DROUGHT, FREEZING, AND NITROGEN STRESS AS FACTORS PREDISPOSING PISTACHIO TREES TO INFECTION BY *BOTRYOSPHAERIA OBTUSA* AND *B. DOTHIDEA*

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PREFACE

This dissertation is a compilation of six manuscripts. The introductory chapter is a review on the role of stress in the predisposition of woody plants to infection by fungal pathogens with specific reference to *Botryosphaeria* species.

The development of a selective medium for the isolation of *B. obtusa* and *B. dothidea* from diseased plant material is described in chapter 2.

In Chapter 3, fungicides which could possibly be used in the chemical control of these two pathogens are investigated.

The last three chapters investigate the effect of drought, freezing and nitrogen stress on the predisposition of pistachio seedlings to infection by *B. obtusa* and *B. dothidea.*

Due to the fact that chapters represent manuscripts which are independent entities some redundancy and a lack of continuity between chapters, has been unavoidable.

GENERAL INTRODUCTION

Pistachio (*Pistacia vera* L.) is a woody species belonging to the *Anacardiaceae* family. It is thought to have originated in Central Asia where high summer temperatures, low winter temperatures and very little rainfall are the norm. Irrigation, however, has been identified as an essential element for the successful production of pistachio nuts all over the world. The result is that cultivated pistachio trees are less adapted to extremely dry conditions. Although cultivated pistachio can tolerate low winter temperatures it is sensitive to spring frosts or any sudden drop in temperature. The precise nutrient requirements of cultivated pistachios, especially with regard to nitrogen, is still controversial and incorrect fertilization practices coupled with insufficient irrigation can therefore predispose trees to disease development.

Stress is known as a major factor contributing to disease development caused by *Botryosphaeria* spp. Stresses associated with the predisposition of plants to invasion by *Botryosphaeria* spp. and to subsequent disease development include drought, freezing and defoliation. *Botryosphaeria dothidea* (Moug.:Fr.) Ces. & De Not and *Botryosphaeria obtusa* (Schwein.) Schoemaker are opportunistic pathogens of more than 100 genera of woody plants. *Botryosphaeria dothidea* is an endophyte of woody species such as *Pinus* spp. and *Eucalyptus* spp. It has also been identified as a pathogen that infects plants during periods of drought stress. *Botryosphaeria obtusa* thrives during periods of warm and humid weather and can enter its host via frost cracks caused by freezing injury during winter. Both pathogens have been associated with winter stress and defoliation of *Pseudotsuga menziesii* var. *glauca*. Other *Botryosphaeria* species, such as *B. ribis* Gross. & Duggar, attack *Eucalyptus radiata* trees that have been subjected to water stress. *B. ribis* also causes severe lesions on walnut trees (*Juglans regia*) as a result of water stress.

CHAPTER 1

THE ROLE OF STRESS IN THE PREDISPOSITION OF WOODY PLANTS TO ATTACK BY FUNGAL PATHOGENS WITH SPECIFIC REFERENCE TO *BOTRYOSPHAERIA* SPECIES

INTRODUCTION

In physical science, the term stress is described as any force applied to an object while strain is the resulting change in the object's dimensions (Levitt, 1972; Salisbury & Ross, 1992). Biological stress is any change in the environment that might reduce or adversely change a plant's growth or development; biological strain is the reduced or changed function (Levitt, 1980). It is thus clear that any environmental factor that reduces the growth rate of a plant below the optimum or maximum level for its age, genotype and physiological stage may be regarded as a stress upon the plant, which is itself said to be under strain. If the specific strain disappears on removal of the stress it may be said, by analogy with physical systems, to be elastic. If the strain remains after the removal of the stress it is plastic, resulting in injury (Levitt, 1972; Ayres, 1984).

Sorauer (1974), who clearly recognized the importance of environmental factors and stress in relation to plant diseases, first introduced concept of predisposition due to stresses imposed by the environment. One of the first and most relevant definitions of predisposition come from Gäumann (1950) who defined predisposition as a host's "disposition" or "proneness" to disease due to external stress conditions in the plant prior to infection. Factors such as drought, freezing, nutrients, pH, light intensity, salinity and pollution can have significant effects on the predisposition of plants to disease and thereby have a direct influence on disease control (Schoeneweiss, 1975; Schoeneweiss, 1981; Boyer, 1995).

The genus *Botryosphaeria* was established by Cesati & de Notaris in 1863. Species belonging to this genus are well known, throughout the world, as a group of organisms attacking a wide variety of hosts (Barnard *et al.*, 1987; Luque & Girbal, 1989;

Sutton & Arauz, 1991; Michailides, 1991; Smith *et al.*, 1994; Swart & Botes, 1995; Travis *et al.*, 1999). Two species in particular, *Botryosphaeria dothidea* (Moug.:Fr.) Ces. & De Not and *Botryosphaeria obtusa* (Schwein.) Schoemaker, are known opportunistic pathogens of more than 100 genera of woody plants (Sutton & Arauz, 1991; Michailides, 1991; Swart & Botes, 1995; Travis *et al.*, 1999). Both species are predominately canker and die-back pathogens and stress is known as a major factor contributing to diseases caused by them. This review will examine the effect of environmental stress, with specific reference to drought, freezing and nitrogen (N) stress, on diseases of woody plants caused by these and other *Botryosphaeria* species.

DROUGHT STRESS

Drought stress arises when soil water is significantly less than the maximum tendency of plants to lose water as determined by the evaporative demand of the atmosphere. Consequently, water stress can result from either a shortage of rain/irrigation, a soil that is sandy, retaining little water in the root zone or an atmosphere that is hot, dry and windy (Horst, 1990; Salisbury & Ross, 1992; Pelah *et al.*, 1997).

Water plays an essential role in both cellular and whole-plant metabolism. Any decrease in water availability has an immediate and serious effect on processes ranging from photosynthesis to solute transport and accumulation (Ayres, 1984; Salisbury & Ross, 1992; Boyer, 1995; Germanà, 1997; Girona *et al.*, 1997). Lack of sufficient water reduces plant growth directly and indirectly. A reduction in cell division and cell enlargement due to a loss in turgor as well as the reduction of CO_2 assimilation will lead to reduced growth (Kramer, 1983; Turner, 1986). The production of growth regulators such as abscisic acid (ABA) increases during drought stress resulting in an increase in the size of stomata and a subsequent decline in transpiration which will also indirectly lead to a reduction in growth (Loveys, 1984; Tardieu & Davies, 1992). Drought also affects the process of photosynthesis through a reduction in solute transport, stomatal closure and enzyme activity of plants (Boyer, 1976; Bates & Hall, 1981; Kramer, 1983; Salisbury & Ross, 1992).

Prolonged drought stress results in physiological strain on plants which can lead to plants being predisposed to attack by fungal pathogens (Bertrand *et al.*, 1976; Schoeneweiss, 1981; Ayres, 1984; Bèlanger *et al.*, 1989; Boyer, 1995; Blodgett *et al.*, 1997). It is well documented that water stress enhances disease initiation and progress in various host-pathogen combinations (Hepting, 1963; Schoeneweiss, 1974; Schoeneweiss, 1975; Schoeneweiss, 1981; Schoeneweiss, 1983; Appel & Stipes, 1984; Madar & Kimchi, 1989; Klepzig *et al.*, 1996). Fungi such as *Fusarium oxysporum* f. sp. *melonis* (Jorge-Silva *et al.*, 1989), *Diplodia pinea* f. sp. *cupressi* (Madar & Kimchi, 1989) and *Sphaeropsis sapinea* (Blodgett *et al.*, 1997) have been shown to attack their respective hosts when the host is subjected to water stress. The higher the level of stress the more serious the resulting infection.

Pathogens attacking plants that are weakened or predisposed by stress are usually non-aggressive and referred to as opportunistic or facultative parasites that occupy an ephemeral niche within their host (Schoeneweiss, 1981; Houston, 1992; Klepzig *et al.*, 1996). Such pathogens are usually incapable of causing disease in healthy plants but can accelerate the decline of stressed plants sometimes resulting in mortality (Houston, 1992; Manion & Lachange, 1992; Smith *et al.*, 1994; Smith *et al.*, 1996a). *Botryosphaeria spp.*

have been reported as facultative parasites (Schoeneweiss, 1981; Britton & Hendrix, 1986) and stress is known to be a contributing factor to disease development caused by these species. Stresses that have been reported to predispose plants to invasion by *Botryosphaeria* spp. and to subsequent disease development include drought, freezing and defoliation (Crist & Schoeneweiss, 1975; Wene & Schoeneweiss, 1980; Pusey, 1989). Drought stress was identified as the major predisposing factor involved in dogwood (*Cornus florida* L.)(Mullen *et al.*, 1991), sycamores (*Acer pseudoplatanus* L.) (Lewis & Van Arsdel, 1978), and mango (*Mangifera indica*) (Johnson *et al.*, 1992) to attack by *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. (syn. = *Botryodiplodia theobromae*)(anamorph: *Botryodiplodia rhodina* (Cooke) Arx (syn. = *Physalospora rhodina* Cooke). The pathogen was also identified as a canker causing agent of drought stressed dogwood (*Cornus florida* L.) trees (Mullen *et al.*, 1991) while *Botryosphaeria ribis* Gross & Duggar causes serious cankers in drought-stressed peach (*Prunus persica*) (Weaver, 1974), thornless blackberry (*Rubus* spp.) (Maas & Uecker, 1984) and macadamia (*Macadamia integrifolia*) (Herbert & Grech, 1985).

Botryosphaeria dothidea, reported as an endophyte occurring in exotic pine and eucalyptus species in South Africa (Smith *et al.*, 1994; Smith *et al.*, 1996a; Smith *et al.*, 1996b), attacks its hosts during periods of drought stress. *Dothiorella dominicana* and *D. mangiferae,* both Botryosphaeria anamorph (Johnson *et al.*, 1991), also attack mango trees (*Mangifera indica*) that are subjected to water stress. Both species have been isolated as endophytes from the stems of mangos (Johnson *et al.*, 1991). *Dothiorella dothidea* also caused cankers on water stressed Douglas-fir trees (*Pseudotsuga menziesii*) (Shahin & Claflin, 1980). *B. obtusa* has not been associated with stress in the same degree as *B. dothidea*. This can be due to the fact that *B. obtusa* thrives during warm and humid weather (Beisel *et al.*, 1984; Arauz & Sutton, 1990a; Arauz & Sutton, 1990b). Canker on apple trees (Holmes & Rich, 1969) caused by *B. obtusa* has, however, been associated with drought stress. The anamorph of *B. obtusa*, *Sphaeropsis* sp., has been associated with drought stress on numerous occasions (Wingfield, 1987; Swart & Wingfield, 1991).

Attenuation of drought stress

Drought stress can be reduced in many ways, but certain factors should be taken into account when attempting this (Schoeneweiss, 1981; Goldhamer & Smith, 1995). Plant age, species/genotype (Kramer & Kozlowski, 1979; Bélanger *et al.*, 1989; Germanà 1997), growth stage, physiological status, evapotranspiration and planting density (Levitt, 1972; Blodgett *et al.*, 1997) should all be considered. One of the most effective means of controlling drought stress related diseases is the identification of a drought resistant or drought tolerant cultivar that will be able to survive the predisposing period (Bachelard, 1986; Karunaratne *et al.*, 1991).

All plants can withstand some degree of drought stress (Salisbury & Ross, 1987) and it is common practice for plants to be hardened-off in autumn by reducing irrigation so that they are less susceptible to freezing injury (Kramer & Kozlowski, 1979; Salisbury & Ross, 1987). However, the longer a plant is exposed to this condition the more susceptible the plant becomes to attack by opportunistic pathogens (Schoeneweiss, 1975; Schoeneweiss, 1981). Plant age is also a very important factor. An older tree consumes more water than a younger one and with increasing age greater susceptibility to water stress occurs (Kramer & Kozlowski, 1979; Salisbury and Ross, 1987).

When applying irrigation to trees continuous adjustment according to the needs of the plant through the use of growth models is recommended. Growth models, however, require specific plant growth input parameters, which are not always readily available, for all crops and conditions (Jovanovic *et al.*, 1999; Jovanovic & Annandale, 2000). Plant density also plays and important role in susceptibility of plants to stress since it results in greater competition for nutrients and water (Kramer & Kozlowski, 1979; Salisbury & Ross, 1987). Blodgett *et al.* (1997) speculated that water stress caused by the competing vegetation appeared to be the dominant factor involved in the increased disease severity due to water stress and reported an increase of disease in red pine (*Pinus resinos*) caused by *Sphaeropsis sapinea*. Fertilization also plays an important role. High nitrogen levels leads to more vigorous growth of a plant resulting in greater susceptibility to drought stress (Mengel & Kirkby, 1987; Abrol, 1989; Groninger *et al.*, 1995). Another important measure for the reduction of drought stress is the application of mulch where the surface is covered in order to lower evapotranspiration from the soil surface (Joseph *et al.*, 1994; Paris *et al.*, 1997; Pliszka *et al.*, 1997).

CHILLING AND FREEZING STRESS

Stress induced by low temperatures is categorized into chilling stress and freezing stress (Körner & Larcher, 1988; Kramer & Kozlowski, 1979; Hällgren & Öquist, 1990). Chilling stress, which occurs above freezing point, usually results in an elastic strain but may become a plastic strain depending on length of exposure and the temperature threshold. Plastic strain is an irreversible condition where the plant is damaged to such an extent that it cannot recover even when the stress is eliminated. Freezing stress commonly results in a plastic strain (Levitt, 1980; Ayres, 1984).

Since stress resulting from low temperature is often elastic and disease resulting from it may be reversible in time, providing host tissue is not killed directly during exposure, the plant is able to survive the stress (Levitt, 1972; Schoeneweiss, 1975; Levitt, 1980). The time frame for the predisposition is very important. Schoeneweiss (1981) showed that the longer the period of exposure to chilling the more susceptible a plant is to attack by opportunistic pathogens.

Direct injuries such as frost rings and frost cracks lead to weakened plants and result in portals for infection by pathogens (Kramer & Kozlowski, 1979; Hällgren & Öquist, 1990). Frost rings occur during the growing season as a result of frost, which injures the cambium. Such rings consist of an inner part comprised of cells killed by the frost (usually xylem cells and differentiating cambial derivatives) and an outer part of abnormal xylem cells produced after frost. The structure of the frost rings varies among species as well as with the severity of the frost and the activity of the cambium. The location of a frost ring often dates the frost, early spring frosts causing the abnormal cells to be localized in the early formed portion of the annual growth increment. Frost rings occur more frequently in thin-barked trees than in those with characteristic thick bark. Small twigs are injured more easily than thicker branches and the latter are injured more than the stems (Kramer & Kozlowski, 1979; Hällgren & Öquist, 1990). Frost cracks and winter scald (sunscorch) lesions develop on stems of trees when alternating freezing and thawing occurs during the winter and early spring. The lesions can be recognized by dead bark patches, which sometimes peel end expose the wood, or by sunken cankers, which become sites for fungal attacks (Kramer & Kozlowski, 1979; Salisbury & Ross, 1992). Rapid freezing of the stems after sundown is largely responsible for winter sunscald lesions. The bark on the south side of a tree may be 20°C warmer at midday than the north side, suggesting that the south side undergoes violent temperature fluctuations resulting in uneven expansion of the bark tissue (Kramer & Kozlowski, 1979; Hällgren & Öquist, 1990; Salisbury & Ross, 1992).

Mechanisms of freezing injury

Many mechanisms have been proposed to account for the specific mode of action that chilling has in predisposing plants to disease (Kramer & Kozlowski, 1979; Graham & Patterson, 1982; Hällgren & Öquist, 1990). As temperatures drop in plants, lipids in cellular membranes solidify (crystallize) at critical temperatures that are determined by the ratio of saturated to unsaturated fatty acids. The critical temperature for transition from a liquid to a crystalline form is often equivalent to the temperature that causes chilling damage. Development of tolerance to chilling temperatures in chilling-sensitive plants apparently involves changes in this ratio. An increase in the proportion of unsaturated fatty acids or in the quantity of sterols causes membranes to remain functional at lower temperatures (Graham & Patterson, 1982; Salisbury & Ross, 1992).

Threshold temperatures of –20 to –30 °C have been identified for the predisposition of trees to freezing or chilling stress. Plants kept at these temperatures for short periods of time showed significantly higher disease susceptibility than control plants (Schoeneweiss, 1974; Schoeneweiss & Wene, 1977; Schoeneweiss, 1981). The degree to which plant tissue experiences freezing or chilling stress is also influenced by the duration of sub-zero temperatures and often symptoms will only develop after extended periods of cold exposure. According to Cameron & Dixon (1997) *Magnolia soulangiana* trees kept at a temperature of –4 °C for 4 hours showed no fatalities. When the freezing period (-4 °C) was extended to 12 hours, 33% of the trees died and after 36 hours exposure all the trees in the treatment were dead. Similar results were observed with *Acer palmatum* and *Euphorbia griffithii* (Cameron & Dixon, 1997). Hardened plants are able to survive short periods of freezing temperatures, but there is a maximum threshold which varies between species after which the plant is injured and subsequently predisposed to disease (Schoeneweiss & Wene, 1977).

Kable *et al.* (1967) showed severe losses on sweet cherry due to *Cytospora* canker following winter injury. Similar observations were made on *Rhamnus frangula* infected with *Tubercularia ulmea* (Schoeneweiss, 1975), *Euonymus alatus* infected with *Nectria cinnabarina* (Schoeneweiss & Wene, 1977) and *Eucalyptus* spp. infected by *Botryosphaeria dothidea* (Smith *et al.*, 1994). Schoeneweiss (1983) showed that although blue spruce (*Picea pungens*) showed longer lesions when subjected to freezing stress of –20 to –30 °C for 30 min, it did not differ significantly from the control trees kept at a constant temperature of 24°C. Similar results to those obtained by Schoeneweiss (1983) were obtained on peach trees (Weaver, 1978) and other woody plants (Schoeneweiss, 1981).

Schoeneweiss (1981) indicated that threshold temperature is very important for freezing damage to occur and if it is not reached stress may be reversible and the plant will be able to survive. Under severe conditions, when threshold temperature is reached, stress is plastic or irreversible, resulting in the death of the plant (Schoeneweiss, 1981). Visible symptoms such as wilting, cracks or browning of the phloem tissue are not always an indication of predisposition as demonstrated with *Rhamnus frangula* predisposed to attack by *Tubercularia ulmea* (Schoeneweiss 1974) and with stem cankers of *Euonymus alatus* caused by *Nectria cinnabarina* (Schoeneweiss & Wene, 1977) where the plants showed clear fungal infestation without any symptoms of freezing damage beforehand.

Plants that are infected with non-aggressive, opportunistic pathogens that have an endophytic phase may become predisposed to disease caused by these endophytic pathogens when subjected to low temperatures (Schoeneweiss, 1975; Wene & Schoeneweiss, 1977). Diseases caused by *Botryosphaeria spp.* have often been associated with predisposing environmental stresses such as chilling or freezing. *Botryosphaeria rhodina* (anamorph: *Lasiodiplodia theobromae*) causes cankers on the stems of dogwood trees as a result of freezing stress (*Cornus florida* L.) (Mullen *et al.*, 1991). Britton & Hendrix (1986) showed that peach (*Prunus persica*) trees were more susceptible to attack by *B. dothidea* when subjected to freezing stress. Similar results were observed on *Eucalyptus* spp. (Smith *et al.*, 1994) and apples (Ellis, 1998) infected with *B. dothidea*. Sometimes it is not freezing stress that directly predisposes plants to infection but mechanical damage resulting from frost cracks which are ideal infection portals for opportunistic fungi (Beisel *et al.*, 1984; Venkatasubbaiah *et al.*, 1991; Michailides, 1991).

Attenuation of freezing stress

The most effective way of preventing freezing stress of woody species is to subject the plants to a period of hardening-off prior to the occurrence of winter (Kramer & Kozlowski, 1979; Salisbury & Ross, 1989). Practices such as irrigation during autumn and high levels of nitrogen (N) fertilization in late summer can prevent hardening-off from taking place and result in winter or freezing injury (Schoeneweiss, 1974; Perkins *et al.*, 1991). A reduction in irrigation should, however, be implemented gradually since it can lead to drought stress in actively growing plants which in turn can result in plants that are more susceptible to freezing stress (Schoeneweiss, 1978; Cameron & Dixon, 1997).

Other measures include hormonal treatments with abscisic acid (Skriver & Mundy, 1990; Surànyi, 1991), spraying with chemicals such as sucrose and boric acid (Milovankic *et al.*, 1990; Porwal & Singh, 1990), heat and steam treatment (Jiao & Wang, 1991) and the planting of frost resistant cultivars (Anisko & Lindstrom, 1995; Okie & Pusey, 1996; Ellis, 1998; Travis *et al.*, 1999). Although expensive, the use of mechanical barriers in the form of plastic, cheesecloth, vinyl or polyethylene have also been used as preventative measures against frost damage (Zhu, 1991; Jiao & Wang, 1991; Igarashi, 1994).

NITROGEN STRESS

Compounds containing nitrogen constitute only a small proportion of the total dry weight of woody plants, but are extremely important physiologically. The nitrogen content of the foliage amounts to about 1.0 to 1.2% of the dry weight of trees (Kramer & Kozlowski, 1979). Nitrogen compounds however, play an extremely important role in the physiological and biochemical processes of plants (Mengel & Kirkby, 1987; Hageman & Below, 1989; Abrol, 1989). Nitrogen occurs in amides, amino acids, nucleic acids, nucleotides and other nitrogenous bases, hormones, vitamins and alkaloids (Mengel & Kirkby, 1987; Abrol, 1989) and is probably the most limiting factor following water tress for normal plant growth (Kramer & Kozlowski, 1979; Salisbury & Ross, 1992).

Plants suffering from nitrogen deficiency display general chlorosis, especially of the older leaves (Mengel & Kirkby, 1987; Abrol, 1989). On fruit bearing trees, N deficiency can lead to delayed bud break, short and thin shoots, smaller leaves and early senescence (Crane & Maranto, 1988; Brown, 1995). Plants grown with excess nitrogen usually have dark leaves and show an abundance of foliage, usually with a root system of minimal size and therefore a high shoot-to-root ratio (Abrol, 1989; Salisbury & Ross, 1992). High levels of nitrogen can thus lead to plants that are predisposed to drought (Paine *et al.*, 1992) and freezing stress (Brooks, 1992; Cline, 1994; Smith *et al.*, 1994; Boyce, 1995).

High and low levels of nitrogen not only lead to predisposition of plants to drought and freezing, but also to attack by pathogens (Entry *et al.*, 1990; Entry *et al.*, 1991a; Entry *et al.*, 1991b; Chambers *et al.*, 1993; R'Houma *et al.*, 1998). *Armillaria ostoyae* and *Phytophthora* spp. are opportunistic pathogens that attack Douglas-fir (*Pseudotsuga menziesii*) and *Chamaecyparis nootkatensis* when trees are subjected to periods of nitrogen deficiency (Hennon *et al.*, 1990; Entry *et al.*, 1990; Entry *et al.* 1991a; Entry *et al.*, 1991b). The addition of N fertilizers can reduce the susceptibility of many plant species to disease. Enebak *et al.* (1990) demonstrated a lower incidence of damping-off in white pine seedlings caused by various species of *Fusarium, Rhizoctonia* and *Pythium* spp. with higher levels of nitrogen. Huang & Kuhlman (1991) decreased infection by *Rhizoctonia* spp. of slash pine seedlings by adding N fertilizers.

Mechanisms of nitrogen stress

High levels of N can directly increase the susceptibility to both obligate and facultative parasites, either by increasing tissue succulence and facilitating entry, or by providing some complex N source. Solel & Bruck (1988) demonstrated that high levels of N predisposed loblolly pine (*Pinus elliottii*) seedlings to pitch canker and proposed that the disease enhancing effect could result from an increased availability of N to the pathogen, either via plant metabolites or directly as unincorporated, ionic N. *Botrytis* stem necrosis on grapevines has also been associated with high levels of N (Chambers *et al.*, 1993; R'Houma *et al.*, 1998).

High levels of nitrogen can also play an indirect role in disease predisposition. Increased levels of N lead to lower levels of phenols, which are responsible for the inhibition of *Armillaria ostayae* in Douglas-fir (*Pseudotsuga menziesii* var. *menziesii*) (Wargo, 1984; Entry & Cromack, 1988). The application of high levels of N thus results in less inhibition of *A. ostayae* and a higher disease incidence.

Attenuation of nitrogen stress

Nutrient usage and fertilizer practices are more difficult to determine in tree crops than in annual crop species mostly due to a lack of knowledge of the size of nutrient storage pools in trees and their contribution to spring growth (Rosecrance *et al.*, 1998).

New practices such as nitrogen fertilization via low-volume irrigation systems (Kallsen *et al.*, 1999) accentuate the importance of a well-planned fertilizer program for cultivated crops (Mengel & Kirkby, 1987; Abrol, 1989).

Many factors need to be taken into account when determining an ideal fertilizer program. Solel & Bruck (1988) reported that careful monitoring of the N content of needles before deciding on a fertilization program could probably prevent pitch canker on loblolly pine seedlings. In pistachio, nitrogen and phosphorus demand decreases during the vegetative period while Ca, K, and Mg increase. Nitrogen content of the leaves is however higher during the vegetation period than during fruit set, shooting and leaf formation (Idem & Gezerel, 1995; Caruso *et al.*, 1996). It is therefore clear that more nitrogen is being used during the fruit set, shooting and leaf formation period and therefore higher levels should only be applied during this period (Caruso *et al.*, 1995; Idem & Gezerel, 1995; Caruso *et al.*, 1996).

CONCLUSIONS

Stress imposed by abiotic and biotic factors has many adverse effects on plant metabolism, growth and development. One very important effect is the enhanced susceptibility of plants to weak or opportunistic pathogens. Abiotic and biotic factors interact by distributing the energy balance of the plant but the mechanisms of such interactions remain unstudied and an understanding of how stress predisposes plants to disease is still very vague. Recent research has given various new perspectives to the confusing etiology of stress-related disease and many unanswered questions still exist.

A comprehensive study of environmental factors that impose stress in plants is important in establishing a sustainable disease and pest management program. Most experiments to investigate the effects of stress on disease susceptibility have however, not been designed to separate predisposition from other effects. Embarking on studies of the effects of individual biotic and abiotic factors involved in stress is therefore crucial in order to understand the precise mechanisms involved. This should first be done under intense controlled conditions and later verified under field conditions.

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CHAPTER 2

A SELECTIVE MEDIUM FOR THE ISOLATION OF *BOTRYOSPHAERIA OBTUSA* AND *B. DOTHIDEA* FROM WOODY PISTACHIO TISSUE

ABSTRACT

Botryosphaeria obtusa and *B. dothidea* are often associated with diseased pistachio trees in South Africa, but isolation of these fungi from diseased tissue on culture media is impeded by contamination of media with various saprophytic fungi. A selective medium for their isolation was therefore developed. Seven fungicides were tested for their effect on the growth of *B. obtusa* and *B. dothidea in vitro*. Four of the least inhibitory fungicides were subsequently selected for further evaluation on 14 of the fastest growing fungal species recovered as contaminants from diseased pistachio tissue. Tannic acid was least inhibitory to the target fungi, but suppressed most of the contaminant fungi including *Mucor* sp., *Trichoderma* sp. and *Alternaria tenuissima*. The putative selective medium was then tested by conducting isolations from various possible sources of *B. dothidea* and *B. obtusa* in pistachio orchards. *B. dothidea* was isolated from the debri, bark, diseased and asymptomatic tissue without hindrance by extraneous fungi. Isolation of this fungus from asymptomatic tissue suggests an endophytic lifestyle. *B. obtusa*, however, was only isolated from cankers on the stems of diseased pistachio trees.

INTRODUCTION

Botryosphaeria panicle and shoot blight caused by the anamorph stage (*Dothiorella* sp.) of *Botryosphaeria dothidea* (Moug.:Fr.) Ces. & de Not. (syn. *B. ribis* Gross. & Duggar), is one of the most devastating diseases of pistachio trees in California (Crane & Maranto, 1988; Michailides, 1991; Michailides & Morgan, 1992; Michailides *et al.*, 1998). It was only recently, however, that Swart & Blodgett (1998) first reported *B. dothidea* as the causal agent of disease of pistachio trees in South Africa. The pathogen has also been associated with diseases of *Eucalyptus, Pinus* and *Protea* spp. in South Africa, specifically as an endophyte (Von Broembsen & Van der Merwe, 1990; Smith *et al.*, 1994; Smith *et al.*, 1996a; Smith *et al.*, 1998). *Botryosphaeria obtusa* (Schwein.) Schoemaker has to date only been reported in South Africa as a canker causing pathogen of pistachios (Swart & Botes, 1995). The presence of both pathogens in pistachio orchards in South Africa is associated with predisposing environmental factors such as drought, frost damage and the application of nitrogen fertilizers. Studies of their epidemiology are therefore required in order to formulate management practices aimed at limiting the damage they cause.

The isolation of *B. dothidea* and *B. obtusa* from soil, plant debri, bark and diseased wood is severely hampered by the presence of fast growing saprophytic fungi and bacteria which contaminate culture media. Michailides (1991) used a lactic acid solution (2.5 ml of a 25% solution of lactic acid per liter of potato-dextrose agar medium) for the isolation of *B. dothidea* from diseased plant parts from male and female pistachio trees (i.e., petioles, leaves, panicles, fruits and buds). Using this medium we still encountered problems due to the presence of *Trichoderma* spp. and *Penicillium* spp. This paper describes the development of a selective medium for the isolation of *B. obtusa* and *B. dothidea* and compares its efficiency with that of a nonselective basal medium.

MATERIALS AND METHODS

Screening for fungal activity

In previous studies in which selective media had been developed for the isolation of *Sphaeropsis sapinea* (Fr.) Dyko & Sutton (*Diplodia pinea* (Desm.) Kickx) (Swart *et al.*, 1987), *Cryphonectria cubensis* (Bruner) C.S. Hodges (Conradie *et al.*, 1992) and *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. (Cilliers *et al.*, 1994) from pine and eucalyptus tissue, both *Botryosphaeria* spp. were observed as being relatively tolerant of tannic acid, gallic acid, o-phenylphenol, chlorothalonil, tridemorph, rose bengal and Na_2HCO_3. These chemicals were consequently tested for their inhibitory effect towards 14 contaminant fungi associated with the isolation of *B. dothidea* and *B. obtusa* from bark and woody tissue. Fungi tested were *Trichoderma sp.* (CDN 120), *Cerebella andropogonis* (Ces.) Schol-Schwarz (CDN 71), *Fusarium oxysporum* Schlechtend.: Fr. (CCP 331), *Dreshlera sp.* (CCP 25), *Aspergillus niger* Tiegh. (CCP 174), *Mucor sp.* (CDN 78), *Phoma sp.* (CCP 136), *Alternaria alternata* (Fr.: Fr) Kiessl. (CDN 310), *Alternaria tenuissima* (Kunze: Fr.) (CDN 311), *Botrytis cinerea* Pers.: Fr. (CCP 8), *Penicillium glabrum* (Wehmer) Westling (CCP 176), *Cytospora sp.* (CCP 21), and *Epicoccum purpurascens* Ehrenb. (CCP 310). The species were all obtained from the culture collection of the Department of Plant Pathology at the University of the Orange Free State.

Stock solutions of each chosen compound were made with distilled water. Tannic acid, rose bengal and gallic acid were added before autoclaving the water for 20 minutes at 120°C. Chlorothalonil, o-phenyl-phenol, tridemorph and Na_2HCO_3 were added to autoclaved water that had cooled to room temperature (25°C). A basal agar medium (20g Difco malt extract, 15g Difco technical agar in 1000 ml of distilled water) was amended with stock solutions of each compound following autoclaving for 20 minutes and cooling to 50°C. Final concentrations of compounds in the respective agar media were: tannic acid (5000 μg/ml); gallic acid (2 g/l); *o*-phenyl-phenol (5 μg/ml); chlorothalonil (2.5 μg/ml); tridemorph (1 μg/ml); rose bengal (50μg/ml), furalaxyl (150 μg/ml) and Na_2HCO_3 (5 mg/l).

The amended agar medium was agitated for 2 min to allow for even mixing of a particular compound before 20 ml of each medium was poured into 90-mm petri plates and allowed to solidify. Colonized 5-mm agar plugs of fungi from the periphery of actively growing colonies on potato-dextrose agar (39g Difco potato-dextrose agar plus 1000 ml distilled water) were transferred to the center of each assay plate. Four replicate plates per fungus per compound were used. The basal medium served as a control treatment in all trials. The colony diameter on each Petri plate was recorded as the mean of two perpendicular measurements after 96 hr of incubation at 25°C.

A selective medium consisting of 3000 μg/ml tannic acid, 2g/l gallic acid, 50 μg/ml rose bengal, 150 μg/ml furalaxyl and 500μl/l streptomycin was formulated (Fig. 2). The concentration of the tannic acid was reduced because the agar did not solidify at a concentration of 5000 μg/ml. Streptomycin was added to all compounds tested individually as well as to the selective medium and the basal medium that served as control treatment.

Verification of the selective medium

Soil, bark and debri: Samples of soil, bark and debri were collected from five different blocks (selected at random from 25 blocks in a 400 ha area) in pistachio orchards near Prieska, Northern Cape province, South Africa during April of 1999. Twenty (20) trees were randomly selected in each block. The samples from each block were mixed and one uniform sample was taken. This was done for the soil, bark and debri samples. Soil was collected 100 mm deep and 200 mm from the base of the tree. Debri was gathered in a radius of 300 mm around the base of the tree. Bark samples were collected by scraping off the outer bark 300 mm above soil level. All samples were individually blended in 250 ml sterile water. A 1:1000 dilution of sample suspension (1 ml of sample suspension in 999 ml agar) was made with both the selective and basal media. The agar was poured into Petri plates and left to solidify before being incubated at 25°C for two days. Developing fungal colonies were removed every day for six days and transferred to 1.5% water agar (15g Difco technical agar in 1000ml distilled water) overlaid with 3-5 sterile pine needles to aid sporulation. Plates were then incubated in a growth chamber at 25°C for 7 days after which they were removed and placed under "black-light" at room temperature to induce sporulation. Sporulating fungi were identified using a light microscope.

Pine bark growth medium: A pine bark medium (Bark Enterprise Pty., Brits, South Africa) used to propagate pistachio plants in the nursery at Prieska was screened to determine if *Botryosphaeria* spp. were present in the medium. Small pieces of bark, previously rinsed for 5 min in sterile water, were placed on the selective medium in culture plates. For the control treatment, bark was rinsed and placed on 1.5% MEA (10g Difco malt extract, 15g Difco technical agar in 1000ml water). In a second trial, bark was blended in sterile water from which a 10x dilution was made and 100 ml of this dilution was added to 900 ml selective medium, which had been cooled to 45 °C. The 100 ml dilution was added to 900 ml 1% malt extract agar for a control treatment. The medium was subsequently poured into Petri plates and incubated at 25 °C. Transfers were made every 24 hours for a period of 6 days from all the resulting fungal colonies to 1.5% MEA (20g Difco malt extract, 15g Difco technical agar and 1000 ml distilled water) to allow for sporulation. In instances where colonies did not sporulate, they were transferred to 1.5% water agar (15g Difco Technical agar in 1000ml of distilled water) overlaid with autoclaved pine needles and incubated under "black light" at 19°C for 5-14 days.

Diseased tissue: Stems with cankers and twigs showing die-back symptoms were collected from pistachio orchards. Small pieces of wood (ca. 4 mm^2 were cut from the advancing edge of cankers and placed directly on the selective medium. For the control treatment, pieces of tissue were placed on the basal medium. Transfers were made every 24 hours for a period of 7 to 14 days from resulting fungal colonies to 1.5 % MEA to allow for sporulation. In instances where colonies did not sporulate, they were transferred to 1.5% water agar (15g Difco Technical agar in 1000ml of distilled water) overlaid with autoclaved pine needles and incubated under "black light" at 19°C for 5-14 days. Additional twigs and branches with die-back symptoms were treated in the same manner except for the fact that tissue was removed from the outer and inner bark as well as the wood.

Asymptomatic tissue: Healthy shoots, leaves and branches of 4-year-old pistachio trees were collected in a pistachio orchard throughout the year and incubated at 5°C for a maximum of 48 hours before being processed in the laboratory. Small pieces of tissue were cut from the outer bark, inner bark (phloem) and xylem tissue. Tissue pieces were surface-disinfected in a 20% NaOCl solution for 60 seconds, blotted dry and plated onto the selective medium. In the control treatment bark tissues were placed on the basal medium. After incubation at 25°C for 4-20 days emerging fungal colonies were identified.

RESULTS

Screening for fungal inhibition

Trichoderma sp., *Mucor* sp., *Cerebella andropogonis, Fusarium equiseti, Dreshlera* sp. and *Epicoccum purpurascens* were completely inhibited by addition of tannic acid (5000 µg/ml) (Fig. 1). Gallic acid (2 g/l) significantly inhibited radial growth of *Fusarium oxysporum, Phoma* sp. and both *Alternaria* species in relation to the two *Botryosphaeria* species. Rose bengal (50 µg/ml) strongly inhibited radial growth of all species except for *Mucor* sp., *Trichoderma* sp. and the two *Botryosphaeria* spp. Furalaxyl (150 µg/ml) inhibited the radial growth of *Fusarium oxysporum* and *Epicoccum purpurascens* (Fig. 1).

Verification of selective medium

Soil, bark and debri: Of 538 fungal isolates recovered from debri on the basal medium, 15% were isolates of *B. dothidea* compared to 2.5% from bark. The remainder of the debri isolates consisted of *Phoma* spp. (28.5%), *Fusarium* spp. (24%), *Alternaria tenuissima* (17.5%), *Epicoccum purpurascens* (10%), *Drechslera biseptata* (2.5%) and *Trichoderma* spp. (2.5%). The rest of the isolates from bark consisted of *Alternaria tenuissima* (44.47%), *Epicoccum purpurascens* (13.33), *Phoma spp.* (20%), *Fusarium spp.* (15%) and *Mucor spp.* (4.7%). On the selective medium, where fewer fungal isolates were obtained from debri (256). Of these, 28.7% were *B. dothidea.* Bark samples yielded 217 isolates of which 15.6% were *B. dothidea.* The remainder of the bark samples yielded *Alternaria tenuissima* (46%), *Phoma* spp. (19.9%), *Fusarium* spp. (12.5%), *Mycelia sterilia* (2.5%) and *Trichoderma* spp. (1.5%). *Epicoccum purpurascens* and *Mucor* spp. were also isolated in lower frequencies (2%). With the basal medium 695 isolates of the following fungi were isolated: *Trichoderma* spp. (26%), *Fusarium* spp. (19.8%), *Phoma* spp. (15.6%), *Alternaria tenuissima* (13.5%), *Mucor* spp. (8.3%), *Penicillium* spp. (6.3%), *Mycelia sterilia* (4.2%), *Epicoccum purpurascens* (2.1%), *Cerebella andropogonis* (2.1%) and *Pythium* spp. (2.1%). Using the selective medium *Trichoderma* spp., *Mucor* spp., *Penicillium* spp., *Mycelia sterilia*, *Pythium* spp. and *Epicoccum purpurascens* were completely inhibited. The remaining isolates (367) consisted of *Alternaria tenuissima* (73%), *Fusarium* spp. (17%), *Phoma* spp. (10%). Neither *B. obtusa* nor *B. dothidea* was isolated from soil samples with or without the selective medium.

Diseased plant tissue: On the selective medium *B. dothidea* was obtained in 87% of 278 isolations made from diseased twigs and shoots. The remaining 13% of isolations were exclusively *Phomopsis* spp. The basal medium yielded *B. dothidea* 32% of a total number of 467 isolations from twigs and shoots with the remaining isolations yielding *Mucor* spp., *Trichoderma* spp., *Penicillium* spp. and *Alternaria tenuissima*. *B. obtusa* was recovered 85% of 307 isolations from stem cankers on the selective medium with *A. tenuissima* comprising the remaining 15% of these isolations. The basal medium yielded *B. obtusa* 28% of 648 isolations with remaining isolates comprised of contaminants such as *Mucor* spp. (32%) *Trichoderma* spp. (27%) *A. tenuissima* (9.8%) and *Penicillium* spp. (3.2%).

Asymptomatic tissue: Of the 785 isolates obtained from asymptomatic bark, stem and twig tissue on the selective medium, 70% were *A. tenuissima* and 15% *B. dothidea*. The remainder consisted of *Trichoderma* spp. and *Penicillium* spp. Of 637 isolations on the basal medium only 1.5% of isolates recovered were *B. dothidea* and the remainder *Alternaria tenuissima* (62%), *Mucor* spp. (28%), *Trichoderma* spp. (5.5%) and *Penicillium* spp. (3%). On both the basal and selective medium, *B. dothidea* isolates were only isolated from the outer and inner bark but never from the wood. *B. obtusa* was never isolated from asymptomatic tissue.

Pine bark growth medium: Of a total of 875 isolations made from the pine bark growth medium neither *B. obtusa* nor *B. dothidea* were isolated on either the basal medium nor the selective medium. *A. tenuissima*, *Mucor* spp. and *Penicillium* spp. predominated.

DISCUSSION

Numerous media have been developed for the selective isolation of fungi. Principles involved in the development of these media include either inhibition or enhancement of fungal growth (Tsao, 1970). The selective medium here is based on the inhibition of the competing fungi species. In developing a selective medium, it is crucial to restrict the growth of rapid proliferating fungi and fungi that produce masses of easily dispersed spores. In this respect, *Trichoderma* spp., *Mucor* spp., *A. niger*, and *Penicillium* spp. were particularly important, since we frequently isolated these species from pistachio tissue in association with *B. obtusa* and *B. dothidea*. Tannic acid was employed as the main inhibitory compound while other compounds were added to inhibit those fungal species that were not inhibited by tannic acid. The three fastest growing contaminant fungi were *Trichoderma sp.*, *A. niger* and *Mucor sp.*, all of which were effectively inhibited by tannic acid. The lactic acid medium used by Michailides (1991) was most effective against the *Mucor* spp., but did not inhibit *Trichoderma* and *Penicillium* sufficient enough for *Botryosphaeria* spp. to be isolated.

Synergism among compounds in a selective medium can sometimes prevent successful isolation of the target organism (Tsao, 1970). In the case of the present medium, however, synergism between gallic acid (2 g/l), furalaxyl (150 μg/ml) and rose bengal (50 μg/ml) completely inhibited the growth of *A. niger* and *C. eucalypticola* without negatively influencing the growth of *B. obtusa* and *B. dothidea*.

B. obtusa was only isolated from the diseased pistachio tissue, and not from soil, debri or asymptomatic tissue. The isolation of *B. dothidea* from asymptomatic pistachio tissue suggests an endophytic or latent phase. This is consistent with the isolation of *B.*

dothidea from asymptomatic tissue of *Pinus* and *Eucalyptus* spp. in South Africa (Fisher *et al.*, 1993; Smith *et al.*, 1996a; Smith *et al.*, 1996b). Neither *B. dothidea* nor *B. obtusa* was isolated from the bark medium used for the propagation of the pistachio trees in the nursery, which had been sterilized using methyl bromide. This confirmed that both pathogens were not being dispersed by growth media from the nursery.

The selective medium described here provides an efficient means for isolating *B. dothidea* from diseased and asymptomatic woody tissue and debri. Isolation of the two *Botryosphaeria* spp. from the diseased tissue on the basal medium was normally severely hampered by *Mucor* spp., *Trichoderma* spp., *A. niger* and *Penicillium* spp. contaminating the medium to such an extent that no isolations from the two *Botryosphaeria* species could be successfully obtained. Although some fungi did manage to grow on the selective medium (Fig. 2), their colony diameters were significantly smaller than that of *B. obtusa and B. dothidea* thus allowing for easy recovery of pure cultures.

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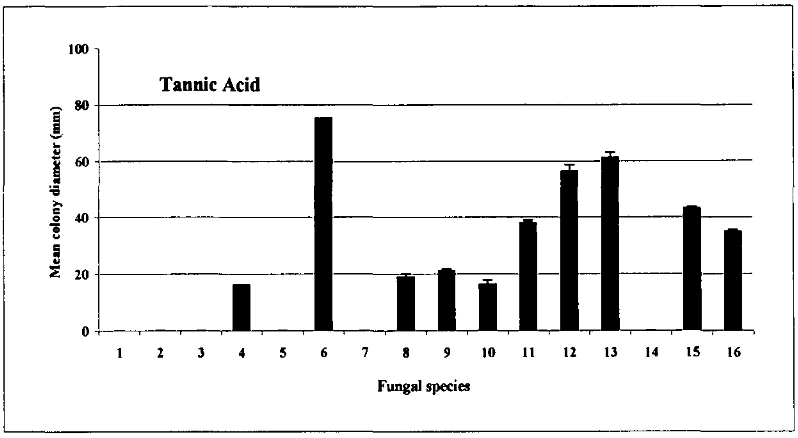
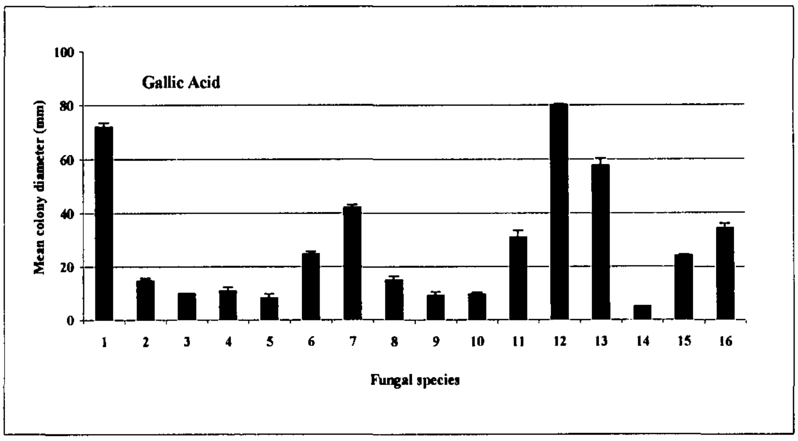
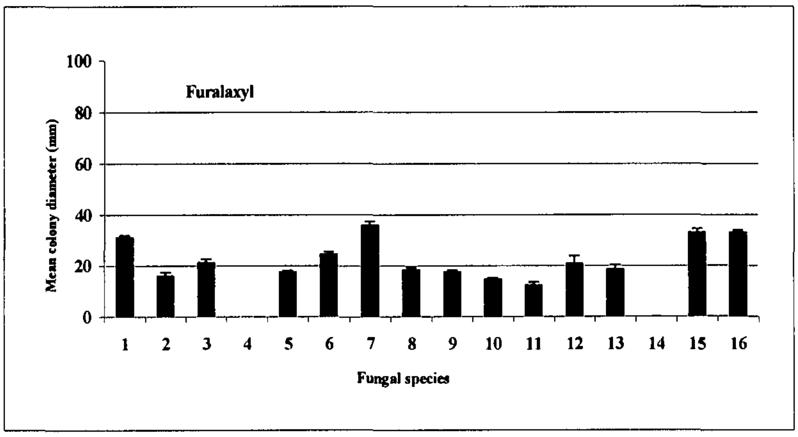
Figure 1: Mean colony diameter of 15 species of fungi on each of the four individual fungicidal constituents comprising the Botryosphaeria selective medium. The species are as follows: 1) *Trichoderma* sp.; 2) *Cerebella andropogonis*; 3) *Fusarium equiseti*; 4) *Fusarium oxysporum*; 5) *Dreshlera* sp.; 6) *Aspergillus niger*; 7) *Mucor* sp.; 8) *Phoma* sp.; 9) *Alternaria alternata*; 10) *Alternaria tenuissima*; 11) *Botrytis cinerea*; 12) *Penicillium glabrum*; 13) *Cytospora* sp.; 14) *Epicoccum purpurascens*; 15) *Botryosphaeria obtusa*; 16) *Botryosphaeria dothidea*. Each bar is denoted with a standard deviation ($P \leq 0.05$) bar to show significant differences.


Tannic Acid
Mean colony diameter (mm)
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Fungal species


Gallic Acid
Mean colony diameter (mm)
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Fungal species


Furalaxyl
Mean colony diameter (mm)
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Fungal species

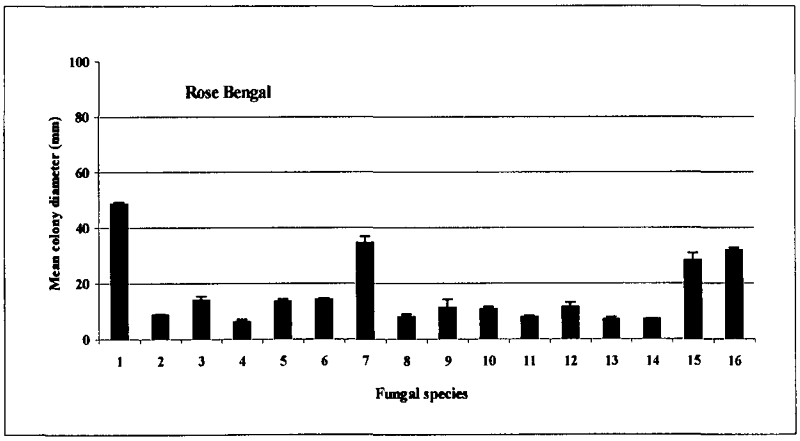
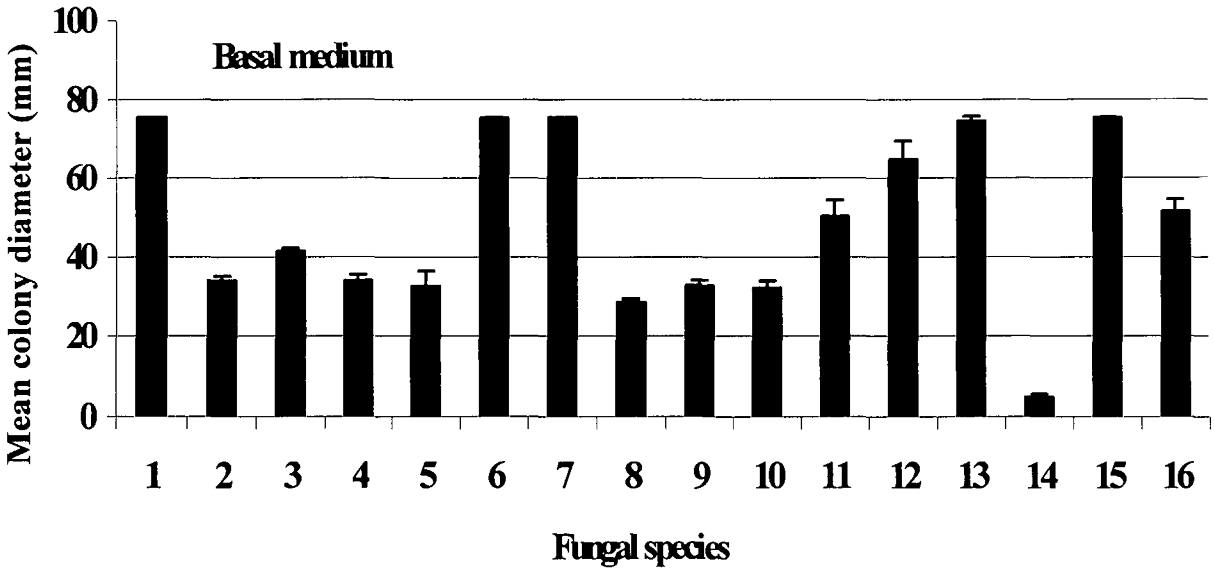
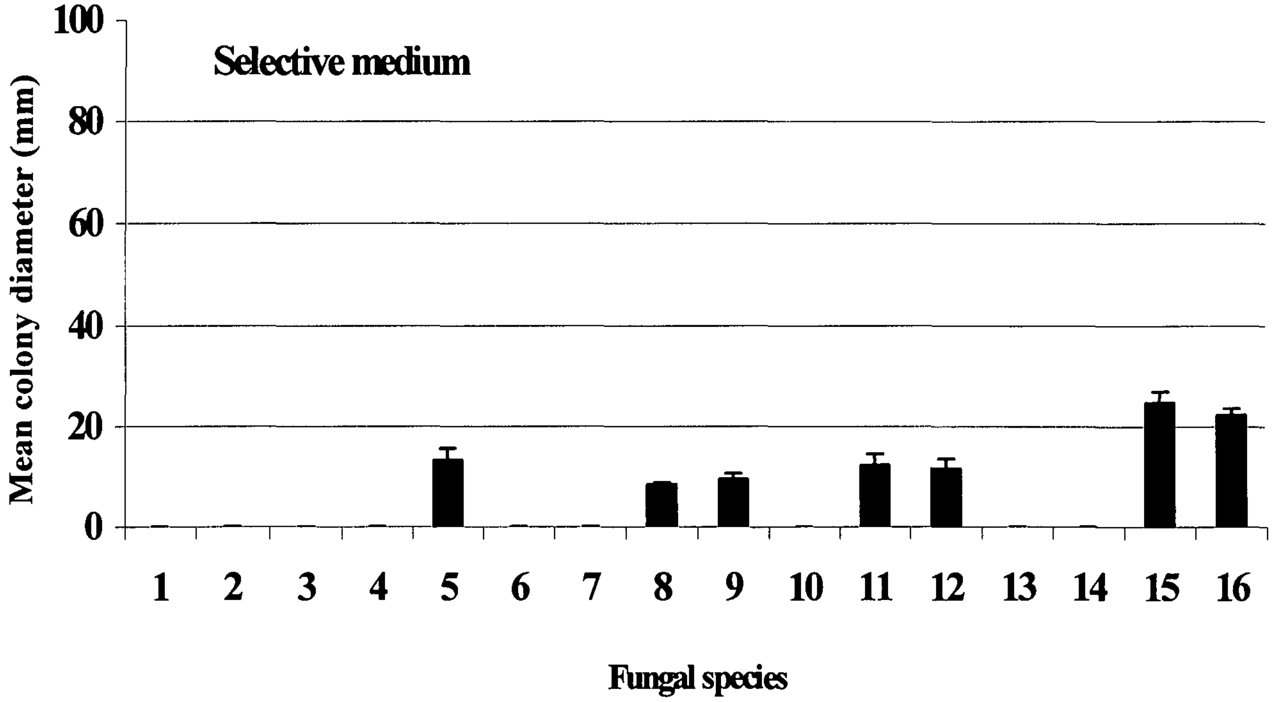

Rose Bengal
Mean colony diameter (mm)
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Fungal species

Figure 2: Comparative colony growth of 15 fungal species on basal medium (10g Difco malt extract, 15g Difco Technical agar and 1000ml of distilled water) used as control and selective medium: 1) *Trichoderma* sp.; 2) *Cerebella andropogonis*; 3) *Fusarium equiseti*; 4) *Fusarium oxysporum*; 5) *Dreshlera* sp.; 6) *Aspergillus niger*; 7) *Mucor* sp.; 8) *Phoma* sp.; 9) *Alternaria alternata*; 10) *Alternaria tenuissima*; 11) *Botrytis cinerea*; 12) *Penicillium glabrum*; 13) *Cytospora* sp.; 14) *Epicoccum purpurascens*; 15) *Botryosphaeria obtusa*; 16) *Botryosphaeria dothidea*. Each bar is denoted with a standard deviation bar to show significant difference ($P \leq 0.05$).


Basal medium
Mean colony diameter (mm)
0
20
40
60
80
100
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
Fungal species


Selective medium
Mean colony diameter (mm)
0
20
40
60
80
100
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
Fungal species

CHAPTER 3

IN VITRO EFFECT OF FUNGICIDES ON ISOLATES OF *BOTRYOSPHAERIA OBTUSA* AND *B. DOTHIDEA* COLLECTED FROM PISTACHIO ORCHARDS

ABSTRACT

Botryosphaeria obtusa and *B. dothidea* are important pathogens of pistachio (*Pistacia vera* L) for which no chemical control strategy currently exists in South Africa. Reduction of radial colony diameter of isolates of both pathogens was determined on fungicide-amended potato-dextrose agar media. Fungicides were tested at the following concentrations: 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 25 and 50 µg active ingredient/ml in order to determine EC_{50} values. EC_{50} values (µg/ml) for *B. obtusa* and *B. dothidea* respectively, were as follows: chlorothalonil (5.34; 9.4), furalaxyl (348.31; 437.69), kresoxim-methyl (5.05; 96.02), benomyl (0.011; 0.024), carbendazim/difenoconazole (0.007; 0.041), difenoconazole (0.014; 0.031) and tebuconazole (0.025; 0.034). From these results it would appear that the application of benomyl or carbendazim/difenoconazole during bloom and tebuconazole and difenoconazole applied monthly throughout the season, combined with effective sanitation measures, should be a successful management strategy for controlling Botryosphaeria diseases in South African pistachio orchards.

INTRODUCTION

Botryosphaeria panicle and shoot blight caused by *Botryosphaeria dothidea* (Moug.:Fr.) Ces. & de Not. (syn. *B. ribis* Gross. & Duggar) is one of the most devastating diseases of pistachio (*Pistacia vera L.*) in California (Crane & Maranto, 1988; Michailides, 1991; Michailides & Morgan, 1992; Michailides *et al.*, 1998). Serious diseases of blueberry (Cline & Milholland, 1992), apples (Parker & Sutton, 1993a), chaparral (Brooks & Ferrin, 1994), pears (Ko *et al.*, 1996) and camphor (Deng & Guo, 1998) have also been reported to be caused by this fungus. In southern Africa, *B. dothidea*, has been associated with die-back of *Eucalyptus* spp. (Smith *et al.*, 1994; Smith *et al.*, 1996a; Whittle & Ratten, 1997, Smith *et al.*, 1998), *Pinus* spp. (Smith *et al.*, 1994; Smith *et al.*, 1996b; Smith *et al.*, 1998) and *Protea* spp. (Von Broembsen & Van der Merwe, 1990). Swart & Blodgett (1998) first reported *B. dothidea*, causing panicle and shoot blight, on pistachio trees in the Prieska area of South Africa.

Botryosphaeria obtusa (Schwein.) Shoemaker (syn. *Physalospora obtusa* (Schwein) Cooke) is reported to be a disease causing agent of peach (Britton & Hendrix, 1982; Pusey, 1989), apple (Arauz & Sutton, 1990; Ellis, 1998) and grapevine (Contesini & Faretra, 1991) causing a variety of symptoms. On apple it causes leaf spot or "frog-eye", blossom-end rot and black fruit rot (Venhatasubbaiah *et al.*, 1991; Ellis, 1998; Travis *et al.*, 1999) and in peach orchards, tree gummosis (Britton & Hendrix, 1986; Pusey, 1989). Swart & Botes (1995) first reported stem canker of pistachio caused by *B. obtusa*.

Both *B. dothidea* and *B. obtusa* have the potential to cause major losses in pistachio orchards in South Africa. Effective control of these pathogens is therefore a prerequisite for the successful cultivation of the crop in South Africa. Chemical control of *B. obtusa* and *B. dothidea* on woody plants is well documented. Benomyl, thiophanate methyl, carbendazim, mancozeb, Bordeaux mixture, tiabendazole, tebuconazole and vinclozolin allow for excellent control of *B. dothidea* (Cline & Milholland, 1992; Parker & Sutton, 1993b; Ko *et al.*, 1996; Deng & Guo, 1998). Benomyl, applied during full bloom is currently the only fungicide registered in California for the control of Botryosphaeria panicle or shoot blight (Michailides & Morgan, 1992; Michailides *et al.*, 1995; Michailides, 1999). Tebuconazole, although not registered as yet, is also recommended as soon as symptoms are detected in orchards (Parker & Sutton, 1993b; Michailides, 1999). Recently another fungicide Stratego®, a pre-mixture of propiconazole and trifloxystrobin, provided the best control of the disease in California, especially when applied from early June to mid August 1998 (Michailides et al., 1999). Fungicides that have been effectively used against *B. obtusa* include benomyl, bitertanol, flusilazole, myclobutanil, penconazole, carbendazim, tridemorf and tebuconazole (Beisel *et al.*, 1984; Brown II & Britton, 1986; Sharma, 1988; Sutton & Arauz, 1990; Contesini & Faretra, 1991; Travis *et al.*, 1999). In the present study, the potential of various other fungicides to prevent diseases caused by South African isolates of *B. obtusa* and *B. dothidea* was determined by means of *in vitro* studies.

MATERIALS AND METHODS

Six monoconidial isolates that originated from the anamorphs of *Botryosphaeria obtusa* (anamorph: *Sphaeropsis* sp.) and six of *B. dothidea (*anamorph*: Fusicoccum aesculi)*, collected from cankered stems and blighted shoots of pistachio trees in Prieska, South Africa, were used in all screening trials. Autoclaved potato-dextrose agar (PDA) (Difco) was cooled to 45°C and amended with aqueous stock solutions of each of the following fungicides: kresoxim-methyl (Stroby®–500 g/kg WP), tebuconazole (Folicur®–250 g/l EC), difenoconazole (Score®–250 g/l EC), benomyl (Benlate®-500 g/kg WP), furalaxyl (Fongarid®-250g/kg WP), chlorothalonil (Bravo®-500g/l EC) and carbendazim/difenoconazole (Eria®-187.5 g/l EC). In the first experiment, fungicides were added to the PDA in the following concentrations: 0.5, 1, 5, 10, 25 and 50 µg a.i./ ml. Each treatment was replicated three times and the experiment was repeated. In a separate experiment, benomyl, tebuconazole, carbendazim/difenoconazole and difenoconazole were tested at lower concentrations of 0.01, 0.05, 0.1, 0.5, 1 and 5 µg a.i./ml.

Isolates were allowed to grow for 6 days on PDA prior to a 5-mm-diameter agar plug being removed with a cork-borer and placed mycelium side down onto 90 mm plates containing 25 ml fungicide amended agar. Plates were incubated at 25 °C and radial colony growth was determined by calculating the mean of two perpendicular measurements of colony diameter on each plate after 72 hours. Inhibition of colony growth compared to the unamended control treatment was determined. The percentage a.i. resulting in 50% inhibition of colony diameter (effective concentration (EC_{50}) compared to the control was determined for each isolate by plotting inhibition values transformed to probits against the log concentration of fungicides (Bliss, 1935).

Variances between trials in respective experiments were tested for homogeneity using Bartlett's test (Bartlett, 1937) and a two-way analysis of variance (ANOVA) was performed on the pooled data. The statistical program NCSS 2000 (BMDP Statistical

Software Inc., Los Angeles, CA) was used for analyses of variance and for calculating standard deviations of treatment means. The EC_{50} value of each fungicide was determined and Tukey's HSD procedure for comparison of means (Steele & Torrie, 1980) was applied where a factorial analysis of variance showed significant variation between treatments.

RESULTS

Benomyl, carbendazim/difenoconazole, difenoconazole and tebuconazole strongly inhibited radial growth of both *B. obtusa* and *B. dothidea* on PDA (Fig. 1). There was no significant difference between these fungicides for both *Botryosphaeria* species. (Fig. 1 and 2). The mean EC_{50} values of these fungicides were 0.011, 0.007, 0.014 and 0.025 µg/ml respectively for *B. obtusa* and 0.0237, 0.0409, 0.031 and 0.0338 µg/ml for *B. dothidea* (Table. 1). Chlorothalonil and kresoxim-methyl also had low EC_{50} values (Fig. 2). The mean EC_{50} values for these two fungicides were 5.34 and 5.05 µg/ml for *B. obtusa* and 9.4 and 96.02 µg/ml for *B. dothidea*, respectively (Table 2). There was no significant difference between the two fungicides towards *B. obtusa* (Fig 1) although chlorothalonil showed significantly lower EC_{50} values for *B. dothidea* (Fig 2). Furalaxyl was the least effective fungicide with mean EC_{50} values of 348.31 and 437.69 µg/ml respectively for *B. obtusa* and *B. dothidea* (Table 2). With the exception of furalaxyl, there were no significant differences between the isolates of the two *Botryosphaeria* sp. (Tables 1 and 2). The largest difference between isolates was observed for *B. dothidea* where the EC_{50} was 92.02 for isolate CWS178 while that for isolate CWS192 was 1630.81µg/ml (Table 2). The same tendency was observed with *B. obtusa* isolates.

There was a significantly higher EC_{50} value for *B. dothidea* (Fig. 1 and 2) on all the fungicides tested except tebuconazole and chlorothalonil, which both had very low EC_{50} values for both fungi.

DISCUSSION

The fungicide that was the most inhibitory in this study was Eria®, a mixture of carbendazim and difenoconazole. Consistent with published literature, the three systemic fungicides, tebuconazole, difenoconazole and benomyl also displayed significant levels of inhibition to both *Botryosphaeria* species. The EC_{50} values of benomyl and tebuconazole are similar to those obtained by Arauz & Sutton (1990) and Parker & Sutton (1993b). Arauz & Sutton (1990) reported *in vitro* inhibition of *B. obtusa* with benomyl, bitertanol, flusilazole and tebuconazole. Tebuconazole has also shown effective control of *B. obtusa* on apple trees (Sutton & Arauz, 1990).

These fungicides are sprayed to control shoot blight of pistachio caused by *B. dothidea* in California (Worthing & Hance, 1991; Michailides, 1999). Tebuconazole is applied four times during the growing season and benomyl is sprayed during bloom in the spring (Worthing & Hance, 1991; Michailides *et al.*, 1995). Michailides & Morgan (1992) however observed variable results when benomyl was applied in different pistachio orchards. This variable control might be attributed to genetic variation in *B. dothidea*. Differences in the benomyl EC_{50} values of different *B. dothidea* isolates, ranging from 0.0074 to 0.0173, a difference of 43%. Although these two isolates differ the difference between the six isolates were not significant and therefore the data was pooled after doing Bartlett's test. There are numerous recent reports of resistance to benomyl by various fungi (McGrath, 1996; Romero & Sutton, 1998; Everts, 1999). Differences in the sensitivity among isolates of both species were also observed for other fungicides tested, but were only significant for furalaxyl.

According to Lyr (1995), variability in fungicide sensitivity within one fungal species is a natural phenomenon when the species has had no previous contact with a particular fungicide, and no shifts in the level of resistance have occurred. The concentration decided on should therefore be low enough to make the application economically viable, but high enough to ensure that resistance does not develop. It follows that a fungus, which becomes resistant to benomyl, can also become resistant to carbendazim, which is also a benzimidazole fungicide (cross-resistance) (Martin & Worthing, 1976; Worthing & Hance, 1991). In some cases this phenomenon can however lead to higher sensitivity to the same group of fungicides (negative cross-resistance) (Sisler, 1988).

A significant difference in sensitivity between the two *Botryosphaeria* species towards all the fungicides tested, except tebuconazole and chlorothalonil, suggests the possible use of these traits to distinguish between the two species using a differential medium. In all instances, but especially in the case of benomyl and kresoxim-methyl, *B. obtusa* had lower EC_{50} values than *B. dothidea*.

It has been proven that *B. dothidea* is an endophyte in *Eucalyptus* spp. and *Pinus* spp. (Smith *et al.*, 1994; Smith *et al.*, 1996a; Smith *et al.*, 1996b; Smith *et al.*, 1998). We have also isolated *B. dothidea* as an endophyte from pistachio bark tissue (see Chapter 2). Due to this phenomenon it is possible that the use of a systemic fungicide might be more effective than contact fungicide applied on the surface of a plant. This should however first be proven under filed conditions.

The time of fungicide application is very important and seasonal differences between the two species will have to be taken into consideration. *B. dothidea* predominates in summer and fall while *B. obtusa* predominates during winter and spring (Britton & Hendrix, 1982; Britton & Hendrix, 1986; Brown II & Britton, 1986; Biggs & Britton, 1988). Benomyl therefore, should be applied during bloom (spring) for the control of *B. dothidea* (Michailides & Morgan, 1992, Michailides *et al.*, 1999). Tebuconazole should be applied during late summer for the control of *B. obtusa* (Sutton & Arauz, 1990). Travis *et al.* (1999) showed that the longer the time period which elapsed between fungicide applications the higher the incidence of disease. He suggested that fungicides should therefore be applied throughout the active growing period for the control of *B. obtusa*.

From the results presented here, it would appear that the application of fungicides by means of sprinkler irrigation should be investigated to control both *Botryosphaeria* species. A benomyl application during bloom and tebuconazole applied in four in-season sprays throughout the season should effectively control both pathogens (Beisel *et al.*, 1984; Brown II & Britton, 1986; Michailides, 1992; Michailides *et al*, 1995; Cline *et al.*, 1997). Stratego®, although not yet available in South Africa, also seems to be an effective control measure (Michailides *et al.*, 1999). The judicious application of these fungicides combined with effective sanitation practices, should therefore form part of a successful disease management plan for Botryosphaeria diseases in South African pistachio orchards (Michailides, 1991; Ellis, 1998).

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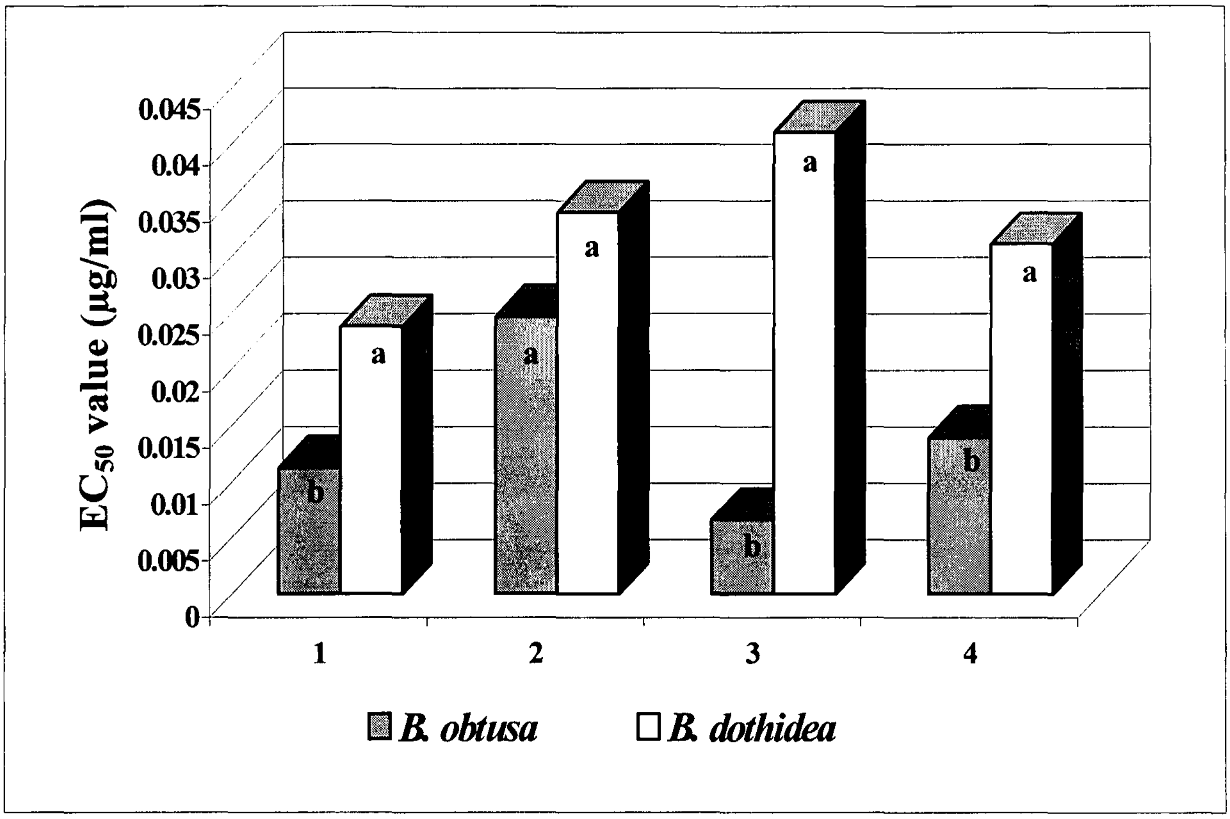
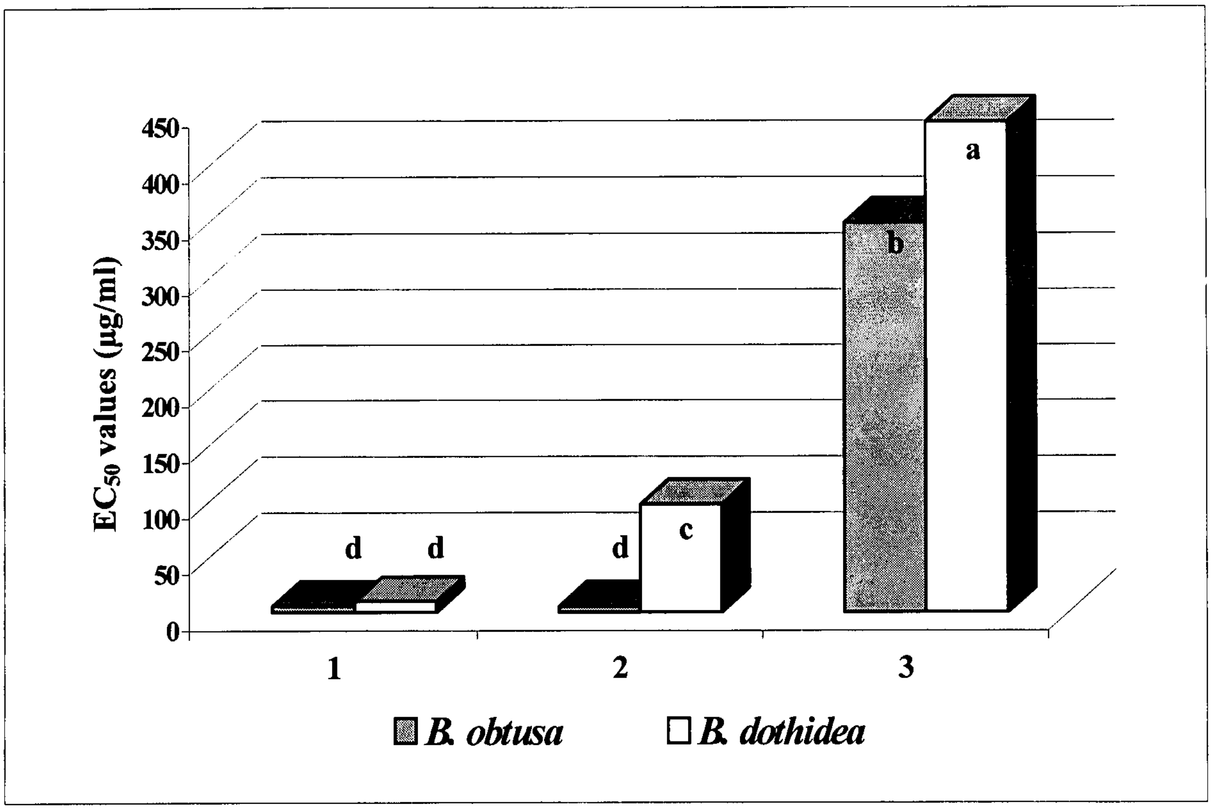
Figure 1: Mean EC_{50} values of the four most effective fungicides tested against *Botryosphaeria obtusa* and *Botryosphaeria dothidea.* Bars denoted by different letters are significantly different ($P<0.05$) according to Tukey's HSD procedure.



Number	Fungicide
1	Benomyl
2	Tebuconazole
3	Carbendazim/Difenoconazole
4	Difenoconazole

Figure 2: Mean EC_{50} values of the three least effective fungicides tested against *Botryosphaeria obtusa* and *Botryosphaeria dothidea*. Bars denoted by different letters are significantly different ($P<0.05$) according to Tukey's HSD procedure.



Number	Fungicide
1	Chlorothalonil
2	Kresoxim-methyl
3	Furalaxyl

Table 1: Mean EC_{50} values recorded for the four most effective fungicides against isolates of *Botryosphaeria obtusa* and *Botryosphaeria dothidea*. Standard deviation values are in brackets ($P<0.05$).

Mean EC_{50} values per fungicide*				
Isolate	**Benomyl**	**Tebuconazole**	**Carbendazim/ Difenoconazole**	**Difenoconazole**
B. obtusa				
CWS154	0.011(±0.0007)	0.0235(±0.0004)	0.0081(±0.0021)	0.0157(±0.0036)
CWS155	0.0098(±0.0019)	0.0242(±0.0042)	0.0062(±0.0004)	0.0064(±0.0012)
CWS156	0.0083(±0.0013)	0.0245(±0.0020)	0.0044(±0.0007)	0.0121(±0.0037)
CWS158	0.0173(±0.0040)	0.0237(±0.0046)	0.0062(±0.0016)	0.0141(±0.0007)
CWS192	0.0145(±0.0015)	0.0273(±0.0020)	0.01(±0.0012)	0.0079(±0.0021)
CWS178	0.0074(±0.0008)	0.0245(±0.0027)	0.0048(±0.0048)	0.0275(±0.0124)
B. dothidea				
JB 12-6	0.6864(±0.0959)	0.0017(±0.0006)	0.0464(±0.0065)	0.0017(±0.0006)
CWS92	0.2142(±0.1531)	0.034(±0.0022)	0.0243(±0.0028)	0.0340(±0.0022)
CWS93	0.2376(±0.0334)	0.0276(±0.0015)	0.0474(±0.0149)	0.0276(±0.0015)
CWS182	0.025(±0.0032)	0.053(±0.013)	0.0187(±0.0094)	0.0530(±0.013)
CWS192	0.1023(±0.0278)	0.0468(±0.0043)	0.0574(±0.0056)	0.0468(±0.0043)
CWS178	0.1583(±0.0204)	0.0231(±0.0038)	0.0512(±0.0046)	0.0231(±0.0038)

*Values are in μg active ingredient/ml

*Standard deviation values are in parentheses

Table 2: Mean EC_{50} values recorded for the three least effective fungicides against isolates of *Botryosphaeria obtusa* and *Botryosphaeria dothidea*. Standard deviation values are in brackets ($P<0.05$).

Mean EC_{50} values per fungicide*			
Isolate	**Furalaxyl**	**Chlorothalonil**	**Kresoxim-methyl**
B. obtusa			
CWS154	282.033(±10.069)	3.2169(±0.305)	2.4066(±0.4043)
CWS155	270.5696(±27.1647)	7.0184(±0.5175)	1.2756(±0.2304)
CWS156	148.516(±40.2947)	5.3051(±0.4207)	1.8632(±0.3869)
CWS158	213.9047(±87.1306)	0.5323(±0.0822)	2.4227(±0.1888)
CWS192	1030.8556(±354.577)	3.8014(±0.2283)	1.6488(±0.2267)
CWS178	143.9721(±19.782)	12.1703(±2.2627)	20.6815(±1.9168)
B. dothidea			
JB 12-6	136.4531(±29.4257)	6.0715(±1.1965)	27.6901(±6901)
CWS92	107.8857(±23.5564)	4.2133(±0.951)	212.7739(±75.5735)
CWS93	507.7178(±157.7579)	24.2637(±1.421)	47.903(±19.2632
CWS182	151.2395(±70.6427)	0.9049(±0.3287)	46.8305(±14.4891)
CWS192	1630.8153(±322.5989)	0.7801(±0.0402)	0.495(±0.0065
CWS178	92.0238(±29.6787)	20.1904(±7.3864)	240.4304(±64.7393)

*Values are in µg active ingredient/ml

*Standard deviation values are in parentheses

CHAPTER 4

INFLUENCE OF WATER STRESS ON THE SUSCEPTIBILITY OF PISTACHIO SEEDLINGS TO INFECTION BY *BOTRYOSPHAERIA OBTUSA* AND *B. DOTHIDEA*

ABSTRACT

Pistachios are a new crop in South Africa with considerable commercial potential providing constraints such as disease can be overcome. The role of environmental stress in the predisposition of trees to infection by facultative parasites is particularly important. *Botryosphaeria obtusa* was identified as a pathogen causing cankers in pistachio orchards in 1995 and *B. dothidea* was isolated from blighted shoots in the same orchards in 1998. Both pathogens were associated with drought stress. Trials were conducted with *B. obtusa* and *B. dothidea* to determine the effect of water potential on radial growth of the two species *in vitro* on agar media. Although the control treatments did not differ, *B. obtusa* grew significantly faster than *B. dothidea* at all water potentials except for –5 MPa. In greenhouse trials, rootstocks of *P. integerrima, P. terebinthus* and Clone II (*P. integerrima* X *P. atlantica*) that had been stressed by withholding water for six weeks, were inoculated with mycelium of *B. obtusa* and *B. dothidea.* Stressed trees were kept at a water potential of –4.0 to –6.0 MPa while control trees were kept at field water capacity. In contrast to its slower *in vitro* growth rate compared to *B. obtusa, B. dothidea* caused the longer lesions. All stressed *P. terebinthus* plants inoculated with *B. dothidea* showed significantly longer lesions than control trees, while *P. integerrima* displayed the greatest resistance to the pathogen as expressed by short lesions.

INTRODUCTION

Pistachio (*Pistacia vera* L.) is a native of Central Asia, and is thus adapted to the hot, dry summers of high desert regions. Although it is a relatively drought tolerant plant, successful commercial production requires irrigation (Crane & Maranto, 1988; Ferguson & Arpaia, 1990). In the semi-arid Prieska area of the Northern Cape province of South Africa where pistachio is being cultivated for export purposes, an intensive micro-irrigation system with water drawn from the Orange River is being employed (Nofal, 1997; Jooste, 1998). Trees, however, are hardened-off prior to the onset of winter by terminating irrigation at the end of April. Stress associated with the end of irrigation could predispose trees to disease.

Organisms that attack plants which are weakened or predisposed by environmental stress are normally facultative parasites or opportunistic pathogens which often occupy an ephemeral niche within their host (Schoeneweiss, 1981; Houston, 1992; Klepzig *et al.*, 1996). They are usually incapable of causing disease in healthy hosts but can, however, kill or accelerate the decline of stressed trees (Houston, 1992; Manion & Lachange, 1992; Smith *et al.*, 1994; Smith *et al.*, 1996a).

Botryosphaeria species have often been reported as facultative parasites (Schoeneweiss, 1981;Britton & Hendrix 1986) and physiological stress of host plants is a major factor contributing to disease caused by these fungi. Such stresses include drought, freezing and defoliation (Crist & Schoeneweiss, 1975; Wene & Schoeneweiss, 1980; Pusey, 1989). The present study investigated the effect of water stress on the radial growth of *B. obtusa* and *B. dothidea in vitro*, and their pathogenicity on three genotypes of pistachio following artificial inoculations in the glasshouse.

MATERIALS AND METHODS

***In vitro* water stress:** Water stress was artificially induced using the method described by Kidd et al. (1977) and Koske & Tessier (1986) where polyethylene glycol 4000 (mol wt = 3000-3700) is incorporated into a liquid nutrient medium. The percentage of PEG 4000 needed to establish water potential at a given level is determined by the following equation: % PEG 4000 = 23.8 log x –0.83 where x is the desired water potential (Mexal & Reid, 1973).

Glass bars (5 mm diameter) were placed in a 90-mm petri plate, and a disk of fine nylon mesh cut to the diameter of the petri dish was suspended on the bars. Thirty ml of sterilized, single-strength modified Melin-Norkrans (MMN) solution (Marx, 1969) was used to saturate the nylon mesh cloth but not submerge it. A stock solution of 60% PEG 4000 was used to adjust the water potential of the Melin-Norkrans solution as follows: -0.5, -1, -2, -3, -4 and -5 MPa. Each treatment was replicated three times and the experiment was repeated.

One isolates (most pathogenic from the isolates in chapter 3) of the two *Botryosphaeria* species were cultured on potato-dextrose agar (PDA) (39g Difco potato dextrose agar) for 6 days prior to a 5-mm-diameter agar plug being removed with a cork-borer and placed with the mycelium side down onto the nylon mesh suspended in the various solutions. Plates were incubated at 25°C and colony diameter was determined by calculating the mean of two perpendicular measurements after 96 hours. The experiment was conducted twice and variances among trials were tested for homogeneity using Bartlett's test (Bartlett, 1937) before a two-way analysis of variance (ANOVA) was performed on the pooled data using the statistical program NCSS 2000 (BMDP Statistical Software Inc., Los Angeles, CA).

Glasshouse trials: Two-year-old potted plants (15l pots) of *Pistacia terebinthus*, *P. integerrima* and Clone II (*P. integerrima* x *P. atlantica*) were selected and placed in a glasshouse under controlled environmental conditions. The trees were planted in a 50% sand and 50% peat medium. Day (13 hours) temperatures in the glasshouse did not exceed 30°C while night (11 hours) temperatures never dropped below 10°C. Two treatments, with 12 trees of each rootstock in each treatment, were used and the experiment was conducted twice. A complete randomized design was employed in both experiments. In the first treatment the trees were stressed by withholding water until the leaf water potential, measured using the pressure chamber method developed by Scholander et al. (1964), was between –4.5 and –6.0 MPa. In the second treatment (control), trees were kept at field water capacity by watering the trees thoroughly every other day and maintaining their average leaf water potential between –1.0 and –1.2 MPa for the following six weeks. Water potential measurements of leaves were done between 05:00 and 05:30 (before sunrise) every second day for the duration of the trial. Plants were fertilized with a mixture (10g/liter water) containing N (5.6%), P (4.6%), K (2.74%), Mg (3%), S (11.2%), Fe (0.069%), Mn (0.03%), Zn (0.027%), B (0.044), Cu (0.014%), Mo (0.009%).

After six weeks the trees were inoculated with mycelium of the two respective *Botryosphaeria* species. For each species the fastest growing isolate from the fungicide trials were (Chapter 3 – CWS 155 and 12-6 JB) was chosen and mycelium was produced in a growth chamber at 25°C on Difco potato-dextrose agar (39 g PDA in 1000 ml of distilled water) overlaid with sterile cotton-gauze strips (10 x 50 mm) for 14 days. Stems (10 cm from the soil) were wounded by scraping off a small length of bark (2 x 5 mm) with a scalpel and then wrapping colonized gauze around the inoculation site. Parafilm "M" (American National Can^{TM}, Chicago, IL, 60631) was subsequently wrapped around the inoculation site to prevent desiccation of the inoculum. After inoculation the trees were maintained at the respective stress and control leaf water potential levels for another three weeks after which inoculation sites were uncovered and the length of resulting cambial lesions was determined.

Variances between trials were tested for homogeneity using Bartlett's test (Bartlett, 1937) before data were combined and an analysis of variance (ANOVA) was performed using NCSS200 (BMDP Statistical Software Inc., Los Angeles, CA). Tukey's HSD (Steele & Torrie, 1980) procedure for the comparison of means was applied where the factorial ANOVA showed significant variation between treatments.

RESULTS

***In vitro* water stress**: Increasingly negative water potential of agar media usually had a marked positive effect on the colony diameter of both fungi, especially on B. *obtusa*, which grew significantly faster ($P < 0.05$) than *B. dothidea* at all water potentials between -0.5 and –4 MPa (Fig. 1). The optimum growth rate for each fungus achieved at –2 MPa. The mean colony diameter of *B. obtusa* at –2 MPa was almost three times greater than in the control treatment. At –5 MPa very little mycelial growth occurred for both fungi (Fig. 1).

Glasshouse trials: The greatest mean length of lesion result of inoculation with *B. obtusa* and *B. dothidea* on stressed plants of *P. terebinthus* and the shortest on *P.*

integerimma (Fig. 2). Mean lesion length caused by *B. dothidea* on stressed plants of all three genotypes were significantly longer ($P < 0.01$) than on unstressed plants. Results of inoculations with *B. obtusa* on *P. integerrima* and Clone II respectively, did not differ between stressed and unstressed treatments.

DISCUSSION

The present study demonstrates that drought stress has an exacerbating effect on the pathogenicity of *B. dothidea* on all cultivars of potted pistachio plants in the glasshouse. The effect of drought stress on the pathogenicity of *B. obtusa,* however, was only apparent in *P. terebinthus.* Contradicting this results was demonstrated by two *Botryosphaeria* species *vitro* where the growth decreased at lower water potentials. Both fungi grew fastest *in vitro* at –2 MPa from where there was a steady decline to –5 MPa where virtually no mycelial growth took place (Fig. 1). Stressed plants had been kept at a leaf water capacity of –4.5 to –6.0 MPa and serious symptoms of wilting were observed, suggesting significant drought stress on these plants. Schoeneweiss (1981; 1983) reported that woody plants remain resistant to attack by *B. dothidea* until the plant water potential reaches a threshold level of –1.2 MPa. From this threshold, the extent of fungal colonization was found to increase with decreasing water potential.

The fact that *B. obtusa* grew significantly faster than *B. dothidea in vitro* is contrast to the significantly ($P < 0.05$) smaller lesions which *B. obtusa* caused on non-stressed and stressed plants of the two most resistant cultivars (Fig. 2). The difference between the two pathogens was greatest in non-stressed *P. terebinthus* plants despite the fact that they grew equally fast in stressed *P. terebinthus* plants. The latter is by far the most susceptible of the three cultivars, however, a factor, which probably negates the differentiating effect, that water stress has on the two pathogens. This disparity in growth of the two pathogens in the host is probably related to the fact that *B. dothidea* has been more commonly associated with disease of drought stressed woody plants than *B. obtusa* (Schoeneweiss 1981; Fisher *et al.*, 1993; Smith *et al.*, 1996a; Smith *et al.*, 1996b). As far as could be determined, *B. obtusa* has only been associated with a single instance of canker on drought stressed apple trees (Holmes & Rich, 1969) and reportedly prefers warm, moist conditions for optimal growth (McPartland & Schoeneweiss, 1984; Beisel et al., 1984; Arauz & Sutton, 1990a; Arauz & Sutton, 1990b; Smith et al., 1994).

The results presented here suggest that not hardening-off trees prior to winter, and irrigating them throughout winter, might help to prevent disease caused by opportunistic pathogens such as *Botryosphaeria* spp. However, this must be seen in the context of winter injury (Schoeneweiss, 1974; Perkins et al., 1991) which may then result and predispose trees to disease caused by these pathogens (see next chapter). One possible measure might therefore be to lengthen the hardening-off period by slowly decreasing the water supply and not ending it abruptly as is currently being practiced in pistachio orchards in South Africa.

Genetic variation among pistachio rootstocks should also be considered as an additional control measure. *P. terebinthus* is the most susceptible rootstock when predisposed by drought. The results of this study suggest that *P. integerrima* is the most resistant rootstock, closely followed by Clone II. By integrating the use of resistant material with a well-planned irrigation program, and possible chemical application, a significant decrease in the incidence and impact of *Botryosphaeria* diseases in South African pistachio orchards is possible.

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Figure 1: Influence of *in vitro* water potential on the growth of *B. obtusa* and *B. dothidea*. Standard deviation bars indicate a significant difference between treatments ($P \leq 0.05$).

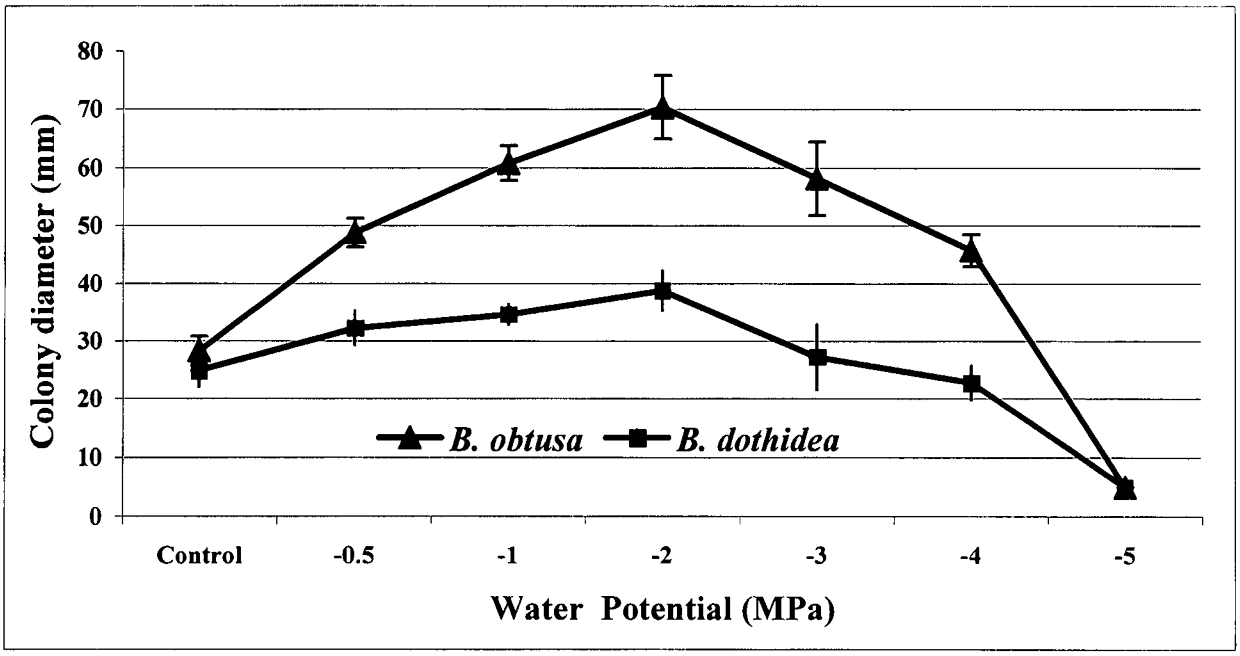
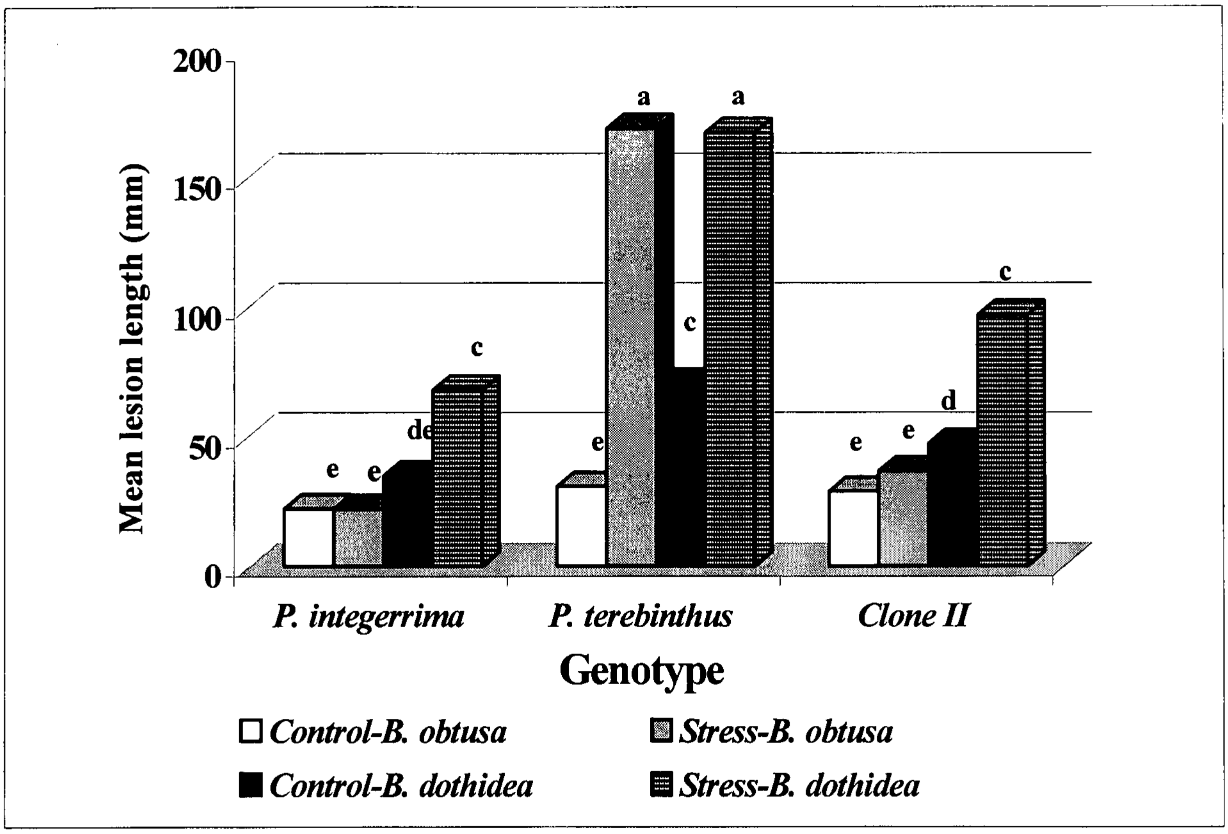

Colony diameter (mm)
80
70
60
50
40
30
20
10
0
B. obtusa
B. dothidea
Control
-0.5
-1
-2
-3
-4
-5
Water Potential (MPa)

Figure 2: The susceptibility of potted plants of three pistachio genotypes to *Botryosphaeria obtusa* and *B. dothidea* under induced water stress. Bars denoted by different letters are significantly different ($P<0.05$) according to Tukey's HSD procedure.


200
150
100
50
0
Mean lesion length (mm)
a
a
c
c
c
d
de
e
e
e
e
e
P. integerrima
P. terebinthus
Clone II
Genotype
□ Control-B. obtusa
▨ Stress-B. obtusa
■ Control-B. dothidea
▤ Stress-B. dothidea

CHAPTER 5

INFLUENCE OF COLD STRESS ON THE SUSCEPTIBILITY OF PISTACHIO SEEDLINGS TO INFECTION BY *BOTRYOSPHAERIA OBTUSA* AND *B. DOTHIDEA*

ABSTRACT

Isolations from diseased trees in 1995 and 1998 proved that *Botryosphaeria obtusa* and *B. dothidea* could cause serious damage to pistachio trees under commercial production in South Africa. An important factor that can predispose trees to these pathogens is frost damage. Early frost in autumn and late frost in spring are problems encountered in the Prieska area of the Northern Cape province of South Africa where pistachios are cultivated. Three different pistachio rootstocks (*Pistacia terebinthus, P. integerrima* and Clone II (*P. integerrima* X *P. atlantica*) were subjected to a sudden drop in temperature under controlled environmental conditions to determine if chilling can predispose stem tissue to faster colonisation by *B. obtusa* and *B. dothidea*. Following a three week acclimation period, two-year-old potted plants were exposed to an initial temperature of 10°C for 8 hours that was subsequently reduced to –7°C for 60 minutes. Plants were then inoculated on stems through small wounds with the two *Botryosphaeria* spp. and the resulting cambial lesions were measured after 3 weeks. There was a significant difference between stressed and non-stressed (control) trees of *P. integerrima* and Clone II. *Pistacia integerrima* was the most susceptible rootstock and *P. terebinthus* the least susceptible. *Botryosphaeria dothidea* caused the most severe lesions in all treatments.

INTRODUCTION

Pistachio (*Pistacia vera* L.) originated in Central Asia where high summer temperatures, very little rainfall and low winter temperatures are the rule (Crane & Maranto, 1988; Küden *et al.*, 1992; Küden *et al.*, 1995). Certain parts of South Africa such as the Northern Cape province in which long, hot summers and cold winters occur which are ideally suited for pistachio cultivation. Although pistachios can tolerate low winter temperatures, it is sensitive to early frosts or a sudden drop in temperature before plants have become acclimated (Crane & Maranto, 1988; Ferguson *et al.*, 1989; Nofal, 1997). Plants exposed to such conditions become predisposed to attack by non-aggressive, opportunistic pathogens, particularly stem canker fungi (Schoeneweiss, 1974; Schoeneweiss & Wene, 1977; Weaver, 1978; Schoeneweiss; 1981). In recent years considerable damage has been caused by *B. obtusa*, and to a lesser extent *B. dothidea*, to pistachio rootstocks in commercial orchards near Prieska, Northern Cape province (Swart & Botes, 1995; Swart & Blodgett, 1998). Differences in the susceptibility to frost damage between three genotypes of rootstock have also been observed in these orchards. This has led to speculation regarding the possible role of frost damage in predisposing trees to attack by these two species of *Botryosphaeria*.

Freezing stress can lead to frost cracks, which are ideal sites for fungal infection (Kramer & Kozlowski, 1979, Salisbury & Ross, 1992) although freezing stress is not a prerequisite for infection. The metabolism of a plant is disrupted during freezing stress resulting in reduced plant growth (Salisbury & Ross, 1992). Environmental stress is a major factor contributing to disease development caused by *Botryosphaeria* spp., particularly drought, freezing and defoliation (Crist & Schoeneweiss, 1975; Wene &

Schoeneweiss; 1980; Pusey, 1989). *Botryosphaeria dothidea* (Moug.:Fr.) Ces. & De Not and *Botryosphaeria obtusa* (Schwein.) Schoemaker are known to attack more than 100 genera of woody plants (Sinclair *et al.*, 1987; Sutton & Arauz, 1991; Michailides, 1991; Swart & Botes, 1995; Travis *et al.*, 1999b).

Botryosphaeria dothidea is an endophyte of woody species such as *Pinus* spp. and *Eucalyptus* spp. and has been shown too readily colonize host tissue that has suffered frost damage (Fisher *et al.*, 1993; Smith *et al.*, 1996a; Smith *et al.*, 1996b). *B. dothidea* was also isolated from symptomless pistachio stem tissue in South Africa which confirms its endophytic status (results to be published). *Botryosphaeria obtusa,* which thrives during periods of warm and humid weather (Beisel et al., 1984; Arauz & Sutton, 1990a; Arauz & Sutton, 1990b) has been shown to enter woody tissue via frost cracks (Beisel et al., 1984; Venkatasubbaiah et al., 1991; Michailides, 1991).

The aim of the present study was to determine the relative importance of these two *Botryosphaeria* species in causing stem canker of pistachio rootstocks that have been exposed to freezing damage. The two main objectives of this study were: a) to determine the effect of different temperatures on radial growth of the two *Botryosphaeria* species *in vitro,* and b) to determine the effect of below freezing temperatures as a factor predisposing rootstocks of pistachio to disease caused by *B. obtusa* and *B. dothidea* on three different.

MATERIALS AND METHODS

***In vitro* growth of fungi.** Four monoconidial isolates of each of *B. obtusa* and *B. dothidea* were cultured for six days on malt extract agar (MEA)(20g Difco malt extract, 15g Difco technical agar in 1000 ml distilled water). A 5-mm-diameter agar plug was subsequently removed from each fungal colony with a cork-borer and placed mycelium side down on 90 mm plates containing 25 ml MEA before plates were incubated at 15, 20, 25, 30 and 35°C respectively, for 72 hours. Mean colony diameter of each isolate grown at each different temperature was measured along perpendicular lines drawn on the bottom of each plate. Each treatment was replicated four times and the experiment was conducted twice.

Variances between trials were tested for homogeneity using Bartlett's test (Bartlett, 1937), and a two-way analysis of variance (ANOVA) was performed on the pooled data. The statistical program NCSS 2000 (BMDP Statistical Software Inc., Los Angeles, CA) was used for analyses of variance and for calculating standard deviations of treatment means. Tukey's HSD procedure for the comparison of means (Steele & Torrie, 1980) was applied, where a factorial analysis of variance showed significant variation between treatments.

Growth chamber inoculations. Two-year-old potted plants of *Pistacia integerrima, P. terebinthus* and Clone II (*Pistacia integerrima* x *P. atlantica)* were pre-conditioned in a growth chamber (Conviron^R – Model PGW36, Conviron, Winnipeg, Manitoba, Canada, R3H0R9) by maintaining plants at a constant day temperature of 25°C for 10 hours and a night temperature of 10°C for 14 hours over a period of 2 weeks. Following this, plants were subjected to a sudden decrease in night temperature from 10°C to –7°C and kept at this temperature for 60 min during three consecutive nights prior to artificial inoculation with *B. obtusa* and *B. dothidea*. The drop from 10°C to –7°C was done within 70 minutes. This drop over the specific period was already measured in the pistachio orchards in Prieska. Stems were wounded 10 cm above the soil medium, by lightly scraping off a length of bark (2 x 5 mm) with a scalpel. Autoclaved strips of gauze (10 x 50 mm) that had been colonized by either *B. dothidea* (12-6 JB) or *B. obtusa* (CWS 155) after having been incubated on potato dextrose agar (PDA) at 25°C for 14 days were wrapped around each wound. Parafilm "M" (American National Can™, Chicago, IL., 60631) was then wrapped around each inoculation site to prevent desiccation of the inoculum. Inoculated plants were then maintained at 25°C day temperature and 10°C night temperature for 3 weeks. Each treatment had 12 replications and the experiment was conducted twice. The control treatment comprised plants that had not been subjected to –7°C, but were inoculated in the same manner as described above.

Three weeks after inoculation, bark surrounding each inoculation site was scraped off using a scalpel before the length of each resulting cambial lesion was measured. Variances among the two trials were tested for homogeneity using Bartlett's test (Bartlett, 1937), and a two-way ANOVA was performed on the pooled data using the statistical programme described above. Tukey's HSD procedure for comparison of means (Steele & Torrie, 1980) was applied to indicate significant variation between treatments.

RESULTS

***In vitro* growth of fungi.** *B. obtusa* showed significantly faster ($P<0.01$) colony development than *B. dothidea* at all temperatures except 35 °C. At this temperature its growth was almost completely inhibited while *B. dothidea* still showed minimal growth (Fig. 1). Optimum temperature for growth of both fungi was between 25 and 30°C.

Growth chamber inoculations. Results revealed significant differences in cambial lesion lengths between stressed and non-stressed treatments, as well as between host and pathogen genotypes (Fig. 2). Stressed plants had significantly ($P < 0.01$) longer cambial lesions than non-stressed control plants for *P. integerrima* and Clone II but not for *P. terebinthus*. The latter species was generally most resistant to both pathogens in both stressed and non-stressed treatments while *P. integerrima* was the most susceptible species.

There was a significant difference ($P < 0.01$) between *B. obtusa* and *B. dothidea* in their ability to colonize the tissue of the three pistachio genotypes. *Botryosphaeria dothidea* caused significantly longer lesions than *B. obtusa* ($P < 0.01$) for all treatments (Fig. 2). The longest lesions (mean = 100.37 mm) resulted from inoculation of cold stressed *P. integerrima* plants with *B. dothidea* (Fig. 2). The shortest lesions resulted from inoculation of control plants with *B. obtusa*.

DISCUSSION

The sudden exposure of pistachio plants to very low temperatures has an exacerbating effect on the ability of opportunistic pathogens such as *B. dothidea and B. obtusa* to colonize cambial tissue (Fig. 2). This greater susceptibility of pistachio plants to *B. obtusa* and *B. dothidea*, following exposure to freezing stress, is largely consistent with genotypic differences that exist between host genotypes in the absence of freezing stress. The most resistant rootstock to both pathogens was *P. terebinthus*. This could be attributed to it either being more tolerant to the pathogens or more tolerant to chilling which predispose tissue to pathogen attack and colonisation. This was however not proven in the experiments. More research on this subject is needed. Okie & Pusey (1996) also found differences between peach cultivars with regard to their susceptibility to chilling and attack by *B. dothidea*. Travis *et al.* (1999a) reported differences in the susceptibility of different apple cultivars to attack by *B. obtusa* following chilling injury. *P. integerrima* was the most susceptible rootstock irrespective of it being subjected to freezing stress or not.

The results of the present study also demonstrate distinct differences between *B. dothidea* and *B. obtusa* with regard to their ability to grow at different temperatures *in vitro* as well as their ability to colonize living pistachio tissue following freezing stress. Although *B. obtusa* grew faster than *B. dothidea* at temperatures up to ±32°C, its growth declined markedly above this temperature. This is consistent with the findings of numerous other workers such as Britton & Hendrix (1986) who suggested that *B. dothidea* predominates in apple trees in the summer as a result of its greater competitiveness at high temperatures, but that as soon as temperatures drop in winter months, *B. obtusa* becomes predominant. In the present study this trend was also evident from all isolations from field material (Chapter 2) where *B. dothidea* was mostly isolated during the latter part of summer and autumn. Most *B. obtusa* isolations were made during spring and at the beginning of summer suggesting that infection had taken place during winter. The cankers were always on the lower part of the stem where frost damage and frost cracks were most severe.

Another interesting observation made in the present study is the disparity that existed between *B. obtusa* and *B. dothidea* as far as their growth rate *in vitro* and in pistachio tissue is concerned. In studies conducted *in vitro, B. obtusa* grew significantly faster than *B. dothidea* at temperatures between 15 to 30°C. However, following the artificial inoculation of plants, *B. dothidea* consistently caused longer lesions in both control and stress treatments. This would seem to be consistent with findings of Britton & Hendrix (1986) who suggested that *B. obtusa* is a relatively weak canker pathogen on apple, acting more as a secondary invader. According to these authors, *B. dothidea* is generally a more aggressive pathogen in host tissue than *B. obtusa*.

It is a common practice in many parts of the world to pre-condition or "harden-off" fruit trees prior to the onset of winter by decreasing irrigation or nitrogen fertilization in order to prevent cold damage or freezing stress (Schoeneweiss, 1974; Perkins et al., 1991). The gradual decrease of irrigation is important because if done too rapidly drought stress can result in actively growing plants which in turn can lead to even greater susceptibility to freezing stress (Schoeneweiss, 1978; Cameron & Dixon, 1997).

The most efficient method of preventing freezing injury of pistachio trees in South Africa has been to cover the lower part of the stem with soil and to paint the stem white. White stems apparently ensure a more even distribution of heat at sunrise, avoiding excessive warming of the stem only on the eastern side resulting in uneven expansion of the bark tissue, which leads to cracks. In 1998, however, early frost resulted in losses of up to 85% in *P. integerrima* stands due to the fact that trees had not been covered in time. In *P. terebinthus* stands only 6% of the trees were lost due to the early frost. This observation is consistent with data of the trials conducted on potted plants in the glasshouse and suggests that *P. terebinthus* is a better rootstock to plant due to the fact that it is more resistant to freezing temperatures and inherently more resistant to attack by *B. obtusa* and *B. dothidea*.

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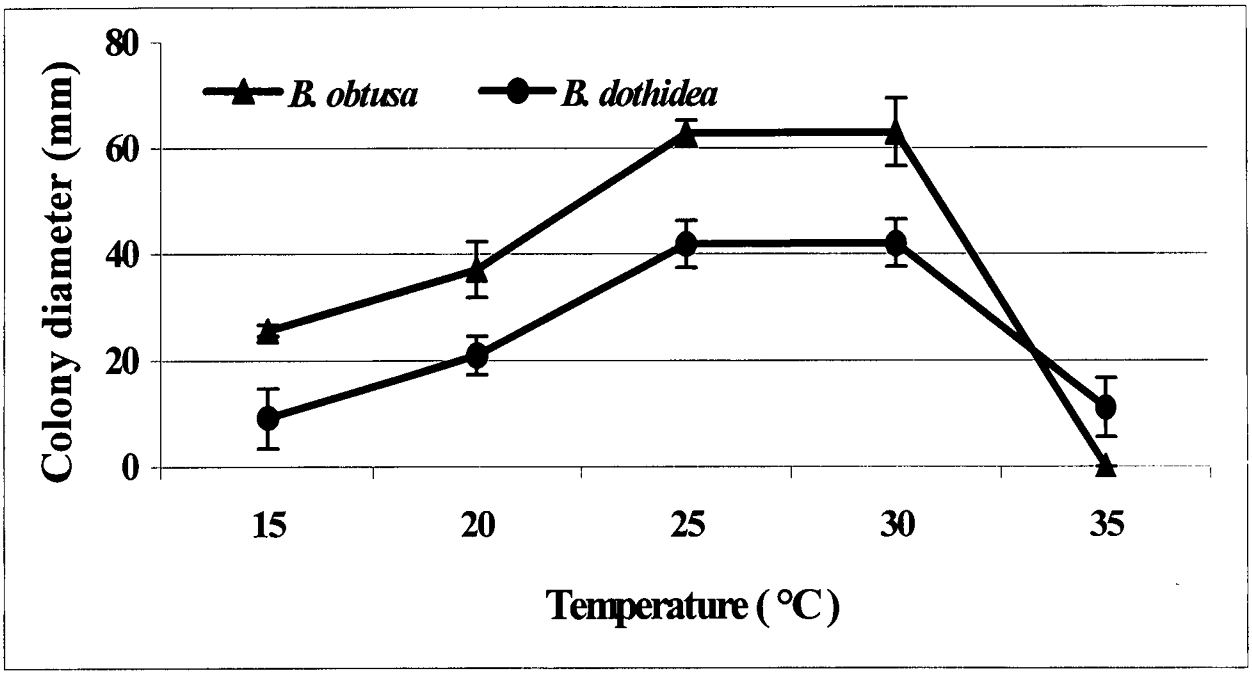
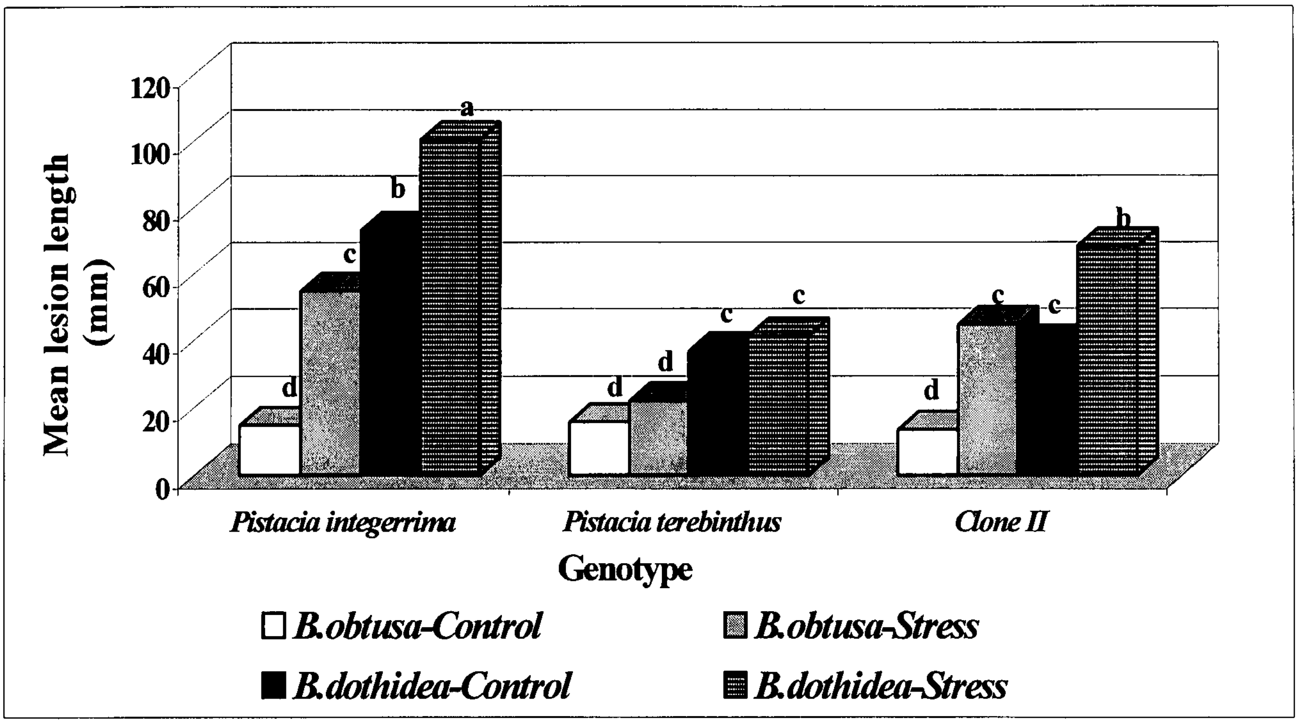
Figure 1: Effect of temperature on growth of *B. obtusa* and *B. dothidea*. Standard deviation bars show significance between the different treatments ($P \leq 0.05$).



80
60
40
20
0
Colony diameter (mm)
B. obtusa
B. dothidea
15
20
25
30
35
Temperature (°C)

Figure 2: The susceptibility of three pistachio genotypes to *Botryosphaeria obtusa* and *B. dothidea* under conditions of freezing stress. Bars denoted by different letters are significantly different ($P<0.05$) according to Tukey's HSD procedure.


Mean lesion length (mm)
120
100
80
60
40
20
0
d
c
b
a
d
d
c
c
d
c
c
b
Pistacia integerrima
Pistacia terebinthus
Clone II
Genotype
□ B.obtusa-Control
▨ B.obtusa-Stress
■ B.dothidea-Control
▤ B.dothidea-Stress

CHAPTER 6

INFLUENCE OF NITROGEN FERTILIZATION ON SUSCEPTIBILITY OF PISTACHIO SEEDLINGS TO *BOTRYOSPHAERIA OBTUSA* AND *B. DOTHIDEA*

ABSTRACT

Nitrogen is routinely applied to pistachio trees from a very young age in commercial pistachio orchards in the semi-arid Northern Cape province of South Africa, in order to boost growth. Increasing reports of canker caused by *Botryosphaeria obtusa* and *B. dothidea* in recent years has led to speculation regarding the possible role of nitrogen fertilization on disease incidence and severity. The present study investigated the effect of high levels of nitrogen on the susceptibility of three pistachio rootstock genotypes to *B. obtusa* and *B. dothidea* in the glasshouse. Double the normal field application of nitrogen was applied to 2-year-old potted plants of *Pistacia integerrima, P. terebinthus* and Clone II (*P. integerrima* X *P. atlantica*) for a period of three weeks. Plants were subsequently inoculated with either *B. obtusa* or *B. dothidea* respectively and the length of resulting cambial lesions was measured after three weeks. Plants receiving the same amount of nitrogen usually applied in pistachio orchards served as control treatment. Most treatments showed a significant decrease in the length of cambial lesions in plants that received high levels of nitrogen. Inoculations with *B. dothidea* generally resulted in longer lesions than *B. obtusa* for both levels of N applied for all three genotypes. The most significant differences between treatments were recorded for *P. terebinthus*.

INTRODUCTION

Pistachio (*Pistacia vera* L.) is a new commercial crop in South Africa with considerable commercial potential providing constraints such as disease can be overcome, particularly diseases that are predisposed by environmental stress. The role of soil nitrogen (N), in the predisposition of woody plants to diseases caused by facultative parasites has been well documented (Mengel & Kirkby, 1987; Abrol, 1989; Hennon *et al.*, 1990; Entry *et al.*, 1990; Entry *et al.* 1991a; Entry *et al.*, 1991b; R'Houma *et al.*, 1998). On the one hand, the addition of nitrogen to white pine seedlings has been reported to drastically reduce the incidence of damping-off caused by various species of *Fusarium, Rhizoctonia* and *Pythium (*Enebak *et al.*, 1990; Huang & Kuhlman, 1991). On the other hand, high levels of N can also increase the susceptibility of plants to both obligate and facultative parasites, either by increasing tissue succulence and facilitating entry, or by providing a complex N source (Solel & Bruck, 1989). These phenomena accentuate the importance of a well-planned fertilizer programme in pistachio orchards.

Solel & Bruck (1989) proposed that the disease enhancing effect associated with high N content in stem tissues of loblolly pine seedlings resulted from an increased availability of N to *Fusarium oxysporum* f. sp. *pini*, either via plant metabolites or directly as unincorporated, ionic N. Stem necrosis of grapevines caused by *Botrytis cinerea* have on various occasions been associated with high levels of N (Chambers *et al.*, 1993; R'Houma *et al.*, 1998). Increased levels of N leads to lower levels of phenols (Entry & Cromack, 1988) which are in turn responsible for the inhibition of *A. ostoyae in vitro* (Wargo, 1984; Entry & Cromack, 1988). Thus, by applying high levels of N, the incidence of *A. ostayae* will theoretically be increased.

High concentrations of nitrogen can not only predispose plants directly to disease, but also to secondary stress factors which can in turn lead to increased disease incidence. It has been shown that drought tolerant woody plants are more susceptible to drought stress after high levels of N are applied (Paine *et al.* 1992; Harvey & Van den Driessche, 1997). High concentrations of N increases the growth rate of plants which results in large amounts of succulent tissue that is more susceptible to winter injury and consequently predisposes plants to fungal infection (Brooks, 1992; Cline, 1994; Smith *et al.*, 1994; Boyce, 1995).

In commercial pistachio orchards situated near Prieska in the Northern Cape province of South Africa, high concentrations of nitrogen are routinely applied to pistachio trees from a very young age. Fertilization and irrigation with water from the Orange River serves to significantly boost growth and yield in this semi-desert region. Increasing reports of canker caused by *Botryosphaeria obtusa* (Sutton & Arauz, 1991; Swart & Botes, 1995; Travis *et al.*, 1999) and *B. dothidea* (Fisher *et al.*, 1993; Smith *et al.*, 1996a; Smith *et al.*, 1996b) in recent years has, however, led to speculation regarding the possible role of over optimal nitrogen fertilization on disease incidence and intensity. The objective of the present study was to determine the effect of high concentrations of nitrogen on the susceptibility of three pistachio genotypes to colonization by *B. obtusa* and *B. dothidea* in the glasshouse.

MATERIALS AND METHODS

Potted (151 pots) three-year-old pistachio plants of three rootstock genotypes (*Pistacia integerrima, P. terebinthus* and Clone II [*P. integerrima* x *P. atlantica*]) were fertilized each week with limestone ammonium nitrate (LAN 28, Kynoch Fertilizer Co., South Africa) containing an effective 28% nitrogen over a period of three weeks. The trees were planted in sand which was washed first before plant to ensure that there is no nutrients in it. Each genotype received two applications of LAN 28 per plant - 26 g and 60g, respectively. This resulted in a nitrogen application of 7.3 g for the lower limit and 14.56 g for the higher limit. The total amount of nitrogen applied was therefore 21.9 g and 43.68 g, respectively for the two treatments. A normal micro nutrient feed was applied before the treatment start to ensure no deficiencies. Trees received 150 ml of water every other day for the duration of the trial. The trees were maintained at field water capacity.

Three weeks from the start of the fertilization regime, plants were inoculated with mycelium of *B. obtusa* and *B. dothidea*. Stems were wounded 10 cm above the soil medium, by lightly scraping off a length of bark (2 x 5 mm) with a scalpel. Autoclaved strips of gauze (10 x 50 mm) that had been colonized by either *B. dothidea* (12-6 JB) or *B. obtusa* (CWS 155) after having been incubated on potato dextrose agar (PDA) at 25°C for 14 days were wrapped around each wound. Parafilm "M" (American National Can^{TM}, Chicago, IL, 60631) was subsequently wrapped around the inoculation site to prevent desiccation of the inoculum. Three weeks after inoculation the inoculation sites were uncovered and the length of resulting cambial lesions was measured. Each treatment had 12 replications and the experiment was conducted twice.

Variances between trials were tested for homogeneity using Bartlett's test (Bartlett, 1937) before data were combined and an analysis of variance (ANOVA) was performed using NCSS200 (BMDP Statistical Software Inc., Los Angeles, CA). Tukey's

HSD (Steele & Torrie, 1980) procedure for the comparison of means was applied where the factorial ANOVA showed significant variation between treatments.

RESULTS

Significantly shorter mean lesion lengths resulted in plants that received high concentrations of nitrogen (Fig. 1). *B. dothidea* produced longer lesions than *B. obtusa* for both levels of N applied in all three genotypes. There was a significant difference ($P < 0.05$) in lesion lenght of *B. obtusa* between the two levels of N for all three rootstock genotypes. For *B. dothidea,* however, this difference was only significant in *P. terebinthus* in which there was a very marked reduction in lesion length of *B. dothidea* at the higher N level.

No significant differences were observed among the three host genotypes for *B. obtusa*. This was in sharp contrast to *B. dothidea* where *P. terebinthus* had significantly longer lesions than the other two rootstocks at the lower N level, but significantly shorter lesions at the higher level.

DISCUSSION

Higher levels of nitrogen application significantly reduced mean lesion length on trees inoculated with *B. obtusa* in all three genotypes as well as the *B. dothidea -P. terebinthus* treatment. There was also a reduction for the *B. dothidea–P. integerrima* treatment, although not significant. This enhancement phenomenon is consistent with the demonstrated pathogenicity of *Fusarium subglutinans* on *Pinus elliottii* seedlings (Solel & Bruck, 1989) and that of *Fusarium*, *Rhizoctonia* and *Pythium* species on *Pinus strobus* (Enebak *et al.*, 1990). High levels of nitrogen result in a lower sugar content in plants

(Mengel & Kirkby, 1992) and fungi readily utilize sugar (Griffin, 1994). This has been given as a factor contributing to reduced fungal growth in plants subjected to high levels of N (Wallander & Nylan, 1992).

The above findings are in contradiction to the interaction of *Armillaria ostoyae* on *Pseudotsuga menziesii* and *Cylindrocarpon didymum* on *Chamaecyparis nootkatensis* where higher than normal nitrogen levels increased disease severity (Hennon *et al.*, 1990; Entry *et al.*, 1990; Entry *et al.*, 1991a; Entry *et al.*, 1991b). These authors demonstrated that high nitrogen levels not only lead to lower sugar levels in plants, but also to lower levels of phenolic compounds which are responsible for the inhibition of *Armillaria ostoyae*.

Although controversy surrounds the correct nutritional requirements of pistachio (Wolpert, 1986; Ferguson & Maranto, 1988) it has been determined that 70-80% of supplied N is re-directed to the fruit (Weinbaum & Murakoa, 1989). Tekin et al. (1995) found that by applying 800 g of N per tree there was a significant increase in yield in comparison to 400 g/tree. Crane & Maranto (1988) postulated that pistachios are not a "luxury consumer" of N when abundantly available in the soil, which suggests that the probability of pistachio trees suffering from over abundant N application is low.

The level of N application to pistachio orchards in South Africa is far lower than what is considered the norm in other parts of the world. Rainfed pistachios in Turkey receive 140 kg N per ha (Caruso et al., 1996) while the best results in a fertilizer trial done by Tekin et al. (1995) was with an application of 800 g of nitrogen per tree with a additional 60 kg of manure per tree in conjunction with irrigation. This suggests that what was deemed to be an over abundance of N in our trials could in fact have been less than optimal and that higher levels could reduce the effect of the two pathogens even more. This possibility would be consistent with the fact that cankers caused by *B. obtusa* are absent from Californian pistachio orchards.

The results presented here suggest that increased nitrogen application and the planting of resistant *P. terebinthus* rootstocks in South Africa should control disease caused by the two *Botryosphaeria* species. However, plants receiving excess nitrogen usually have an over-abundance of foliage, a minimal sized root system and a high shoot-to-root ratio (Abrol, 1989; Salisbury & Ross, 1992). High levels of nitrogen can thus lead to plants that are predisposed to drought (Paine *et al.*, 1992) and freezing stress (Brooks, 1992; Cline, 1994; Smith *et al.*, 1994; Boyce, 1995) which in turn could predispose them to disease.

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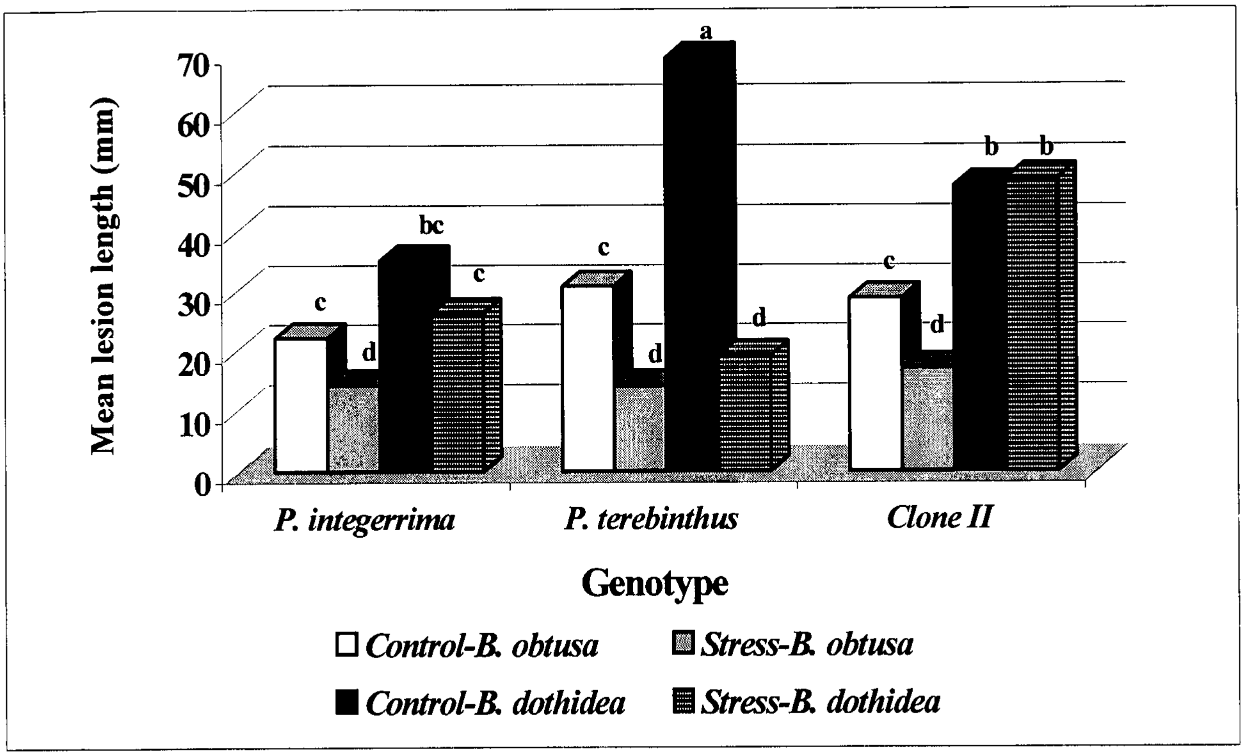
Figure 1: Mean lesion length of three pistachio genotypes inoculated with *Botryosphaeria obtusa* and *B. dothidea* following two levels of nitrogen application. Bars denoted by different letters are significantly different ($P < 0.05$) according to Tukey's HSD procedure.


Mean lesion length (mm)
70
60
50
40
30
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10
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c
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c
d
a
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c
d
b
b
P. integerrima
P. terebinthus
Clone II
Genotype
□ Control-B. obtusa
▨ Stress-B. obtusa
■ Control-B. dothidea
▤ Stress-B. dothidea

SUMMARY

Drought, freezing and nitrogen stress are environmental factors known to predispose woody plants to infection by numerous fungal species. The main and ultimate objective of the present study was to identify possible stress factors that predispose commercially pistachio (*Pistacia* spp.) trees to infection and colonization by *Botryosphaeria obtusa* and *B. dothidea* in order to develop a sustainable disease control programme for South African pistachio producers.

It was found that these stress conditions significantly affected the susceptibility of three genotypes of potted pistachio plants to infection and colonization by *B. obtusa* and *B. dothidea*.

In the glasshouse, drought stress trials with potted *Pistacia terebinthus* plants artificially inoculated with *B. dothidea* showed significantly longer lesions than control treatments while *P. integerrima* displayed the greatest resistance to the pathogen. Although *B. dothidea* caused more severe cambial lesions in glasshouse trials, it showed a slower growth rate than *B. obtusa* under *in vitro* water stress conditions.

In freezing stress trials performed in a growth chamber, there was a highly significant difference between the stressed and non-stressed plants of *P. integerrima* and Clone II (*P. integerrima x P. atlantica*). *P. integerrima* was the most susceptible rootstock and *P. terebinthus* the least susceptible. *B. dothidea* caused the most severe cambial lesions in all treatments.

In nitrogen stress trials, most treatments showed a significant decrease in the length of cambial lesions at a higher level of applied nitrogen (28%). *B dothidea* was

generally more pathogenic than *B. obtusa* for both levels of N applied. The most significant differences between nitrogen levels were recorded for *P. terebinthus*.

From the above results it is clear that drought and freezing stress have a significant effect on the predisposition of pistachio plants to *B. obtusa* and *B. dothidea*. Drought stress occurs in pistachio orchards due to a very short hardening off period lasting two to three weeks. By postponing this period not only can drought stress be prevented but also freezing stress. Current nitrogen levels applied in the pistachio orchards are much lower than those applied in other parts of the world. Applying higher levels of nitrogen could thus possibly prevent the occurrence of *Botryosphaeria* diseases.

The development of a selective medium for the pure isolation of *B. obtusa* and *B. dothidea* from diseased tissue and other sources enabled the isolation of *B. dothidea* from debri, bark, diseased and asymptomatic tissue without hindrance by extraneous fungi. *B. obtusa*, however, was only isolated from active cankers on the stems of pistachio trees. Isolation of *B. dothidea* from asymptomatic plant tissue suggests an endophytic or latent phase.

Trials were conducted *in vitro* with different fungicides to determine other possible fungicides that could be employed for the control of the two *Botryosphaeria* species. Eria®, a mixture of carbendazim and difenoconazole displayed the highest inhibition. Benomyl or the Eria® applied during bloom, and tebuconazole in combination with difenoconazole on a monthly basis throughout the season, should thus successfully control both *Botryosphaeria* diseases in South African orchards.

KEYWORDS: Pistachio; *Pistacia* spp.; *Botryosphaeria*; stress; drought; freezing; nitrogen.

SAMEVATTING

Droogte, koue en stikstof stremming is bekende omgewingsfaktore wat houtagtige spesies meer vatbaar maak vir infeksie deur verskeie swamme. Die hoofdoel van die huidige studie was om stremmings faktore te identifiseer wat kommersieële pistachio bome meer vatbaat maak vir infeksie deur *Botryosphaeria obtusa* en *B. dothidea*. Die doel hiervan is om 'n effektiewe siektebeheerprogram vir pistachio produksie in Suid-Afrika te ontwikkel.

Droogte en koue stremming is geïdentifieseer as faktore wat die vatbaarheid verhoog terwyl stikstof stremming 'n duidelike afname in infeksie getoon het. Daar was duidelike verskille tussen verskillende pistachio genotipes.

Pistacia terebinthus plante het in glashuisproewe betekenisvolle langer letsels as kontrole plante getoon terwyl *P. integerrima* die hoogste weerstand onder droogte toestande getoon het. *B. dothidea* het deurgaans die langste letsels veroorsaak al het die betrokke swam stadiger gegroei as *B. obtusa* onder droogte toestande *in vitro*.

Betekenisvolle verskille het tussen gestremde en nie-gestemde plante van *P. integerrima* en Kloon II (*P. integerrima* x *P. terebinthus*) voorgekom, toe plante in groeikabinette aan koue stremming onderwerp is. Gestremde plante het in die bogenoemde behandelings deurgaans langer letsels getoon. *P. integerrima* het die langste letsels getoon terwyl *P. terebinthus* die beste weerstand onder die toestande getoon het. *B. dothidea* het weereens die langste letsels in al die behandelings getoon.

Tydens die stikstof proewe was daar deurgaans korter letsels by die verhoogde stikstof peil. *B. dothidea* het langer letsels as *B. obtusa* veroorsaak in al die betrokke

behandelings. Die mees betekenisvolle verskil tussen die stikstof behandelings was in die geval van *P. terebinthus*.

Die resultate van hierdie studie toon duidelik dat droogte en koue stremming belangrike faktore is as dit kom by predisponering van pistachio bome teenoor siektes. Droogte stremming kom op die oomblik gereeld voor in pistachio boorde in Suid-Afrika as gevolg van die feit dat water vroeg in die herfs heeltemal getermineer word om die bome af te hard vir die winter. 'n Langer afhardings periode wat minder skok vir die plante meebring sal definitief 'n positiewe effek op siektevoorkoms hê. Huidige stikstofpeile wat toegedien word is heelwat laer as die wat in ander dele van die wêreld toegedien word. 'n Hoër stikstofpeil sal nie net lei tot beter groei nie, maar ook moontlik tot minder infeksie deur *Botryosphaeria* spesies.

Die ontwikkeling van 'n selektiewe medium vir die suiwer isolasie van *B. obtusa* en *B. dothidea* het gelei tot die suksesvolle isolasie van *B. dothidea* uit dooie pistachio materiaal, bas, geïnfekteerde weefsel en gesonde weefsel sonder dat die proses deur enige saprofietiese swamme belemmer is. Die feit dat *B. dothidea* uit gesonde, symtoomlose weefsel geïsoleer is dui op 'n moontlike endofitiese of latente leefwyse. *B. obtusa* is slegs uit aktiewe kankers van volwasse bome geïsoleer.

Proewe is *in vitro* uitgevoer om moontlike nuwe swammiddels te identifiseer vir die beheer van die twee *Botryosphaeria* spesies. Eria®, 'n mengsel van carbendazim en difenoconazole het die beste resultate getoon. Benomyl or Eria®, toegedien tydens bloeiselvorming en opgevolg deur tebuconazole in kombinasie met difenoconazole op 'n maandelikse basis deur die seisoen, sal moontlik *Botryosphaeria*-siektes in Suid-Afrikaanse boorde die beste beheer.

SLEUTELWOORDE: Pistachio; *Pistacia* spp.; *Botryosphaeria*; predisponering; stremming; droogte; koue; stikstof.