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Shikha Srivastava
Honolulu, Hawai’i, USA.
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Abstract

Commercially grown orchids are among the most valuable ornamental crops produced in the United States. Hawaii is one of the three largest orchids-growing states in the nation, producing the greatest variety of tropical species and unique intergeneric hybrids. A decrease in orchid production has been observed in Hawaii in association with spot and blights of shoots and flowers, as well as root rot that result in rapid death of juveniles and slow decline of mature plants. Such decline is now commonly observed in nearly all nurseries across the Oahu and the Island of Hawaii. Fungal pathogens are speculated to be one of the important sources of diseases in orchids in Hawaii. In the present study 16 fungal genera were isolated from 60 plant samples collected from Oahu and the Island of Hawaii. *Fusarium* was the most prevalent genus, with 78% of the isolates and frequently associated with several orchid cultivars, including over 9 genera and hybrids. Eleven *Fusarium* species, namely *F. proliferatum*, *F. oxysporum*, *F. solani*, *F. subglutinans*, *F. poae*, *F. begoniae*, *F. bulbicola*, *F. anthropilum*, *F. denticulatum*, *F. circinatum* and *F. semitectum* were found. *Fusarium oxysporum* was identified and was the most commonly isolated species from all the samples followed by *F. proliferatum* and *F. solani*. All *Fusarium* species were tested for pathogenicity on four orchid genera. Among the 11 *Fusarium* species, 6 were found pathogenic on 4 orchid genera. Three species, *F. proliferatum*, *F. oxysporum* and *F. solani*, showed moderate to high virulence on *Dendrobium*, *Cymbidium* and *Miltonia*. *Fusarium circinatum* and *F. poae* were low to moderate virulent on *Dendrobium* and *Cymbidium* whereas *F. begoniae* caused very low virulence on *Dendrobium* and *Miltonia*. *Cattleya* was susceptible to *F. oxysporum* only. Pathogenicity assays of *Fusarium* isolates on different cultivars of orchids resulted in identification of pathogenic *Fusarium* species was
followed by testing efficacy of three fungicides, Pyraclostrobin, a.i. at 25%, Azoxystrobin a.i. at 50% and Triticonazole, a.i. at 20% on mycelial inhibition on four most prevalent *Fusarium* species namely- *F. proliferatum*, *F. oxysporum*, *F. solani* and *F. subglutinans*. Triticonazole was the most effective fungicide in the *in vitro* tests and was further evaluated for its efficacy in on *Dendrobium* plants *in vivo* against the most aggressive species of *Fusarium*, *F. proliferatum* under greenhouse conditions. This dissertation provides a thorough study to identify pathogenic *Fusarium* species on orchids and provides information regarding pathogenicity on orchid genera other than *Dendrobium*, and viable chemical methods to manage *Fusarium* species on orchids in Hawaii.
CHAPTER 1
Characterization and Management of Different \textit{Fusarium} Species Associated with Orchids Cultivated in Hawai‘i

1. Introduction

Orchids are among the most beautiful, delicate, and exotic ornamental flowers. They belong to the Orchideaceae which is the largest flowering plant family, consisting of about 700 genera with over 20,000 species worldwide (Staples and Herbst, 2005). Due to high degree of genetic compatibility among different genera and species, hybrids of orchids can be created which increases the availability of different varieties of flowers. The first artificial orchid hybrid flowered in 1856 in England by John Dominy, called \textit{Calanthe dominii}, which resulted from crossing \textit{Calanthe masuca} with the pollen of \textit{Calanthe furcata} (Veitch, 1906). Since then the number of hybrids registered has grown to approximately 100,000 (Pridgeon, 2006). Orchids are relatively slow-growing, long term perennial crops, which require two to ten years to attain reproductive maturity. Depending on the type of orchid, mature plants may flower continuously, or only once or twice a year. Flowers are either born in sprays of long inflorescences, as floral clusters, or as single flowers which can last from one day to more than ten weeks (Hew \textit{et al.} 1997; Kumaria, 2001). Different genera of orchids are bred for their unique blossom characteristics, including color, pattern, size, scent, and shape, and for their spray characteristics, such as length, longevity, and flower number that make them marketable (Lenz, 1988; Kamemoto, 1999). Plants are either grown for cut flower sales or for retail as ornamental potted plants (Hew \textit{et al}. 1997). Commercially grown tropical orchids include species of \textit{Vanda}, \textit{Cattleya}, \textit{Dendrobium}, \textit{Oncidium}, and \textit{Papilionanthe} (Withner, 1988; Hew \textit{et al}. 1997). Temperate commercial orchids include species of \textit{Cymbidium}, \textit{Spathoglottis}, \textit{Dendrobium},

Orchids are monocots that are long-lived herbaceous plants with two growth forms, either a sympodial growth, with a terminal shoot that produces new growth from the old shoot base, or monopodial growth, with a single growing point on a non-terminal shoot (Hew *et al.*, 1997; Staples and Herbst, 2005). Orchids are also classified into two growth types as epiphytic and terrestrial. Epiphytes are those that spend most of their lives growing on other plants absorbing moisture and nutrients from the atmosphere, bark, moss, or plant debris. Terrestrial orchids on the other hand spend most of their lives rooted in the soil. There is one another type of orchids known as Lithophytes, which is actually a subset of epiphytes, this type lives on thin layers of detritus on rocks.

Evolutionarily, orchids are among the most advanced group of monocotyledonous plants with different adaptive mechanisms. One of the most developed adaptations includes the highly modified floral structures, related to specific insect pollinators. Other adaptations include water uptake and storage, and associations with specific fungi for their nutritional requirements, including those of seed germination. For water uptake and storage, orchid roots consists of a spongy hydrophilic type, or many layers of cells called the velamen, whose primary function is to capture and hold water, increase nutrition absorption and reduce water loss from the cortex (Pridgeon, 2006).

Orchids can grow in every possible habitat worldwide except the oceans. The majority of orchids grow naturally in the tropics (Burnett, 1985; Kumaria, 2001; Raabe, 1981). Due to the tremendous variability in geography and tropical climate of the Hawaiian Islands, this location offers ideal places for commercial orchid cultivation. It is interesting to note that, although the
tropical climate in Hawaii is ideal for many orchids, native or endemic orchids are extremely rare in the Islands. There are only three endemic and four naturalized species of orchids found in the state: Endemic species of orchids are: *Anoectochilus sandwicensis* Lindl., *Liparis hawaiensis* H. Mann, and *Platanthera holochila* (Hillebr.) Kraenzl. While the four naturalized species of orchids are: *Arundina graminifolia* (D. Don) Hochr or Bamboo orchid, *Epidendrum × obrienianum* Rolfe. or Scarlet butterfly or baby orchid, *Phaius tankervilleae* (L'Heritier) Blume or Chinese ground orchid, nun's orchid, or nun's-hood and *Spathoglottis plicata* Blume or Malayan or Philippine ground orchid (Wagner et al., 1999; Wood, 2006). Orchids are an important ornamental crop in many countries due to their unique and attractive flowers (Hew et al., 1997).

1.1 Orchid production:

The ornamental orchids first reached Europe from the New World in 1731. After 1821, the commercial cultivation of orchids started in greenhouses in London. Currently, many countries of the world are involved in cultivation of orchids such as United States, Britain, Japan, China, Taiwan, Thailand, Australia and Singapore. Orchids are grown for two main reasons, first for export of cut flowers to supply the international market, and for the marketing of plants of different sizes, particularly those that are near flowering, and secondly for the ornamental plants suppling for the domestic market of each country. Thailand is one of the most specialized in the production of orchid flowers, with exports totaling $40 million for the year 2001. Among the most common orchids grown for cut flowers or ornamental plants are *Cattleya, Dendrobium, Epidendrum, Paphiopedilum, Phalaenopsis, Vanda, Brassia, Cymbidium, Laelia, Miltonia, Oncidium, Encyclia*, and *Coelogyne*. However, the highest proportions of current cultivars of
orchids, are those created through artificial hybridization between two or more species, and often from different genera. (Anon. Economic Importance, 2010). In the United States, most of the orchids grown are not for export. In 2005, 18 million potted orchids produced in the U. S. were sold at wholesale, with an average value of $8.00 per pot (USDA 2006 b). In the U.S. for 2010 the largest state producers of potted orchids were California ($63 million), Florida ($47 million), and Hawaii ($17 million) (USDA, 2006a), (Anon. Michigan, 2010; Anon. Flor.Crop.Sum., 2011). Orchids are currently one of the most important ornamental crops in the United States with a total wholesale value of $191 million in 2012 (Anon. Floriculture Statistics Bulletin, 2014).

Hawaiian potted orchid prices have increased gradually in the past decade. During this period, the value of potted orchid imports has risen almost five times, and is projected to continue to grow in the near future. Today, Taiwan is the major orchid supplier to the United States (55%), and has increased their import value to 32% from 2005 to $18 million in 2006. This increase was driven by the trade liberalization of the orchid markets with Taiwan, particularly the specifications regarding approved media and pest free procedure (Anon. HAAS, 2010). Other important exporters to the United States include Thailand, the Netherlands, and Canada, with a combined value of $10.3 million (31%) in 2006 (Anon. HAAS, 2008; Palma et al., 2010).

The Island of Hawaii is the primary contributor to Hawaii’s orchid economy, producing over 50% of the state’s orchids in 2008 (Anon. HAAS, 2010). In 2010, total sales ranked orchids as 16th most important agricultural commodity in Hawaii (USDA, NASS 2010, Annual Statistical Bulletin), with a majority of sales on Dendrobium, Cymbidium, Vanda, Phalaenopsis, and Oncidium. There were a total of 32 producers of Dendrobium floral sprays, 58 growers of potted Dendrobium plants, 22 producers of Oncidium sprays, 23 producers of Phalaenopsis
plants and 7 growers of _Cymbidium_ sprays. Nearly 90 orchid producers also grew a wide variety of genera and intergeneric crosses, including _Miltonia, Cattleya, Vanda, Odontoglossum, Brassolaeliocattleya, Ascocenda_, and _Epidendrum_. In 2011 the preliminary value of floriculture and nursery products are estimated at $69.6 million in Hawaii although, the sale of Hawaiian orchids is continuously declining in the last few years. The geographical location of Hawaii provides ideal conditions and the perfect natural environment for orchids to grow all year round. However, a decline in orchid production has been observed partly due to increasing competition from foreign suppliers and larger domestic producers. Another important reason for decline in production of orchids is frequent attack of pathogens. Currently, there are limited complete studies on different diseases associated with orchids. This dissertation will focus to study on different fungal genera on orchids in Hawaii.

1.2 _Fusarium_ as an orchid pathogen worldwide

_Fusarium_ is a highly destructive pathogen that economically limits the production of several crops. Throughout the world _Fusarium_ has been found to be associated with orchids both as pathogens and non-pathogens. The pathogenic _Fusarium_ is now being considered as one of the most limiting reasons for the production of high quality orchids and incidence of _Fusarium_ diseases has been steadily increasing in many production facilities worldwide. The non-pathogenic _Fusarium_ species associated with orchids are mostly decomposers (Booth, 1971) and/or are mutualists, which help in seed germination and seedling coloration (Vujanovic et al., 2000). The important _Fusarium_ species that have been established as pathogens includes _F. oxysporum, F. proliferatum, F. solani, F. subglutinans_, and _F. fractiflexum_.


*Fusarium oxysporum* is a complex species composed of at least 70 host-specific, phytopathogenic strains (Armstrong and Armstrong, 1981; Booth, 1971) and a vast number of saprophytic strains. Due to a relatively high level of biodiversity in this species, it has the ability to adapt to environmental changes and form new pathogenic strains over a short period of time (White *et al.*, 2001). It is also the most economically important species in the *Fusarium* genus given its numerous hosts and the level of loss that is produced on infected plants. Many *F. oxysporum* isolates appear to be host specific, which has resulted in the subdivision of the species into *formae speciales* and races that reflect the apparent plant pathogenic specialization. (Leslie and Summerell, 2006).

Despite the broad host range of the species, host specialization of the isolates is the characteristic that attracted the most attention from plant pathologists (Leslie and Summerell, 2006). Isolates having same or similar host ranges are recognized as a *forma specialis*, with more than 70 *formae speciales* described (Armstrong and Armstrong *et al.*, 1981; Booth, 1971). More often than not, host range is restricted to a few plant species. For example, *F. oxysporum* f. sp. *lycopersici* causes disease only in plants of the genus *Lycopersicon* (Rowe, 1980). However, some *formae speciales* have broader host ranges, such as *F. oxysporum* f. sp. *radicislycopersici*, which cause disease on hosts from several plant families, including tomato (*Lycopersicon esculentum* Mill.), cucumber (*Cucumis sativus* L.), zucchini (*Cucurbita pepo* L.), beet (*Beta vulgaris* L.), asparagus (*Asparagus officinalis* L.) and onion (*Allium cepa* L.) in the greenhouse (Kim *et al.*, 2002; Menzies *et al.*, 1990; Rowe, 1980).

*Fusarium oxysporum* Shlechtendahl emend. Snyder and Hansen is the most widely documented and one of the most destructive pathogenic *Fusarium* on orchids. This species is found worldwide, in various countries, including the U.S. (Fungal Database), Australia (Burnett,
1985), Taiwan (Huang et al., 2013), Korea (Kim et al. 2002; Lee et al., 2002), India (Yadav et al., 2010), Malaysia (Latifah et al., 2009), and Japan (Hadley et al., 1987). Fusarium oxysporum infects through the roots, causing root rot, wilt and sometimes stem rot (Latifah et al., 2009), which kills the plant by systemic infection of the vascular system, creating severe deficiencies in nutrients and water (Broadhurst and Hartill, 1996; Burnett, 1985; Hadley et al., 1987, Ichikawa et al., 2003). Susceptible genera to F. oxysporum include Cattleya, Cymbidium, Dendrobium, Oncidium and Phalaenopsis (Burnett, 1985; Hadley et al., 1987; Kim et al., 2002) There are some reports of F. oxypsorum f. sp. cattleyae (Pedroso et al., 2011; Jeong et al., 2004) whereas other reports lack a forma specialis designation (Hadley et al., 1987). Fusarium oxysporum f. sp. vanilla is another example of forma specialis description which is pathogenic to vanilla and causes stem rot in Indonesia (Pinaria et al., 2010) and Hawaii but formae specialis has not been established in Hawaii.

Fusarium proliferatum (Matsushima) Nirenberg, [teleomorph Gibberella intermedia (Kuhlman) Samuels, Nirenberg and Seifert], is synonymous with G. fujikuroi mating population D, previously F. moniliforme (Booth, 1971). This species causes black and yellow leaf spot on Cymbidium, Ondontioda, Dendrobium, and Cattleya, and root rot on Cymbidium and Phalaenopsis. Fusarium proliferatum was reported as an orchids pathogen in Japan (Ichikawa and Aoki 2000; Zakaria et al., 2004), Australia (Benyon et al., 1996) and was associated with diseased orchids in Germany (Nirenberg and O’donell, 1998) as well as in Korea (Chang et al., 1998; Kim et al., 2002).

Fusarium solani (Martius) Appel and Wollenweber emend. Snyder and Hansen with the teleomorph Haemanectria haematococca (Berkeley and Broome) Samuals and Nirenberg, is synonymous with Nectria haematococca, and causes localized root rot which then expands and
kills the host. *Fusarium solani* has been found to infect both *Cymbidium* (Benyon *et al*., 1996) and *Dendrobium* in Australia (Simmonds, 1966), and *Cattleya* hybrids in California (French, 1989). In addition, *F. solani* has been associated with root and pseudobulb rot of *Cymbidium* in Japan (Ichikawa and Heideki, 1998). Based on restriction enzymes specific for each special form, a mapping data was published (Suga *et al*., 2000). The digestion pattern of the rDNA-ITS region with these restriction enzymes was able to distinguish *F. solani* into 10 ‘forma specialis’ including *batatas*, *cucubitae*, *eumartii*, *mori*, *phaseoli*, *piperis*, *pisi*, *radicicola*, *robiniae* and *xanthoxyli* having specificity to infect sweet potato, pumpkin, potato, mulberry, french bean, pepper, pea, gladiolus, black locust, and Japanese pepper (Suga *et al*., 2000). Recently a new formae specialis of *F. solani* has been reported to cause yellowing of leaves in *Phalaenopsis* in Taiwan and identified as *F. solani* f. sp. *phalaenopsis* (Chung *et al*., 2011).

*Fusarium subglutinans* (Wollenweber and Reinking) Nelson, Toussoun, and Marases, [teleomorph *Gibberella subglutinans* Nelson, Toussoun and Marasas, is synonymous with *G. fujikuroi* mating population E, previously reported as *F. moniliforme* var. *subglutinans* (Booth 1971) has only been found to infect *Cymbidium* orchids. *Fusarium subglutinans* causes leaf spot (Broadhurst and Hartill, 1996; Hadley, 1987; Ichikawa and Aoki, 2000), seedling soft rot (Burnett, 1986), shoot rot and pseudobulb rot (Broadhurst and Hartill, 1996), and has been associated with flower blight (Hadley, 1987). This species has been found as an orchid pathogen in the USA (Burnett, 1986), New Zealand (Broadhurst and Hartill, 1996), Japan (Ichikawa and Aoki, 2000), and Italy (D’Agliano and Carrai, 1994).

*Fusarium fractiflexum* (Aoki *et al*., 2001) is a relatively newly described *Fusarium* species which was originally isolated from orchids. It has been found to cause yellow leaf spot on *Cymbidium* orchids in Japan (Ichikawa *et al*.,1998; Aoki *et al*., 2001).
*Fusarium moniliforme* can be also found as a foliar orchid pathogen in the literature (Booth, 1971; Burnett, 1986; Hadley, 1987). In 2003 the name *F. moniliforme* was removed entirely, since it had an overly broad species concept (Seifert et al., 2003). *Fusarium verticilliodes* has precedence for the species name of isolates previously referred to as *F. moniliforme* as described by Nelson et al. (Nelson et al., 1983) and Gerlach and Nierenberg in 1982 (Seifert et al., 2003). Over the years, the species definition of *F. moniliforme* has included over 35 phylogenetically distinct species (Booth, 1971; O’Donell et al., 1998; Snyder and Hansen, 1954), which is now considered as the *Gibberella fujikuroi* species complex (GFC) (Leslie and Summerell, 2006; Seifert et al., 2003). Species in the GFC include *F. subglutinans*, previously *F. moniliforme* var. *subglutinans*, (Ichikawa and Aoki, 2000), *F. proliferatum*, and *F. fractiflexum* (Booth, 1971). *Fusarium moniliforme* isolates associated with orchids were described to have polyphialides and microconidia in chains (Peschke, 1977). These characteristics currently are attributed to *F. proliferatum* (Leslie and Summerell, 2006).

In addition to the above species, *F. avenaceum* and *F. semitectum* have been isolated from diseased *Cattleya* hybrids in Chile and were assumed to be pathogens (Sepulveda and Goykovic, 1999). More than one species of *Fusarium* causing dry rot on Cymbidium orchids in Korea is also reported (Lee et al. 2002). There are also multiple reports of unidentified *Fusarium* species recovered from diseased *Cymbidium* orchids, in both India (Sharma et al., 1998) and Korea (Lee et al., 1998).

Three of these five species reported on orchids, *F. oxysporum, F. solani,* and *F. proliferatum* were reported in Hawaii (Swett, 2007) mostly occurring on the commercial nurseries of the Island of Hawaii. Swett reported a total of eight species including *F. oxysporum, F. solani, F. proliferatum, F. subglutinans* – like, *F. culmorum, F. sporotrichioides, F.*
avenaceum and F. sambucinum on the basis of morphology. Molecular analysis confirmed the identity of F. oxysporum, F. solani, and F. proliferatum (Swett, 2007). There are some unidentified Fusarium species that also had been found to be associated with orchids in Hawaii (Raabe et al., 1981), but their pathogenicity is not yet established. Hence, a comprehensive study is required to further identify new Fusarium species based on their morphology as well as genetic analysis and their pathogenicity should be established on different orchid genera.

**1.3 Fusarial Orchid Diseases in Hawaii**

*Fusarium* was first found in Hawaii's orchid crops in the early 1990's, causing foliar rot of field grown Dendrobium orchids in nurseries on Oahu. Since then, it has spread tremendously both in geographic locations and in host range (Swett, 2007). Presently, *Fusarium* wilt appears to be more frequent and severe in commercial nurseries and is found both on field grown and potted plants on Oahu and the Island of Hawaii (Swett, 2007). Island of Hawaii commercial nurseries that have adopted newer production technology and pot formulations for fast production cycles, are found to be more susceptible in terms of *Fusarium* frequency and severity (Swett, 2007). Increased disease incidence caused growers to raise chemical applications in an attempt to control disease. In general the infected plants are treated with fungicides after *Fusarium* has already established itself and the symptoms were decreasing plant quality. This late application of fungicides is often ineffective against established Fusarial diseases and unnecessarily increases the chemical load and causes wastage of resources. Moreover, such application gives an opportunity to the pathogen to develop resistance against some fungicides. Actual losses due to *Fusarium* wilt have not been calculated, although Swett found *Fusarium* is associated with different genera of orchids that include Dendrobium, Oncidium, Cymbidium, Vanda, Cattleya.
and hybrids of these genera. In this current study of primarily Oahu orchid farms, *F. proliferatum* was found to be the most prevalent *Fusarium* sp. associated with different orchid genera in preliminary isolations followed by *F. oxysporum* and *F. solani* respectively. *Fusarium* has been found associated with spots and blights of leaves, flowers, sheaths, and pseudobulbs, as well as root rot and damping-off of seedlings (Uchida, 1999). Young, expanding leaves were observed to have greater susceptibility to infection than older foliar tissues. Orchid production is affected by these diseases as they reduce both, quantity and quality of marketable flowers. They also reduce plant vigor, and causes blemishes, which decreases the value of potted ornamental plants. However, there have been several other *Fusarium* species found as orchid pathogens in other countries, which might also be found potential pathogens and it is necessary to make a comprehensive study of *Fusarium* associated with orchids and to establish an etiology of Fusarial disease (Uchida, 1999). Further a major problem of all orchid diseases is the cross infectivity of *Fusarium* species with different genera. This will be the first study employing *Cymbidium, Miltonia* and *Cattleya*. This will demonstrate that isolates pathogenic on one host genus may be pathogenic on others.

### 1.4 Taxonomic Considerations in Determining the Etiology of Fusarial Diseases

Identification of *Fusarium* species is one of the greatest challenges for any plant pathologist because *Fusarium* taxonomy has been constantly changing over a period of several decades and still continues to change. Few species have been well characterized and depending upon the definition of species used, the number of species identified lies between nine to just over a thousand during the last century (Summerell *et al.*, 2003). With the publication by Gerlach
and Nirenberg in 1980s (Broadhurst and Hartill, 1996; Summerell et al., 2003) and Nelson et al. in 1983 (Summerell et al., 2003), the morphological concept of species were finally established and were widely accepted and successfully used by numerous practitioners. It is very important to understand how species of *Fusarium* are characterized and identified, both at the genus and species levels for the more effective study of the members of this genus present on orchids in Hawaii. In *Fusarium* there are currently three different basic species concepts being employed: morphological, biological and phylogenetic (Summerell et al., 2003). Until the 1990’s, *Fusarium* species taxonomy was based primarily on the morphological species concept (Leslie and Summerell, 2006; Summerell et al., 2003). Morphological identification is the most commonly employed species concept for *Fusarium*. The genus *Fusarium* is characterized by the production of 2-6 celled canoe-shaped asexual spores called macroconidia (Seifert, 2001). However, the production of these macroconidia cannot be used alone to describe all members of the group, since macroconidia like spore formation is not unique to the *Fusarium* genus and because some *Fusarium* species produce macroconidia very rarely and they may never be observed.

Therefore, the combinations of various morphological characters are necessary to define the species in the *Fusarium* genus. These characteristics include formation of phialides (an open-ended, tubular or flask-like conidiophore that produces phialoconidia.) that are either branched (polyphialides) or unbranched (monophialides), production of microconidia (1-3 celled spores) in chains or false heads (clusters), macroconidia borne singly or in clusters called sporodochia, and production of survival spores, called chlamydospores as well as presence or absence of macroconidia, conidia size and shape (including shape of apical and foot cells), sporodochia coloration, and reverse colony color. Based on these expanded morphological
characteristics, the genus *Fusarium* can be more reliably identified (Seifert, 2001; Leslie and Summerell, 2006; Summerell *et al.*, 2003)

In addition to these, some other characters also have been used for species delimitation like, circinate (coiled) hyphae are important for *F. circinatum*. Mesoconidia is a term that was proposed by Pascoe to describe the fusoid conidia that are typically produced by some species of *Fusarium* from polyphialides in aerial mycelium on Carnation Leaf agar (CLA) or Spezieller Nahrstoffarmer Agar (SNA) (Pascoe, 1990). Mesoconidial size is typically in between the macroconidia and microconidia and often lacks a foot shaped or notched basal cell. They generally protrude from the aerial mycelium and sometimes give the appearance of “rabbit ears” under the microscope. The species that produce mesoconidia include *F. compatoceros*, *F. chlamydosporum* and *F. semitectum* (Leslie and Summerell, 2006)

The biological species concept is based on the cross-fertility of its members, and the ability to form viable and fertile progeny (Leslie and Summerell, 2006; Summerell *et al.*, 2003). When the sexual stage is produced, it provides valuable confirmation of the species identity. However, this species concept is limited since the sexual stage of the heterothallic species can only be used to define species for which both mating populations are found or identified mating types can be used.

The phylogenetic species concept defines species relationships based on DNA sequences of select gene regions which are considered to be reflective of evolutionary changes at the species level (Leslie and Summerell, 2006; Summerell *et al.*, 2003). Many genes are used when characterizing species, to give better representation of the entire genome evolution (Villa *et al.*, 2006). These gene regions are usually conserved and are responsible for fundamental biological
functions, that includes the internal transcribed spacer unit (ITS), β-tubulin (BT), and transcription elongation factor (TEF 1-α) (Geiser et al., 2005; Leslie and Summerell, 2006; O'Donnell et al., 1998; O'Donnell et al., 2000). Molecular analysis is quickly becoming a standard for characterization and identification of Fusarium species since it offers the most reliable and consistent detection of differences between groups (Leslie and Summerell, 2006; Summerell et al., 2003). Using molecular analysis, species are identified based on the sequence similarities of unknown isolates to species in National Center of Biotechnology Information (NCBI) database sites, and a Fusarium database that has been created to improve the accuracy of molecular identification of Fusarium species (Fusarium database, Geiser et al., 2004).

1.5 Overview on Methods of Control Options Available for Control of Fusarium on Orchids in Hawaii

Breaking the disease cycle is the main focus of any management practice. Initiation of the disease cycle occurs with the dispersion of spores from a contaminated source to a healthy host.

Fusarium species produces two types of asexual spores, macroconidia and microconidia that are the primary means of dispersal, sometimes sexual spores (ascospores) can also play an important role (Doohan et al., 2003). These asexual spores can disperse by wind and water splash, primarily from overhead watering in nurseries (Doohan et al., 2003, Ichikawa et al., 2003). Insects and tools also play a major role in the spread of these spores as insects comes in the contact of infected material and move the spores (Agrios, 2000). Once in contact with the host, the spore germinates with moisture present and penetrates the tissue, entering the host through the epidermis. The pathogen infects the tissue, feeding on cellular material, growing, and producing spores that are then dispersed, continuing the disease cycle. If a host is absent, thick walled survival spores, called chlamydospores, can develop from cells in the hyphae and/or the
macroconidia for some species (Summerell et al., 2001). Chlamydospores enable survival in plant parts (such as root matter) or in the soil or potting medium for many years (Summerell et al., 2001).

Spread of *Fusarium* among greenhouse- or field-grown orchids can be reduced by various cultural practices, including removal of infected material (Agrios, 2000; Garibaldi and Gullino, 1990; Gullino et al., 2002), regulation of overhead watering and wind movement (when possible) (Doohan et al., 2003; Ichikawa et al., 2003; Leonhardt et al., 1999), and management of vectors (Wang and Jeffers, 2002), along with other sanitary practices. *Fusarium* infection and reproduction can also be prevented through the use of resistant cultivars (Agrios, 2000; Rose and Parker, 2003). *Fusarium* germination growth and infection can also be inhibited by anti-fungal compounds, including chemical fungicides and biological controls (Agrios, 2000). Some of the most successful practices in controlling fusarial diseases on nursery crops have involved the use of anti-fungal compounds or fungicides.

1.5.1 Chemical fungicides:

Fungicides are chemical or synthetic agents that consist of toxic compounds that inhibit the fungal growth and proliferation thereby controlling the infection/disease caused by fungi. Based on mode of action, fungicides are classified as either broad spectrum or specific. A broad spectrum fungicide inhibits or diminishes growth and proliferation of a wide range of fungal pathogens, and includes captan, sulfur, and mancozeb. While, others are specific in their action and act on a very narrow range of fungi, e.g. mefenoxam is effective only against oomycetes like *Phytophthora*. Alternatively, fungicides can also be described as protective or as curative, or as an eradicant. Protectives are applied to host plant before the pathogenic fungal spore are
expected to land on the surface of leaf, they are mostly applied on healthy plants. Examples of protectants include mancozeb, coppers, and chlorothalonil. Protectants generally are not effective once the fungus grows into plant tissues. Curative fungicides are generally present inside the plant body and acts on those pathogens that have already penetrated the plant, thus the host plants are already infected. Dodine, triflumizole, or myclobutanil are common curative fungicides that are applied against apple scab fungus *Venturia inaequalis*. Eradicants are more effective on pathogens and remains for longer periods on plants and even kills the over wintering pathogens. Some fungicides are also systemic in their mode of action that is they are absorbed and translocated to different plant parts.

Many fungicides are available for *Fusarium* disease control in nursery crops. For example captan, carbadexim, thiram and carboxim that has been found effective against *Fusarium oxysporum* and has been very effective in controlling rots caused by *F. oxysporum* in many ornamental plants such as gladiolus and vegetable crops like cucumber (Mishra et al., 2000).

Most of the fungicides that interfere with vital metabolic pathways of fungi are relative stable compounds. Strobilurins are compounds that inhibit fungal mitochondrial respiration, and have been effective in *Fusarium* control. Heritage the commercial formulation of strobulerins, is registered for control of *Fusarium* on more than 100 ornamental crops, including chrysanthemums (*Chrysathemum* spp.), carnation (*Dianthus caryophyllus*), geranium (*Pelargonium* spp.), and rose (*Rosa* spp.) (Ram et al., 2004; Swett, 2007).

In Hawaii, there are very few fungicides available for control of *Fusarium* on orchids. According to NSPIRS data for the State of Hawaii (Anonymous, NSPIRS, 2013) only following five products are registerd:
1. Manganese ethylenebis (dithiocarbamate) (Dupont Mnex Fungicide).

2. Zinc ion and manganese ethylenebisdithiocarbamate, coordination product (Dupont Manzate Flowable Fungicide, Protect DF Turf and Ornamental Fungicide, Ferti-Lome Consan 20).

3. Alkyl* dimethyl benzyl ammonium chloride (Floralife microbloc Greenhouse Disinfectant, Physan 20).

4. 1-(4-chlorophenoxy)-3, 3- dimethyl1 – 1 – (1H – 1, 2, 4 – triazol – yl) -2 – butanone (Bayleton Flo Turf and Ornamental Fungicide, Green Light Fung-Away Systemic Fungicide).

5. QST 713 strain of Bacillus subtilis (Serenade Mix, Cease, Rhapsody).

Hence, there is an urgent requirement of seeking effective control measures they may include screening of more fungicides and evaluation of some other control agents such as essential oils or biocontrol methods.;

1.5.2 Fungicide resistance:

Fungicide resistance is a major problem for effective disease management and is very common among many plant pathogenic fungi throughout the world. Many new fungicides are at a high risk for the development of fungal resistance (Pscheidt, 2013). Pathogen populations contain naturally occurring resistant types in very low numbers in nature but this is because there is normally no adaptive advantage of this trait in the absence of the particular fungicide. However, when a fungus population is treated with a specific fungicide, the resistant strains will have a great adaptive advantage and are left with less competition, so, their population increases and either a part of population or sometime the entire population may become dominant and
resistance for that particular fungicide. Fungicides with very specific site of activity are most likely prone for the development of resistance in a fungal population than the fungicides having a variety of antifungal actions or multi sites of activity (Schnabel, 2013; Bertrand and Padgett, 1997).

1.5.3 Alternative approaches:

The best approach to fungicide resistance management is to use a program consisting of both combinations and alternating of fungicides with different modes of action as well as to use fungicides with other non-fungicide approaches such as, use of essential oils or biocontrol.

Antimicrobial activity of numerous plant essential oils and their volatile components on plant pathogenic bacteria, fungi, nematodes and virus have been extensively investigated in the past decade (Bowers and Locke, 2004; Ji et al., 2007; Kishore et al., 2007; Oka et al., 2000; Perez and Lewis, 2006; Reitz et al., 2008) and it has shown promising results. Previous studies have shown that essential oils of basil (*Ocimum basilicum*) and sweet fennel (*Foeniculum vulgare*) at 0.6 % and 0.8 % showed the strongest mycelium growth inhibition of *F. moniliforme* and *F. proliferatum*. Use of essential oils as biofumigants could prove to be an effective alternative approach for the management of different *Fusarium* species.
Hypothesis

We hypothesize that different *Fusarium* spp. are pathogenic to different genera of orchids cultivated in Hawaii.

Objectives:

1. Identify different fungal genera associated with orchids in Hawaii.
2. Confirm the identity and pathogenicity of *Fusarium oxysporum*, *Fusarium proliferatum*, and *Fusarium solani* on orchids.
3. Isolate and identify other *Fusarium* species and determine pathogenicity of *Fusarium* species on orchids.
4. Manage *Fusarium* species using chemical controls.
5. Investigate the concept of *F. oxysporum* forma specialis.
CHAPTER 2

Fungal Genera Associated With Orchid Diseases in Hawaii

Abstract

Orchids are among the major horticulture crops of economic importance in Hawaii. Many fungal genera have been found to be associated with orchids that live as saprotrophs and pathogens. However, a systematic study is needed to identify and characterize the association of various fungal genera and orchids. In the present study 16 different fungal genera were isolated from 60 plant samples collected from Oahu and the Island of Hawaii and *Fusarium* was the most common genus, frequently associated with several orchid cultivars, including over 20 different genera and hybrids, such as *Dendrobium*, *Cymbidium*, *Oncidium*, *Miltonia*, *Phalaenopsis*, *Miltonia* hybrids (e.g., *Miltonidium*), *Vuylstekaria* (*Oncidium* and *Miltonia* hybrids), *Cymbidium* hybrids (*Valerie Absolonova # 57 X Valley Zenith ’Concord’*) and *Cattleya* hybrids (e.g., *Brassolaeliocattleya*). A total of 480 isolates of *Fusarium* were recovered from the plant samples, which was 78% of all fungi isolated, indicating that *Fusarium* species are the primary pathogens associated with foliar and root diseases. This study also confirms the previous study by Swett (2007), that *Fusarium* species were the most common fungi associated with several orchid diseases in Hawaii. Of the 16 genera that were identified, ten have been previously documented as orchid pathogens worldwide. *Fusarium* was the most frequently recovered genus followed by *Colletotrichum* and *Phyllosticta* distributed across the islands.
2.1 Introduction

In the past twenty years, several new fungal diseases have emerged on orchids in Hawaii, some of which appear to be caused by *Fusarium* (Uchida, 1994; Uchida, 1999). *Fusarium* associated diseases on orchids are prevalent in many parts of Oahu and the Island of Hawaii (Swett, 2007). These diseases occur on many orchid genera and inter-generic hybrids. Disease symptoms associated with *Fusarium* includes rot on roots and pseudo bulbs, spots and blights of leaves and sheaths, in which both seedlings and mature plants were studied (Uchida, 1994; Uchida, 1999). Sixteen fungal genera were identified associated with diseased orchids in Hawaii in which few of them were already reported as potential pathogens of orchids in different parts of the world (Uchida, 1994), rest of them were saprotrophs. Almost all parts of the diseased orchids plant samples collected on the basis of symptoms produced *Fusarium* thus establishing their common relationship with diseased orchids. Less information is available of these new diseases therefore the objective of this study was to collect diseased samples of orchids and to identify the fungi associated with those plants showing disease symptoms.

The objective of this study was to (1) Collect samples from commercial orchid nurseries, with a focus on nurseries on Oahu and the Island of Hawaii, where the majority of orchids are produced as well as from hobbyists and retail centers. (2) Isolate different fungal genera associated and identify major fungal genera associated with them.
2.2 Materials and Methods

2.2.1 Sample collection

Diseased orchid tissue samples were collected from different locations on Oahu and the Island of Hawaii (Table 2.1). Overall, 60 plant samples were collected, representing 20 different genera and inter-generic hybrids (here after referred as hybrids).

Table 2.1 List of Fungal genera isolated from diseased samples collected from Oahu and the Island of Hawaii.

<table>
<thead>
<tr>
<th>Fungal genera</th>
<th>Location of isolation</th>
<th>No. of isolates</th>
<th>Percent share</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Fusarium</em></td>
<td>Oahu, Island of Hawaii</td>
<td>480</td>
<td>78.7</td>
</tr>
<tr>
<td><em>Collectrotrichum</em></td>
<td>Oahu, Island of Hawaii</td>
<td>32</td>
<td>5.3</td>
</tr>
<tr>
<td><em>Phyllosticta</em></td>
<td>Oahu</td>
<td>13</td>
<td>2.1</td>
</tr>
<tr>
<td><em>Pestalotiopsis</em></td>
<td>Oahu</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td><em>Penicillium</em></td>
<td>Oahu</td>
<td>11</td>
<td>1.9</td>
</tr>
<tr>
<td><em>Trichoderma</em></td>
<td>Oahu</td>
<td>36</td>
<td>6</td>
</tr>
<tr>
<td><em>Lasiodiplodia</em></td>
<td>Oahu</td>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td><em>Alternaria</em></td>
<td>Oahu</td>
<td>4</td>
<td>0.7</td>
</tr>
<tr>
<td><em>Rhizoctonia</em></td>
<td>Island of Hawaii</td>
<td>10</td>
<td>1.6</td>
</tr>
<tr>
<td><em>Phytophthora</em></td>
<td>Oahu</td>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td><em>Phoma-like</em></td>
<td>Oahu</td>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td><em>Phoma</em></td>
<td>Oahu</td>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td><em>Pseudocercospora</em></td>
<td>Oahu</td>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td><em>Dactylella</em></td>
<td>Oahu</td>
<td>3</td>
<td>0.5</td>
</tr>
<tr>
<td><em>Botrytis</em></td>
<td>Oahu</td>
<td>2</td>
<td>0.3</td>
</tr>
<tr>
<td><em>Cladosporium</em></td>
<td>Oahu</td>
<td>1</td>
<td>0.1</td>
</tr>
</tbody>
</table>
For each genera or hybrid, from 1 to 20 plants were sampled. Diseased parts of individual plants were used for isolation with each plant represented by a single sample. Those plants that showed typical symptoms of disease most likely to be caused by other pathogens such as bacteria or viruses were not sampled. Samples were collected in plastic bags labeled with name of plant, date of sampling and location of sampling and then symptoms were photographed (Fig 2.1 to 2.5) and plant part, type of lesion, color, and location on the plant part (e.g., side of leaf, base of bulb, etc.) infected were documented.
Fig 2.1. Symptoms associated with *Fusarium* on Oncidium X Miltoniopsis seedling, which led to plant death. (A) Leaf rot, leaf sheath rot and root rot (B) Leaf sheath rot (C) Root rot (D) Pseudobulb and root rot.
Fig 2.2. Symptoms on *Miltonia* seedling. (A) Leaf spots. (B) Root rot and root rot leading to stem rot.

Fig. 2.3. Symptoms on *Dendrobium* shoot which led to plant death. (A) Leaf spots. (B) Leaf spots, leaf blight and leaf sheath blight. (C) Leaf wilt. (D) Leaf sheath blight and leaf spots.
Fig 2.4. Symptoms on *Vuylstekeara* seedling, which led to plant death. (A) Spots and sheath rots (B) Root rot leading to sheath and heart rot.

Fig 2.5. Symptoms on *Cymbidium* hybrid (*Valerie Absolonova # 57 X Valley Zenith ‘Concord’) which led to plant death (A) Leaf sheath rot (B) Root rot.
2.2.2 Isolation and purification

For each diseased plant, the interphase tissue of an individual lesion or rotted area on each leaf, leaf sheath, root, rhizome, or pseudo bulb was dissected, washed with liquid soap, rinsed with running tap water, and cut into approximately 5 to 10 mm pieces, and surface disinfested in 10% bleach solution for 5 to 10 seconds. Samples were placed on water agar (WA) (18 g Difco Bacto agar/ liter of distilled water) and incubated at room temperature (24°C) with approximately 12 hours ambient light, from one week to a maximum of one month depending on the growth rate of fungi. Plates were observed twice a week for the emergence of new fungal hyphae. Pure cultures of all fungi were established by single, hyphal-tip isolation (SH) or single-spore isolation (SS) and grown on 10% vegetable juice agar (VA) (100 ml Campbell's V8 juice, 0.24 g calcium carbonate, 18 g Bacto agar, and 900 ml of water). For *Fusarium* isolates, the cultures were further purified by single-spore isolation on WA and three single-spore isolates were transferred to VA per single tip isolate. Single-spore (*Fusarium*) or single-tip (non *Fusarium*) cultures were grown 5 to 10 days under constant cool fluorescent light and tubed in 15cm long tubes with VA slants. Each isolate was collected from a separate symptom from different parts of each sample. Thus, several isolates of a species or more than one species could be recovered from one plant, from leaf spots on the same leaves, leaf spots on different leaves, or root rot.

2.2.3 Morphological identification

For morphological identification, the purified culture of fungus were grown on VA under continuous cool fluorescent irradiation at 23 to 25°C for 5 to 30 days and were routinely examined microscopically for genus identification. *Fusarium* isolates were further identified to
species as per the description presented in ‘The Fusarium, A laboratory Manual’ by Leslie and Summerell (2006). Single-spore cultures of putative Fusarium were grown on carnation leaf agar (CLA, 2% WA with 4-6 sterile carnation leaf pieces added to the surface) and Potato Dextrose Agar (PDA, 39 g Difco potato dextrose agar, 1 g Bacto agar, and 1 liter distilled water) at 24°C under continuous fluorescent irradiation. Cultures were examined after 5-10 days for formation of microconidia in chains or false heads, and production of monophialides (either short or long) or polyphialides. Chlamydospore production was examined between and 5 and 45 days. Isolates were cultured on CLA to assess sporodochia production and on PDA, to characterize reverse colony color.

2.3 Results
2.3.1 Fungal Genera Isolated

Sixty plant samples showing disease symptoms were collected from different nurseries on Oahu and the Island of Hawaii and a total 610 fungal isolates were obtained, representing 16 fungal genera. Among them, Fusarium was the most commonly isolated genus with a total of 480 isolates. Symptoms associated with Fusarium included rot of roots, pseudobulbs, and rhizomes, as well as spots and blights on leaves and sheaths. In addition, 130 isolates of other fungal genera were isolated from 22 % of plants (Tables 2.2) in which few of them had already been reported as pathogens on orchids. Previously reported pathogens were isolated and includes Colletotrichum, Phytophthora, Alternaria, Phyllosticta, and Botrytis (Uchida, 1994). Apart from known pathogens, a few common saprotrophs were also isolated such as Penicillium, Cladosporium, and Trichoderma.
Table 2.2: Fungal genera isolated from diseased orchid samples collected during study.

<table>
<thead>
<tr>
<th>Fungal genera</th>
<th>Plant Parts</th>
<th>%</th>
<th>Location of Isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Leaves</td>
<td>Pseudo bulb</td>
<td>Roots</td>
</tr>
<tr>
<td><em>Fusarium</em></td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td><em>Collectrotrichum</em></td>
<td>√</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Phylosticta</em></td>
<td>√</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Pestalotiopsis</em></td>
<td>√</td>
<td>√</td>
<td>-</td>
</tr>
<tr>
<td><em>Penicillium</em></td>
<td>√</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Trichoderma</em></td>
<td>√</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td><em>Lasiodiplodia</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Alternaria</em></td>
<td>√</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Rhizoctonia</em></td>
<td>-</td>
<td>-</td>
<td>√</td>
</tr>
<tr>
<td><em>Phytophthora</em></td>
<td>-</td>
<td>√</td>
<td>-</td>
</tr>
<tr>
<td><em>Phoma</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Pseudocercospora</em></td>
<td>√</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Dactylella</em></td>
<td>√</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Botrytis</em></td>
<td>√</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Cladosporium</em></td>
<td>√</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Spermospora</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**X:** Likely to be saprotrophic.

**Y:** Small spored (μ) and in chains.

**Z:** Isolates were binucleate indicating their non-parasite nature.
2.4 Discussion

Different fungal genera were isolated from different plant samples in which *Fusarium* spp. *Colletotrichum, Phytophthora, Phyllosticta, Botrytis* and *Pseudocercospora* were reported as pathogenic in Hawaii (Uchida, 1994), while others were present as probable saprotrophs. Presence of different species of *Fusarium* was dominant among the different genera of fungi isolated. This information is similar with a previous study reported by Swett (2007). The study by Swett was conducted on samples collected from different nurseries on the Island of Hawaii. In this study, *Fusarium* was the most commonly isolated genus and was isolated from all samples of plants and *Fusarium* was present on each sample collected from both islands. *Fusarium* was associated with several orchids, including over nine different genera and hybrids, such as *Dendrobium, Cymbidium, Oncidium, Miltonia, Phalaenopsis, Miltonia* hybrids (e.g., *Miltonidium*), *Vuylstkaaria* (Oncidium and Miltonia hybrids), *Cymbidium* hybrids (Valerie Absolonova # 57 X Valley Zenith ‘Concord’) and *Cattleya* hybrids (e.g., *Brassolaeliocattleya*). Overall, *Fusarium* was recovered from symptomatic plants approximately 13 times more frequently than any other genus, indicating that *Fusarium* species are the primary pathogens associated with these foliar and root diseases on those orchids sampled. This study demonstrated that *Fusarium* species were the most common fungi associated with several orchid diseases in Hawaii. The *Fusarium* species were widely distributed. Of the sixteen genera identified, six have been previously documented as orchid pathogens worldwide.
CHAPTER 3

Characterization of Various *Fusarium* Species and Confirmation of Their Pathogenicity to Orchids.

Abstract

Orchids are one of the most important agricultural commodities in the state of Hawaii, grossing $14.5 million in 2011 (HASS, 2011). A considerable loss of crop occurs due to disease caused by various pathogens especially fungi. Among fungi, *Fusarium* was found to be the most frequently isolated genus from diseased orchids. Symptoms caused by various *Fusarium* species are leaf spots, leaf rot as well as wilt. In this study major orchid genera affected by *Fusarium* are *Dendrobium*, *Cymbidium*, *Miltonia* and *Cattleya*. Eleven *Fusarium* species, *F. proliferatum*, *F. oxysporum*, *F. solani*, *F. begoniae*, *F. bulbicola*, *F. circinatum*, *F. poae*, *F. semitectum*, *F. anthophilum*, *F. denticulatum* and *F. subglutinans* associated with orchids were identified morphologically and by using TEF-1α gene sequence analysis. Three *Fusarium* species, *F. proliferatum*, *F. oxysporum* and *F. solani* were found, to be frequently moderate to highly virulent to *Dendrobium*, *Cymbidium* and *Miltonia* causing leaf spots and blights as initial symptoms followed by leaf sheath rot, leaf rot and wilt as advanced symptoms. All the three *Fusarium* species were isolated frequently from different orchid genera such as *Dendrobium*, *Cymbidium* and *Cattleya* showing a strong correlation between the disease and these *Fusarium* species. Three other *Fusarium* species were low to moderate virulent on *Dendrobium* and *Cymbidium*. *Fusarium poae* showed very low to low virulence on *Dendrobium* and *Cymbidium* causing leaf spots. While *F. circinatum* was moderately high to highly virulent on *Cymbidium* causing leaf spots as initial symptoms that progressed into blight and rot of youngest leaves but it showed low virulence on *Dendrobium* caused only leaf spots. *Fusarium begoniae* showed very
low virulence on Miltonia causing only leaf spots. *Fusarium anthophilum, F. bulbicola, F. denticulatum* and *F. semitectum* did not produce infection on all the four orchid genera tested (*Dendrobium, Cymbidium, Miltonia* and *Cattleya*). Identification of pathogenic species of *Fusarium* on orchids will help in tailoring more efficient and feasible interventions to manage the decrease in orchid production.
3.1 Introduction

Orchids are one of the most commercially valuable ornamental crop in the United States generating revenue of approximately 191 million dollars annually (Anonymous, floriculture crops summery, 2012). Hawaii is one of the leading producers of orchids in the United States where it is an important cash crop having a value of around $ 14.5 million in 2011 (HASS, 2011). Hawaiian orchids occupy a unique place due their genetic diversity that results in the continuous development of new varieties of orchids.

For past several years a consistent reduction in production of orchids in Hawaii has been observed in spite of its growing demand in the United States and the world (HASS, 2011). Several abiotic and biotic factors are considered responsible for the decrease in crop yield. Orchids are frequently found to be associated with various fungi, some of which are saprotrophs whereas others are pathogens (Umata, 1998). Fungal pathogens and the diseases caused by them often result in stunted growth, wilting and foliar damage in form of spots on leaves and loss of new growth.

*Fusarium* species have long been recognized as both symptomless endophytes and pathogens of orchids around the world. In 1955, *Fusarium* was first identified as pathogens on orchids in United States (Foster 1955), it took over 40 years before pathogenic *Fusarium* species were isolated from various parts of the world (Benyon et al., 1996; Broadhurst and Hartill, 1996; Ichikawa et al., 1998; Chung et al., 2011; Xiao et al., 2012). In a previous study, *Fusarium proliferatum*, *F. oxysporum* and *F. solani* were reported as pathogenic to *Dendrobiums* in the island of Hawaii (Swett, 2007). Other studies have also identified *F. subgutinans* and *F. fractiflexum* to be pathogenic on orchids in different parts of the world (Ichikawa and Aoki,
Further a major problem of all orchid diseases is the cross infectivity of *Fusarium* species with different genera. Hence there was a need to visit the commercial nurseries on Oahu and the Island of Hawaii and to study the possibility of emergence of new *Fusarium* species. This will be the first study employing *Cymbidium*, *Miltonia* and *Cattleya* and will demonstrate the potential cross-infectivity of *Fusarium* isolates.

The objective of this study was to:

1. Describe the symptoms formed from, *F. proliferatum*, *F. oxysporum* and *F. solani* from diseased orchids on Oahu.

2. Identify three species and confirm the pathogenicity of three *Fusarium* species on orchids.

3. Identify several other unidentified *Fusarium* species and test the pathogenicity of *Fusarium* species isolated.

### 3.2 Materials and Methods

#### 3.2.1 Sample collection

Diseased orchid tissue samples were collected from different locations, on Oahu and a few on the Island of Hawaii. Samples were placed in plastic bags labeled with name of plant, date of sampling and location. Overall, 60 plant samples were collected, representing nine different orchid genera and inter-generic hybrids (hereafter referred as ‘hybrids’). Symptoms were photographed and plant part, type of lesion, color, and location on the plant part (e.g., side of leaf, base of bulb, etc) were noted (Chapter 2).
3.2.2 Isolation and purification

For a given diseased plant, the interphase tissue of individual lesions or rotted areas on each leaf, leaf sheath, root, rhizome, or pseudo bulbs were dissected. Isolates were collected from various symptomatic regions on the different parts of same plant sample. Samples were individually washed in mild soap water, rinsed in running tap water, cut into approximately 5 to 10 mm pieces, and surface disinfested in 10% bleach solution for 5 to 10 seconds. Samples were placed on water agar (WA) (18 g Difco Bacto agar/ liter of distilled water) and incubated at room temperature with approximately 12 hours ambient light, from 72 hours to a maximum one month depending on the growth rate of fungi. Plates were observed every 3 days then once per week after 2 weeks for the emergence of fungal hyphae. Pure cultures of all fungi were established by single hyphal tip isolation (SH) then single-spore (SS) and grown on 10% vegetable juice agar (VA) (100 ml Campbell's V8 juice, 0.24 g calcium carbonate, 18 g Bacto agar, and 900 ml of water). For *Fusarium* isolates, the cultures were further purified by single-spore isolation (SS) on WA and three single-spore isolates were transferred to VA per single-tip isolate. Single-spore of *Fusarium* or single-tip of non *Fusarium* cultures were grown 5 to 10 days under constant cool fluorescent irradiation and tubed in 15 cm tubes with VA slants.

For long-term storage, isolates were stored on silica gel in small 13 X 100 mm screw-cap tubes. Tubes containing silica gel and 7% skimmed milk were sterilized and were shaked with the suspension of *Fusarium* spores of isolates and then refrigerated at 4°C in desiccated boxes.

3.2.3 Morphological identification

For morphological identification, the purified culture of fungus were grown on VA under continuous cool fluorescent irradiation at 23 to 25°C for 5 to 30 days and were routinely
examined microscopically for genus identification using a Baush and Laumb Dissection microscope and Olympus BX43 Manual System Microscope. *Fusarium* isolates were further identified to species using Leslie and Summerell (2006). Single-spore cultures of putative *Fusarium* were grown on carnation leaf agar (CLA solidified, 2% W A with 4-6 sterile carnation leaf pieces added to the surface) and Potato dextrose agar (PDA, 39 g Difco potato dextrose agar, 1 g Bacto agar, and 1 liter distilled water), at 24°C under continuous fluorescent irradiation. Cultures were examined after 5-10 days for formation of microconidia in chains or false heads, and production of monophialides (either short or long) or polyphialides on WA and CLA. Chlamydosporic production was examined between 5 and 45 days WA and CLA. Isolates were cultured on CLA to assess sporodochia production, mesoconidia and microconidia formation and on PDA, to characterize reverse colony color.
3.2.4 Molecular Identification

For those *Fusarium* species that were present in high frequencies, molecular analysis was used to confirm morphological identification. For molecular analysis, the translation elongation factor *TEF la* gene was sequenced for one to five representative isolates from each morphological species.

Mycelium was grown for 3-4 days on 10% VA, at 24°C with continuous cool fluorescent irradiation. Total genomic DNA was isolated from mycelial tissues using the phenol-chloroform extraction method (Srivastava A., 2013). Approximately 3-5 mg of mycelium was gently scrapped with a sterile spatula and was grown for 3 days in 50 ml of PDB (20 gram in a 1000 ml) media in an incubator shaker at 25°C. Approximately 0.2 g of mycelium was harvested, filtered and semi-dried with paper towels, and ground into fine powder with a mortar and pestle in the presence of liquid nitrogen. The samples were mixed with 900 µl of extraction buffer (0.7 M NaCl, 50 mM Tris-HCl, pH 8.0, and 10 mM EDTA) and 10 µl of Rnase A (Sigma-Aldrich) at 100 mg/ml and incubated at 60°C for 30 min with intermittent mixing. Water (900µl) was added to the tube and DNA was extracted with 2 ml of Tris-saturated phenol, followed by chloroform extraction. DNA was separated from the solution by adding a 0.75v/v of volume of isopropanol at room temperature followed by centrifuging at 1200 rpm for 10 min at room temperature. The pellet containing DNA was washed with 70% ethanol. After drying, DNA samples were dissolved in 80-100µl ultrapure nuclease free water (Gibco, Invitrogen Inc. Carlsbad, CA, U.S.A.). The quality and quantity of DNA was estimated by running them in 1% gel and taking spectrophotometric readings (Eppendorf AG, Germany) respectively.

*TEF I-α* primers efl (forward primer; 5’-ATGGGTAAGGA(A/G)GACAAGAC-3’) and ef2 (reverse primer; 5’-GGA(G/A)GTACCAGT(G/C)ATCATGT-3’ (Geiser *et al*., 2004) were
used to amplify an approximately 690 base pairs (bp) portion of the TEF I-a gene by polymerase chain reaction (PCR) in a Mastercycler gradient 5331 thermal cycle (Ependorf, Westbury, New York). PCR conditions consisted of initial denaturation for 5 minutes at 95°C, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 1:30 minutes, and extension for 2 minutes at 72 °C, with a final extension at 72 °C for 7 minutes, and an ending storage temperature of 4°C. PCR products were purified (USB ExoSAP-IT, Affymetrix Inc) and used for sequencing. Sequences were generated with the SP6 reverse primer, using an ABI 3730 DNA analyzer, and sequence homologies were determined by Basic Local Alignment Search Tool (BLAST) search in the FUSARIUM database 1.0 curated at University of Pennsylvania and National Centre for Biotechnology Information (NCBI) database. Only those BLAST hit was considered for identification that aligned at 95-100% identity with one or more accessions with strong supporting E-value of ≥ 0.

3.2.5 Pathogenicity Tests

Pathogenicity tests were conducted on Dendrobium (UH 800), Valerie Cymbidium (Valerie Absolonova 57x valley Zenith 'Concord' #1), Miltonia (Milt. (Amber X Lynne Waihee) ‘Cream Puff’) and potted Cattleya. Plants were kept in a screened, solid covered glasshouse. Plants were watered at least once per day, and fertilized with Osmocote 14-14-14, a slow release fertilizer, every month. Dendrobiums were transplanted every six months to encourage young shoot production.

In foliar pathogenicity tests, plants were selected which had a young, partially unfolded shoot. Foliar spray method and glass fiber disc method were used for the inoculation. In the glass fiber disc method, sterilized glass fiber discs of 10 mm diameter (Glass fiber prefilters,
Millipore) were used for the inoculation. Conidial suspension of *Fusarium* isolates were used at a concentration of $10^7$ conidia per ml. Glass fiber discs were dipped into the suspension and then put on the youngest three leaves of a young shoot. Two discs were used on each leaf, one on the more mature expanded half of the leaf and another on the younger base of leaf to determine which part is more susceptible to the pathogen. For testing each isolate, had three replicates, and a control was used, and the youngest three leaves were inoculated (two sites on each leaf were inoculated, one on the expanded side and another near the base of leaf), and each test was repeated twice for *Dendrobium* and *Cattleya* while test was repeated once for *Cymbidium* and *Miltonia*.

In the foliar spray method, young shoot of host plant was sprayed with the conidial suspension of *Fusarium* isolate, at a concentration of $10^7$ conidia per ml until the inoculum dripped (about 15 ml per plant). The inoculated plants were placed for 48 hours in plastic bags for humidity for the conidia germination and the penetration of the leaf surfaces. After 48 hours plants were unbagged and observed every 4\textsuperscript{th} day for the production of symptoms. A scale was developed for *Dendrobium* for the rating of symptoms having a range of 0 to 5 (Fig. 3.1):

**Level 0**: Healthy shoot with no symptoms.

**Level 1**: Less than 5 spots per inoculated leaf.

**Level 2**: 5-10 spots per inoculated leaf.

**Level 3**: More than 10 spots or blight per inoculated leaf.

**Level 4**: Initiation of leaf base rot or leaf sheath rot.

**Level 5**: Leaf base rot and wilt of youngest 1 to 2 leaves.
Fig. 3.1 Symptom range showing different levels of disease on *Dendrobium*. (Level 0) Healthy shoot (Level 1) Less than five chlorotic spots that formed necrotic, spots with time. (Level 2) Five to ten necrotic spots. (Level 3) More than ten necrotic spots or blight. (Level 4) Initiation of leaf rot and leaf sheath rot. (Level 5) Leaf base rot and wilt of youngest leaf.

Observations were made for 12 weeks. Then the observations were tabulated and analyzed. Throughout the pathogenicity tests, plants were kept in the same type of environment. Beginning 2 days after inoculation, plants were watered daily, and fertilized monthly with Osmocote 14-14-14. At the end of each test, isolations were made from all symptoms produced in the test, to accurately evaluate whether the *Fusarium* species inoculated was the cause of disease. Isolations were conducted as described above. Plates were incubated at room temperature with cool fluorescent light. Single tip or single-spore cultures were prepared for each isolate for identification and grown from WA, CLA and PDA.
3.3. Results

3.3.1 High frequency of *Fusarium* species.

From 60 diseased plants a total of 480 isolates were obtained for identification. Based on morphological characteristics and TEF1α gene sequence analysis, 11 *Fusarium* species were identified from different orchid genera (Table 3.1 and Fig 3.2). A strong relationship exists between the diseased orchids and isolated *Fusarium* species (Table 3.3). Among the different species of *Fusarium* identified, *F. oxysporum* was the most common species. It was isolated from 9 genera of orchids showing symptoms of disease. The second most common *Fusarium* species was *F. proliferatum* which was present in 6 genera. However it was interesting to note that in terms of number of isolates *F. proliferatum* was most prominent with maximum number of 209 out of 480 isolates and registered presence in nearly 50% of total plant samples and were closely followed by *F. oxysporum*, which was present in 141 isolates making up nearly 30% of total isolates. *Fusarium solani* with 16% percent of isolates was third most frequently isolated *Fusarium* species (Table 3.1).

*Cymbidium* was host of 7 of 11 *Fusarium* species while *Dendrobium* and *Phalaenopsis* were the second most infected orchids with *Fusarium* having 6 of 11 *Fusarium* species. However *Dendrobium* registered maximum number of isolates of *F. proliferatum* indicating that they were very susceptible to *F. proliferatum*. Interestingly, *Cattleya* does not appear to be a host of many *Fusarium* species but is very prone to infection by *F. oxysporum* (Table 3.1to 3.4).
Table 3.1 *Fusarium* species isolated from different orchid samples.

<table>
<thead>
<tr>
<th><em>Fusarium</em> sp.</th>
<th>No. of pathogens</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. proliferatum</em></td>
<td>209</td>
<td>43.5</td>
</tr>
<tr>
<td><em>F. oxysporum</em></td>
<td>141</td>
<td>29.4</td>
</tr>
<tr>
<td><em>F. solani</em></td>
<td>81</td>
<td>16.9</td>
</tr>
<tr>
<td><em>F. begoniae</em></td>
<td>21</td>
<td>4.38</td>
</tr>
<tr>
<td><em>F. poae</em></td>
<td>10</td>
<td>2.08</td>
</tr>
<tr>
<td><em>F. circinatum</em></td>
<td>4</td>
<td>0.83</td>
</tr>
<tr>
<td><em>F. subglutinans</em></td>
<td>4</td>
<td>0.83</td>
</tr>
<tr>
<td><em>F. anthophilum</em></td>
<td>3</td>
<td>0.63</td>
</tr>
<tr>
<td><em>F. bulbicola</em></td>
<td>3</td>
<td>0.63</td>
</tr>
<tr>
<td><em>F. denticulatum</em></td>
<td>2</td>
<td>0.42</td>
</tr>
<tr>
<td><em>F. semitectum</em></td>
<td>2</td>
<td>0.42</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>480</strong></td>
<td><strong>100</strong></td>
</tr>
</tbody>
</table>

*Fig. 3.2:* The distribution of *Fusarium* species isolated from orchids. The isolates were identified on the basis of morphological characteristics and by sequencing conserved translation elongation factor (TEF1α) gene followed by using Basic Local Alignment Search Tool (BLAST) for aligning to the reference database curated at NCBI and Fungal DB version 1.0 database. Sequence with 95-100% identity and E value \( \geq 0.00 \) were only considered for identification.
*Fusarium proliferatum* isolates were morphologically characterized by three to four septate slender thin-walled relatively straight macroconidia and production of microconidia in chains from polyphialides (Fig. 3.3). Chlamydospores were absent and generally violet pigments were produced on PDA as reverse colony color.

**Fig. 3.3** Important morphological features used for identification of *Fusarium proliferatum*. (A) Macroconidia are slender, almost straight and usually 3 to 4 septate and are found in pale orange sporodochia; (B) Microconidia are club shaped with a flattened base and nonseptate; (C) Microconidia *in situ* on false heads and chains as indicated by arrows. (D) Polyphialides as indicated by arrows. Scale bar for A-C = 50 μm and for D = 25 μm.
*Fusarium oxysporum* isolates were characterized by the production of single celled and terminal or intercalary chlamydospores and microconidia in false heads on short monophialides with usually three septate straight to slightly curved and thin walled macroconidia on CLA (Fig. 3.4). Chlamydospores formed in macroconidia also. Colony morphology on PDA varies widely and isolates produce white to pale violet pigmentation as reverse colony color.

Fig. 3.4. Important morphological features used for identification of *Fusarium oxysporum*. (A) Macroconidia are short to medium in length, falcate to almost straight, thin walled and usually 3 septate and are found in pale orange with usually abundant sporodochia; (B) Microconidia are usually nonseptate, oval, elliptical or reniform; (C) Microconidia *in situ* in false heads on short monophialides as indicated by arrow. (D) Abundant chlamydospores as indicated by arrow, (E) Chlamydospores shown in inset are terminal and single celled. Scale bar for A-D = 50 μm and for E = 25 μm.
*Fusarium solani* isolates were characterized by the production of 2-celled and terminal chlamydospores on short lateral branches and microconidia in false heads on long monophialides with characteristic usually five to seven septate relatively wide, straight, stout and robust macroconidia (Fig. 3.5). White to cream cultures were usually found on PDA sometimes light greenish pigment was also found on reverse colony color.

![Image of morphological features](image-url)

**Fig. 3.5** Important morphological features used for identification of *Fusarium solani*. (A) Macroconidia are relatively straight to slightly curved and usually 3 to 7 septate with rounded ends and are found abundantly in cream and less frequently in blue or green sporodochia (B) Microconidia are oval, ellipsoidal or reniform, 0 to 3 septate, (C) Microconidia *in situ* on CLA in false heads on long monophialides as indicated by arrow. (D) Abundant chlamydospores are one or two celled and terminal as indicated by arrow. Scale bar for A-D = 50 μm
Key characters to identify *F. poae* were globose to napiform microconidia, non-septate to one septate microconidia produced on urn-shaped monophialides with a distinct collarette. The short, branched conidiophore gave the appearance of a “bunch of grapes”. Reverse colony pigmentation on PDA was reddish-brown. Macroconidia were rare and found on the carnation leaves and the surface of both CLA and PDA. Macroconidia were slender, relatively short, falcate, usually three to four septate with curved and tapering apical cell and well developed foot cell (Fig. 3.6).

**Fig. 3.6.** Important morphological features used for identification of *Fusarium poae*. (A) Macroconidia are rare, relatively short, falcate and 1 to 3 septate; as indicated by arrow (B) Microconidia are globose to napiform and non-septate as indicated by arrow; (C) Microconidia *in situ* on CLA are found on urn shaped monophialides and appears to be a “bunch of grapes” as indicated by arrow. (D, E) Microconidia *in situ* on CLA are found on urn shaped monophialides with a distinct collarette and appears to be a “bunch of grapes” as indicated by arrows. **A to D:** Scale bar for A-D = 50 μm and for E = 25 μm
The most distinctive character to identify *F. semitectum* was the abundant production of straight, spindle-shaped mesoconidia from polyphialides in the aerial mycelia that gave the “rabbit ear” appearance (Fig. 3.7). Off white to beige or brown pigmentation appeared on PDA.

**Fig. 3.7.** Important morphological features used for identification of *Fusarium semitectum.* (A) Macroconidia are slightly curved and formed in orange sporodochia and usually 3 to 5 septate and usually rare as indicated by black arrows. Mesoconidia are straight, spindle shaped and usually 3 to 5 septate as indicated by red arrows; (B) Mesoconidia *in situ* on CLA and are found in the aerial mycelia and often have the appearance of “rabbit ears” as indicated by arrow. Scale for A-B = 50 µm.
The key character to identify *F. denticulatum* were the formation of microconidia and mesoconidia from denticulate (finger like) polyphialides in the aerial mycelia. Macroconidia were slender, slightly falcate and thin walled, usually 3 to 4 septate and are found in orange sporodochia (Fig. 3.8). Colony color as well as the reverse colony color were orange on PDA.

**Fig. 3.8.** Important morphological features used for identification of *Fusarium denticulatum*. (A) Macroconidia are slender, slightly falcate and thin walled, usually 3 to 4 septate and are found in orange sporodochia, (B) Microconidia are long oval to obovoid and 0 to 1 septate as indicated by arrow, (C) Microconidia *in situ* on CLA are found on false heads from monophialides. (D) Denticulate (finger-like) mono and polyphialides as indicated by arrow. Scale bar for A-C = 50 μm and for D = 25 μm.
**Fusarium subglutinans-like or GFSC (Gibberella fuzikuroi species complex):** Isolates produced relatively slender, thin and slightly curved macroconidia that are typical with GFSC with microconidia in false heads from polyphialides and chlamydospores were absent in general. However, each species showed some other specific morphological characters that differentiated them from each other, such as prostrate or erect mycelium, hyphal coils etc. Five species of GFSC (*F. subglutinans, F. anthophilum, F. begonia, F. bulbicola* and *F. circinatum*) were seperated based upon these characteristics.
*Fusarium subglutinans* produced relatively slender, thin and slightly curved usually 3-septate macroconidia that are typical with GFSC with abundant oval, non-septate microconidia in false heads from mono and polyphialides on aerial mycelia and chlamydospores were absent (Fig. 3.9). Initially abundant mycelial growth was white that turned violet as the culture ages with violet reverse colony color.

**Fig. 3.9.** Important morphological features used for identification of *Fusarium subglutinans*. (A) Macroconidia are slender, slightly falcate and thin walled, usually 3 to 4 septate and are found in tan to orange sporodochia. (B) Microconidia are oval shaped and nonseptate. (C, D) Microconidia *in situ* on CLA are found on false heads from mono- and polyphialides as indicated by arrow in D and E. Scale bar for A-D = 50 μm and for E = 25 μm.
*Fusarium anthophilum*, produced relatively slender, thin and slightly curved macroconidia that are typical with GFSC. Microconidia were the most important character, with globose or pyriform, 0–septate microconidia, formed on monophialides and branched and unbranched polyphialides. False heads were present but chains were absent (Fig. 3.10). Violet pigments were found on PDA as reverse colony color.

![Fig. 3.10 Important morphological features used for identification of *Fusarium anthophilum*.](image-url)

(A, B) Macroconidia are slender, slightly falcate and thin walled, usually 3 to 4 septate, (C) Ovoid and globose microconidia *in situ* on CLA on monophialides and polyphialides as indicated by arrows, (D) Long-oval microconidia as indicated by arrow; (E) Globose microconidia *in situ* on CLA as indicated by arrow; (F) Microconidia are globose and nonseptate as indicated by arrow. Scale bar for A-F = 50 μm.
*Fusarium begoniae* produced relatively slender, thin and slightly curved macroconidia that are typical with GFSC and nonseptate, abundant obovoid microconidia produced on both monophialides and polyphialides in false heads. Prostrate form and limited branching of the phialides were important characters (Fig. 3.11). White mycelium with grayish-yellow pigmentation was formed on PDA as reverse colony color.

![Images of macroconidia and microconidia](image)

**Fig. 3.11.** Important morphological features used for identification of *Fusarium begoniae* (A) Macroconidia are slender, slightly falcate and thin walled, usually 3-4 septate, found in tan to orange sporodochia, (B) Microconidia are oval to obovoid and usually nonseptate but occasionally 1 to 2 septate, (C, D) Microconidia in situ on CLA are found on false heads from mono and polyphialides usually found on prostrate mycelium as indicated by arrows. Scale bar for A-C = 50 μm and for D = 25 μm.
*Fusarium bulbicola* produced relatively slender, thin and slightly curved macroconidia that are typical with GFSC. Nonseptate obovoid microconidia were abundant and produced on both monophialides and polyphialides in false heads. Their aerial mycelia were mostly erect and frequently branched (Fig. 3.12). White mycelium with reddish pigmentation as reverse colony color on PDA was produced.

**Fig. 3.12.** Important morphological features used for identification of *Fusarium bulbicola*. (A) Macroconidia are slender, slightly falcate and thin walled, usually 3 to 4 septate and are found in tan to orange sporodochia as indicated by arrow, (B) Microconidia are long oval to obovoid and 0 to 1 septate as indicated by arrow; (C, D) Microconidia *in situ* on CLA are found on false heads from mono- and polyphialides usually found on erect mycelium. Scale bar for A-D = 50 μm.
Macroconidia were typical with GFSC and found on sporodochia that were rare and sparse. Microconidia were produced on erect false heads from mono and polyphialides. The key character to identify *F. circinatum* was the formation of abundant coiled hyphae from which the species epithet is derived (Fig. 3.13). Coiled hyphae is also found in *F. pseudocircinatum* but microconidia sometimes form short chains on prostrate conidiophores that were not found in these isolates (Leslie and Summerell, 2006). White mycelium with violet pigmentation is found on PDA that grows relatively rapidly as reverse colony color.

Fig. 3.13. Important morphological features used for identification of *Fusarium circinatum*. (A) Macroconidia are slender, slightly falcate and thin walled, usually 3 septate, (B) Microconidia are long oval to obovoid and non-septate. (C) Microconidia in *situ* on CLA showing mono and polyphialides. (D) Showing extensive proliferation of polyphialides on slide. E: Hyphal coils as indicated by arrows. Scale bar for A, B, C and E = 50µm, and for D= 25 µm.
### Table 3.2 Summary of morphological characteristics of different *Fusarium* species.

<table>
<thead>
<tr>
<th>Macroconidia</th>
<th>Microconidia</th>
<th>Chlamydospores</th>
<th>Colony</th>
<th>Specific character</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Size μ</strong></td>
<td><strong>Shape</strong></td>
<td><strong>Septa</strong></td>
<td><strong>Apical</strong></td>
<td><strong>Foot</strong></td>
<td><strong>FH/Ch</strong></td>
</tr>
<tr>
<td>35-40 X</td>
<td>Thin walled, relatively straight</td>
<td>3 to 4</td>
<td>Curved</td>
<td>poorly developed</td>
<td>Conidia in chain</td>
</tr>
<tr>
<td>25-35 X</td>
<td>Thin walled, relatively straight</td>
<td>3 to 4</td>
<td>Curved</td>
<td>poorly developed</td>
<td>Conidia in chains</td>
</tr>
<tr>
<td>55-60 X</td>
<td>Wide, straight, stout &amp; thick walled</td>
<td>3 to 6</td>
<td>Blunt &amp; rounded</td>
<td>Foot shaped</td>
<td>False heads</td>
</tr>
<tr>
<td>35-40 X</td>
<td>Relatively slender, curved upper side straight underside</td>
<td>3 to 5</td>
<td>Pointed</td>
<td>Foot shaped</td>
<td>False heads</td>
</tr>
<tr>
<td>45-49 X</td>
<td>Relatively slender</td>
<td>3 to 4</td>
<td>Curved</td>
<td>Foot shaped</td>
<td>False heads</td>
</tr>
<tr>
<td>55 X</td>
<td>Pointed thin</td>
<td>3 to 4</td>
<td>Curved</td>
<td>pointed</td>
<td>False heads</td>
</tr>
<tr>
<td>40-42 X</td>
<td>Pointed thin</td>
<td>3 to 4</td>
<td>Curved</td>
<td>pointed</td>
<td>False heads</td>
</tr>
<tr>
<td>40 X</td>
<td>Pointed thin</td>
<td>3 to 4</td>
<td>Curved</td>
<td>pointed</td>
<td>False heads</td>
</tr>
<tr>
<td>30-36 X</td>
<td>Relatively slender, falcate, thin walled</td>
<td>3 to 4</td>
<td>Elongate and tapering</td>
<td>poorly developed</td>
<td>False heads</td>
</tr>
<tr>
<td>30-36 X</td>
<td>Relatively slender, falcate, thin walled</td>
<td>3 to 4</td>
<td>Curved</td>
<td>poorly developed</td>
<td>False heads</td>
</tr>
<tr>
<td>30-36 X</td>
<td>Relatively slender, falcate, thin walled</td>
<td>3 to 4</td>
<td>Curved</td>
<td>poorly developed</td>
<td>False heads</td>
</tr>
</tbody>
</table>

1. False heads (FH) and presence of chains (Ch).
2. Average length and width.
3. Monophialides (M) and polyphialides (P).
4. Absent (A) and present (P).
5. Reverse colony color on PDA.
Table 3.3 *Fusarium* species isolated from nine genera of orchids.

<table>
<thead>
<tr>
<th>Fusarium sp. (*)</th>
<th>Dendrobium</th>
<th>Cymbidium</th>
<th>Miltonia</th>
<th>Oncidium</th>
<th>Phalaenopsis</th>
<th>Cattleya</th>
<th>Brassolaelio cattleya</th>
<th>Vuylstekaria</th>
<th>Valerie</th>
<th>Cymbidium</th>
</tr>
</thead>
<tbody>
<tr>
<td>F. anthophilum (2)</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>F. begoniae (3)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>14</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>F. bulbicola (1)</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>F. circinatum (2)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>F. denticulatum (1)</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>F. oxysporum (9)</td>
<td>10</td>
<td>1</td>
<td>15</td>
<td>11</td>
<td>10</td>
<td>40</td>
<td>2</td>
<td>24</td>
<td>26</td>
<td>0</td>
</tr>
<tr>
<td>F. poae (3)</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>F. proliferatum (6)</td>
<td>162</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>17</td>
<td>4</td>
<td>5</td>
<td>16</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>F. semitectum (1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>F. solani (5)</td>
<td>5</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>33</td>
<td>0</td>
<td>17</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>F. subglutinans (2)</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Number of plants having Fusarium species</td>
<td>5</td>
<td>7</td>
<td>1</td>
<td>3</td>
<td>6</td>
<td>2</td>
<td>5</td>
<td>4</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

*: Number of orchid genera from which the *Fusarium* species was isolated.
Table 3.4: Identification of *Fusarium* species isolated from diseased orchids.

<table>
<thead>
<tr>
<th>Isolate No.</th>
<th>Morphological</th>
<th>NCBI BLAST Result&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Accession&lt;sup&gt;*c&lt;/sup&gt;</th>
<th><em>Fusarium</em> Database Result&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Accession No.*</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK-273</td>
<td><em>F. solani</em></td>
<td><em>F. solani</em></td>
<td>DQ247447.1</td>
<td><em>F. solani</em></td>
<td>FD_01371_EF-1a</td>
<td><em>F. solani</em></td>
</tr>
<tr>
<td>SK-275</td>
<td><em>F. anthophilum</em></td>
<td><em>F. anthophilum</em></td>
<td>AF160292.1</td>
<td><em>F. anthophilum</em></td>
<td>FD_01297_EF-1a</td>
<td><em>F. anthophilum</em></td>
</tr>
<tr>
<td>SK-359&lt;sup&gt;#&lt;/sup&gt;</td>
<td><em>F. semitectum</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td><em>F. semitectum</em></td>
</tr>
<tr>
<td>SK-499</td>
<td><em>F. proliferatum</em></td>
<td><em>F. proliferatum</em></td>
<td>AF336913.1</td>
<td><em>F. proliferatum</em></td>
<td>FD_01380_EF-1a</td>
<td><em>F. proliferatum</em></td>
</tr>
<tr>
<td>SK-533</td>
<td><em>F. poae</em></td>
<td><em>F. poae</em></td>
<td>HE802674.1</td>
<td><em>F. poae</em></td>
<td>FD_01308_EF-1a</td>
<td><em>F. poae</em></td>
</tr>
<tr>
<td>SK-535</td>
<td><em>F. denticulatum</em></td>
<td><em>F. denticulatum</em></td>
<td>AF160269.1</td>
<td><em>F. denticulatum</em></td>
<td>FD_01178_EF-1a</td>
<td><em>F. denticulatum</em></td>
</tr>
<tr>
<td>SK-556</td>
<td><em>F. begonia</em></td>
<td><em>F. begoniae</em></td>
<td>AF160293.1</td>
<td><em>F. begoniae</em></td>
<td>FD_00940_EF-1a</td>
<td><em>F. begoniae</em></td>
</tr>
<tr>
<td>SK-570</td>
<td><em>F. oxysporum</em></td>
<td><em>F. oxysporum</em></td>
<td>JQ809659.1</td>
<td><em>F. oxysporum SC</em></td>
<td>FD_00442_EF_1a</td>
<td><em>F. oxysporum</em></td>
</tr>
<tr>
<td>SK-661</td>
<td><em>F. bulbicola</em></td>
<td><em>F. bulbicola</em></td>
<td>AF160294.1</td>
<td><em>F. bulbicola</em></td>
<td>FD_01295_EF-1a</td>
<td><em>F. bulbicola</em></td>
</tr>
<tr>
<td>SK-925</td>
<td><em>F. subglutinans</em></td>
<td><em>F. subglutinans</em></td>
<td>JX867945.1</td>
<td><em>F. bulbicola</em></td>
<td>FD_01295_EF-1a</td>
<td><em>F. subglutinans</em></td>
</tr>
<tr>
<td>SK-955</td>
<td><em>F. circinatum</em></td>
<td><em>F. circinatum</em></td>
<td>KC514058.1</td>
<td><em>F. circinatum</em></td>
<td>FD_01034_EF-1a</td>
<td><em>F. circinatum</em></td>
</tr>
</tbody>
</table>

<sup>a</sup> Identification of isolates on the basis of their morphological characteristics observed under the microscope and colony characteristics.

<sup>b</sup> Conserved TEF1a gene was amplified and sequenced from isolates and aligned to NCBI database using algorithm Basic Local Alignment Search Tool (BLAST). The BLAST hit with maximum identity was considered for identification.

<sup>c</sup> Accession number of identified isolate in NCBI Genbank.

<sup>d</sup> To further ascertain finding the TEF1a sequence were also blasted with *Fusarium* specific database curated at www.isolate.Fusariumdb.org

<sup>#</sup> This isolate was not identified using molecular tools however, two separate studies done at Uchida’s lab and in Fusarium Workshop at Kansas State University have identified it on the basis of morphological structures.

* E value ≥ 0

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3.3.2 Pathogenicity Results

_Fusarium proliferatum, F. oxysporum and F. solani_ were pathogenic on _Dendrobium_, _Cymbidium_ and _Miltonia_. _Fusarium proliferatum_ was the most common and moderate to highly virulent followed by _F. oxysporum_ and _F. solani_ on _Dendrobiums_, while _F. oxysporum_ and _F. solani_ caused moderate to moderately high virulence on _Cymbidiums_ and _Miltonia_. _Fusarium oxysporum_ caused high virulence on _Cattleya_ while, the isolates of _F. proliferatum_ and _F. solani_ did not produced any disease symptoms on _Cattleya_ (Table 3.5 to 3.9).

Table 3.5: _Fusarium_ isolates tested for pathogenicity on _Dendrobium*X.

<table>
<thead>
<tr>
<th><em>Fusarium</em> species</th>
<th>Isolate No.</th>
<th>Host isolated from</th>
<th>Symp. Levely</th>
<th>Virulence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. proliferatum</em></td>
<td>SK-165</td>
<td><em>Dendrobium</em></td>
<td>5</td>
<td>High</td>
</tr>
<tr>
<td><em>F. proliferatum</em></td>
<td>SK-259</td>
<td><em>Dendrobium</em></td>
<td>5</td>
<td>High</td>
</tr>
<tr>
<td><em>F. proliferatum</em></td>
<td>SK-499</td>
<td><em>Dendrobium</em></td>
<td>5</td>
<td>High</td>
</tr>
<tr>
<td><em>F. proliferatum</em></td>
<td>SK-524</td>
<td><em>Dendrobium</em></td>
<td>5</td>
<td>High</td>
</tr>
<tr>
<td><em>F. proliferatum</em></td>
<td>SK-975</td>
<td><em>Dendrobium</em></td>
<td>5</td>
<td>High</td>
</tr>
<tr>
<td><em>F. proliferatum</em></td>
<td>SK-268</td>
<td><em>Cymbidium</em></td>
<td>4</td>
<td>Moderately high</td>
</tr>
<tr>
<td><em>F. proliferatum</em></td>
<td>SK-1024</td>
<td><em>Vuylstkearea</em></td>
<td>3</td>
<td>Moderate</td>
</tr>
<tr>
<td><em>F. oxysporum</em></td>
<td>SK-570</td>
<td><em>Cattleya</em></td>
<td>5</td>
<td>High</td>
</tr>
<tr>
<td><em>F. oxysporum</em></td>
<td>SK-601</td>
<td><em>Cattleya</em></td>
<td>0</td>
<td>No infection</td>
</tr>
<tr>
<td><em>F. oxysporum</em></td>
<td>SK-624</td>
<td><em>Dendrobium</em></td>
<td>5</td>
<td>High</td>
</tr>
<tr>
<td><em>F. oxysporum</em></td>
<td>SK-727</td>
<td><em>Phalaenopsis</em></td>
<td>3</td>
<td>Moderate</td>
</tr>
<tr>
<td><em>F. oxysporum</em></td>
<td>SK-819</td>
<td><em>Dendrobium</em></td>
<td>5</td>
<td>High</td>
</tr>
<tr>
<td><em>F. oxysporum</em></td>
<td>SK-985</td>
<td><em>Miltonia</em></td>
<td>2</td>
<td>Low</td>
</tr>
<tr>
<td><em>F. oxysporum</em></td>
<td>SK-826</td>
<td><em>Dendrobium</em></td>
<td>5</td>
<td>High</td>
</tr>
<tr>
<td><em>F. solani</em></td>
<td>SK-625</td>
<td><em>Dendrobium</em></td>
<td>5</td>
<td>High</td>
</tr>
<tr>
<td><em>F. solani</em></td>
<td>SK-645</td>
<td><em>Nobile Dendrobium</em></td>
<td>4</td>
<td>Moderately high</td>
</tr>
<tr>
<td><em>F. solani</em></td>
<td>SK-358</td>
<td><em>Brassolaeliocattleya</em></td>
<td>4</td>
<td>Moderately high</td>
</tr>
<tr>
<td><em>F. solani</em></td>
<td>SK-362</td>
<td><em>Brassolaeliocattleya</em></td>
<td>5</td>
<td>High</td>
</tr>
<tr>
<td><em>F. solani</em></td>
<td>SK-360</td>
<td><em>Brassolaeliocattleya</em></td>
<td>0</td>
<td>No infection</td>
</tr>
<tr>
<td><em>F. solani</em></td>
<td>SK-1066</td>
<td><em>Vuylstkearea</em></td>
<td>0</td>
<td>No infection</td>
</tr>
<tr>
<td><em>F. solani</em></td>
<td>SK-273</td>
<td><em>Cymbidium</em></td>
<td>3</td>
<td>Moderate</td>
</tr>
<tr>
<td><em>Fusarium</em> species</td>
<td>Isolate No.</td>
<td>Host isolated from</td>
<td>Symp. Level\textsuperscript{Y}</td>
<td>Virulence\textsuperscript{Z}</td>
</tr>
<tr>
<td>-------------------</td>
<td>-------------</td>
<td>--------------------------</td>
<td>-------------------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td><em>F. poae</em></td>
<td>SK-533</td>
<td>Cymbidium</td>
<td>3</td>
<td>Moderate</td>
</tr>
<tr>
<td><em>F. poae</em></td>
<td>SK-532</td>
<td>Cymbidium</td>
<td>2</td>
<td>Low</td>
</tr>
<tr>
<td><em>F. poae</em></td>
<td>SK-282</td>
<td>Brassolaeliocattleya</td>
<td>2</td>
<td>Low</td>
</tr>
<tr>
<td><em>F. poae</em></td>
<td>SK-290</td>
<td>Brassolaeliocattleya</td>
<td>0</td>
<td>No infection</td>
</tr>
<tr>
<td><em>F. poae</em></td>
<td>SK-639</td>
<td>Nobile Dendrobium</td>
<td>1</td>
<td>Very low</td>
</tr>
<tr>
<td><em>F. begoniae</em></td>
<td>SK-937</td>
<td>Oncidium</td>
<td>2</td>
<td>Low</td>
</tr>
<tr>
<td><em>F. begoniae</em></td>
<td>SK-1013</td>
<td>Vuylstekeara</td>
<td>1</td>
<td>Very low</td>
</tr>
<tr>
<td><em>F. bulbicola</em></td>
<td>SK-659</td>
<td>Nobile Dendrobium</td>
<td>0</td>
<td>No infection</td>
</tr>
<tr>
<td><em>F. bulbicola</em></td>
<td>SK-661</td>
<td>Nobile Dendrobium</td>
<td>0</td>
<td>No infection</td>
</tr>
<tr>
<td><em>F. anthophilum</em></td>
<td>SK-275</td>
<td>Cymbidium</td>
<td>0</td>
<td>No infection</td>
</tr>
<tr>
<td><em>F. anthophilum</em></td>
<td>SK-936</td>
<td>Oncidium</td>
<td>0</td>
<td>No infection</td>
</tr>
<tr>
<td><em>F. circinatum</em></td>
<td>SK-955</td>
<td>Phalaenopsis</td>
<td>1</td>
<td>Very low</td>
</tr>
<tr>
<td><em>F. circinatum</em></td>
<td>SK-1051</td>
<td>Cymbidium</td>
<td>2</td>
<td>Low</td>
</tr>
<tr>
<td><em>F. denticulatum</em></td>
<td>SK-525</td>
<td>Cymbidium</td>
<td>0</td>
<td>No infection</td>
</tr>
<tr>
<td><em>F. denticulatum</em></td>
<td>SK-535</td>
<td>Cymbidium</td>
<td>0</td>
<td>No infection</td>
</tr>
<tr>
<td><em>F. semitectum</em></td>
<td>SK-355</td>
<td>Brassolaeliocattleya</td>
<td>0</td>
<td>No infection</td>
</tr>
<tr>
<td><em>F. semitectum</em></td>
<td>SK-359</td>
<td>Brassolaeliocattleya</td>
<td>0</td>
<td>No infection</td>
</tr>
<tr>
<td><em>F. subglutinans</em></td>
<td>SK-925</td>
<td>Oncidium</td>
<td>0</td>
<td>No infection</td>
</tr>
</tbody>
</table>

\textsuperscript{X}: two sites on each plant were inoculated with $1 \times 10^7$ spores of fungi. The inoculated plants were kept in humid chamber for 48 hours. Observations for disease symptoms were recorded after 48 hours and 72 hours of inoculation and subsequently regularly at four days of interval. Symptoms levels were assigned based on the observations recorded at the end of three weeks after inoculation and virulence levels were made. All pathogenicity assays were repeated twice in triplicate.

\textsuperscript{Y}: A symptom scale was developed to assess the severity of disease. Each diseased plant was given a symptom level number between 0 to 5, where 0 is the healthy plant showing no disease symptom. Level 1 is given to plants that show less than five chlorotic spots that formed necrotic spots with time. Plants with five to ten necrotic spots are given symptom level 2. More than ten necrotic spots or blight containing plants showed symptom level of 3. Initiation of leaf rot and leaf sheath rot is given symptom level 4. Finally, plants with leaf base rot and wilt of youngest leaf showed symptom level 5. Figure 3.13 illustrates each of the symptom level.

\textsuperscript{Z}: Rating of virulence was based upon the scale of symptom levels shown below:

Level 0 = No infection; Level 1 = Very low virulence; Level 2 = Low virulence; Level 3 = Moderate virulence; Level 4 = Moderately high virulence and Level 5 = High virulence.
Table 3.6: *Fusarium* isolates tested for pathogenicity on *Cymbidium*.

<table>
<thead>
<tr>
<th><em>Fusarium</em> species</th>
<th>Isolate No.</th>
<th>Host isolated from</th>
<th>Symp. Level(^Y)</th>
<th>Virulence</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. solani</em></td>
<td>SK-273</td>
<td><em>Cymbidium</em></td>
<td>4</td>
<td>Moderately high</td>
</tr>
<tr>
<td><em>F. solani</em></td>
<td>SK-358</td>
<td><em>Brassolaeliocattleya</em></td>
<td>4</td>
<td>Moderately high</td>
</tr>
<tr>
<td><em>F. proliferatum</em></td>
<td>SK-499</td>
<td><em>Dendrobium</em></td>
<td>2</td>
<td>Low</td>
</tr>
<tr>
<td><em>F. proliferatum</em></td>
<td>SK-268</td>
<td><em>Cymbidium</em></td>
<td>4</td>
<td>Moderately high</td>
</tr>
<tr>
<td><em>F. poae</em></td>
<td>SK-533</td>
<td><em>Cymbidium</em></td>
<td>3</td>
<td>Moderate</td>
</tr>
<tr>
<td><em>F. circinatum</em></td>
<td>SK-1051</td>
<td><em>Cymbidium</em></td>
<td>5</td>
<td>High</td>
</tr>
<tr>
<td><em>F. circinatum</em></td>
<td>SK-1053</td>
<td><em>Cymbidium</em></td>
<td>5</td>
<td>High</td>
</tr>
<tr>
<td><em>F. oxysporum</em></td>
<td>SK-1037</td>
<td><em>Cymbidium</em></td>
<td>3</td>
<td>Moderate</td>
</tr>
<tr>
<td><em>F. oxysporum</em></td>
<td>SK-1032</td>
<td><em>Cymbidium</em></td>
<td>3</td>
<td>Moderate</td>
</tr>
<tr>
<td><em>F. proliferatum</em></td>
<td>SK-430</td>
<td><em>Cymbidium</em></td>
<td>2</td>
<td>Low</td>
</tr>
<tr>
<td><em>F. proliferatum</em></td>
<td>SK-268</td>
<td><em>Cymbidium</em></td>
<td>2</td>
<td>Low</td>
</tr>
<tr>
<td><em>F. denticulatum</em></td>
<td>SK-525</td>
<td><em>Cymbidium</em></td>
<td>0</td>
<td>No infection</td>
</tr>
<tr>
<td><em>F. denticulatum</em></td>
<td>SK-535</td>
<td><em>Cymbidium</em></td>
<td>0</td>
<td>No infection</td>
</tr>
</tbody>
</table>

\(^X\): two sites on each plant were inoculated with \(1 \times 10^7\) spores of fungi. The inoculated plants were kept in humid chamber for 48 hours. Observations for disease symptoms were recorded after 48 hours and 72 hours of inoculation and subsequently regularly at four days of interval. Symptoms levels were assigned based on the observations recorded at the end of three weeks after inoculation. All pathogenicity assays were repeated twice in triplicate.

\(^Y\): A symptom scale was developed to assess the severity of disease. Each diseased plant was given a symptom level number between 0 to 5, where 0 is the healthy plant showing no disease symptom. Level 1 is given to plants that show less than five chlorotic spots that formed necrotic spots with time. Plants with five to ten necrotic spots are given symptom level 2. More than ten necrotic spots or blight containing plants showed symptom level of 3. Initiation of leaf rot and leaf sheath rot is given symptom level 4. Finally, plants with leaf base rot and wilt of youngest leaf showed symptom level 5. Figure 3.13 illustrates each of the symptom level.

\(^Z\): Rating of virulence was based upon the scale of symptom levels shown below:

Level 0 = No infection; Level 1 = Very low virulence; Level 2 = Low virulence; Level 3 = Moderate virulence; Level 4 = Moderately high virulence and Level 5 = High virulence.
Table 3.7: *Fusarium* isolates tested for pathogenicity on *Miltonia*.

<table>
<thead>
<tr>
<th>Fusarium species</th>
<th>Isolate No.</th>
<th>Host isolated from</th>
<th>Symp. Level&lt;sup&gt;Y&lt;/sup&gt;</th>
<th>Virulence&lt;sup&gt;Z&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. solani</em></td>
<td>SK-1021</td>
<td>Vuylstekeara</td>
<td>3</td>
<td>Moderate</td>
</tr>
<tr>
<td><em>F. proliferatum</em></td>
<td>SK-1024</td>
<td>Vuylstekeara</td>
<td>3</td>
<td>Moderate</td>
</tr>
<tr>
<td><em>F. oxysporum</em></td>
<td>SK-985</td>
<td>Vuylstekeara</td>
<td>3</td>
<td>Moderate</td>
</tr>
<tr>
<td><em>F. oxysporum</em></td>
<td>SK-1009</td>
<td>Vuylstekeara</td>
<td>3</td>
<td>Moderate</td>
</tr>
<tr>
<td><em>F. proliferatum</em></td>
<td>SK-499</td>
<td>Dendrobium</td>
<td>1</td>
<td>Very low</td>
</tr>
<tr>
<td><em>F. begoniae</em></td>
<td>SK-1013</td>
<td>Vuylstekeara</td>
<td>2</td>
<td>Low</td>
</tr>
<tr>
<td><em>F. oxysporum</em></td>
<td>SK-1032</td>
<td>Vuylstekeara</td>
<td>0</td>
<td>No infection</td>
</tr>
<tr>
<td><em>F. poae</em></td>
<td>SK-533</td>
<td>Cymbidium</td>
<td>0</td>
<td>No infection</td>
</tr>
</tbody>
</table>

X: Two sites on each plant were inoculated with $1 \times 10^7$ spores of fungi. The inoculated plants were kept in a humid chamber for 48 hours. Observations for disease symptoms were recorded after 48 hours and 72 hours of inoculation and subsequently regularly at four days of interval. Symptoms levels were assigned based on the observations recorded at the end of three weeks after inoculation. All pathogenicity assays were repeated twice in triplicate.

Y: A symptom scale was developed to assess the severity of disease. Each diseased plant was given a symptom level number between 0 to 5, where 0 is the healthy plant showing no disease symptom. Level 1 is given to plants that show less than five chlorotic spots that formed necrotic spots with time. Plants with five to ten necrotic spots are given symptom level 2. More than ten necrotic spots or blight containing plants showed symptom level of 3. Initiation of leaf rot and leaf sheath rot is given symptom level 4. Finally, plants with leaf base rot and wilt of youngest leaf showed symptom level 5. Figure 3.13 illustrates each of the symptom level.

Z: Rating of virulence was based upon the scale of symptom levels shown below:

Level 0 = No infection; Level 1 = Very low virulence; Level 2 = Low virulence; Level 3 = Moderate virulence; Level 4 = Moderately high virulence and Level 5 = High virulence.
Table 3.8: *Fusarium* isolates tested for pathogenicity on *Cattleya*.

<table>
<thead>
<tr>
<th><em>Fusarium</em> species</th>
<th>Isolate No.</th>
<th>Host isolated from</th>
<th>Symp. Level(^Y)</th>
<th>Virulence(^Z)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. proliferatum</em></td>
<td>SK-579</td>
<td><em>Cattleya</em></td>
<td>0</td>
<td>No infection</td>
</tr>
<tr>
<td><em>F. oxysporum</em></td>
<td>SK-570</td>
<td><em>Cattleya</em></td>
<td>5</td>
<td>High</td>
</tr>
<tr>
<td><em>F. oxysporum</em></td>
<td>SK-601</td>
<td><em>Cattleya</em></td>
<td>5</td>
<td>High</td>
</tr>
<tr>
<td><em>F. proliferatum</em></td>
<td>SK-619</td>
<td><em>Cattleya</em></td>
<td>0</td>
<td>No infection</td>
</tr>
<tr>
<td><em>F. solani</em></td>
<td>SK-358</td>
<td><em>Brassolaeliocattleya</em></td>
<td>0</td>
<td>No infection</td>
</tr>
<tr>
<td><em>F. solani</em></td>
<td>SK-362</td>
<td><em>Brassolaeliocattleya</em></td>
<td>0</td>
<td>No infection</td>
</tr>
</tbody>
</table>

\(^X\): Two sites on each plant were inoculated with \(1 \times 10^7\) spores of fungi. The inoculated plants were kept in humid chamber for 48 hours. Observations for disease symptoms were recorded after 48 hours and 72 hours of inoculation and subsequently regularly at four days of interval. Symptoms levels were assigned based on the observations recorded at the end of three weeks after inoculation. All pathogenicity assays were repeated twice in triplicate.

\(^Y\): A symptom scale was developed to assess the severity of disease. Each diseased plant was given a symptom level number between 0 to 5, where 0 is the healthy plant showing no disease symptom. Level 1 is given to plants that show less than five chlorotic spots that formed necrotic spots with time. Plants with five to ten necrotic spots are given symptom level 2. More than ten necrotic spots or blight containing plants showed symptom level of 3. Initiation of leaf rot and leaf sheath rot is given symptom level 4. Finally, plants with leaf base rot and wilt of youngest leaf showed symptom level 5. Figure 3.13 illustrates each of the symptom level.

\(^Z\): Rating of virulence was based upon the scale of symptom levels shown below:

Level 0 = No infection; Level 1 = Very low virulence; Level 2 = Low virulence; Level 3 = Moderate virulence; Level 4 = Moderately high virulence and Level 5 = High virulence.
Table 3.9 Different *Fusarium* species isolated from the samples of Oahu and the Island of Hawaii.

<table>
<thead>
<tr>
<th>Fusarium sp.</th>
<th>Oahu</th>
<th>Island of Hawaii</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. anthophilum</em></td>
<td>✔</td>
<td></td>
</tr>
<tr>
<td><em>F. begoniae</em></td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td><em>F. bulbicola</em></td>
<td>✔</td>
<td></td>
</tr>
<tr>
<td><em>F. circinatum</em></td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td><em>F. denticulatum</em></td>
<td>✔</td>
<td></td>
</tr>
<tr>
<td><em>F. oxysporum</em></td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td><em>F. poae</em></td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td><em>F. proliferatum</em></td>
<td>✔</td>
<td></td>
</tr>
<tr>
<td><em>F. semitectum</em></td>
<td>✔</td>
<td></td>
</tr>
<tr>
<td><em>F. solani</em></td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td><em>F. subglutinans</em></td>
<td>✔</td>
<td></td>
</tr>
</tbody>
</table>

*Fusarium proliferatum*, *F. oxysporum* and *F. solani* produced typical leaf spots that sometimes coalesced to form blights that also progressed in to leaf sheaths. When the base of youngest leaves start rotting causing the wilting of youngest leaves occur and slowly the whole shoot become infected with in 6 to 12 weeks for *F. proliferatum*, *F. oxysporum* and *F. solani* on *Dendrobium* and Cymbidium (Fig. 3.14 and 15). *Cattleya* also developed leaf spots, leaf sheath rot and leaf base rot followed by wilt of youngest inoculated leaves. *Miltonia* produced leaf spots, leaf blight and marginal necrosis of leaf and leaf sheath. Leaf base rot and wilt was not produced in *Miltonia* even after 14 weeks (Fig. 3.16).
Fig 3.14 Foliar symptoms produced by *Fusarium* species on *Dendrobium*. (A) Healthy shoot. (B) Leaf spots (C) Leaf blight (D) Leaf sheath blight (E) Wilt of youngest leaves. (F and G) Showing progression of infection inside the stem.

Fig 3.15. Foliar symptoms on *Cymbidium* produced by *Fusarium* species (A) Healthy shoot (Rating = 0). (B) Less than five chlorotic spots that formed necrotic, spots with time (Rating = 1). (C) Five to ten necrotic spots (Rating = 2). (D) More than ten necrotic spots or blight (Rating = 3). (E) Initiation of leaf rot and leaf sheath rot (Rating = 4). (F) Leaf base rot and wilt of youngest leaf (Rating = 5).
Fig. 3.16. Foliar symptoms on *Miltonia* produced by *Fusarium* species (A) Healthy shoot (B) Less than five chlorotic spots that formed necrotic, spots with time. (C) Marginal necrosis on leaf. (D) Marginal necrosis progressing towards leaf sheath. (E) Severe necrotic leaf spots. (F) Leaf blight.

3.4 Discussion

Isolations made from all diseased plants representing 9 genera and hybrids collected from both islands showed the presence of *Fusarium* on them. Typical symptoms of fungal disease were evident on all of the plants used for isolation except for one sample of *Cymbidium* that had symptoms of viral infection along with leaf rot and root rot. *Fusarium oxysporum* was later isolated from this plant. Although *Fusarium* spp were the major pathogens, few additional fungal genera were also recovered. Nevertheless, *Fusarium* was recovered from symptomatic plants thirteen times more frequently than any other genus, indicating the high incidence of *Fusarium* species.

A total of 480 isolates of different *Fusarium* species were isolated from nine different genera and hybrids of orchids, collected from 60 samples. Most of the diseased samples obtained
for the isolation belonged to *Dendrobium* followed by *Cymbidium* and *Miltonia*. The reason for their dominance is that since *Dendrobium*, *Cymbidium* and *Miltonia*, because of their high marketability as potted orchids are very popular among growers in Hawaii (Palma *et al.*, 2010) and hence the numbers of samples were more in comparison to other genera of orchids. Diseased samples of *Dendrobium* were maximum in number followed by *Cymbidium* and five samples from rest of each genera. Similarly, numbers of isolates of *F. proliferatum* were high followed by *F. oxysporum* and *F. solani* and *F. proliferatum* isolates were more common on *Dendrobium*. *Fusarium oxysporum* presents an interesting scenario where though it is isolated from smaller number of isolates but it is found to be the most common species among orchids as it was isolated from nine genera of orchids while *F. proliferatum* was isolated from six genera.

Further six species of *Fusarium*, *Fusarium oxysporum*, *F. proliferatum*, *F. solani*, *F. poae*, *F. circinatum* and *F. begoniae* were present in both islands, whereas *F. anthropilum*, *F. denticulatum*, *F. semitectum* and *F. subglutinans* were present only on Oahu while *F. bulbicola* was found only on Island of Hawaii (Table 3.9).

Morphological characteristics for identification of *Fusarium* had been a useful tool for plant pathologists, but tiny variation in the morphological characters in response to the environment always created challenging situations for the precise identification of species. This study highlights the advantages of combining molecular analysis together with morphological methods for *Fusarium* species identification, especially of uncharacterized or rare species, which continue to emerge within this diverse and taxonomically challenging group (Leslie, and Summerell, 2006). With the advent of newer molecular tools such as DNA sequencing, functional genomics and because sequence analysis are becoming inexpensive and
experimentally available, the accurate and robust identification of *Fusarium* species has become increasingly possible. The two fungal genome databases, at the National Center for Biotechnology Information (NCBI) and Fusarium–ID Version 1.0, curated at National Institutes of Health (NIH) and University of Pennsylvania respectively has huge deposition of fungal genome sequences. Although enormous fungal genomes have been sequenced and the sequences are available in the databases still both databases are not comprehensive enough for example the Fusarium–ID Version 1.0 mostly relies on few conserved sequences such TEF1α, Tubulin and, ITS etc. which are excellent and usually gives good results in terms of molecular identification but many times using such few and specific sequences does not give any hits. Such problems can be mitigated by increasing the coverage of sequenced regions and making them available for alignment. The continuous molecular studies will make molecular identification more specific and a reliable tool for identification.

*Fusarium proliferatum, F. oxysporum, F. solani, and F. subglutinans* have all been previously known as orchid pathogens in the literature (Benyon *et al*, 1996, Broadhurst and Hartill, 1996, D’Agliano *et al*, 1994, Ichikawa, and Aoki, 2000), therefore these three except *F. subglutinans* were the most frequently isolated *Fusarium* species, indicated that one or more are causing the foliar and root diseases found on orchids in Hawaii. Swett in a survey focused on the commercial nurseries of Island of Hawaii and also confirmed the identity of the same three species *Fusarium proliferatum, F. oxysporum* and *F. solani* and found them to be pathogenic to *Dendrobium* orchids in addition to other unidentified *Fusarium* species (Swett, 2007). Current results indicate that *Fusarium* species may be able to infect the same genera and hybrids as well as they are also having a capability of cross infection i.e. they are also having a potential to infect other genera and hybrids. This may increase the incidence of these diseases in the field, since
only two or three species are required to be present in a place to infect a very wide range of hosts, and eleven species need not be present to have high disease frequencies. The presence of several *Fusarium* species on the same individual plant may suggest that *Fusarium* species are able to co-infect without competing with one another, on a single individual for example *F. oxysporum* and *F. circinatum* were isolated from the same *Cymbidium* plant and were found pathogenic on the same variety of *Cymbidium*.

*Fusarium poae*, *F. begoniae* and *F. circinatum* were identified as pathogens on orchids (Table: 3.5 to 3.7). Nevertheless, it is likely that less commonly isolated three species of *Fusarium, F. bulbicola, F. denticulatum* and *F. semitectum* that generally exists as saprotrophs can be weak pathogens because in the past, many important *Fusarium* pathogens have been overlooked because they were thought to be saprotrophs [e.g., *F. proliferatum* on Asparagus (Elmer, 2001)]. It would therefore be valuable to also assess the ability of these less frequent species to infect orchids by isolating them from *Cymbidium* and Nobile *Dendrobium* samples and testing their pathogenicity on the same cultivars from which isolations were made to support Koch’s Postulates and establish their possible role as pathogens.

In this study, several new symptoms and host associations were observed between *Fusarium* species and orchids genera. *Fusarium* species are, in general, not foliar pathogens and are rather root or soil borne pathogens. Foliar assays were done to assess the ability of pathogenic *Fusarium* species to penetrate the waxy orchid leaf surface and to evaluate the variation in symptoms produced by them in comparison to when they infect via roots. Further, studies of such variation also helps in evaluating the effect of plant age, infection court on susceptibility of plants towards pathogens. Also because *Fusarium* is usually known as a vascular pathogen that initially infects roots and then spreads in the other parts of plant via the
vascular system therefore foliar inoculation of *Fusarium* on orchids will also aid in assessing whether pathogens are having a capability to colonize in the opposite direction from leaves to stem.

Several new host pathogen association were also noted during the study, *Fusarium poae*, *F. begoniae*, *F. anthropilum*, *F. bulbicola*, *F. denticulatum*, and *F. circinatum* was were the new species found to be associated with several orchid hosts. *Fusarium poae* was found associated with *Cymbidium, Brassolaliocattleya* and *Nobile Dendrobium*, while *F. begoniae* was associated with *Oncidiums, Phalaenopsis* and *Vuylstekeara*. *Fusarium anthropilum* was found associated with *Cymbidium* and *Oncidium* while *F. circinatum* was associated with *Cymbidium* and *Phalaenopsis*. *Fusarium bulbicola*, and *F. denticulatum* was found associated with only one genus of orchids, *Nobile Dendrobium* and *Cymbidium* respectively. All genera are are commonly propagated by seeds or mericlons; however, nobile *Dendrobium*, which is a fragrant hanging type is propagated by cuttings and has the potential to assimilate pathogens, this genera is often a host for viral diseases. To this end the precise identification of *Fusarium* species especially those that are pathogenic creates an opportunity for plant pathologists to devise efficient management practices like testing of different fungicide and other ways to control proliferation of pathogens. Also, more surveys and isolations from different islands especially on the Islands of Maui, and Kauai is needed for identification of pathogenic and non-pathogenic species and to further evaluate their dispersal across the state.

### 3.5 Conclusions

This study confirmed that *Fusarium* species were the most common fungi associated with orchid diseases in Hawaii. The *Fusarium* species were widely distributed on both, the island of
Hawaii and Oahu. *Fusarium oxysporum* was the most frequently recovered species followed by *F. proliferatum* and *F. solani*. *Fusarium proliferatum* and *F. oxysporum* were highly virulent on *Dendrobiums* followed by *F. solani*. Three *Fusarium* species, *F. proliferatum*, *F. oxysporum* and *F. solani* were frequently moderate to highly virulent on *Dendrobium*, *Cymbidium* and *Miltonia* causing leaf spots and blights as initial symptoms followed by leaf sheath rot, leaf rot and wilt as advanced symptoms. While three other *Fusarium* species were also found to be to low to moderately virulent on *Dendrobium* and *Cymbidium*. *Fusarium poae* showed very low to low virulence on *Dendrobium* and *Cymbidium* causing only leaf spots while *F. circinatum* was moderate to highly virulent on *Cymbidium* causing leaf spots as initial symptoms that progressed in to blight and rot of youngest leaves but it showed low virulence on *Dendrobium* causing only leaf spots. *Fusarium begonii* caused very low to low virulence on *Miltonia* causing only leaf spots. *Fusarium anthophilum*, *F. bulbicola*, *F. denticulatum* and *F. semitectum* did not caused infection on all the three genera of orchids but other genera need to be tested. Identification of pathogenic species of *Fusarium* orchids will help in tailoring more efficient and feasible interventions to manage the consistent decrease in orchid production. This is the first research study of pathogenicity on different orchid genera other than *Dendrobium*. High costs were spent to establish *Miltonia*, *Cymbidium* and *Cattleya* for pathogenicity tests. With time this host list can be expanded and more genera can be tested.
CHAPTER 4

Management of *Fusarium* Species Using Fungicides

Abstract

Pathogenicity assays with isolated *Fusarium* on different cultivars of orchids resulted in identification of pathogenic *Fusarium* species with varying degrees of virulence. The information generated from pathogenicity assays has provided an insight of frequency of pathogenic *Fusarium* on orchids in Hawaii. Such knowledge can be utilized to devise efficient management practices targeted towards specific pathogenic *Fusarium* species. There are very few registered chemicals for the management of different *Fusarium* species in Hawaii. Therefore, there is an eminent need to test new fungicides needed to manage these *Fusarium* species. Chemical agents like fungicides are one of the most attractive options for immediate and effective control of *Fusarium* diseases. Three fungicides, Pyraclostrobin, a.i. at 25%, Azoxyastrobin a.i. at 50%, and Triticonazole, a.i. at 20% were tested in vitro for their effect on mycelial inhibition on *Fusarium proliferatum*, *F. oxysporum*, *F. solani* and *F. subglutinans*. Triticonazole came up as the most effective fungicide in the in vitro tests and was further evaluated for its efficacy in in vivo on *Dendrobium* plants against the most aggressive species of *Fusarium*, *F. proliferatum* under greenhouse conditions. Results obtained in vivo were similar to in vitro tests and hence Triticonazole can be considered as an effective management tool for *Fusarium* pathogens on *Dendrobium*. This study is important as it attempts to identify viable chemical methods to manage *Fusarium* sp. on orchids in Hawaii.
4.1 Introduction

Orchids are one of the most important agricultural commodities in the state of Hawaii. However, its production is continuously on decline due to many reasons one of them being diseases caused by various pathogenic fungi mainly by members of the *Fusarium* genus. Six *Fusarium* species, *F. proliferatum*, *F. oxysporum*, *F. solani*, *F. subglutinans*, *F. circinatum* and *F. poae* are currently widespread pathogens on orchids in Hawaii (Chapter 2 and 3). All species are able to cause leaf spots on different orchids. *Fusarium proliferatum*, *F. oxysporum*, *F. solani* appears to be the most widespread and causing leaf base rot and wilt on *Dendrobium*, *Cymbidium* and *Miltonia*. *Fusarium oxysporum* is also highly pathogenic to *Cattleya* (Chapter 3).

The disease caused by these pathogens results in severe economic impacts on the orchid industry as they reduce flower yields, kill plants, and cause cosmetic damage to potted ornamentals. Currently, commercial growers have few options for controlling these pathogens and are seeking effective strategies for disease prevention and reduction. In other host systems, various control methods have been successful in reducing diseases caused by *Fusarium* in nursery crops (Milus and Parson, 1994; Ram *et al.*, 2004; Rose and Parker, 2003). Some of the most successful practices have involved the use of fungicides.

Fungicides are chemical or synthetic agents that consist of toxic compounds that inhibit the fungal growth and proliferation thereby controlling the infection/disease caused by fungi. Based on the mode of action, fungicides can be classified as either broad spectrum or specific. A broad spectrum fungicide inhibits or diminishes growth and proliferation of a wide range of fungal pathogens, examples includes Captan (N-trichtoromethylthio-cyclohexene-1, 2-dicarboximide), Sulfur, and Mancozeb (Magnese and Zinc Ethylene bisdithiocarbamate) (Russel, 2005). Whereas, others are specific in their action that acts on a very narrow range of fungi e.g.
mefenoxam is effective only against oomycetes like *Phytophthora*. Alternatively, fungicides can also be described as protective, curative, or eradicant. Protective are applied to host plant before the pathogenic fungal spore are expected to land on the surface of leaf, they are mostly applied on healthy plants. Examples of protectants include mancozeb, coppers, and chlorothalonil (Russel, 2005). Protectants generally are not effective once the fungus grows into plant tissues. Curative fungicides generally systematically translocate throughout the plant and inhibit pathogens that have already infected the host plant. Dodine, triflumizole, or myclobutanil are common curative fungicides that are applied against apple scab fungus (Pscheidt, 2013) which are also systemic in mode of action. Eradicants are more effective on pathogens and remains for longer periods on plants and even kills the over wintering pathogens, for example lime sulfur can be used during the dormant season to kill overwintering fruiting bodies (cleistothecia) of grape powdery mildew.

Many fungicides are available against *Fusarium* disease control in nursery crops. For example Captan (N-trichtoromethylthio-cyclohexene-1,2-dicarboximide), Carbendaxin (Benzimidazole), Thiram (Tetramethylthiuram disulfide) and carboxin (Tetramethylthiuram disulfide) that has been found effective against *Fusarium oxysporum* and has been effective in controlling rots caused by *F. oxysporum* in many ornamental plants such as gladiolus (Mishra *et al.*, 2000; Ram *et al.*, 2004) and vegetable crops like cucumber (Rose and Parker, 2003).

Most of the fungicides inhibits on the vital metabolic pathways of fungi and are relative stable compounds. Strobilurins are compounds that inhibit fungal mitochondrial respiration, and have been effective in *Fusarium* control. Azoxystrobin, developed from a compound originally isolated from mushrooms, is a broad spectrum reduced risk compound marketed as either Heritage (for ornamentals) or Quadris (for vegetables) (Swett, 2007). Azoxystrobin is a broad
spectrum fungicide with protectant, curative, eradicant and systemic properties. It is absorbed through the roots and translocated in the xylem to the stems and leaves, or through leaf surfaces to the leaf tips and growing edges. The mode of action is by inhibition of mitochondrial respiration in fungi. It inhibits spore germination, mycelial growth, and spore production of fungi. Heritage is registered for control of *Fusarium* on more than 100 ornamental crops, including chrysanthemums (*Chrysathemum* spp.), carnation (*Dianthus caryophyllus*), geranium (*Pelargonium* spp.), and rose (*Rosa* spp.) (Ram *et al.*, 2004; Swett, 2007).

The triazole family of fungicides were introduced in the 1980s consists of numerous members – difenoconazole, fenbuconazole, myclobutanil, propiconazole, tebuconazole, tetraconazole, triadimefon, and triticonazole. They are important tools against diseases of turfgrasses, vegetables, citrus, field crops and ornamental plants. They are applied as foliar sprays and seed treatments, but are diverse in use, as they may be applied as protectant or curative treatments. Generally trizole fungicides are applied as a curative treatment and applications must be made early in the fungal infection cycle. Once the fungus begins to produce spores on an infected plant, the triazoles are not effective. Triazoles are systemic and readily taken up by leaves and move within the leaf and movement within the plant is through xylem. The triazoles are very specific in their mode of action – they inhibit the biosynthesis of sterol, a critical component for the integrity of fungal cell membranes. Because their site of action is very specific, there are resistance concerns. Applicators of triazoles are advised to rotate fungicide chemical families in their disease management programs, not simply rotate to a different member of the triazole family (Fishel, 2011).

In Hawaii, there are very few fungicides available for control of *Fusarium* on
orchids. According to NSPIRS data for the State of Hawaii (Anonymous, NSPIRS, 2013) only following five products are registered:

1. Manganese ethylenebis (dithiocarbamate) (DuPont Mnex Fungicide).
2. Zinc ion and manganese ethylenebisdithiocarbamate, coordination product (DuPont Manzate Flowable Fungicide, Protect DF Turf and Ornamental Fungicide, Ferti-Lome Consan 20).
3. Alkyl* dimethyl benzyl ammonium chloride (Floralife microbloc Greenhouse Disinfectant, Physan 20).
4. 1-(4-chlorophenoxy) -3, 3 – dimethyl1 – 1 –(1H – 1, 2, 4 – triazol – yl) -2 – butanone (Bayleton Flo Turf and Ornamental Fungicide, Green Light Fung-Away Systemic Fungicide).
5. QST 713 strain of *Bacillus subtilis* (Serenade Mix, Cease, Rhapsody).

Hence there is an urgent need of seeking effective control measures they may include screening of more fungicides and evaluation of some other control agents such as essential oils or bio-control methods.

The objective of this research was to:

1. Identify fungicides for effective control of *Fusarium* diseases on orchids in vitro.
2. Identify effective fungicide or *Fusarium* control on orchids.
4.2 Materials and Methods

4.2.1 Fungal spores and plants

Pathogenicity assay with fungicides were performed on *Dendrobium* orchids (UH 800). Healthy four leaves stage young shoots of *Dendrobium* were used for the *in vivo* test. These plants were grown on wood chips in six inches pots, under controlled conditions in greenhouse. Plants were watered at least once per day, and fertilized with Osmocote 14-14-14, a slow release fertilizer, every month. Only healthy and symptomless plants were included in the test. Each culture was maintained on 10% V8 juice agar and transferred every 2 weeks. The spores were collected from the identified fungal samples and re-cultured on VA plates and used *in vitro* and *in vivo* tests as described in following sections.

4.2.2 Fungicides

Three fungicides namely Empress, BAS 500 WU F (250 SC), Heritage, BAS 9164 3 F (50 WG) and Trinity, BAS 595 16 F were provided by BASF commercial vendor. Empress and Trinity were in liquid form with 250 g/l and 200 g/l concentration of active ingredient. Heritage was in wettable granule form with 50 % concentration of active ingredient (Table 4.1). They were subsequently diluted to the concentrations as suggested by the manufacturer before in vitro and *in vivo* testing.
Table 4.1: Fungicides studied for their efficacy against *Fusarium* sp on orchids.

<table>
<thead>
<tr>
<th>Trade name</th>
<th>Chemical family (group)</th>
<th>Active ingredient</th>
<th>FRAC Group$^x$</th>
<th>Mode of action</th>
<th>Topical mode of action$^y$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empress</td>
<td>Strobilurin (Methoxy-carbamates)</td>
<td>Pyraclostrobin</td>
<td>11</td>
<td>Respiration</td>
<td>Localized penetrant</td>
</tr>
<tr>
<td>Heritage</td>
<td>Strobilurin Methoxyacrylates</td>
<td>Azoxystrobin</td>
<td>11</td>
<td>Respiration</td>
<td>Acropetal penetrant</td>
</tr>
<tr>
<td>Trinity</td>
<td>Triazoles</td>
<td>Triticonazole</td>
<td>3</td>
<td>Dimethylatin-on inhibitor</td>
<td>Acropetal penetrant</td>
</tr>
</tbody>
</table>

$^x$Fungicide Resistance Action Committee Codes (FRAC): fungicides with the same FRAC code have the same mode of action.

$^y$1. Localized penetrants form a protective barrier on the plant surfaces and permeate into the plant leaf in the area in which it was deposited. These fungicides have some curative activity, but do not move upward or downward in the plant.

2. Acropetal penetrants form a protective barrier on the plant, permeate into the plant, and move upward in the plant's xylem. These fungicides have protective activity including new growth, and have good curative activity.

4.2.3 *In vitro* assays

*In vitro* assays were conducted to evaluate fungicide inhibition of mycelial growth. The concentrations of the fungicides tested were at 1.0 ppm, 3.0 ppm, 10.0 ppm, 30.0 ppm and 100.0 ppm (parts per million) active ingredients (a.i.) in 2% vegetable juice (VA) (18 g Bacto agar, 20 ml Campbell's V8, and 962 ml deionized water) and 2% VA plate (no fungicide) for the normal growth control. To prepare media, the 2% VA was autoclaved and allowed to cool to approximately 70°C before the fungicide was added. The plates were hand poured to uniform thickness (20 ml in each 95 × 15 mm petri plate). To avoid possible light degradation of the chemical compounds, plates were stored in the dark, and used within 48 hours of preparation.
Each culture was maintained on 10% V8 juice agar and transferred every 2 weeks. For the fungicide assay tests *Fusarium* isolates were grown on 2% V8 agar for 3 days. The mycelial growth was cut with a 7mm diameter cork borer and transferred aseptically to the plates containing the fungicides. Plates were incubated at 24°C in dark boxes, with each treatment in a separate box. Each concentration had 3 plates. Each *Fusarium* isolate was inoculated on 3 locations per plate and 3 measurements were taken for each colony at 3 and 5 days. Each measurement consisted of the length between the edge of the inoculum plug and the edge of the colony. The three longest lengths were measured and then their average was calculated. The raw data represents these average measurements (i.e three measurements per inoculum in a plate and three inoculum per plate = 9 measurements).

Six replicate plates per isolate-fungicide-concentration combination were tested in the experiment and the entire experiment was conducted thrice. A total of three fungicides were examined. The fungicides Empress (pyraclostrobin, a.i. at 25%), Heritage (azoxystrobin, a.i. at 50%) and Trinity (triticonazole, a.i. at 20%) were tested against one isolate of each of the four *Fusarium* species (*F. proliferatum*, *F. oxysporum*, *F. solani*, and *F. subglutinans*). The data were plotted against the chemical concentrations tested.

### 4.2.4 In vivo assays:

Three concentrations of fungicide Triticonazole were tested on *Dendrobiums* according to the directions of the manufacturer. Plants were sprayed with 8, 12, and 24 fl.oz/100 gallon. Each test was set in a random complete block design with five replications and of five treatment groups:
1. Water control (WC).
2. Concentration A (8 fl.oz/100 gallon or 0.625 ml/liter a.i.).
3. Concentration B (12 fl.oz/100 gallon or 0.938 ml/liter a.i.).
4. Concentration C (24 fl.oz/100 gallon or 1.880 ml/liter a.i.).
5. Inoculum control (IC).

The experiment was conducted in a greenhouse at the University of Hawaii Magoon Research Facility. *Dendrobium* plants were grown in 6 inches pots. Five young shoots of *Dendrobiums* (i.e. three leaf stage shoots) were sprayed with the designated fungicide concentration until run off. Leaves on each shoot were marked to identify treated area. The controls were sprayed with distilled water only. The plants were not watered for 24 hours to allow the fungicide to dry on the plants then plants were watered daily. Third and sixth day after fungicide treatment, plants were observed for any toxic effect of fungicide on plants. On the seventh day after fungicide treatment, the plants were inoculated with an inoculum suspension of conidia of *F. proliferatum* at a concentration of $10^7$ spores/ml by using the glass fiber disc method (Chapter 3). The youngest two leaves were inoculated at the base of leaves (two discs were put at the base per leaf on the both sides of mid vein). Discs soaked in distilled autoclaved water were used to inoculate the leaves of water control plants. Inoculated plants were placed in a sealed plastic bag for maintaining 100% humidity. After 48 hours the plants were removed from the bags and maintained in the green house for disease development. Disease ratings were taken every week until 8 weeks post inoculation using a scale as described previously.
4.2.5 Statistical analysis

Data collected for the in vitro test were subjected to 3×3×6 (trials × fungicide × concentration) factorial analysis of variance (ANOVA) using Proc Mixed in SAS (Statistical Analysis System, version 9.0, SAS Institute Inc., Cary, NC, USA). Means were separated by Tukey t-test wherever appropriate at $P \leq 0.05$.

Data collected for the in vivo tests were subjected to 4 × 5 (trials × treatments) ANOVA using Proc Mixed in SAS. Means were separated using Tukey t-test wherever appropriate at $P \leq 0.05$.

4.3 Results

4.3.1 Efficacy of fungicides:

4.3.1.1 In vitro tests

Analysis of variance of the in vitro test indicated that trial effect was not significant (Table 4.2), thus data from all three trials were pooled. Significant differences among the treatments ($P < 0.01$) and the concentrations ($P < 0.01$) were detected (Table 4.2). Triticonazole appeared to be the most effective fungicide followed by Pyraclostrobin and Azoxystrobin. Triticonazole inhibited more than 50 % of mycelial growth (Figs. 4.1 and 2). At 100 ppm concentration all the three fungicides were able to inhibit more than 50% mycelial growth for *F. proliferatum*, *F. oxysporum*, and *F. subglutinans*. Azoxystrobin was inhibiting 36 % mycelial growth for *F. solani* but Pyraclostrobin was even inhibiting 60 % mycelial growth of *F. solani* (Figure 4.1 and 2).
Table 4.2: In vitro data analysis for fungicides using ANOVA

<table>
<thead>
<tr>
<th>Effect</th>
<th>Num DF</th>
<th>Den DF</th>
<th>F Value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial</td>
<td>2</td>
<td>943</td>
<td>0.14</td>
<td>0.8679</td>
</tr>
<tr>
<td>Trt</td>
<td>2</td>
<td>943</td>
<td>94194.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Conc</td>
<td>5</td>
<td>943</td>
<td>52534.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Trt*Conc</td>
<td>10</td>
<td>943</td>
<td>5207.86</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Trial*Trt</td>
<td>4</td>
<td>943</td>
<td>0.33</td>
<td>0.8592</td>
</tr>
</tbody>
</table>

Pyraclostrobin suppressed 68% of the growth of all Fusarium species tested at 100 ppm as compared to 0 ppm. Similarly Azoxystrobin at 100 ppm showed approximately a reduction of 50% while Triticonazole showed more potency with a reduction of approximately 92% (Figs 4.1 and 4.2).
Fig. 4.1. Mycelial growth of A) *Fusarium proliferatum*, B) *Fusarium oxysporum*, C) *Fusarium solani* and D) *Fusarium subglutinans*, affected by three fungicides. Means are average of 108 replications. Means followed by the same letter are not different according to Tukey-Kramer Range Test (*P* < 0.05).
Fig. 4.2. Mycelial growth of A) *Fusarium proliferatum*, B) *Fusarium oxysporum*, C) *Fusarium solani* and D) *Fusarium subglutinans*, affected by concentrations of three fungicides. Means are average of 54 replications. Means followed by the same letter are not different based on Tukey-Kramer Range Test ($P < 0.05$).

4.3.1.2 *In vivo* tests

Table 4.3: In vivo data analysis for fungicides using ANOVA

<table>
<thead>
<tr>
<th>Effect</th>
<th>Num DF</th>
<th>Den DF</th>
<th>F value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial</td>
<td>3</td>
<td>737</td>
<td>7.07</td>
<td>0.0001</td>
</tr>
<tr>
<td>Trt</td>
<td>4</td>
<td>737</td>
<td>929.56</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Date</td>
<td>7</td>
<td>737</td>
<td>48.50</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>date*trt</td>
<td>28</td>
<td>737</td>
<td>38.77</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Trial*trt</td>
<td>12</td>
<td>737</td>
<td>4.04</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

The analysis of variance revealed significant results ($P > 0.05$) for four trials in *in vivo* experiments, and the data were analysed separately (Table 4.3). There were significant differences
among the treatments (P <0.01), and interaction between trial and treatments were also significant (Table 4.3).

**Fig. 4.3:** Disease ranking of (0 = Healthy shoot with no symptoms, 1= Less than 5 spots per inoculated leaf, 2 = 5-10 spots per inoculated leaf, 3 = More than 10 spots or blight per inoculated leaf 4 = Initiation of leaf base rot or leaf sheath rot and 5 = Leaf base rot and wilt of youngest leaf.) *Fusarium proliferatum* on *Dendrobium* plants treated by three concentrations of Trinity fungicide. IC is inoculum control without fungicide and WC is water control (without fungicide and without inoculum). Means are average of repeated measures of disease rankings over eight weeks for the five replications. Means followed by the same letter for each trial were not significantly different according to Tukey-Kramer Range Test (P < 0.05).

*In vivo* tests of Triticonazole fungicide with three concentrations on *Dendrobium* plants produced the most effective results. In all four trials, at concentration 8 fl.oz/100 gallon all plants in general showed no sign of infection with exception to few plants 1-3 where plants showed 2-4 spots on inoculated leaves (equivalent to level = 1) while with concentration 12 fl.oz/100 gallon
one plant out of five replicates was rotted (equivalent to level = 5). Plants treated at concentration 24 fl.oz/100 gallon produced no infection on all the five replicates and were equivalent to the results produced by water control. While inoculum control were those plants that were inoculated but not treated with fungicide, produced expected disease symptoms. Results across all the four trial were significantly constant.

The results clearly show that Triticonazole was very effective in controlling *Fusarium proliferatum* even at the lowest concentration (8 fl.oz/100 gallon or 0.625 ml/liter a.i.) and as the plants did not produced any symptoms of disease and were similar to that of plants under water control treatment set. Water control was negative control that was treated with sterilized distilled water only and was not inoculated so it was expected that those plants will not produce any disease and the results were according to our expectations. Inoculum control (IC) was the positive control and was not treated with any fungicide thus, it was expected to produce disease symptoms and those disease symptoms were increased with time making significant variation.

In general, time plays a significant role in appearance of disease. We have observed that weak pathogens often produced disease symptoms quite late after inoculation. In order to see whether the fungicides have decreased the virulence or have completely stopped / disabled the pathogen we continued to monitor the plants for 8 weeks. None of the plants with any of the treatment 12 fl.oz/100 gallon, or 24 fl.oz/100 gallon produced any symptoms on the plants signifying that all 3 fungicidess are very effective at these concentrations.

4.4 Discussion

*Fusarium* species produce two types of asexual spores, macroconidia and microconidia that are the primary means of dispersal, however sometimes sexual spores (ascospores) are also
produced (Doohan et al., 2003). These asexual and sexual spores can be dispersed by wind and water splash, primarily from overhead watering in nurseries (Doohan et al., 2003; Ichikawa et al., 2003). Insects and farm implements also play a major role in the spread of these spores which comes in the contact of infected material (Agrios, 2000). Once in contact with the host the spore germinates with moisture and penetrates the tissue, entering the host. The pathogen infects the tissue, feeding on cellular material, growing, and producing spores that are then dispersed, continuing the disease cycle. If a host is absent, thick walled survival spores, called chlamydospores, may develop from cells in the hyphae and/or the macroconidia in some species (Summerell et al., 2001). Chlamydospores can allow for survival in plant parts (such as root matter or debris) or in the soil or potting medium for many years (Summerell et al., 2001).

Spread of Fusarium in greenhouse or field grown orchids may be reduced by various cultural practices, including removal of infected material (sanitation) (Agrios, 2000; Garibaldi and Gullino, 1990; Gullino et al., 2002), regulation of overhead watering and wind (when possible) (Doohan et al., 2003; Ichikawa et al., 2003; Leonhardt et al., 1999), and management of vectors (Wang and Jeffers, 2002). Among other practices Fusarium infection and reproduction can also be prevented through the use of resistant cultivars (Agrios, 2000; Rose and Parker, 2003). Fusarium germination, growth and infection can also be inhibited by anti-fungal compounds, including chemical fungicides and biological agents (Agrios, 2000). Some of the most successful practices in controlling fusarial diseases on nursery crops have involved the use of anti-fungal compounds or fungicides.

Results from the present study clearly indicate the efficacy of chemicals in controlling Fusarium species on orchids. The possibility of judicious use of chemical control agents like fungicides can be partnered with integrated pest management strategy for developing methods
for efficient control. The most effective fungicide for *Fusarium* on orchids was Triticonazole followed by Pyraclostrobin (Empress) and Azoxystrobin (Heritage). All the fungicides tested in this study are not registered in Hawaii till now and this is the first report that shows their efficacy in management of *Fusarium* species a major group of pathogens on orchids. The results from this study establishes Triticonazole as very effective in inhibiting the mycelial growth in both in vitro and in vivo conditions. Hence, Triticonazole is effective in controlling *F. proliferatum* infections on *Dendrobiums* even at the lowest concentration.

Fungicide resistance has emerged as a major problem in disease control and it is very common among many plant pathogenic fungi throughout the world. Fungicides are at a high risk for the development of fungicide resistance. Pathogen populations contain naturally occurring resistant types in very low numbers in nature but normally there is no adaptive advantage of this trait in the absence of the particular fungicide. However, when a fungus population is treated with a specific fungicide repeatedly and indiscriminately, the resistant strains will have a great adaptive advantage and face less competition, so, their population increases and either a part of population or sometimes entire population may become dominant and resistant for that particular fungicide. Fungicides with very specific site of activity are most likely prone for the development of resistance in a fungal population than the fungicides having a variety of antifungal actions or multi sites of activity (Schnabel, 2013; Bertrand and Padgett, 1997).

In this study three fungicides were tested *in vitro*, all of them showed reduction in mycelial growth for more than 50%, for four species of *Fusarium*. Two of them (Pyraclostrobin and Azoxystrobin) belonged to same chemical family group, the Strobularins that inhibits mitochondrial respiration while Triticonazole belonged to Triazoles that is having a different mode of action by inhibiting sterol biosynthesis. Pyraclostrobin worked as a localized penetrant
while Azoxystrobin and Triticonazole acted as apical penetrant so that they can move towards the new growth after absorption by the plant parts.

The best approach to fungicide resistance management is to use a program consisting of both combinations and alternating of fungicides with different modes of action as well as to use fungicides with other non-fungicide approaches such as, use of essential oils or bio-control. Information generated by this study would be useful to develop a fungicide spray program by using all the three fungicides used in this study as well as other fungicides already registered for *Fusarium* on orchids in Hawaii.
CHAPTER 5

Investigation of Host Specificity of *F. oxysporum* Isolated from Orchids.

Abstract

*Fusarium oxysporum* consists of many important pathogens that cause vascular wilt diseases and root rots. Despite the broad host range, host specialization (*formae speciales*) of the isolates is a very important characteristic of *Fusarium oxysporum*. The objective of this study was to determine host ranges of *F. oxysporum* isolated from *Cattleya*, which cause leaf spots, blight, leaf rot and foliar wilt on *Cattleya*; and to determine whether these isolates are specifically pathogenic to orchids or whether they are pathogenic on some other vegetable crops known to be hosts. Two isolates were tested with one that was *Cattleya* specific while another was pathogenic to both *Cattleya* and *Dendrobium*. We tested 12 vegetable crops that are usually very susceptible to *F. oxysporum* namely tomato, asparagus, eggplant, pea, pepper, soybean, cabbage, coriander, cucumber, chickpea, okra and green beans. All the inoculated plants as well as control plants remained healthy during the 8 weeks of observation showing the orchid isolates were not pathogenic to them.
5.1 Introduction:

The genus *Fusarium* includes many species that cause plant diseases, such as vascular wilts, root, stalk and cob rots, collar rot of seedlings, and rots of tubers, bulbs and corms (Anon. Cornell University, 2006). *Fusarium oxysporum*, an important member of genus *Fusarium*, consists of many important pathogens (*formae speciales*) that cause vascular wilt diseases and some root rots. However, *F. oxysporum* also includes many saprophytic strains which colonize diseased roots after the pathogen has killed root tissues. Some of these saprophytic species may also colonize the outer cells of the roots as endophytes causing no damage.

Over the past 80 years, *Fusarium oxysporum* has received considerable attention from plant pathologists because of its pathogenic ability on a wide range of valuable plants. Despite the broad host range of the species, host specialization of the isolates is the characteristic that attracted the most attention from plant pathologist (Leslie and Summerell, 2006). Isolates having same or similar host ranges are recognized in to a *forma specialis*, with more than 70 *formae speciales* have been described (Armstrong *et al*., 1981; Booth, 1971). More often than not, host range is restricted to a few plant species. For example, *F. oxysporum* f. sp. *lycopersici* causes disease only in plants of the genus *Lycopersicon* (Rowe, 1980). However, some *formae speciales* have broader host ranges, such as *F. oxysporum* f. sp.*radicislycopersici*, which cause disease on hosts from several plant families, including tomato (*Lycopersicon esculentum* Mill.), cucumber (*Cucumis sativus* L.), zucchini (*Cucurbita pepo* L.), beet (*Beta vulgaris* L.), asparagus (*Asparagus officinalis* L.) and onion (*Allium cepa* L.) in the greenhouse (Kim *et al*., 2001; Menzies *et al*., 1990; Rowe, 1980).

Vascular wilt diseases caused by *Fusarium* are important problems in all the parts of world as well as in Hawaii. In Hawaii, hosts of *Fusarium oxysporum* include: potato, sugarcane,
garden bean, cowpea, Prickly pear, cultivated zinnia, pansy, Assam rattlebox, Baby's breath, and Musa sp. (Raabe et al., 1981). Several Formae specialis of *Fusarium oxysporum* have been reported which includes: *Fusarium oxysporum* f.sp. *asparagi* (*Fusarium* yellows of asparagus); f.sp. *callistephi* (wilt of China aster); f.sp. *cubense* (Panama disease/wilt of banana); f.sp. *dianthi* (wilt of carnation); f.sp. *koae* (of koa); f.sp. *lycopersici* (wilt of tomato); f.sp. *melonis* (*Fusarium* wilt of muskmelon); f.sp. *niveum* (*Fusarium* wilt of watermelon); f.sp. *pisi* (of edible-podded pea); f.sp. *tracheiphilum* (wilt of Glycine max); and f.sp. *zingiber I* (*Fusarium* yellows of ginger) (Raabe et al., 1981) (Gonsalves and Ferreira, 1993; Trujillo, 1963) (Table 5.1).

Although, *F. oxysporum* and its various formae speciales often causes the following symptoms: vascular wilt, yellows, corm rot, root rot, and damping-off the most important of these is vascular wilt (Agrios, 1988; Smith et al., 1988). Strains that are rather poorly specialized may induce yellows, rot, and damping-off, rather than the more severe isolate that causes vascular wilt (Smith et al., 1988) on non specific hosts.

In general, initiation of the symptom of *Fusarium* wilts on tomato appears as slight vein clearing or yellowing of leaves on many hosts such as cucurbits and legumes, followed by epinasty (downward drooping) of the older leaves. Plants infected by *F. oxysporum*, at the seedling stage may wilt and die soon after symptoms appear. In older plants, vein clearing and leaf drooping are often followed by stunting, yellowing of the lower leaves, wilting of leaves and young stems, defoliation, marginal necrosis of remaining leaves, and finally death of the entire plant (Kistler, 2001; Agrios, 1988). Browning of the vascular tissue is strong evidence of *Fusarium* wilt. Further, on older plants of tomato, symptoms generally become more apparent
during the period between blossoming and fruit maturation (Jones et al., 1982; Smith et al., 1988).

In potato (*Solanum tuberosum*) *Fusarium* wilt symptoms are also similar to that on tomato. Vein clearing on outer leaflets and drooping of leaf petioles occurs during the initial stages of infection. If the infection persists, the lower leaves wilt, turns yellow and die and finally the whole plant is killed. The hallmark characteristics of the infection caused by *Fusarium* is observed when the main stem is cut, dark, chocolate-brown streaks may be seen running lengthwise through the stem (Miller et al., 2014).

In pepper (*Capsicum annum*), the lower leaves do not show any immediate effect of infection and remains unaffected until roots and the base of the stem have already started to decay. Dark brown, sunken, and eventually girdling cankers may be seen at the base of the pepper plant. If the infection remains unchecked the leaves followed by entire plant is killed by decay. While in eggplant, wilting progresses from lower to upper leaves, followed by collapse of the plant (Miller et al., 2014)

In okra (*Abelmoschus esculentus*), the leaves are affected and show the initial symptoms of infection. The color of leaves turns yellow probably because of blockade of water transportation system resulting in wilting and finally shedding of leaves. Usually the lower leaves are the first affected. When a stem or the main root is cut crosswise, brown discolouration is usually found in the ring just beneath the bark. Wilting of plants is mostly gradual (Anon., infonet, 2014).
In beans (*Phaseolus vulgaris*), the medium aged or plants shows susceptibility to *Fusarium* infections in comparison to younger plants. The infection begins as a yellowing and wilting of the lower leaves. The infection progresses up the plant until the entire plant turns yellow (Anon., infonet, 2014).

In cucumber (*Cucumis sativus*), old plants also show susceptibility towards *Fusarium* pathogens. Infected plants shows infection first on older leaves as yellowing which spreads to younger leaves. The yellowing of leaves is closely accompanied by stunting and wilting. Once the wilting starts to appear the death of plants may occurs within a week of time (Pscheidt, 2014).

Diseased plants of cilantro (*Coriandrum sativum*) show poor and stunted growth, lower foliage turns yellow with reddish tinges, and plants wilt during warmer times of the day. The main stem, crown, and taproot exhibits vascular discoloration. Unchecked infection results in death of plant mainly due to severe wilt (Koike, 2005).

The initial symptoms on cabbage (*Brassica oleracea* L. var. *capitata*) plant shows yellowing of the lower leaves, often on one side of the plant. These leaves later turn brown and drop off. The growth of *Fusarium* on the water conducting tissue results in discoloration and is regarded as the typical characteristics of *Fusarium* infection. As the infection continues to persists the entire plant may turn yellow followed by wilting and ultimately death of the plant. (Anon., UC IPM Online, 2014).

Infected plants of asparagus (*Asparagus officinalis*) are characterized by one to several stunted, bright yellow leaves. A reddish brown vascular discoloration, which may extend into the
crown, is present at the base of stalks infected by *Fusarium oxysporum* f. sp. *asparagi* (Anon., UC IPM Online, 2014.).

In pea (*Pisum sativum*), yellowing of the lower leaves and a stunting or dwarfing of plant are the major symptoms observed in plants with *Fusarium* infection. The stipules and margins of the leaflets curl downward and inward. The stems at or near the soil line may be slightly thickened and brittle. A cross section of the lower stem often reveals a lemon to orange-brown and finally black discoloration of the water-conducting tissue (xylem) within the stem (Anon., IPM, 1988.)

In chickpea (*Cicer arietinum*), yellowing and wilting of whole plants are typical symptoms of *Fusarium* infection. Vascular discoloration due to blockage in water conducting tissues occurs from the roots to the young stems, followed by a yellowing and wilting of the leaves before the plant dies due to necrosis (Mc Taggart, 2007).
Table 5.1 *Fusarium* vascular wilt pathogens and their associated diseases are:

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. oxysporum</em> f. sp. <em>cubense</em></td>
<td><em>Fusarium</em> wilt of banana (Panama disease)</td>
</tr>
<tr>
<td><em>F. oxysporum</em> f. sp. <em>dianthi</em></td>
<td><em>Fusarium</em> wilt of carnations</td>
</tr>
<tr>
<td><em>F. oxysporum</em> f. sp. <em>zingiberi</em></td>
<td><em>Fusarium</em> wilt of ginger</td>
</tr>
<tr>
<td><em>F. oxysporum</em> f. sp. <em>callistephi</em></td>
<td><em>Fusarium</em> wilt of asters</td>
</tr>
<tr>
<td><em>F. oxysporum</em> f. sp. <em>niveum</em></td>
<td><em>Fusarium</em> wilt of watermelon</td>
</tr>
<tr>
<td><em>F. oxysporum</em> f. sp. <em>pisi</em></td>
<td><em>Fusarium</em> wilt of peas</td>
</tr>
<tr>
<td><em>F. oxysporum</em> f. sp. <em>lycopersici</em></td>
<td><em>Fusarium</em> wilt of tomato</td>
</tr>
</tbody>
</table>

The objective of this study was to determine host ranges of *F. oxysporum* isolated from *Cattleya*, which cause leaf spots, blight, leaf rot and foliar wilt on *Cattleya*; and to determine whether these isolates are specifically pathogenic to orchids or in addition, are they pathogenic on some other vegetable crops. Two isolates were tested one was *Cattleya* specific and another was pathogenic to both *Cattleya* and *Dendrobium*. We tested twelve vegetable crops that are usually very susceptible for *F. oxysporum* namely tomato, asparagus, eggplant, pea, pepper, soybean, cabbage, coriander, cucumber, chickpea, okra and green beans.

5.2 Materials and Methods

5.2.1 Selection of test plants:

For testing the concept of *F. oxysporum* formae specialis we selected twelve vegetable crops that are usually very susceptible to *F. oxysporum* namely tomato (*Solanum lycopersicum*), asparagus (*Asparagus officinalis*), eggplant (*Solanum melongena*), pea (*Pisum sativum*), pepper (*Capsicum annuum*), soybean (*Glycine max*), cabbage (*Brassica oleracea L. var. capitata*), coriander (*Coriandrum sativum*), cucumber (*Cucumis sativus*), chickpea (*Cicer arietinum*), okra (*Abelmoschus esculentus*) and green beans (*Phaseolus vulgaris*). Economically important cultivated crops as well as plant species reported to be highly susceptible to *F. oxysporum*
diseases in the tropics were included for this test. A more robust test using *F. oxysporum* isolates from each host was not possible as isolates were not available.

### 5.2.2 Materials and conditions of pot experiments:

Seeds of the selected crops for host range studies were purchased from a commercial vendor (Johny’s Selected Seeds Company, Fairfield, ME, USA). During the selection of seeds care was taken not to use seeds that were bred for *Fusarium* resistance. Six inch pots were used to sow the seeds and Sunshine mix band # 4 (Sun Gro Horticulture, Canada) was used as the media. Osmocote (14:14:14) a slow release fertilizer, was applied at fifteen days intervals to make sure that the plants remain healthy and there will be no yellowing due to nutritional deficiency. Fifteen to twenty seeds were sown per 6” pots. These were thinned to a single plant per pot after the appearance of cotyledons. Two isolates of *F. oxysporum* (SK-570 and SK-601) were used that were isolated from *Cattleya*. SK-601 was *Cattleya* specific but SK-570 was not because it caused disease on *Dendrobium* and *Cattleya*. Each culture was maintained on 10% V8 juice agar and transferred every 2 weeks. Each isolate was cultured on fresh 10 % V8 juice agar media for 3 days under cool fluorescent light. Conidial suspension of *Fusarium* isolates were used at a concentration of $10^7$ conidia per ml.

### 5.2.3 Experimental design:

For each plant 15 plants were inoculated in three sets of five with each of the *F. oxysporum* isolate at $10^7$ spores/ ml concentration with 15 ml of suspension was added to each pot. Five plants were tested with distilled water and served as controls. Each seedling was inoculated at the two-true-leaf stage. Observations were recorded every 4th day for the
production of any symptom on the foliar phase of each host for 8 weeks. During this period of observation plants were watered daily and fertilized with Osmocote 14:14:14, every 2 weeks. After 8 weeks plants were unpotted, roots were rinsed, examined, and observed for any discoloration, browning or rot. Three plants were selected randomly and for any root discoloration and roots were cut, surface sterilized with 10% bleach and plated to re-isolate *F. oxysporum*. The whole set of experiment was repeated twice.

5.3 Results

5.3.1 Investigation of host specificity of *F. oxysporum* from *Cattleya*.

None of the plants showed any symptoms representing 12 different genera and species of plants inoculated with *Fusarium oxysporum*. Figures 5.1 to 5.5 shows the status of infection on plants tested in comparison to the water control. The figures also show the status of infection roots when plants were removed from pots, roots were rinsed and observed for any sign of disease. After eight weeks of inoculation. The figures of six plant types are representation of the pathogenicity assays done. The remaining six other plant sets also showed similar results (data not shown). Only *Trichoderma, Spermospora* and binucleate *Rhizoctonia solani* - like fungi were recovered and all are generally considered as saprotrophs.
Fig. 5.1 Three plants of okra were inoculated with 15 ml of $10^7$ spores of *Fusarium oxysporum* (Test plants) and sterile water was added to one plant (Control). (A) After eight weeks of inoculation, none of the plants (test or control) showed any symptoms or sign of disease. (B) The plants were then removed from pots; roots were rinsed and observed any sign of disease. (C) The roots of both control and test plants did not showed any signs of disease symptoms.

Fig. 5.2 Three plants of chickpea were inoculated with 15 ml of $10^7$ spores of *Fusarium oxysporum* (Test plants) and sterile water was added to one plant (Control). (A) After eight weeks of inoculation, none of the plants (test or control) showed any symptoms or sign of disease. (B) The plants were then removed from pots; roots were rinsed and observed any sign of disease. (C) The roots of both control and test plants did not showed any signs of disease symptoms.
Fig. 5.3 Three plants of tomato were inoculated with 15 ml of $10^7$ spores of *Fusarium oxysporum* (Test plants) and sterile water was added to one plant (Control). (A) After eight weeks of inoculation, none of the plants (test or control) showed any symptoms or sign of disease. (B) The plants were then removed from pots, roots were rinsed and observed any sign of disease. (C) The roots of both control and test plants did not showed any signs of disease symptoms.

Fig. 5.4 Three plants of coriander were inoculated with 15 ml of $10^7$ spores of *Fusarium oxysporum* (Test plants) and sterile water was added to one plant (Control). (A) After eight weeks of inoculation, none of the plants (test or control) showed any symptoms or sign of disease. (B) The plants were then removed from pots; roots were rinsed and observed any sign of disease. (C) The roots of both control and test plants did not showed any signs of disease symptoms.
Three plants of cucumber were inoculated with 15 ml of $10^7$ spores of *Fusarium oxysporum* (Test plants) and sterile water was added to one plant (Control). (A) After eight weeks of inoculation, none of the plants (test or control) showed any symptoms or sign of disease. (B) The plants were then removed from pots; roots were rinsed and observed any sign of disease. (C) The roots of both control and test plants did not showed any signs of disease symptoms.

### 5.4 Discussion

SK-570 and SK-601 did not caused disease on any of the tested plants. The inability of *F. oxysporum* isolate no. SK-570 to produce disease symptoms on vegetable crops suggest that this isolate is specific to orchids because this isolate was also pathogenic for *Dendrobiums*. SK-601 showed pathogenicity only on *Cattleya* but not on *Dendrobiums* or on vegetable crops. This suggests that SK-601 is specific to *Cattleya (F. oxysporum f.sp. cattleya).*
The accurate identification of pathogen up to strain level and its host range is essential for determination of effective management practices. *Fusarium oxysporum* is a complex species as it consists of several morphologically indistinguishable pathogenic and non-pathogenic strains. One of the initial ways to distinguish different strains of pathogenic *F. oxysporum* is based on their host specificity and hence is assigned to different *forma specialis*. Currently pathogenicity assays or bioassays are one of the most suitable methods for determination of *forma specialis*. But such methods are often time consuming and labor intensive. Also in case of certain pathogens that cannot be cultured on artificial media it is usually difficult to perform bioassays with them. The development of several molecular techniques has created ways to make such identification studies in much quicker and economical fashion. Molecular genetics methods such as those using first generation of markers such as Random Amplification of Polymorphic DNA (RAPD) or Restriction Fragment Length Polymorphism (RFLP) and second generation molecular markers methods such using Internal Transcribed Spacer (ITS) regions and (Single nucleotide polymorphism (SNPs) have opened new wide avenues for determination as well as studying phylogenetic relationships among different *forma specialis* and strains. However there are some limitations of using molecular techniques too, such as poor understanding of genetic basis of host’s specificity. Furthermore discrimination of *Fusarium oxysporum* isolates also becomes complicated by polyphyletic nature of *forma specialis* (Lievens et al 2008). Nevertheless molecular can be useful in generating initial information regarding the determination and differentiation of *forma specialis* which can be further experimentally confirmed by the pathogenicity assays.
Conclusion and Impact of Study

Hawaii is one of the America’s leading producers of orchids, producing the greatest variety of tropical species and unique intergeneric hybrids. Geographical location of Hawaii provides ideal natural environment for orchids to grow year round, but there has been a consistent decline in production of orchids have been observed in Hawaii and elsewhere in world, largely due to fungal pathogens. Precise identification of fungal pathogens is the first step needed for designing of proper and efficient management systems.

The present study reports sixteen different fungal genera associated with diseased orchids and *Fusarium* was the most prevalent fungal genus among them representing 78% isolates from symptomatic plants confirming that *Fusarium* species were the most common fungi associated with orchid diseases in Hawaii and suggesting that different *Fusarium* species is the major reason behind the decline of quantity and quality of orchids in Hawaii. Eleven species of *Fusarium* were isolated and identified associated with orchids. *Fusarium oxysporum* was the most frequently recovered species followed by *F. proliferatum* and *F. solani*. *Fusarium proliferatum* and *F. oxysporum* were highly virulent on *Dendrobium* followed by *F. solani* which was moderately virulent. Three *Fusarium* species, *F. proliferatum*, *F. oxysporum* and *F. solani* were frequently moderate to highly virulent to *Dendrobium, Cymbidium* and *Miltonia* causing leaf spots and blights as initial symptoms followed by leaf sheath rot, leaf rot and wilt as advanced symptoms. While, three other *Fusarium* species were also found to be very low to moderately virulent on *Dendrobium* and *Cymbidium*. *Fusarium poae* showed low virulence on *Dendrobium* and *Cymbidium* causing only leaf spots while *F. circinatum* was moderate to highly virulent on *Cymbidium* causing leaf spots as initial symptoms that progressed in to blight and rot.
of youngest leaves but it showed low virulence on *Dendrobium* causing only leaf spots. *Fusarium begoniae* showed very low virulence on *Miltonia* causing only leaf spots. *Fusarium anthophilum, F. bulbicola, F. denticulatum and F. semitectum* did not caused disease on all the three genera of orchids.

The information generated from pathogenicity assays was followed by testing the efficacy of three new fungicides, Empress (Pyraclostrobin, a.i. at 25%), Heritage (Azoxystrobin a.i. at 50%) and Trinity (Triticonazole, a.i. at 20%), *in vitro* for their effect on mycelial inhibition on four most prevalent *Fusarium* species namely- *Fusarium proliferatum, F. oxysporum, F. solani* and *F. subglutinans*. Results from the fungicide testing clearly indicate the efficacy of chemicals in controlling *Fusarium* species on orchids. The most effective fungicide for *Fusarium* on orchids was Trinity (Triticonazole) followed by Empress (Pyraclostrobin) and Heritage (Azoxystrobin). At 100 ppm concentration all the three fungicides showed reduction of more than 50% mycelial growth in *F. proliferatum, F. oxysporum, and F. subglutinans*. Heritage inhibited 36% mycelial growth of *F. solani* while Empress was found to inhibit up to 60% mycelial growth of *F. solani*.

Trinity appeared as the most effective fungicide in the *in vitro* tests and was further evaluated for its efficacy in *in vivo* on *Dendrobium* plants against the most virulent species of *Fusarium, F. proliferatum* under greenhouse conditions. Results obtained *in vivo* were similar to *in vitro* tests and hence Trinity can be considered as an effective management tool for *Fusarium* pathogens on *Dendrobium*.

All the fungicides tested in this study are not registered in Hawaii till now and this is the first report that shows their efficacy in management of *Fusarium* species, a major group of
pathogens on orchids. Among three tested fungicides two of them (Empress and Heritage) belonged to same chemical family groups strobularins that inhibits mitochondrial respiration while Trinity belonged to triazoles that inhibits sterol biosynthesis. Empress worked as a localized penetrant while Heritage and Trinity acted as Apical penetrant so that they can move towards the new growth after absorption by the plant parts.

The use of chemical control agents like fungicides along with integrated pest management strategy can be developed as a pragmatic method for efficient control the fungal diseases on Orchids. The combinations and alternating of fungicides of different modes of action as well as to use fungicides with other non-fungicide approaches such as, use of essential oils or bio-control can also be considered as an attractive method of control. Information generated by this study would be useful to develop a fungicide spray program by using all the three fungicides used in this study as well as other fungicides already registered for Fusarium on orchids in Hawaii.
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