

Bio-control of root rot disease in vanilla

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BIO-CONTROL OF ROOT ROT DISEASE IN VANILLA

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A thesis submitted in partial fulfilment of the
requirements of the University of Wolverhampton
for the degree of Doctor of Philosophy

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in collaboration with the Yunnan Agricultural University

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ABSTRACT

Fusarium oxysporum Schl. var. *vanillae* (Tucker) Gondon is known to cause root rot in *Vanilla planifolia* Andrews in most regions where it is grown, including the major plantations in Xishuangbanna, Yunnan Province of China. This is of serious economic concern to the Province since the vanilla flavouring extractable from the beans of the plant is a valuable food product and an important export commodity.

There are no fungicides registered for the control of *Fusarium* root rot and the only available chemical control methods are ineffective and cause serious contamination of the soil. Breeding for resistance is difficult when no dominant gene is known or where little information is available on fungal pathogenicity. Biocontrol is the main alternative for disease control in this crop, an attractive approach because of increasing concerns for environmental protection. The investigation considers two biocontrol strategies: first the introduction of virulent, antagonistic, non-pathogenic strains, closely-related to the pathogen, to overcome pathogenic populations in infected soils; second the use of essential oils with antimicrobial properties when applied to infected soils.

Pathogenicity tests have been done on 81 out of 87 *F. oxysporum* isolates collected in Yunnan Province. Among these, 32 isolates were non-pathogenic and 49 were pathogenic. The pathogenicity results showed the complexity of *F. oxysporum* in Yunnan. Seventeen isolates were recovered from the Daluo plantation, of which 14 were pathogenic isolates and 3 non-pathogenic isolates; 26 from the Menglun plantation, in which 12 were pathogenic and 14 were non-pathogenic; 18 isolates from the Manjingdai plantation, in which 12 isolates were pathogenic, whilst the other 6 were non-pathogenic and 20 were obtained from the plantation in Hekou

County, of which 11 were pathogenic isolates and 9 were non-pathogenic.

Genetic diversity within this population of *F. oxysporum* has been investigated with respect to vegetative compatibility and to determine the relationship between VCGs and virulence. The VCG results showed that the 87 strains of *Fusarium oxysporum* f.sp *vanillae* isolated from Yunnan Province were complex. They could be distributed into 12 different VCGs and that a direct relationship between VCGs group and virulence could not be drawn.

Two non-pathogenic strains, ML-5-2 and HK-5b-4-1, have been screened from 87 strains as candidate biocontrol agents by pathogenicity and VCG, which are self-incompatible and closely related to the pathogens. These two strains were effective in vanilla root rot control in controlled environments, but their effects in field experiments were less conclusive. Seven essential oils, which have long been regarded as having inhibitory effects on pathogens in nature, have also been investigated as biocontrol agents. Three oils, cinnamon oil, thyme oil and clove oil, were effective in inhibiting the growth of pathogen *in vitro*. These oils may develop into useful components of different management strategies with non-pathogenic strains. For the future, consideration will need to be given to the mechanism(s) of the interaction of the antagonistic components with the soil microbe population and host plant and also to appropriate formulation, to take account of soil type, crop status, cultural practices, environmental and economic factors. Biocontrol methods have considerable potential but must be acceptable to farmers as part of an overall crop management programme.

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List of Acronyms

BCA	Biological Control Agents
CK	Control
h	hour (s)
l	Litre
min	minute
ml	millilitre
mM	millimole
nit	nitrate nonutilizing
PCR	Polymerase chain reaction
µg	microgram
µl	microlitre
<i>vic</i>	vegetative incompatibility
VCG	vegetative compatible group

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CHAPTER 1

GENERAL INTRODUCTION

1.1 INTRODUCTION

1.1.1 Importance, Geographic Distribution and Cultivation of Vanilla

Vanilla (*Vanilla planifolia* Andrews) is a climbing terrestrial orchid suitable for the warm humid tropics (Figure 1.1). It is the second most expensive flavouring spice after saffron. The United States is the biggest consumer of vanilla followed by Germany, France, Canada, Australia and Japan. It is an example of one of the few contributions of the Western Hemisphere to the world of spices (Kumar, 2004; Pesach, 2005).

Vanilla, a native of South Eastern Mexico and other parts of Central America, was domesticated about 1000 years ago. During the 18th century, vanilla vine cuttings were taken out of Mexico to Europe and other parts of the world. Today the major vanilla growing areas are Madagascar, Mexico, the Comoro Islands, Reunion Island, Uganda, Java, The Philippines, Papua New Guinea, Fiji, Jamaica, Costa Rica, China and India (Anon., 2005; Anon., 2003; Ruan *et al.*, 2002; Menz and Fleming, 1989; Pesach, 2005). Vanilla is a natural alternative to synthetic vanillin. Vanillin (4-hydroxy-3-methoxybenzaldehyde) is the major component of natural vanilla, which is one of the most widely used and important flavour and aroma molecules (Walton *et al.*, 2003). The source of vanillin is the bean, or pod, of the Vanilla plant, of which the principal species is *Vanilla planifolia* Andrews, syn. *V. fragrans* (Salisb. Ames) (Walton, 2003). Vanillin in fact occurs in trace amounts in other plants, including commercial products such as tobacco; however, the pods of the vanilla orchid still remain the only commercial source of natural vanillin (Walton, 2003). This crop is grown in Yunnan Province, P.R. China, where the most serious disease affecting production is root rot disease, the subject of this study.



Figure 1.1 Luxurious growth and healthy vanilla plants (left) and vanilla beans (right).

Ecological requirements: Vanilla can be grown up to an altitude of 1500 m above mean sea level. It is well-adapted to the humid tropics. The optimum temperature for the successful growth of vanilla ranges from 25-32 °C. A well-distributed rainfall of 150-300 mm throughout the 10 months of the year, with dry periods during flowering and harvesting is most ideal (Frankel *et al.*, 2004; Kumar, 2004; Anon., 2005). Well-drained loose and friable soil, rich in organic matter sustains a good crop of vanilla. In open areas it can be raised with support stands providing a little, but sufficient, shade.

Crop nutrition: Three approaches in crop nutrition; organic farming, integrated nutrient management and chemical farming are practiced to supply nutrients for crop growth (Kumar, 2004). In organic farming organic manures, both bulky and concentrated as well as bioinoculants, are liberally used to sustain soil health. In integrated nutrient management, three components: organic manures, inorganic fertilizers and bioinoculants are integrated (Frankel, 2004). Intensive use of chemicals alone is used in chemical farming which results in pollution of the environment and several other ill effects. The organic farming approach is best for vanilla gardens (Kumar, 2004)

Flowering and artificial pollination: Vanilla starts flowering from the second or third year of planting. Manipulation of shade and slight wilting of vines encourage flowering. The normal flowering season is from December to March and there is generally only one flowering season in a year. It takes 45 days from the initiation of the inflorescence to opening of its first flower. The racemose inflorescence in the leaf axil consists of ~15-20 greenish yellow flowers (Figure 1.2). Self pollination rarely occurs in vanilla because a structure called a rostellum prevents the stigma coming into direct contact with the pollen grains. Consequently, hand pollination is

frequently used. Artificial pollination should start from early morning and be completed before noon as the flower closes in the afternoon. The retention of the flower and enlargement of the ovary are indications of the success of artificial pollination. Even though every plant produces 18-20 inflorescences, only 10-12 inflorescences are allowed to develop and in each inflorescence only 10-15 flowers alone are hand pollinated to ensure the formation of high quality beans (Figure 1.3). Generally, only one flower opens a day and flowering continues for about three weeks. Pods take about eight to nine months to attain maturity. During flowering, chemicals should not be sprayed, as this may result in scorching of ovaries and subsequent scab formation in beans (Frankel, 2004).

Harvesting and processing: Generally harvesting begins in October and ends in December. Each pod is harvested separately by looking at its colour. Mature pods at the appropriate stage are picked when they are flavourful and contain more vanillin and their commercial value is fixed based on the length of the pod. The beans then go through a curing, drying and resting process for several months. The processed beans (Figure 1.4) are sorted, graded, bundled and wrapped in paraffin paper and preserved for the development of desired bean qualities, especially flavour and aroma. Each vanilla bean is handled many times before it is ready to use, making it one of the most labour-intensive agricultural products in the world. It is therefore a very valuable commodity and the production of vanilla is an important economic activity (Frankel, 2004).



Figure 1.2 Flower of *Vanilla planifolia*.



Figure 1.3 Vanilla flower and unripe fruits.



Figure 1.4 Vanilla processed pods.

(http://kfunigraz.ac.at/~jatzer/engl/generic_frame.html?vani_pla.html)

Uses and applications: Records cited in Kumar (2004) mentioned that vanilla was used in medicines as a mild sedative in earlier days. Now, it is no longer considered as a medicinal plant. Both natural and synthetic vanillin are extensively used in the food industry as a flavouring agent for making cakes, chocolates, biscuits, ice creams as well as a wide variety of other foods. The perfume industries also make use of vanillin. The price of synthetic vanillin is far below that of natural vanillin. Consumers prefer natural vanillin as the essence of its synthetic counterpart is in no way comparable to that of the natural flavour (Kumar, 2004).

Vanilla is in great demand all over the world. The world export market for natural vanilla is valued at around \$422 million with nearly 50 per cent sourced from the leading producer country Madagascar (Partos, 2006). Thus, the political situation, natural disasters, and disease occurrence in Madagascar determine the price of vanilla in the international market. A steep escalation in the price has been observed since 2000. It is estimated that in the international market, the price of cured beans may range from 20-500 USD (\$) per kilogram. On the commodities market, vanilla beans cost importers about \$33 per kilogram in 2000 and by 2003 were at an all-time high of \$400-\$500 per kilogram (Paul, 2003). According to ITC/UN statistics the total global demand for vanilla is about 2000 to 3000 metric tonnes a year with the world market for vanilla beans highly concentrated in a few developed countries (Partos, 2006).

Vanilla Species: Plants of the genus *Vanilla* of the *Orchidaceae* are part of the largest family of flowering plants in the world, with over 35,000 species worldwide. More than 50 species of vanilla have been described, but only three are important as sources of natural vanillin, namely, *Vanilla fragrans* (Salisb) Ames [Syn *Vanilla planifolia* Andrews] (Mexican vanilla), *Vanilla pompona* Shiede (West Indian vanilla)

and *Vanilla tahitensis* J. M. Moore (Tahiti vanilla). Of these, *V. planifolia* is the most preferred and hence the most common to be commercially cultivated, whereas only a very small amount of beans are commercially produced from *Vanilla tahitensis* (Anon., 2003; Kumar, 1992; Stern and Judd, 1999).

Vanilla planifolia is indigenous to South East Mexico, Guatemala and other parts of Central America, growing wild as a climber in the forests (Besse *et al.*, 2004). Its cultivation on a systematic basis began with its introduction into Java, the Seychelles, Tahiti, the Comoro Islands, Martinique, Madagascar and Uganda in the 19th century and early part of the 20th century. It was introduced into India in 1835 and is now cultivated mainly in the southern states of Kerala, Tamil Nadu and Karnataka (Anon., 2003).

Vanilla planifolia was first introduced into China (Fujian and Hainan provinces) in 1960, and then introduced to the Xishuangbanna Prefecture in Yunnan Province between 1963 and 1975. There are ~133 ha under cultivation in Xishuangbanna and it is becoming an increasingly important cash crop (Figure 1.5; Ruan *et al.*, 2002).



Figure 1.5 Healthy vanilla plantation in Xishuangbanna Prefecture, Yunnan Province, P.R.China.

1.1.2 Agriculture and Vanilla Plantations in Yunnan Province

China is considered as one of the biodiversity countries in the world (Chen, 1992). Many important crops, such as soybean, rice, barley, tea and apples originated here. Besides these, there are many wild species and ancestral forms of cultivated plants, such as wild soybean, wild barley and wild rice. In China, 7% of the world's cropland has to support 22% of the global population. Therefore, agrobiodiversity play a vital role in agricultural production and livelihood. However, China is a vast country with complex topogeography and climate. It changes from frigid, temperate to tropical zones from north to south. Farmers utilize agrobiodiversity in their management and employ a wide range of different cultivation techniques.

Yunnan is a frontier province in South West China. It encompasses an area of 394,000 square kilometers, covering 4.1% of China's territory. It borders on Guizhou Province and the Guangxi Zhuang Autonomous Region in the east, Sichuan Province to the north, the Tibetan Autonomous Region in the northwest, Myanmar in the west and both Laos and Vietnam in the south. Yunnan encompasses a wide range of environments, including tropical and subtropical rainforest, temperate uplands and cool highlands of the Hengduan and Gaoligong Mountains of the Himalayan range. Approximately 94% of the total area is mountainous and hilly terrain, with river valleys comprising the remaining 6%, which are the most suitable for agriculture. It boasts the largest variety of flora in China and is known as the "Kingdom of Flora". Due to its unique climate and geographical environment, Yunnan is also home to a wide variety of wildlife (Chen and Dao, 2001).

By the end of 2005, Yunnan's population had reached 44.5 million, of which over 13.95 million are ethnic minorities. Of the 55 ethnic minority groups in China, 51 can be found in Yunnan. Twenty-five ethnic minority groups live in compact

communities, each with a population of over 5,000. Of these minority groups, 15 are indigenous to Yunnan. Historically, these cultural minorities have practiced shifting cultivation, which depends on clearing native forests to plant agricultural crops, especially in the mountainous tropical and subtropical areas, and shifting cultivation is still the most important production method for the people among mountainous groups. For example, in Xishuangbanna Prefecture, it is estimated that shifting cultivation produces food for up to 20% of the people and shifting cultivation fields make up about 46,000 ha, which is 37.85% of the total farming land of the prefecture (Chen and Dao, 2001).

Agriculture can be viewed as supportive of biodiversity conservation since the same places that produce our food and other living essentials are the sources of much biodiversity. Agriculture forms a powerful basis for personal, experiential development and connection to a setting or landscape (Lockwood, 1999). The Yunnan experience of cash cropping and intensification of agriculture is an alternative to shifting cultivation and the differences are instructive (Fu *et al.*, 2000). Tea from plantations is a traditional commercial product for the mountainous cultural groups. Rubber plantations started in the mid 1970s, initially by state farms and were then extensively developed by individual family farming from the early 1980s. The rapid development of the economy and private land ownership policy has provided opportunities to further diversify cash cropping. Coffee, sugarcane, passionfruit, pomelom, citrus and Chinese cardamom have all been rapidly extended since the 1990s (Chen and Dao, 2001). Vanilla is another important cash crop developed over this period.

Vanilla, being an orchid vine, originated in the rainforests; it requires a constant tropical environment to grow well. As a result, most people grow orchids in

greenhouses where they can control the temperature, shade and humidity. In Yunnan Province, vanilla can be raised either as a monocrop or intercrop in coconut and areca nut gardens. Being a vine, it grows by climbing over some existing tree, pole, or other supports. It can be grown on a wood (on trees), in a plantation (on trees or poles; possibly alternating with rows of sugar cane), or in a "shader", in increasing orders of productivity. It is usually trained on a trellis or on low branching, rough barked trees like *Glyricidia maculata*, *Plumeria alba* Jack (*Artocarpus heterophyllus*), *Erythrina* spp. or on dead stands. In some places, areca nut is also used for trailing vanilla. The standards have to be planted well in advance at a spacing of 1.2-1.5 m within rows and 2.5-3.0 m between rows. Approximately 1600-2000 stands can be accommodated in a hectare. If dead stands are used, shade should be provided to the vines initially by planting banana or other suitable plants. The vanilla plants should be allowed to trail horizontally on poles/trellis tied to trees after trailing to a height of 1.5-2.0 m or coiled around the branches so as to facilitate pollination and harvesting. If left alone, it will grow as high as possible on the support; every year, growers fold the higher parts of the plant downwards so that the plant stays at heights accessible by a standing human. Flowering will not occur as long as the vines climb upward. The shade trees should be regularly pruned to maintain a light shade. The pruned leaves and branches can be applied as mulch (Anno, 2002; 2005).

1.1.3 General Disease and Pest Management

Most agricultural practices have exploited a broad ecological and biophysical system for the purpose of both subsistence and commercial production. A range of activities in the Xishuangbanna area includes mainly shifting cultivation, forest product collection, cultivation under native forests, permanent agriculture and cash crop plantation. The management system depended on a cultural group's religion before 1949. Since then community ownership appeared followed by an individual household responsibility system since the early 1980s, when farmers received land for household cultivation. Consequently, the decision-making system has been totally shifted from a collective quota system, village-based, community system to individual farmers (Guo and Long, 1998).

Vanilla is susceptible to many fungal and viral diseases, so to prevent diseases spreading, the following instructions have been frequently recommended by local extension advisors:

It is best to avoid excessive use of manure, mulch and irrigation, and cut, remove and burn disease affected plant parts. Do not use planting materials procured from infected gardens and avoid close planting of vines and over-crowding. Do not use implements, which have been used on disease-affected plants on healthy plants without thoroughly washing and cleaning them (Anon., 2005). Moreover, to prevent the occurrence of diseases, Bordeaux mixture (1%), Bavistin (0.2%) and copper oxy chloride (0.2%) should be sprayed (Kumar, 2004).

Biocontrol methods have also been suggested for disease management. The use of beneficial organisms such as *Trichoderma* and *Pseudomonas* has recommended preventing and controlling the growth of the pathogen (Lu *et al.*, 2004; Saravanan *et al.*, 2004). Application of these organisms has also been reported to increase the

resistance of the vanilla plant against pathogenic fungi. There are also some other chemical fungicides, such as sulphur-based fungicides and phosphites available in the market (Ruan *et al.*, 2002). However, it is recommended that chemical fungicides and beneficial organisms should not be used simultaneously over short intervals (Kumar, 2004).

1.1.4 Specific Diseases of Vanilla and Plant Protection

A number of serious diseases affect the vanilla plant, with pathogens infecting various parts of the vines, which could arise from complex infections by different pathogens in natural environments (Ruan *et al.*, 2002). The following describe some severe vanilla diseases.

Stem rot: The infection caused by *Fusarium oxysporum* and *F. solani* starts at the leaf axil and spreads to the nodal inter region resulting in rotting and drying of the stem above the point of infection (Ruan *et al.*, 2002; Summerell *et al.*, 2003). The fungus also causes leaf rot symptoms on the plant.

Anthracnose disease: The disease is caused by *Colletotrichum gloeosporioides* Penz. It attacks the stem apex, leaves and roots and results in wilting and falling of the fruits. Small brown spots appear on leaves, which eventually form into bigger spots. It is prevalent during the monsoon season. Great damage was caused in the Seychelles in the 1890s, when the vines were planted too close together (Anon., 2002).

Phytophthora blight: *Phytophthora* causes rotting of beans, leaves and stems. In severe cases all the beans in a bunch are completely rotten. The disease is more severe during the monsoon, especially in shaded plantations and poorly drained soils.

Sclerotium rot disease: The disease affects leaves and is caused by *Sclerotium rolfsii*. The beans develop reddish brown sunken lesions initially and later the entire bean rots. In severe cases all the beans in the bunch are covered with a thick white feathery fungal mat, at the distal end of the leaves (Anon., 2002).

Dry rot disease: Dry rot affects all parts of the plant. The disease is caused by *Fusarium* and *Rhizoctonia sp.* (Ruan *et al.*, 2002). Shrinking of stems, roots and leaves followed by yellowing are the common symptoms. Though the disease occurs

throughout the year, its severity is greater during the summer. Intermittent drying of stems and leaves occurs at advanced stages of the disease.

Bacterial soft-rot disease: This disease infects leaf and shoot, and shows soft-rot symptoms. It is caused by *Erwinia carotovora* pv. *carotovora* (Jones) Bergey (Wen and Li, 1992).

Viral disease: Virus identification was based on enzyme-linked immunosorbent assay (ELISA) and, for potyviruses, also on the sequence of part of the coat protein and inoculation assays (Grisoni *et al.*, 2004). The prevalent viral diseases are Cymbidium Mosaic Virus (CyMV), odontoglossum ring-spot virus (ORSV), Vanilla Mosaic Virus (VaMV) and Vanilla Necrosis Virus (VNV) (Grisoni *et al.*, 2004). Plants infected with CyMV and ORSV are usually symptomless, occasionally mild mottles or mild chlorotic streaks are observed on leaves of *Vanilla fragrans* and *V. tahitensis*. VNV was first described in 1984 in French Polynesia on *V. tahitensis* showing leaf mosaic and distortion on younger leaves with diffuse chlorotic patches, and necrotic lesions on older leaves and on stems, causing defoliation and death.

Root rot disease: Root rot disease causes serious damage to many vanilla plantations and was found to be caused by *F. oxysporum* (Tucker, 1927), which was named *Fusarium oxysporum* Schlecht. f.sp. *vanillae* (Tucker), synonym *F. batatas* Woll.Var. *vanillae* (Tucker) (Gordon, 1965). It is classified as Nectriaceae, Hypocreales, Sordariomycetidae, Ascomycetes, and Ascomycota (Gordon, 1965). In Puerto Rico, Tucker (1927) made the first detailed study of the disease, determining its aetiology, and naming the pathogenic organism. Root rot has been a limiting factor to vanilla production in Puerto Rico and has also been reported from other countries (Pearson *et al.*, 1991). It is currently causing serious crop losses in the Xishuangbanna Prefecture of Yunnan, a tropical rain forest area near the borders of

Laos and Myanmar (Ruan *et al.*, 2002).

In the early stages of the disease, browning and eventual death of the underground roots occurs followed by browning and death of aerial roots (Figure 1.6). The fungus attacks aerial roots of vanilla when they reach infested soil. The portion of the root in the leaf litter or mulch turns dark brown and decays, but the aerial part may remain healthy and produce new root branches, which will, in turn, be infected as they reach the ground. This proliferation of new root branches to re-establish soil contact weakens the plant, and older portions usually die as they exhaust their capacity for root proliferation. The stems and leaves become flaccid and the stems begin to shrivel and eventually droop and dry out (Tucker, 1927).

Inspection of the aerial root systems in the field suggested that most of the infection occurred at the growing tips of the root branches and main axes. With few exceptions, the site of infection was at the root tip (Alconero, 1968). The disease appears to progress from the tips toward the thicker and more mature portions of the root. The heavier parts of the roots usually are not damaged unless decay is general and the thinner portions were already affected (Alconero, 1968). Histological observation of the affected roots suggested that *F. oxysporum* f. sp. *vanillae* invaded the roots mainly through wounds caused by insects, nematodes or other agents. Direct penetration of the epidermis was occasionally observed in roots (Alconero, 1968; Tucker and Talbot, 2001). The disease was more prevalent in plantations during the monsoon season. The length of time from infection to death depends upon the size and vigour of the plant and the severity of infection. Some diseased plants may live several years (Tucker, 1927; Ruan *et al.*, 2002). The pathogen can survive in the soil for many years and will attack vanilla plants as soon as they are planted in infested soil (Ruan *et al.*, 2000). When the pathogen becomes established in the soil,

whole plantations may be destroyed (Figure 1.7). It becomes impossible to grow any vanilla cultivars as growers do not have a practical and economical solution to control this pathogen.

There are no fungicides registered for the control of *Fusarium* root rot. The available chemical control methods are not efficient or are difficult to apply, especially under field conditions where direct application to roots may not be possible. Drenching of the soil with anti-microbial chemicals which inhibit or destroy the pathogen may also cause considerable damage to other living organisms and leave the soil seriously contaminated (Shiraishi, 2003). Applications have to be repeated and become uneconomic. The main theme of this study is the exploration of alternative control strategies based on biological control (biocontrol) methods. With biocontrol, living agents, including other organisms, systems and processes are used to control or eliminate diseases in ways which are typically less damaging to the environment than other methods (Weller, 1988). These methods frequently leave fewer residues and can be more sustainable. Very little detailed work has been published on biocontrol strategies for vanilla (see Chapters 4 and 5).

The aim of this study is to develop and investigate the potential of biocontrol agents for the management of root rot disease in vanilla. A two component strategy will be explored, based on soil inoculation of non-pathogenic strains of the disease-causing fungus and treatment with the use of natural essential oils possessing anti-microbial activity. The objectives of the research are described below:

1. To review the literature on vanilla and the management of root rot disease.
2. To test the pathogenicity of isolates of *F. oxysporum* found in Yunnan Province in order to identify non-pathogenic strains which could act as biocontrol agents.
3. To investigate the genetic diversity of the isolates identified in Yunnan Province,

to determine the vegetative compatible groups (VCGs) as part of the differentiation of non-pathogenic and pathogenic strains, leading to further identification of potential biocontrol strains.

4. To investigate the effects of selected essential oils, as part of a possible biocontrol system, on the inhibition of isolate growth and the development of the disease.
5. To investigate the effects of non-pathogenic strains on the development of the disease in controlled environments and the field.
6. To discuss the results obtained and draw conclusion on the outcomes of the experimental programme.



Figure 1.6 Vanilla plants infected with root rot disease in the fields (left) and infected vanilla roots (right); note the symptoms of root wilt.



Figure 1.7 Vanilla plantation destroyed by root rot disease in the Daluo plantation of Xishuangbanna.

CHAPTER 2

***FUSARIUM OXYSPORUM* FORMA SPECIALIS *VANILLAE* AND PATHOGENICITY TESTING**

2.1 INTRODUCTION

2.1.1 *Fusarium oxysporum*

Fusarium oxysporum was characterised as the first *Fusarium* plant pathogen and it is still the most important pathogenic *Fusarium* species, causing more economic damage to agricultural crops than any other plant pathogen (Gordon, 1965). It is considered a common soil-borne plant pathogen with a worldwide distribution (Windels, 1993; Chen and Swart, 2001). It causes vascular wilts in a wide variety of plant species by directly penetrating roots and colonizing the vascular tissue (Inoue *et al.*, 2001). The economic damage caused by *Fusarium* wilt diseases has inspired considerable research on those species affecting important crops. Another common characteristic of *F. oxysporum* strains is the ability to parasitize plant roots, usually without inducing symptoms. This capability is non-specific and pathogenic strains can colonize roots of plants in which they will not cause disease (Gordon *et al.*, 1997).

Strains of *F. oxysporum* have been divided into formae speciales on the basis of virulence on a particular host or group of hosts (Correll, 1991). Within *F. oxysporum* there is a high level of host specificity with over 120 described formae speciales capable of causing vascular wilt diseases of many agricultural crops. Many formae speciales demonstrate a race structure. Further subdivisions of formae speciales into races are often made based on virulence to a particular set of differential host cultivars that vary in disease resistance (Armstrong and Armstrong, 1981).

Understanding the races of the pathogen is essential to guide the development of appropriate strategies for disease management that will enhance durability of *Fusarium* wilt resistance. It is not possible to differentiate one pathogenic formae speciales from another without the use of a suitable host plant (Belabid *et al.*, 2004).

Hence although virulence has been an extremely useful characteristic for differentiating strains of *F. oxysporum*, there are some inherent problems associated with characterizing strains based solely on pathogenicity.

Fusarium oxysporum Schlecht. f. sp. *Vanillae* (Tucker), synonym *F. batatas* Woll. Var. *vanillae* (Tucker) causes *Fusarium* root rot disease of vanilla (1965). This forma specialis has not been divided into different races based on pathogenicity to a set of differential cultivars and there is still no standard to identify different strains. While recognizing these difficulties, as an essential first step in developing potential biocontrol agents, non-pathogenic strains which are closely related to forms causing the disease, must be identified. Typically, large numbers of isolates from different sources have to be screened for pathogenicity using crop plant specimens in a standardized test.

2.1.2 *Fusarium* Resistance in Vanilla

The vanilla crop of choice is *Vanillae planifolia*, however, its cultivation is severely affected by *F. oxysporum* root rot infection. Laboratory inoculations suggest that the aerial root reaction to wounding and infection is neither rapid nor strong enough to block invasion by the fungus (Alconero, 1968). The fungus grows rapidly in the tissues and within a few days may destroy the entire root. Some root rot disease resistant species of vanilla are known, including *V. pompona*, *V. phaeantha* Rchb. f. and *V. barbellata* Rchb. f. and they can be hybridised with *V. frangrance* (Nielsen *et al.*, 1999). Greenhouse trials indicated that *V. phaeantha* was markedly resistant to vanilla root rot and that *V. planifolia* was the most susceptible species tested with *V. pomposa* and *V. barbellata* showing intermediate resistance. Hybrids of *V. tahitensis* and *V. planifolia* were classified as of intermediate resistance (Nielsen, 2000). There was different physiological reaction in different varieties when the plants were injured. Alconero (1968) reported that in wounded roots of the resistant variety, *V. phaeantha*, cells collapse sooner and secrete crystal-bearing idioblasts heavily. A larger number of idioblasts in the resistant species form dark secretions several cells from the wound site.

However, *V. phaeantha* have fruits of poor quality and short lengths that do not meet commercial criteria and plants often flower sparsely and tend to drop their fruits before maturity. By contrast, *V. planifolia* produces an adequate supply of flowers and retain its fruits until maturity (Anon., 2002). Hybrids produced between *V. phaeantha* and *V. planifolia* to date have not given the high grade products available from *V. planifolia*, but are similar to the less valuable fruits obtained from the other species.

Therefore, while there is potential for a breeding strategy for resistance to *F.*

oxysporum, almost nothing is known about the pathogenic nature of *F. oxysporum* in vanilla and there are difficulties in breeding. To develop breeding criteria, it would be important to know if there are any different races within the formae speciales and what variation exists within the pathogenic and non-pathogenic races of *F. oxysporum*. Moreover, *F. oxysporum* isolates are highly variable because of both their genetic characteristics and their response to changes in their environment (Nielsen *et al.*, 1999). Mutations can cause changes in the pathogenicity of the isolates because which result in loss of virulence. Hence, resistant varieties of vanilla have not been identified to date (Kosmiatin *et al.*, 2005).

Pathogenicity testing: Saprophytic forms of *F. oxysporum* cannot be distinguished from pathogenic isolates based on cultural or morphological characteristics for any of the *Fusaria*. Since *Fusarium* species commonly invade necrotic tissues in soil, isolation from diseased tissue is no guarantee that a pathogen has been isolated, thus, pathogenicity tests become essential. The methods used in pathogenicity tests are important when comparing results of various workers. Lack of agreement often is aggravated by a diversity of approaches used by researchers, hence, uniform testing is essential (Windels, 1993).

A number of factors are known to affect the results of pathogenicity testing. Fungal cultures, ideally, should be recently isolated from diseased tissue, prepared from single conidium and maintained by lyophilization. *F. oxysporum* is highly variable in virulence. Cultures may become avirulent if they have been routinely subcultured on rich media leading to mutation (Nielsen *et al.*, 1999). Concentrations of inoculum can affect the severity of diseases caused by *F. oxysporum* (Burgess *et al.*, 1981). Also the soil selected for pathogenicity tests should have physical and chemical properties similar to those where the disease occurred.

Plant age at the time of inoculation can affect disease severity determinations. It is important to maintain comparable plant ages in order to obtain consistent results. Armstrong and Armstrong (1975) found that inoculation of plants with wilt *Fusarium* in the early seedling stage may result in damping-off disease and an inaccurate evaluation of mature plant reaction. However, the propagation of vanilla from seed is extremely difficult. Various workers have attempted propagation by single node cuttings using growth regulators. Initially, one-node vanilla cuttings were taken from the field grown plant and were used to test the pathogenicity of *F. oxysporum* (Alconero, 1968; Ruan *et al.*, 1998). When the root was ~10 cm long, it was lightly cut to the cortex and then inoculated with a suspension of mycelium. The cuttings were placed in soil for 1 week before being scored for disease symptoms (Ruan *et al.*, 1998). Plants should be grown under environmental conditions similar to those during the cropping season where the disease occurs. Plants should be grown in deep containers that allow relatively normal growth and development, with temperatures and humidity that favoured disease occurrence. Vanilla stem and root rot disease is favoured by temperatures approaching 28°C with a humidity of up to 90%.

2.2 MATERIALS AND METHODS

2.2.1 Fungal Isolation

Fusarium oxysporum was isolated from infected vanilla plant tissue, debris and soil samples, which were collected from Manjingdai, Menglun, Daluo and Meiqi plantations in the Xishuangbanna area and Hekou County of South West Yunnan Province.

Freshly infected tissues were selected for isolation. The infected tissue was cut into small pieces (~2 x 2 x 2 mm) to limit the number of fungi growing from each piece (Burgess *et al.*, 1981). Infected tissues were washed with tap water to remove all visible soil, surface disinfected with 70% ethanol or 0.2% sodium hypochlorite for 1 min, and then were damp-dried on absorbent paper towels. A low nutrient, half-strength water agar medium was used for initial isolations by transferring the segments on to it (Bao and Lazarovits, 2002; Booth, 1971; Freeman *et al.*, 2002). Penicillin (50 µg/ml) or streptomycin (50 µg/ml) was added to the medium to prevent bacteria growth. Emerging colonies were transferred onto potato sucrose agar (PSA, see Appendix) and *Fusarium*-like isolates were purified using dilution plating to isolate single-spore colonies. Newly isolated *Fusarium* strains were examined for their morphological characteristics and identified using the system described by Nielsen *et al.* (1999), and preserved on sterile filter paper. Fungal cultures were maintained on PSA medium at 27°C in the dark. For the production of fungal spore inoculum, 50 ml of Capze's medium (see Appendix) was inoculated with 10⁷ microconidia and incubated on a rotary shaker (150 rpm) for 5 days at 27°C in the dark. Conidia (~90% microconidia) were harvested by filtering the culture through sterile microcloth and the spore concentration was measured using a haemocytometer under a light microscope.

When the fungus was isolated from soil samples, the soil dilution method was used. The soil samples were air-dried before placing on a PSA medium for direct isolation (Fang, 1998). Soil samples were pulverized in a micromill for 30 sec, and then passed through a 250 μm screen, and then 10 mg of soil was deposited uniformly onto the PSA plates. Plates were incubated for 2-4 days at 27°C in the dark. Single colonies were examined for their morphological characteristics and identified, then transferred to a new potato sucrose agar medium for purification.

2.2.2 Isolates Maintenance and Storage

Fungal isolates were cultured on PSA. Isolates were subcultured by transferring a small block of agar from the edge of a colony onto a fresh plate. Plates were incubated at 27°C in the dark (Fang, 1998). For the production of a fungal spore inoculum, flask cultures with Capze's medium were incubated at 27°C, with constant shaking at 200 rpm in the dark. Conidia were harvested by filtering the culture through sterile microcloth, then suspended in 50% (v/v) glycerol and stored at -80°C or as dry pellets preserved on sterile filter paper stored at -80°C.

2.2.3 Identification of *Fusarium oxysporum* f. sp. *Vanillae*

Purified colonies were subcultured to PSA for 7-10 days before any attempts were made to identify them. The plate containing the PSA culture was examined microscopically for the features of the fungus (Summerell *et al.*, 2003). If microconidia and chlamydospores were present, their shape, size and the manner in which they were formed were noted; examination was also carried out on the hyphae, on the surface of the agar, or embedded in the agar. Microscope slide preparations were made for detailed examinations of macroconidia, microconidia (if present) and the conidiogenous cells producing the microconidia (if present) (Booth, 1984). This information was used to identify most species accurately.

2.2.4 Vanilla Cultivars

The vanilla species used was *Vanillae planifolia*, which is normally grown in Xishuangbanna plantations. In order to obtain disease-free planting materials for pathogenicity testing, tissue culture seedlings were obtained from the Kunming Institute of Botany, Chinese Academy of Sciences. The seedlings for large-scale propagation were grown for 3 months before use, having a height of 10 cm with 5-6 leaves and 3-4 roots.

Vanilla *in vitro* Propagation

The seedlings were subcultured in Murashige and Skoog (MS, see Appendix) basal medium (Murashige and Skoog, 1962) every 4 weeks (Geetha and Shetty, 2000). The proliferating clusters were cultured in MS basal medium with 0.25 mg/L BA and 0.05 mg/L IAA and 2% sucrose for elongation of shoots. They were incubated at 28°C with a 12 h photoperiod utilizing a light intensity of 2500 lux from cool white fluorescent tubes under 90% relative humidity for 1 month to yield 8-10 cm long seedlings. Then the seedlings were cultured onto MS basal with 1.0 mg/L IAA and 0.1 mg/L NAA and 2% sucrose for formation of root initials for 2 months, producing 3-4 roots.

2.2.5 Pathogenicity Test on Vanilla Plants

The pathogenicity testing of the isolates was performed using a modification of the method of Alconero (Alconero, 1968; Gure *et al.*, 2005; Wen and Li, 1992). Fungal isolates were grown in 100 ml Capze's liquid medium in a 250 ml flask incubated on a rotary shaker (150 rpm) for 5-7 days at 27°C in the darkness. A sufficient volume of culture was used to give a suspension of conidia and mycelial fragments of 10^6 conidia /ml.

Pathogenicity tests were carried out using a root-dip inoculation method (Fang, 1998). Vanilla seedlings were uprooted gently from the medium. The root was lightly injured with a sterile scalpel, the wound site was dipped in a spore suspension containing 10^6 spores /ml for 5 min, dried briefly on a paper towel and then transplanted into a pot containing water agar medium (see Appendix). Wounded, non-inoculated roots were used as controls and were soaked in distilled water. Plants were left in a controlled environment at 28°C with a 12 h dark/12 h light cycle. Relative humidity was maintained close to 100%. Experiments using this method were repeated four times for each fungal isolate.

Disease development was evaluated every 5 days for 1 month before symptoms were scored using a disease severity index on a scale of 0-5 (Fang, 1998): 0, no symptoms; 1, root tips or injured root turned brown, rot occurred with the length of the injured section less than $\frac{1}{4}$ of total length; 2, root turned brown, rot occurred with the length of the injured section more than $\frac{1}{4}$ of total length, but less than $\frac{1}{2}$ of total length; 3, root turned brown and rot occurred with the length of the injured section more than $\frac{1}{4}$ of total length, but less than $\frac{3}{4}$ of total length; 4, all roots turned brown with rot; 5, all roots turned brown with rot and the pathological symptoms extended to the stem base. Using this scale, the pathogenic ability of isolates can be

directly compared. Strongly pathogenic isolates (3+) cause the roots to be injured on the scale 3-5, where the number of these roots are more than $\frac{1}{2}$ of total injured roots; moderately pathogenic isolates (2+) cause the roots to be injured on the scale 3-5, where the number of these roots are less than $\frac{1}{2}$ of total injured roots; hypovirulent isolates (+) cause the roots to be slightly injured on the scale of scale 1-2; non-pathogenic isolates (-) failed to cause root disease.

2.3 RESULTS

2.3.1 Identification of *Fusarium oxysporum* f. sp. *Vanillae*

Based on the information of morphological characteristic on the isolates provided by Booth (1971), two major *Fusarium* species, *F. oxysporum* and *F. solani*, were identified.

All isolates of *F. oxysporum* produced abundant, single-celled, oval or oblong microconidia (Figure 2.1a). Short and unbranched microconidiophores, and long, four to five septate, slightly falcate macroconidia were the most important morphological characteristics in identifying *F. oxysporum*. Chlamydo spores were usually terminal and one or two celled. Isolates of *F. solani* produced mostly sparse, single-celled, oval or oblong microconidia (Figure 2.1b). Microconidia (Figure 2.1c) were characteristically produced on long microconidiophores. Chlamydo spores were intercalary and terminal. Macroconidia (Figure 2.1d) were abundant, medium to long, stout, and three to four septate. The isolates had different colony characteristics and pigmentation on PSA. The aerial mycelium of *F. oxysporum* isolates ranged from white to purple and the undersurface of colonies ranged from white to dark purple. The aerial mycelium and the undersurface of colonies for *F. solani* isolates ranged from cream to tan.

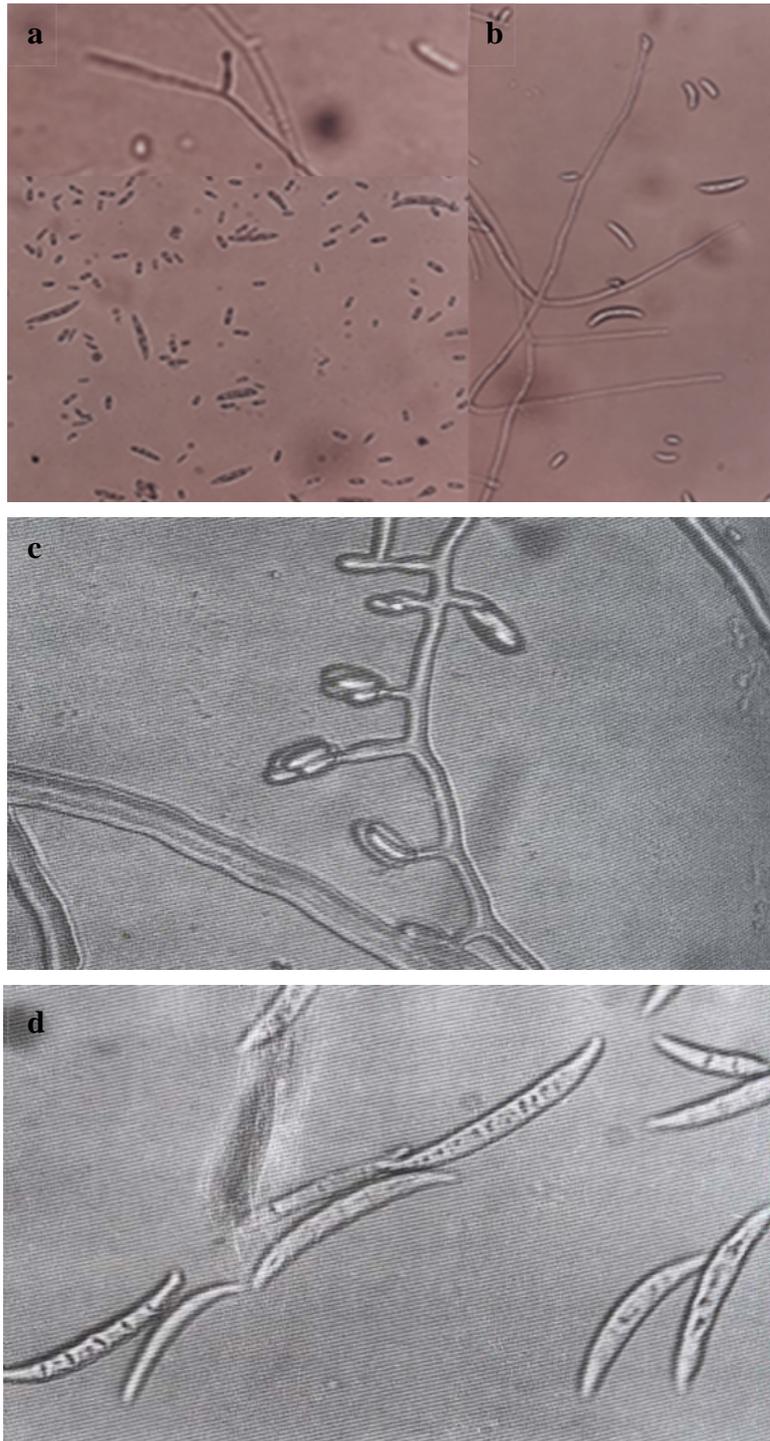


Figure 2.1 Microconidia and Macroconidia of *F. oxysporum* and *F. solani*

a: microconidia and macroconidia of *F. oxysporum*.

b: sparse, single-celled, oval or oblong microconidia and abundant, medium to long, stout, and three- to four- septate macroconidia of *F. solani*. Microconidia were characteristically produced on long microconidiophores.

c: abundant, single-celled, oval or oblong microconidia and short and unbranched microconidiophores of *F.oxysporum*.

d: long, four to five-septate, slightly falcate macroconidia of *F. oxysporum*.

2.3.2 Isolation of *Fusarium oxysporum* f. sp. *vanillae*

Of the 252 isolates of *F. oxysporum* and *F. solani* obtained from infected tissues and soils, 174 were *F. oxysporum* (69%) and 78 were *F. solani* (31%). Of the 210 isolates from the Xishuangbanna area, 148 were *F. oxysporum* (70.5%) and 62 were *F. solani* (29.5%). Of these, 56 were from Menlung Plantation [40 were *F. oxysporum* (71.4%), and 16 were *F. solani* (28.6%)], 55 from Manjingdai Plantation [46 were *F. oxysporum* (83.6%), and 9 were *F. solani* (16.4%)], 40 from Daluo Plantation [30 were *F. oxysporum* (75%), and 10 were *F. solani* (25%)], and 59 from Meiqi Plantation [32 were *F. oxysporum* (54.2%), and 27 were *F. solani* (45.8%)]. Of the 42 isolates from Hekou County, 26 were *F. oxysporum* (62%) and 16 were *F. solani* (38%). *F. oxysporum* was the predominant fungus (69%) in all the vanilla plantation areas in Yunnan Province (Table 2.1). Vanilla stem and root rot was diagnosed to be caused by the fungal pathogen *Fusarium oxysporum* f. sp. *vanillae*. This accords with previous studies (Ruan *et al.*, 1998). Many pathogens were directly isolated from infected vanilla plants or soils from Manjingdai, Menglun, Daluo plantations in the Xishuangbanna area or plantations in the Hekou County of Yunnan Province (Figure 2.2).



Figure 2.2 The distribution of vanilla plantations in Yunnan Province.
 Note the red area showing Manjingdai, Menglun, Daluo plantations in the Xishuangbanna area and in Hekou County in the Honghe area.

Table 2.1 Isolates of *Fusarium oxysporum* and *F. solani* obtained from infected tissues of plants grown in naturally infested field soil from the Xishuangbanna area and Hekou County

Origin	Source	Number of isolates	<i>F. oxysporum</i>		<i>F. solani</i>	
			Number	Percent	Number	Percent
Mengjindai	Infected tissues or soils	55	46	83.6	9	16.4
Daluo	Infected tissues or soils	40	30	75	10	25
Menglun	Infected tissues or soils	56	40	71.4	16	28.6
Meiqi	Infected tissues or soils	59	32	54.2	27	45.8
Hekou	Infected soils	42	26	61.9	16	39.1
Total		252	174	69	78	31

2.3.3 Virulence of *Fusarium oxysporum*

Of 81 isolates of *F. oxysporum* f. sp. *vanillae* tested, 32 isolates (39.5%) were rated as non-pathogenic, 49 isolates (60.5%) were rated as pathogenic (Table 2.2a-d). The pathogenicity results showed the different pathogenic abilities of isolates. Twenty-five strains (30.9%) were rated as strongly pathogenic isolates (3+) which caused the seedlings to produce severe symptoms, most of the roots turned brown with rot, at times the pathological symptom even extended to the stem base. Eight isolates (9.88%) showed moderate pathogenicity (2+), where less than 50% of total roots showed severe symptoms; 16 isolates (19.7%) were hypovirulent isolates (+) which gave the root-tips slight disease or on some roots no symptoms at all. Thirty-two strains (39.5%) failed to cause root injury (-) and they were classified as non-pathogenic isolates.

Of the 81 isolates of *F. oxysporum*, 17 isolates were recovered from the Daluo plantation, of which 14 isolates were rated as pathogenic isolates (82.3%) and 3 as non-pathogenic isolates (17.6%); 26 from the Menglun plantation, in which 12 isolated were pathogenic (46.1%) and 14 isolates were non-pathogenic (53.8%); 18 isolates from the Manjingdai plantation, in which 12 isolates were pathogenic (66.7%), whilst the other 6 isolates were non-pathogenic (33.3%); of the 20 isolates obtained from the plantation in Hekou County, 11 isolates were pathogenic (55%) and 9 were non-pathogenic (45%). A further six isolates were taken from the Meiqi plantation, but because of practical difficulties it was not possible to complete pathogenicity testing (Table 2.2e).

Table 2.2 Geographic origins and the pathogenicity of isolates of *Fusarium oxysporum vanillae* used in the study

2.2a Isolates from Daluo plantation in the Xishuangbanna area

Isolate Number	Strains	Geographical origins	Species	Virulence rating
1	DL-1	Daluo plantation	<i>F. oxysporum</i>	-
2	DL-1-1	Daluo plantation	<i>F. oxysporum</i>	3+
3	DL-1-2	Daluo plantation	<i>F. oxysporum</i>	3+
4	DL-2-1	Daluo plantation	<i>F. oxysporum</i>	3+
5	DL-2-3	Daluo plantation	<i>F. oxysporum</i>	3+
6	DL-2-4	Daluo plantation	<i>F. oxysporum</i>	2+
7	DL-2-5	Daluo plantation	<i>F. oxysporum</i>	3+
8	DL-2-7	Daluo plantation	<i>F. oxysporum</i>	-
9	DL-2-9	Daluo plantation	<i>F. oxysporum</i>	+
10	DL-2-12	Daluo plantation	<i>F. oxysporum</i>	2+
11	DL-2-15	Daluo plantation	<i>F. oxysporum</i>	3+
12	DL-3	Daluo plantation	<i>F. oxysporum</i>	-
13	DL-3-1	Daluo plantation	<i>F. oxysporum</i>	+
14	DL-3-2	Daluo plantation	<i>F. oxysporum</i>	3+
15	DL-4-1	Daluo plantation	<i>F. oxysporum</i>	3+
16	DL-4B	Daluo plantation	<i>F. oxysporum</i>	3+
17	DL-13b	Daluo plantation	<i>F. oxysporum</i>	+

2.2b Isolates from Menglun plantation in the Xishuangbanna area

Isolate Number	Strains	Geographical origins	Species	Virulence rating
18	ML-1-7	Menglun plantation	<i>F. oxysporum</i>	3+
19	ML-1-8	Menglun plantation	<i>F. oxysporum</i>	2+
20	ML-2-4	Menglun plantation	<i>F. oxysporum</i>	-
21	ML-2a	Menglun plantation	<i>F. oxysporum</i>	-
22	ML-2d	Menglun plantation	<i>F. oxysporum</i>	+
23	ML-3-1	Menglun plantation	<i>F. oxysporum</i>	3+
24	ML-3-3	Menglun plantation	<i>F. oxysporum</i>	3+
25	ML-4-1	Menglun plantation	<i>F. oxysporum</i>	2+
26	ML-5-1	Menglun plantation	<i>F. oxysporum</i>	+
27	ML-5-2	Menglun plantation	<i>F. oxysporum</i>	-
28	ML-6-1	Menglun plantation	<i>F. oxysporum</i>	3+
29	ML-6-5	Menglun plantation	<i>F. oxysporum</i>	-
30	ML-7-1	Menglun plantation	<i>F. oxysporum</i>	-
31	ML-7-2	Menglun plantation	<i>F. oxysporum</i>	-
32	ML-7-3	Menglun plantation	<i>F. oxysporum</i>	-
33	ML-7-4	Menglun plantation	<i>F. oxysporum</i>	2+
34	ML-7-7	Menglun plantation	<i>F. oxysporum</i>	+
35	ML-7-8	Menglun plantation	<i>F. oxysporum</i>	-
36	ML-7-9	Menglun plantation	<i>F. oxysporum</i>	-
37	ML-7-10	Menglun plantation	<i>F. oxysporum</i>	2+
38	ML-8-1	Menglun plantation	<i>F. oxysporum</i>	3+
39	ML-8-2	Menglun plantation	<i>F. oxysporum</i>	-
40	ML-8-4	Menglun plantation	<i>F. oxysporum</i>	-
41	ML-13	Menglun plantation	<i>F. oxysporum</i>	-
42	ML-13b	Menglun plantation	<i>F. oxysporum</i>	-
43	ML-13d	Menglun plantation	<i>F. oxysporum</i>	-

2.2c Isolates from Manjingdai plantation in the Xishuangbanna area

Isolate Number	Strains	Geographical origins	Species	Virulence rating
44	MJD-2	Manjingdai plantation	<i>F. oxysporum</i>	-
45	MJD-2-1	Manjingdai plantation	<i>F. oxysporum</i>	-
46	MJD-2-2	Manjingdai plantation	<i>F. oxysporum</i>	+
47	MJD-2B	Manjingdai plantation	<i>F. oxysporum</i>	-
48	MJD-2C-1	Manjingdai plantation	<i>F. oxysporum</i>	3+
49	MJD-2C-2	Manjingdai plantation	<i>F. oxysporum</i>	+
50	MJD-2D-1	Manjingdai plantation	<i>F. oxysporum</i>	3+
51	MJD-2e	Manjingdai plantation	<i>F. oxysporum</i>	3+
52	MJD-3A-1	Manjingdai plantation	<i>F. oxysporum</i>	+
53	MJD-3A-3	Manjingdai plantation	<i>F. oxysporum</i>	-
54	MJD-3B	Manjingdai plantation	<i>F. oxysporum</i>	-
55	MJD-3B-1	Manjingdai plantation	<i>F. oxysporum</i>	3+
56	MJD-3C	Manjingdai plantation	<i>F. oxysporum</i>	+
57	MJD-4	Manjingdai plantation	<i>F. oxysporum</i>	-
58	MJD-4-3	Manjingdai plantation	<i>F. oxysporum</i>	+
59	MJD-4B-1	Manjingdai plantation	<i>F. oxysporum</i>	3+
60	MJD-4i	Manjingdai plantation	<i>F. oxysporum</i>	3+
61	MJD-5G-1	Manjingdai plantation	<i>F. oxysporum</i>	2+

2.2d Isolates from plantations in Hekou County

Isolate Number	Strains	Geographical origins	Species	Virulence rating
62	HK-1b-1	Hekou county	<i>F. oxysporum</i>	3+
63	HK-1b-2	Hekou county	<i>F. oxysporum</i>	+
64	HK-1b-3	Hekou county	<i>F. oxysporum</i>	3+
65	HK-2a-1	Hekou county	<i>F. oxysporum</i>	+
66	HK-2a-4-1	Hekou county	<i>F. oxysporum</i>	+
67	HK-2b-2	Hekou county	<i>F. oxysporum</i>	-
68	HK-3a-1	Hekou county	<i>F. oxysporum</i>	3+
69	HK-3a-3	Hekou county	<i>F. oxysporum</i>	-
70	HK-5a-2	Hekou county	<i>F. oxysporum</i>	-
71	HK-5a-3	Hekou county	<i>F. oxysporum</i>	-
72	HK-5a-4	Hekou county	<i>F. oxysporum</i>	3+
73	HK-5b-4-1	Hekou county	<i>F. oxysporum</i>	-
74	HK-6a-2	Hekou county	<i>F. oxysporum</i>	3+
75	HK-6a-3	Hekou county	<i>F. oxysporum</i>	+
76	HK-7a-1	Hekou county	<i>F. oxysporum</i>	-
77	HK-8a-1	Hekou county	<i>F. oxysporum</i>	-
78	HK-8a-2	Hekou county	<i>F. oxysporum</i>	-
79	HK-9a-1	Hekou county	<i>F. oxysporum</i>	-
80	HK-9a-2	Hekou county	<i>F. oxysporum</i>	2+
81	HK-10b-1	Hekou county	<i>F. oxysporum</i>	+

2.2e Isolates from Meiqi plantation in the Xishuangbanna area

Isolate Number	Strains	Geographical origins	species	Virulence rating
82	MQ-R5	Meiqi Plantation	<i>F. oxysporum</i>	N. D
83	MQ-R12	Meiqi Plantation	<i>F. oxysporum</i>	N. D
84	MQ-R13	Meiqi Plantation	<i>F. oxysporum</i>	N. D
85	MQ-SL4	Meiqi Plantation	<i>F. oxysporum</i>	N. D
86	MQ-sm14	Meiqi Plantation	<i>F. oxysporum</i>	N. D
87	MQ-sm19	Meiqi Plantation	<i>F. oxysporum</i>	N. D

Note: The virulence rating of each isolate was determined according to the disease severity ratings: 3+, strongly pathogenic strains giving severe disease; 2+, moderately pathogenic strains giving moderate disease; +, hypovirulent pathogenic strains giving slight or no disease; -, non-pathogenic strains giving no disease; N.D: not determined.

2.4 DISCUSSION

The results of the isolations of *F. oxysporum* from diseased vanilla plants grown in naturally infested soil, coupled with pathogenicity tests, indicated that strongly pathogenic and moderately pathogenic forms of *F. oxysporum* were primary and frequent colonizers of vanilla tissues. *F. solani* isolates were less frequently obtained. Some isolates of *F. oxysporum* were found to be non-pathogenic colonizers of vanilla plants. Moreover, 16 hypovirulent *F. oxysporum* isolates were recovered from the infected tissues and soils. Sneh *et al.* (2002) noted that hypovirulent isolates could provide plants immunity against diseases caused by virulent isolates of the same species via a number of mechanisms, such as induction of plant resistance, transmission of dRNA mycoviruses and mutating genes of pathogens to render them non-pathogenic epiphytic mutualists. Thus, hypopathogenic strains would have potential as biocontrol agents. This will need further research in vanilla. Alabouvette *et al.* had also studied the biocontrol effects and mechanisms of non-pathogenic isolates (Alabouvette *et al.*, 1992; 1995; 1999; Trouvelot *et al.*, 2002; Olivain and Alabouvette, 1997; 1999; Lemanceau *et al.*, 1991). Non-pathogenic and hypovirulent *F. oxysporum* strains were isolated from specific field soils which may result in a natural biological control of disease (Alabouvette *et al.*, 1985; Roebroek and Mes, 1992; Scher and Baker 1980; Kilic, 1997; Sneh, 1998). According to the results in this study, the occurrence of non-pathogenic strains was highest in the plantations of Menglun and Hekou County. If hypovirulent isolates are also included, the percentage of potentially-useful isolates may be even higher. The percentage of non-pathogenic and hypovirulent isolates in the plantations of Hekou County (70%) is more than that of in the plantations of Daluo (35.3%), Menglun (65.4%), and Manjingdai (61.1%) plantation. In investigating the sources of the isolates, 20 were

from the plantation in Hekou County were isolated from soil (Table 2.1), whilst other providers were unable to differentiate their source, labeling them as isolated from both infected roots and soils.

These results demonstrate that non-pathogenic strains of *F. oxysporum* can be isolated from plantations where the disease occurs. The strains have potential for use in biocontrol strategies for the management of the disease. The cause(s) of the variations in occurrence of non-pathogenic strains in different areas requires further study.

CHAPTER 3

VEGETATIVE COMPATIBILITY TESTING OF *FUSARIUM*

OXYSPORUM F. SP. VANILLAE

3.1 INTRODUCTION

3.1.1 The Heterokaryon and Parasexual Recombination

The term heterokaryosis was first used to describe the association of nuclei of opposite mating in the same hyphae in *Phycomyces nitens* in 1912 (reviewed by Parmeter *et al.*, 1963). Since then, the term has been used to describe any system where unlike nuclei are found in common vegetative hyphae. Heterokaryon formation between different fungal individuals is an important component of many fungal life cycles and may serve as the first step in the parasexual cycle and the transmission of hypovirulent factors such as dsRNAs (Leslie, 1993). Puhalla (1984) examined anastomosis in *Fusarium apii* by pairing colour mutants. He found that there was no nuclear migration within the heterokaryon and that hyphal tips, 2-3 mm ahead of the anastomosed cells, were homokaryotic. This state was also reported in *F. moniliforme* (Puhalla and Spieth, 1983), where genetic complementation was restricted to the anastomosed cells and products of this complementation were translocated to the homokaryotic areas of the mycelium. This phenomenon of heterokaryon formation may be a mechanism for liberating diversity previously “stored” in the heterokaryon. Heterokaryon formation has potential benefits of mitotic genetic exchange (parasexual cycle) or may increase biomass for co-operation in physiological efforts such as resource exploitation or asexual/sexual reproduction (Leslie, 1993; Di Primo *et al.*, 2001).

The term parasexual cycle was first used by Pontecorvo *et al.* (1953) to describe the genetic process in *Aspergillus nidulans*, which involves heterokaryon formation, fusion of two unlike haploid nuclei to give a diploid heterozygous nucleus, and mitotic recombination. Genetically dissimilar nuclei are intermingled within a common cytoplasm in which nuclear fusion may occur. When genotypically different

haploid nuclei are involved, a heterozygous diploid nucleus results. During the multiplication of such nuclei, mitotic crossing over or haploidization or both may occur and spores may eventually be obtained which have either diploid nuclei homozygous for certain genetic markers or haploid nuclei containing new combinations of genetic markers (Tuveson and Garbere, 1961; Leslie, 1993).

Since its first demonstration by Pontecorvo *et al.* (1953), parasexual recombination has been detected in many ascomycetes, basidiomycetes and deuteromycetes, suggesting that parasexual processes are widespread in fungi (Glass *et al.*, 2000; Leslie *et al.*, 1996). Leslie (1993) suggested that a genetic analysis of factors controlling or determining pathogenicity in the imperfect fungus *Fusarium oxysporum* f. sp. *pisi* might be accomplished by using the parasexual cycle. Heterokaryosis may be especially important in imperfect fungi since it presents an opportunity for parasexual recombination. It was believed that parasexuality explains the diversity found in many fungi and especially the origin of new races of plant pathogenic fungi (Leslie *et al.*, 1996). Theoretically, recombination between different races of a pathogen could give rise to new combinations of virulence and avirulence genes (Gomez-Gomez, *et al.*, 2002; Pontecorvo *et al.*, 1953).

3.1.2 Vegetative Compatibility Groups

In many fungi, sexual and vegetative heterokaryons are quite distinct from one to another (Leslie, 1993). Strains capable of forming a successful sexual heterokaryon may be unable to form a successful vegetative heterokaryon and *vice versa* (Di Primo *et al.*, 2001). Strains that are capable of forming these types of heterokaryons are referred to as “sexually” or “vegetatively” compatible, respectively (Correll *et al.*, 1986a,b; 1987). Vegetative compatibility (VC) is the ability of two fungal isolates to fuse when grown in close proximity. Groups of isolates that share identical alleles at corresponding loci are known as vegetative compatibility groups (VCGs). Hence in an asexual organism, such as *F. oxysporum*, vegetative compatibility is an important mechanism of generating genetic diversity and vegetative compatibility analysis is an indicator of the genetic relatedness of isolates (Glass, 2000). Strains that are vegetative compatible with one another are described as members of the same vegetative compatibility group, or VCG.

Sexual compatibility is usually governed by one or more mating-type loci that may have two or more alleles. Vegetative compatibility is mediated by multiple vegetative incompatibility loci (*vic* loci) (Leslie, 1993). In most cases vegetative compatibility is homogenic, that is to say, two fungal isolates are vegetative compatible only if the alleles of each of their corresponding loci are identical. Hence, vegetative compatible strains are more likely to be genetically similar than incompatible strains (Puhalla, 1985). For example, strains of fungus that are vegetative compatible are quite similar with respect to such traits as colony size, antibiotic production, virulence and isozyme patterns (Correll *et al.*, 1986).

Vegetative compatibility has been useful in characterizing population structures of asexual fungi, including *F. oxysporum* (Appel and Gordon, 1994; Elias and

Schneider, 1991), *Colletotrichum* spp. and *Veniciliium* spp. (Correll *et al.*, 1987). Fungal strains able to undergo mutual hyphal anastomosis and form viable heterokaryons are assigned to the same VCG. Without sexual recombination, members of each VCG will form a genetically isolated subpopulation that will be subjected to standard population genetic forces, such as selection, mutation, migration and drift. Moreover, members within a VCG have the potential to exchange genetic material through parasexual recombination (Pasquali *et al.*, 2005).

During the mid-1980s, Puhalla (1985) first attempted to distinguish and classify strains of *F. oxysporum* by genetic means, rather than by morphology and/or host range. Using a technique previously applied to *Aspergillus*, Puhalla created auxotrophic mutants unable to use nitrate as a sole nitrogen source. These *nit* mutants, affected at different steps in the pathway involved in nitrogen reduction, were paired on minimal medium. If pairs of isolates fused to produce a heterokaryon, complementation resulted in wild-type growth. Such isolates were determined to be in the same VCG. The four or five-digit numbering system for VCG designation, proposed by Puhalla (1985), is still in use. Kisterler *et al.* (1998) and Katan (1999a,b) promoted the standardization of the numbering system of VCGs in *F. oxysporum* and numbered 38 formae speciales (f. sp) of *F. oxysporum* which have been subjected to VCG analysis.

VCG analysis has a number of advantages for this investigation. Firstly, it is a good method to assess the genetic diversity of a population of *Fusarium* and studies of populations using VCGs as a means to measure diversity have become widespread (Elias and Schneider, 1992; Kistle, 1997; Lori *et al.*, 2004; Plyler *et al.*, 2000; Vakalounakis and Fragkiadakis, 1999). Since the mid-1980s, an increasing number of formae speciales have also been characterized by the VCG method, following the

approach and procedure described by Puhalla (1995) and Leslie (1993). Secondly, it is relatively easy and cheap to perform VCG on a large number of isolates, compared to molecular analyses (Mes *et al.*, 1999; Morita *et al.*, 2005), such as DNA fingerprinting (Namike *et al.*, 1994; 1998; 2001; Fernandez *et al.*, 1994; Kawabe *et al.*, 2005), RAPD (Kalc *et al.*, 1996; Kerényi *et al.*, 1997), AFLP and similar other techniques.

In addition, spontaneous vegetative compatibility allows for the characterization of the non-pathogenic portion of the population. *Fusarium oxysporum* is distributed widely in natural and cultured soils and non-pathogenic *F. oxysporum* isolates are noted as biological control agents (Fravel *et al.*, 2003). So an understanding of diversity in this portion of the population must be developed. It was strongly suggested that research on vegetative compatibility by using nitrate nonutilizing (*nit*) mutant isolates should be used for identification of non-pathogenic *F. oxysporum* strains for future use as biological control agents (Correll *et al.*, 1987; Liu *et al.*, 1995; Tombe *et al.*, 1994). It is because within the last 25 years, the population studies of plant pathogenic fungi has been important, in which vegetative compatibility serves as a polymorphic marker (Leslie and Klein, 1996). However, vegetative compatibility can also be used as a model for self-nonsel recognition, as a tool in the construction of genetic maps and the rapid isogenization of strains; and, when the interaction mechanism is understood, as a novel target for antifungal agents (Leslie and Klein, 1996).

3.1.3 Heterokaryon Self-incompatibility (HSI) and Its Genetic Control

Although there are obvious benefits to filamentous fungi to form heterokaryons, a genetic mechanism exists that restricts heterokaryon formation between two genetically different individuals in many fungi (Jacobson and Gordon, 1988; Glass *et al.*, 2000; Leslie, 1993). Hyphal fusion does occur between incompatible strains, but this is followed rapidly by cell death. Those isolates that do not form viable vegetative heterokaryons are vegetatively incompatible, which sometimes can be assayed as a “barrage” of hypertrophic mycelium where the isolates meet. In strongly interacting strains a marked line of necrotic mycelium can be observed, although there may be also lesser interactions.

In nature vegetative incompatibility is widespread and its function maybe to prevent the spread of mycoviruses and the fungi may enjoy the benefits of complementation or heterosis (Leslie, 1993), which can make the isolates survive when having parasexual reproduction. In genetics, vegetative compatibility can be complex, and sexually compatible isolates are often vegetative incompatible (Leslie, 1996). It has been said that vegetative incompatibility is determined by nuclear gene loci known as *vic* or *het* genes that are dispersed throughout the genome and responsible for the VCG phenotype (Leslie *et al.*, 1996). Two isolates of a species are vegetative compatible if they have identical alleles at corresponding *vic* loci. That is to say, if individuals differ in specificity at one or more *vic* loci, heterokaryotic fusion cells are usually compartmentalized and undergo death by the lytic process, a phenomenon called vegetative incompatibility or heterokaryon incompatibility (Leslie, 1993). In *Cryphonectria parasitica* (Huber, 1996), vegetative incompatibility prevents stable heterokaryon formation and it is controlled by at least six unlinked vegetative incompatibility (*vic*) loci. *Nerospora crassa* has 10 such loci, with two

alleles at each locus (reviewed by Leslie, 1993). *Fusarium moniliforme* is the species most closely related to *F. oxysporum* that has been investigated. So far only two alleles have been discovered at each locus in *Fusarium moniliforme*, the number of loci is at least eight (Puhalla, 1985). The high degree of specificity required by this system explains the widespread occurrence of vegetative incompatibility found in the wild.

It is important, therefore, to identify vegetative incompatible strains. Vegetative incompatibility occurs if individuals differ in specificity at one or more *vic* loci and there are strains carrying mutations that prevent them from fusing to form heterokaryons, even with themselves. All crosses between two HSI strains, examined to date, are all female sterile (Leslie, 1993). The female-sterile mutants are at a selective disadvantage every time sexual reproduction occurs, and must have an advantage during vegetative propagation to persist at a significant frequency. When a high frequency of female-sterile strains is observed in field populations, it indicates that vegetative propagation is a significant component of the fungus' natural history (Leslie *et al.*, 1996). These strains can lead to an incorrect diagnosis of vegetative incompatibility since they will usually not form heterokaryons with any other strains. It is important to identify such strains to prevent the over-estimation of the number of VCGs within a population.

To sum up, knowledge of vegetative compatibility (or vegetative incompatibility) is important for two reasons. First, in some instances it may be a relatively objective test for genetic similarity, because in ascomycetes with better characterized genetic systems vegetative compatibility is known to be the result of the action of alleles at several distinct loci. For example, in *Neurospora crassa* heterokaryon compatibility is conferred by at least 11 loci dispersed on 5

chromosomes (Glass *et al.*, 2000). For two strains of *N. crassa* to be compatible, alleles at all loci must be identical. Thus, the more closely related two isolates are genetically similar, then the more likely they are to be vegetatively compatible. By inference, only isolates of *F. oxysporum* that are very similar genetically (and isoallelic at all presumptive heterokaryon compatibility loci) would be members of the same VCG. Therefore, VCG determination may reflect genetic similarities, although not the degree of genetic differences, among isolates of the species.

Second, and perhaps a more important aspect of VCG, is what it might imply about the reproductive strategy and, ultimately, the population structure of the fungus. *Fusarium oxysporum* is presumed to reproduce strictly by asexual means and strains related by clonal descent should be within the same VCG, because clonally derived strains would be isogenic, or nearly so, and, therefore, vegetatively compatible (unless they happen to be vegetatively self-incompatible (Leslie, 1993)). Additionally, isolates within separate clonal lineages and in different VCGs might then be isolated genetically due to vegetative incompatibility. Clonal reproduction and, more importantly, the lack of meiotic recombination would greatly limit or preclude reassortment of genes for heterokaryon incompatibility. Indeed, a population structure of *F. oxysporum* consisting of distinct clonal lineages corresponding to distinct VCGs suggests the absence of genetic recombination between members of those VCGs/lineages (Kistler, 1997).

3.1.4 Analysis of *nit* Mutants

Most fungi can utilize nitrate as a nitrogen source by reducing it to ammonium via nitrate reductase and nitrite reductase (Fig. 3.1). Some fungi unable to utilize nitrate, such as the higher Basidiomycetes, the Saprolegniaceae and the Blastocladales, apparently cannot synthesize nitrate reductase. Chlorate, a nitrate analogue, has been very useful for studying nitrate assimilation in fungi as well as bacteria, algae and plants. The reduction of chlorate to chlorite by nitrate reductase can presumably result in chlorate toxicity in these organisms. In general, chlorate-sensitive strains can reduce nitrate to nitrite but chlorate-resistant strains cannot (Correll *et al.*, 1987). *Nit* mutants have been selected from *F. oxysporum* without mutagen treatment. Therefore, a *nit* mutant can be used to assess heterokaryon formation and vegetative compatibility.

Nit mutants have been recovered from a number of fungi, including *F. oxysporum*, *Nerospora crassa*, *Ustilago maydis*, *A. nidulans*, *A. flavus*, *F. moniliforme* and *F. solani*. All the *nit* mutants recovered from *F. oxysporum* could be divided into three distinct phenotypic classes. These classes presumably represent a mutation at a nitrate reductase structural locus (*nit1*) (Table 3.1), or a nitrate-assimilation pathway-specific regulatory locus (*nit3*) or loci that affect the assembly of a molybdenum-containing cofactor necessary for nitrate reductase activity (NitM) (Correll *et al.*, 1987).

Vegetative compatibility groupings have been widely investigated in pathogenic *Fusarium oxysporum* formae speciales (Correll *et al.*, 1986b). Since no sexual stage is known for *F. oxysporum*, the only possible mechanism for genetic exchange would be parasexuality. Since parasexuality in nature relies on heterokaryosis, it could only occur between members of the same VCG. Each VCG is, therefore, a genetically

isolated population. The main reason for the interest shown in VCGs is the hope that VCGs will be correlated with *formae speciales* or even races of a pathogen. VCG analysis, which is relatively easy to carry out experimentally, would therefore circumvent the need for lengthy and unreliable pathogenicity tests (Correll *et al.*, 1986b; 1991; Elena and Pappas, 2002; Fernandez *et al.*, 1994; Kondo *et al.*, 1997).

On the other hand, it is more preferable if vegetative incompatibility phenotypes that are naturally occurring genetic markers could be used to differentiate strains of *F. oxysporum*. To determine if two isolates are vegetative compatible or vegetative incompatible, the isolates must be marked with complementary mutants so that heterokaryotic growth can be detected on a selective medium.

The objectives of VCG analysis were to investigate genetic diversity within the population of *F. oxysporum* f. sp. *vanillae* with respect to the vegetative compatibility, to determine the relationship between VCGs and virulence, and the relationship between pathogenic and non-pathogenic strains.

Figure 3.1 The Nitrate and hypoxanthine assimilation pathways

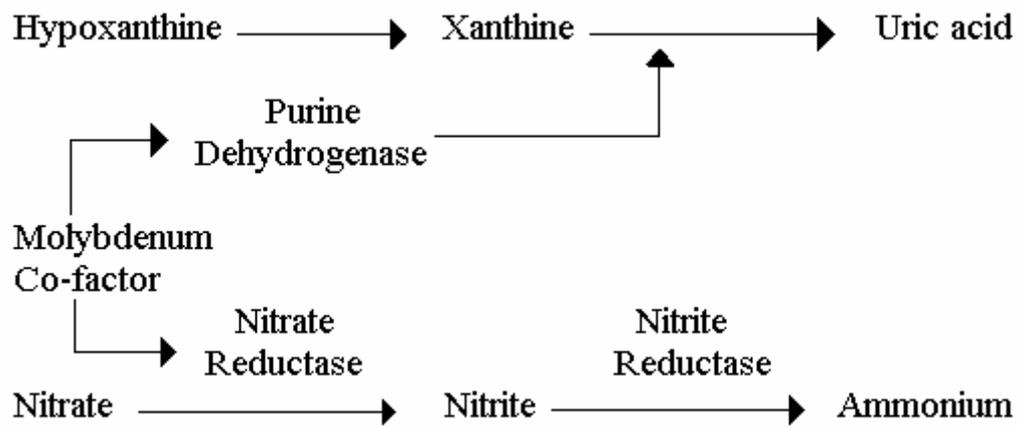


Table 3.1 Identification of nitrate non-utilizing (*nit*) mutants from *Fusarium oxysporum* by growth on different nitrogen sources

Mutation	Mutant designation	Growth on nitrogen source*		
		Nitrate	Nitrite	Hypoxanthine
None	Wild type	+	+	+
Nitrate reductase structural locus	<i>nit1</i>	-	+	+
Nitrate-assimilation pathway-specific regulatory locus	<i>nit3</i>	-	-	+
Molybdenum cofactor loci	NitM	-	+	-

* Growth on basal medium with various nitrogen sources;
 + = Typical wild-type growth, - = thin growth with no aerial mycelium.

3.2 Materials and methods

3.2.1 Fungal Strains

See Section 2.3.3.

3.2.2 Culture Media

Media for the maintenance of wild type isolates was as detailed in Section 2.2.2. For all experiments involving the isolation or testing of chlorate resistant mutants, a minimal medium (MM), supplemented according to the intended use of the medium (Puhalla, 1985) was used.

Minimal medium (per litre distilled water): sucrose, 30 g; NaNO₃, 2g; KH₂PO₄, 1g; MgSO₄·7H₂O; 0.5 g; KCl, 0.5 g; agar, 20 g; and sterile trace elements solution, 0.2 ml.

Trace elements solution (per 95 ml distilled water): citric acid, 5 g; ZnSO₄·7H₂O, 5 g; FeSO₄·7H₂O, 4.75 g, Fe (NH₄)₂(SO₄)·6H₂O, 1 g; CuSO₄·5H₂O, 250 mg; MnSO₄·H₂O, 50 mg; H₃BO₃, 50 mg; NaMoO₄·2H₂O, 50 mg; the pH was adjusted to 5.5 before autoclaving at 120°C for 20 minutes.

Three chlorate-containing media were used to recover *nit* mutants: 1) MMC was prepared by adding the following to 1L of minimal medium: KClO₃, 15-25 g; L-asparagine, 1.6 g; 2) KPS was prepared by adding 15-25 g KClO₃ to PSA, and 3) WAC was water agar medium plus 15-25 g KClO₃.

Nitrite medium (NMM): MM without NaNO₃ was amended with 0.5 g/L NaNO₂.

Hypoxanthine medium (HMM): MM without NaNO₃ was amended with 0.2 g/L hypoxanthine.

3.2.3 Isolation of *nit* Mutants

A small 3 mm³ block of mycelia from PSA cultures of each isolate was inoculated at four points on to 9-cm diameter plates containing KPS, MMC or WAC (Puhalla 1985, Correll *et al.* 1987, Glass 2000, Gordon 1997, Liu *et al.*, 1995) and incubated at 27°C in the dark. Spontaneous fast-growing chlorate-resistant sectors were recovered from some *F. oxysporum* isolates when cultured on MMC medium with 1.5%, 2.0%, or 2.5% chlorate for the first time. The isolates which did not produce chlorate-resistant sectors were then inoculated on PSA medium with 1.5%, 1.8%, 2.0% or 2.5% chlorate. If mutants were not recovered on this medium then the isolates were incubated on WAC medium with 2.5%, 3.0%, 3.5%, 4.0% or 4.5% chlorate. The plates were examined periodically for the appearance of fast-growing sectors from the initially restricted colony. Those sectors which were considered chlorate resistant were transferred to a minimal medium containing NaNO₃ as the sole nitrogen source and incubated at 27°C for 5 days. Very thin, but normally expansive growth with little or no aerial mycelium on MM, indicated that the sectors were also unable to reduce nitrate and were considered to be *nit* mutants.

3.2.4 *Nit* mutant Phenotypes Identification

The *nit* mutants were assigned to different phenotypic classes (*nit1*, *nit3*, and NitM) on the basis of their growth on media containing one of three different nitrogen sources: 1) nitrate medium = MM, 2) nitrite media = NMM, 3) hypoxanthine media = HMM. To determine the physiological phenotype, a mycelial transfer (2 mm³ MM block) of the *nit* mutant was put on each of the three media (Correll *et al.* 1987). The phenotypes of the nitrate nonutilizing mutants from *F. oxysporum* were determined by their colony morphology on media containing nitrate, nitrite or hypoxanthine as the sole nitrogen sources (Table 3.1). The plates were

incubated at 27°C in the dark.

3.2.5 Complementation Tests

Vegetative compatibility was determined by pairing *nit* mutants on MM medium. Heterokaryon formation between mutants was identified by the formation of a wild-type mycelium growth at the contact zone. Pairings were made by placing mycelia from each *nit* mutant 1-3 cm apart on MM plates (9 cm diameter). The plates were incubated as described above and cultures were observed for growth periodically over 4 weeks. At least two compatible NitM mutants from each of the strains participated in the compatibility pairings with *nit1*, *nit3* or NitM type mutants from other strains in all possible combinations to determine the number of VCGs present among 87 isolates of *F. oxysporum* f. sp. *vanillae*. Complementation was evident by the formation of dense prototrophic growth where the mutants had made contact and anastomosed. Heterokaryon formation was recorded for up to 4 weeks. The complementation pairings were carried out at least twice for each isolate with different *nit* mutants.

3. 3 Results

3.3.1 *Nit* Mutant Generation

Chlorate resistant mutants were readily obtained from all isolates with the exception of DL-13b from which only a single mutant could be obtained. The majority of spontaneous fast-growing chlorate-resistant sectors recovered was unable to utilize nitrate as a sole nitrogen source (Fig. 3.2) and consequently grew as thin expansive colonies with no aerial mycelium on MM (Fig. 3.3). These sectors were designated *nit* mutants. A few sectors were recovered from each strain that were chlorate-resistant but able to utilize nitrate. The frequency at which chlorate-resistant sectors were produced depended upon the strains and the amount of chlorate in the medium, and generally *nit* mutants were easily generated from most isolates. A total of 818 nitrate non-utilizing (*nit*) mutants were recovered from 87 strains of *F. oxysporum* cultured on three media, KPS, MMC, or WAC and more than one mutant was generated for each strain. Nearly 49% of *nit* mutants were generated by isolates inoculated on WAC medium; 32.6% *nit* mutants on KPS medium; and 18.4% on MMC medium.



Figure 3.2 Growth of strain ML-2-4 on water agar medium with 1.5% chlorate after 5 days of incubation at 27°C. Red elliptical areas are the chlorate-resistant sectors.



Figure 3.3 Growth of *nit* mutants from strains DL-1-7 and DL-1-8 on minimal medium after 5 days of incubation at 27°C. Note the normally expansive, but very thin growth.

3.3.2 *Nit* Mutant Phenotypes Identification

The phenotypes of the *nit* mutants from *F. oxysporum* were determined by their colony morphology on media containing nitrate, nitrite or hypoxanthine as sole nitrogen sources. The *nit* mutants could be divided into three phenotypic classes: *nit1*, *nit3*, and NitM (Table 3.1). Growth was assessed as being wild type or starved (thin expansive colonies with no aerial mycelium)

The three *nit* mutant classes were not recovered with equal frequency with the majority of *nit* mutants recovered being *nit1* mutants irrespective of which media was used. The *nit1* mutants were approximately three times as common as the NitM and *nit3* mutants (Figures 3.4 and 3.5). Among the 818 *nit* mutants, there were 539 *nit1* mutants, 128 *nit3* mutants, and 151 NitM mutants obtained (Table 3.2). Not all three phenotypes were recovered from all isolates, and the frequency of occurrence of each mutant type varied from isolate to isolate.

The frequency of *nit1* mutants recovered was considerably higher on KPS than on MMC and WAC. Consequently, the relative frequency of both the *nit3* and NitM mutants recovered was also considerably higher on MMC or WAC than KPS. *nit1* was recovered from 74 isolates, while *nit3* was recovered from only 31 isolates, 49 isolates failed to yield NitM mutants, although they were repeatedly exposed to different media and different concentration of potassium chlorate media.

The frequency of different *nit* mutant recovered varied slightly amongst different plantations. *Nit1* mutants still had the highest occurrence obtained. One hundred and forty-seven mutants were *nit1* (71.7%) out of 205 *nit* mutants obtained from the Daluo plantation isolates (Table 3.2a); 139 mutants were *nit1* (61.8%) out of 225 *nit* mutants from the Menglun Plantation isolates (Table 3.2b), and 134 mutants were *nit1* (65.36%) out of 205 *nit* mutants obtained from the Manjingdai plantation

isolates (Table 3.2c); 90 were *nit1* mutants (62.5%) out of 144 *nit* mutants obtained from the plantation isolates in Hekou County (Table 3.2d); 29 were *nit1* mutants out of 40 *nit* mutants obtained from the plantation isolates in Meiqi County (Table 3.2e). Whether the isolates were pathogenic or non-pathogenic, *nit* mutants could be easily obtained and their phenotypes shown to have no relationship to their pathogenicity (Table 3.2).

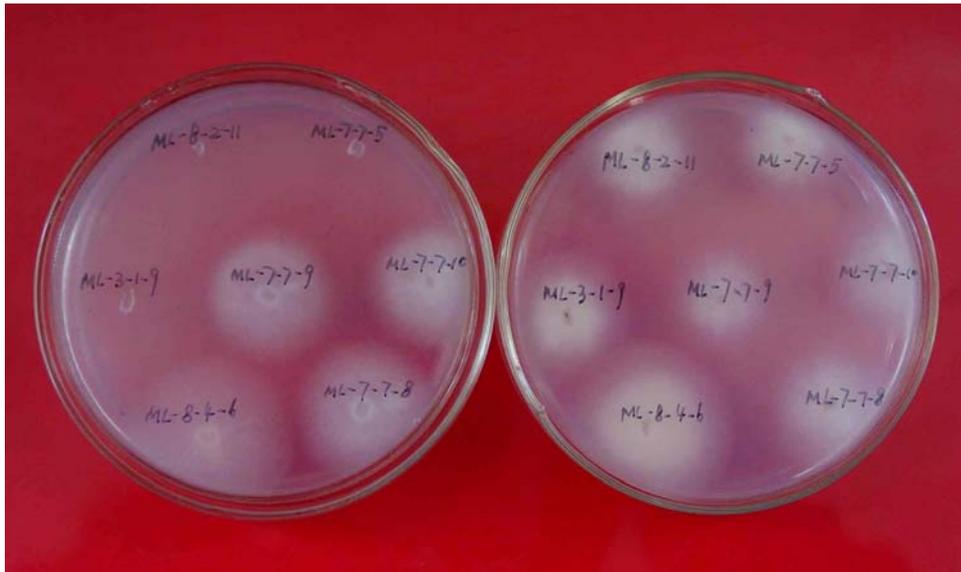


Figure 3.4 Growth of *nit* mutant phenotypes from strains ML-8-2, ML-7-7, ML-8-4 and ML-3-1 on two different nitrogen sources, hypoxanthine (left) and nitrate (right), after 3 days of incubation at 27°C.

Note the growth on NitM mutants of ML-8-2-11, ML-7-7-5 and ML-3-1-9; and *nit1* of ML-7-7-8, ML-7-7-9, ML-7-7-10 and ML-8-4-6. It shows the thin growth of NitM mutants on hypoxanthine medium (left) and hyphal wild growth on Nitrite medium (right); hyphal wild growth of *nit1* mutants on both media.

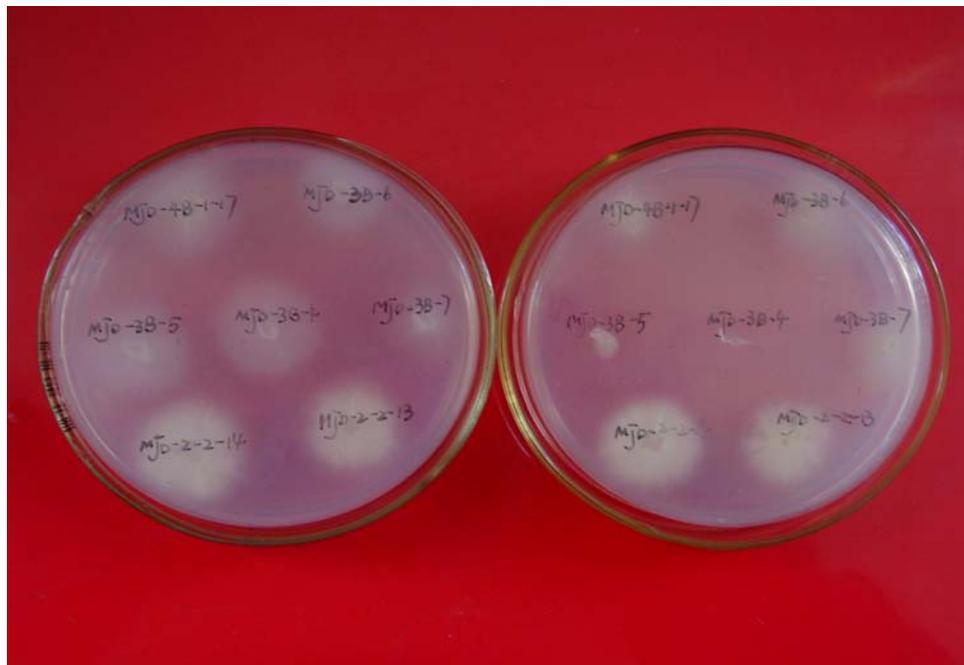


Figure 3.5 Growth of *nit3* mutants of MJD-3B-4 and MJD-3B-5 on nitrite medium (right). Wild growth of *nit1* mutants of MJD-4B-1-17, MJD-3B-6, MJD-3B-7, MJD-2-2-13 and MJD-2-2-14 on hypoxanthine medium (left);

It shows the hyphal wild growth of *nit3* mutants on hypoxanthine medium (left) and thin growth on Nitrite medium (right), and hyphal wild growth of *nit1* mutants on both media.

Table 3.2 Vegetative Compatibility Groups of *Fusarium oxysporum***3.2a Phenotypes of *nit* mutants recovered from Daluo plantation in the Xishuangbanna area**

No.	Strains	Virulence rating	VCGs*	Number of <i>nit</i> mutants examined	Classes of complementary <i>nit</i> mutants		
					<i>nit1</i>	<i>nit3</i>	NitM
1	DL-1	-	0201	17	17	0	0
2	DL-1-1	3+	0202	9	9	0	0
3	DL-1-2	3+	0201	10	10	0	0
4	DL-2-1	3+	020-HSI	11	11	0	0
5	DL-2-3	3+	0200	9	4	0	5
6	DL-2-4	2+	0200	10	0	0	10
7	DL-2-5	3+	0204	9	6	0	3
8	DL-2-7	-	0200	12	8	0	4
9	DL-2-9	+	0200	21	12	0	9
10	DL-2-12	2+	0205	7	5	0	2
11	DL-2-15	3+	020-HSI	11	5	6	0
12	DL-3	-	0206	23	12	11	0
13	DL-3-1	+	020-HSI	19	19	0	0
14	DL-3-2	3+	0207	10	5	5	0
15	DL-4-1	3+	0200	19	19	0	0
16	DL-4B	3+	0200	7	5	2	0
17	DL-13b	+	020**	1	0	0	1
Total				205	147	24	34

*Following the numbering systems adopted by Puhalla (1985) and further standardized by Kanta (1999) and Kisterler *et al.* (1998), these VCGs were designated as 020- for which the host is vanilla. According to the isolates' number of complementation, they are divided into 1-11 groups. Designation 020-HSI is for those isolates that were incomplementary with other isolates.

** Undetermined *nit* mutant phenotypes.

3.2b Phenotypes of *nit* mutants recovered from Menglun plantation in the Xishuangbanna area

No.	Strains	Virulence rating	VCGs	Number of <i>nit</i> mutants examined	Classes of complementary <i>nit</i> mutants		
					<i>nit1</i>	<i>nit3</i>	NitM
18	ML-1-7	3+	0200	1	1	0	0
19	ML-1-8	2+	0201	2	2	0	0
20	ML-2-4	-	0200	11	4	0	7
21	ML-2a	-	0200	2	0	0	2
22	ML-2d	+	020*	12	0	12	0
23	ML-3-1	3+	0200	16	4	0	12
24	ML-3-3	3+	0200	3	3	0	0
25	ML-4-1	2+	0208	7	1	6	0
26	ML-5-1	+	0200	16	16	0	0
27	ML-5-2	-	020-HSI	9	0	0	9
28	ML-6-1	3+	0200	10	10	0	0
29	ML-6-5	-	0200	13	10	3	0
30	ML-7-1	-	0200	10	5	5	0
31	ML-7-2	-	020-HSI	6	5	0	1
32	ML-7-3	-	0200	6	6	0	0
33	ML-7-4	2+	0202	14	14	0	0
34	ML-7-7	+	0200	11	7	0	4
35	ML-7-8	-	020-HSI	7	6	0	1
36	ML-7-9	-	0201	3	2	1	0
37	ML-7-10	2+	020-HSI	12	12	0	0
38	ML-8-1	3+	020-HSI	10	0	2	8
39	ML-8-2	-	0200	8	8	0	0
40	ML-8-4	-	0201	6	1	0	5
41	ML-13	-	0201	6	4	0	2
42	ML-13b	-	0201	9	6	3	0
43	ML-13d	-	0209	15	12	3	0
Total				225	139	35	51

3.2c Phenotypes of *nit* mutants recovered from Manjingdai plantation in the Xishuangbanna area

No.	Strains	Virulence rating	VCG	Number of <i>nit</i> mutant examined	Classes of complementary <i>nit</i> mutants		
					<i>nit1</i>	<i>nit3</i>	NitM
44	MJD-2	-	0201	5	4	0	1
45	MJD-2-1	-	0201	20	20	0	0
46	MJD-2-2	+	020-HSI	22	20	2	0
47	MJD-2B	-	0203	17	14	3	0
48	MJD-2C-1	3+	020-HSI	6	2	0	4
49	MJD-2C-2	+	020-HSI	4	4	0	0
50	MJD-2D-1	3+	020-HSI	2	2	0	0
51	MJD-2e	3+	0203	16	13	3	0
52	MJD-3A-1	+	020-HSI	10	0	0	10
53	MJD-3A-3	-	0200	8	0	0	8
54	MJD-3B	-	0201	6	2	2	2
55	MJD-3B-1	3+	0201	12	7	0	5
56	MJD-3C	+	0200	15	12	3	0
57	MJD-4	-	0201	12	9	2	1
58	MJD-4-3	+	0202	21	0	21	0
59	MJD-4B-1	3+	0202	18	18	0	0
60	MJD-4i	3+	0200	8	7	1	0
61	MJD-5G-1	2+	0200	2	0	2	0
Total				204	134	39	31

3.2d Phenotypes of *nit* mutants recovered from plantations in Hekou County

No.	Strains	Virulence rating	VCG	Number of <i>nit</i> mutant examined	Classes of complementary <i>nit</i> mutants		
					<i>nit1</i>	<i>nit3</i>	NitM
62	HK-1b-1	3+	0202	3	2	0	1
63	HK-1b-2	+	0202	5	0	1	4
64	HK-1b-3	3+	0201	18	18	0	0
65	HK-2a-1	+	0200	8	5	0	3
66	HK-2a-4-1	+	0201	2	0	0	2
67	HK-2b-2	-	02010	6	4	2	0
68	HK-3a-1	3+	02011	5	5	0	0
69	HK-3a-3	-	0200	2	2	0	0
70	HK-5a-2	-	0201	12	7	5	0
71	HK-5a-3	-	0200	7	1	6	0
72	HK-5a-4	3+	0201	8	8	0	0
73	HK-5b-4-1	-	020-HSI	7	7	0	0
74	HK-6a-2	3+	0200	17	5	4	8
75	HK-6a-3	+	0200	7	0	4	3
76	HK-7a-1	-	0201	4	3	0	1
77	HK-8a-1	-	0201	5	4	0	1
78	HK-8a-2	-	0200	8	4	0	4
79	HK-9a-1	-	0201	7	3	0	4
80	HK-9a-2	2+	0201	8	8	0	0
81	HK-10b-1	+	0200	5	4	1	0
Total				144	90	23	31

3.2e Phenotypes of *nit* mutants recovered from Meiqi plantation in the Xishuangbanna area

No.	Strains	Virulence rating	VCG	Number of <i>nit</i> mutant examined	Classes of complementary <i>nit</i> mutants		
					<i>nit1</i>	<i>nit3</i>	NitM
82	MQ-R5	N. D	0200	9	7	0	2
83	MQ-R12	N. D	0200	10	9	1	0
84	MQ-R13	N. D	0200	5	2	2	1
85	MQ-SL4	N. D	0200	5	5	0	0
86	MQ-sm14	N. D	0200	7	6	0	1
87	MQ-sm19	N. D	020*	4	0	4	0
Total				40	29	7	4

As a result (Table 3.2), 12 vegetative compatibility groups (VCGs) were found among 87 isolates analysed for vegetative compatibility. One predominant VCG containing 34 isolates, a second VCG containing 20 isolates, a third VCG containing 6 isolates, and 2 isolates were in a fourth VCG, while ML-2-5, DL-2-12, DL-3, DL-3-2, ML-4-1, ML-13d, HK-2b-2, HK-3A-1, HK-5b-4-1 were self-compatible, but were not complementary with other *nit* mutants in all combinations. Following the numbering systems adopted by Puhalla (1985) and further standardized by Kanta (1999) and Kisterler *et al.* (1998), these VCGs were designated as 0200-02011. The distributions of all of the VCGs were complex which includes isolates from different geographic origins and includes pathogenic and non-pathogenic strains as well. Vegetative compatible group 0200 included 34 isolates (Table 3.2) which were from different plantations including 6 isolates from Daluo, 12 from Menglun, 4 from Manjingdai, 7 from Hekou and 5 from Meiqi plantations. These showed different pathogenicities on vanilla and included 18 pathogenic strains, 11 non-pathogenic strains and 5 as yet not tested for virulence. VCG 0201 included 20 strains: 2 from Daluo, 5 each from Menglun and Manjingdai, 8 from Hekou plantations. VCG 0202 included 6 strains and VCG 0203 included only 2 strains from MJD-2B and MJD-2e. Others consisted of single-isolate VCGs. Twenty-six non-pathogenic strains were placed in 6 VCGs, with the 3 isolates from Daluo placed in three VCGs: 0200, 0201 and 0206; 8 from Menglun were placed in 3 VCGs: 0200, 0201 and 0209, 6 from Manjingdai were placed in three VCGs: 0200, 0201 and 0203 and 8 from Hekou County were placed in four VCGs: 0200, 0201, 0202 and 0211.

In some cases, the *nit* mutants of the tested isolates and the tester strains formed strong heterokaryons. The complementary *nit* mutants chosen as testers of VCG0200 were MQ-R5-8 (NitM, parental isolate MQ-R5) and ML-7-1-3 (*nit3*,

parental isolate ML-7-1); the testers of VCG0201 were HK-7A-1-4 (NitM, parental isolate HK-7A-1) and HK-9A-1-6 (NitM, parental isolate HK-9A-1); the tester of VCG0202 was MJD-4-3-5 (NitM, parental isolate MJD-4-3). All the other *nit* mutants generated by other isolates were paired with testers. MQ-sm19, ML-2d had only the *nit3* phenotype, DL-13b created only one NitM mutant, but they did not form heterokaryon among all combinations, so there was no evidence to identify which VCG they were in or whether they were self-incompatible.

3.3.3 Complementation Analysis

Physiological complementation between *nit* mutants with different phenotypes was indicated by the development of dense aerial growth where the mycelia of the *nit* mutant colonies came in contact and anastomosed to form a heterokaryon (Figures 3.6 and 3.7). The mutants of each isolate were first paired among themselves on MM to reveal complementation within the isolate. Complementation occurred among *nit* mutants with different phenotypes (Table 3.3). The number of NitM mutants obtained from chlorate substrates was evaluated to compare the efficacy of different media in the production of *nit* mutants useful for VCG analysis. In agreement with previous reports (Correll, 1991), many NitM mutants were produced on MMC. Complementation occurred more rapidly and growth of the resulting heterokaryon was more robust in pairings of NitM with *nit1* or *nit3* mutants than in pairings of *nit1* with *nit3* mutants. NitM was more vigorous when pairing with *nit1*, *nit3* and NitM type mutants in all possible combinations and the majority of complementation groups were identified among the NitM mutants. Two complementary *nit* mutants (*nit1* and *nit3*, *nit1* and *nit1*, NitM and NitM) that formed robust heterokaryons with other mutants were selected as tester strains when heterokaryons did not form after pairing among NitM combination (Figures 3.6 and 3.7). When *nit1* and *nit3* mutants were paired, subsequent complementation was not evident for 2-3 weeks. Frequently, even after 3 weeks, the complementation reaction was weak (very little aerial mycelium). In some pairings of *nit1* with *nit3* mutants recovered from the same parental strain, no complementation was observed. Some *nit1* mutants were able to complement one another, as could some NitM mutants. No complementation was observed between any of the *nit3* mutants (Figures 3.8 and 3.9).

Table 3.3 Complementation Reaction between Nitrate Non-utilizing Mutants of *F. oxysporum*

	<i>nit1</i>	<i>nit3</i>	NitM
<i>nit1</i>	- or ±	± or -	+
<i>nit3</i>	± or -	-	+
NitM	+	+	+ or -

+ = Complementation occurs readily;

± = weak and slows complementation occurs;

- = no complementation occurs.



Figure 3.6 A pairing between the complementary mutants, *nit1* (1-7 and 9) and *NitM* (8 and 10) mutants of strain MQ-R5 in plates after 11 days of incubation at 27°C. The heavy, white line of growth where the two mutant colonies contact indicates heterokaryon formation. The line has already overgrown the inoculum blocks of the *nit* mutants.

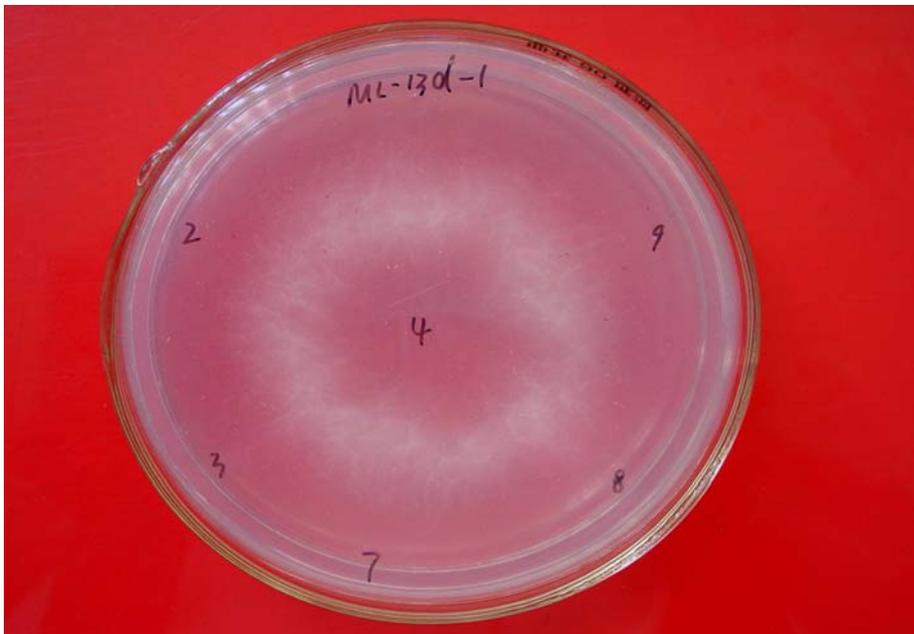


Figure 3.7 A pairing between the complementary mutants, *nit1* (1-3 and 7-9) and *nit3* (4) mutants of strain ML-13d in plates after 11 days of incubation at 27°C. The heavy, white line of growth where the two mutant colonies contact indicates heterokaryon formation.

3.3.4 Heterokaryon Self-incompatibility

No complementation between any *nit* mutant of ML-5-2 (9 NitM type mutants), ML-7-2 (5 *nit1* and 1 NitM), ML-7-8 (6 *nit1* and 1 NitM), ML-7-10 (12 *nit1*), ML-8-1 (2 *nit3* and 8 NitM), DL-2-1 (11 *nit1*), DL-2-15 (5 *nit1* and 6 *nit3*), DL-3-1 (19 *nit1*), MJD-2-2 (20 *nit1* and 2 *nit3*), MJD-2C-1 (2 *nit1* and 4 NitM), MJD-2C-2 (4 *nit1*), MJD-3A-1 (10 NitM), MJD-2D-1 (2 *nit1*), HK-5b-4-1 (7 *nit1*) was observed, even after repeated attempts. Two types of mutants of themselves were obtained from ML-7-2, ML-7-8, ML-8-1, DL-2-15, MJD-2-2 and MJD-2C-1. They were considered to be heterokaryon self-incompatible. However, with ML-5-2, ML-7-1, DL-2-1, DL-3-1, MJD-2C-2, MJD-3A-1, MJD-2D-1 and HK-5b-4-1 only one type of mutant was recovered, either *nit1* or NitM despite different chlorate concentration and different media being used. When no complementation occurred between the mutants of these isolates in themselves, they were also considered as showing heterokaryon self-incompatibility. Among these strains, ML-5-2, ML-7-2, ML-7-8 and HK-5b-4-1 were non-pathogenic isolates, so HSI is not exclusive to pathogenic strains.



Figure 3.8 A pairing between the complementary mutants among different isolates: *nit1* mutants of isolate ML-3-3 (1) and MJD-3c (2) paired with *nit3* mutant (4) of strain ML-7-1 in plates after 10 days of incubation at 27°C. NitM mutant of isolate ML-5-2 (3) failed to form heterokaryons between combinations.



Figure 3.9 A pairing between the complementary mutants among different isolates: *nit1* mutant (1) of isolate ML-1-8 and *nit3* mutant (2) of HK-5a-2 pairing with NitM mutant (3) of strain HK-9A-1 in plates after 10 days of incubation at 27°C.

3.4 Discussion

3.4.1 *nit* Mutants in *Fusarium*

The use of mutants to force heterokaryons was necessary because no other protocol is applicable to *F. oxysporum* f. sp. *vanillae*. Chlorate, a nitrate analogue, has been used widely for studying heterokaryosis in *Fusarium* (Puhalla, 1985). In general, the growth of chlorate-sensitive strains is restricted by the internal reduction of chlorate to toxic chlorite by nitrate reductase. Chlorate-resistant strains either do not take up chlorate or are unable to reduce chlorate to chlorite (Liu and Sundheim, 1995). Nitrate non-utilizing mutants are usually unable to reduce chlorate to chlorite because of a lesion at one or more of the loci that control nitrate reductase, thus rendering them chlorate-resistant. This class of mutants has several advantages over other autotrophic mutations. Chlorate resistant mutants appear spontaneously, without the need for mutagenesis. This avoids the problems that mutagenesis can cause, for example gratuitous mutations in regions of the genome other than the target gene, such as in genes affecting growth rate or even in genes responsible for vegetative compatibility or avirulence (Correll *et al.*, 1987). The mutants appear very quickly, usually in less than seven days and the frequency of mutation is high.

Moreover, the genotype of a particular mutant is also rapid and straightforward to assign. Phenotypic test involves putting the isolates onto a variety of nitrogen sources. The three mutant genotypes used in this study were *nit1*, *nit3* and *nitM*. Chlorate-resistant sectors were obtained at frequencies that depended on both the strain and the amount of chlorate in the medium. The three *nit* mutant classes were not recovered with equal frequency, with *nit1* mutants usually more than twice as common as the *NitM* mutants (Table 3.2).

3.4.2 Genetic Diversity within *Fusarium oxysporum*

Puhalla (1985) found 16 VCGs among 21 pathogenic isolates of *F. oxysporum* with 6 different formae specialis. Genetic and physiological characters had also been analysed to assess the diversity within pathogenic and non-pathogenic populations of *F. oxysporum* (Appel and Gordon, 1994; Elena and Pappas, 2002). In this study, there were 12 VCGs among 87 randomly selected isolates and among 30 non-pathogenic strains, 7 VCGs were obtained in this study. These data would suggest that there may be a greater VCG diversity within *F. oxysporum*. In the study, more diversity was also found in the non-pathogenic strains, which is consistent with previous reports.

Strains recovered from soil as saprophytes are almost always diverse with respect to VCG. By using a combined approach to determine genetic relationships, vegetative compatibility is probably an excellent indicator of a close genetic relationship but that vegetative incompatibility do not need to indicate a large genetic difference. Puhalla (1985) suggested that vegetative compatibility could be used to subdivide populations into different VCGs and that these subdivisions were correlated with pathogenicity, which assumes that the pathogens rarely participate in recombination events that could lead to reassortment of the *vic* alleles to yield new VCG phenotypes. In that case, VCG and pathogenicity are coincidentally correlated rather than related by a cause-and-effect association. Twenty years after the original proposal a few conclusions can be drawn. First, there is a strong correlation between pathogenicity and VCG in some pathogenic *formae speciales* of *F. oxysporum*, e.g. *melonis*, with most members of the individual *forma specialis* confined to one or a few VCGs (Aha *et al.*, 1998; Jacobson and Gordon, 1988; Vakalounakis and Fragkiadakis, 1999). The correlation is weak, or nonexistent, in others, e.g. *asparagi*

and *lycopersici*, and the members of these formae speciales belong to a relatively large number of VCGs (Elias, 1991).

In the study, with the formae speciales *vanillae*, vegetative compatible groups do not correlate with pathogenicity, as the group includes pathogenic and non-pathogenic strains. The finding of non-pathogenic strains that are vegetatively compatible with pathogenic strains is not new (Appel and Gordon, 1994; Gordon and Okamoto, 1992), but the identification of the pathogenic and non-pathogenic strains by other techniques such as molecular marker still need to be developed (McFadden *et al.*, 2006). Without sexual reproduction, and therefore exchange of genetic material, the strains of *F. oxysporum* which were vegetatively incompatible may have become genetically isolated subpopulations (Jacobson and Gordon, 1988). If this were the case, we would expect to find biological and ecological differences between VCGs in the various non-pathogenic *F. oxysporum* populations. Alabouvette *et al.* (1985) have shown that non-pathogenic strains of *F. oxysporum* can reduce disease incidence and severity of several vascular wilt diseases induced by various formae speciales of *F. oxysporum*. According to the work employing soils suppressive to *Fusarium* vascular wilt diseases (Alabouvette, 1999), differentiating strains among the non-pathogenic *F. oxysporum* isolates is very important. Little is known, however, about the ecological significance and the population dynamics of the non-pathogenic strains of *F. oxysporum*, particularly at the subspecies level. Vegetative compatibility and VCGs are naturally occurring genetic markers. They could provide a means of identifying and characterizing the various subpopulations of the *F. oxysporum*.

3.4.3 Vegetative Self-Incompatibility

Thirteen isolates in this study were vegetatively self-incompatible. When these isolates were paired in all combinations, no heterokaryons were formed. Whitehead (1991) observed macroscopically a barrage that appeared to form between two vegetatively self-incompatible mutants in *F. oxysporum* f. sp. *pisi*. Jacobson and Gordon (1988) reported that HSI phenotype in *F. oxysporum* f. sp. *melonis* resulted from the inability of an isolate to initiate or complete heterokaryon formation. This conclusion was also drawn by Correll *et al.* (1987) who examined the HSI phenotype in *Fusarium moniliforme*. They found that HSI isolates produced fewer hyphal fusions. They postulated three possible reasons for this, either these isolates are defective in enzymes associated with hyphal branching or these isolates are reduced in their number of fimbriae or that these isolates lack a diffusible substance that signals nearby hyphae and stimulates anastomosis. It is postulated here that in HSI isolates of *F. oxysporum* f. sp. *vanillae*, a modification of this third possibility is likely, however, further research is needed to confirm this.

3.4.4 Applications of VCGs Analysis

VCGs are useful for the analysis of fungal populations. VCGs are a direct multigenetic assessment of a trait of adaptive importance within fungal populations. The laboratory analysis of VCGs with complementary *nitrate*-non-utilizing (*nit*) mutants is technically simple and requires little more than basic microbiological materials (Correll *et al.*, 1987). VCGs are well-suited for measuring genotypic diversity, e.g. the frequency of different genotypes within a population, and for determining if two strains are identical to one another. However, the VCG technique is not a panacea for population analyses of pathogenic fungi. VCG analyses are not appropriate for determining if strains belong to different biological species or for assessing differences that occur above the species level (Leslie, 1993). Although the VCG technique is useful for measuring genotypic diversity, it is not useful for assessing the levels of allele frequencies, e.g. the frequencies of alleles at different *vic* loci. Similarly, although the VCG technique is a powerful tool for determining clonality, it cannot be used for ascertaining the degree of relatedness. Finally, the detection of heterokaryosis may be complicated by alleles that prevent formation of a heterokaryon, even if the component strains are vegetatively compatible, e.g. mutants at heterokaryon self-incompatibility (*hsi*) loci. When compared with some other multilocus techniques, such as DNA fingerprint probes, VCGs require less technical equipment and laboratory sophistication, but may not sample as many loci and require more effort to interpret.

Nitrogen-responsive genes (Divon *et al.*, 2006) had been reported involved in regulating fungal nutrition-genes and fitness during *Fusarium oxysporum* pathogenesis. We need to understand the role *vic* loci play in the life cycle is at least as important. These studies could lead to the development of ‘universally compatible’

strains. Such strains could be used as delivery vehicles for mycoviruses or other intracellular biocontrol agents into a population that was otherwise so subdivided by VCG as to make biological control by such transmission impossible (Kistler, 1997). For more direct pathogen control, agents that simulate an incompatible reaction might be used to induce the physiological killing response, providing a novel set of antifungal compounds. If the response is limited, it might be possible to eliminate pathogenic strains while leaving their non-pathogenic, and perhaps beneficial, relatives untouched. If the response mechanism is more general, then it might be possible to identify species-specific or a cluster-of-species-specific antifungal agents. In essence these compounds would trigger a 'suicide' reaction in the target, and could be environmentally friendly, quite specific in their action, and applicable in settings other than just plant protection, e.g. medical and veterinary uses. Studies of vegetative compatibility have greatly advanced our understanding of populations of fungal pathogens, but progress resulting from an analysis of the mode of action of the *vic loci* will probably be responsible for even greater changes in plant pathology in the coming decade (Correll, 1991).

VCG, coupled with the integrated use of powerful molecular tools, has greatly helped in our current understanding of the pathology, population biology and race relationships of this organism (Correll, 1991). However, with regard to *F. oxysporum* our understanding of this complex fungal pathogen is far from complete.

CHAPTER 4

IN VITRO* ACTIVITY OF SEVERAL ESSENTIAL OILS AGAINST *FUSARIUM OXYSPRUM* AND INVESTIGATION OF VANILLA ROOT ROT DISEASE CONTROL BY ESSENTIAL OILS *IN VIVO

4.1 INTRODUCTION

4.1.1 Antimicrobial Properties of Essential Oils and their Role in Biocontrol

Many natural compounds isolated from plants have been shown to have wider biological activities and the essential oils from aromatic and medicinal plants are particularly interesting (Anupama *et al.*, 2005; Deans and Ritchie, 1987; Gundidza *et al.*, 1992). The prospect of an increased use of natural plant products in food flavouring and preservation, or pharmaceutical and fragrance industries may speed up the development of new antimicrobial agents for use in crop protection that are safe for humans and environment, readily available and renewable sources (Anon., 2006; Elgayyar *et al.*, 2001; Janssen *et al.*, 1987).

Essential oils can be defined as volatile organic constituents of fragrant plant matter. They are concentrated in various parts of the plant, for example, cinnamon oil in bark, garlic oil in bulbs, peppermint oil in leaves, fennel oil in seeds (Burt, 2004; Ranasinghe *et al.*, 2002; Wang *et al.*, 2005). They are extracted from plants by steam distillation, cold pressing or by using organic solvents. Such oils were originally called essential because they were thought to represent the very essence of odour and flavour (Deans and Ritchie, 1987).

The spices and herbs such as thyme, clove, cinnamon, bay, oregano, garlic and lemongrass provide a potentially rich source of novel biocides and preservatives. Early cultures recognized the value of using spices and herbs in the flavour and aroma of foods, also in preserving foods and for their medicinal value (Dorman and Deans, 2000; Skandamis and Nychas, 2000). Traditional Pakistani dishes are enriched with a number of exotic spices and herbs (Rahman *et al.*, 1999). The antimicrobial properties of essential oils have been used for thousands of years empirically (Aziz *et al.*, 1998; Ultee *et al.*, 1998; Shelef, 1983). However they have

only recently been scientifically shown to contain antimicrobial compounds active against a wide range of bacteria, yeasts and moulds (Deans and Ritchie, 1987; Bowers and Locke, 2000; Flori, *et al.*, 1999; Janssen *et al.*, 1987; Porter and Wilkins, 1999; Panizzi, 1993; Zambononelli, 1996).

Tea tree oil is an example of an essential oil with known antimicrobial properties (Nenoff *et al.*, 1996). It is obtained by steam distillation of the leaves of *Melaleuca alternifolia*, a tree which is a member of the family *Myrtaceae* which is native to Australia (Cox *et al.*, 2000). It is known to be antibacterial, antifungal, antiviral, anti-inflammatory and analgesic. The major antimicrobial components in tea tree oil have been determined (Brophy *et al.*, 1989). These include terpinen-4-ol, a monoterpenoid alcohol, α - and β -terpinenes and various other compounds.

Essential oils are products of plant secondary metabolism. Their function in plants is not well understood. Odours of flowers probably aid in natural selection by acting as attractants for certain insects. Leaf oils, wood oils, and root oils may serve to protect against plant parasites or insects (Dev, *et al.*, 2004). Exudates that appear when the trunk of a tree is injured prevent loss of sap and act as a protective seal against parasites and disease organisms. A few essential oils are involved in plant metabolism (reviewed by Edwards-Jones, 2001). By contrast, however, the antimicrobial action of essential oils *in vitro* is well documented in the literature (Gould, 1996; Lambert *et al.*, 2001; Montes-Belmont and Carvajal, 1998).

The chemical characteristics of the essential oils and their relation to antimicrobial properties have been studied for many years. Some are low molecular weight phenolic compounds that are present within spice and herbs (Gould, 1996). Some are hydrocarbons, frequently terpenes (Edwards-Jones, 2001; Wedge *et al.*, 2000), while others are oxygenated compounds such as alcohols, ketones and esters.

Terpenes in essential oils are usually monoterpenes consisting of two isoprene units or, less commonly, sesquiterpenes consisting of three isoprene units (Grayer and Harborne, 1994).

Essential oils from many plant families have now been demonstrated to have antimicrobial properties. Interestingly, different plant families exhibit varying degrees of effectiveness depending on the strain (AI-Shuneigat *et al.*, 2005; Bhattacharjee *et al.*, 2005; Carson and Riley, 1995; Cosentina *et al.*, 1999; EI-Hadrami *et al.*, 2005; Fiori *et al.*, 2000; Krauze-Baranowska, 2002). This is due to the particular molecular structures found in each type of oil, which penetrate biophysical entities (different tissues, cell walls, mucous membranes, etc.), to varying degrees (Cox *et al.*, 2000; Fitzgerald *et al.*, 2004). The effect of each strain depends also on the type of each different microbe, such as bacteria, fungi and viruses.

Antibacterial properties: This is the most widely studied area of essential oils: the capacity of essential oils to inhibit bacterial growth is now almost irrefutable (Harkental, 1999; Inouye *et al.*, 2001a; Kalemba and Kunicka, 2003). Knowledge of the antibacterial components of essential oils is of prime importance. For example, in the essential oil of *Satureja montana*, carvacrol is the active molecule; paracymene, also usually present in this essential oil, does not possess protective properties (Aziz *et al.*, 1998).

The molecules with the highest anti-bacterial coefficient are: carvacrol, thymol and eugenol; all three are phenols (Cosentino *et al.*, 1999). Cinnamic aldehyde has an anti-bacterial activity comparable to phenols. Alcohols with 10 carbon atoms (or monoterpenols) are the next most potent: geraniol, linalool, thujanol and myrcenol, terpineol, menthol and piperitol are the most well-known (Harkental, 1999). They are

reliable, broad-spectrum molecules, which are useful in numerous cases of bacterial infections.

Aldehydes also have antibacterial properties; the most widely used are neral and geranial (citral), citronellal and cuminal. Ketones and terpenes also have been shown to have potential, but terpenes are called atmospheric antiseptic agents and typically evaporate into the air when being used (Marino *et al.*, 1999; Porter and Wilkin, 1999).

Antifungal properties: The molecular groups with the strongest antibacterial action are also active on fungi. A wide variety of essential oils are known to possess antifungal properties and in many cases this activity is due to the presence of active monoterpene constituents (Guynot *et al.*, 2003; Knobloch *et al.*, 1988; Mahmoud, 1994). Fundamental studies have also revealed the anti-fungal activity of alcohols and sesquiterpenic lactones (Alam *et al.*, 2004; Fraternali *et al.*, 2004; Larrondo *et al.*, 1995; Mangena and Muyima, 1999; Muller-Riebau *et al.*, 1995). Rahman *et al.* (1999) had studied the composition of essential oils yielded from *Amomum subulatum*, *Cinnamomum verum*, *Coriandrum sativum*, *Cuminum cyminum*, *Elettaria cardamomum*, *Myristica fragrans* (Mace) and *Myristica fragrans* (Nutmeg) which contained 1,8-cineole, cinnamaldehyde, linalool, cuminaldehyde, 1,8-cineole, terpinen-4-ol, respectively, as the main determined constituents.

Singh *et al.* (1980) found that essential oils from *Cymbopogon martinii*, *C. oliveri*, and *Trachyspermum ammi* exhibited strong antifungal activity against *Helminthosporium oryzae*. Soliman and Badeaa (2002) had tested anti-fungal activities of essential oils of thyme, cinnamon, anise and spearmint against *Aspergillus flavus*, *A. parasiticus*, *A. ochraceus* and *Fusarium moniliforme*. The results showed some extensive inhibition of fungal growth by different essential oils;

thyme and cinnamon had more adverse effect on fungal development than others.

Antiviral properties: Over 20 years ago, antiviral properties of essential oils from many plant families have been demonstrated. More than 100 species of the Lamiaceae family have anti-viral effect (Anon., 2006). There are many essential oils exhibiting antiviral effects including Melissa, tea tree, juniper, eucalyptus, thyme, palmarosa, lavender, rosemary, clove, laurel, cinnamon bark, anise, rose, lemongrass, geranium, neroli, bergamot, clary sage and dill show varying levels of antiviral activity (Anon., 2006; Cavanagh and Wikinson, 2002). As with other activities, the antiviral effect of an essential oil is determined by the particular molecular components of the oil. It has been found that the particular molecular structures in each type of oil exhibit varying degrees of effectiveness (Cosentino *et al.*, 1999). For example, anethol found in anise; alpha sabinene found in tea tree and laurel; beta-caryophyllene found in lavender, rosemary and thyme; linalool and carvone found in dill, cinnamic aldehyde found in cinnamon bark; citral found in Melissa and lemongrass; citronellol found in rose and geranium; eugenol found in clove; gamma-terpinene found in juniper, eucalyptus, niaouli and tea tree; linalol found in lavender and neroli; linalyl acetate found in clary, sage, lavender and bergamot (Anon., 2006; Soliman and Badeaa, 2002). The effect on each virus strain depends also on the virus structure, such as enveloped, non-enveloped, molecular symmetry, etc. (Porter *et al.*, 1999)

4.1.2 The Application of Essential Oils

Essential oils are utilized as flavour ingredients in a wide variety of food confectionary, beverage and perfume products. Because of their antiseptic, antispasmodic and antimicrobial properties, they are also used for medicinal purposes (Panizzi *et al.*, 1993). Clove oil is a topical analgesic, especially useful in dentistry (Velluti *et al.*, 2003). Tea tree, eucalyptus, and sandalwood oils are used as antiseptics and disinfectants (Brophy *et al.*, 1989; Carson *et al.*, 1995; Cox *et al.*, 2000; Hammer *et al.*, 1998; 2002; 2003; Harkental, 1999). In recent years, several reports have been published concerning the composition and the biocontrol properties of *Thymus* essential oils (Stahl-Biskup, 1991; Senatore, 1996).

Essential oils and their constituents have been used as biological agents for their therapeutic and cytotoxic activities (Viollon and Chaumont, 1994), as well as toxicity against plant pathogenic fungi and insects (Bankole and Adebajo, 1995). These studies have emphasized the existence of marked chemical differences among oils extracted from different species or varieties. These variations are likely to influence the antimicrobial activity of the oil and are generally a function of three factors: genetically determined properties, the age of the plant and the environment (Fitzgerald *et al.*, 2004; Lambert *et al.*, 2001).

There has been an increasing interest recently in the use of natural substances, and concerns about the safety of synthetic compounds have encouraged more detailed studies of plant resources. This interest is aimed primarily at finding new and safe antimicrobial agents from natural sources to prevent deterioration of foods and to minimize oxidative damage to living cells. New antimicrobial compounds were isolated from various spices and herbs and their structures were determined by chemical and spectroscopic means (Bhattacharjee *et al.*, 2005). Examples of new

antimicrobial agents include: phenolic diterpenes from rosemary and sage, phenolic carboxylic acids from oregano, biphenyls and flavonoids from thyme, phenolic amides from pepper and chilli pepper, and diarylheptanoids from ginger (Nakatani, 1992). All the phenolic compounds have effective antimicrobial activities.

4.1.3 Methods for the Determination of the Antimicrobial Activity of Essential Oils *in Vitro*

Initial screening of potential antimicrobial compounds from plants may be performed with pure substances or crude extracts (Cowan, 1999). The methods used for the two types of products are similar. Two classical methods used to screen antimicrobial susceptibility are the dilution assay, based either on agar or liquid broth (Manou *et al.*, 1998) and the agar diffusion method by paper disc and well (Navarro *et al.*, 1996). Turbidimetric and impedimetric monitoring method of microorganism growth in the presence of tested essential oils had also been used (reviewed by Kalemba and Kunicka, 2003). Adaptations such as the agar overlay method may also be used. In some cases, the inoculated plates or tubes are exposed to UV light (Taylor *et al.*, 1996) to screen for the presence of light-sensitizing photochemicals. Antifungal phytochemicals can be analysed by a spore germination assay. Samples of plant extracts or pure compounds can be added to fungal spores collected from solid cultures, placed on glass slides, and incubated at an appropriate temperature (25°C for 24 h). Slides are then fixed in lacto phenol-cotton blue and observed microscopically for spore germination (Rana *et al.*, 1997).

After initial screening, more detailed studies of their antibiotic effects should be conducted (Si *et al.*, 2006). At this stage, more specific media can be used and the minimum inhibitory concentrations (MICs) can be effectively compared to those of a wider range of currently used antibiotics (Chorianopoulos *et al.*, 2006). When they are to be considered for use in the food industry or plant disease control, although the majority of the essential oils are classified or generally recognized as safe, their use especially in foods as preservatives is often limited due to flavour considerations, since effective antimicrobial doses may exceed organoleptically acceptable levels

(Navarro *et al.*, 1996). Therefore, there is an increasing demand for accurate knowledge of MICs of essential oils to enable a balance between the sensory acceptability and antimicrobial efficacy.

The MIC of essential oils can be tested by both *in vitro* and *in vivo* studies. The former evaluation methods can be divided into groups such as diffusion, dilutions, impedance and optical density methods (Carson and Riley, 1995; Tassou *et al.*, 2000). The MIC data published in earlier years had largely been obtained using the agar dilution method (Suhr and Nielsen, 2003), while only a few studies had used National Committee for Clinical Laboratory Standards (NCCLS method) (Hammer, 2002; Carson *et al.*, 1995). Although NCCLS methods are widely accepted for determining *in vitro* susceptibilities of micro-organisms to antimicrobial agents, how well essential oils, or indeed antifungal activities, fit within these protocols is questionable. For example, in the case of turbidity, interference due to insufficient dissolution of the test compounds can be overcome to an extent with specific metabolic indicator stains (Chand *et al.*, 1994; Mann and Markham, 1998). Among these methods, the dilution method provides more quantitative results (Inouye *et al.*, 2001b; Manou *et al.*, 1998) while results obtained with other methods may not be comparable between different experiments (Tassou *et al.*, 2000).

Lambert and Pearson (2000) introduced and evaluated an innovative technique for assessment of MIC for chemical biocides using the Bioscreen Microbiological Growth Analyser. The advantages of this method rely on the simultaneous examination of multiple preservative concentrations and subsequent determination of MIC based on mathematical processing. There is still no report that this methodology has been applied to the examination of the MIC of essential oils.

Comparisons of studies that have used different methodologies are difficult,

especially regarding MIC and the need for uniform and reliable procedures when testing activity has been emphasized (Mann *et al.*, 2000). The extensively used method is diffusion from discs, drops, wells or double plate (Zaika, 1988; Mangena and Muyima, 1999) or agar and broth dilution for mixing the agent into the growth media (Cosentino *et al.*, 1999). Unfortunately, results obtained by the different methods using the same oils are not always comparable. As a result, the limitations within each of the methods should be taken into account.

4.1.4 Mode of Anti-microbial Action of Essential Oils

Although the antimicrobial activity of essential oils from spices has been reviewed recently (Ultee *et al.*, 1998; Lambert, 2001; Velluti, 2001), their mechanism of action against micro-organisms has not been studied in great detail. For example, terpenoids are the main compounds in many essential oils, such as tea tree (Hammer *et al.*, 1998; 2002, Cox *et al.*, 2000), cinnamon, and citronella (Marino, 1999; Porter, 1999; Ramezani *et al.*, 2002). However, the mechanism of action of terpenes is not fully understood, but it is speculated to involve membrane disruption by the lipophilic compounds.

The mode of action of nearly all antimicrobials can be classed into one or more of the following groups: (a) reaction with the cell membrane, (b) inactivation of essential enzymes, or (c) destruction or inactivation of genetic material (Fitzgerald *et al.*, 2004). Gould (1996) found that the antimicrobial activity of essential oils can sometimes be due to the low molecular weight phenolic compounds that are present within them. Phenolic compounds are hydrophobic in nature and are, therefore, regarded as membrane active (Sikkema *et al.*, 1994, 1995).

In general, studies on the mechanism of action of essential oils have used a common methodology that attempts to illustrate deleterious effects on cellular membranes, i.e. permeability and proton motive force (Cox *et al.*, 1998; Helander *et al.*, 1998; Ultee *et al.*, 1999). According to Conner and Beuchat (1984) the antimicrobial action of some essential oils may be due to impairment of a variety of enzyme systems, including those involved in energy production and structural component synthesis. Edwards-Jones (2005) proposed the mode of action of tea tree oil – primarily disruption of the cell membrane which causes leakage of potassium ions. Other possible modes of action include inhibition of respiration found in

Escherichia coli, *Staphylococcus aureus* and *Candida albicans* (Cox, 2000).

Several studies have also shown that monoterpenes exert membrane-damaging effects (reviewed by Cox, 2000). Examination of *E. coli* cells using electron microscopy after exposure to tea tree oil revealed a loss of cellular electron-dense material and coagulation of cytoplasmic constituents. Accordingly, Mendoza *et al.* (1997) found that increasing the hydrophilicity of kaurene diterpenoids by addition of a methyl group drastically reduced their antimicrobial activity.

Recent mode of action studies using plant essential oils (thyme and tea tree) or some of their phenolic constituents (carvacrol, eugenol and thymol) against several pathogenic bacteria, fungi and yeasts have shown that their activity resides in their ability to perturb the cell membrane resulting in the loss of chemiosmotic control leading to cell death (Cox *et al.*, 2000; Lambert *et al.*, 2001; Burt and Reinders, 2003; Walsh *et al.*, 2003). Cox *et al.* (2000) reported that exposing the micro-organisms to minimum inhibitory concentrations of tea tree oil inhibited respiration and increased the permeability of bacterial cytoplasmic and yeast plasma membranes as indicated by uptake of propidium iodide. Tea tree oil stimulates leakage of cellular potassium ions and inhibits respiration in *E. coli* cell suspensions, providing evidence of a lethal action related to cytoplasmic membrane damage (Cox *et al.*, 2000). Differences in the susceptibility of the test organisms to tea tree oil were also observed and these are interpreted in terms of variations in the rate of monoterpene penetration through cell wall and cell membrane structures. The ability of tea tree oil to disrupt the permeability barrier of cell membrane structures and the accompanying loss of chemiosmotic control is the most likely source of its lethal action at minimum inhibitory levels.

The antimicrobial activity of essential oils as antifungal agents was tested in this

work, with the ultimate aim of using plant essential oils to inhibit growth of soil-borne pathogens of vanilla by examining whether essential oils show antifungal activity against pathogenic isolates using the modified agar dilution assay, with agar as an oil stabilizer, under sealed conditions. Overall, the present study sought to: (1) assess the antifungal properties of seven essential oils and the MIC of three essential oils; and (2) determine the susceptibility of pathogenic fungi isolated from vanilla plants to these oils.

4.2 MATERIALS AND METHODS

4.2.1 Materials

4.2.1.1 *In Vitro* Pre-screening of Essential Oils for Effective Inhibition of *F. oxysporum* sp. *vanillae*

Three pathogenic *F. oxysporum* sp. *vanillae* isolates, ML-7-10, ML-8-1 and DL-4-1, isolated from the Xishuangbanna plantation in Yunnan Province were selected for testing effectiveness of essential oils on inhibiting *F. oxysporum* *in vitro*. These three strains grew well on the PSA media. Among them, ML-7-10 was moderately pathogenic causing vanilla root rot disease when inoculated on the healthy vanilla plants. The other two isolates were strongly pathogenic.

Seven essential oils were used to test the inhibiting effect on *F. oxysporum* including camphor, citronella, cinnamon, orange, clove, tea tree and peppermint oil. Potato sucrose agar (PSA), and Capez's medium (see Section 2.2.1) were used for the growth and analysis of strains in the study.

4.2.1.2 Inhibitory Effects of Selected Essential Oils on *F. oxysporum* *in Vitro*

Three isolates of *F. oxysporum* sp. *vanillae* isolated from the plantation in Xishuangbanna were selected. They have different pathogenic ability on vanilla including DL-4-1, the strongest pathogenic strain; ML-7-10, medium pathogenic strain; and ML-5-2, non-pathogenic. The last one is a potential non-pathogenic biocontrol agent which was identified by the VCG method.

Three effective essential oils selected by initial screening, cinnamon oil, citronella oil and clove oil, were used in the test. Two media were used, potato and sucrose (PS) medium and potato, sucrose and agar (PSA) medium (see Section 2.2.1) to test the ability of these three essential oils on inhibiting the growth of fungus.

4.2.1.3 Experiment for Essential Oils Inhibiting Effect on Vanilla Root Rot in a Controlled Environment.

The strongly pathogenic strain, DL-4-1, was used for the inoculation of healthy vanilla seedlings which were grown by tissue culture methods (see Section 2.2.4) Two essential oils, cinnamon oil and clove oil, were used in the study. Garden soil for growing the vanilla plants was prepared by autoclaving.

4.2.2 METHODS

4.2.2.1 Pre-screening of Effective Essential Oils on Inhibiting *F. oxysporum* sp. *vanillae* in Vitro - Test for Spore germination

The antimicrobial properties of essential oils were studied against several pathogenic *Fusarium* pathogens. Seven oils were dissolved in acetone to give concentrations of 50, 100, 200, 400, 600, 800 and 1600 ppm. The negative control treatment received the same volume of acetone only.

The diluted essential oil or control solution, 15 µl, was dropped into a concave microscope slide, and then 100 µl spore suspension (10^6 Microspore/ml) was added. Each treatment was repeated four times. The slide was incubated in the dark at 27°C. Spore germination was tested after 12-16 h, and assessed on 100-150 spores under the microscope and the number of spore germinated recorded. Calculation of the percentage of spore germination and inhibition were as follows:

Percentage spore germination = [Number of spores germination / total number of spores] × 100%

Percentage inhibition = [(percentage of control group – percentage of treated group) / rate of control group] × 100%

4.2.2.2 Pre-screening of Effective Essential Oils on Inhibiting *F. oxysporum* sp. *vanillae* in Vitro - Test for Hyphal Growth of *F. oxysporum* Inhibited by Essential Oils.

Potato sucrose agar (PSA) medium 9 ml and diluted essential oil 1 ml were added into petri dishes, the control treatment received the same quantity of acetone. A disk of mycelia (DL-4-1; d = 4.0mm) taken from the rim of 7-day-old culture grown on PSA media plates was placed in the middle of each plate and incubated at 27°C. The diameters of the colonies were measured after 7 days. The experiment was

conducted in quadruplicate. The percentage of inhibition was calculated by the following formula:

$$\text{Percentage of inhibition} = (\text{diameter of mycelium colony with control} - \text{diameter of mycelium colony with treatment} / \text{diameter of mycelium colony with control}) \times 100.$$

4.2.2.3 Inhibition Effect of Selected Essential Oils on *F. oxysporum* in Vitro - Determination of Minimum Inhibitory Concentration (MICs) Using an Agar Dilution Method

Three oils were dissolved by absolute ethyl alcohol to give concentrations of 50, 100, 200, 400, 600, 800, 1600, 3200 and 6400 ppm and were added to the culture medium at a temperature of 40-45°C and poured into 9 cm diameter petri dishes (Zambonelli *et al.*, 1996). The negative control treatment received the equal quantity of absolute ethyl alcohol.

PSA medium 9 ml and diluted essential oil 1 ml were added into 9 cm diameter petri dishes, the control treatment received the same quantity of alcohol. A disk of mycelium (d = 4.0 mm) taken from the rim of 7-day-old cultures on PSA media plates was placed in the middle of each plate and incubated at 27°C. The colony diameter was measured 3 days after incubation and test was terminated when the plate surface of the control treatment with water was covered by the fungus. The growth values were obtained and then converted into the inhibition percentage of the mycelia growth in relation to the control treatment. Each treatment was repeated four times and the experiment was conducted in triplicate. MICs were defined as the lowest concentration of the essential oils that inhibited the visible formation of colonies.

4.2.2.4 Inhibition Effect of Selected Essential Oils on *F. oxysporum* in Vitro - Determination of Antimicrobial Activities of the Oils Using a Liquid Medium Dilution Method

Three oils were dissolved as previously (Section 4.2.2.3) over the range 50–6400 ppm, the negative control treatment received the same quantity of absolute ethyl alcohol.

Potato sucrose liquid medium (see Appendix), 8 ml, and diluted essential oil, 1 ml, were added in a test-tube (Zhou, 2002). To each tube was added 1 ml of 10^7 microspores/ml spore suspension, which was incubated for 7 days at 27°C. The samples were centrifuged after 10 days and the change of volume of the liquid culture medium was measured along with the dried weight of the pellets. All tests were conducted in triplicate. The concentration of the hyphal growth and the percentage of inhibition were calculated by the following formulae:

Concentration of the hyphal growth = $(10 - \text{volume of supernatant}) / 10 \times 100\%$

Percentage of inhibition = $(\text{concentration of the hyphal growth with control} - \text{concentration of the hyphal growth with treatment}) / \text{concentration of the hyphal growth with control}$.

4.2.2.5 Test for Essential Oils Inhibiting Effect on Vanilla Root Rot in a Controlled Environment

Spores of the pathogenic isolate were collected from potato sucrose liquid medium incubated at 27°C for 7 days. The spore concentration was determined with a haemocytometer and adjusted to the required concentration using sterile water.

Clove and cinnamon oils were dissolved in acetone. The final concentrations of 50, 100, 200, 400, 600, 800, 1600, 3200 and 6400 ppm were tested.

Vanilla seedlings grown by tissue culture for 3 months (see Section 2.2.4) were

inoculated by the root dipping method. The roots were immersed in 1×10^6 microspore/ml suspension of pathogenic strain DL-4-1 for 5 minutes, and then transplanted into potted soil (500 ml) which was previously infested with pathogenic strain DL-4-1 (1×10^6 microspore / ml of soil). Each pot had two seedlings and the pots were placed in a growth room at 27°C (12h day length). After 48 h, 20 ml diluted essential oils were added into soil. The control treatment received 1ml of acetone. Each pot was placed separately to avoid interference with each other and each treatment was repeated four times. Disease development was evaluated every 15 days for 1 month using a disease severity index, as in Section 2.2.5.

4.3 RESULTS

4.3.1 Inhibition of *F. oxysporum* Spore Germination by Selected Essential Oils

All seven oils demonstrated the ability to inhibit spore germination of *F. oxysporum* isolates, with different degrees of effectiveness. The essential oils responsible for the greatest inhibition of the spore germination of all three isolates were cinnamon oil, followed by clove oil, citronella oil and camphor oil (Table 4.1). Cinnamon oil showed 100% inhibition of spore germination of all three *F. oxysporum* isolates at a concentration of 200 ppm. Even at a low concentration of 100 ppm, it had a pronounced inhibition. A clear correlation was observed between concentrations of cinnamon, clove and citronella oils and percent inhibition of spores for all the pathogens tested (Figure 4.1). Tea tree oil and orange oil showed relatively weak inhibitory effects and mint oil was the weakest.

Different isolates were affected differently by the essential oils. The essential oils demonstrated apparent inhibition ability on the medium pathogenic strain ML-7-10, whereas, with the strong pathogenic strain ML-8-1, and especially with DL-4-1, the spore germination was higher than with medium pathogenic strain ML-7-10 (Table 4.1). In the negative control without essential oils, the strong pathogenic strains, DL-4-1 (Figure 4.1) and ML-8-1 (Figure 4.2), always showed higher rate of growth in terms of colony diameter and more dense hyphal development than with the medium pathogenic strain, ML-7-10 (Figure 4.3).

Table 4.1 Inhibitory effects of seven essential oils on spore germination of three different isolates, DL-4-1, ML-8-1, ML-7-10

Essential oils	Concentration	Percent of spore germination (%)		
		Mean (Std. Deviation)		
		DL-4-1	ML-8-1	ML-7-10
cinnamon oil	50ppm	87.01(8.80)	5.01 (1.00)	42.63 (17.00)
	100ppm	25.46 (10.75)	1.07 (0.51)	6.67 (4.49)
	200ppm	2.58 (1.97)	0	0
	400ppm	0	0	0
	800ppm	0	0	0
	1600ppm	0	0	0
camphor oil	50ppm	90.24 (0.95)	81.54 (2.63)	47.63 (12.36)
	100ppm	92.63 (1.40)	77.95 (9.59)	38.68 (10.69)
	200ppm	92.05 (6.73)	71.51 (15.69)	42.44 (17.20)
	400ppm	89.36 (1.42)	46.33 (4.12)	31.12 (12.89)
	800ppm	84.96 (1.97)	16.34 (2.66)	13.73 (1.38)
	1600ppm	41.36 (6.98)	0	0
clove oil	50ppm	90.35 (5.04)	78.95 (5.44)	63.79 (22.61)
	100ppm	90.16 (6.35)	61.92 (3.48)	51.41 (16.32)
	200ppm	83.37 (1.52)	56.08 (5.00)	31.47 (12.29)
	400ppm	81.17 (4.11)	22.81 (2.14)	9.66 (4.81)
	800ppm	22.76 (5.12)	0.59 (0.45)	1.12 (0.80)
	1600ppm	0.37 (0.43)	0	0
citronella oil	50ppm	84.33 (8.28)	80.59 (1.68)	61.79 (17.71)
	100ppm	81.09 (2.78)	72.31 (1.08)	67.57 (20.14)
	200ppm	72.40 (0.98)	54.20 (3.72)	60.41 (20.50)
	400ppm	65.73 (2.97)	38.02 (6.94)	45.47 (18.61)
	800ppm	67.15 (4.32)	10.76 (1.89)	40.17 (10.32)
	1600ppm	40.58 (1.90)	0	14.01 (6.25)
tea tree oil	50ppm	96.35 (1.21)	77.17 (1.40)	62.63 (12.50)
	100ppm	94.10 (2.03)	64.81 (0.98)	58.15 (22.16)
	200ppm	86.09 (2.12)	54.60 (3.14)	54.23 (26.92)
	400ppm	72.34 (2.92)	43.57 (0.46)	50.47 (4.10)
	800ppm	82.93 (1.59)	43.64 (20.61)	46.95 (40.10)
	1600ppm	86.03 (2.29)	39.39 (3.12)	38.16 (32.11)
orange oil	50ppm	72.32 (4.07)	74.21 (1.45)	60.34 (18.60)
	100ppm	81.79 (2.86)	64.49 (2.56)	54.69 (19.93)
	200ppm	84.54 (1.06)	58.18 (2.71)	56.97 (23.40)
	400ppm	85.62 (0.28)	55.61 (1.45)	56.06 (24.25)
	800ppm	86.54 (2.25)	36.49 (1.59)	52.25 (26.28)
	1600ppm	26.61 (1.68)	8.96a (1.56)	19.02 (7.18)
mint oil	50ppm	94.61 (1.79)	88.36 (3.69)	71.32 (17.86)
	100ppm	93.58 (2.64)	80.32 (2.63)	70.11 (16.89)
	200ppm	94.52 (2.07)	57.80 (3.49)	68.33 (19.47)
	400ppm	80.28 (4.01)	55.74 (2.90)	65.36 (18.81)
	800ppm	66.06 (5.95)	53.70 (1.31)	59.73 (22.04)
	1600ppm	53.60 (0.68)	35.96 (1.75)	57.63 (32.78)
water control		95.34 (1.61)	72.32 (6.16)	85.64 (2.72)
acetone control		95.74 (2.16)	77.63 (5.92)	76.08 (10.86)

Note: All tests were conducted in triplicate.

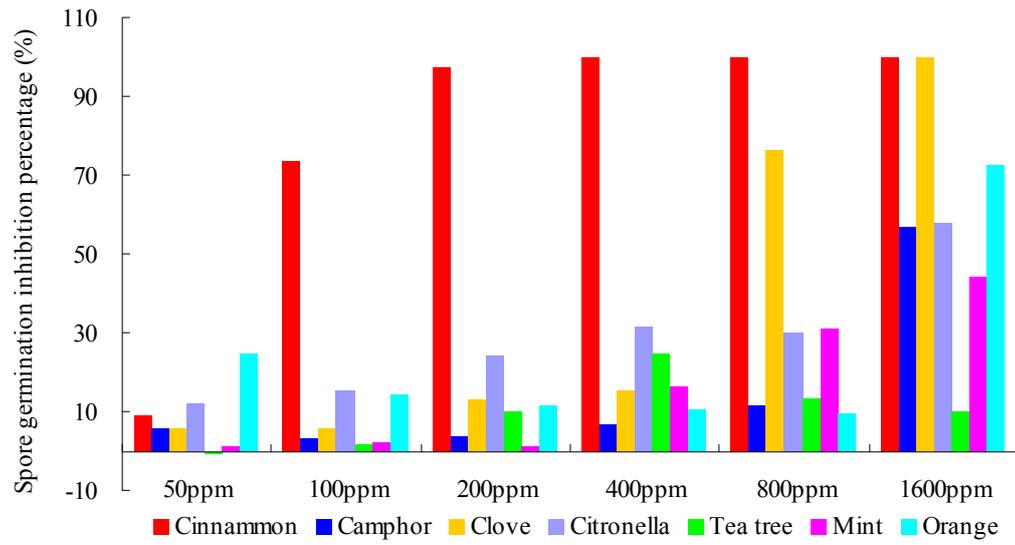


Figure 4.1 Inhibitory effects of seven essential oils at various concentrations on spore germination of isolate DL-4-1.

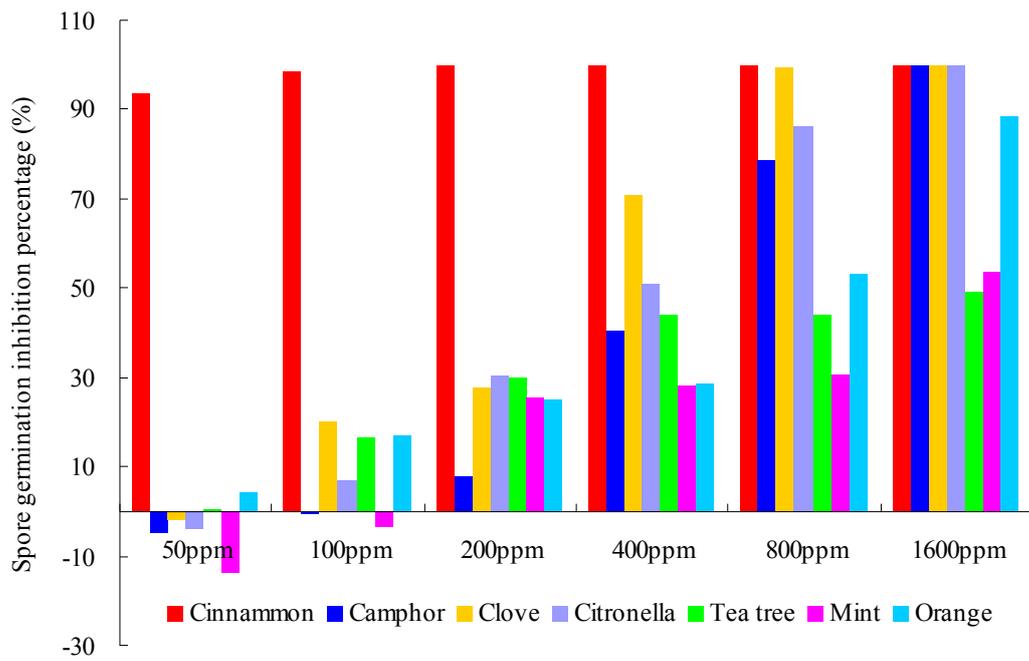


Figure 4.2 Inhibitory effects of seven essential oils at various concentrations on spore germination of isolate ML-8-1.

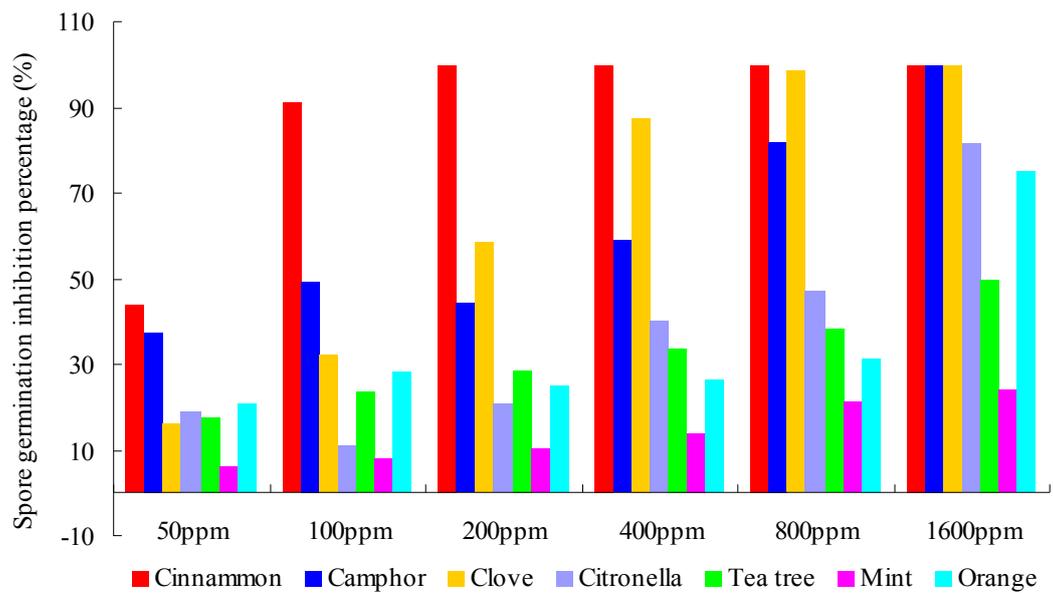


Figure 4.3 Inhibitory effects of seven essential oils at various concentrations on spore germination of isolate ML-7-10.

4.3.2 Comparison of Seven Essential Oils in Inhibition of *F. oxysporum* Hyphal Growth

All treatments inhibited mycelia growth. The inhibition ranged from the lowest inhibitory percent 7.31% by tea tree oil at 50 ppm to the high of 100% with 400 ppm and higher concentrations of cinnamon, 800 ppm and above of clove and 1600 ppm of camphor and citronella (Figure 4.4). Similarly, inhibition of growth rate was noted in relation to germination of spores. Essential oils of cinnamon, camphor, clove and citronella were highly effective on inhibiting the hyphal growth and treatments with these four essential oils resulted in no hyphal growth when the concentration was 400 ppm of cinnamon, 800 ppm of clove, 1600 ppm of camphor and citronella, respectively (Table 4.2).

Table 4.2 Inhibitory effects of seven essential oils on hyphal growth of DL-4-1

Concentration	Diameter of hyphal growth (cm)						
	Mean (Std. Deviation)						
	Cinnamon	Camphor	Clove	Citronella	Tea tree	Mint	Orange
50ppm	4.50 (0.50)	6.07 (0.23)	5.33 (0.23)	4.40 (0.10)	6.77 (0.25)	6.23 (0.25)	6.53 (0.25)
control	9.00	9.00	9.00	9.00	9.00	9.00	9.00
100ppm	6.07 (0.12)	5.60 (0.10)	3.60 (0.10)	4.60 (0.30)	6.10 (0.36)	6.07 (0.40)	5.70 (0.10)
control	9.00	9.00	9.00	9.00	9.00	9.00	9.00
200ppm	6.07 (0.12)	6.57 (0.51)	2.9 (0.06)	3.50 (0.00)	6.03 (0.06)	6.70 (0.10)	5.67 (0.15)
control	9.00	9.00	9.00	9.00	9.00	9.00	9.00
400ppm	0	4.50 (0.10)	2.13 (0.12)	2.73 (0.06)	6.70 (0.10)	6.20 (0.10)	5.20 (0.10)
control	9.00	9.00	9.00	9.00	9.00	9.00	9.00
800ppm	0	2.07 (0.40)	0	2.90 (0.17)	5.60 (0.10)	2.87 (0.15)	4.40 (0.10)
control	9.00	9.00	9.00	9.00	9.00	9.00	9.00
1600ppm	0	0	0	0	5.97 (0.15)	2.67 (0.15)	3.70 (0.10)

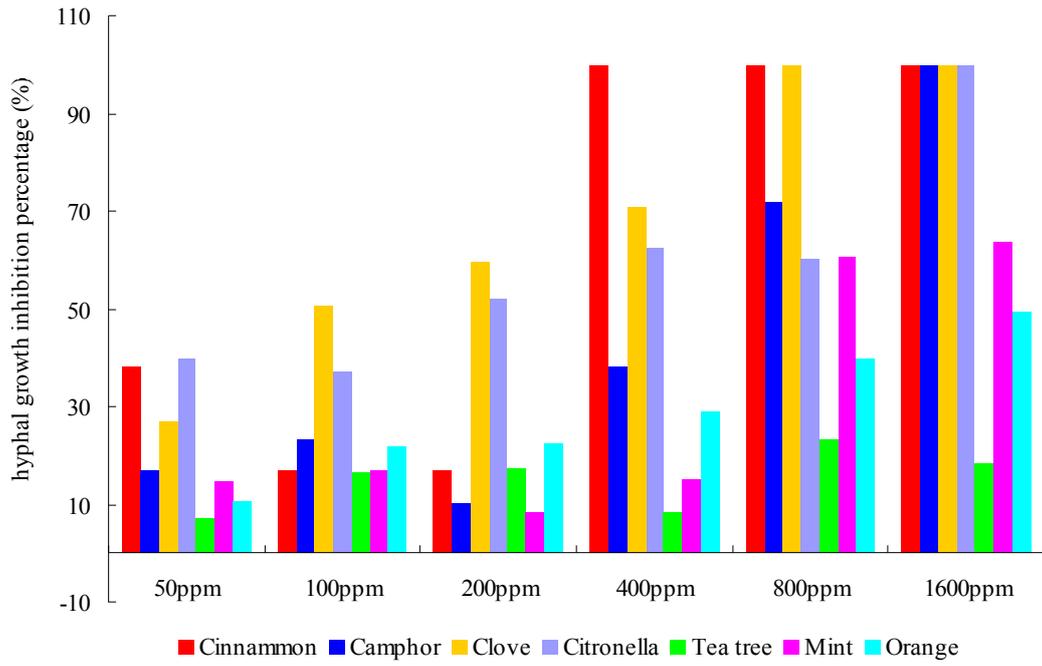


Figure 4.4 Inhibitory effects of seven essential oils on hyphal growth of DL-4-1 by colony diameter testing.

4.3.3 Determination of Minimum Inhibitory Concentration (MICs) Using Agar Dilution Method Based on the Incubation Time

The results of MICs using the agar dilution method are shown in Table 4.3. The three essential oils, cinnamon, clove and citronella, reduced the hyphal growth of the pathogens. Cinnamon oil was the most effective followed by clove oil and then citronella oil.

MIC was defined as the lowest concentration of the essential oils that inhibited the visible formation of colonies. MIC of the essential oil was strain specific (Table 4.4). Pathogenic strain DL-4-1 was most resistant followed by ML-7-10 and ML-5-2. Strain DL-4-1 showed the most active growth and the MIC was the highest value. When treated with cinnamon oil, MIC was 1600 ppm, while for clove oil or citronella oil, their MICs were both 3200 ppm. ML-7-10 was a less pathogenic strain than DL-4-1. The MIC for this strain was 800 ppm, 800 ppm and 1600 ppm, respectively, when treated by cinnamon oil, clove oil and citronella oil. ML-5-2 was a non-pathogenic strain and the MIC of 200 ppm was lower than that for other two pathogens.

Table 4.3 The mean diameter of the mycelia grown in the agar media to determine the minimum inhibitory concentration (MIC).

	Cinnamon oil (cm)			Clove oil (cm)			Citronella oil (cm)			Water Control (cm)		
	DL-4-1	ML-7-10	ML-5-2	DL-4-1	ML-7-10	ML-5-2	DL-4-1	ML-7-10	ML-5-2	DL-4-1	ML-7-10	ML-5-2
50ppm	2.1	1.86	0.7	2.17	1.4	1	2.93	2.4	1.2	4.33	4.1	2.9
100ppm	1.97	1.6	0.4	1.97	1.3	0.73	2.43	2.17	0.9	3.7	3.27	2.27
200ppm	1.1	1.4	/	1.83	1.13	0	2.1	1.23	0	2.7	2.4	2
400ppm	0.9	0.9	0	1.2	0.7	0	1.63	1.1	0	2.59	2.1	1.6
800ppm	0.6	0	0	1.23	0	0	1.53	0.7	0	2.29	1.93	1.4
1600ppm	0	0	0	0.43	0	0	0.87	0	0	1.73	1.1	0.9
3200ppm	0	0	0	0	0	0	0	0	0	1.17	0.9	0.63
6400ppm	0	0	0	0	0	0	0	0	0	1.1	0.63	0.4

Table 4.4 MIC values determined by the agar diluted method for three essential oils against *F. oxysporum*

isolates	MIC values (ppm)		
	Cinnamon oil	Clove oil	Citronella oil
DL-4-1	1600	3200	3200
ML-7-10	800	800	1600
ML-5-2	200	200	200

4.3.4 Determination of Antimicrobial Activities of the Oils Using a Liquid Medium Dilution Method Based on the Incubation Time

The antimicrobial activities of essential oils were determined by using a liquid medium dilution method based on the incubation time. The volumes of the supernatant were measured after 10 days incubation by centrifugation. The concentrations of the hyphal growth and the percentage inhibition of the essential oils (Figure 4.5) which could evaluate the antimicrobial activities were calculated by the formula described above (see Section 4.2.2.2).

Three essential oils, cinnamon, clove and citronella were all clearly inhibitory towards the fungal isolates. Cinnamon oil still showed the greatest inhibitory ability amongst the three essential oils tested. Clove oil was the second and the weakest was citronella (Figure 4.5). Concentrations of oil were the most important factor and were positively correlated with growth inhibition of the fungus, with higher concentrations giving higher inhibition. However, differences between fungi were notable, with non-pathogenic isolates showing the highest inhibitory effects, corresponding to greatest reduction of hyphal growth. All three essential oils exhibited sustained inhibitory effects against the selected fungi with time.

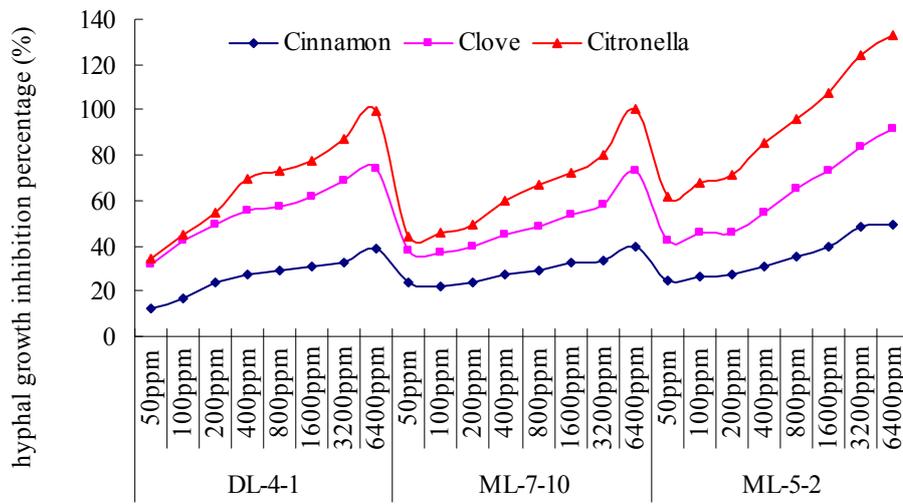


Figure 4.5 Inhibitory effects of three essential oils on hyphal growth of three isolates, DL-4-1, ML-7-10 and ML-5-2 by hyphal concentration testing.

4.3.5 Effects of Essential Oils on Vanilla Root Rot in a Controlled Environment

Two essential oils, cinnamon and clove oils were selected to test their inhibitory efficacy on root rot disease in controlled environment, following the results of their inhibitory effects on spore germination and hyphal growth. Control treatment with acetone injured the vanilla plants and increased the disease incidence among all the treatments (Table 4.5). Cinnamon and clove oils showed efficacy in controlling the vanilla root rot disease at a concentration of 400 ppm. The disease incidences of both were 33.30% and the disease severity index were 0.145 and 0.154, respectively. At higher and lower concentrations the disease incidence and severity increased markedly. The severity of plant disease when treated with clove oil was less than with cinnamon oil (Table 4.5). The results indicated the potential for developing new and safer types of antimicrobial agents for crop protection. However, the inconsistent results at both higher and lower concentration of essential oils are problematic and require further study. Acetone appears to have adversely affected plants in all treatments and a less damaging solvent should be considered.

Table 4.5 Biocontrol effects of cinnamon oil and clove oil on root rot of vanilla

Essential oils	concentrations	Disease incidence (%)	severity	Disease severity index (10^{-2})
Cinnamon oil	50ppm	75.00	+	0.728
	100ppm	57.70	+	0.552
	200ppm	50.00	+	0.471
	400ppm	33.30	+	0.145
	800ppm	46.60	+++	0.783
	1600ppm	77.70	+++	0.825
Clove oil	50ppm	66.60	+	0.633
	100ppm	66.60	+	0.582
	200ppm	47.60	+	0.482
	400ppm	33.30	++	0.154
	800ppm	56.60	+++	0.622
	1600ppm	74.30	+++	0.778
Control with acetone		83.30	+++	0.778

For severity of damaged tissue:

+: lightly injured, but did not affect normal growth;

++: moderate injury, but can be recovered;

+++: highly injured, markedly affects the normal growth of the plant.

4.4 DISCUSSION

4.4.1 Antifungal Activity of Essential Oils

The results obtained in this study demonstrate that essential oils have activity against *Fusarium oxysporum*, including pathogenic and non-pathogenic isolates. The fungicidal values obtained for isolates in this study are similar to those published previously (Zambonelli *et al.*, 1996; Venturini and Oria, 2002). The main methodological differences were the inoculum size and test medium, which may have contributed to the slight differences in results obtained by each method. The susceptibility data obtained for fungi against essential oils by the broth micro-dilution method showed that all isolates were inhibited in the range of 50 ppm to 6400 ppm of essential oils and were killed in the range of 200 to 6400 ppm. It is difficult to compare results to previous studies, because very few studies investigated the activity of essential oil against *Fusarium* fungi and also because of differences in methodology. Previously published studies have commonly used the agar dilution method, which gives an indication of the activity of essential oils against different fungi but does not give specific, quantifiable data.

The agar dilution method has commonly been used for investigating the activity of essential oils against fungi (Mishra and Dubey, 1994). Delespaul *et al.* (2000) studied different methods to determine MICs on filamentous fungi, *Microsprum canis* and *M. gypseum* and showed that the MICs determined by the agar dilution method were higher than those obtained by the broth dilution method. This may be due to differences in the degree of contact between the oil and organism between the two methods. Presumably the organisms come into direct contact with the oil in the broth medium compared to agar. Other differences that may account for the different results include the length of incubation of tests (four days compared to 14 days). Also,

solid agar may be a more favourable growth medium for the fungi, as compared to liquid. Niewerth *et al.* (1998) compared MICs for dermatophytes using both agar and broth methods and also found that MICs were consistently higher by the agar method,⁹ although they did not speculate as to why this effect occurred.

It is reported that the filamentous fungi appeared relatively difficult to kill *in vitro* by essential oils. The differences in inhibitory effects on bacteria or yeasts and fungi demonstrate that to a certain degree, essential oils display higher inhibitory ability on bacteria or yeast than on fungi (Hammer *et al.*, 2002). It is possible that fungal spores are more resistant to essential oils than bacterial spores. However, there is no evidence to confirm this assumption. By contrast, Russell (cited by Hammer *et al.*, 2002) commented that fungi were often more susceptible than bacterial spores. On the basis of the results of the experiments in the study and with some knowledge of the pathogenesis of fungal infections, it is likely that treating fungal infections with essential oils will require a relatively high concentration of oil and a reasonably long duration of treatment. It is important that careful consideration should be given to the application medium as this can affect treatment efficacy.

The experiments in a controlled environment showed that at the relatively low application concentration of 400 ppm, essential oils could effectively control vanilla disease. No previous reports on the toxic activity of essential oils on vanilla plants *in vivo* exist. These results raise the possibility of developing new and safer types of antimicrobial agents for the control of the disease. But if essential oils are to be successfully developed for plant disease control, then further work needs to be performed at the greenhouse level and in the field. The natural environment in the field is a very complex parameter to consider because of variations in micro-organisms, climate, wind and rainfall, etc. These factors will influence the

inhibition of fungal growth. Essential oils will also inhibit other beneficial micro-organisms in the soil which are good for maintaining the balance of the rhizosphere (Vokou, 1999). Moreover, the volatile property of essential oils would be affected by the soil environment. Most of all, the cost of essential oil could be prohibitive when using it for biocontrol methods.

4.4.2 Implications and Further Work

This study has provided some knowledge on the *in vitro* susceptibility of fungi to cinnamon, camphor, citronella, clove, tea tree, mint and orange oils. Further work is required to determine the *in vitro* susceptibility to essential oils of additional soil micro-organisms, such as *Fusarium sonali*, *Trichoderma* spp., *Pseudomonas* spp. The importance of this is that these organisms are found in soil, and the presence of some of these species may be associated with the disease severity. The testing of these species will also determine the range of filamentous fungi and bacterial that are susceptible to essential oils.

MICs should be determined by comparable method. Scientists from different fields are investigating plant product with an eye to their antimicrobial usefulness. Laboratories in the world have found literally thousands of phytochemicals which have inhibitory effects on all types of micro-organisms *in vitro* (Cowan, 1999). Cowan has also proposed that more of these compounds should be subjected to plant, animal and human studies to determine their effectiveness in whole-organism systems, including in particular toxicity studies, as well as an examination of their effects on beneficial microbiota. It would be advantageous to standardize methods of extraction and *in vitro* testing, so that the search could be more systematic and interpretation of results would be facilitated (Cowan, 1999).

The MIC studies will add to our understanding of the activity of essential oils against fungi. Once these mostly descriptive studies are complete, investigations into the mechanism of action of essential oil are warranted. Alternative mechanisms of infection prevention and treatment should be included in initial activity screenings. Disruption of adhesion is one example of an anti-infection activity not commonly screened for currently. Attention to these issues could usher in a much needed new

era of chemotherapeutic treatment of infection by using plant-derived principles. Also, an understanding of the mechanisms causing the inhibition and killing of fungi will provide a basis for the formulation and design of products for specific fungal conditions, and the use of essential oils *in vivo*.

The results of this study demonstrate unequivocally that essential oils have antifungal activity. Although not all of the factors contributing to the *in vivo* efficacy have been elucidated, the data provided here demonstrate that essential oils do have promise as an antifungal biocontrol agent. These data benefit the crop protection industry by providing the basis on which essential oil could be developed and formulated for soil applications.

CHAPTER 5

EVALUATION OF THE EFFECTS OF NON-PATHOGENIC STRAINS OF *FUSARIUM OXYSPORUM* ON VANILLA ROOT ROT DISEASE IN CONTROLLED ENVIRONMENT AND FIELD EXPERIMENTS

5.1 INTRODUCTION

5.1.1 Biocontrol of *Fusarium* Wilt and the Use of Non-Pathogenic *Fusarium oxysporum*

The species *Fusarium* is well represented among the communities of soil-borne fungi in every type of soil all over the world (Alabouvette *et al.*, 1993). This species is also considered a normal constituent of the fungal communities in the rhizosphere of plants. All strains of *F. oxysporum* are saprophytic and able to grow and survive for long periods on organic matter in soil and in the rhizosphere of many plant species (Gordon and Martyn, 1997). Moreover, some strains of *F. oxysporum* are pathogenic to various plant species and they penetrate into the roots inducing either root-rots or tracheomycosis when they invade the vascular system. Many other strains can penetrate roots, but do not invade the vascular system or cause disease (Olivain and Alabouvette, 1997). The wilt-inducing strains of *F. oxysporum* are responsible for severe damage on many economically important plant species. Research has provided convincing evidence that root health and vigour are directly related to plant productivity (Gilbert *et al.*, 2000; Handelsman and Stabb, 1996). As a consequence, root disease control has become one of the most challenging research areas in the context of plant productivity improvement. This has led to the concept that manipulating the rhizosphere in such a way that is beneficial to micro-organisms with antagonistic eliciting properties would protect roots from the deleterious effect of soil-borne pathogens (Weller, 1988).

Current management of *Fusarium* wilt involves chemical soil fumigation and cultivation of resistant cultivars. The broad-spectrum biocides used to fumigate soil before planting, particularly methyl bromide, are environmentally damaging (Flood, 2003; Fravel *et al.*, 2003). The most cost effective and environmentally safe method

of control is the use of resistant cultivars if they are available. However, breeding for resistance can be very difficult and time consuming when no dominant gene is known. In addition, new races of the pathogen can develop overcoming host resistance. Biocontrol has been reported to have potential for the management of soil-borne diseases (Larkin and Fravel, 1998). The difficulty in controlling *Fusarium* wilt and root rot diseases by other methods has stimulated research in this area (Fravel, 2005).

One of the goals of the use of biocontrol in agriculture is to avoid the pitfalls associated with overuse of synthetic pesticides, including the development of resistance in pest populations. An attractive feature of biocontrol strategies is that populations of pathogens developing resistance to antagonistic products produced by biocontrol agents is likely to be very slow (Jenkins and Grzywacz, 2000). There are two reasons why this may be so. First, most biocontrol agents produce more than one antimicrobial component and resistance to multiple antimicrobial factors should occur only at a very low frequency. Second, total exposure of the pathogen population to the antimicrobial factor is low because, in general, the populations of biocontrol agents are localized on the root. Therefore, selection pressures are minimized. Nevertheless, if sufficient selection pressure is applied to pathogens, the appearance of strains that are not controlled by biocontrol agents is inevitable (Siddiqui, 2006). The use of antimicrobial agents in human medicine and agriculture has shown that selection pressures drive the evolution of resistance faster than might have been expected (Handelsman and Stabb, 1996; Jiménez-Gasco *et al.*, 2002; John *et al.*, 1999). This has increased focus on strategies based on multiple biocontrol agents.

Weller (1988) proposed the manipulation of the rhizosphere to benefit

micro-organisms which produce antagonistic compounds that would protect roots from the deleterious effect of soil-borne pathogens. Support for this came from the discovery of suppressive soils in which the active microflora naturally control the disease-causing activities of pathogen populations (Weller *et al.*, 2002). A variety of soil microorganisms have demonstrated activity in controlling various soil-borne plant pathogens, including *Fusarium* wilt and root rot pathogens. *Fusarium* wilt-suppressive soils are known to occur in many regions of the world, and suppression has generally been shown to be of biological origin (Alabouvette, 1999; Weller *et al.*, 2002; Zhang *et al.*, 1996; Díaz *et al.*, 2005). *Fusarium*-suppressive soils, in addition to pathogenic strains which are responsible for the disease, also contain large populations of non-pathogenic *F. oxysporum* (Alabouvette *et al.*, 1993; Nel *et al.*, 2003). Both pathogenic and non-pathogenic strains are able to persist through saprophytic growth on organic matter in soil (Edel *et al.*, 1997). Antagonists recovered from *Fusarium* wilt-suppressive soils, especially non-pathogenic *F. oxysporum*, have been used to reduce *Fusarium* wilt diseases of several different crops (Alabouvette *et al.*, 1993; Larkin and Fravel, 1998; Minuto *et al.*, 1995; Park *et al.*, 1988; Paulitz *et al.*, 1987; Postma and Rattink, 1992; Silva and Bettioli, 2005; Olivain and Alabouvette, 1999). Indeed, the biological control achieved with the combination of antagonism *Pseudomonas* spp. and non-pathogenic *F. oxysporum* was more efficient and consistent than that recorded after inoculation of the *Pseudomonas* sp. or non-pathogenic *F. oxysporum* alone (Lemanceau *et al.*, 1991; 1992; Park *et al.*, 1988).

Other biocontrol microbes, such as species of *Gliocladium*, *Trichoderma*, *Streptomyces*, and *Bacillus* have been used to control a variety of fungal pathogens, including *Rhizoctonia*, *Pythium*, *Sclerotinia*, *Sclerotium* and *Fusarium* spp. and may

also be effective against *Fusarium* wilt diseases (Marois and Mitchell, 1981; Zhang *et al.*, 1996). *Stilbella aciculosa*, *Trichoderma hamatum* and *Bacillus subtilis* are being developed to control the primary foliar diseases, *Botrytis* and powdery mildew (Paulitz *et al.*, 1987; Lewis and Papavizas, 1993; 1996; Ongena *et al.*, 2005; Wilson *et al.*, 1997). *Pseudomonas* has been adapted for the control of *Pythium* diseases in hydroponics (Harman, 1991; Lewis, 1996) and *Pseudozyma flocculosa* has also been used for the control of powdery mildew (Mandeeel and Baker, 1991; Larkin and Fravel, 1998; Lugtenberg and Dekker, 1999). In addition, several lesser-known genera of biocontrol fungi, including *Laetisaria*, *Stilbella*, *Cladorrhinum*, and *Penicillium* spp., have been used to control soil-borne pathogens and may also have activity against *Fusarium* diseases (Lewis *et al.*, 1993; 1996). Rhizobacterial strains of *Pseudomonas*, *Gliocladium*, and *Bacillus* spp. also have been used to reduce disease caused by a variety of soil-borne pathogens, including *Fusarium* spp. (Bolwerk *et al.*, 2003; Duffy *et al.*, 1996; Fridlender *et al.*, 1993; Liu *et al.*, 1995; Laville *et al.*, 1991; Lemanceau *et al.*, 1992; Ongena *et al.*, 2005; Raaijmakers, 1995; Schouten *et al.*, 2004; Van Peer *et al.*, 1991; Zhang *et al.*, 2005). Moreover, fluorescent *Pseudomonas* spp. was demonstrated to be involved in soil suppressiveness (Lemanceau and Alabouvette, 1991) and has been reported to control *Fusarium* diseases in different crops (Bolwerk *et al.*, 2005; Lemanceau *et al.*, 1991, 1992; Park *et al.*, 1988; Paulitz *et al.*, 1987; Stutz *et al.*, 1986). They have been applied to seeds and rhizosphere soil or other plant parts for biological control (Alabouvette, 1999; Weller, 1988). *Trichoderma* spp. are naturally occurring soil inhabiting fungi and have been shown to be important antagonists to plant root pathogenic fungi, including *Fusarium* and *Pythium* (Green *et al.*, 1999; Hermosa *et al.*, 2000; Lu *et al.*, 2004; Sivan and Chet, 1986; 1989; Brunner *et al.*, 2005;

Benhamou *et al.*, 2002).

Biocontrol of *Fusarium* wilts by application of antagonistic fungi and bacteria isolated from suppressive soils has been carried out during the last two decades all over the world (Fravel *et al.*, 2003; Larkin and Fravel, 1998; Lemanceau, 1992; Park, 1988). Unsurprisingly, of the antagonistic micro-organisms obtained from such soils that have been evaluated to control *Fusarium* wilt, the most promising are non-pathogenic *F. oxysporum* isolates (Alabouvette *et al.*, 1993; Edel *et al.*, 1997; Lemanceau *et al.*, 1991; 1993; Olivain and Alabouvette, 1999; Larkin and Fravel, 1999; 2002). Biocontrol of *Fusarium* wilt diseases through non-pathogenic soil isolates of *Fusarium* spp. has shown potential as an alternative disease management strategy (Alabouvette *et al.*, 1985; Alabouvette and Couteaudier, 1992; Blok *et al.*, 1997; Edel *et al.*, 1997; Lemanceau and Alabouvette, 1991; Lemanceau *et al.*, 1993; Olivain and Alabouvette, 1997, 1999) and the role of non-pathogenic *F. oxysporum* in the natural suppressiveness of some soils to *Fusarium* wilts has been established (Alabouvette *et al.*, 1985; 1999; Larkin and Fravel, 1999; Mazzola, 2002; Scher and Baker, 1980; Shim and Lee, 1990; Weller *et al.*, 2002). This has led to more detailed screening of potentially effective strains as biocontrol agents (Alabouvette *et al.*, 1993; 1998; Paulitz *et al.*, 1987). Selected isolates of avirulent or non-pathogenic *F. oxysporum* have successfully reduced the incidence of *Fusarium* wilt in numerous crops in greenhouse and field trials (Alabouvette *et al.*, 1993; Larkin and Fravel, 1998; 1999; 2002). Larkin and Fravel (1998) studied the efficacy of several strains of non-pathogenic *Fusarium* spp. isolated from a wilt-suppressive soil in controlling *Fusarium* wilt of tomato in greenhouse tests. Selected isolates were equally effective at reducing wilt disease in repeated tests, with reductions in disease incidence of 60 to 80% relative to the pathogen-infested controls. They (1999) had also demonstrated

that the mechanism of action of biocontrol by two non-pathogenic isolates (CS-1 and CS-20) involved induced systemic resistance and that these isolates effectively reduced disease incidence at low biocontrol inoculum densities and high pathogen densities. *Fusarium* wilt control in suppressive soils has also been attributed to the occurrence of non-pathogenic *Fusarium* strains, such as Fo47 (Alabouvette *et al.*, 1993; Fuchs *et al.*, 1999; Lemanceau *et al.*, 1992; 1993; Trouvelot *et al.*, 2002). The wild-type non-pathogenic *F. oxysporum* Fo47 was previously isolated from soil in France that suppressed *Fusarium* wilt; the efficacy of Fo47 in reducing the severity of *Fusarium* wilt demonstrated on tomato and carnation (Olivain and Alabouvette, 1997; 1999; Lemanceau, 1991; 1992; Fuchs, 1999). Subsequently, Fo47b10, a mutant of Fo47 that is resistant to benomyl has been obtained and was equally effective in the control of *Fusarium* wilt (Lemanceau *et al.*, 1992). These results indicated a high potential for development of non-pathogenic isolates as biocontrol agents.

Vanilla plants are affected by several diseases, including *Fusarium* root rot disease, caused by *Fusarium oxysporum* Schlecht. f. sp. *vanillae* (Tucker), which can cause serious economic losses. The management of this disease depends on the integration of different control methods including chemical, genetic, cultural management or microbial control (Johansson *et al.*, 2004). However, the management of this disease suffers from the same problems as the methods used to control *Fusarium* wilt disease. They are either not very efficient or are difficult to apply. Thus alternative methods of controlling the disease are needed. Biological control, especially by using non-pathogenic strains, could be a promising method for vanilla root rot disease control, because this is a soil-borne disease with many similarities to wilt disease.

5.1.2 Mechanism of Suppression of *Fusarium* Diseases by Non-pathogenic Strains

Disease suppression by biocontrol agents is a result of the sustained manifestation of interactions among the plant, pathogen, biocontrol agent, and environment (Gilbert *et al.*, 1994; Handelsman and Stabb, 1996; Maor and Shirasu, 2005; Sen, 2003; Salerno *et al.*, 2004; Sartoratto, *et al.*, 1980). Despite the potential use of biocontrol agents in agricultural applications, biological control remains one of the most poorly understood areas of plant–microbe interactions (Handelsman and Stabb, 1996). However, promising model systems for studies of such interactions have become available with the recent successes found in protecting diverse crop plants from *Fusarium* wilts using non-pathogenic *Fusarium oxysporum* (Alabouvette, 1995; Larkin and Fravel, 1999).

A number of possibilities have been proposed to be involved in the suppression of *F. oxysporum* by the antagonistic micro-organisms. Competition for nutrients was proposed as a mechanism for suppression by both non-pathogenic *F. oxysporum* and fluorescent *Pseudomonas* spp. (Alabouvette *et al.*, 1993; 1998; 1999). Weller (1988) concluded that pathogen suppression was due to nutrient competition between pathogenic and saprophytic *F. oxysporum*. Numerous studies have established that fluorescent *Pseudomonas* spp. is efficient competitors for ferric iron (Elad and Baker, 1985; Leong, 1986). Alabouvette (1999) suggested that the efficacy of non-pathogenic *F. oxysporum* strains in controlling *Fusarium* wilt is related to their ability to compete for carbon and iron. Greater efficacy in disease suppression by the association of non-pathogenic *F. oxysporum* and fluorescent *Pseudomonas* spp. could be due to the combination of carbon and iron competition. Schneider (1984) and Mandeel and Baker (1991) proposed that suppression was due to competition for

infection sites and colonization at the root surface. The role of competition including saprophytic competition for nutrients in the soil and rhizosphere and competition for infection sites on and in the root had been summarized (Chin *et al.*, 2000; Fravel *et al.*, 2003; Marois and Mitchell, 1981).

Lemanceau *et al.* (1992) had suggested that pseudobactin production by *Pseudomonas putida* was responsible for the improved biocontrol of *Fusarium* wilt achieved by the association of non-pathogenic *F. oxysporum* Fo47 and *P. putida* compared with the separate application of each antagonistic organism. These results suggested that pseudobactin-mediated competition for iron increases the efficacy of the antagonistic activity of non-pathogenic *F. oxysporum* Fo47 against pathogenic *F. oxysporum*. To assess the value of this hypothesis, an *in vitro* bioassay had been done by Lemanceau *et al.* (1993). The results showed both non-pathogenic and pathogenic *F. oxysporum* reduced each other's growth when grown together. However, in these coinoculation experiments, pathogenic *F. oxysporum* was relatively more inhibited in its growth than non-pathogenic *F. oxysporum* Fo47. This fungal antagonism appears to be mainly associated with the competition for glucose. Pseudobactin reduced the growth of both *F. oxysporum* strains, whereas ferric pseudobactin did not; antagonism by pseudobactin was then related to competition for iron. However, the pathogenic *F. oxysporum* strain was more sensitive to this antagonism than the non-pathogenic strain. Pseudobactin reduced the efficiency of glucose metabolism by the fungi. These results suggest that pseudobactin increases the intensity of the antagonism of non-pathogenic *F. oxysporum* Fo47 against pathogenic *F. oxysporum* by making pathogenic *F. oxysporum* more sensitive to the glucose competition by Fo47. The antagonism of non-pathogenic *F. oxysporum* against pathogenic *F. oxysporum* strongly depends on the ratio of non-pathogenic to pathogenic *F.*

oxysporum densities: the higher this ratio, the stronger the antagonism (Alabouvette *et al.*, 1998).

Recently, induced systemic resistance (ISR) has been proposed as the possible mechanisms of action associated with non-pathogenic isolates of *Fusarium* spp. (Bao and Lazarovits, 2001; Brunner *et al.*, 2005; Elmer, 2005; He and Wolyn, 2005; Zhang *et al.*, 2005). ISR refers to plant-induced resistance through the rapid stimulation of a general cascade of nonspecific defence responses, including structural barriers and pathogenesis-related proteins, such as chitinases and β -1,3-glucanases (Singh *et al.*, 1999; Larkin and Fravel, 1999; Fuchs *et al.*, 1997). ISR involves the colonization of roots by certain organisms, resulting in some level of systemic resistance to the pathogen in the host plant. This phenomenon has been observed with *F. oxysporum* strains (Inoue *et al.*, 2002; Olivain and Alabouvette, 1997; 1999; Postma and Luttikholt, 1996). Fuchs *et al.* (1997) attributed the biocontrol activity of the non-pathogenic strain Fo47 to induced resistance in tomato, correlated with an increased activity of chitinase, β ,1–3 glucanase and β ,1– 4 glucosidase. Tamietti *et al.* (1993) found increased activity of several plant enzymes related to plant defence reactions in tomato plants transplanted in sterilized soil infested with a strain of non-pathogenic *F. oxysporum*. Rep showed that the non-pathogenic strain Fo47, although not very effective in inducing systemic resistance in tomato induced an increase of plant resistance (PR) proteins (Rep *et al.*, 2004; 2005). By using root inducing transferred-DNA-transformed pea roots, Benhamou and Garand (2001) demonstrated that pea root inoculation with non-pathogenic *Fusarium* strain Fo47 triggered a set of plant defence reactions that resulted in the elaboration of permeability barriers and in the creation of a fungitoxic environment that protected the roots by barricading the fungus in the outermost root tissues.

Inhibition effect of fungal pathogens on plant roots has many sources, the common element being a high level of soil microbial activity associated with decomposing organic matter (Shim and Lee, 1990). In soils more suppressive to *Fusarium*, the numbers of total bacteria and total fungi were higher than soils that were less suppressive, while organic matter content of suppressive soils was twice that of non-suppressive soils. Plants grown in *Fusarium* suppressive soils were shown to have enzymes that inhibited the growth of pathogenic *Fusarium* in the absence of non-pathogenic *Fusarium* (Tamietti *et al.*, 1993).

5.1.3 Problems Using Biocontrol Methods

Although numerous mechanisms for disease control have been proposed, there is not yet sufficient information to fully exploit non-pathogenic *F. oxysporum* strains for commercial use. This lack of information is mostly related to difficulties involved in studying interactions between the pathogenic *Fusarium* and the non-pathogenic *F. oxysporum* strain in the rhizosphere and soil. It is not only impossible to measure populations of the two organisms on roots, but it is also very difficult to differentiate them quantitatively from each other, or from the numerous other morphologically identical *F. oxysporum* strains that are common residents on plants and in soil. In biocontrol, root colonization is presumed to be important for many antagonists, but the inability to accurately assess colonization has hampered research efforts (Bao and Lazarovits, 2001; Paparu *et al.*, 2006; Paulitz and Bélanger, 2001). Thus, the location of the non-pathogenic *F. oxysporum* strain either on or in a plant root, the extent of its presence in comparison to root biomass or to the pathogen, and the behaviours of both the non-pathogenic *F. oxysporum* strain and the pathogenic when introduced together remain unclear.

Moreover, although significant disease reductions were obtained in a controlled environment, many non-pathogenic *F. oxysporum* strains were less effective as crop protectants under field conditions (Larkin and Fravel, 1998; Postma and Luttikholt, 1996). The efficacy may vary under a wide range of environmental, cultural and biotic conditions. Given that establishment of a threshold density of an antagonist is a key factor in biocontrol, the rationale of using a mixture of antagonist isolates is a logical approach, because mixtures are more closely mimic microbial communities and have multiple mechanisms of disease suppression (Aguilar and Barea, 1996). For example, the complexity of the soil ecosystem is a constraint that makes biological

control of root pathogens by introducing antagonists (Weller, 1988).

Many attempts at biological control have resulted in inconsistent or unsatisfactory disease control under varying environmental conditions and locations (Alabouvette *et al.*, 1993, 1995, 1998; Cook, 1993; Herva's *et al.*, 1998; Larkin and Fravel, 2002; Schisler *et al.*, 2000). This inconsistency may be partly related to a general lack of understanding of how these biocontrol systems work and under what conditions they may or may not be expected to function (Marois *et al.*, 1981; Reid *et al.*, 2002). This has resulted in the introduction of biocontrol organisms into environments in which they are ecologically unsuited (Larkin and Fravel, 2002). Any individual biocontrol organism can only be expected to perform within a limited set of physical, biological and environmental conditions. Yet, in most cases, these conditions are not adequately defined. Basic environmental conditions, such as temperature, moisture, sunlight, and soil physical and chemical characteristics, can greatly affect the physiology of the host plant and subsequent disease development, as well as alter the interactions among plant, pathogen and biocontrol agent in various ways (Larkin and Fravel, 2002). This is in addition to potential direct effects on the pathogen and biocontrol organisms and other soil microbes, and all of these effects may influence efficacy of biological control.

Other conditions related to the specific pathosystem involved, such as the occurrence of different pathogenic races and variability in disease resistance and susceptibility among host cultivars, also affect the disease response and may influence biological control (Larkin and Fravel, 2002; Marois *et al.*, 1981; Reid *et al.*, 2002). For biocontrol to be effective, biocontrol agents must control all races of the pathogen which cause host cultivars diseased, as well as be capable of controlling disease on many different host cultivars with varying levels of disease susceptibility

and resistance. This race and cultivar effect may be particularly important to determine for biocontrol agents because induced resistance has been determined to be involved in their biocontrol interaction. Consequently, response to these biocontrol isolates and the capability for induced resistance may vary greatly among cultivars (Larkin and Fravel, 2002; Liu *et al.*, 1995).

Overall, before biocontrol by agents can be practically implemented, it is essential to determine how biological control may be affected by changing environmental conditions. It is important to learn as much as possible regarding the ecology of these biocontrol organisms and their interactions with the pathogen, host plant, soil and rhizosphere microbial communities, and their surrounding environments (Handelsman and Stabb, 1996).

5.1.4 Development of Potential Non-pathogenic Strains as Biocontrol Agents

Although many different biocontrol strains have shown potential for some degree of control of *Fusarium* diseases, strains which can provide truly effective control of *Fusarium* wilt and the potential for effective implementation in commercial agriculture have not yet been identified (Alabouvette *et al.*, 1998; Schisler *et al.* 2000). It has been suggested that micro-organisms isolated from the root or rhizosphere of a specific crop may be better adapted to that crop and may provide better control of diseases than organisms originally isolated from other plant species (Cook, 1993). Such plant associated micro-organisms may make better biocontrol agents because they are already closely associated with and adapted to the plant or plant part as well as the particular environmental conditions in which they serve function. The screening of such locally adapted strains has yielded improved biocontrol in some cases (Larkin and Fravel, 1998; Cook, 1993).

The use of combinations of multiple antagonistic organisms may also provide improved disease control compared to the use of single organisms (Duffy *et al.*, 1996; Larkin and Fravel, 1998). Multiple organisms may enhance the level and consistency of control by providing multiple mechanisms of action, a more stable rhizosphere community, and effectiveness over a wider range of environmental conditions. In particular, combinations of fungi and bacteria may provide protection at different times or under different conditions, and occupy different or complementary niches (Lugtenberg and Dekkers, 1999; Larkin and Fravel, 1998; Weller, 1988). Such combinations may overcome inconsistencies in the performance of individual isolates. Several researchers have observed improved disease control using various combinations of multiple compatible biocontrol organisms (Ali Siddiqui *et al.*, 2001; Dissanayake and Hoy, 1999; Duffy *et al.*, 1996; Elmer, 2004). Lemanceau (1991)

and Alabouvette *et al.* (1993) have demonstrated enhanced biocontrol of *Fusarium* wilt by combining certain non-pathogenic strains of *F. oxysporum* with fluorescent strains of *Pseudomonas*.

Although biocontrol still represents a very small portion of disease management, some of these uses are significant. If the goal of biocontrol research is to place biocontrol products in the growers' hands, then perhaps there needs to be more communication between public researchers and industry in the early stages of development (Brunner *et al.*, 2005). Over the past 25 years, the approach to biocontrol research has evolved toward being more ecologically holistic and more oriented toward both production systems and industry's concerns (Elmer, 2004). This evolution is likely to continue. In particular, research in production, formulation and delivery could greatly assist in commercialization of biocontrol agents. More research is needed in integrating biocontrol agents into production systems, such as in rotating biocontrol with chemical pesticides and in calculating these into forecast models to choose whether to apply a chemical pesticide or biocontrol (Fravel, 2005). Continued research in biocontrol is needed to contribute to the movement toward sustainable agriculture and simply to ensure that alternatives are available if other management tools fail or are lost.

The research detailed here was conducted as a first step toward the development of effective biological control as an alternative strategy for the management of vanilla *Fusarium* root rot disease. The objective of this work was to evaluate the efficiency of non-pathogenic *F. oxysporum* isolates for biological control of vanilla root rot disease caused by *F. chuoxyosporum* f. sp. *vanillae* and to screen them for their biocontrol capabilities. In addition, preliminary investigations on the potential benefits of combining various antagonistic organisms also used for the control of

Fusarium wilt were performed.

5.2 MATERIAL AND METHODS

5.2.1 Materials

Two non-pathogenic isolates, ML-5-2 and HK-5b-4-1, were investigated for their inhibition effect on the vanilla root rot disease.

Pathogenic isolates, DL-4-1, was a strong pathogenic strain inoculated on the healthy vanilla thereby causing vanilla root rot disease.

TAC, a biocontrol agent product from a Yunnan Agricultural University project, has been tested through greenhouse and field trials on other crops such as chilli, lily and pseudoginseng for many years and was produced by the Wolin Company of Yunnan Province, China. TAC includes 750 g/ha of *Pseudomonas*, 1500 g/ha of *Bacillus*, 600 g/ha of *Trichoderma* and chemical mixtures which include 750 g/ha of metalaxyl and mancozeb and 80 g/ha of hymexazol. TAC and chemical fungicides (metalaxyl and mancozeb) were used as positive controls in this study.

Pseudomonas, *Bacillus* and *Trichoderma* used in the treatments were screened by Yunnan Agricultural University and were produced by Wolin Company, Yunnan, China.

5.2.2 Investigating the Effect of Non-pathogenic Strains on Vanilla Root Rot Disease in a Controlled Environment

Spores of two non-pathogenic strains and one pathogenic strain were prepared in liquid medium at 27°C for 7 days. Spores density was counted with a hemacytometer under the microscope and adjusted to the required inoculum concentration with sterile water.

Vanilla plants were collected from the plantation in Hekou County of Yunnan. Plants were cut into 15 cm pieces and placed in tap water for 2 months to produce roots and then planted in sterile soil for 1 month. Vanilla seedlings produced from 3

months tissue culture were transferred to sterilized soil to grow for 15 days. The pots were placed in a controlled environment at 27°C (12 h day length) and were watered daily prior to inoculation.

Inoculation studies were conducted as a set of treatments to examine the response of vanilla cuttings or vanilla seedlings to separate and simultaneous inoculation with pathogenic isolate and different non-pathogenic isolates (ML-5-2 and HK-5b-4-1) and to different inoculum ratios between pathogenic and non-pathogenic isolates. The experiments were conducted with 3 replicates and 5-9 roots of 3 cuttings and seedlings formed each replicate. Twenty-two treatments were utilized to determine the inhibition effect of non-pathogenic isolates. Treatment 1: The roots of vanilla plants were immersed in 1×10^6 microspore/ml suspension of the pathogenic strain DL-4-1 for 5 minutes, and then planted in soil previously infested with this pathogenic strain (1×10^6 microspore/ml of soil). Three days later, 1×10^6 microspore/ml microspore spore suspension of the non-pathogenic strain, ML-5-2, was inoculated. Treatment 2: The roots of vanilla plants were immersed in 1×10^6 microspore/ml spore suspension of pathogenic strain DL-4-1 for 5 minutes, and then planted in soil previously infested with this pathogenic strain (1×10^6 microspore/ml of soil). Three days later, 1×10^7 microspore/ml spore suspension of the non-pathogenic strain, ML-5-2, was inoculated. Treatment 3: The roots of vanilla plants were immersed in 1×10^6 microspore/ml spore suspension of pathogenic strain DL-4-1 for 5 minutes, and then planted in soil previously infested with this pathogenic strain (1×10^6 microspore/ml of soil). Three days later, 1×10^8 microspore/ml microspore spore suspension of the non-pathogenic strain, ML-5-2, was inoculated. Treatment 4: The roots of vanilla plants were immersed in 1×10^6 microspore/ml spore suspension of pathogenic strain DL-4-1 and 1×10^6

microspore/ml spore suspension of non-pathogenic strain ML-5-2, and then planted in soil previously infested with pathogenic strain DL-4-1 (1×10^6 microspore/ml of soil) and non-pathogenic strain ML-5-2 (1×10^6 microspore/ml of soil). Treatment 5: The roots of vanilla plants were immersed in 1×10^6 microspore/ml spore suspension of pathogenic strain DL-4-1 and 1×10^7 microspore/ml microspore spore suspension of non-pathogenic strain ML-5-2, and then planted in soil previously infested with pathogenic strain DL-4-1 (1×10^6 microspore/ml of soil) and non-pathogenic strain ML-5-2 (1×10^7 microspore/ml of soil). Treatment 6: The roots of vanilla plants were immersed in 1×10^6 microspore/ml spore suspension of pathogenic strain DL-4-1 and 1×10^8 microspore/ml spore suspension of non-pathogenic strain ML-5-2, and then planted in soil previously infested with pathogenic strain DL-4-1 (1×10^6 microspore/ml of soil) non-pathogenic strain ML-5-2 (1×10^8 microspore/ml of soil) at the same time. Treatment 7: The roots of vanilla plants were immersed in 1×10^6 microspore/ml spore suspension of non-pathogenic strain ML-5-2 for 5 minutes, and then planted in soil previously infested with non-pathogenic strain ML-5-2 (1×10^6 microspore/ml of soil). Three days later, 1×10^6 microspore/ml spore suspension of pathogenic strain DL-4-1 was inoculated. Treatment 8: The roots of vanilla plants were immersed in 1×10^7 microspore/ml spore suspension of non-pathogenic strain, ML-5-2 for 5 minutes, and then planted in soil previously infested with non-pathogenic strain ML-5-2 (1×10^7 microspore/ml of soil). Three days later, 1×10^6 microspore/ml spore suspension of pathogenic strain DL-4-1 was inoculated. Treatment 9: The roots of vanilla plants were immersed in 1×10^8 microspore/ml spore suspension of non-pathogenic strain, ML-5-2 for 5 minutes, and then planted in soil previously infested with non-pathogenic strain ML-5-2 (1×10^8 microspore/ml of soil). Three days later, 1×10^6 microspore/ml spore suspension of pathogenic

strain DL-4-1 was inoculated. The next 9 treatments (10-18) were as described above but carried out using another non-pathogenic strain, HK-5b-4-1. Metalaxyl and mancozeb (treatment 19) and TAC (treatment 20) were dripped into soil three days later after 1×10^6 microspore/ml of pathogenic strain had been inoculated. In addition to the non-pathogenic *F. oxysporum* isolates, the assay included a non-inoculated control that received sterile water only (treatment 21) and a control treated with 1×10^6 microspore/ml of pathogenic strain DL-4-1 (treatment 22). The plants were grown in the growth control. Water was drained from the pots 5 days after inoculation. Plants were then watered daily beginning 6 days after inoculation.

Evaluation of the development of the disease was performed at 40 days (vanilla cuttings) and 30 days (vanilla seedlings) after the inoculation with pathogenic strain. The inoculated plant was carefully removed from each pot, washed and evaluated for disease severity using the following disease severity index on a scale of 0-5 (Fang, 1998):

0 = no symptoms; 1= root tips or injured root turned brown, rot with the length of the injured section less than $\frac{1}{4}$; 2 = root turned brown, rot with the length of the injured section more than $\frac{1}{4}$, but less than $\frac{1}{2}$ of total length; 3 = root turned brown and rot with the length of the injured section more than $\frac{1}{4}$, but less than $\frac{3}{4}$ of total length; 4 = all roots turned brown and rot; 5 = all roots turned brown, rot and the pathological symptom extended to the stem base. The disease incidence (DI), the disease severity index (DSI) and the control effect of non-pathogenic strains were calculated by the following formula:

- a. $DI = 100 \times \text{number of disease root} / \text{total number of investigated roots}$.
- b. $DSI = 100 \times \Sigma (\text{total number of each diseased root} \times \text{value of relative scale}) / \text{total number of investigated roots} \times 5$.

$$c. \text{ Control Effect} = 100 \times (C_1 - T_1) / C_1.$$

Where C_1 equals disease severity index without any treatment with inoculated pathogenic strains to cause injured plants, and T_1 equals disease severity index following different treatments.

5.2.3 Investigation of the Effects of Non-pathogenic Strain on Vanilla Root Rot Disease in the Field

Non-pathogenic inoculum was prepared from twice-autoclaved wheat seed (50 g seed 100 mL⁻¹ H₂O) inoculated with non-pathogenic strain ML-5-2 and incubated at 27°C in a rotary shaker for 10 days and air-dried. Concentration of conidia was counted with a haemocytometer under the microscope.

The field plots were established at Manjingdai vanilla plantation in Xishuangbanna that had been planted vanilla for 5 years. The planting had declined due to *Fusarium* root rot disease. In the field 2×2 m² plots were laid out and planted to 20-30 plants. Plots were arranged in a randomized blocked factorial design with three replicates per treatment. Plots were managed by customary cultural practices for vanilla in the area.

Eighteen treatments were conducted to examine the response to non-pathogenic isolates of ML-5-2 at different concentrations and in different combination with other medications, Treatment 1, 2, 3: Non-pathogenic ML-5-2 treated at three concentrations of 1500 g/ha, 3000g /ha, 4500 g/ha; Treatment 4, 5, 6: ML-5-2 at three different concentrations of 1500 g/ha, 3000 g/ha and 4500 g/ha and with *Pseudomonas* (1500 g/ha); Treatment 7, 8, 9: ML-5-2 at three different concentrations of 1500 g/ha, 3000 g/ha and 4500 g/ha and with *Bacillus* (1500 g/ha); Treatment 10, 11, 12: ML-5-2 at three different concentrations of 1500 g/ha, 3000 g/ha and 4500 g/ha and with *Pseudomonas* (750 g/ha) + *Bacillus* (750 g/ha);

Treatment 13, 14, 15: ML-5-2 at three different concentrations of 1500g/ha, 3000g/ha and 4500g/ha and plus *Pseudomonas* (750g/ha) + *Bacillus* (1500g/ha) + *Trichoderma* (600g/ha); Treatment 16: Biocontrol agent, TAC alone (1500 g/ha); Treatment 17: Chemical fungicides, metalaxyl and mancozeb (1500g/ha); Treatment 18: a non-treated plot served as control. Wheat seeds with non-pathogenic isolate were directly planted in the soil around the vanilla roots, and other medications were dissolved in 3000 ml water and sprayed around the roots of the plants.

The investigations of disease were conducted twice. One was basal disease severity investigation measured before treatment. Another was carried out 30 days later after treatment. All the aerial roots touching the soil were counted. The evaluation of disease severity was as described above (see Section 5.2.2). The calculation of the disease incidence and the disease severity index were the same as above also, but the control effect of non-pathogenic strains was calculated by the following formula:

$$\text{Control Effect} = 100 \times [1 - (C_0 \times T_1) / (C_1 \times T_0)].$$

Where C_0 = disease severity index for the control plot before treatment.

T_0 = disease severity index for the treatment plot before treatment.

C_1 = disease severity index for the untreated control 4 weeks later after treatment began.

T_1 = disease severity index for the treated plot with different treatments 4 weeks later after treatment began.

5.2.4 Data Analysis

Data were analysed with SPSS System Software Package, version 10.0. Analyses were based on means for each treatment and the data were compared by the Duncan's multiple range test at 5% probability.

5.3 RESULTS

5.3.1 Biocontrol Effects of Two Non-pathogenic *Fusarium oxysporum* f.sp. *vanillae* Isolates

In the experiments for determination of the effect of the two non-pathogenic strains on controlling vanilla root rot disease by using vanilla cuttings and vanilla seedlings, both non-pathogenic isolates were able to reduce the progress of disease symptoms to some extent, and showed the similar control effects both on vanilla cuttings and vanilla seedlings.

The vanilla cuttings or seedlings had no symptom of root rot when investigations were initiated. Vanilla cuttings or seedlings whose root systems were immersed in the conidial suspension of non-pathogenic *F. oxysporum* isolates ML-5-2, and HK-5b-4-1, did not show symptoms of vascular disease and developed normally after 3 days before they were inoculated with pathogenic strain DL-4-1. However, vanilla cuttings and vanilla seedlings whose root systems were immersed in the conidial suspension of pathogenic *F. oxysporum* isolate DL-4-1, developed disease rapidly within 3 days prior to being inoculated with non-pathogenic strain ML-4-1 and HK-5b-4-1.

The data in Figure 5.1 showed that two non-pathogenic strains, ML-5-2 and HK-5b-4-1, were effective on controlling vanilla root rot disease when inoculated onto vanilla cuttings in the controlled environment. Non-pathogenic strain ML-5-2 reduced disease incidence from 91.67% to 76.85% and reduced disease severity index from 75.83% to 50.11% compare to non-treated controls when inoculated on vanilla cuttings (Table 5.1). The Non-pathogenic strain HK-5b-4-1 reduced disease incidence from 91.67% to 81.48% and reduced disease severity index from 75.83% to 60% compare to to non-treated controls (Table 5.1). Disease incidence and disease

severity index showed significant differences among different treatments when inoculated on vanilla cuttings ($P < 0.01$, Table 5.2).

Data in Figure 5.2 showed that both non-pathogenic strains, ML-5-2 and HK-5b-4-1, were effective on controlling vanilla root rot disease when inoculated onto vanilla seedlings in the controlled environment. Non-pathogenic strain ML-5-2 reduced disease incidence from 44.44% to 25.92% and reduced disease severity index from 39.26% to 23.80% compare to non-treated controls (Table 5.1). Non-pathogenic strain HK-5b-4-1 reduced disease incidence from 44.44% to 33.33% and reduced disease severity index 39.26% to 28.89% relative to non-treated controls. Disease incidence and disease severity index also showed significant differences among different treatments when inoculated onto vanilla seedlings ($P < 0.01$, Table 5.2).

Table 5.1 Effects of the different treatments on vanilla root rot disease incidence, disease severity index and biocontrol effects in a controlled environment

Treat- ments	Vanilla cuttings			Vanilla seedlings		
	Disease Incidence Mean (%) ± S.D. (Std. Deviation)	Disease Severity Index Mean (%) ± S.D.	Control Effect (%)	Disease Incidence Mean (%) ± S.D.	Disease Severity Index Mean (%) ± S.D.	Control Effect (%)
1	95.83 c ± 7.22	66.20 f ± 1.58	12.70	42.13 fgh ± 4.01	32.31 fg hi ± 1.12	17.70
2	91.53 bc ± 7.50	64.12 ef ± 1.77	15.44	36.11 def ± 2.41	31.20 efg ± 1.25	20.53
3	86.90 abc ± 1.03	61.67 de ± 2.89	18.67	34.72 cdef ± 2.41	30.00 efg ± 1.11	23.59
4	84.26 abc ± 13.70	58.43 d ± 1.37d	17.4	33.33 bcde ± 0.00	28.15 cde ± 2.56	28.30
5	87.96 abc ± 12.53	53.52 c ± 3.06	29.42	33.32 bcde ± 0.00	28.15 cde ± 1.28	28.30
6	76.85 ab ± 11.23	52.22 c ± 1.93	31.14	30.55 bcd ± 4.81	26.02 bcd ± 3.24	33.72
7	80.09 abc ± 6.56	51.94 c ± 1.73	31.50	27.78 abc ± 4.81	24.72 bc ± 2.10	37.04
8	87.96 abc ± 12.53	50.11 c ± 3.17	33.34	25.92 ab ± 6.41	24.44 abc ± 3.85	37.75
9	87.96 abc ± 12.53	50.56 c ± 4.19	33.91	26.85 ab ± 5.78	23.80 ab ± 2.49	39.38
10	92.13 bc ± 6.85	75.00 h ± 2.50	1.09	42.13 fgh ± 4.01	36.95 jk ± 1.21	5.88
11	88.43 abc ± 11.14	74.54 h ± 1.77	1.70	44.44 gh ± 0.00	36.30 ijk ± 1.28	7.54
12	88.43 abc ± 11.14	72.87 gh ± 1.97	3.90	42.13 fgh ± 4.01	36.02 ijk ± 3.05	8.25
13	92.13 bc ± 6.85	73.79 gh ± 1.58	2.69	44.44 gh ± 0.00	36.30 ijk ± 1.28	7.53
14	92.13 bc ± 6.85	71.94 gh ± 1.73	5.13	42.13 fgh ± 4.01	33.71 ghij ± 3.21	14.13
15	88.43 abc ± 11.14	70.09 g ± 3.05	7.57	39.81 efg ± 4.01	31.94 efgh ± 1.73	18.64
16	87.96 abc ± 12.53	65.79 f ± 0.84	10.03	42.13 fgh ± 4.01	35.28 hijk ± 2.65	10.13
17	84.72 abc ± 6.05	65.46 ef ± 2.84	10.92	37.03 defg ± 6.41	33.33 ghij ± 2.23	15.10
18	81.48 abc ± 6.41	60.00 d ± 0.00	15.83	33.33 bcde ± 0.00	28.89 def ± 2.22	26.41
19	71.03 a ± 4.18	33.37 a ± 0.89	55.99	22.22 a ± 0.00	20.74 a ± 1.28	47.17
20	80.56 ab ± 7.35	43.89 b ± 1.47	42.15	25.92 ab ± 6.41	23.70 ab ± 2.57	39.63
21	91.67 bc ± 7.22	75.83 h ± 3.82		48.15 h ± 6.42	39.26 k ± 1.28	
22	0.00	0.00		0.00	0.00	

Note: All tests were conducted in triplicate. The mean values of different variables followed by different letters are significantly different at 5% level with Duncan test.

Table 5.2 Summary of an ANOVA to compare the biocontrol effects of the different treatments on disease severity index

	isolates	Vanilla cuttings		Vanilla seedlings	
		F Value	P Value	F Value	P Value
DI	ML-5-2	0.930	0.516	4.840	0.003
	HK-5b-4-1	0.381	0.930	2.904	0.029
DSI	ML-5-2	17.405	<0.001	5.369	<0.001
	HK-5b-4-1	33.635	<0.001	4.197	0.005

DI: Disease Incidence; DSI: Disease Severity Index

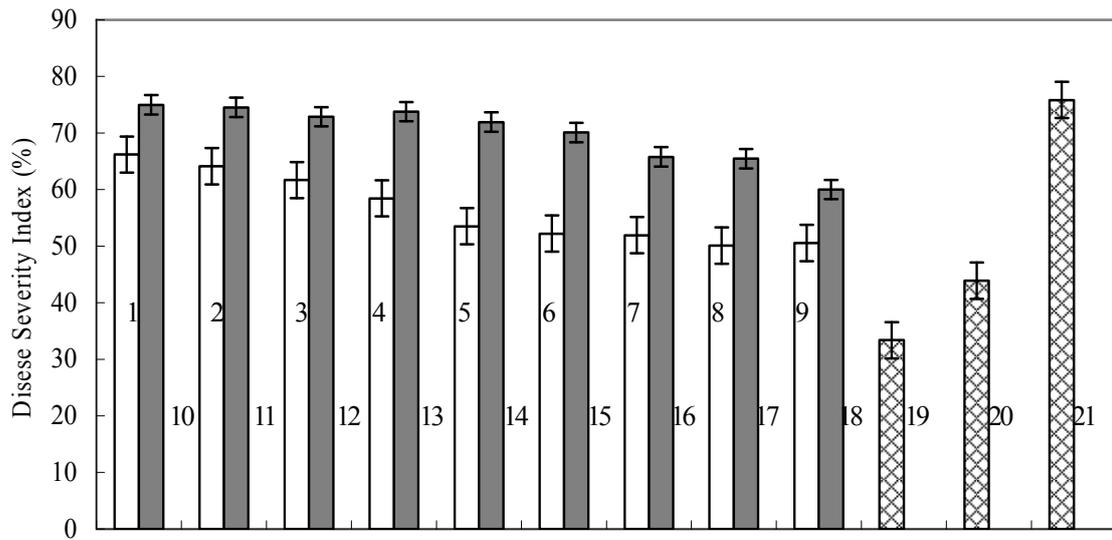


Figure 5.1 Effect of different non-pathogenic isolates on the disease severity index of vanilla cuttings in a controlled environment

The non-pathogenic *F. oxysporum* strains were inoculated at a concentration of 10^6 , 10^7 , or 10^8 conidia/ml, respectively and at different times which included 3 days later than pathogenic strain inoculation, simultaneously with pathogenic strains and 3 days prior to pathogenic strain inoculation. The experiment included also an uninoculated control (no disease), as well as a treatment in which only DL-4-1 was present and two treatments with biocontrol agent TAC and with chemical fungicide. The details of treatments 1-21 are given in 5.2.3. Error bars represent standard errors. All tests were conducted in triplicate.

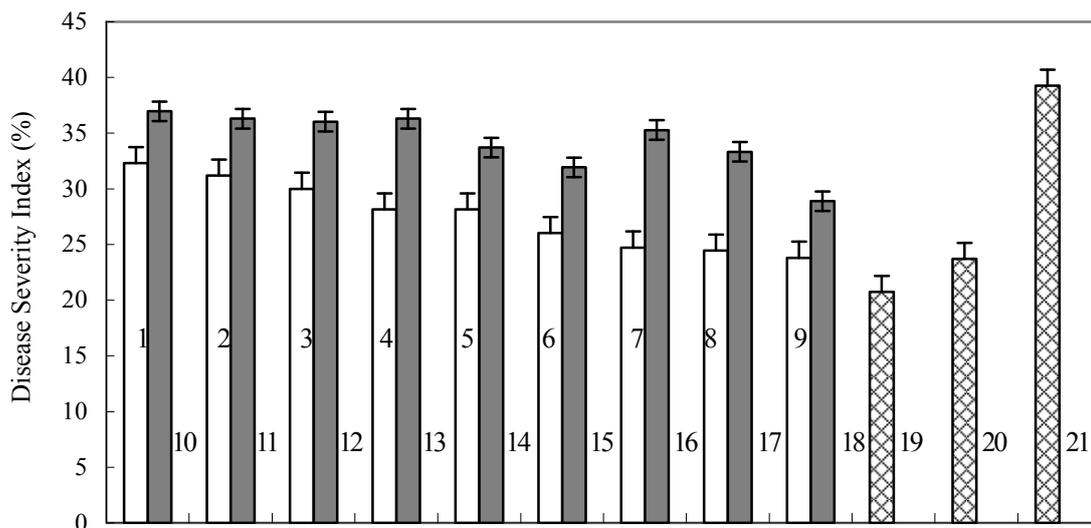


Figure 5.2 Effect of different non-pathogenic isolates on disease severity index on vanilla seedlings in a controlled environment

The non-pathogenic *F. oxysporum* strains were inoculated at the concentration of 10^6 , 10^7 , or 10^8 conidia/ml, respectively and at different times included 3 days later after pathogenic strains inoculated, simultaneously with pathogenic strains and 3 days before pathogenic strains inoculated. The experiment included also an uninoculated control (no disease), as well as a treatment in which only DL-4-1 was present and two treatments with biocontrol agent TAC and with chemical fungicide. The details of treatments 1-21 are given in 5.2.3. Error bars represent standard errors. All tests were conducted in triplicate.

However, comparing the biocontrol effects of the two different non-pathogenic strains, ML-5-2 was more effective than non-pathogenic strain HK-5b-4-1 when inoculated either prior to pathogenic strain DL-4-1, or subsequently or simultaneously (Figures 5.1 and 5.2). When inoculation was carried out on vanilla plants with HK-5b-4-1, there was no significant difference between treatment and control. The biocontrol effects of the non-pathogenic strain, HK-5b-4-1 were 1.09% and 5.88% on cuttings and seedlings, respectively. Even when inoculated with the pathogenic strain simultaneously, the biocontrol effects of HK-5b-4-1 were only 1.70% and 7.54%, respectively (Table 5.1).

5.3.2 Comparison of Biocontrol Effects of Non-pathogenic *Fusarium oxysporum* f.sp. *vanillae* at Different Inoculation Times

Both non-pathogenic isolates have the ability to control vanilla root rot disease on vanilla cuttings and seedlings in the growth control. The effects of both non-pathogenic *F. oxysporum* strains on disease severity index were statistically significant ($P < 0.05$, Table 5.2). Moreover, control effects were more pronounced when inoculation of non-pathogenic strains occurred three days before inoculation of the pathogenic strain, and also were more effective than inoculating the pathogenic strain and non-pathogenic strains simultaneously (Table 5.1). Comparing the control effect when the concentration of non-pathogenic strain ML-5-2 was 10 times greater than that of the pathogenic strain (10^6 conidial spore/ml), for example, treatments 2, 5 and 8 on vanilla cuttings where the pathogenic strain was inoculated three days later than the inoculation of non-pathogenic strains (Table 5.1), the disease severity index and the control effect of non-pathogenic strain ML-5-2 were 50.11% and 33.34%, respectively, whereas when the pathogenic strain and non-pathogenic strains were inoculated simultaneously, the disease severity index and the control effect of non-pathogenic strain ML-5-2 were 53.52% and 29.42%, respectively.

When the pathogenic strain was inoculated three days earlier before non-pathogenic strain inoculation, the disease severity index and the control effect of non-pathogenic strain ML-5-2 were 64.12% and 15.44%, respectively. The same results were obtained on vanilla seedlings and the other treatments (treatments 1, 4 and 7; treatments 3, 6 and 9) at the same concentration ratio of non-pathogenic and pathogenic isolates, and also for another non-pathogenic isolates HK-5b-4-1 (10, 13 and 16; 11, 14 and 17; 12, 15 and 18 treatments) on vanilla cuttings and vanilla seedlings.

5.3.3 Comparison of the Biocontrol Effects of Non-pathogenic *Fusarium oxysporum* f.sp. *vanillae* at Different Concentrations of Inoculation

Non-pathogenic isolates had proved to be more effective at protecting vanilla when it was inoculated first, followed three days later by inoculation of pathogenic strain. In the study of the effect of increasing non-pathogenic inoculum levels from 10^6 to 10^7 or 10^8 microspore/ml, it was found that whether non-pathogenic isolates were inoculated after pathogenic isolates (treatments 1, 2 and 3 for ML-5-2; treatment 10, 11 and 12 for HK-5b-4-1) or before pathogenic isolates (treatments 7, 8 and 9 for ML-5-2; treatment 16, 17 and 18 for HK-5b-4-1) or at the same time as pathogenic isolates (treatment 4, 5 and 6 for ML-5-2; treatment 13, 14 and 15 for HK-5b-4-1) on vanilla cuttings or vanilla seedlings, the control effect of non-pathogenic isolates increased slightly (Table 5.1 and Table 5.2). However, the effects on disease severity index were not statistically significant with different inoculum of 10^6 , 10^7 and 10^8 of non-pathogenic isolates ML-5-2 ($P = 0.160$; 0.140 ; 0.054), nor with different inoculation time on vanilla cuttings, respectively; $P = 0.054$; 0.067 ; 0.061 , or different inoculation time on vanilla seedlings, respectively.

Similarly results were not statistically significant when inoculum of 10^6 , 10^7 and 10^8 of non-pathogenic isolates HK-5b-4-1 on vanilla cuttings ($P = 0.053$ and 0.111 , respectively) were inoculated either prior to pathogenic strains or simultaneously, except for the case when inoculum 10^8 of HK-5b-4-1 were inoculated after three days ($P < 0.05$, Table 5.1). Moreover, there was significant difference when an inoculum of 10^8 of non-pathogenic strains HK-5b-4-1 was inoculated either prior or simultaneously with pathogenic strain DL-4-1 on vanilla seedlings ($P < 0.05$, Table 5.1) but no such significant effect when it was inoculated three days later after DL-4-1 inoculated ($P = 0.095$).

Both non-pathogenic strains inoculated simultaneously with the pathogen reduced disease severity at all three inoculum ratios (Treatments 4, 5 and 6; treatments 13, 14 and 15, Table 5.1). However, biocontrol effects with the higher ratios of ML-5-2 or HK-5B-4-1 (ratios of 10:1 and 100:1) were significantly improved. With the increasing inoculum ratio of ML-5-2 or HK-5b-4-1 to DL-4-1 (1:1, 10:1, and 100:1), the Disease Incidence was 84.26% at a ratio of 1:1, 87.96% at a ratio of 10:1 and 76.85% at a ratio of 100:1, respectively, on vanilla cuttings, and the Control Effect increased from 17.4 at a ratio of 1:1, 29.42% at a ratio of 10:1 and 31.14% at a ratio of 100:1, respectively. The same results were obtained with vanilla seedlings (Table 5.1).

5.3.4 Effect of TAC and Chemical Fungicides on *Fusarium* Root Rot Disease in the Field

The use of chemical fungicides poses urgent problems in environmental protection through the potential health and safety risks, leading to their decreased uses. However, the chemical fungicides (metalaxyl and mancozeb) still showed better effectiveness in controlling vanilla root rot disease than non-pathogenic isolates. The control effect was 55.99% when fungicides were sprayed on the infected vanilla cuttings and 47.17% when on vanilla seedlings and the effect on the disease incidence and disease severity index was significantly reduced by 33.37% and 20.74%, respectively ($P < 0.05$, Table 5.1 and Table 5.2).

Treatment with a combination biocontrol agent TAC significantly controlled *Fusarium* root rot of vanilla in the field, reducing disease incidence on vanilla cuttings and vanilla seedlings by 80.56% and 25.92%, respectively, and disease severity index by 43.89% and 23.70%, respectively.

The results showed non-pathogenic strains delayed the progression of the disease to some extent and indicate that non-pathogenic *Fusarium* isolates, particularly ML-5-2, have potential for further development as biocontrol agents, and that practical implementation of biocontrol of *Fusarium* wilt disease in the field would be feasible and further study should be directed to this end.

5.3.5 The Biocontrol Effects of Non-pathogenic Isolates on Vanilla Root Rot in the Field

5.3.5.1 Effects of Non-pathogenic *F. oxysporum* ML-5-2 on *Fusarium* Root Rot Disease in the Field

To determine the effects of non-pathogenic strains in suppression of *Fusarium* root rot disease, non-pathogenic strain DL-5-2 was chosen over HK-5b-4-1 because of its improved ability to control *Fusarium* root rot at different inoculum concentrations and at different combination with other treatments in the field (see Table 5.2, treatments 8, 9 with treatments 17, 18). Comparison of effects of the non-pathogenic strain in different treatments on disease incidence and disease severity index showed that there is slight decrease in the percentage of infected plants (Table 5.3, Figure 5.3). Disease severity showed inconsistent results and increases and decreases of disease severity index were not correlated with uncorrelated to non-pathogenic strain concentrations or combination with other treatments (Table 5.3, Figure 5.4). Non-pathogenic strain ML-5-2 used in addition to the other medications, *Pseudomonas*, *Bacillus* and *Trichoderma* did not show any synergistic effects on the disease control. The control effects also fluctuated with change of varying concentrations and different combinations of the non-pathogenic strain (Figure 5.5). Due to the complexity of interactions in the soil, it is not possible to deduce the efficacy of non-pathogenic strain in controlling vanilla root rot disease from this study.

Table 5.3 Effect of non-pathogenic isolates on vanilla root rot disease control in the field

Treatments	Increasing rate of Disease Incidence Mean (Std. Deviation)	Increasing Rate of Disease Severity Index Mean (Std. Deviation)	Rate of Control Effect Mean (Std. Deviation)
1	0.78ab (0.51)	1.49a (1.30)	-0.15a (0.60)
2	0.11ab (0.0586)	0.32a (0.20)	0.39a (0.0901)
3	1.15ab (1.02)	1.04a (0.93)	0.633a (0.43)
4	0.33ab (0.15)	0.49a (0.27)	0.32a (0.12)
5	0.63ab (0.46)	1.75a (0.26)	-0.27a (0.12)
6	0.59ab (0.80)	0.81a (0.68)	0.16a (0.31)
7	0.86ab (1.04)	1.33a (1.08)	0.07a (0.50)
8	0.50ab (0.32)	0.61a (0.16)	0.26a (0.0702)
9	0.54ab (0.70)	0.61a (0.59)	0.26a (0.27)
10	0.39ab (0.0495)	0.95a (0.0778)	0.11a (0.0354)
11	0.095ab (0.19)	0.085a (0.18)	0.50a (0.0849)
12	2.65c (1.15)	3.69b (2.52)	-1.16b (1.17)
13	0.065a (0.13)	0.12a (0.21)	0.49a (0.0919)
14	0.68ab (0.55)	0.78a (0.58)	0.18a (0.27)
15	0.56ab (0.32)	1.43a (1.59)	-0.12a (0.74)
16	-0.14a (0.0757)	0.12a (0.40)	0.48a (0.19)
17	0.31ab (0.60)	0.85a (1.24)	0.15a (0.57)
18	1.39b (0.51)	1.31a (0.47)	

The mean values of different variables followed by different letters are significantly different at 5% level with Duncan's Multiple Range Test. All tests were conducted in triplicate.

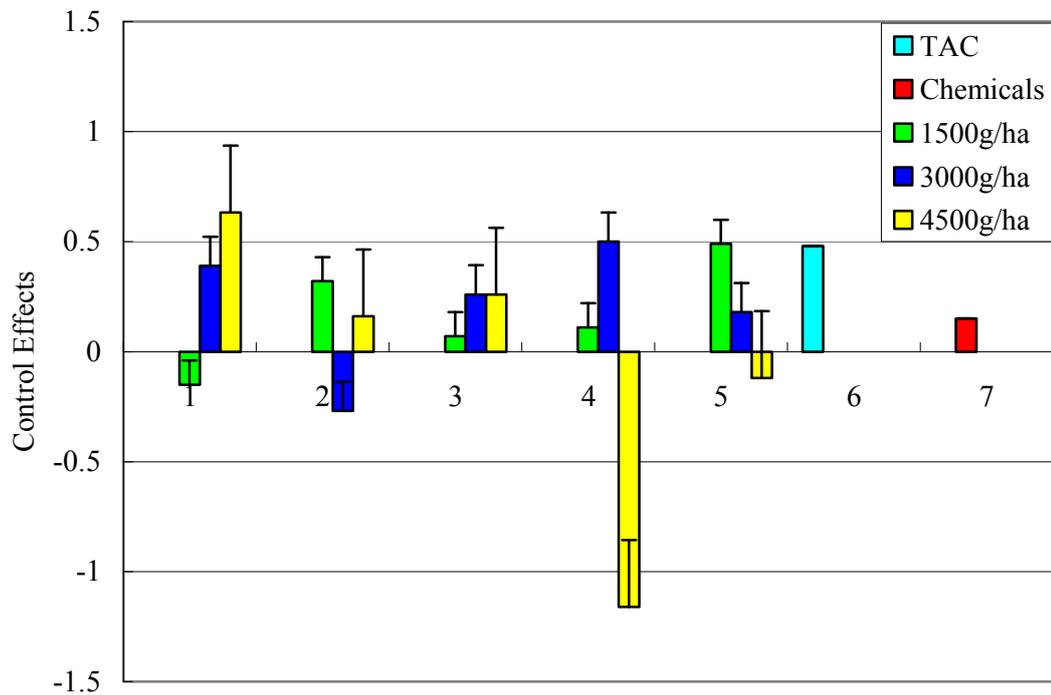


Figure 5.3 Effect of a non-pathogenic strain at different concentrations and combinations on disease control in the field

Non-pathogenic strain ML-5-2 was treated at different concentration and different combination with other medications. 1) ML-5-2 only treated at different concentrations; 2) ML-5-2 at different concentrations with *Pseudomonas* (1500g/ha); 3) ML-5-2 at different concentrations and with *Bacillus* (1500g/ha); 4) ML-5-2 at different concentrations and with *Pseudomonas* (750g/ha) + *Bacillus* (750g/ha); 5) ML-5-2 at different concentrations and with *Pseudomonas* (750g/ha) + *Bacillus* (1500g/ha) + *Trichoderma* (600g/ha); 6) bio-control agent, TAC (1500g/ha); 7) chemical fungicide- metalaxyl and mancozeb (1500g/ha); 8) a non-treated plot served as control. Error bars represent standard errors. All tests were conducted in triplicate.

5.3.5.2 Effect of TAC and Chemical Fungicide on *Fusarium* Root Rot Disease in the Field

For the chemical fungicides, metalaxyl and mancozeb, plant disease severity was not consistent (Table 5.3), although the control effect showed slight efficacy.

The combination treatment with biocontrol agent TAC gave significantly better control of *Fusarium* root rot of vanilla in the field, reducing disease incidence and disease severity (Table 5.3, Figure 5.3).

The results in the field showed non-pathogenic strains delayed the progression of the disease to some extent; however, further work is needed to study the role and efficacy of *F. oxysporum* non-pathogenic ML-5-2 on *Fusarium* wilt disease in the field. Before biological control on its own can be practically implemented, it is essential to determine how biological control may be affected by changing environmental conditions. Overall, it is important to learn as much as possible about the ecology of the biocontrol organism and its interactions with the pathogen, host plant, soil and rhizosphere microbial communities, and their surrounding environments (Handelsman and Stabb, 1996).

5.4 DISCUSSION

5.4.1 Identification and Development of Effective Strains of Non-pathogenic *F. oxysporum*

The first step in developing a biocontrol method is screening for an effective strain. The success of all subsequent steps depends on how well this first step is accomplished and some screening methods will only identify biocontrol organisms with a particular mode of action. Many studies dealing with non-pathogenic *F. oxysporum* have shown that not all the non-pathogenic strains are effective in controlling *Fusarium* wilts.

In this study, we screened two potential candidate non-pathogenic *F.oxysporum* strains. A good candidate *F. oxysporum* for use as a biocontrol agent should be non-pathogenic, closely related to the pathogenic isolates and vegetatively self-incompatible (Fuchs *et al.*, 1999; Fravel, 2005). Non-pathogenic strain ML-5-2 and HK-5b-4-1 were selected on the basis of the results of Vegetative Compatible Group analysis (VCG), supported by the genetic analysis of strains by using rep-PCR technique (these results were obtained by another group). The mechanisms of suppression of *Fusarium* diseases by non-pathogenic strains include microbial competition for nutrients (Alabouvette *et al.*, 1993; Olivain *et al.*, 2006), competition for infection sites and root colonization. The selected non-pathogenic strains which have similar genetic backgrounds with the pathogenic strain but self-vegetative incompatible would have the priority in occupying the infection sites over other strains.

Since there is currently no known genetic marker to identify biocontrol strains, the only available and reliable method to screen for efficient strains is a bioassay in which the potential biocontrol agents are confronted with the pathogen in the

presence of the host-plant and disease incidence or severity are monitored. While very labour-intensive and time consuming, the advantage of such a bioassay is that it takes into account most of the possible interactions among micro-organisms, and between micro-organisms and the plant, that can lead to biocontrol. In general, the closer the screening method is to the production system, the greater the chances are for success (Larkin and Fravel, 1999). Most non-pathogenic *Fusaria* provide control that is at least slightly better than the conventional disease control treatments (Fravel, 2005). Thus, tests must be repeated several times under different environmental conditions (soil type, temperature, moisture), and on different cultivars to determine reliability of the biocontrol. The two strains in the study both showed slight effectiveness on *Fusarium* root rot control in the controlled environment on vanilla cuttings and seedlings.

Similarly, tests are needed to determine the inoculum density relationships between the pathogen and biocontrol agent. In addition to providing information on how much biocontrol agent is needed to achieve disease control, inoculum density tests often provide the first clues about the mechanism of action, because competitors usually need to be present in populations greater than that of the pathogen to achieve control while those that induce resistance can achieve control when present in populations lower than that of the pathogen.

The effects of increased inoculum levels from 10^6 to 10^7 or 10^8 conidia of non-pathogenic isolates have been compared, for a given density of the pathogen. The efficacy of control by non-pathogenic *F. oxysporum* isolates ML-5-2 and HK-5b-4-1 was related to their inoculum density. As the ratio of non-pathogenic *F. oxysporum* to pathogenic *F. oxysporum* was increased, the disease was better suppressed, but there were no significant differences on biocontrol effects between

conidia inocula of 10^7 and 10^8 . The addition of a higher dose of antagonist formulation may not have increased biocontrol activity, probably because of a decreasing amount of pathogen inactivation occurring per unit of biocontrol agent at the high densities.

Johnson (1994) had proposed that the amount of disease suppression obtained with a biocontrol agent depends on the density of the agent, the density of the pathogen, how efficiently individual units of the agents render units of the pathogen ineffective, and the proportion of the pathogen population that potentially is affected by the agent. Raaijmakers *et al.* (1995) also reported a threshold dose of antagonist inoculum below which large changes in disease suppression resulted from small decreases in antagonist dose. The high inoculum densities of the nonpathogen necessary to achieve biological control would be difficult to reach under agricultural production conditions, which is why, until now, the use of non-pathogenic *F. oxysporum* has been mostly limited to crops cultivated in potting mixtures or soil-less systems.

When the vanilla cutting or seedling root systems were immersed in inocula of non-pathogenic *F. oxysporum* isolates and the plants were grown in soil infested with pathogenic *F. oxysporum* f. sp. *vanillae* isolates DL-4-1, both non-pathogenic *F. oxysporum* isolates showed efficiency in controlling the disease; plants showed lower disease severity. These results agree with Chini *et al.* (2000), Postma and Rattink (1992) and Minuto *et al.* (1995), who reported that non-pathogenic *Fusarium* spp. isolates, introduced by root immersion before transplanting, were efficient in colonizing the rhizosphere and in controlling *Fusarium* wilt. There are reports of non-pathogenic *F. oxysporum* acting by competing for infection sites and for nutrients, and by induction of resistance (Mandee and Baker, 1991; Alabouvette and

Couteaudier, 1992; Larkin and Fravel, 1999; Benhamou *et al.*, 2001). In order to improve the efficiency of the biocontrol, it is necessary to identify the best method for applying the agent, e.g. by varying the depth of the application or by applying the non-pathogenic inoculum to a range of different soil types in which the vanilla is grown.

In the field experiments, the use of combined agents improved disease control over the non-pathogenic isolates applied alone, but the results were not consistent or stable. This is maybe because synergistic effects occur in the control of the disease with the biocontrol agents in combination (Whipps, 2001). Further research on the potential advantages of using combinations of these effective antagonists under varying stress conditions is needed.

5.4.2 Effect of the Dynamic Soil Environment on Root Rot Disease Control by the Biocontrol Method

Biocontrol of soil-borne diseases is particularly complex because these diseases occur in the dynamic environment at the interface of root and soil known as the rhizosphere, which is defined as the region surrounding a root that is affected by it. The rhizosphere is typified by rapid change, intense microbial activity and high populations of bacteria compared with non-rhizosphere soil (Fravel, 2005). Plants release metabolically active cells from their roots and deposit as much as 20% of the carbon allocated to roots in the rhizosphere, suggesting a highly evolved relationship between the plant and rhizosphere micro-organisms. Root exudates can attract pathogens, and pathogen is reduced by the increased numbers and types of micro-organisms around the root (Iqbal, *et al.*, 2005; Gilbert *et al.*, 1994). The rhizosphere is subject to dramatic changes on a short temporal scale-rain events and daytime drought can result in fluctuations in salt concentration, pH, osmotic potential, water potential and soil structure. Chitinolytic enzymes have been considered important role in the biological control of soil-borne pathogens because of their ability to degrade fungal cell walls, of which a major component is chitin (Hoitink and Fahy, 1986).

Over longer temporal scales, the rhizosphere can change due to root growth, interactions with other soil biota and weathering processes. It is the dynamic nature of the rhizosphere that makes it an interesting setting for the interactions that lead to disease and biocontrol of disease (Handelsman and Stabb, 1996). However, in controlled environments, the high value of the crops and the limited number of registered fungicides offer a unique niche for the biocontrol of plant diseases. In the future, biocontrol of diseases could predominate over chemical pesticides for crops

grown in glasshouses.

In order to succeed in the biocontrol of a soil-borne disease, the complexity of the root-soil interface must be accommodated, involving ultimately the study of entire communities, if we are to understand the essential interactions in field soil. The challenge in elucidating mechanisms of biocontrol is in reducing the complexity to address tractable scientific questions. One of the most effective approaches towards the identification of critical variables in a complex system has been the use of genetic analyses for the study of microbial populations in the field (Hatsch *et al.*, 2002; 2004; Hermosa *et al.*, 2000; Summerell *et al.*, 2003; Chiocchetti *et al.*, 1999; Edel *et al.*, 1996). Using these techniques, changes in specific populations, both pathogenic and non-pathogenic, can be monitored in the natural environment - measurements which have not been possible using conventional techniques, which could not distinguish between closely-related populations.

5.4.3 Commercial Development for Biocontrol Agents

For biocontrol methods to be implemented commercially on a practical level, it is necessary to understand more fully the ecology of these biocontrol agents and their interactions with the pathogen, host plant, and surrounding soil and rhizosphere microbial communities (Alabouvette *et al.*, 1998; Handelsman and Stabb, 1996; Larkin and Fravel, 2002; Fravel, 2005). Ideally, the antagonists must be ecologically fit to survive and function within the particular conditions of the crop ecosystem to which they are applied. Moreover, the antagonists must be present at adequate population levels and be capable of effectively interacting with the pathogen or host plant to provide acceptable disease control. Understanding the mechanisms of action involved in biocontrol processes is of primary importance in establishing these characteristics and can provide much insight into where and when the interaction occurs and how the pathogen will be affected. The mechanism of action will also determine, to a large degree, how the antagonist will need to be applied and managed, as well as suggesting potential strategies that may improve control (Larkin and Fravel, 1999).

Although the number of biocontrol products is increasing, these products still represent only about 1% of agricultural chemical sales. Yet these are important contributions because biocontrol agents offer disease management alternatives with different mechanisms of action to chemical pesticides. Trends in research include the increased use of bio-rational screening processes to identify micro-organisms with potential for biocontrol, increased testing under semi-commercial and commercial production conditions, increased emphasis on combining biocontrol strains with each other and with other control methods, integrating biocontrol into an overall system.

Our present understanding of soil and root associated microbial communities

and activities come at a time of growing concern at the extent of soil erosion and degradation in intensively managed agroecosystems around the world. These concerns and proposed solutions (e.g. raising organic matter content of soils under cultivation) were identified in resolutions passed following much debate among soil scientists at the 17th World Congress of Soil Sciences (WCSS) held in Bangkok, Thailand, in August 2002:

<http://www.iuss.org/Resolutions%2017th%20WCSS.htm>

While maintaining crop yield in countries with highly developed agriculture, long-term NPK fertilizer application is now seen as the prime cause for the elevated nutrient status of ground water supplies and eutrophication of water bodies. In developing countries, exhaustion of agricultural land is often compensated for through expansion into native grassland or forest ecosystems. Non-sustainable slash-and-burn cropping regimes further exacerbate the problems of soil degradation and habitat loss. These crop production regimes adversely affect diversity and functioning of beneficial soil- and root-associated microbiota. The resulting loss of soil structure leads to reduced water and nutrient carrying capacity that has obvious negative ramifications for soil fertility and maintenance of plant production.

A greater appreciation of the importance of root-associated microbes in maintaining soil structure has come from studies identifying significant quantities of a glycoprotein, glomalin, produced by root symbiotic arbuscular mycorrhizal fungi in stable, well-structured field and native forest soils (Aguilar and Barea, 1996; Green *et al.*, 1999; Van Elsas *et al.*, 2000). Smith (2002) stressed the need to better integrate information on root and soil microbe distribution dynamics and activities with known spatial and physicochemical properties of soils. This should be achieved through greater collaborative efforts between biologists, soil chemists and physicists. Such

collaboration will be essential if the full potential of biocontrol methods is to be realised.

5.4.4 Future Research

The use of non-pathogenic strains of *F. oxysporum* to control *Fusarium* wilt has been reported for many crops including: basil (Fravel and Larkin, 2002), carnation (Postma *et al.*, 1996), cucumber (Mandeel and Baker, 1991), cyclamen (Minuto *et al.*, 1995), flax (Alabouvette *et al.*, 1993), melon (Rouxel *et al.*, 1979), tomato (Lemanceau and Alabouvette, 1991) and watermelon (Larkin *et al.*, 1996). Among these papers, only a few presented data that were obtained under commercial production conditions. The main reason for the low adoption of biological control is the lack of consistent performance data on the application of non-pathogenic strains under production conditions.

Cook *et al.* (1996) list several areas of concern for the development of biocontrol methods: pathogenicity to non-target organisms; extrapolation of experimental studies into production systems; lack of information on mechanisms of action; the need for risk assessment; genetic improvement of biocontrol agents and displacement of non-target micro-organisms. They point out however that, based on available experience, any adverse effects from biocontrol are likely to be short-term and can be eliminated by terminating use of the biocontrol agent. By contrast, many of the conventional crop protection strategies in agriculture have produced significant long-term adverse affects.

Olivain *et al.* (1997) compared the early physiological responses of flax cells challenged with conidia of strains of pathogenic and non-pathogenic *Fusarium oxysporum*. The results observed with the pathogenic strain were typical of those described in the case of the compatible reaction and results observed with Fo47 were similar to those observed in the case of the incompatible reaction when a pathogen is interacting with a resistant cultivar. The non-pathogenic strain elicits early defence

reactions restricting its growth into the root. These preliminary results open the way to new research in the field of the plant–microbial interactions leading to the identification of the biochemical pathways triggered by the non-pathogenic strains. It is conceivable that understanding what is triggered in the induced resistance discussed above may lead to being able to trigger this in the absence of a biocontrol agent, thus avoiding the problems inherent in production, formulation and delivery of a living agent.

Gullino *et al.* (1995) pointed out the need for data on which to base informed decisions about the risk involved in releasing non-pathogenic *F. oxysporum*. They found release of non-pathogenic *F. oxysporum* to have negligible non-target effects with respect to persistence and survival, effect on indigenous microbial communities, and genetic stability and transfer. Because some *Fusaria* produce mycotoxins, it is important to establish that those used as biocontrol agents do not produce toxins. With *F. oxysporum* in particular, the concern arises as to whether the biocontrol agent is widely non-pathogenic, or whether it may be pathogenic on a species of plant on which it has not yet been tested. Further, given the lack of understanding about how new races of the pathogen arise, there is some concern that the biocontrol agent could become pathogenic. Further research is required here.

In addition to field studies integrating biological control into commercial production systems, genetic data are needed to allay these fears. But progress in the development of non-pathogenic *Fusaria* as biological control agents needs a co-operative research effort in all these fields from basic understanding of the mechanisms to practical field studies of the environmental conditions influencing the efficacy of the control. A more thorough understanding of the genetics, biology and ecology of biocontrol agents and their modes of action will enable optimal

exploitation of these fungi for disease control (Delgado-Jarana *et al.*, 2005; Deng *et al.*, 2006). These issues are interrelated since molecular techniques can provide insights in each of these areas. Molecular genetics offers new tools for unraveling mechanisms and understanding genetic relationships among *Fusaria* (Zhu-Salzman, *et al.*, 2005). For example, mutants of Fo47 showing either an increased or a decreased biocontrol capacity have been identified (Larkin *et al.*, 1999). More research using molecular genetics is needed to determine the genetic relationships among pathogenic, biocontrol and saprophytic *Fusaria*, as well as to elucidate the genetic determinants of pathogenicity and biological control ability. Identification of genes involved in biological control should assist in making the method more consistent and in optimization of control. Identification of genes involved in biological control may also facilitate screening for new biocontrol agents or genetic improvement of current agents.

CHAPTER 6

DISCUSSION AND CONCLUSIONS

6.1 Biocontrol Agents Used in Vanilla Disease Control (soil-borne disease control)

Soil-borne plant pathogens causing root and crown rots, wilts and damping-off are major yield-limiting factors in the production of food, fibre and ornamental crops (Weller *et al.*, 2002). Most soil-borne pathogens are difficult to control by conventional strategies, such as the use of resistant host cultivars and synthetic fungicides. The lack of reliable chemical controls, the occurrence of fungicide resistance in pathogens and the breakdown or circumvention of host resistance by pathogen populations are among the key factors underlying efforts to develop other control measures (Weller *et al.*, 2002). The search for alternative strategies also has been stimulated by public concerns about the adverse effects of soil fumigants such as methyl bromide on the environment and human health.

It has been demonstrated that natural disease-suppressive soils probably are the best examples of indigenous microflora effectively protecting plants against soil-borne pathogens, including *Fusarium oxysporum* (Alabouvette *et al.*, 1993). Suppressive soils initially become apparent because the incidence or severity of disease is lower than expected for the prevailing environment or as compared to that in surrounding soils. Biocontrol involves harnessing such disease-suppressive micro-organisms to improve plant health. In this study, two non-pathogenic *Fusarium oxysporum* strains have been isolated from vanilla root rot disease-infested areas, which, to some extent, have been effective in reducing disease incidence. Possibly they were isolated from suppressive soils, although it is not known in all cases where the samples were taken from - soil or soil containing fragments of vanilla plants. In future work it will be very important to differentiate the precise source of isolates.

Disease suppression by biocontrol agents is the sustained manifestation of

interactions among the plant, the pathogen, the biocontrol agent, the microbial community on and around the plant and the physical environment (Handelsman and Stabb, 1996). Even in model laboratory systems, the study of biocontrol involves interactions among a minimum of three organisms. Therefore, despite its potential in agricultural applications, biocontrol is one of the most poorly understood areas of plant-microbe interactions (Handelsman and Stabb, 1996). The complexity of these systems has influenced the acceptance of biocontrol as a means of controlling plant diseases in two ways.

Firstly, practical results with biocontrol have been variable. Thus, despite some stunning successes with biocontrol agents in agriculture, there remains a general scepticism born of past failures (Cook and Baker, 1983; Weller, 1988). Secondly, progress in understanding an entire system has been slow. Biocontrol of soil-borne diseases is particularly complex because these diseases occur in the dynamic environment at the interface of root and soil, known as the rhizosphere. The rhizosphere is typified by rapid change, intense microbial activity and high populations of bacteria compared with non-rhizosphere soil (Shim and Lee, 1990). Plants release metabolically active cells from their roots and deposit as much as 20% of the carbon allocated to roots in the rhizosphere, suggesting a highly evolved relationship between the plant and rhizosphere micro-organisms (Siddiqui, 2006). The rhizosphere is subject to dramatic changes on a short temporal scale, rain events and daytime drought can result in fluctuations in salt concentration, pH, osmotic potential, water potential and soil structure. Over longer time scales, the rhizosphere can change due to root growth, interactions with other soil biota and weathering processes (Siddiqui, 2006). It is the dynamic nature of the rhizosphere that makes it an interesting setting for the interactions that lead to disease and biocontrol of disease

(as reviewed by Alabouvette *et al.*, 1993).

Moreover, much work has been done by introducing representative strains of different genera, generally increasing the level of soil suppressiveness in most cases (Mazzola, 2002; Anjaiah, 2003). However, introduction of large biocontrol populations is unlikely to reproduce the microbial community structure and interactions that occur naturally in suppressive soils. The complexity of the interactions involved in biocontrol and the wide range of environmental conditions found globally in agriculture make it unlikely that any one strain will suppress even a single disease in all settings. Hence, there is a need to seek new biocontrol strains, particularly strains adapted to the site where they will be used. The effort will continue to be labour-intensive and will not always be able to make use of existing knowledge of biocontrol mechanisms. In this study, biocontrol agent products, TAC, were used for investigation in the field which showed to be more effective than non-pathogenic fungal strains used alone (see also Section 6.6). This mechanism should be investigated. Possibly the intimate and complementary association between three different micro-organisms provided enhanced disease suppression mediated by intense competition for iron via siderophores produced by the *pseudomonads* in the mixture.

In the past, there has been some success in identifying diverse biocontrol strains that suppress plant pathogens (Alabouvette, 1999; Hermosa *et al.*, 2000). However, the limitation of these approaches is that widespread use of organisms that share a mechanism of biocontrol will increase the selection for resistance; therefore, this approach must be coupled with an understanding of the mechanism and frequency of resistance and of strategies to avoid resistance developing. Fluorescent strains have been identified from geographically and chemically diverse soils (Laville *et al.*, 1991;

Zuber *et al.*, 2003). Strains that produce the same antibiotic can be phenotypically diverse and may express additional traits useful under particular conditions. Some of these strains may provide effective control in certain soils in certain geographic regions or on particular crops. In addition, the genetic diversity of these strains may be exploited by combining them in mixed inoculants. Certain mixtures of fluorescent *pseudomonads*, or fluorescent *pseudomonads* and fungi, suppressed disease more effectively than did single strain inoculants (Park *et al.*, 1988; Duffy *et al.*, 1996).

6.2 Pathogenicity Testing

Difficulties in diagnosis are due to: (1) the ubiquitous presence of fungal pathogens that may lead to false positive test results and (2) difficulties in the evaluation of the aetiological significance of these pathogens. The relatively small number of effective antifungal agents reflects to a large extent the fact that many aspects of fungal physiology and virulence are poorly understood (Namiki *et al.*, 1998; 2001; Correll *et al.*, 1986a). The methods of molecular genetics provide effective tools for the diagnosis of mycoses and may also contribute to the identification of new targets for antifungal agents by genetic analyses of fungal virulence. Molecular genetic methods have been developed for strain identification (Chiocchetti *et al.*, 1999; 2001; Edel *et al.*, 1996; Hatsch *et al.*, 2002; 2004; Summerell *et al.*, 2003). This success indicates a general use of molecular genetics for the analysis of fungal pathogenesis.

Very little work has been performed on the analysis of *F. oxysporum* f.sp. *vanillae* and therefore comparisons of the results to other work on *F. oxysporum* f.sp. *vanillae* are difficult to make. However, extensive work has been performed on other formae specialis, for example, Vakalounakis and Fragkiadakis (1999) analysed VCG grouping in *F. oxysporum* obtained from cucumber. They found that of 34 isolates obtained from cucumber roots showing stem or root rot, 10 were non-pathogenic, while when Bao *et al.* (2002) analysed 43 *F. oxysporum* isolates, 23 were non-pathogenic to tomato plants while 20 were pathogenic. This variability in isolating either pathogenic or non-pathogenic isolates was also seen in the results presented here which probably indicate that little significance can be drawn from this observation. Such variation may even be influenced by the collection and culturing techniques used when preparing the isolates.

Lori *et al.* (2004) analysed the genetic diversity among 151 *F. oxysporum* f. sp. *dianthi* isolates obtained from soils and carnation plants in Argentina. They were characterized using vegetative compatibility group (VCG), intergenic spacer (IGS) typing, and pathogenicity tests on carnation. Twenty-two isolates were pathogenic on carnation, had the same IGS type, and belonged to a single VCG (0021). The 129 remaining isolates were non-pathogenic on carnation and sorted into 23 IGS types and 97 VCGs. Studies expanded to include both the pathogenic and the non-pathogenic diversity of *F. oxysporum* on a large scale might change current perceptions of *F. oxysporum* and may increase understanding of the origin of the pathogenic diversity in this complex fungal species. In this study, pathogenicity tests have been performed on 81 isolates from different plantations in Yunnan Province. Among these, 32 isolates were non-pathogenic and 49 were pathogenic. These results showed the variability and complexity of isolates in Yunnan.

6.3 VCGs Analysis

Puhalla (1985) found a correlation between the vegetative compatibility group (VCG) and formae speciales of *F. oxysporum*, i.e., members of a VCG belong to the same forma specialis. He suggested that when the sexual stage and meiotic recombination in *F. oxysporum* were lost in evolution, the loci that determine vegetative incompatibility and virulence became closely linked. Therefore, VCG analysis of populations of *F. oxysporum* has been used as a powerful tool to distinguish between non-pathogenic and pathogenic populations of the same host species (Puhalla, 1985), classify races (Correll, 1991) and to assess genetic homogeneity among populations (Katan *et al.*, 1996). Furthermore, VCG analysis has been used to estimate the virulence level of the isolates in *F. moniliforme* (Pasquali *et al.*, 2005) and *Fusarium oxysporum* f. sp. *Cucumerinum* (Ahn *et al.*, 1998). In this study, non-pathogens that were vegetatively compatible with the pathogen were not closely related to the pathogen based on standard molecular analyses. Thus, contrary to the finding by Puhalla (1985), non-pathogens and pathogens may share common alleles at vegetative compatibility loci by factors other than recent clonal derivation from a common ancestor.

Although extensive studies on VCG with races, formae speciales and virulence of *F. oxysporum* have been conducted (Robin *et al.*, 2000), no reports are available on *F. oxysporum* f. sp. *vanilla*. The objectives of this study were to investigate genetic diversity within the population of *F. oxysporum* f. sp. *vanilla* with respect to its vegetative compatibility and to determine the relationship between VCGs and virulence. The results showed that the strains of *Fusarium oxysporum* f.sp *vanillae* isolated from Yunnan Province were complex, could be distributed into 12 different VCGs and that a direct relationship between VCGs group and virulence could not be

drawn.

VCGs are well-suited for measuring genotypic diversity, e.g. the frequency of different genotypes within a population, and for determining if two strains are identical to one another (Vakalounakis and Fragkiadakis, 1999). However, the VCG technique is not a panacea for population analyses of pathogenic fungi. VCG analyses are not appropriate for determining if strains belong to different biological species nor for assessing differences that occur above the species level nor useful for assessing the levels of allele frequencies, e.g. those at different *vic* loci. Finally, the detection of heterokaryosis may be complicated by the presence of alleles that prevent formation of a heterokaryon, even if the component strains are vegetatively compatible, e.g. mutants at *heterokaryon self-incompatibility (hsi)* loci. When compared with some other multiloci techniques, such as DNA fingerprint probes, VCGs analyses require less technical equipment and laboratory sophistication, but may not be able to analyse enough samples and require more effort to interpret because the basic biological mechanisms underlying VCGs are poorly understood.

F. oxysporum (which reproduces asexually) tends to have smaller numbers of VCG groups with larger numbers of individuals within it (Kistler *et al.*, 1998). We identified 12 VCGs, which is comparable to those found in other formae specialis (Katan *et al.*, 1999; Kistler *et al.*, 1998). Most of the isolates appear within groups 0200 and 0201, therefore, this would indicate a high degree of clonality. The fact that the different VCG groups are identified across the diverse locations could also have implications as to how this disease originated in the Chinese plantations. One explanation for the rapid appearance of the wilt disease is that one type of pathogenic *F. oxysporum* gave rise to *F. oxysporum* f.sp *vanillae* and there is some evidence for this occurring although recent origins from a non-pathogen have not been recorded

(Gordon and Marlyn, 1997). Contrary to this, most new occurrences of *Fusarium* wilt are the result of introduction rather than local origins of the pathogen types. This was confirmed by the work of Vakalounakis *et al.* (2005) and in this study here. It is also apparent in this study that a common VCG was found in the isolates from different plantations in Yunnan Province. Greater insight into this would be possible by a more detailed molecular genetic analysis coupled to the existing VCG data. Analysis such as DNA sequencing, DNA fingerprinting, AFLP and RAPD should be done to further demonstrate the clonality and genetic variation within the VCG groups (Vakalounakis and Fragkiadakis, 1999; Belabid *et al.*, 2004; Kalc Wright *et al.*, 1996; Kerenyi *et al.*, 1997). Appel and Gordon (1994) sampled populations of pathogenic and non-pathogenic isolates of *F. oxysporum* from soils in Maryland and California. Isolates were assigned to VCGs and grouped according to restriction maps of mtDNA and the intergenic spacer (IGS) region of the nuclear rDNA. In general, VCGs were aligned with distinctive mtDNA and IGS haplotypes, but a few exceptions were noted. In one case, a pathogenic isolate in VCG 0131 shared the mtDNA and IGS haplotype characteristics of pathogenic isolates from VCG 0134. Several other examples showed that non pathogenic isolates could be vegetatively compatible with pathogenic isolates but differ in their IGS and/or mtDNA haplotype. Therefore, this correlation between VCGs and underlying genetic similarities among strains is not consistent. Non-pathogenic strains further characterized by mtDNA analysis would be a promising method for studying genetic variation among *F. oxysporum* strains.

6.4 Essential Oils Results and their Potential Use

The use of essential oils in disease control is expected to increase in the future because of the rise of biocontrol methods, which has stimulated the use and development of products derived from plants. International standardization of the composition of commercially available essential oils would, however, be essential for reliable applications. Scientists from divergent fields are investigating plants anew with an eye to their antimicrobial usefulness. A sense of urgency accompanies the search as the pace of species extinction continues (Cowan, 1999). This study found three essential oils that have inhibitory effects on *F. oxysporum* f.sp. *vanillae* *in vitro*. More of these types of compounds should be subjected to animal and human studies to determine their effectiveness in whole-organism systems, including in particular toxicity studies, as well as an examination of their effects on normal beneficial microbiota. It would be advantageous to standardize methods of extraction and *in vitro* testing so that the search could be more systematic and interpretation of results would also be facilitated. Also, alternative mechanisms of infection prevention and treatment should be included in initial activity screenings. Disruption of adhesion is one example of an anti-bacterial activity not commonly screened for currently. Attention to these issues could usher in a much needed new era of chemotherapeutic treatment of infection by using plant-derived components.

The soil-borne fungus under investigation here causes root rot by infecting plants through the roots and growing internally (Ruan *et al.*, 2000). As the roots become non-functional, the entire plant wilts and dies as the pathogen moves into the stem. Currently, pre-planting soil fumigation and fungicide applications are used to control soil-borne pathogens on high-value crops (Noling, 2002). However, because of the prohibition of the major fumigant, methyl bromide, the long-term

goal of the research here is to develop and evaluate new or existing alternative control methods for soil-borne plant pathogens to replace methyl bromide and fungicides in crop production systems. Biologically based and environmentally safe alternatives, such as biocontrol agents, natural plant products, and cultural methods, are being investigated for possible use as components in integrated management programmes.

Many plants and plant products have been reported to possess pathogen and pest control properties (Graver, 1994). In most reports, however, the efficacy of plant extracts and plant essential oils has been evaluated only *in vitro*, and efficacy data in soil are lacking. If natural plant products can reduce populations of soil-borne plant pathogens and control disease development, then these plant extracts have potential as environmentally sound alternatives to methyl bromide in integrated management programmes (Messenger *et al.*, 2000).

Several formulated botanical extracts were shown here to effectively reduce soil populations of *F. oxysporum* and increase symptomless plant stand in controlled experiments. The experiments in this study were conducted under controlled conditions. In future it will be necessary to determine if these essential oils are also efficacious when applied to field conditions. These products are or were being developed for foliar application or other uses, and little or no data exist for their activity in soil. As such, a range of dilutions of the various formulations can be prepared for soil treatment and attempts made to optimize the conditions for decreasing pathogen survival in the laboratory and then reducing disease development when applied in the greenhouse. Under these conditions, this study was able to determine with some confidence if any of the essential oils were effective and deserving of further research. This *in situ* approach would be closer to

production conditions than *in vitro* assays. Based on the results here, several extracts, especially clove, cinnamon and citronella or their combinations, warrant further investigation. It is unlikely that these products by themselves in their current formulations can replace methyl bromide or other fumigants and pesticides in all situations. However, these essential oils may develop into useful components of different management strategies. For the future, several parameters need to be developed in order to scale up to field conditions, such as delivery method, appropriate formulation for delivery and soil type, rate, cultural practices and the economic factors involved. Consideration will need to be given to the mechanism of the interaction of the product with the pathogen population and the host plant.

6.5 Non-pathogenic Strains and Their Potential as Biocontrol Agents

Non-pathogenic isolates of *F. oxysporum* are the main candidates for biological control of *Fusarium* wilts and root rots (Alabouvette *et al.*, 1993), but a great diversity exists among the natural populations of these micro-organisms in relation to their ability to control the diseases. As little or no published work relates specifically to root rot in vanilla, most discussion must be based on the closely-related studies on vascular wilts and rots in general. As discussed in Section 6.1, one step to solve is the isolation and screening of effective strains of non-pathogenic *F. oxysporum* and fluorescent *pseudomonads*. Among a large collection of bacteria, fungi and actinomycetes isolated from the suppressive soil, only non-pathogenic *F. oxysporum* isolates consistently suppressed the disease in both microwave-treated and natural soil, emphasizing that a highly specific *F. oxysporum* interaction is required to combat pathogenic events. Induced systemic resistance was the primary mode of action for several isolates (Fuchs, 1997; 1999; He, 2002; Brunner, 2005), but it is not yet clear if the mechanism is similar to that described for induced systemic resistance by rhizobacteria. Early work by Alabouvette and co-workers (1993) and later work by Larkin and Fravel (1998) clearly indicated that strains of non-pathogenic *F. oxysporum* differ considerably in their efficacy against *Fusarium* wilt. Other researchers who have also studied non-pathogenic strains as biocontrol agents suggested that the composition of non-pathogenic *F. oxysporum* populations remained relatively stable over a considerable period of time (Van Elsas *et al.*, 2000; Cook, 1993), consistent with the known long-standing nature of the suppressiveness of the soils.

Another issue is whether or not, after inoculation, the non-pathogenic strain is capable of penetrating intact host tissues and recolonising the host internally, which

is important for their suitability as biological control agents. Successful colonization is necessary for efficient biological control of the disease. Using GUS-transformed strains, Olivain and Alabouvette (1999) clearly showed that both a pathogenic and a non-pathogenic strain were able to actively colonise the surface of the tomato root.

Moreover, in order to improve the efficacy of non-pathogenic strains, the levels of non-pathogenic strains in the soil were important (Fravel, 2005; Alabouvette, 1999). The proportion of the root colonised and the efficacy of biological control depend on the inoculum ratio of the pathogenic and the non-pathogenic strains. Lemanceau (1993) showed that the antagonism of non-pathogenic *F. oxysporum* against pathogenic *F. oxysporum* strongly depended on the ratio of non-pathogenic to pathogenic *F. oxysporum* densities: the higher this ratio, the stronger the antagonism. Alabouvette (1995) also studied the ratio of the non-pathogenic strains and the pathogenic strains. When the ratio was 10:1, the effect of the non-pathogenic strains was better than the ratios of 1:1 and 100:1. Larkin and Fravel (1999) used this dose-response relationship to characterize the mechanisms of action by non-pathogenic strains. In this study, when the ratio of the non-pathogenic strains and the pathogenic strains was increased to 100:1, the efficacy of controlling vanilla root rot disease also increased.

There is a hypothesis that specific infection sites exist on the root for *F. oxysporum*. Mandeel and Baker (1991) postulated that the root surface had a finite number of infection sites that could be protected by increasing the inoculum density of the non-pathogenic strain. However, Olivain *et al.* (2006) used confocal laser scanning microscopy to visualize the colonization of tomato roots in heat-treated soil, to observe the interactions between a non-pathogenic strain and a pathogenic strain inoculated onto tomato roots in soil. When inoculated separately, both fungi

colonized the entire root surface, with the exception of the apical zone. When both strains were introduced together, they both colonized the root surface and were observed at the same locations. When the non-pathogenic strain was introduced at a higher concentration than the pathogenic strain, it colonized much of the root surface, but hyphae of the pathogenic strain could still be observed at the same location on the root. These results are not consistent with the hypothesis that specific infection sites exist on the root for *F. oxysporum* and instead support the hypothesis that competition occurs for nutrients rather than for infection sites.

Non-pathogenic *F. oxysporum* isolates have been shown to be efficient in reducing the severity of the disease and maintaining normal plant development (Silva and Bettiol, 2005). In this study, two non-pathogenic strains, ML-5-2 and HK-5b-4-1 were efficient in vanilla root rot control. These results agree with Postma and Rattink (1992), and Minuto *et al.* (1995), who reported that non-pathogenic *Fusarium* spp. isolates, introduced by root immersion before transplanting, were efficient in colonizing the rhizosphere and in controlling *Fusarium* wilt. These results all provide evidence of the antagonistic activity of non-pathogenic *F. oxysporum* isolates in controlling vascular wilt.

In order to enhance the effectiveness to control vascular wilt caused by *F. oxysporum* with non-pathogenic *F. oxysporum*, it is necessary to study the best methods for applying the non-pathogenic strain, i.e., by enhancing the ability of large quantities of non-pathogen inoculum to survive unfavourable environmental conditions and to efficiently disperse along the root. This raises the question, which kind of method would be useful for disease control? Inoculating the non-pathogenic *F. oxysporum* strain deep in the soil or applying on the surface? Silva and Bettiol (2005) evaluated the efficiency of non-pathogenic isolates in controlling vascular

wilt caused by *F. oxysporum* f. sp. *lycopersici* in tomato (*Lycopersicon esculentum*) seedling of cv. Viradoro. In order to determine the effect of non-pathogenic *F. oxysporum* isolates in tomato plants and the efficiency of the non-pathogenic *F. oxysporum* isolates in controlling *Fusarium* wilt, the root system of 30-day-old seedlings was immersed in conidial suspensions of each isolate and the seedlings were transplanted to a cultivation substrate. For comparison tomato seedling roots were also immersed in a conidial suspension of each isolate and then transplanted into substrates previously infested with isolates of *F. oxysporum* f.sp. *lycopersici*. Thirty-five days after transplanting it was observed that the non-pathogenic *F. oxysporum* isolates were not pathogenic to the cv. Viradoro nor did they affect seedling development. The non-pathogenic *F. oxysporum* isolates were efficient in reducing the severity of the disease and maintaining normal plant development. These results provide evidence of the antagonistic activity of non-pathogenic *F. oxysporum* isolates in controlling vascular wilt caused by *F. oxysporum* f. sp. *lycopersici* in tomato and indicate a mechanism that occupation of root sites by non-pathogenic isolates might be critical in providing protective action against pathogenic strains.

Rodríguez *et al.* (2006) evaluated the antagonistic activity of *Fusarium oxysporum* S6 non-pathogenic fungal strain against the phytopathogenic fungus *Sclerotinia sclerotiorum*. *F. oxysporum* (S6) is a good candidate for the biocontrol of *S. sclerotiorum* in soybean. The metabolite responsible for the activity was isolated by chromatographic techniques, purified and identified by spectroscopic methods as cyclosporine A. The antifungal activity against the pathogen was correlated with the presence of this metabolite by a dilution assay and then quantified. Cyclosporine A caused both growth inhibition and suppression of sclerotia formation. In a

greenhouse assay, a significant increase in the number of surviving soybean plants was observed when *S. sclerotiorum* and *F. oxysporum* (S6) were inoculated together when compared with plants inoculated with *S. sclerotiorum* alone. *Fusarium oxysporum* (S6) may be a good fungal biological control agent for *S. sclerotiorum* and cyclosporine A is the responsible metabolite involved in its antagonistic activity *in vitro*.

6.6 Integrated Strategies or Combined Methods for Vanilla Disease Control

An important result observed in this study was that a combination of three different micro-organisms plus chemicals (TAC) was more effective for control of vanilla root rot disease than non-pathogenic strains used alone. This suggests that an integrated strategy for the control of *F. oxysporum* should be developed that includes using a weather-based disease forecasting system and soil drenches of a chemical fungicide. Biocontrol agents have been applied with reduced rates of chemical fungicides or bactericides for control of disease in the field (Fravel, 2005). The use of a chemical fungicide may have to be considered under expected high disease pressure, with slow or no disease progress application of biocontrol agents is more suitable. Martin and Bull (2002) concluded that while single microbial inoculants provided some control for specific diseases of strawberry, they could not currently provide the broad-spectrum control needed to replace methyl bromide. They further indicated that cost-effective biocontrol strategies will probably require development of an integrated systems approach. The authors also pointed out that the longer-term goal of complete methyl bromide replacement requires focusing on more sustainable systems that incorporate cultural practices, monitoring host resistance and biological approaches. It is suggested here that the same concept should be used in the control of vanilla disease.

The application of biological control organisms in combinations has often yielded better results than when the organisms have been applied individually (Nel *et al.*, 2006a,b; Herva's, 1998). For instance, the combination of non-pathogenic *F. oxysporum* and *Pseudomonas* spp. enhanced disease suppression of *Fusarium* wilt of carnation (Lemanceau *et al.*, 1992), and pseudobactin 358 production by *Pseudomonas putid* increased the intensity of the antagonism of non-pathogenic *F.*

oxysporum against pathogenic *F. oxysporum*. Park *et al.* (1988) showed that a combined application of fluorescent *P. putida* and a non-pathogenic *F. oxysporum* strain was effective in suppression of *Fusarium* wilt of cucumber, although each was ineffective when applied separately. Combining fluorescent *Pseudomonas* with an effective non-pathogenic *Fusarium* strain has been shown to give better and more consistent protection against crown rot disease of tomato (Alabouvette *et al.*, 1993). De Boer *et al.* (2005) showed that the combination of two compatible *Pseudomonas* spp. resulted in better disease suppression of *Fusarium* wilt of radish than a single strain.

Although biocontrol agents were combined in this study, the combinations only consisted of either *Trichoderma* or mycorrhizae and it would be interesting to try other combinations. The use of biocontrol agents as a component of an integrated disease management programme depends on many factors, both biotic and abiotic, including consistency of performance of the individual biological control agents (Weller, 1988; Fravel *et al.*, 2003; Ros *et al.*, 2005). Elmer (2004) studied the abilities of non-pathogenic strains of *Fusarium oxysporum* and rock salt (NaCl) to suppress *Fusarium* crown and root rot of asparagus in replanted fields. NaCl can stimulate asparagus growth, suppress *Fusarium* crown and root rot and reduce slug damage. Furthermore, applications of NaCl have benefited asparagus that was cropped in replanted fields. It is most beneficial when plantings are under stress from drought or disease (reviewed by Elmer, 2004). However, greenhouse studies have also shown that applications of NaCl could reduce the number of colonies of *Fusarium* spp. in the root. Hence, it becomes important to understand whether or not combining NaCl with non-pathogenic strains of *F. oxysporum* will promote the re-establishment of asparagus in the field or if they will adversely affect each other

and the application of NaCl did not hinder the suppressive ability of the non-pathogenic strains (reviewed by Elmer, 2004). Consistent biocontrol traits of non-pathogenic strains would be needed for the asparagus disease control.

Mixtures of compatible biocontrol agents are an ecologically sound approach to biocontrol of soil-borne diseases, especially when used in combination with partial resistance. Mixtures of different species of micro-organisms may result in better plant colonization and better adapted to environmental changes. It may present a large number of pathogen suppressive mechanisms, and may protect against a broad range of pathogens (Duffy *et al.*, 1996). However, several areas need improvement if the maximum potential of mixtures of micro-organisms is to be attained. These include improved formulations, more efficacious strains, and appropriate adjuvants for biocontrol agents. Better delivery systems also are needed as well as more information about specific cultivar responses to the presence of beneficial micro-organisms.

6.7 Limitations and Prospects for Further work

As described previously in this study, many factors affect the implementation of biocontrol agents on controlling vanilla root rot disease.

1) Stability of the two non-pathogenic strains of *F. oxysporum* is variable even after one generation; it is an open question whether they still have the vegetative incompatible ability with other strains when put into effect in the field.

2) The results for pathogenicity testing were not consistent, although conducted three times, making it difficult to consistently deduce the relationship between VCGs and virulence.

3) The growth of the vanilla plants is time-consuming, prolonging the time of experiments and increasing the difficulties of the study. Greater variation occurs as the experimental period lengthens.

4) Because of the complexity of biocontrol agents when being used for disease control in the field, it has to be anticipated that results in the field may be different to those found in controlled environment experiments.

In future work, the mechanisms of action should be investigated for the non-pathogenic strains. Perhaps the intimate and complementary association between the micro-organisms provided enhanced disease suppression, mediated by competition for iron via siderophores produced by the *pseudomonads* in combination. One possible alternative hypothesis is that the suppression of disease by the non-pathogenic *Fusarium* strain is related to reductions in both population density and metabolic activity of the pathogen on the root surface simply through competition for iron; there may be further contributions to this suppression by *Pseudomonas*, thereby enhancing the biological activity of the non-pathogenic *F. oxysporum* strain. It has been reported that iron nutrition of plants in calcareous soils

can be effectively regulated by the application of iron chelated by natural or synthetic water-soluble chelating agents (Crowley *et al.*, 1991). The effectiveness of the chelates used was in the order of their capacity to maintain iron in soluble form in the soil solution. Thus, chelation of iron in the soil can be used to increase competition for iron.

Successful biocontrol of plant disease requires an intricate array of interactions. Understanding these interactions at the molecular and ecological levels will make possible the rational development of biocontrol strategies for agriculture (Chini *et al.*, 2000). Application of genetic analysis to micro-organisms involved in biocontrol has led to substantial progress in understanding the microbial metabolites and regulatory genes involved in biocontrol. Ecological analyses have begun to describe the responses of microbial communities to introduction of biocontrol agents (Gutterson *et al.*, 1986; Handelsman and Stabb, 1996). The integrated use of genetic, molecular and ecological approaches will form the basis for significant future advances in biocontrol research. In particular, additional effort in three particular areas, detailed below, will be essential for developing a more complete understanding of biocontrol and making more practical use of biocontrol strategies for agriculture (Weller *et al.*, 2002; Handelsman and Stabb, 1996).

Firstly, understanding the mechanisms of pathogen resistance to the action of biocontrol agents is critical to sustain disease suppression with long-term use. Strategies to minimize resistance and prevent its spread should be designed.

The second area that is ripe for study is examining the genetic diversity within species of both biocontrol agents and the host plant. Exploitation of genetic variation among members of a microbial species that suppresses disease may provide a solution to the intrinsic variability across space and time that has been observed with

many biocontrol agents (Edel *et al.*, 2001). The genetics of the host should be exploited for supportiveness of biocontrol, and hospitality to biocontrol agents should be enhanced through directed breeding or genetic modification of the host plant.

The third and most challenging area of research needed to explain the biological context for biocontrol is microbial community ecology (Handelsman and Stabb, 1996). A better understanding of the microbial interactions that enhance or detract from biocontrol will determine the long-term success of biocontrol. In particular, attention needs to be paid to non-culturable members of the root-associated and soil communities because these micro-organisms may be numerically dominant and have not yet been studied. Molecular methods developed for the study of micro-organisms in their natural environment are key tools for the study of the influences of the microbial community on biocontrol (Attitalla *et al.*, 2004; Pantou and Typas, 2005).

6.8 Conclusions

From the results of this study, four main conclusions can be drawn:

First, pathogenicity testing was undertaken using 81 isolates of *F. oxysporum* f.sp. *vanillae*, 32 (36.8%) isolates were rated as non-pathogenic and 49 (56.3%) isolates were rated as pathogenic. From the Daluo plantation 17 isolates were recovered, of which 14 isolates were rated as pathogenic (82.35%) and three as non-pathogenic (17.65%); A total of 26 isolates were recovered from the Menglun plantation, of which 12 were pathogenic (46.15%) and 14 isolates were non-pathogenic (53.85%), in addition 18 isolates recovered from the Manjingdai plantation with 12 isolates being pathogenic (66.67%), whilst the other 6 isolates were non-pathogenic (33.33%). Finally, 20 isolates were obtained from the plantation in Hekou County, of which 11 isolates were pathogenic isolates (55%) and 9 were non-pathogenic (45%). The pathogenicity results emphasize the astonishing predominance of pathogenic isolates in Yunnan, presumably only developed since the introduction of the plant. There is no significant difference in the ratio of pathogenic and non-pathogenic isolates among three different plantations in the Xishuangbanna area.

Second, the genetic structure of the differences between the sites *F. oxysporum* isolates was analysed by VCGs. Among the isolates tested, one predominant VCG was found, containing 36 isolates and a second large VCG, containing 21 isolates. Two smaller VCGs were determined with one containing six isolates, and the other only two isolates. Following the numbering systems adopted by Puhalla (1985) and further standardized by Gordon (1999) and Kisterler *et al.* (1998), these VCGs were designated within 0200-02011. All the *nit* mutants generated from the other isolates were paired with the tester strains. From isolates MQ-sm19, ML-2d, DL-13b, only

the *nit3* phenotype could be produced and there was no evidence to identify which VCG they were in or whether they were self-incompatible. These results showed the isolates from Yunnan Province were complex in genetic diversity, but revealed no correlation between VCGs and virulence. In the same VCG group, there were different pathogenic type of strains and yet the same pathogenic type strains could be found in different VCGs. There is no correlation that can be drawn between pathogenicity and VCGs.

Third, in the investigation here, the activity of several essential oils, against *F. oxysporum* and of vanilla root rot disease *in vivo* was determined. The results showed all seven oils - cinnamon, camphor, clove, citronella, tea tree, orange and mint oils, demonstrated the ability to inhibit spore germination of *F. oxysporum* isolates with different degrees of effectiveness. The essential oil responsible for the greatest inhibition of the spore germination of all three isolates tested was cinnamon oil, followed by clove oil, citronella oil and camphor oil. Cinnamon oil showed 100% inhibition of germination when the concentration was 200 ppm. Even at a low concentration of 100 ppm, it gave a pronounced inhibition. Three other essential oils (clove, citronella and camphor) were also effective and all showed that an increase in concentration leads to increased inhibition. This is an important finding that deserves much further work to bring it to a fruitful conclusion.

Fourthly, as to the evaluation of the effects of non-pathogenic strains on vanilla root rot disease control in growth controlled environments and in the field experiment, two effective non-pathogenic isolates ML-5-2 and HK-5b-4-1 were screened and selected from 81 strains as candidate biocontrol agents. These two strains were non-pathogenic, self-incompatible and genetically related to other strains isolated from the Yunnan Province. Again this is a very important finding,

whose particular significance must be exploited to the full in future work. In experiments to determine the effect of the two non-pathogenic strains on controlling vanilla root rot disease, using both vanilla cuttings and vanilla seedlings, it was found that both of these non-pathogenic strains were able to reduce the progress of disease symptoms to some extent. This effect was found on both vanilla cuttings and vanilla seedlings for both isolates.

6.9 Future Development of Biocontrol Methods and Perspectives

The goal of biocontrol research is to provide additional tools for disease management. Biocontrol can be used in situations where currently no chemical control is available, where conventional pesticides cannot be used due to re-entry or residue concerns, or where the product must be certified organic.

Although the number of biocontrol products is increasing, these products still represent only about 1% of agricultural chemical sales. For comparison, fungicides represent approximately 15% of pesticide sales (Fravel, 2005). Yet 10% is important because biocontrol agents offer disease management alternatives with different mechanisms of action to chemical pesticides. Increasingly research is being performed to identify micro-organisms with potential for biocontrol. There is increased emphasis on combining biocontrol strains with each other and with other control methods, so integrating biocontrol into an overall system.

Biocontrol of soil-borne pathogens traditionally has been studied as a three-way interaction between the pathogen, the host and the biocontrol agent, with little attention to other interspecies interactions in the rhizosphere. To fully understand the nature of soil suppressiveness, however, the ecological context within which the major players act also must be considered (Weller *et al.*, 2002; Bainton *et al.*, 2004). With specific suppressiveness, one or a few groups of micro-organisms may be responsible for biocontrol activity, but interactions with other members of the rhizosphere community can significantly modulate the degree of suppressiveness observed (Gilbert *et al.*, 1994). Indeed, there are probably hundreds, if not thousands, of genotypically distinct microbial species inhabiting the rhizosphere of individual plants, and biotic as well as abiotic variables influencing both the structure and activities of rhizosphere populations including those of the pathogens and their

antagonists. A clear understanding of the biological factors responsible for soil suppressiveness may require broader knowledge of the identity, relative abundance, and biological activity of the phylogenetically diverse microbial populations that inhabit the rhizosphere.

From a scientific viewpoint, biocontrol is more likely to be successful in some pathosystems than others. Particular times in the cropping cycle with low biological diversity and at least some degree of environmental control are logical targets (Fravel, 2005). Classical concepts in control should also be remembered, such as determining when and where the pathogen is most vulnerable. Some reasons why biocontrol agents fail to become commercial products are that scientists outside industry often (a) overestimate the power of environmental concerns as economic drivers; (b) lack sufficient knowledge of grower needs, registration strategy and competitive forces; (c) have naive ideas about positioning and market strategy; and (d) underestimate registration costs and difficulties (reviewed by Fravel, 2005). Fravel (2005) adds that insufficient cost-performance and poor shelf life are other reasons why promising micro-organisms do not become products. The limit for development of the biocontrol agent is it is so expensive that it is impossible to clearly define what the product must do to be commercially acceptable.

Although many useful biocontrol agents were first identified through *in vitro* inhibition tests, several researchers have reported no correlation between *in vitro* inhibition tests and field performance of biocontrol agents (Fravel, 1988; 2005; Akköprü, 2005; Sen, 2003). In this study, similar problems were observed with the candidate biocontrol agents showing efficient control of vanilla root rot disease in controlled environments, while the results in the field were far more complex. Hence, the potential biocontrol agents should always be tested in the field in different

environments. They should be designed to be robustly tested in multiple locations and seasons and tested in locations naturally infested with pathogens.

Understanding how the biocontrol agent works can facilitate optimization of control, as well as helping to screen for more efficient strains of the agent. The mechanisms of some biocontrol agents are now understood in detail (Dekkers *et al.*, 1998; 2000; Mazzola, 2002; James *et al.*, 1993; Larkin and Fravel, 1999). Understanding of the role of ecology and microecology has also increased (Fravel, 2005). A more thorough understanding of ecology can help understand which problems to work on, how to approach them, when and where to apply the biocontrol agent, and how to predict situations in which biocontrol agents would not be expected to work.

Continued research in biocontrol is needed to contribute towards more sustainable agriculture and to ensure that alternatives are available if other management tools fail or are lost. The latest research into the root–microbe–soil interface has already, and will continue, to provide us with new tools to promote sustainable agricultural and forestry practices. Traditional management techniques have to be used but, in the future, will incorporate the new products of plant and microbial biotechnology research and development.

What has been provided here are exciting leads to follow from a combination of essential oils decreasing *Fusarium* pathogenicity on vanilla, plus the development of two non-pathogenic isolates that appear competitive against the causative organism and thirdly a biocontrol cocktail of agents that appears particularly potent. It is by incorporating these kinds of steady and promising advances into programmed strategies in field conditions that we can hope to overcome the ravages of the pathogen on the valuable vanilla plantations growing in China.

Production of agricultural crops dominates Yunnan's economy, in part because of its frontier location, difficult access to the rest of China, its mountainous topography, poor rural development, its rich natural biodiversity and the diversity of cultural minority groups that farm its slopes. It contains a great repository of agricultural knowledge, developed through long creativity and experimentation with crops by small holder farmers (Chen and Dao, 2001). Ultimately, the successful implementation of any new advance depends on the acceptability of that development to the farmers and the decisions they make in growing their crops. These decisions are taken in relation to a complex set of environmental and socioeconomic influences on the farmer. Final, practical assessments of biocontrol methods will be made within this context.

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APPENDIX

Media used in the study:

1. Potato sucrose agar (PSA) (per litre distilled H₂O):

Potatoes	200 g
Sucrose	20 g
Agar	20 g
2. Potato sucrose liquid medium (PS) (per litre distilled H₂O):

PSA without agar
3. Water agar medium (WA) (per litre distilled H₂O):

Agar	20g
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4. Capez's medium (per litre distilled H₂O):

NaNO ₃	2.00 g
K ₂ HPO ₄	1.00 g
KCL	0.50 g
MgSO ₄ .7H ₂ O	0.50 g
FeSO ₄	0.01 g
Sacrose	30 g
5. Murashige and Skoog (MS) basal medium:

NH ₄ NO ₃	1.601
KNO ₃	2.022
CaCl ₂ .2H ₂ O	0.441
KH ₂ PO ₄	0.204
MgSO ₄ .7H ₂ O	0.370
MnSO ₄ .4H ₂ O	0.022
FeNa.EDTA	0.038
H ₃ BO ₃	6.2mg
ZnSO ₄ .7H ₂ O	8.6mg
Na ₂ MoO ₄ .2H ₂ O	0.25mg
KI	0.83mg
CuSO ₄ .5H ₂ O	0.025mg
CoCl ₂ .6H ₂ O	0.24mg

6. Minimal medium (MM) (Puhalla 1985) (per litre distilled H₂O):

Sucrose	30 g
NaNO ₃	2 g
KH ₂ PO ₄	1 g
MgSO ₄ ·7H ₂ O	0.5 g
KCl	0.5 g
Trace elements solution	0.2 mL (Sterile)
Agar	20 g

7. Trace elements solution (per 95mL distilled H₂O):

Citric acid	5 g
ZnSO ₄ ·7H ₂ O	5 g
FeSO ₄ ·7H ₂ O	4.75 g
Fe(NH ₄) ₂ (SO ₄) · 6H ₂ O	1 g
CuSO ₄ ·5H ₂ O	250 mg
MnSO ₄ ·H ₂ O	50 mg
H ₃ BO ₃	50 mg
NaMoO ₄ ·2H ₂ O	50 mg

The pH was adjusted to 5.5 before autoclaving at 120⁰C for 20 minutes.

8. Three chlorate-containing (*nit*) media were used to recover nitrate-nonutilizing (*nit*) mutants:

a) MMC was prepared by adding the following to 1L of minimal medium:

KClO ₃	15-25 g
L-asparagine	1.6 g

b) KPS was prepared by adding 15-25g KClO₃ to PSA.

c) WAC was water agar medium plus 15-25g KClO₃.

9. Nitrite medium (NMM):

MM without NaNO₃ was amended with 0.5g/L NaNO₂.

10. Hypoxanthine medium (HMM):

MM without NaNO₃ was amended with 0.2g/L hypoxanthine.