hoog and Horré (2002) stated, the A. *tenuissima* isolate could have been misidentified in their culture collection, or alternatively, but unlikely, an incorrect isolate was dispatched by CBS.

Inconsistency between present results and those of other workers may be due to Hong *et al*. (2006) using type species from the Simmons collection (*A. alternata*, EGS 34-016; *A. tenuissima*, EGS 34-015; *A. arborescens*, EGS 39-128 & *A. infectoria*, EGS 27-193) while this study used type species from CBS. In their study De Hoog & Horré (2002) stated that: "In the CBS culture collection, made up of strains primarily identified by morphology, 10% of the *Alternaria* strains have been misidentified. Some species have nearly identical ITS sequences. They are mostly also morphologically similar and are frequently found on the same host plant. Information on whether these are separate species was not gained in the course of this study".

However, despite the contrasting results obtained in this study, the AFLP technique was successfully employed to differentiate between isolates obtained from different source material of pistachio. This is the first report on molecular analysis of *Alternaria* spp. found on pistachio trees in South Africa. Results shed important light on the possible identity of the *Alternaria* isolates. Results indicated that all tested isolates were genetically the most related to *A. arborescens* and *A. alternata*. To further clarify the identity of the isolates, future studies should include more type isolates of different species and specifically type isolates of *A. infectoria*. *A. infectoria* is often isolated together with *A. alternata*, *A. arborescens* and *A. tenuissima* from pistachio, but it was found to be non-pathogenic (Pryor & Michailides, 2002). It may also be profitable to include the specific type cultures used by Hong *et al*. (2006) and compare these with the CBS cultures and isolates from the South African orchards.

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Table 2.1 Origins of *Alternaria* isolates used throughout the study with the proposed morphotypes

Isolate number	*Alternaria* sp.	Collection number	Source	Morphotype[a]
1	*Alternaria arborescens*	CBS02605	*Lycopersicon esculentum*	1
2	*A. arborescens*	CBS09730	Stem lesion of *L. esculentum*	1
3	*A. alternata*	CBS105.24	Leaf spot *Solanum tuberosum*	2
4	*A. alternata*	CBS101.13	Unknown	2
5	*A. brassicae*	CBS106.36	Leaf spot *Brassica oleracea*	4
6	*A. brassicae*	CBS102.24	Leaf spot *Cochlearia officinalis*	4
7	*A. tenuissima*	CBS878.95	*Arachis hypogeae*	3
8	*A. tenuissima var godetiae*	CBS117.44	*Godetia* sp.	3
9	*A. citri*	CBS106.27	Fruit of *Citrus sinensis*	4
10	*A. citri*	CBS107.27	Fruit of *Citrus limonium*	4
11	*Alternaria* sp.	AN11	Asymptomatic nuts	1
12	*Alternaria* sp.	AN12	Asymptomatic nuts	1
13	*Alternaria* sp.	AN13	Asymptomatic nuts	1
14	*Alternaria* sp.	AB14	Asymptomatic nuts	1
15	*Alternaria* sp.	AN15	Asymptomatic nuts	1
16	*Alternaria* sp.	AN16	Asymptomatic nuts	1
17	*Alternaria* sp.	AN17	Asymptomatic nuts	1
18	*Alternaria* sp.	AN18	Asymptomatic nuts	1
19	*Alternaria* sp.	AN19	Asymptomatic nuts	1
20	*Alternaria* sp.	AN20	Asymptomatic nuts	1
21	*Alternaria* sp.	AN21	Asymptomatic nuts	1
22	*Alternaria* sp.	AN22	Asymptomatic nuts	1
23	*Alternaria* sp.	AN23	Asymptomatic nuts	1
24	*Alternaria* sp.	AN24	Asymptomatic nuts	2
25	*Alternaria* sp.	AN25	Asymptomatic nuts	2
26	*Alternaria* sp.	AN26	Asymptomatic nuts	2
27	*Alternaria* sp.	AN27	Asymptomatic nuts	2
28	*Alternaria* sp.	AN28	Asymptomatic nuts	2
29	*Alternaria* sp.	AN29	Asymptomatic nuts	2
30	*Alternaria* sp.	AN30	Asymptomatic nuts	2
31	*Alternaria* sp.	AN31	Asymptomatic nuts	2
32	*Alternaria* sp.	AN32	Asymptomatic nuts	2
33	*Alternaria* sp.	AN33	Asymptomatic nuts	2
34	*Alternaria* sp.	AN34	Asymptomatic nuts	2
35	*Alternaria* sp.	AN35	Asymptomatic nuts	2
36	*Alternaria* sp.	AN36	Asymptomatic nuts	3
37	*Alternaria* sp.	AN37	Asymptomatic nuts	3
38	*Alternaria* sp.	AN38	Asymptomatic nuts	3
39	*Alternaria* sp.	AN39	Asymptomatic nuts	3
40	*Alternaria* sp.	AN40	Asymptomatic nuts	3
41	*Alternaria* sp.	AN41	Asymptomatic nuts	3
42	*Alternaria* sp.	AN42	Asymptomatic nuts	3
43	*Alternaria* sp.	AN43	Asymptomatic nuts	3
44	*Alternaria* sp.	AN44	Asymptomatic nuts	3
45	*Alternaria* sp.	AN45	Asymptomatic nuts	3
46	*Alternaria* sp.	AN46	Asymptomatic nuts	3

47	*Alternaria* sp.	AN47	Asymptomatic nuts	3
48	*Alternaria* sp.	AN48	Asymptomatic nuts	3
49	*Alternaria* sp.	AN49	Asymptomatic nuts	3
50	*Alternaria* sp.	AN50	Asymptomatic nuts	4
51	*Alternaria* sp.	AN51	Asymptomatic nuts	4
52	*Alternaria* sp.	AN52	Asymptomatic nuts	4
53	*Alternaria* sp.	AN53	Asymptomatic nuts	4
54	*Alternaria* sp.	AN54	Asymptomatic nuts	4
55	*Alternaria* sp.	AN55	Asymptomatic nuts	4
56	*Alternaria* sp.	AN56	Asymptomatic nuts	4
57	*Alternaria* sp.	AN57	Asymptomatic nuts	4
58	*Alternaria* sp.	AN58	Asymptomatic nuts	4
59	*Alternaria* sp.	AN59	Asymptomatic nuts	4
60	*Alternaria* sp.	AN60	Asymptomatic nuts	4
61	*Alternaria* sp.	CAA101	Diseased nuts	1
62	*Alternaria* sp.	CAA102	Diseased nuts	2
63	*Alternaria* sp.	CAA103	Diseased nuts	4
64	*Alternaria* sp.	CAA104	Diseased nuts	3
65	*Alternaria* sp.	CAA105	Diseased nuts	1
66	*Alternaria* sp.	CAA230	ONFIT leaves	2
67	*Alternaria* sp.	CAA232	ONFIT leaves	1
68	*Alternaria* sp.	CAA233	ONFIT leaves	4
69	*Alternaria* sp.	CAA235	ONFIT leaves	1
70	*Alternaria* sp.	CAA238	ONFIT leaves	1
71	*Alternaria* sp.	CAA703	Asymptomatic flower buds	1
72	*Alternaria* sp.	CAA716	Asymptomatic flower buds	2
73	*Alternaria* sp.	CAA250	Asymptomatic flower buds	2
74	*Alternaria* sp.	CAA409	Asymptomatic flower buds	3
75	*Alternaria* sp.	CAA249	Asymptomatic flower buds	2
76	*Alternaria* sp.	TB1	ONFIT twigs	3
77	*Alternaria* sp.	TB2	ONFIT twigs	1
78	*Alternaria* sp.	TB3	ONFIT twigs	2
79	*Alternaria* sp.	TB4	ONFIT twigs	1
80	*Alternaria* sp.	TB5	ONFIT twigs	4

[a] - See Table 2.3 and Fig 2.1

CBS - Centraalbureau voor Schimmelcultures

AN - Author isolation

CAA - Culture collection at Plant Pathology Division, Dept of Plant Science, University of the Free State, South Africa

TB - Collection of Mr Terekengn, Division of Plant Pathology, Dept of Plant Science, University of the Free State, South Africa

Table 2.2 *Eco*RI and *Mse*I adapter, pre-amplification primer and selective amplification primer sequences used in AFLP analysis (Hong *et al*., 2006)

Enzyme	Type	Sequence (5'-3')
*Eco*RI	Adapter - F Adapter - R	CTCGTAGACTGCGTACC AATTGGTACGCAGTCTAC
*Mse*I	Adapter - F Adapter - R	GACGATGAGTCCTGAG TACTCAGGACTCAT
*Eco*RI	Pre-amplification primer	GACTGCGTACCAATTC
*Mse*I	Pre-amplification primer	GATGAGTCCTGAGTAA
*Eco*RI	Selective primer	GACTGCGTACCAATTCNN NN = AC; AG; TT
*Mse*I	Selective primer	GATGAGTCCTGAGTAANN NN = AG; AT; TT

Table 2.3. Morphological observations of *Alternaria* isolates grown on acidified weak potato dextrose agar (AWPDA) at 25°C.

Morpho-type	Top colour	Top pattern	Bottom colour	Bottom pattern	Agar discolouration	Spore arrangement
1	Dark green	Faint to strong rings, dark to pale green	Green and salmon	Rings	None	Non-branched
2	Yellow to pale green	Smooth to faint rings	Salmon	Smooth to faint rings	None	Very few branched
3	Light to pale green	Smooth to faint rings	Orange	Smooth to faint rings	Orange	Branched
4	Beige, pale- and dark green	Flames*	Beige, green/grey	Flames	None	Branched

*Flames = wedge shaped areas, raised and often darker than the surrounding growth

Figure 2.1 Morphological differences between *Alternaria* isolates recovered from asymptomatic pistachio leaves on AWPDA at 25° C

1 - Morphotype group one
2 - Morphotype group two
3 - Morphotype group three
4 - Morphotype group four
a - top view
b - bottom view

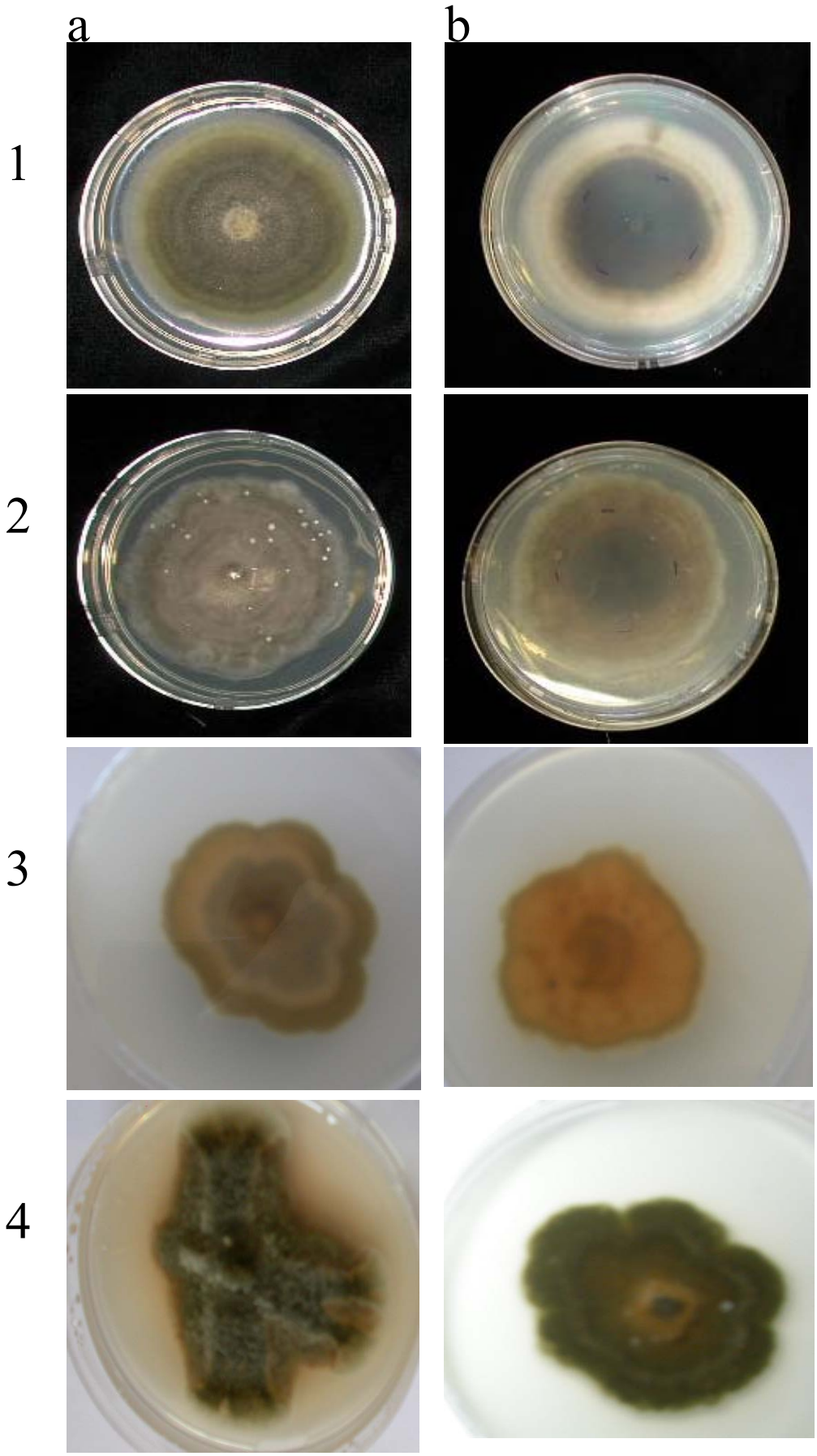

a
b
1
2
3
4

Figure 2.2 Branching pattern of conidial chains of morphotypes 1 and 2 (a) and morphotypes 3 and 4 (b) were similar to those portrayed in this figure.

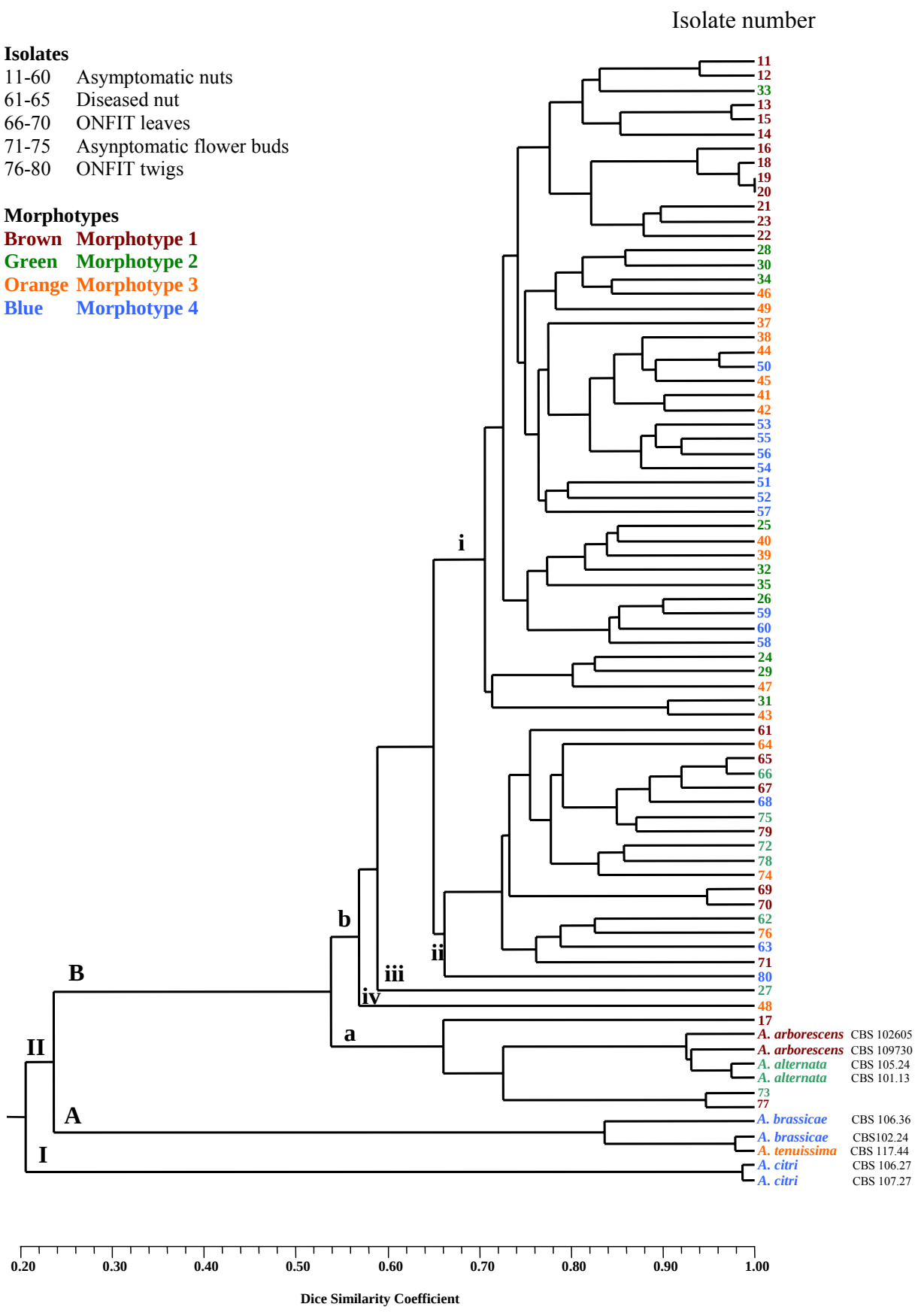
a

(www.environmentalpublichealth.org/mold.htm)

b

(Roberts, 2005).

Figure 2.3 Dendrogram of *Alternaria* isolates from AFLP data, showing the origin of isolates and morphotypes. See Table 2.1 for the identification of isolates


Isolate number
Isolates
11-60 Asymptomatic nuts
61-65 Diseased nut
66-70 ONFIT leaves
71-75 Asynptomatic flower buds
76-80 ONFIT twigs
Morphotypes
Brown Morphotype 1
Green Morphotype 2
Orange Morphotype 3
Blue Morphotype 4
11
12
33
13
15
14
16
18
19
20
21
23
22
28
30
34
46
49
37
38
44
50
45
41
42
53
55
56
54
51
52
57
25
40
39
32
35
26
59
60
58
24
29
47
31
43
61
64
65
66
67
68
75
79
72
78
74
69
70
62
76
63
71
80
27
48
17
A. arborescens CBS 102605
A. arborescens CBS 109730
A. alternata CBS 105.24
A. alternata CBS 101.13
73
77
A. brassicae CBS 106.36
A. brassicae CBS102.24
A. tenuissima CBS 117.44
A. citri CBS 106.27
A. citri CBS 107.27
i
ii
iii
iv
a
b
B
II
A
I
0.20
0.30
0.40
0.50
0.60
0.70
0.80
0.90
1.00
Dice Similarity Coefficient

CHAPTER 3

Effect of Fungicides on *Alternaria* Infection of Pistachio Orchards in South Africa

Introduction

Alternaria spp. have the potential to cause major losses in pistachio orchards in South Africa. Effective control of this pathogen is therefore a prerequisite for the successful cultivation of the crop. These fungi have been recovered from numerous types of pistachio tissue including healthy leaf and flower buds, young flowers, diseased twigs and healthy leaves (Michailides, Morgan & Doster, 1995; Belisario *et al.,* 1999; Ash, & Lanoiselet, 2001; Michailides & Teviotdale, 2003; Belisario, *et al.,* 2004; Michailides, & Morgan, 2004; Wright *et al.,* 2004, Hong *et al.,* 2006).

Alternaria spp. spend part of their life cycle within asymptomatic plant tissue although they are not true endophytes (Kim *et al*., 2001; Christensen. & Bennett, 2005). These fungi can therefore be said to be in a state of quiescence or latency, in the colonized tissue (Chen, Brown & Cleveland, 2004; Evans, Michailides & Morgan, 1999). Pryor & Michailides (2002) reported that *Alternaria* spp. are consistently isolated from surface sterilised asymptomatic flower and leaf buds of pistachio. Devi et al., (1995) showed the presence of the fungus within plant tissue both through stimulation of growth in the laboratory by plating asymptomatic tissue under conditions favourable for fungal growth and by electron microscopy. *Alternaria* spp. can survive within seemingly healthy pistachio tissue until environmental conditions change or certain physiological changes take place within the plant itself, enabling the fungus to become pathogenic (Schardl & Phillips, 1997; Michailides *et al*., 1995). Infection therefore, takes place long before symptoms develop, an important fact if the use of chemical fungicides to combat the pathogen are to be considered.

Chemical control of *Alternaria* spp. on woody plants is well documented (Michailides & Morgan, 1993; Michailides *et al.,* 1995; Everett, & Neilson, 1996; Pryor & Michailides, 2002). In California Alternaria late blight on pistachio was successfully controlled with cupric hydroxide (Kocide 101®) and cuprous oxide (Nordox®) (Michailides & Morgan, 1993). Michailides & Morgan (1993) reported a reduction in fruit staining caused by *Alternaria* spp. with benomyl. Cupric hydroxide and benomyl were registered for use against Alternaria late blight on pistachio in the USA (Michailides *et al.,* 1995). Michailides, Morgan & Felts, (1999) reported excellent control of Alternaria late blight on pistachio with the strobilurin fungicide azoxystobin (Abound®). Ma & Felts, Michailides (2003a) and Ma & Michailides

(2004a; 2004b) subsequently reported that certain *Alternaria* isolates had become resistant to this fungicide. Azoxystrobin (Abound®), iprodione (Rovral®), and tebuconazole (Elite®) were found to be effective against *Alternaria* spp. on pistachio in California by Solel, Oren & Kimchi (1997) and later by Pryor & Michailides (2002). Michailides & Teviotdale (2003) reported increasing resistance of *Alternaria* spp. on pistachio to the strobilurin group of fungicides. These authors found the best control of Alternaria diseases with boscalid + pyraclostrobin (Pristine®), azoxystrobin (Abound®), pyraclostrobin (Cabrio®), trifloxystrobin (Flint®) and cyprodinil + fludioxonil (Switch®), used in a rotation program.

Since 2001 the United States Department of Agriculture has registered 143 fungicides for use on pistachio (CERIS, 2007). No fungicide is currently registered for use against *Alternaria* spp. on pistachio in South Africa (Nel, Krause & Khelawanlal, 2003).

Due to the quiescent nature of the opportunistic *Alternaria* spp. that cause disease on pistachio, the time of fungicide application is as critical as the formulation and concentration of the chemicals (Ansari, Khan & Muheet, 1990; Bhatia, Roberts & Timmer, 2003; Survilienė & Dambrauskienė, 2006). The objectives of this study were a) to determine the efficacy of individual fungicides against *Alternaria* spp. isolates *in vitro* and the effect of combination fungicide regimes *in vivo* on the Sirora, Ariyeh and Shufra cultivars cultivated in South Africa and b) to determine the influence of the time of application of the fungicides on the incidence of *Alternaria* spp.

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Materials and Methods

In vitro *reaction of isolates to fungicides*:

Eleven fungicides (Table 3.1) were tested for their activity against *Alternaria* spp. *in vitro*. *Alternaria* isolates were obtained during ONFIT experiments (material is frozen overnight, then incubated at room temperature, and any fungal growth isolated) from nuts, displaying similar colony morphology and branching of conidial chains. Seven monoconidial isolates were chosen from the Shufra cultivar (CAA# 121, 122, 123, 125, 126, 127 and 128), and three from Ariyeh (CAA# 108, 115 and 155). The number identifies the isolate as belonging to the culture collection of *Alternaria* spp. kept at the Plant Pathology Division of the Department of Plant Science at the University of the Free State. After autoclaved potato dextrose agar (PDA) (Biolab) was cooled to approximately 48°C, aqueous stock solutions (Table 3.1) of the

various fungicides were added to the agar. Agar plugs with a diameter of 5 mm of the *Alternaria* isolates were plated onto the amended PDA, with standard PDA as control. Four plates of each fungicide and the respective controls were used and the experiment was repeated four times. Plates were incubated at room temperature (ca. 25°C) for 11 days whereafter the diameters of the colonies were measured in two directions and averaged. Data were expressed as the percentage inhibition compared to the control treatment. Analysis of variance was carried out using the NCSS statistical program (Hintze, 2001), and means were separated using the Tukey-Kramer Multiple-Comparison Test (Steel & Torry, 1980).

Fungicide dilutions.

The four best performing fungicides from the above experiment (Table 3.2) were diluted as indicated and tested against the three most resistant *Alternaria* isolates (CAA# 126, 127 and 128). The above procedure was followed and the experiment was repeated four times.

Effect of fungicides in orchards.

A fungicide application programme using seven of the fungicides tested *in vitro* (Table 3.3), was implemented in the Green Valley Nuts pistachio orchards. Five blocks of each of three cultivars, Sirora, Ariyeh and Shufra, each containing three trees, were treated with the fungicide combinations. The trees ranged in age from five to eight years and were commercially productive.

Asymptomatic fruit was sampled on the 6th of February 2006 and at harvest on the 6th of March 2006. Ten nuts from each tree were surface sterilized with 75% (v\v) ethanol for 30 s. The stylar end was excised, sterilized with NaOCl for 3 minutes, and plated on acidified weak potato dextrose agar (AWPDA) (Andrews, 1996; Bilgrami & Ghaffer, 1997; Hong & Pryor, 2004). Growth of *Alternaria* spp. from the tissue sample was microscopically confirmed. Data were analysed as the isolation frequency of *Alternari*a spp., expressed as a percentage of the samples.

Data were averaged per tree and a two factorial design statistical analysis conducted, with main factors cultivar and fungicide treatment. Analysis of variance was carried out using the NCSS statistical program (Hintze, 2001), and means were separated using the Tukey-Kramer Multiple-Comparison Test (Steel & Torry 1980).

Orchard observations

During harvest the percentage of nuts with discolouration at the apical end was quantified using three clusters from each of the fungicide treated trees of the Sirora and Shufra cultivars. The amount of discoloured nuts from each of the clusters in each of the five blocks were recorded and the percentage incidence of discolouration was determined. Isolations were made from the discoloured areas of randomly selected nuts after surface sterilization as above.

Results

In vitro *reaction of isolates to fungicides*

ANOVA indicated that the efficacy of the fungicides *in vitro* differed significantly, as did the response of the isolates to fungicides. A significant ($p \leq 0.05$) isolate x fungicide interaction was recorded. Pyroclostrobin, tebuconasole + carbendazim, difenoconazole and kresoxim-methyl consistently inhibited linear growth of all isolates by more than 75% (Table 3.4). Boscalid + pyroclostrobin, boscalid and cyprodinil + fludioxonil inhibited linear growth by between 55% and 70% and azoxystrobin, kresoxim methyl + boscalid, benomyl and trifloxistrobin consistently inhibited the linear growth of all isolates by less than 50%. The most resistant isolate was inhibited by an average of 52.71% and the most sensitive by 64.42%

Fungicide dilutions

The isolate x fungicide dilution interaction was significant ($p \leq 0.05$), indicating that the three isolates reacted differently to the diluted fungicides used in this study. Cyprodinil + fludioxonil totally inhibited fungal growth even at 25% of the recommended rate (Table 3.5). Tebuconasole + carbendazim totally inhibited growth all isolates at 75% of the recommend rate, but not at 50% and 25%. Boscalid-pyraclostrobin showed more than 80% inhibition at all rates over all isolates. Difenoconazole provided more than 80% inhibition in the growth of the isolates diluted to up to 50% of the recommend rate.

Effect of fungicides in orchards

During mid-season sampling of nuts in sprayed orchards, treatments 13 and 14 consistently showed a lower incidence of *Alternaria* spp. than the control treatment 16 (Tables 3.3 & 3.6).

Treatments 12, 17, 18 and 19 showed a higher incidence of *Alternaria* spp. than the control treatment 16. Treatments 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 15 did not differ significantly ($p \leq 0.05$) from treatments 12, 13, 14, 16, 17, 18 and 19.

An increase of between 12% and 20% in the isolation frequency of *Alternaria* spp. in fruit tissue was observed between samples examined at midseason and at harvest (Tables 3.3, 3.7 & Fig. 3.1). Treatments 13 and 14 had the lowest incidence of infection with treatments 5, 6, 10, 15, 18 and 19 having higher isolation frequency than the control. The other treatments did not differ significantly at the 95% level from treatments 5, 6, 10, 15, 18 and 19.

Orchard observations

Observations in the orchards from the treated Sirora cultivar showed that treatments 9, 12 and 15 produced significantly less discolouration than treatments 1, 8, 7, 13, 14, 16, 17 and 19 (Fig. 3.2). The worst three treatments were 13, 14 and 16 with discolouration on the nuts being significantly higher than that obtained with treatments 2, 3, 4, 5, 6, 9, 11, 12, 15 and 18. ANOVA indicated that these treatments did not significantly differ at the 95% confidence lever. With the Shufra cultivar no significant differences in visual discolouration of the nuts over all the treatments were observed. Sirora had a significantly ($p \leq 0.05$) higher incidence of observed discolouration than Shufra. Between 25% and 50% of all observed Sirora nuts showed discolouration, while less than 5% of Shufra nuts showed discolouration. Isolations indicated that all discoloured areas were colonized by *Alternaria* spp.

Discussion

Boscalid-pyraclostrobin, tebuconasole-carbendazim, difenoconazole and cyprodinil-fludioxonil provided more than 75% inhibition *in vitro* over all the isolates tested. These findings contrast with those of other authors who found both tebuconasole and difenoconazole to be ineffective against *Alternaria* spp. on pistachio *in vitro* (Solel *et al.,* 1997). Azoxystrobin, trifloxistrobin, pyraclostrobin and benomyl were consistently less effective in their inhibition of *Alternaria* spp. *in vitro* which is consistent with findings by Michailides *et al*. (2005) and Adaskaveg *et al*. (2007). Michailides *et al*. (2005) found resistance of *Alternaria* spp. against azoxystrobin (Amistar®) where the fungus was previously exposed to the fungicide. Michailides & Morgan (2004) also found benomyl (Benlate®) to be totally ineffective against *Alternaria* spp. isolated from pistachio nuts. Adaskaveg *et al*. (2007)

reported field resistance to strobilurin containing fungicides including azoxystrobin®, trifloxistrobin® and pyraclostrobin®. The differences between the reaction of the different isolates in this and other studies are important as they indicate the possible build-up of resistance in the fungus population and this must be taken into account during the development of a rotational fungicide application program (Michailides *et al*., 2005).

Cyprodinil + fludioxonil, difenoconazole, tebuconasole + carbendazim and boscalid + pyraclostrobin provided significant inhibition at lower than the recommended rates *in vitro*. These dilutions need to be tested *in vivo* to confirm their efficacy. Furthermore, the potential cost saving provided by reduced application rates, has to be balanced against the probability of fungicide resistance associated with a lower concentration of active ingredients (Watkins & Klemme, 1948; Wiedman & Appleby, 1972; Kenyon, Dixon & Helfer, 1997; Calabrese & Baldwin, 1998). Kenyon *et al*. (1997) found increased sporulation of an *Erysiphe* sp. when treated with three fungicides (propiconazole, pyrazophos and triadimenol) with concentrations less than 1 ppm. Calabrese and Baldwin (1998) made a comprehensive study of 4 000 articles dealing with hormesis and found it to be a common biological observation. Thus further studies are essential to quantify all aspects of the pathogen responses to reduced application rates in South African pistachio orchards.

A number of treatments showed an isolation frequency of *Alternaria* spp. greater than the control treatment (Table 3.6 & 3.7) *in vivo*. This is possibly due to the effect of hormesis with growth being stimulated by the fungicides that were not lethal to it (Watkins & Klemme, 1948; Weidman & Appleby, 1972; Kenyon *et al*., 1997; Calabrese & Baldwin, 1998). This is also consistent with findings by Solel *et al*. (1997) that showed that captan and folpan-prochloraz stimulated Alternaria brown spot of *Minneola tangelo,* caused by *Alternaria alternata* pathovar *citri* at reduced rates.

The efficacy of treatments 13 and 14 in the pistachio orchards are probably due to the inclusion of all the fungicides that proved effective against *Alternaria* isolates *in vitro*. The efficacy of sequential use of fungicides from different chemical groups is to be expected and is consistent with studies conducted by Solel *et al*. (1997) and Mathivanan & Prabavathy (2006) who found a combination of carbendazim and mancozeb to be more efficacious in controlling *A. helianthi* on sunflowers, than either of the fungicides independently. Doubling

the dosage used in treatment 13 in treatment 14 did not shown a significantly higher ($p \leq 0.05$) inhibition of *Alternaria* spp. and is therefore not cost effective. Rotation of fungicides and avoiding single chemicals or groups of chemicals play an important role in the prevention of resistance to chemical fungicides (Ma *et al*., 2003b).

The choice of cultivar and rootstock for a specific geographical area is vital for profitable production of pistachio (Chao, Parfit & Michailides, 2001; Beede & Ferguson, 2002; Holtz, 2002; Epstein *et al*. 2004; Ferguson, Polito & Kallsen, 2005). *Alternaria* spp. are a large constraint to pistachio production (Doster & Michailides, 1999), and it is therefore essential that a cultivar which exhibits resistance to infection while still producing high levels of good quality nuts is used.

Visual observation of nut discolouration on the Sirora cultivar indicated that the best fungicide treatments were 9, 12 and 15, with 13, 14 and 16 (control) being the worst. This is in direct contrast to laboratory results where treatments 13 and 14 had the lowest incidence of quiescent opportunistic *Alternaria* spp, and where the control gave lower results than treatments 12, 17, 18 and 19. Averaged over all treatments, 38% of the harvested nuts were discoloured, while 86% of harvested non-discoloured (asymptomatic) nuts were found to contain latent *Alternaria* spp. In the Shufra cultivar no significant differences in discolouration occurred over all the treatments. Discolouration varied from a maximum of 5% to as little as 1.6% with an average of 2.3% which is in sharp contrast to the 85% of non-discoloured nuts from which quiescent *Alternaria* spp. were isolated. The discrepancy between the incidence of quiescent *Alternaria* spp and observed discolouration caused by *Alternaria* spp. may be explained in terms of microclimate or resistance of individual trees which extends the latency of the quiescent *Alternaria* spp. (Prusky, 1996; Evans *et al*., 1999; Pryor & Michailides, 2002). Another explanation could be that the isolated organism is not pathogenic, such as *Alternaria infectoria* which does not cause disease on pistachio, but which is often isolated from pistachio tissue (Pryor & Michailides, 2002).

As discoloured nuts are seen as an indication of infection by *Alternaria* spp. and are discarded at harvest (Doster & Michailides, 1999), this raises the issue as to whether the *Alternaria* spp. isolated from asymptomatic nuts lead to disease at harvest or only under post-harvest conditions (Prusky, 1996). Evans *et al*. (1999) also considers staining an indication of

infection early in the growing season which stays latent until just before or after harvest. Doster & Michailides (1999) warns that seemingly healthy nuts which are quiescently infected could be processed and marketed while containing toxins formed by *Alternaria* spp. A range of host specific toxins, used to facilitate infection by these fungi, have been identified. Tentoxin, tenuazonic acid, alternariols, altertoxin, altenuene, alternariol monomethyl ether, AK toxin and altersetin have been isolated from a variety of hosts, and have been proven to be cytotoxic to mammals (Yekeler *et al*., 2001; Andersen, Kroger, & Roberts, 2002, Logrieco *et al*., 2003). Consumption of *Alternaria alternata* contaminated food has been implicated in elevated levels of oesophageal cancer (Schrader *et al*., 2001).

Results from the previous chapter indicate that 95% of the *Alternaria* spp. isolated from asymptomatic nuts from pistachio orchards in South Africa belong to the alternata/arborescens group. It can be postulated that the same would hold true of the *Alternaria* spp. isolated during the fungicide and canopy wetting experiments. During the canopy wetting experiments (Chapter 4), asymptomatic flower buds which had not been treated (control) exhibited between 25% and 50% infection by quiescent *Alternaria* spp. Asymptomatic mature nuts harvested from the control treatment in the fungicide experiments showed an infection rate of more than 80% (Table 3.7). It has been postulated that opportunistic *Alternaria* spp. infect the flower buds and only become pathogenic later in the growth of the nuts (Ngugi & Scherm, 2006). It is therefore critical that the orchards be treated with the most effective fungicides on a rotational basis from before flower bud formation, right up to before harvest (Doster & Michailides, 1999; Brazauskiené & Petraitiené, 2004; Meena et al., 2004; Ferguson *et al*., 2005).

The use of chemical fungicides is still the fastest and most efficacious way of controlling fungal diseases. This study emphasised the importance of the factors involved in the successful use of chemical fungicides. In order to achieve successful control it is essential to use the correct active ingredient at the right concentration, applied at the correct time. Use of different families of chemicals in a rotational and/or repetitive programme, will prevent fungi from developing resistance to a given active ingredient (Doster & Michailides, 1999).

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Table 3.1 Fungicides used during *in vitro* testing of *Alternaria* isolates

Trade name	Company	Active ingredient	Concentration of active ingredient (a.i.) in formulation	Concentration (ai) used in experiment	Mode of action
Amistar®	Syngenta	azoxystrobin*	250 g kg^{-1}	200 µg ml^{-1}	Systemic and contact
Bellis®	BASF	boscalid + pyraclostrobin	252 g kg^{-1}+ 128 g kg^{-1}	125 µg ml^{-1}	Systemic and contact
Cabrio®	BASF	pyraclostrobin	200 g kg^{-1}	100 µg ml^{-1}	Systemic and contact
Canthus®	BASF	boscalid*	500 g kg^{-1}	400 µg ml^{-1}	Systemic
Colliss®	BASF	kresoxim methyl + boscalid	100 g ℓ^{-1}+ 200 g ℓ^{-1}	30 µl ml^{-1}	Systemic and contact
Folicur®	Bayer	tebuconasole + carbendazim	167 g ℓ^{-1}+ 167 g ℓ^{-1}	187.5 µg ml^{-1}	Systemic
Switch®	Syngenta	cyprodinil + fludioxonil*	750 g kg^{-1} + 250 g kg^{-1}	200 µg ml^{-1}	Systemic and contact
Score®	Syngenta	difenoconazole	250 g ℓ^{-1}	150 µg ml^{-1}	Systemic
Benlate®	Dupont	benomyl*	500 g kg^{-1}	1000 µg ml^{-1}	Systemic
Flint®	Bayer	trifloxistrobin	500 g kg^{-1}	50 µg ml^{-1}	Systemic and contact
Stroby®	BASF	kresoxim-methyl	500 g ℓ^{-1}	75 µg ml^{-1}	Systemic and contact

* Registered for use on pistachio in the USA

Table 3.2. The four most effective fungicides used at percentages of the recommended rate applied in 100 l water

Trade Name	Active ingredient	Recommended rate	75% of recommended rate	50% of recommended rate	25% of recommended rate
Switch®	cyprodinil + fludioxonil	15 g + 5 g	11.25 g + 3.75 g	7.5 g + 2.5 g	3.75 g + 1.25 g
Bellis®	boscalid + pyraclostrobin	3.15 g + 1.6 g	2.36 g + 1.2 g	1.575 g + 0.8 g	0.79 g + 0.4 g
Score®	difenoconazole	15 g	11.25 g	7.5 g	3.75 g
Folicur®	tebuconasole + carbendazim	9.375 g + 9.375 g	7.03 g + 7.03 g	4.69 g + 4.69 g	2.35 g + 2.35 g

Table 3.3 Spray program used in pistachio orchards by Green Valley Nuts.

Date	Treatment									
	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10
19/09/2005										
26/09/2005	Cyprondinil + fludioxonil®		Cyprondinil + fludioxonil®		Cyprondinil + fludioxonil®		Cyprondinil + fludioxonil®		Cyprondinil + fludioxonil®	
10/03/2005	Cyprondinil + fludioxonil®		Cyprondinil + fludioxonil®		Cyprondinil + fludioxonil®		Cyprondinil + fludioxonil®		Cyprondinil + fludioxonil®	
10/10/2005										
17/10/2005										
24/10/2005										
31/10/2005										
11/07/2005										
21/11/2005	Tebuconasole + carbendazin®	Tebuconasole + carbendazin®								
28/11/2005										
05/12/2005	Tebuconasole + carbendazin®	Tebuconasole + carbendazin®	Tebuconasole + carbendazin®	Tebuconasole + carbendazin®	Tebuconasole + carbendazin®	Tebuconasole + carbendazin®				
12/12/2005										
19/12/2005	Tebuconasole + carbendazin®	Tebuconasole + carbendazin®	Boscalid + pyraclostrobin®	Boscalid + pyraclostrobin®	Tebuconasole + carbendazin®	Tebuconasole + carbendazin®	Azoxystrobin®	Azoxystrobin®	Kresoxim methyl + boscalid®	Kresoxim methyl + boscalid®
26/12/2005										
02/01/2006	Boscalid + pyraclostrobin®	Boscalid + pyraclostrobin®	Boscalid + pyraclostrobin®	Boscalid + pyraclostrobin®	Tebuconasole + carbendazin®	Tebuconasole + carbendazin®	Azoxystrobin®	Azoxystrobin®	Kresoxim methyl + boscalid®	Kresoxim methyl + boscalid®
09/01/2006										
16/01/2006	Boscalid + pyraclostrobin®	Boscalid + pyraclostrobin®	Tebuconasole + carbendazin®	Tebuconasole + carbendazin®	Tebuconasole + carbendazin®	Tebuconasole + carbendazin®	Azoxystrobin®	Azoxystrobin®	Kresoxim methyl + boscalid®	Kresoxim methyl + boscalid®
23/01/2006										
30/01/2006	Boscalid + pyraclostrobin®	Boscalid + pyraclostrobin®								

Date	Treatment								
	#11	#12	#13	#14	#15	#16	#17	#18	#19
19/09/2005									
26/09/2005	Cyprondinil + fludioxonil®		Cyprondinil + fludioxonil®		Cyprondinil + fludioxonil®		Cyprondinil + fludioxonil®		
10/03/2005	Cyprondinil + fludioxonil®		Cyprondinil + fludioxonil®		Cyprondinil + fludioxonil®		Cyprondinil + fludioxonil®		
10/10/2005									
17/10/2005									
24/10/2005									
31/10/2005									
11/07/2005			Cyprondinil + fludioxonil®	Cyprondinil + fludioxonil®					
21/11/2005			Azoxystrobin®	Azoxystrobin®					
28/11/2005									
05/12/2005			Pyraclostrobin®	Pyraclostrobin®					
12/12/2005									
19/12/2005	Cyprondinil + fludioxonil®	Cyprondinil + fludioxonil®	Boscalid®	Boscalid®			Tebuconasole + carbendazin®	Tebuconasole + carbendazin®	Tebuconasole + carbendazin® + Sporekill®
26/12/2005							Tebuconasole + carbendazin®	Tebuconasole + carbendazin®	Tebuconasole + carbendazin® + Sporekill®
02/01/2006	Cyprondinil + fludioxonil®	Cyprondinil + fludioxonil®	Kresoxim methyl + boscalid®	Kresoxim methyl + boscalid®			Boscalid + pyraclostrobin®	Boscalid + pyraclostrobin®	Boscalid + pyraclostrobin® + Sporekill®
09/01/2006							Tebuconasole + carbendazin®	Tebuconasole + carbendazin®	Tebuconasole + carbendazin® + Sporekill®
16/01/2006	Cyprondinil + fludioxonil®	Cyprondinil + fludioxonil®	Boscalid + pyraclostrobin®	Boscalid + pyraclostrobin®					
23/01/2006									
30/01/2006			Tebuconasole + carbendazin®	Tebuconasole + carbendazin®					

Table 3.4. *In vitro* effect of fungicides on colony diameter of *Alternaria* isolates from pistachio as percentage of control, as affected by fungicides.

Fungicide	Isolate										
	1	2	3	4	5	6	7	8	9	10	Mean
azoxystrobin	19.51	22.89	29.41	14.41	14.52	21.24	36.74	28.52	26.14	29.54	24.29
boscalid + pyraclostrobin	55.07	48.98	59.38	52.53	58.52	56.88	63.93	59.63	58.41	54.74	56.81
pyraclostrobin	92.52	91.51	92.25	91.45	90.70	92.84	93.19	92.60	91.85	92.73	92.16
boscalid	72.85	80.10	61.68	67.64	75.74	66.63	74.88	54.33	71.43	73.45	69.87
kresoxim methyl + boscalid	18.48	32.44	42.12	19.86	16.51	19.85	40.22	25.97	24.46	35.19	27.51
tebuconasole + carbendazim	84.27	89.07	78.69	77.45	83.08	81.76	83.21	72.46	85.41	63.61	79.89
cyprodinil + fludioxonil	66.16	75.95	59.06	51.01	70.67	65.91	72.02	50.46	69.26	66.48	64.70
difenoconazole	92.52	91.51	92.25	91.45	90.70	92.84	93.19	91.11	91.85	92.73	92.02
benomyl	19.07	32.41	26.57	18.89	12.56	36.20	41.42	21.93	28.60	31.44	27.21
trifloxistrobin	11.57	14.04	31.82	7.34	19.44	14.90	21.46	17.61	30.72	28.17	19.71
kresoxim-methyl	87.32	91.51	89.29	87.85	88.36	79.79	88.38	86.96	89.46	89.52	87.84
Mean	56.30	60.94	60.23	52.71	56.44	57.16	64.42	54.96	60.69	59.78	
$LSD_{T(0.05)}$	Isolate = 4.13, Fungicide = 4.41, I x F = 5.01										

Table 3.5. *In vitro* effect of various fungicide concentrations on colony diameter of *Alternaria* isolates from pistachio nuts as compaired to the control.

Fungicide concentration a percentage of recommended rate	Isolate			Mean
	1	2	3	
cyprodinil + fludioxonil 100%	100.00	100.00	100.00	100.00
cyprodinil + fludioxonil 75%	100.00	100.00	100.00	100.00
cyprodinil + fludioxonil 50%	100.00	100.00	100.00	100.00
cyprodinil + fludioxonil 25%	100.00	100.00	100.00	100.00
boscalid + pyraclostrobin 100%	89.70	100.00	89.29	93.00
boscalid + pyraclostrobin 75%	88.03	93.68	88.18	89.96
boscalid + pyraclostrobin 50%	84.83	89.88	86.95	87.22
boscalid + pyraclostrobin 25%	89.36	85.17	86.46	87.00
difenoconazole 100%	85.45	84.84	92.96	87.75
difenoconazole 75%	87.57	83.41	90.83	87.27
difenoconazole 50%	86.89	80.43	90.66	85.99
difenoconazole 25%	78.31	86.58	84.16	83.02
tebuconasole + carbendazim 100%	100.00	100.00	100.00	100.00
tebuconasole + carbendazim 75%	100.00	100.00	100.00	100.00
tebuconasole + carbendazim 50%	72.56	74.60	86.04	77.73
tebuconasole + carbendazim 25%	63.49	69.12	69.54	67.39
Mean	89.14	90.48	91.57	
LSD	Isolate = 0.79, Fungicide = 0.92, I x F x C = 4.97			

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Table 3.6 Effect of cultivar and fungicides on the percentage of isolation frequency of *Alternaria* isolates from mid-season pistachio nuts. (For treatments see Table 3.3)

Fungicide treatment	Cultivar			Mean
	Sirora	Ariyeh	Shufra	
1	53.33	64.67	46.67	54.89
2	55.33	73.33	62.00	63.56
3	56.67	80.00	57.33	64.67
4	63.33	80.67	63.33	69.11
5	68.67	84.67	62.67	72.00
6	66.67	88.67	68.67	74.67
7	63.33	78.67	68.67	70.22
8	69.33	75.33	68.67	71.11
9	57.33	80.00	65.33	67.56
10	62.67	77.33	77.33	72.44
11	68.67	76.00	61.33	68.67
12	72.00	83.33	70.67	75.33
13	49.33	64.67	33.33	49.11
14	42.67	68.00	31.33	47.33
15	56.00	82.67	63.33	67.33
16	69.33	86.67	68.00	74.67
17	76.67	82.00	73.33	77.33
18	78.00	88.67	82.67	83.11
19	82.00	93.33	80.67	85.33
	67.93	68.70	69.96	
LSDT(0.05)	Treatment = 13.95. Cultivar = 3.65, T x Cv = NS			

85

Table 3.7 Isolation frequency (%) of *Alternaria* isolates from mature pistachio nuts as fungicide treatments by cultivar. (For treatments see Table 3.3)

Fungicide treatment	Cultivar			Mean
	Sirora	Ariyeh	Shufra	
1	82.67	65.33	81.33	76.44
2	76.00	76.00	70.67	74.22
3	74.67	77.33	84.00	78.67
4	76.00	76.00	89.33	80.44
5	96.00	89.33	93.33	92.89
6	94.67	88.00	88.00	90.22
7	86.67	78.67	92.00	85.78
8	92.00	77.33	89.33	86.22
9	85.33	84.00	86.67	85.33
10	89.33	88.00	94.67	90.67
11	85.33	73.33	85.33	81.33
12	84.00	58.67	70.00	70.89
13	74.67	68.00	74.67	72.44
14	74.67	58.67	70.67	68.00
15	90.67	70.67	86.67	82.67
16	89.33	90.67	84.00	88.00
17	81.33	90.67	89.33	87.11
18	97.33	88.00	90.67	92.00
19	97.33	82.67	93.33	91.11
Mean	77.96	85.68	84.95	
LSDT(0.05)	Treatment = 17.22. Cultivar = 4.51, T x Cv = NS			

Figure 3.1 Comparison of the isolation frequency of *Alternaria* isolates obtained at mid-season and harvest from asymptomatic nuts. (For treatments see Table 3.3)

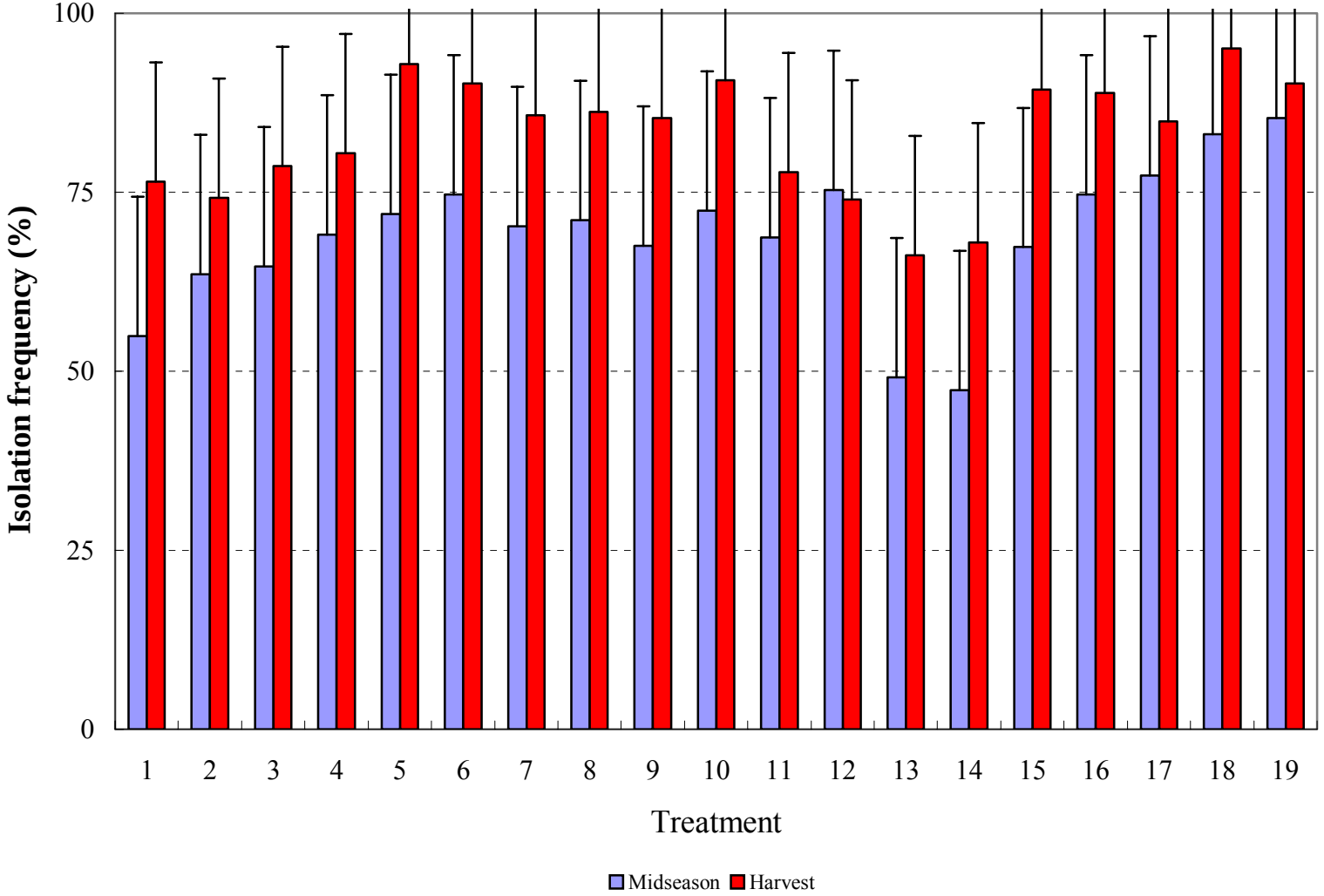
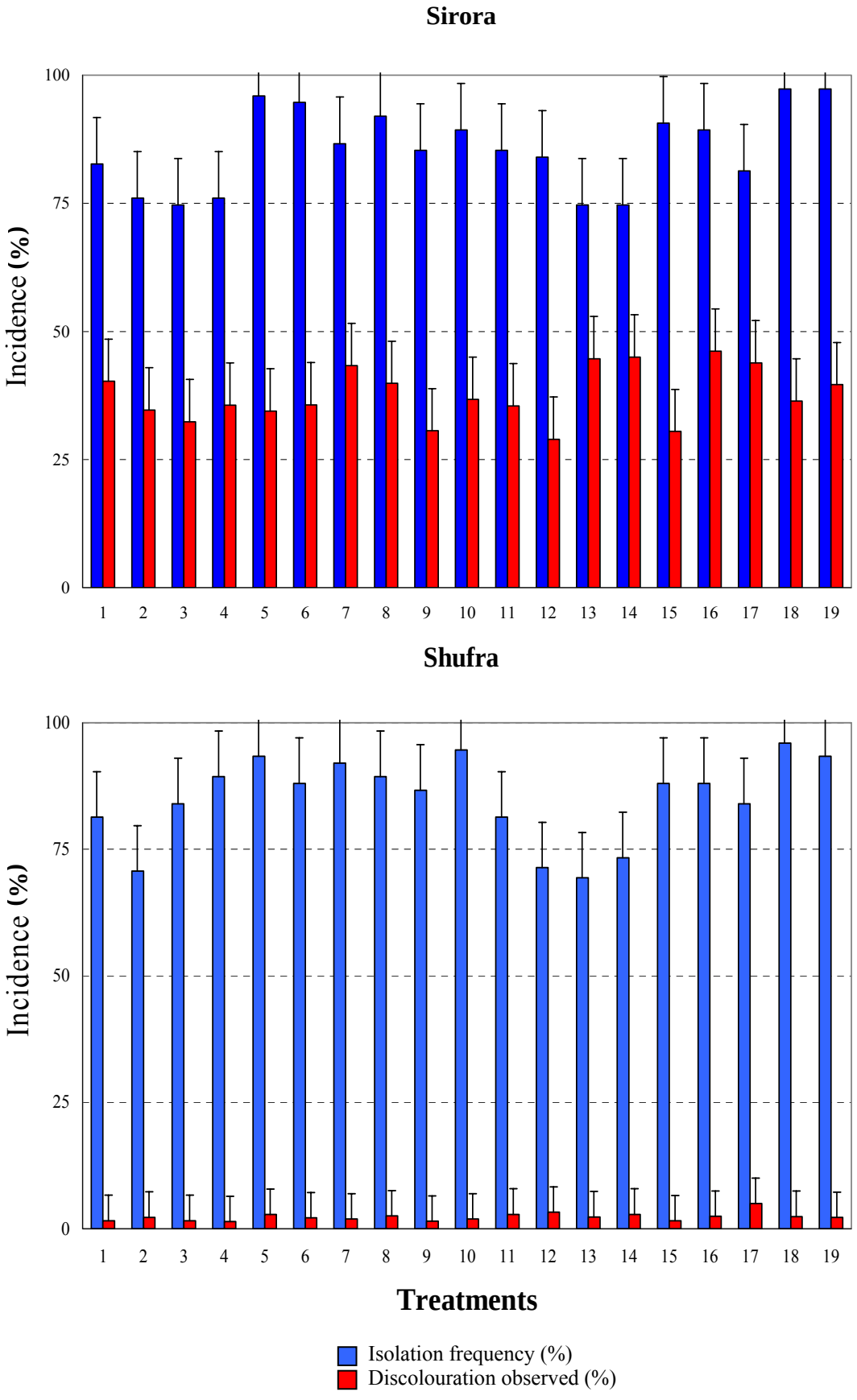

Isolation frequency (%)
100
75
50
25
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
Treatment
Midseason
Harvest

Figure 3.2 Comparison of the isolation frequency of *Alternaria* spp. in asymptomatic nuts and percentage discolouration observed on nuts as a percentage incidence within a cluster, at harvest on the Sirora and Shufra cultivars. (For treatments see Table 3.3)


Sirora
Incidence (%)
100
75
50
25
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Shufra
Incidence (%)
100
75
50
25
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
Treatments
Isolation frequency (%)
Discolouration observed (%)

CHAPTER 4

Effect of Canopy Wetting on the Occurrence of *Alternaria* Infection in Pistachio Orchards in South Africa

Introduction

Pistachios are emerging as an important alternative crop in South Africa. They are cultivated near Prieska in the northern Cape Province (29°, 40'S, 22°, 45'E). Three cultivars, Sirora, Ariyeh and Shufra are being cultivated on different rootstocks; Sirora and Ariyeh on *P. integgerima*, and Shufra on *P. terebinthus* (Muller, 2007). During the 2004/2005 season, blackening at the stylar end of developing pistachio fruits resulted in losses of mature fruits of up to 70% in certain blocks of 6- to 8-year-old trees, (Snyman, 2005). Twig dieback was also noticed in certain blocks where older leaves had become covered by spores of *Alternaria* spp. by the end of summer 2005.

Small-spored catenulate *Alternaria* spp. are common opportunistic pathogens in Italy where they reportedly cause brown apical necrosis (BAN) on English walnut (*Juglans regia*) and grey necrosis on hazelnut (*Corylus avellana*)(Belisario et al., 2002). They are also common in pistachio orchards in California where various symptoms on twigs, leaves and fruit have been reported (Michailides, Morgan & Doster, 1995). Previous studies where isolations were conducted from various types of asymptomatic pistachio tissue (e.g. flower and leaf buds, immature fruits and leaves) in addition to symptomatic fruits and twigs, revealed the presence of *Alternaria* spp. in most instances (present author, results unpublished). Quiescent infection of seeds and green fruits by *A. alternata* is common (Rotem, 1994) and is consistent with the isolation of *Alternaria* spp. from healthy pistachio tissue.

Like most hardwood trees, pistachios require a minimum number of chilling hours in order to break bud dormancy and stimulate fruit set (Arora, Rowlant & Tanino, 2003) and chilling requirements differ between cultivars of most fruit trees (Beede, 2005). The chilling requirement for pistachio varies between 500 and 1000 accumulated hours below 7.2°C (Küden *et al.,* 1995; Beede, 2005; Caruso, 2005) but this is not naturally achieved in the Prieska district where minimum temperatures in winter seldom drop below 10°C (SA Weather Bureau). In an attempt to circumvent the lack of optimal chilling hours, canopy wetting of selected mature trees was implemented during 2005/6 on an experimental basis using sprinkler irrigation which completely wet the trees (Snyman, 2005). This method was preferred to the use of bud-break oils because bud break oils

were found not to increase yield of the pistachio cultivars Sirora and Shufra (Muller, 2007). No reference to the use of sprinkler irrigation for bud break has been found in the literature, except for use on roses to improve yield and stem length (Plaut & Zieslin, 1977).

Introduction of free water into an orchard, whether rain, dew or aerial irrigation, increases the relative humidity which leads to an increase in the incidence of *Alternaria* spp. infection on the plants. The minimum wetting period (WP) for the establishment of various *Alternaria* spp. in host tissue ranges from 3 to 72 h and it is possible that weakly pathogenic species require a longer WP (Rotem, 1994). Similarly, a long WP may be required for infection of resistant organs or cultivars by more virulent species. In nature, *Alternaria* spp. can overcome the adverse effects of intermittent or interrupted WPs by halting germination in dry periods and resuming it during the next WP (Rotem, 1994).

High relative humidity coupled with aerial irrigation also stimulates the release and movement of high levels of potential inoculum from infected abscised leaves (Evans, Michailides & Morgan, 1998; Goldhamer, Michailides & Morgan, 2002; Bhatia, Roberts & Timmer, 2003; Michailides & Teviotdale, 2003). High humidity during non-wetting periods, coupled with temperatures between 23°C and 27°C also facilitates sporulation, germination and infection. Flood irrigation has been shown to significantly increase relative humidity in pistachio orchards in California, and has been directly implicated in causing higher levels of infection by *Alternaria* spp. (Goldhamer *et al.*, 2002), whereas subsurface irrigation reduced the incidence of leaf late blight four fold and fruit infection by 45% due to increased maximum and minimum temperatures and a reduction in the time of dew formation (Goldhamer, 1996) .

Given the possibility that canopy wetting and high RH can create microclimatic conditions that are ideal for quiescent infection of pistachio tissue, the method used for inducing chilling at Prieska, viz. sprinkler irrigation, can also be expected to facilitate the splash dispersal of spores and thereby the onset of infection (Rotem, 1994). The objective of the present study was therefore to firstly, investigate the effect of canopy wetting on the isolation frequency of quiescent infection of pistachio fruits by *Alternaria* spp. and

secondly, to determine whether cultivars differed in terms of their susceptibility to quiescent infection.

Materials and Methods

Layout of trial

Sprinklers were installed in the mid-canopy of individual 6- to 8-year-old trees in five different planting blocks and wetting was either applied during autumn (May/June) or spring (August/September), or during autumn and spring. According to the management wetting took place during the regular irrigation cycle and sufficient water was applied until water ran freely from the tree. Table 4.1 illustrates the temperatures in the orchard during these periods. One cultivar, namely Sirora received an additional treatment during late winter in August, as this cultivar did not react well to the other treatments in earlier studies. Twenty randomly chosen trees of each of the three cultivars, Sirora, Ariyeh and Shufra were selected and subjected to the respective treatments, with the same number of untreated trees serving as control treatment. The soil type over the whole of the orchard was uniform, except for minor differences in clay percentage. Each tree was equidistant from the next.

Isolation from asymptomatic leaf and flower buds

Twigs containing leaf buds as well as twigs containing flower buds were collected from treated and untreated trees before bud break. Eight leaf buds and eight flower buds were harvested per tree. Twig sections were sprayed with 75% (v/v) ethanol and left for 30 s (Andrews, 1996). Each bud was cut off as close to the woody tissue as possible using a heat sterilized scalpel and placed in plastic sieves in a container with 1% (v/v) NaOCl for 3 minutes and subsequently rinsed in sterile water. Each leaf and flower bud was aseptically cut in half longitudinally using a heat sterilized scalpel. The pieces were placed on acidified weak potato dextrose agar (AWPDA) and incubated at 25°C for five to seven days (Hong & Pryor, 2004). Colonies were examined under a dissection microscope for identification of *Alternaria*.

Isolation from fruits

Eight asymptomatic fruits were collected from wetted and control trees of each cultivar during January and at harvest in March, and surface sterilized as previously described for buds. The stylar end of each nut was cut off with a sterilized scalped following surface sterilization, placed on AWPDA and incubated at 25°C until fungal colonies had emerged. Colonies were examined under a dissection microscope for identification of *Alternaria*.

Analysis of data

Data were analyed as a three factor factorial design incorporating cultivar, time of wetting treatment and harvest time. In this case the August wetting treatment for Sirora was omitted from the analysis. Data for each cultivar were then also analysed seperately in order to determine if wetting treatments plaeyd a significant role in infections of each cultivar on their own. Statistical analyses were carried out using NCSS (Hintze, 2001). All results that were significant at the 5% level of significance were further analysed using the Tukey-Kramer Multiple comparison test (Steel & Torry, 1980).

Results

No interactions were significant, therefore only single cultivar analysis was presented.

Isolation from asymptomatic leaf and flower buds

Sirora cultivar

Isolations from all tissue types exhibited no significant differences ($p \leq 0.05$) in isolation frequency of *Alternaria* spp. over all treatment times. Although not significant, both the leaf and the flower buds showed a lower isolation frequency than the control (no treatment) following the August treatment, however, both the midseason and the mature nuts showed a higher isolation frequency with the August treatment (Fig 4.1).

Ariyeh cultivar

No significant differences were observed in the isolation frequency of *Alternaria* spp. in either the leaf or flower buds, but the control treatment showed a lower isolation

frequency from flower buds than any other treatment. Although not significant the spring and autumn + spring treatments exhibited the lowest isolation frequency in the midseason nuts (30% compared to 50%) than both the control and the autumn treatments. The spring treatment shows the lowest isolation frequency of the mature or harvested nuts (50% as compared to 75%) than all three the other treatments (Fig 4.2).

Shufra cultivar

Isolation frequency of *Alternaria* spp. showed no significant differences ($p \leq 0.05$) in this cultivar over all treatments. The leaf and flower buds showed no significant differences in isolation frequency of *Alternaria* spp., although the controls showed a slightly lower isolation frequency. Although not significant, for both the midseason and the mature nuts, all treatments exhibited a higher isolation frequency than the control. (Fig 4.3).

Discussion

Aerial irrigation was implemented at Green Valley Nuts on an experimental basis in order to supplement the number of chilling hours needed for pistachio to break bud dormancy and increase fruit set (Muller, 2007). The present study confirmed that opportunistic *Alternaria* spp. are capable of infecting various pistachio tissues at different stages of development throughout the year. This corresponds with other studies by Evans *et al*., 1998; Pryor & Michailides, 2002. Environmental conditions play an important role in quiescent infection of almonds (Bilgrami & Ghaffer, 1997), pistachio (Evans *et al*., 1998), tangerines (Canihos, Peever & Timmer, 1999), walnuts (Belisario *et al*., 2002) and hazelnuts (Belisario *et al*., 2004) by *Alternaria* spp. Humidity, coupled with temperature, is the most important climatic variable that affects the ability of *Alternaria* spp. to disperse, infect and colonise host tissue (Michailides & Morgan, 1991; Rotem, 1994; Goldhamer 1996; Evans et al., 1998; Timmer et al., 1998; Canihos *et a*l., 1999; Timmer et al., 2000; Blodgett & Swart, 2002; Goldhamer *et al.,* 2002: Bhatia *et al.,* 2003; Michailides & Teviotdale, 2003.

Wetting of a tree canopy results in microclimatic changes which could be conducive to the infection by fungal pathogens. This occurs during prolonged periods of rain,

especially when followed by water pooling in lower lying areas of the orchard, long dew persistence times and aerial or flood irrigation (Timmer *et al*., 1998; Canihos *et al*., 1999; Timmer *et al*., 2000; Michailides & Teviotdale, 2003). As with other types of aerial sprinkler systems or flood irrigation, canopy wetting could contribute to the splash dispersal of spores on infected abscised leafs and increase the incidence of Alternaria apical necrosis (Rotem, 1994; Goldhamer *et al*., 2002; Mila *et al*., 2005). Goldhamer (1996) proved that making use of subsurface or drip irrigation reduces both leaf and fruit infection, and strongly suggested exclusively using this method of irrigation to reduce the incidence of Alternaria late blight on pistachio.

High relative humidity combined with temperatures of between 23°C and 27°C has been shown to increase the incidence of quiescent *Alternaria* spp. infection in pistachio tissue (Evans *et al.,* 1998). The increase in humidity is caused by evaporation of the water from the trees and the soil, depending on the frequency of wetting and how long the soil stayed wet (Goldhamer *et al*., 2002). In the present study similar temperatures occurred during the treatments periods (Table 4.1) when the trees and surrounding soil was completely wet. Results show no significant differences within cultivars over all treatments, and also no significant differences between cultivars. This is consistent with findings in the study of the fungicide effects on the relative resistance of the three cultivars. Given the fact that the mature nut at harvest had had the longest exposure to potential infection by fungi, it would have been expected to exhibit a higher incidence of quiescent infection (Rotem, 1994). There is a general trend of greater *Alternaria* isolation frequency from midseason to mature nuts, but this was not statistically significant.

All isolations in this study were conducted on asymptomatic fruit. Many other quiescently infected crops shows economically important post-harvest deterioration as these pathogens become active with fruit ripening (Michailides et al., 1995; Prusky, 1996; Biles, Bruton, & Zhang, 1999). This is, however, of no consequence in pistachio, as the potentially infected hull is mechanically stripped directly after harvest and any shells exhibiting staining due to fungal infection are removed as part of post harvest processing (Woodroof, 1976; Evans *et al*., 1998; Doster & Michailides, 1999; Ferguson, Polito & Kallsen, 2005). However, if irrigation, irrespective of the method of application, is not

adequate, early split of the nuts occurs (Doster *et al*., 2001) and quiescent infection can spread to the kernel, affecting the tase, and contaminating it with mycotoxins (Yekeler *et al*., 2001; Andersen, Kroger, & Roberts, 2002, Logrieco *et al*., 2003). The results of the current study indicate that canopy wetting in South Africa facilitates budbreak and increased fruit set in the Ariyeh cultivar, and does not cause a singificant increase in quiescent infection by *Alternaria* spp. under the conditions during this study.

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Table 4.1 Temperatures in the orchards during canopy wetting (Muller, 2007)

Treatment time	Month	Ave max °C	Highest °C	Ave min °C	Lowest °C	Average °C
Autumn	May	22	29	6	-2	14
	June	19	24	1	-5	10
Spring	August	21	28	3	-4	12
	September	27	34	7	-1	17

Figure 4.1 Isolation frequency of *Alternaria* spp. from various tissues from the Sirora cultivar exposed to canopy wetting treatments

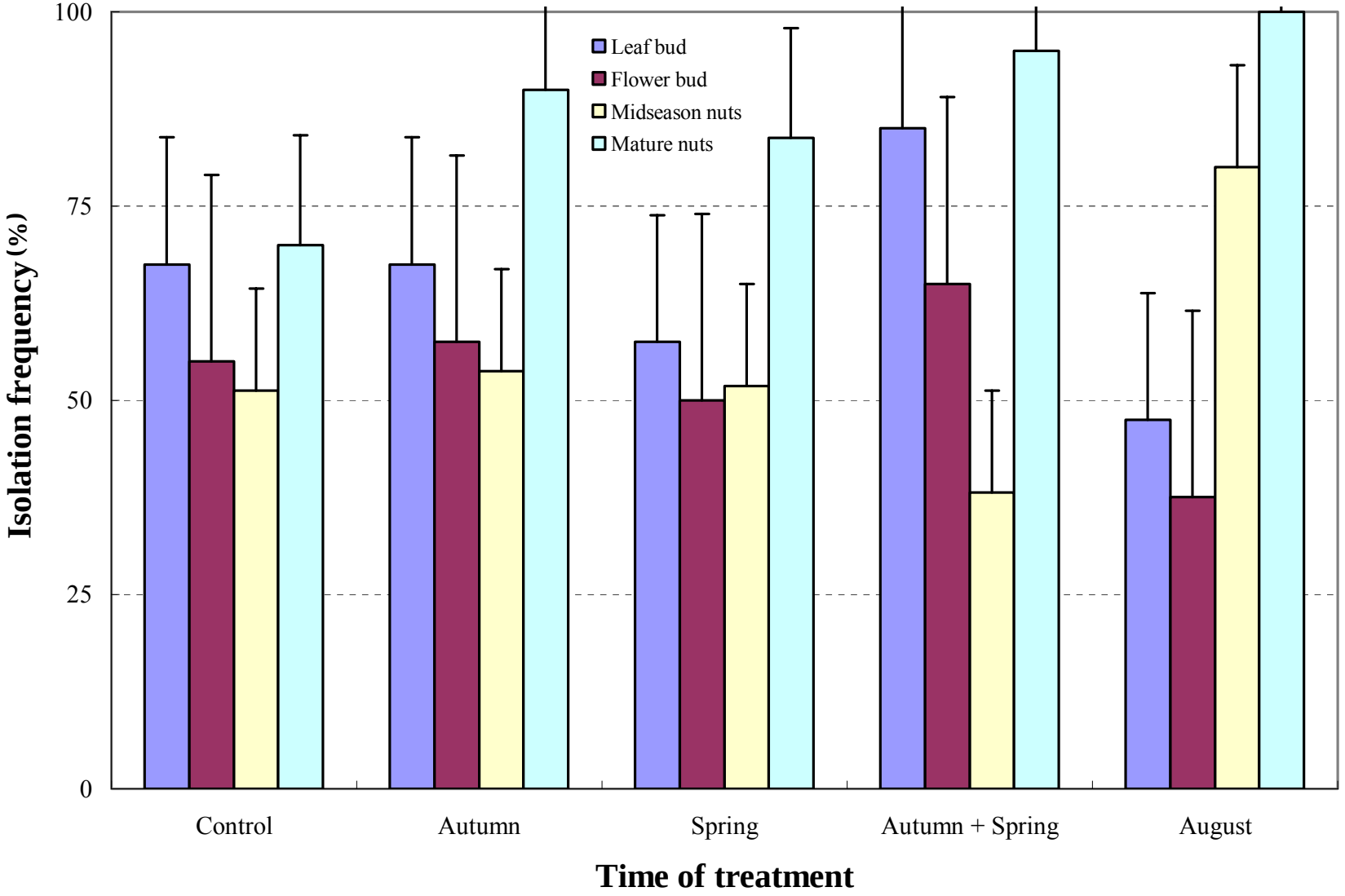

100
75
50
25
0
Isolation frequency (%)
Leaf bud
Flower bud
Midseason nuts
Mature nuts
Control
Autumn
Spring
Autumn + Spring
August
Time of treatment

Figure 4.2 Isolation frequency of *Alternaria* spp. from various tissues from the Ariyeh cultivar exposed to canopy wetting treatments

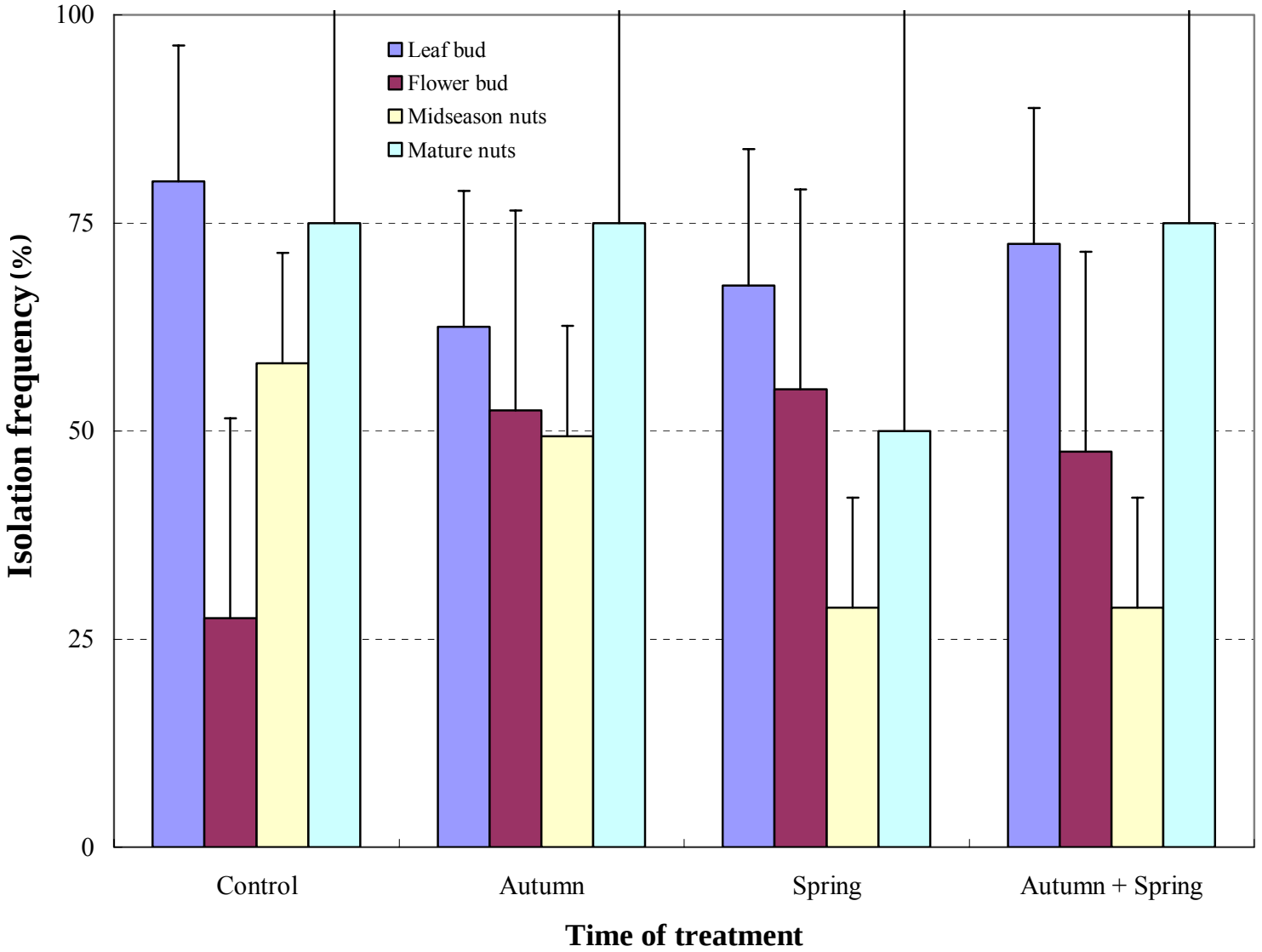
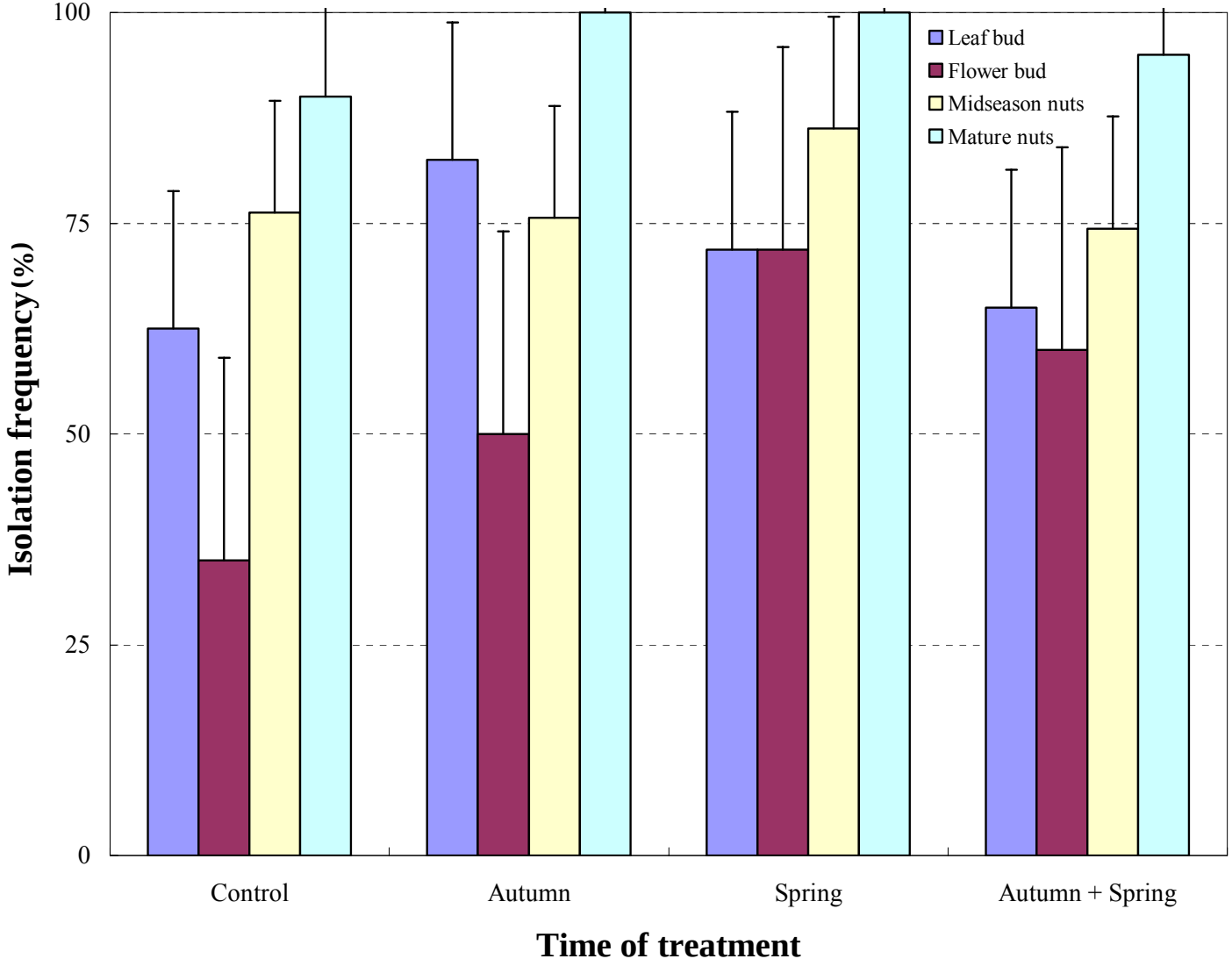

100
75
50
25
0
Isolation frequency (%)
Leaf bud
Flower bud
Midseason nuts
Mature nuts
Control
Autumn
Spring
Autumn + Spring
Time of treatment

Figure 4.3 Isolation frequency of *Alternaria* spp. from various tissues from the Shufra cultivar exposed to canopy wetting treatments


100
75
50
25
0
Isolation frequency(%)
Leaf bud
Flower bud
Midseason nuts
Mature nuts
Control
Autumn
Spring
Autumn + Spring
Time of treatment

SUMMARY

Alternaria spp. were isolated from various types of asymptomatic and symptomatic tissue of pistachio trees in South African orchards. Isolates were subdivided into four morphotypes based on colony morphology. Growth studies at different temperatures however, failed to differentiate the isolates into the same morphotypes. Fifty isolates from asymptomatic nuts, flower buds, leaves and twigs, as well as diseased nuts were submitted to a molecular assay using AFLP. These isolates were compared with five type species of *Alternaria* from the Centraalbureau voor Schimmelcultures. All *Alternaria* isolates from pistachio fell within a single *A. alternata/A. arborescens* group, and did not cluster according to the morphotypes.

Due to the endophytic/quiescent nature of *Alternaria* spp. that cause disease on pistachio, the time of fungicide application is as critical as the formulation and concentration of the chemical used. Fungicide trails were conducted to determine the effect of repeated spraying throughout the year, making use of a system where all fungicides that proved to be effective against *A. alternata* isolates *in vitro* were sequentially used in a spray programme. Primary infection seemed to take place in the young developing flower buds. The use of fungicides early in the season is therefore critical if disease of the nuts is to be prevented. Further sequential sprays continued right up to the time of nuts being harvested. The two best performing fungicided treatments included all the fungicides that was found to be effective against *Alternaria* spp. *in vitro*, used sequencially from early in the season right up to harvesting. Doubling the dosage in the most successful application did not significantly reduce incidence of the fungus in nuts.

Use of bud break oils as done in pistachio orchards in California and Australia was replaced by canopy wetting at Green Valley Nuts in order to increased the number of chilling hours needed for optimal fruit set. Isolations conducted from asymptomattic flower buds, asymptomatic midseason nuts or asymptomatic mature nuts revealed that none of the treatments increased the isolation frequency of quiescent *Alternaria* infection from pistachio tissue.

OPSOMMING

Alternaria spp. is van verskeie tipes simptomatiese en asimptomatiese weefsel van pistachio bome in Suid Afrikaanse boorde geïsoleer. Alle isolate is in vier morfotipes ingedeel. Groei studies by verskillende temperature kon nie die isolate op grond van kolonie morfologie in die morfotipes opdeel nie. Vyftig isolate van asimptomatiese neute, blom- en blaarknoppe, takkies en blare, sowel as van siek neute is molekulêr ontleed. Hiedie isolate is met vyf tipe species van die Centralbureau voor Schimmelculutres vergelyk. Al die *Alternaria* isolate val binne 'n enkele *A. alternata/A. arborescens* groep en het nie volgens die morfotiepes verdeel nie.

As gevolg van die endofitiese/latente aard van *Alternaria* spp. wat siekte op pistachio veroorsaak, is die tyd van swamdoder aanwending net so krities as die samestelling en konsentrasie van die chemikalieë wat aangewend word. Swamdoder proewe is uitgevoer met alle swamdoders wat effektief teen *Alternaria* spp. *in vitro* was, in 'n opeenvolgende bespuitings program om die effek van herhalende toedienings deur die jaar te bepaal. Primêre infeksie blyk in die blomknop stadium plaas te vind en die gebruik van swamdoders vroeg in die seisoen is dus krities as siekte op die neute voorkom wil word. Verder opeenvolgende bespuitings word voorgesit tot oestyd. Die beste twee swamdoder toedienings het gebruik gemaak van al die swamdoders wat effektief was teen *Alternari*a spp. *in vitro*. Die doders is opeenvolgend gebruik van vroeg in die seisoen tot oestyd. Verdubbeling van die dosis van die mees suksesvolle toediening het nie die voorkoms van infeksie in die neute betekenisvol verminder nie.

Die gebruik van botsel stimulerende olies op pistachio soos gebruik in Californië en Australië is vervang met blaardak benatting om die aantal verkoelings ure nodig vir botsel stimulasie te bereik. Geen van die behandelings het die frekwensie van isolasie van *Alternaria* spp. uit asimptomatiese blom knoppe, asimptomatiese groen neute of asimptomatiese ryp neute verhoog nie.