Diversity of *Fusarium* Link and mite species associated with malformed *Syzygium cordatum* Hochst. Ex Krauss (water berry) inflorescences

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DEDICATION

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To my late dad, who was my biggest cheerleader, he didn’t see this dream to fruition.

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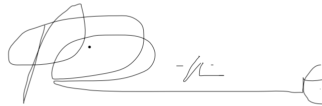
DECLARATION

I, the undersigned, hereby declare that this thesis, which I submit for the degree of PhD in Plant Pathology at the University of Pretoria is my own work and has not been previously submitted for a degree at another university.



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PREFACE

Syzygium cordatum is an evergreen indigenous, water-loving tree species in the Myrtaceae family in South Africa. It is usually found close to water sources such as near streams, on forest margins or in swampy spots and grows best in moist to wet soil. The tree has many functional uses; the fruits are a source of food, the bark is used in traditional medicines for treating stomachache, diarrhea, headaches, tuberculosis and diabetes among others. Recently, floral malformations have been observed among *S. cordatum* trees. The disease causes abnormal development of vegetative shoots and floral panicles resulting in phyllody and hypertrophy similar to disease symptoms in mango malformation which is associated with *Fusarium* and eriophyoid mite species.

The genus *Fusarium* Link is one of the most important fungal genera with a worldwide distribution and contains at least 300 phylogenetically distinct species/species complexes. The species in this genus are broadly distributed in soil, root and plant tissues, and other organic substrates. This ascomycete genus is among the world's most economically destructive plant pathogens, capable of causing diseases of almost all economically important plants. Some of the *Fusarium* species are also opportunistic human pathogens, causing infection of cornea, nails and other diseases. In addition, *Fusarium* fungi can produce a diversity of toxic secondary metabolites, such as trichothecenes, zearalenone, and fumonisins, posing significant threats to food safety and human health. Many of the plant pathogenic and mycotoxin producing fungi are within the *Fusarium fujikuroi* species complex (FFCS) which also includes several species that are linked to mango malformation disease and species that were only isolated from malformed *S. cordatum* inflorescences.

Eriophyoid mites (Acari Eriophyoidea) are phytophagous arthropods which form intimate relationships with their host plants. They can cause malformation by causing galls and blisters. They may also cause malformations indirectly by transmitting plant pathogens such as viruses, phytoplasmas and fungi. Against this backdrop the overall aim of this thesis was to characterize *Fusarium* and mite species associated with malformed inflorescences in *S. cordatum.*

Chapter 1 of this thesis represent a comprehensive review of the current knowledge on the causes of floral malformation. It focuses on information regarding the biotic and abiotic factors, the developmental aspects of the abnormalities and specific examples of the type of malformation they cause. The review also emphasizes *Fusarium* and *Fusarium*-mite interactions as they are most commonly associated with malformations of many economically important plant species.

Considering the importance of *Fusarium* species in floral malformation disease in different plants, the aim of Chapter 2 was to identify *Fusarium* species associated with malformation in *S. cordatum* inflorescences by making use of phylogenetic information of DNA sequences of the translation elongation factor 1 alpha (*TEF1*α) gene. The research focus on the differences in *Fusarium* species diversity between malformed and healthy inflorescences. Results obtained in this study provides valuable information regarding the diversity of species within the *Fusarium* genus. In addition, it provides a blueprint for the possible causes of *S. cordatum* floral malformation. This Chapter has been published as: Mkandawire, R.I., Yilmaz, N., Steenkamp, E.T., Wingfield, M.J. and Fourie, G. (2022). Diversity of *Fusarium* species associated with healthy and malformed *Syzygium cordatum* inflorescences in South Africa. *European Journal of Plant Pathology* 162, 907–926 https://doi.org/10.1007/s10658-021-02447

Chapter 3 of this thesis describes a novel species residing within the *Fusarium incarnatum equiseti* species complex (FIESC). The chapter uses a polyphasic approach by employing a multi-locus phylogenetic, morphological and biological approaches to describe the novel species. It is the first time that a novel species in the *Fusarium* genus has been described from *S. cordatum*.

In chapter 4 the diversity of mites associated with floral malformation in *S. cordatum* is determined. The research compares the diversity of mites in healthy and malformed inflorescences. In addition, it uses the *cytochrome c oxidase subunit 1* gene region to identify mite species and establish a DNA database that could assist in future identification of these species. For the first time, this study provides valuable phylogenetic information of mites associated with malformed inflorescences in *S. cordatum*.

In chapter 5, the results obtained in chapters 2, 3 and 4 are discussed in detail. In addition, it presents some limitations for the research conducted in this study, it makes recommendations for future directions regarding this work and finally draws conclusions regarding the possible causes of floral malformation in *S. cordatum*.

Chapter 1: Literature Review

Causes of Floral Malformation

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Abstract

Floral malformations are abnormalities that occur in floral inflorescences. They are associated with environmental factors, insect pests, and various micro-organisms. Even though floral malformations occur commonly and are well known, knowledge regarding their causes is limited. This review summarizes information regarding the biotic and abiotic factors that are associated with floral malformations. Biotic factors include insects, fungi, bacteria, viruses, nematodes and mites, while abiotic factors include hormonal imbalances, water stress, nutrient status and temperature. Background information of biotic and abiotic factors, the developmental aspects of the abnormalities and specific examples of the type of malformation they cause are provided and discussed in detail. Particular emphasis is given to *Fusarium* and *Fusarium*-mite interactions as they are most commonly associated with malformations and are causal agents of malformation diseases in many economically important plant species.

1 Introduction

The angiosperms are the most abundant and diverse group of all major lineages of plants and the dominant autotrophs with more than 250,000 species described (Herendeen et al., 2017; Groombridge, 1992). Flowering plants occur in the majority of terrestrial habitats, and they provide food and shelter to many organisms that live and depend on them. For humans, flowering plants are a source of food, timber and medicines (Groombridge, 1992). Flowers are sexual structures comprised of reproductive organs, responsible for the formation of fruits and seeds (Dreni and Zhang, 2016). Floral diseases and abnormalities may, therefore, significantly influence propagation, diversity, survival of plants, as well as crop yields.

Floral malformations are abnormalities or modifications in floral development and may lead to developmental changes in inflorescences or floral parts (Meyer, 1966). These developmental changes may be as a result of the pathological effects of infection (i.e., symptoms of disease), the adaptive defense mechanisms by the host, as well as the adaptive manipulation of the propagation parts of flowering plants. Such developmental changes can have huge implications for food availability and agricultural economies.

Information on floral malformations is limited and fragmented. This justifies the overall aim of this review to summarize current knowledge on this topic. The review considers in detail the different types of floral malformations and their causes, focusing on both biotic and abiotic factors. The abiotic include environmental stresses, such as temperature, drought, nutrient stress and hormonal imbalances. The biotic factors include insects, mites, nematodes, fungi, bacteria, phytoplasms and viruses. Of these causes, a specific focus and detailed information is placed on *Fusarium*, their role in causing floral malformation disease and their interactions with mites as these dynamics are commonly associated with floral malformation disease. Furthermore, the interaction between mites and *Fusarium* species is known to increase disease severity in different mite-fusarium disease complexes (Westphal and Manson, 1996).

1.1 Floral Malformations

All floral organs are homologous and each flower starts from a small fraction of undifferentiated cells, which develop into a complex structure with different organs occupying distinct positions (Bemis et al., 2013; Meyerowitz et al., 1989). These different floral organs include the sepals, petals, stamens and carpel (Meyerowitz et al., 1989). In many instances, floral abnormalities or modifications involve unusual development of bracts, which are specialized leaves associated with the flower, or unusual development of sepals, petals and carpels (Meyer, 1966). In other abnormalities, affected floral organs have the capability to metamorphose into different organs (Meyer, 1966). For instance, stamens, calyx and pistils can metamorphose into petals, a phenomenon known as petalody. Petalody has been observed in various species of the plant genera *Gloxinia*, *Mimulus*, *Nicotiana*, *Primula* and *Rhododendron* (Meyer, 1966). Another common form of floral metamorphosis is carpellody, a condition that is characterized by the transformation of stamens into additional carpels, resulting in ovary malformation (Marx and Mishanec, 1964; Meyer, 1966). This floral abnormality has been observed in *Carica papaya* (Kim et al., 2002), as well as *Hibiscus* species (Venkataramani, 1948). Staminody is the rarest form of floral malformation where sepals, petals and calyx metamorphose into stamens (Meyer, 1966). This form of metamorphosis has been observed in *Persea americana* (avocado) (Schroeder, 1940), *Musa sp.* (banana) (Nair and Karunakaran, 1962) *Allium*, *Begonia*, *Phaseolus* and *Prunus* species (Meyer, 1966).

Apart from modifications that are characterized by transformation of floral organs, virescence (Figure 1), hypertrophy and hyperplasia constitute notable forms of floral malformations (Meyer, 1966). Virescence is an abnormality in which floral organs develop abnormal green coloration in floral parts (Dafalla and Cousin, 1988). In the most extreme form, the organ involved is replaced by a foliage-type leaf. Any floral organ such as sepals, petals, pistils and ovules can become phylloid with the exception of the stamen that has the least likelihood to become phylloid (Meyer, 1966; Sim et al., 2004). In some cases, the proliferation of such leafy tissues results in a dense mass, giving the flower an appearance of a broom, and hence the name witches' broom. Witches' broom is more prominent on floral parts and shoots and is characterized by hyperplasia, loss of apical dominance and proliferation of axillary shoots (Aime and Phillips-Mora, 2005; Griffith et al., 2003; Stahel, 1915). Virescence has been observed on *Prunus avium* (sweet cherry) (Engin and Gokbayrak, 2010), *Vicia faba* (faba bean), *Catharanthus roseus* (Dafalla and Cousin, 1988),

Chrysanthemum indicum (chrysanthemum) (Verhoyen et al., 1979) and *Sesame indicum* (sesame) (Khan et al., 2007; Tseng et al., 2014; Win et al., 2010).

Hypertrophy (Figure 1) in floral parts is a condition that is characterized by excessive enlargement of cells within floral parts (Bos, 1960), while hyperplasia is characterized by excessive multiplication of cells within floral parts (Bos, 1963). Both conditions result in larger floral parts than expected under normal developmental conditions. For instance, in brassicas, the inflorescence axis and peduncles become thickened, the petals turn into greenish sepals, the stamens into carpelloid structures and the carpels into sterile structures (Bains and Jhooty, 1979; Rai, 2015; Verma and Bhowmik, 1989). In *Mangifera indica* (mango), hypertrophy is characterized by short and excessively branched and thickened panicles, with more flowers produced than normal (Kumar et al., 1993; Kvas et al., 2008; Marasas et al., 2006; Ploetz, 1994). This condition has also been observed in *Theobroma cacao* (cacao) (Aime and Phillips-Mora, 2005) *Searsia lancea* (karee) (Swanepoel, 2016) as well as *Syzygium cordatum* (water berry) (Kvas et al., 2008).

2 Causes of Floral Malformations

Floral malformations typically occur as a result of hormonal imbalance caused by either or both biotic and abiotic factors. Environmental conditions such as temperature, water stress, and nutrition, as well as biotic factors such as insect damage, infection by fungi, bacteria, phytoplasmas and viruses can disrupt the plant's hormone production. The subsequent hormonal imbalance can lead to the formation of malformed flowers (Engin and Gokbayrak, 2010; Sim et al., 2004).

2.1 Abiotic Factors

2.1.1 Temperature

Different stages of flower development are known to be temperature sensitive (Smith and Zhao, 2016). These include meiosis of pollen mother cells, tapetum development, anther dehiscence/pollen release, anthesis, as well as fertilization (Smith and Zhao, 2016). Floral development is, therefore, influenced by temperature stress, especially in petals and stamens. In *Rosa hybrida* (rose), day and night temperatures of 26°C and 21°C respectively, resulted in the

development of phyllody (Figure 1) four times higher than day and night temperatures of 21°C and 15°C (Chmelnitsky et al., 2001). By contrast, temperatures below 12°C caused the formation of a bullhead phenotype (Moe, 1971; Zieslin, 1968). The latter phenotype is characterized by larger sized flowers buds, increased short petals and petaloids, and a profusion of secondary florets bearing carpels near the base of the flower (Moe, 1971). *Dianthus caryphyllus* (carnation) also produce malformed flowers at temperatures between 5°C and 17°C but rarely occurrs at temperatures above 20°C (Yamane et al., 2018). In this plant, exposure to temperatures below 5°C results in the formation of secondary growing centers within the flower and a marked increase in petal numbers (Garrod and Harris, 1974). In *P. avium* (cherry) exposure to high temperatures (35°C and 25°C, day and night) for more than 15 days resulted in the formation of double pistils (Beppu et al., 2001), whereas temperatures of 25°C also causes floral malformation in *P. persica* (peach) (Beppu et al., 2001)

Solanum lycopersicum (tomato) grown at 17°C and 7°C (day and night), respectively, promoted meristic transformations and alterations in the fusion pattern of floral organs, which resulted in petaloid stamens (Lozano et al., 1998). Low night temperatures (18°C day/15°C night) have also been shown to cause floral malformations in *Capsicum annun* (hot pepper), which is characterized by the formation of abnormal petals, deformed and carpelloid stamens, and deformed gynoecium. In addition, these flowers produced abnormal non-viable pollen (Kohli et al., 1981; Polowick and Sawhney, 1985; Sawhney, 1981, 1983). In stone fruits, high temperatures are also known to be responsible for floral malformations.

In *Triticum aestivum* (wheat), a two-day interval of 36°C and 31°C (day and night) applied three days post floral emergence up to three days post anthesis, results in male sterility due to abnormal pollen grains. Similarly, in *Oryza sativa* (rice), exposure to 35°C and 25°C (day and night) resulted in decreased anther dehiscence and pollen counts (Das et al., 2014). In *O. sativa*, heat stress is also responsible for floral abnormalities in the form of stamen hypoplasia and pistil hyperplasia which causes spikelet sterility and reduced yield (Takeoka et al., 1991), while in *Zea mays* (maize) (Das et al., 2014) and . In chickpea (Devasirvatham et al., 2010; Kaushal et al, 2013), heat stress can result in reduced pollen tube length and reduced pollen germination. In contrast, low temperature stress during the reproductive stage of the plant cause floral abnormalities as a result of flower

abscission, ovule abortion, pollen sterility, pollen tube distortion, degeneration of spikelets, panicle deformation and poor spikelet fertility (Andaya and Mackill, 2003). For example, chilling temperatures of 9°C during reproductive phase in rice results in floral abnormalities characterized by deformation and spikelet degeneration (Andaya and Mackill, 2003; Thakur et al., 2010).

2.1.2 Water Stress

Drought stress is considered to be one of the greatest threats to crop development and crop yield, worldwide (Boyer, 1982). This is also true for reproductive stage development of flowers, which includes flower initiation, anther and pollen development, pistil development, blossom, fertilization and seed development (Saini and Lalonde, 1997). Among these, anther and pollen development is considered the most sensitive to water stress (Saini and Lalonde, 1997). For example, drought stress can result in shortened anther filaments, delayed anther development and dehiscence. In cereals, water stress can cause small and shrivelled anthers that do not dehisce, which results in reduced panicle length and blasted spikelets (Saini and Lalonde, 1997; Westgate et al., 1996). In *R. hybrida*, water stress during the onset of the second 3-leaflet leaf before the separation of 5-leaflet leaf, resulted in either abortion or malformation of flower buds (Chimonidou-Pavlidou, 1995, 2004). The malformed flower buds showed cessation of growth and development, tightly packed stamens, absence of carpels, short petals and stamens (Chimonidou-Pavlidou, 2004).

2.1.3 Nutrient Status

Nutrient status is important for plant development and critical in bud induction, flowering and fruit setting (El Hinnawy, 1956; Loehwing, 1940). For example, application of fertilizers such as nitrogen, phosphorous and potassium in *M. indica* during flower development, resulted in an increase in flower production as well as an increase percentage of flowers produced, compared to non-fertilized treatments (Anees et al., 2011; Thakur et al., 2000). Similarly in *P. persica* (peach) production, soil with poor nutrient status resulted in up to 90% flower abortion, pistil malformations and flower malformations (Crossa-Raynaud et al., 1985). In *Fragaria ananansa* (strawberry), a low soil nutrient status during differentiation of the primary flower bud enhanced phyllody-like symptoms (Lieten, 2000) and low nutrient status during floral induction and differentiation, enhanced other floral abnormalities such as tripartite sepals, elongated receptacles

and phyllody (van den Muijzenberg, 1942). Low nutrient soil content in *Spergularia marina* (salt sandspurry) also resulted in the increase of abnormal flowers, characterised by abnormal anther development (Delesalle and Mazer, 1996).

2.1.4 Hormone regulation

Transition to flowering is a very important process as it determines the plant's survival and its fitness. During this process, different signaling pathways are controlled by different phyto-hormones. These are compounds that are very important in determining flower sexuality, fertility and both male and female organ development (Smith and Zhao, 2016; Yan et al., 2012). These hormones include gibberellic acid, abscicic acid, jasmonate, brassinosteroids, cytokinin nitric acid and ethylene (Conti, 2017; Davis, 2009; Kazan and Lyons, 2015). The presence and/or imbalance of these hormones has an influence, not only on the production but also the quality of flowers produced, and thus an impact on flower anatomy and morphology. For example, in *M. indica* it has been noted that increased ethylene levels resulted in inhibition of the opening of flower buds, less differentiated flowers with fused sepals and petals, poorly developed stamens and carpels and in some cases aborted stamens with fused anther lobes (Singh et al., 2015).

In *M. indica,* it was observed that increased production of hormones such as naphthalene acetic acid and gibberellic acid indirectly increase floral malformation by promoting hyphal growth and conidial germination of *Fusarium* individuals responsible for floral malformation (Ansari et al., 2013). Similar effects have been observed for benzylaminopurine and ethylene (Ansari et al., 2013). These observations suggest that even though the disease may be caused by biotic agents, the increase in production of growth regulators enhances malformation. Understanding the role of growth regulators is, therefore, vital as their presence may promote or inhibit fungal growth, hence influencing malformation development (Ansari et al., 2013).

'Bullhead' malformation in *R. hybrida*, as a result of low temperatures, also showed reduced gibberellin and increased cytokinin activity as compared to normal flowers (Zieslin et al., 1979). Artificial injection of gibberellic acid into the flowers prevented malformation, while injection of benzyladenine reversed malformation (Zieslin et al., 1976). In contrast, flowers that received cytokinin treatment resulted in proliferation of the nectary and increased the appearance of

adventitious florets, which are a characteristic of malformed flowers. Understanding the role of regulators is therefore required in order to control malformation phenotypes (Zieslin, 1968; Zieslin et al., 1979).

Hormonal presence and/or imbalance not only has an influence on floral production, it also determines the sex of the flowers. For example, absence of gibberellic acid is known to cause male sterility in *Arabidopsis thaliana*. Mutant plants, deficient of gibberellic acid are characterized by abnormal microsporogenesis and retarded growth of all floral organs, especially stamens with greatly shortened filaments and severe defects in development (Cheng et al., 2004; Yan et al., 2012). In *Capsicum annum*, gibberellic acid also induces abnormalities in the development of petals and stamens, including the production of non-viable pollen (Kohli et al., 1981; Sawhney, 1981, 1983).

Auxin is another important phytohormone responsible for floral development including floral induction, floral growth and further development processes that lead to seed maturation (Cheng and Zhao, 2007; Krizek, 2011; Smith and Zhao, 2016). Disruption of auxin signaling, biosynthesis, and transport leads to flowers with various abnormalities (Cecchetti et al., 2008; Cheng et al., 2006; Cheng and Zhao, 2007; Nemhauser et al., 1998). In *Arabidopsis* (Cheng et al., 2006; Nemhauser et al., 1998; Wu et al., 2006) and *O. sativa* (Huang et al., 2015), auxin deficient plants have been shown to produce flowers with defects such as shortened petals, gynoecium, and stamen filaments, flowers that are not able to release pollen, as well as abnormal ovules (Cecchetti et al., 2008; Cheng et al., 2006).

2.2 Biotic factors

2.2.1 Bacteria

Among bacteria causing floral malformation, phytoplasmas are the most notable. These bacteria belong to the class Mollicutes. They are characterised by the lack of cell walls and cannot be cultured (Agrios, 2005; Gasparich, 2010; Weisburg et al., 1989). These are mainly transmitted by insects in the families *Cicadellidae* (leafhoppers), *Fulgoridae* (planthoppers), and *Psyllidae*

(psyllids). These insects feed on the phloem tissues of infected plants and alter normal balance of growth regulators (Bertaccini and Duduk, 2009). Plants infected with phytoplasmas exhibit symptoms such as phyllody, virescence, hypertrophy, sterility of flowers, proliferation of axillary buds leading to witches' broom (Lee et al., 2000; Bertaccini, 2007).

Phytoplasmas occur worldwide, infecting plants in different families including those that are economically important (Bertaccini, 2007). For example, *Pyrénées Orientales* stolbur phytoplasma strains have been observed to cause malformation in *Lycopersicon esculentum* (tomato) flowers. These malformations are characterized by green flowers i.e. the loss of normal flower pigment (virescence), the conversion of floral organs into leafy structures (phyllody), sepal hypotrophy, enlarged flower buds, stamens and carpels which lead to flower sterility (Jarausch et al., 2001). Similar symptoms have been observed in other plant species such as *Catharanthus roseus* (periwinkle) (Dafalla and Cousin, 1988; Nejat et al., 2013), *Sesamum indicum* L. (sesame) (Abraham et al., 1977; Khan et al., 2007; Schneider et al., 1995; Tseng et al., 2014; Win et al., 2010) *Cocos nucifera* L (coconut) (McCoy et al., 1983) and *Trifolium* species (clovers) (Chiykowski, 1962; Krczal, 1960).

2.2.2 Viruses

There are more than 2000 plant viruses residing in 21 families (Whitfield et al., 2015), which infect economically important plants, and they represent 47% of new and emerging plant diseases (Anderson et al., 2004). For their transmission, different organisms such as nematodes, fungi, bacteria and insects are involved (Ammar et al., 2009; Hogenhout et al., 2008) although they may also spread from one plant to the other through pollination and vegetative propagation (Bos, 1999). Symptoms of viral infections include mosaic patterns, chlorosis, necrosis, presence of stripes or streaks, leaf rolling and curling, flower deformation, stunting, discoloration or malformation stem pitting and grooving or tumors (Bos, 1999).

One of the most notable floral malformations is caused by *Vigna radiata* (mungbean) yellow mosaic virus. The virus causes severe losses and is wide spread in India, Pakistan and Sri Lanka (Biswas et al., 2008). Apart from mungbean, it also infects other legumes such as *Glycine max* (soybean), *Vigna unguiculata* (cowpea) and *Vigna mungo* (urdbean) causing significant losses as

a result of deformation and curling in flowers (Qazi et al., 2007). Tomato aspermy virus has also been reported to cause mosaic and flower deformation in *Chrysanthemum morifolium* (Florist's daisy, *Asteraceae)*, a very important ornamental plant and an economically important flower crop in India (Raj et al., 1991). Viruses have also been known to be responsible for colour breaking in flowers of different plants. For example, a potyvirus is famously responsible for Tulip breaking virus diseases (Hammond and Chastagner, 1989). In *R. hybrida*, Rose cryptic virus (RCV-1) has been known to cause flower breaking, petal crimping and petals that do not open normally (Hunter, 1966). Finally, virus associated colour breaking has also been observed in *Begoniacea, Geraniaceas, Ranunculacea* and *Primulaceae* (Daughtrey et al., 1995).

2.2.3 Insects

The Hemiptera are considered to be amongst the most important orders of gall forming insects. In this order, gall forming is mainly caused by aphids (Takei et al., 2015; Wool, 2004), scale insects (Hodgson et al., 2013) and psyllids (Yang and Raman, 2007).

Some of the better studied gall forming aphids include species in the genera *Copium* and *Paracopium. Copium* species causes floral malformations in *Teucrium* species whereas *Paracopium* species causes floral malformations in *Clerodendron* species (Meyer, 1987; Raman, 2012; Raman et al., 2005). The aphids induce flower galls with characteristics including fused and hypertrophied corollas that remain closed and thickened cell walls surrounding the hypertrophied parts which results in sterility of both male and female reproductive floral organs (Raman et al., 2005). Other than aphids, the Hemipteran psyllid *Livia juncorum* is responsible for causing virescence and witches' broom on *Junctus articulates* (Jarzembowski et al., 2014).

Dasineura is a genus comprising the tiny gall forming insect in the order Diptera commonly known to infest *Acacia* species, resulting in ovary deformation (Adair et al., 2000). However, linked to the abundance, fecundity and the ability to infest flowers (Degreef, 2002), some of the insect species within this genus insect have the potential to be used as a biological control agent in areas where *Acacia* species are invasive (Adair et al., 2000; Hill and Hulley, 1995). For example, in South Africa successful control of an invasive *Acacia longifolia* has been achieved by using the gall wasp *Trichilogaster acaciaelongifoliae* that infests and causes galls on immature flowers

(Dennill and Donnelly, 1991). *Lygus lineolaris* and other *Lygus* species (*Miridae*) are also known to infest flowers and their feeding causes abnormalities (Butts and Lamb, 1991). This has been observed in *Helianthus annuus* (sunflower) and *Brassica napus* (rapeseed) (Boyd and Lentz, 1994). Other insects that induce galls on flowers include *Dasineura virgaeaureae* on flowerheads of *Solidago virgaurea* (European goldenrot), and *Macrolabis achilae* on flowerheads of *Achillea ptarmica* (European pillotory), which have been recorded in Europe (Bruun et al., 2012). *Dasineura kiefferi* on flower buds of *Hedera helix* (English ivy), *Dasineura dactylidis* on infructuscence of *Dactylis glomerata* (Cocksfoot), *Dasineura cardaminis* on flower buds of *Cardamine amara* (bitter-cress), *Contarinia rumicis* on perianth and the nutlets on *Rumex obtusifolius* (Broad-leaved dock) (Bruun et al., 2014).

2.2.4 Nematodes

Nematodes are ubiquitous and cosmopolitan and can cause substantial losses to agricultural crops. As pathogens of vascular plants, nematodes exploit all parts of the plant (roots, stems, shoots) (Lambert and Bekal, 2002). Foliar infesting nematodes are becoming increasingly important because of their impact on ornamental and agricultural crops (Richardson and Grewal, 1993; de Tomasel and McIntyre, 2001). Some of the most notable foliar nematodes that causes floral deformations reside in the genera of *Ditylenchus* (Tylenchida: Anguinidae) and *Aphelenchoides* (*Tylenchida: Aphelenchoididae*) (Richardson and Grewal, 1993; Brown, 1957; Soh, 1986). *Ditylenchus dipsaci* is a migratory endoparasitic nematode which infests more than 450 plant species. The nematode feeds into parenchymatous tissues that cause the middle lamellae to fall out (Hooper and Southey, 1978). The feeding causes curling and deformation in stems, leaves and flowers in *Allium cepa* (onions), *Medicago sativa* (lucerne), *Trifolium pratense* (red clover), *Vicia faba* (faba beans) and *Phlox drummondii* (annual phlox) (Drees, 1978; Hooper and Southey, 1978). Another inflorescence gall inducing nematode within the same genus is the *Ditylenchus gallaeformans* which induces galls on species in the Melastomataceae with severe symptoms observed in the genus *Miconia* (Santin, 2008). Infestation results into inflorescences that have shortened axes, and do not commonly develop flowers or fruits (Ferreira et al., 2017).

The seed gall nematodes in the genus *Anguina* are gall forming and they inhabit the aerial parts of cereals and forage grasses. For example, *Anguina tritici*, an economically important wheat

pathogen whose infestation and galling results in shorter and wider inflorescence, glumes protruding at an abnormal angle exposing the galls, and hypertrophied ovaries that are light brown to nearly black in color (Ferris, 2013). Similarly, *Anguina agrostis* and *Anguina funesta* are economically important nematodes of *Agrostis stolonifera* (bentgrass) whose inflorescence infestation leads to enlarged glumes, lemmas and paleae. Infestation also results in greenish inflorescences that later become dark purple or dark brown (Alderman et al., 2003; McCay and Ophel, 1993).

Aphelenchoides besseyi is widely distributed in *O. sativa* growing regions (Khan et al., 2012). Its infestation results in distorted and shortened parnicles in *O. sativa* (Todd and Atkins, 1958) and distorted apical growth and crimping of inflorescences in *F. ananassa* (strawberries). In strawberries, similar symptoms have been observed after infestation by *Aphelenchoides fragariae* (Todd and Atkins, 1958; Brown et al., 1993). *Aphelencoides besseyi* is also responsible for causing floral malady in *Polianthes tuberosa* (tuberose) (Khan and Pal, 2001; Khan et al., 2012).

2.2.5 Mites

Phytophagous mites are economically important agricultural, forestry and natural ecosystem pests (Bolland et al., 1998; Jeppson et al., 1975). Of particular importance are species belonging to the families of *Tetranychidae* (spider mites), *Tenuipalpidae* (false spider mites), *Tarsonemidae* (tarsonemid mites) and *Eriophyidae* (gall forming mites). This is because of their potential to cause economic damage, either through direct feeding or by transmitting plant pathogens and viruses. Of the three groups, mites in the *Eriophyidae* are the only group that are responsible for causing galls on plants (Jeppson et al., 1975; Lindquist et al., 1996; Van Leeuwen et al., 2010).

The *Eriophyidae* is a large family of obligatory phytophagous mites comprising over 4000 described species. These species are highly host specific with approximately 80% of them known from a single host species, 95% from one host genus, and 99% from one host family, with non-host specific species feeding on very closely related hosts (Skoracka et al., 2009). In terms of flower malformation, the eriophyid mites are responsible for galling. For example, *Aceria guerreronis* infests *C. nucifera* flowers (Navia et al., 2005). It causes the sepals to lose turgor and it stops development (Lawson-Balagbo et al., 2008; Navia et al., 2013; Oliveira et al., 2012).

Losses of up to 65% have been reported in *L. esculentum* as a result of the tomato russet mite, *Aculopus lucopersici*. Its infestation of flowers causes shriveling and russeting of sepals and petals, which later on leads to defoliation of flowers (Jeppson et al., 1975). *Eriophyes fraxinivus* (*Aceria fraxinivus*) is another important inflorescence galling mite on *Fraxinus excelsior* and its infestations results in flower clusters (Jeppson et al., 1975). *Aceria rhodiolae* is known to induce galls on the flowers, leaves and stems of *Rhodiola rosea* in Europe (Roivainen, 1950), whereas the citrus bud mite, *Aceri sheldoni*, is a serious pest of *Citrus limon* (lemon) and *Citrus sinensis* (orange) trees. It's feeding within the buds invariably results in distortion of shoot growth, excessive and deformed flowers (Boyce and Korsmeier, 1941).

The apple rust mite, *Aculus schlechtendali* is also considered a very important pest with a worldwide distribution, infesting both wild crab apple and cultivated apple. Its infestation on flowers and buds induces galls (Easterbrook, 1996). *Aceria lantana* is known to cause flower deformation of *Lantana camara* by inducing galls on undeveloped flower buds, thus reducing the number of inflorescence, reducing seed production and subsequently sterilizes the plant (Craemer, 1996; Craemer and Neser, 1990). The ability of *Aceria lantana* to damage seed, however, has made it a potential biocontrol agent in areas where *Lantana camara* is considered an invasive weed (Urban et al., 2001a; Urban et al., 2001b).

Infestation by the bud mite, *Aceria pistaciae* on *Pistacia vera* (pistaccio) results in flower stalk brooming and deformation of flower clusters with male flowers modified and female flowers unfertilized (Mehrnejad and Daneshvar, 1991). *Phytoptus avellanae*, commonly called the big bud mite is a very important mite infesting tree nut crops. Its feeding on *Corylus avellana* (hazelnut) degenerative buds induces galls, which result in formation of big buds, resulting in huge economic losses in hazelnut growing regions in the Black sea region. *Cecidophyopsis ribis* is another mite that induces galls resulting in abnormal growth of buds (big buds) in *Ribes nigrum* (blackcurrant). In addition, it transmits blackcurrant reversion virus that renders plants unproductive (Jones, 2000).

2.2.6 Fungal-Floral Associations

Fungal pathogens can infect different floral parts such as petals, sepals, calyx, stamen and pistil. These pathogens consequently cause devastation in many economically important plants (Agrios, 1980, 2005). Infection of flowers by fungi can occur through direct invasion or entry through meristematic cells (Lewandowski et al., 2006). Infection may result in floral deformation such as virescence, phyllody, proliferation of auxilary shoots (side shoots), resulting in 'witches' broom', hyperplasia, hypertrophy, sterility of flowers, compact growth at the end of stems (Bertaccini, 2007; Strauss, 2009) and floral mimicry (Johnson, 1994; Roy, 1993; Roy and Widmer, 1999) (Figure 1). These developmental changes can manifest as symptoms of infection, adaptive defense mechanisms by the plant host, as well as adaptive manipulation of the fungus (Shykoff and Kaltz, 1998). Ultimately, the fungal pathogen alters the morphology of the floral structure or plant part into flower-like structures and they can also alter biochemical processes to assist in insect attraction, for example by producing pollen attractants, pheromone like compounds and defensive compounds (Raguso and Roy, 1998; Roy, 1993; Roy and Widmer, 1999).

Fungi associated with Malformed Inflorescences

Various genera and species of fungi are known to cause flower malformations. For example, *Moniliophthora perniciosa*, causes witches' broom disease. As mentioned above, witches' broom is characterized by hyperplasia, loss of apical dominance and proliferation of axillary shoots and the disease is more prominent on floral parts and shoots (Aime and Phillips-Mora, 2005; Griffith et al., 2003; Stahel, 1915). Witches' broom has also been induced by *Puccinia arrhenatheri* infecting *Berberis vulgaris* (Ngugi and Scherm, 2004). Another rust pathogen, *Puccinia monoica*, also induces formation of leaf rosettes and formation of pseudoflowers at the crown of elongated stems in herbaceous plant hosts. Species in the genus *Exobasidium*, for example, induce galls on leaves, shoots, and cause floral hypertrophy of ericaceous plants. For instance, *E. ferruginea* causes hypertrophy in *Lyonia ferruginea* (Kennedy et al., 2012), while *E. peckii* causes hypertrophy on *L. mariana* (Halsted, 1893).

The oomycete organism *Albugo candida*, that infects over 241 species in 63 genera of the *Cruciferacea* (Biga, 1955; Saharan and Verma, 1992), is known to cause floral hypertrophy and hyperplasia (Saharan and Verma, 1992; Verma, 1987). Apart from crucifers, *A. candida* also

infects amaranthaceae, coleomeceae and caparidaceae (Choi et al., 2011). The ability of the pathogen to cause abnormal growth and malformation has been attributed to the plant's defense response which subsequently alters its morphology. In addition, abnormal growth provides an increased surface area for sporulation. For instance, it induces blister-like pustules that result in twisted, deformed growth of the stem, leaves, or flowers. When mature, the epidermis covering the pustule ruptures, releasing powdery white sporangia that are either carried by wind or splashing water onto neighboring host plants (Kaur, 2013).

Sporisorium species, residing in the *Ustilaginaceae* (Basidiomycota), cause loose smut of the kernels in *Sorghum bicolor* (sorghum), *Sorghum sudanese* (sudan grass), *Saccharum officinarum* (sugarcane) and other crop species in the *Poacee* (Begerow et al., 2006). Infected heads are looser, bushy and a darker green colour than normal, largely due to hypertrophy of the glumes. In some cases fungal infection also reverts floral development to vegetative development (Matheussen et al., 1991). Smut related malformations have also been observed in *Z. maydis* caused by *Ustilago maydis* (Brefort et al., 2009; Schirawski et al., 2006) and in *Buchloe dactyloides* (buffalo grass) caused by *Salmacisia buchloëana* (Chandra and Huff, 2010).

Complete floral reversion leading to phyllody as a result of pathogen infection has also been observed as a result of *Sclerospora graminicola* infection in pearl millets. *Sclerospora graminicola* is considered an important fungal pathogen responsible for causing powdery mildew in *Penniseturn americanum* (pearl millet) (Semisi and Ball, 1989). The infected inflorescence results in proliferation of vegetative tissues, malformation of the ovary and the fused styllodia and abnormal stigmas protruding from diseased florets. The malformation results in phyllody and sterility (Semisi and Ball, 1989).

The rust fungus *Uromyces pisi* causes the development of pseudoflowers, also known as floral mimicry, in *Euphorbia cyparissias* (cypress spurge) (Pfunder and Roy, 2000). In this transformation, the pseudoflowers function to transmit fungal gametes rather than the host's pollen (Naef et al., 2002; Pfunder and Roy, 2000). In contrast, the fungus, produces sexual structures, which are deposited on the lower and upper leaves resembling a flower like structure with a ultraviolet reflectance and a strong sweet scent (Naef et al., 2002). Similar to *P. arrhenatheria*,

these pseudoflowers comprise of spermatogonia, receptive hyphae and they produce more sugary nectar than co-occurring flowers. In addition, the pseudoflowers have a bright yellow ultraviolet reflectance that aids in attraction of insect pollinators to transfer their fungal gametes (Ngugi and Scherm, 2004; Ngugi and Scherm, 2006; Roy, 1993). The rust fungus *Ravenelia macowaniana* is also known to induce large fleshy galls at the apex of seed and flower pedicels on *Vachellia karroo* (previously named *Acacia karroo*) (McGeogh, 1993).

Even though various different fungal pathogens are associated with floral malformations, *Fusarium* species are considered to be very important. The following sections will highlight the *Fusarium* species associated with floral malformations.

***Fusarium* Species Associated with Malformed Inflorescences**

Fusarium associated malformations have been extensively studied in *M. indica* due to their economic impact (Marasas et al., 2006), causing yield loss ranging from 80% to 100% (Ginai, 1965; Ploetz et al., 2002; Sharma and Singh, 2006; Varma et al., 1974). In this plant, floral malformation is mainly a disease of the panicles characterised by short, thickened, enlarged or hypertrophied rachises. In addition, the panicles are greener, with increased branching and with malformed panicles being compact and over-crowded due to the larger flowers (Kvas et al., 2008a; Marasas et al., 2006; Schlosser, 1971; Steenkamp et al., 2000). These panicles have numerous flowers that remain unopened and are predominantly male and rarely bisexual. Flowers are unusually enlarged, sterile and do not bear fruit (Kumar et al., 1993; Marasas et al., 2006; Ploetz et al., 2002).

Different *Fusarium* species are known to be responsible for Mango Malformation Disease MMD (Marasas et al., 2006). Based on pathogenicity tests, *F. mangiferae* (Britz et al., 2002; Freeman et al., 2002; Freeman et al., 1999; Marasas et al., 2006), *F. proliferatum* (Liew et al., 2016; Zhan et al., 2012; Zhan et al., 2010), *F. sterilihyphosum* (Britz et al., 2002), *F. mexicanum* (Rodríguez-Alvarado et al., 2013), *F. tupiense* (Lima et al., 2009; Lima et al., 2012) and *F. subglutinans* (Marasas et al., 2006; Steenkamp et al., 2000) have been implicated in this disease (Liew et al., 2016).

Different *Fusarium* species cause can cause MMD in different countries. For example, *F. sterilihyphosum* causes MMD in Egypt and Brazil, *F. mangiferae* causes malformation in Israel, Egypt and Malaysia (Freeman et al., 1999), *F. mexicanum* and *F. pseudocircinatum* causes MMD in Mexico (Otero-Colina et al., 2010, Freeman et al. 2014a), *F. proliferatum* in Egypt (Haggag et al. 2010) and China (Zhan et al., 2010) and *F. tupiense* in Brazil (Lima et al., 2012). Even though *F. incarnatum, F. oxysporum* and *F. anthophillum* were frequently isolated from mango inflorescences, their aetiology is not clear (Liew et al., 2016).

Fusarium species have been associated with floral abnormalities in various plant species other than mango. For example, *F. pseudocircinatum* is responsible for causing floral malformations in *Tabebuia rosea* (rosy trumpet) which results in malformed panicles with tightly shortened internodes that gives deformed flowers a cauliflower-like appearance in Mexico (Montoya-Martínez et al., 2021). This is the second time *F. pseudocircinatum* has been known to cause floral malformation disease in a plant other than *M. indica* in Mexico (Otero-Colina et al., 2010). There is a possibility that it may have jumped from *M. indica* to *T. rosea* or *T. rosea* to *M. indica*. Host jumps are common in plant fungal pathogens and have been reported in other pathosystems such as *Puccinia psidii*, *Cronartium ribicola* and *Fusarium circinatum* among others (Slippers et al., 2005; Heath et al., 2006). It could also be that one of the plant species is an alternative host and alternative hosts can serve as reservoirs of important plant pathogens which subsequently contribute to disease development (Mourelos et al., 2014; Suproniene et al., 2019).

Fusarium decemcellulare is known to be responsible for causing floral malformations which results in inflorescence over sprouting and enlarged floral peduncles with subsequent drying of floral parts in *Paullinia cupana* (guarana plants) (de Queiroz et al., 2020), *Averrhoa bilimbi* (bilimbi fruit) (Bastos and Santos, 2001*),* and *Anacardium occidentale* (cashew) (Matos et al., 2016). *F. decemcellulare* also causes cushion galls and induces witches broom appearance, a complex floral malformation diseases of *T. cacao* (cacao) which distortes the growth of flower cushions and subsequently affects normal fruiting (del Castillo et al., 2016; Vicente et al., 2012).

Fusarium verticillioides is responsible for *Fusarium* cotton hardlock, a disease in which *Gossypium hirsutum* (cotton) fibres fail to open and can result in yield losses of up to 70% under

severe conditions (Mailhot et al., 2007; Osekre et al., 2009). In contrast, *F. oxysporu*m is the predominant *Fusarium* species isolated from *Cicer arietinum* (chick pea) flowers and it is the main cause of *Fusarium* wilt of chick pea that may result in annual yield losses between 10% to 90% depending on severity (Arunodhayam et al., 2014; Haseeb et al., 2014).

Fusarium species have also been isolated from malformed inflorescences of monocotyledonous plants. For example *F. graminearum* is the causal agent of *Fusarium* head blight, a disease of the inflorescence of *Hordeum vulgare* (barley), *Avena sativa* (oats), *T. aestivum* (wheat), *Secale cereal* (rye) and other small grains (De-Galich, 1997; Parry et al., 1995). *Fusarium graminearum* has also been isolated from grasses such as *Agropyron, Agrostis, Bromus, Calamagrostis, Cenchrus, Cortaderia, , Echinochloa, Glycine, Hierochloe, Lolium, Lycopersicon, Phleum, Poa, Schizachyrium, Secale, Setaria, Sorghum* and *Spartina,* and other crops *Medicago, Cucumis, Trifolium* (Farr et al., 1989; Goswami and Kistler, 2004). Other *Fusarium* species such as *F. poae,* (Lombaert et al., 2003; Schollenberger et al., 2005; Schollenberger et al., 2007), *F. culmorun* (Clear and Patrick, 2006), *F. avenaceum* (Sørensen and Giese, 2013), *F. oxysporum* (Parry et al., 1995; Szunics, 1981; Wilcoxson et al., 1988) *F. solani*, *F. verticilliodes*, *F. subglutinans*, *F. proliferatum*, *F. semitectum*, *F. sporotrichioide,* and *F. equiseti* have also been associated and isolated from inflorescences in cereals, and are also listed among the causal agents of *Fusarium* head blight in small grain cereals (Parry et al., 1995; Torres et al., 2001). Other than cereals, *F. verticillioides* and *F. subglutinans* have been isolated from *Plantago lanceolata* (English plantain) (Alexander, 1984). In addition, *F. oxysporum* and *F. proliferatum* have also been isolated from date palm inflorescence and is responsible for inflorescence rot in *Phoenix dactylifera L.* (date palm) in North Africa, Iraq, Pakistan and most Arabic countries (Abdalla et al., 2000; Abdullah et al., 2010; Rheeder et al., 2002). *F. proliferatum* is listed as the causal agent for *Fusarium* pink rot in, a disease of flowers in *Allium* species (Shin and Kim, 2001).

2.3 Associations of *Fusarium* Species and Mites.

Various studies have suggested that there is an important interaction between mites (Navia et al., 2005; Navia et al., 2013), *Fusarium* species and floral malformations(Liew et al., 2016; Lv et al., 2010; Summanwar et al., 1966). However, the exact role of either the mites or *Fusarium* is not well understood. Different species of phytophagous mites in families such as *Acaridae*,

Siteroptidae, *Tydeidae*, and *Tarsonemidae* are known associates of *Fusarium* species (Hatcher and Paul, 2001). The types of interactions, such as parasitism, antagonism, mutualism and phoresy, however, are variable and they depend on the organisms involved.

In mite-plant-fungal associations, mites can facilitate fungal infection by vectoring pathogen spores or by creating wound sites in plants for fungal penetration (Agrios, 1980; Hatcher and Paul, 2001). *Fusarium mangiferae*, for example, is known to cause floral and bud malformations in *M. indica*, however, the association of *F. mangiferae* with *Aceria mangiferae* (the mango bud mite) is known to increase disease severity (Gamliel-Atinsky et al., 2009). This has mainly been attributed to the mite's involvement in fungal spore dispersal by vectoring the spores into penetration sites and assisting in conidial penetration and thus playing a role in fungal epidemiology (Gamliel-Atinsky et al., 2010; Gamliel-Atinsky et al., 2009).

Similar to the situation in mango, the association of *F. poae* and *Siteroptes avenae* resulted in an increase of *Fusarium* head blight severity as compared to control plants with no mites. In this case the severity was also attributed to the mite's involvement in spore dispersal as they showed to have a sporothecae (sac-like structure) which harbour conidia (Kemp et al., 1996). In South Africa, *F. poae* in association with *S. avena* has been associated with glumes spot on *Triticum aestivum* (wheat). *Fusarium poae* in association with *S. cerealium* (*S. graminum*) is also associated with severity of carnation (*Dianthus cariophyllus)* bud rot (Cooper, 1940; Zhang, 2003) and silver-top disease in wheat (Arnott and Bergis, 1967; Hardison, 1959). The presence of (one sporotheca) sporothecae in these *Siteroptes* species suggests the involvement of mites in *Fusarium* spore dispersal and subsequently disease development (Kemp et al., 1996).

Steneotarsonemus spinki is one of the most important mites in rice cultivation and can cause yield losses of up to 90% by attacking the glumes and parnicles of *O. sativa* (Rao and Prakash, 2003). However, this mite has also been found in association with *F. graminearum* and *F. verticillioides*, the causal agents of rice blast (Jagadiswari and Anand, 2003). In the *S. spinki*'s disease dynamic, the mite plays the role of opening wound spots and thus making it easier for the fungi to penetrate and hence increasing disease severity (Cardenas et al., 2003). The latter species and other

Fusarium species have also been observed in association with the coconut eriophyid mite, *Aceria guerreronis,* which is also a serious pest affecting yield in coconut production areas in India.

3 Conclusions

This review broadly summarizes the developmental changes in floral parts and inflorescences that lead to abnormalities collectively called floral malformation. It is a complex disease which manifests in different morphologies such as bracteody, carpellody, distortions, epinasty, petalody, phyllody, galls and witches' brooms. These floral changes are complex and may be as a result of infection or some type of adaptive defense mechanisms by the host. Various abiotic factors such as temperature, water stress, and nutrition as well as various biotic factors such as insect damage, fungal infection, phytoplasmas, mites and viruses may be attributed to producing malformed inflorescences. However, in many cases, the interaction of abiotic factors (heat and water stress), abiotic and biotic factors (hormonal imbalance and *Fusarium*) or biotic factors (mites and *Fusarium*) will proliferate the disease.

Studies to understand the ecological function, the types of malformation, and the interaction with the causal agents are generally lacking and literature in this area is scanty. There is need to study the type of morphological changes that occur during malformation and their causal agents. For example, the majority of insects cause gall malformation in order to derive nutrition and to find shelter. However, information on the evolution of these galls and characterization of the chemical changes within this transition is not available. While the ecological significance of galls to provide nutrients and shelter in insects is known, future studies should also focus on the chemical changes that lead to these morphological transformations.

This review has provided a foundation for the research chapters in the thesis that follows it. More specifically, the research seeks to understand the diversity of *Fusarium* and mite species associated with *Syzygium cordatum* (Waterberry) Malformation Disease (WMD) which shares striking similarities with Mango Malformation Disease (MMD) i.e. hypertrophied panicles that are thick and shortened and excessively branched. Another similarity between WMD and MMD is the association of the affected inflorescences with eriophyoid mites. Mango Malformation disease (MMD) is known to be associated with *Aceria mangiferae* and this association is known to have

an impact on the disease epidemiology of the disease. On the other hand, the eriophyoid mite species associated with WMD has not yet been identified and its role in causing disease and/or its interaction with *Fusarium* is also unknown.

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a

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Figure 1: Floral malformations in plants

(a) Malformed *Syzygium cordatum* (waterberry) inflorescence **(b)** healthy *Syzygium cordatum* (waterberry) inflorescence (Mkandawire et al., 2021) **(c)** hypetrophy and malformed *Mangifera indica* (mango) inflorescences **(d)** healthy *Mangifera indica* (mango) inflorescences (Pradeep et al., 2011); **(e)** virescence (L) and healthy (R) flowers in *Hydrangea* sp. (Oshima et al., 2013); **(f)** Phyllody in (L) and healthy (R) flowers in *Callistephus chinensis* (China asters) (Bellardi et al., 2018); **(g)** floral mimicry caused by *Puccinia monoica* on *Euphorbiacea* sp. (Rutwoski, 2015).

Chapter 2: Diversity of *Fusarium* species associated with healthy and malformed *Syzygium cordatum* inflorescences in South Africa.

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Diversity of *Fusarium* species associated with healthy and malformed *Syzygium cordatum* inflorescences in South Africa

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Abstract *Syzygium cordatum* (Myrtacease) is one of the most common encountered and widely distributed tree species indigenous to South Africa. This tree is often affected by a malformation disease characterized by grossly misshapen inflorescences that do not bear fruit. Because such symptoms have previously been attributed to *Fusarium* species in other plants, the aim of this study was to determine the diversity of *Fusarium* species associated with *S. cordatum* inflorescences. Healthy and malformed *S. cordatum* inflorescences were collected from Gauteng, Mpumalanga, Western Cape, and KwaZulu-Natal. A total of 118 *Fusarium* isolates were obtained from healthy (19) and malformed (99) inflorescences and identified using the translation elongation factor 1 alpha (*TEF1*α) gene region. The results revealed that 39 isolates belonged to the *Fusarium fujikuroi* species complex (FFSC), 45 isolates to the *Fusarium oxysporum* species complex (FOSC), 33 to the *Fusarium incarnatum-equiseti* species complex (FIESC) and one isolate resided in the *Fusarium chlamydosporum* species complex (FCSC). Phylogenetic analysis separated these isolates into 15 species, of which five (two in the FFSC, three in the FIESC) are new to science. No obvious patterns were found with respect to species recovered in different geographic areas sampled. However, FFSC species, were all recovered only from malformed inflorescences.

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Keywords *Fusarium* · *Syzygium cordatum* · malformation · species diversity

Introduction

Syzygium cordatum (family Myrtaceae) is a tree species indigenous to South Africa that commonly occurs along watercourses on forest margins and in swampy areas. The species is widely planted as an ornamental and used in rural areas for firewood and timber (Dlamini, 1981). In these areas, the fruits are also a source of food, while the bark is used in traditional medicines (Palgrave, 1977; Pooley, 1993; Van Wyk 2011; Van Wyk et al., 1997). Throughout its native range, as well as in urban areas, *S. cordatum* inflorescences are commonly malformed. These malformed structures are associated with the abnormal development of vegetative shoots and floral panicles that are excessively branched and characterized by unusually shortened and thickened rachises. These flowers are typically sterile, are unable to bear fruits or abort prematurely (Rani et al., 2013).

Various biotic and abiotic factors can cause flower malformation, often as a result of hormonal imbalances elicited by these factors (Kaur and Kaur, 2018).

Some of the most important abiotic conditions that trigger malformation include exposure to temperature outside the plant's normal temperature range, drought stress, and nutrient deficiencies (Sim et al., 2004; Engin and Gokbayrak, 2010). Biotic factors resulting in malformation include damage caused by gall-forming insects (e.g., aphids, scale insects, and psyllids) or infection by nematodes and phytophagous mites (Sim et al., 2004). Additionally, infections by various fungi, bacteria, and viruses are also known to cause flower malformation, either as a result of infection or as an adaptive defense mechanism by the host plant (Shykoff and Kaltz, 1998).

Symptoms observed on *S. cordatum*, are similar to those associated with the so-called mango malformation disease (MMD) on *Mangifera indica* (Summanwar et al., 1966; Schlosser, 1971; Marasas et al., 2006). By making use of infection studies, a range of species in the fungal genus *Fusarium* has been identified as causal agents of MMD in various countries of Asia, Africa, the Americas, Europe, and the Middle East. These species include *F. mangiferae* (previously reported as *F. subglutinans*) (Freeman et al., 1999; Steenkamp et al., 2000; Britz et al., 2002; Ploetz et al., 2002; Kvas et al., 2008; Liew et al., 2016), *F. sterilihyphosum* (Steenkamp et al., 2000; Britz et al., 2002), *F. mexicanum* (Otero-Colina et al., 2010), *F. tupiense* (Lima et al., 2012), *F. proliferatum* (Zhan et al., 2010; Liew et al., 2016) and *F. pseudocircinatum* (Freeman et al., 2014; Liew et al., 2016).

Fusarium represents a ubiquitous group of filamentous fungi within the class Ascomycetes. The genus is of significant importance, with estimates suggesting that at least 80% of economically important food crops are affected by pathogens in this group (Leslie and Summerell, 2006a). *Fusarium* species can infect plants at every stage of growth and can cause diseases such as seed, root, stalk, ear and kernel rot, seedling and head blight, as well as, cankers, wilts, and leaf diseases (Leslie and Summerell, 2006a; Summerell and Leslie, 2011). Some *Fusarium* species are also regarded as endophytes or saprotrophs (Summerell et al., 2010). Additionally, some species in this genus pose serious health risks to both humans and animals due to the role they play in producing a variety of toxic secondary metabolites, which can cause acute or chronic diseases (Desjardin, 2006).

The aim of this study was to determine the diversity of *Fusarium* species obtained from *S. cordatum* inflorescences. We hypothesized that several *Fusarium* species would be isolated from this indigenous plant species. We sampled and compared healthy and malformed *S. cordatum* inflorescences. This was done in order to understand which of these *Fusarium* species might contribute to the malformation disease, especially due to the similarity of malformed *S. cordatum* inflorescences to MMD.

Materials and methods

Sample collection and fungal isolations

In 2008, malformed inflorescences were collected from *S. cordatum* trees at various locations across South Africa, including Gauteng, Mpumalanga, Western Cape, and KwaZulu-Natal (Table 1). Thereafter, a second and more comprehensive collection was made in 2016 from trees growing in KwaZulu-Natal (Table 1). During the 2016 survey, both healthy and malformed inflorescences were collected, with five malformed and one healthy inflorescence sampled per tree. In total, 15 *S. cordatum* trees were sampled from different locations, covering the areas of Dukuduku, Mtubatuba, Nyalazi and Mzingazi in addition to Kwambonambi, in the KwaZulu-Natal province.

Prior to fungal isolations, the plant material was surface disinfested by submerging small pieces of an inflorescence in 1% (v/v) sodium hypochlorite solution and then 70% (v/v) ethanol, for 1 min each. Samples were then rinsed in sterile distilled water and plated directly onto Potato Dextrose Agar (PDA; 20g/L PDA, 5g/L Agar; Biolab Diagnostics, Wadeville South Africa) medium and onto *Fusarium* selective medium (FSM) (Nash and Snyder, 1962). Following incubation at 25 °C for 7 days, isolates resembling *Fusarium* were transferred to fresh PDA plates and incubated at 25 °C for 7 days. For each isolate, a spore suspension was then prepared by flooding the plate with sterile water and used to inoculate water agar medium (WA; 20 g/L Agar; Biolab Diagnostics). Single germinating conidia were then used to establish pure cultures for the respective isolates, which were subsequently stored in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (Table 1).

Table 1 Origin and putative species of the isolates from *Syzygium cordatum* used in this study

CMW isolate number	Collection date	Province	Location	Tissue[a]	FID Based ID[b]	%	Phylogeny Based ID	Species complex[c]	GenBank Accession Numbers (TEF1α)
CMW 55303	2016	KwaZulu Natal	Mzingazi	MI	*F. fujikuroi*	100	*F. fujikuroi*	FFSC	MZ966199
CMWF 1156	2008	KwaZulu Natal	Kwambonambi	MI	*F. fujikuroi*	100	*F. fujikuroi*	FFSC	MZ966200
CMWF 1171	2008	KwaZulu Natal	Kwambonambi	MI	*F. fujikuroi*	97,95	*F. fujikuroi*	FFSC	MZ966205
CMWF 1173	2008	KwaZulu Natal	Kwambonambi	MI	*F. fujikuroi*	95,68	*F. fujikuroi*	FFSC	MZ966210
CMWF 894	2008	KwaZulu Natal	Kwambonambi	MI	*F. fujikuroi*	99,69	*F. fujikuroi*	FFSC	MZ966209
CMWF 898	2008	Gauteng	Pretoria	MI	*F. fujikuroi*	100	*F. fujikuroi*	FFSC	MZ966206
CMWF 900	2008	KwaZulu Natal	Penington	MI	*F. fujikuroi*	100	*F. fujikuroi*	FFSC	MZ966204
CMW 55766	2016	KwaZulu Natal	Nyalazi	MI	*F.proliferatum*	100	*F. annulatum*	FFSC	MZ966202
CMWF 954	2008	KwaZulu Natal	Kwambonambi	MI	*F. proliferatum*	94,87	*F. fujikuroi*	FFSC	MZ966207
CMWF 902	2008	KwaZulu Natal	Kwambonambi	MI	*F. fujikuroi*	100	*F. fujikuroi*	FFSC	MZ966203
CMWF 936	2008	KwaZulu Natal	Penington	MI	*F. fujikuroi*	99,7	*F. fujikuroi*	FFSC	MZ966208
CMWF 949	2008	KwaZulu Natal	Kwambonambi	MI	*F. fujikuroi*	95,73	*F. fujikuroi*	FFSC	MZ966201
CMW 55298	2016	KwaZulu Natal	Mtubatuba	MI	*F. proliferatum*	99,39	*F. annulatum*	FFSC	MZ966183
CMW 55301	2016	KwaZulu Natal	Kwambonambi	MI	*F. proliferatum*	99,04	*F. annulatum*	FFSC	MZ966193
CMW 55302	2016	KwaZulu Natal	Kwambonambi	MI	*F. proliferatum*	95,3	*F. annulatum*	FFSC	MZ966196
CMW 55776	2016	KwaZulu Natal	Kwambonambi	MI	*F. proliferatum*	99,04	*F. fujikuroi*	FFSC	MZ966184
CMWF 1000	2008	KwaZulu Natal	Kwambonambi	MI	*F. proliferatum*	99,39	*F. annulatum*	FFSC	MZ966191
CMWF 1009	2008	KwaZulu Natal	Kwambonambi	MI	*F. proliferatum*	98,61	*F. annulatum*	FFSC	MZ966189
CMWF 1132	2008	Mpumalanga	Buffelskloof	MI	*F. proliferatum*	96,12	*F. annulatum*	FFSC	MZ966192
CMWF 1133	2008	KwaZulu Natal	Richards bay	MI	*F. proliferatum*	99,09	*F. annulatum*	FFSC	MZ966197
CMWF 951	2008	Western Cape	Belville	MI	*F. proliferatum*	96,12	*F. annulatum*	FFSC	MZ966194
CMWF 965	2008	KwaZulu Natal	Kwambonambi	MI	*F. proliferatum*	99,72	*F. annulatum*	FFSC	MZ966198
CMWF 966	2008	KwaZulu Natal	Kwambonambi	MI	*F. proliferatum*	97,35	*F. annulatum*	FFSC	MZ966195
CMWF 976	2008	KwaZulu Natal	Kwambonambi	MI	*F. proliferatum*	98,95	*F. annulatum*	FFSC	MZ966190

Table 1 (continued)

CMW isolate number	Collection date	Province	Location	Tissue[a]	FID Based ID[b]	%	Phylogeny Based ID	Species complex[c]	GenBank Accession Numbers (TEF1α)
CMWF 980	2008	KwaZulu Natal	Kwambonambi	MI	*F. proliferatum*	99,85	*F. annulatum*	FFSC	MZ966187
CMWF 990	2008	Gauteng	Pretoria	MI	*F. proliferatum*	97,5	*F. annulatum*	FFSC	MZ966186
CMWF 994	2008	KwaZulu Natal	Kwambonambi	MI	*F. proliferatum*	99,98	*F. annulatum*	FFSC	MZ966185
CMWF 999	2008	KwaZulu Natal	Kwambonambi	MI	*F. proliferatum*	99,53	*F. annulatum*	FFSC	MZ966188
CMWF 1029	2008	KwaZulu Natal	Kwambonambi	MI	*F. sacchari*	99,69	*F. sacchari*	FFSC	MZ966211
CMWF 1139	2008	KwaZulu Natal	Kwambonambi	MI	*F. verticillioides*	97,56	*F. verticillioides*	FFSC	MZ966218
CMW 55299	2016	KwaZulu Natal	Mtubatuba	MI	*F. verticillioides*	99,21	*Fusarium sp. nov.* 2	FFSC	MZ966212
CMW 55753	2016	KwaZulu Natal	Kwambonambi	MI	*F. verticillioides*	98,7	*Fusarium sp. nov.* 2	FFSC	MZ966213
CMW 55769	2016	KwaZulu Natal	Mzingazi	MI	*F. verticillioides*	97,8	*Fusarium sp. nov.* 2	FFSC	MZ966215
CMWF 1151	2008	Gauteng	Pretoria	MI	*F. verticillioides*	98,6	*Fusarium sp. nov.* 2	FFSC	MZ966214
CMWF 956	2008	KwaZulu Natal	Kwambonambi	MI	*F. verticillioides*	97	*Fusarium sp. nov.* 2	FFSC	MZ966217
CMWF 973	2008	KwaZulu Natal	Kwambonambi	MI	*F. verticillioides*	97,51	*Fusarium sp. nov.* 2	FFSC	MZ966216
CMW 55304	2016	KwaZulu Natal	Kwambonambi	MI	*F. fujikuroi*	97,1	*Fusarium sp. nov*. 1	FFSC	MZ966179
CMW 55305	2016	KwaZulu Natal	Dukuduku	MI	*F. fujikuroi*	96	*Fusarium sp. nov*. 1	FFSC	MZ966182
CMWF 924	2008	KwaZulu Natal	Kwambonambi	MI	*F. fujikuroi*	98,21	*Fusarium sp. nov*. 1	FFSC	MZ966181
CMW 55321	2016	KwaZulu Natal	Mzingazi	MI	*F. oxysporum*	95,94	*F. oxysporum*	FOSC	MZ966263
CMWF 1048	2008	Western Cape	Belville	MI	*F. oxysporum*	99,33	*F. oxysporum*	FOSC	MZ966247
CMW 55765	2016	KwaZulu Natal	Mzingazi	MI	*F. oxysporum*	99,84	*F. oxysporum*	FOSC	MZ966254
CMW 55767	2016	KwaZulu Natal	Dukuduku	HI	*F. oxysporum*	98,48	*F. oxysporum*	FOSC	MZ966261
CMWF 1055	2008	Western Cape	Belville	MI	*F. oxysporum*	92,5	*F. oxysporum*	FOSC	MZ966252
CMWF 938	2008	KwaZulu Natal	Kwambonambi	MI	*F. oxysporum*	98,03	*F. oxysporum*	FOSC	MZ966257
CMWF 979	2008	Western Cape	Belville	MI	*F. oxysporum*	97,86	*F. oxysporum*	FOSC	MZ966258
CMWF 1036	2008	Western Cape	Belville	MI	*F. oxysporum*	99,19	*F. oxysporum*	FOSC	MZ966251
CMWF 1043	2008	Western Cape	Belville	MI	*F. oxysporum*	97,95	*F. oxysporum*	FOSC	MZ966259

Table 1 (continued)

CMW isolate number	Collection date	Province	Location	Tissue[a]	FID Based ID[b]	%	Phylogeny Based ID	Species complex[c]	GenBank Accession Numbers (TEF1α)
CMWF 955	2008	KwaZulu Natal	Kwambonambi	MI	*F. oxysporum*	99,7	*F. oxysporum*	FOSC	MZ966255
CMW 55316	2016	KwaZulu Natal	Mzingazi	MI	*F. oxysporum*	98,54	*F. oxysporum*	FOSC	MZ966253
CMWF 985	2008	Western Cape	Belville	MI	*F. oxysporum*	95,55	*F. oxysporum*	FOSC	MZ966262
CMW 55313	2016	KwaZulu Natal	Mzingazi	HI	*F. oxysporum*	99,83	*F. oxysporum*	FOSC	MZ966260
CMW 55314	2016	KwaZulu Natal	Mzingazi	HI	*F. oxysporum*	99,55	*F. oxysporum*	FOSC	MZ966249
CMW 55759	2016	KwaZulu Natal	Kwambonambi	MI	*F. oxysporum*	95,39	*F. oxysporum*	FOSC	MZ966256
CMW 55311	2016	KwaZulu Natal	Kwambonambi	MI	*F. oxysporum*	99,81	*F. oxysporum*	FOSC	MZ966248
CMW 55319	2016	KwaZulu Natal	Kwambonambi	MI	*F. oxysporum*	94,67	*F. oxysporum*	FOSC	MZ966250
CMWF 1058	2008	KwaZulu Natal	Kwambonambi	MI	*F. oxysporum*	98,96	*F. oxysporum*	FOSC	MZ966232
CMW 55312	2016	KwaZulu Natal	Mzingazi	MI	*F. oxysporum*	98,27	*F. oxysporum*	FOSC	MZ966231
CMW 55773	2016	KwaZulu Natal	Dukuduku	HI	*F. oxysporum*	94,31	*F. oxysporum*	FOSC	MZ966243
CMW 55318	2016	KwaZulu Natal	Kwambonambi	MI	*F. oxysporum*	97,88	*F. oxysporum*	FOSC	MZ966233
CMW 55324	2016	KwaZulu Natal	Mzingazi	MI	*F. oxysporum*	98,63	*F. oxysporum*	FOSC	MZ966244
CMW 55778	2016	KwaZulu Natal	Mzingazi	HI	*F. oxysporum*	99,53	*F. oxysporum*	FOSC	MZ966222
CMW 55317	2016	KwaZulu Natal	Nyalazi	MI	*F. oxysporum*	97,46	*F. oxysporum*	FOSC	MZ966228
CMW 55758	2016	KwaZulu Natal	Mzingazi	MI	*F. oxysporum*	98,43	*F. oxysporum*	FOSC	MZ966227
CMW 55756	2016	KwaZulu Natal	Kwambonambi	MI	*F. oxysporum*	98,25	*F. oxysporum*	FOSC	MZ966221
CMW 55323	2016	KwaZulu Natal	Dukuduku	MI	*F. oxysporum*	98,48	*F. oxysporum*	FOSC	MZ966245
CMW 55763	2016	KwaZulu Natal	Mtubatuba	MI	*F. oxysporum*	97,22	*F. oxysporum*	FOSC	MZ966229
CMW 55760	2016	KwaZulu Natal	Mzingazi	MI	*F. oxysporum*	96,03	*F. oxysporum*	FOSC	MZ966236
CMW 55757	2016	KwaZulu Natal	Mtubatuba	MI	*F. oxysporum*	99,7	*F. oxysporum*	FOSC	MZ966226
CMW 55315	2016	KwaZulu Natal	Dukuduku	HI	*F. oxysporum*	99,7	*F. oxysporum*	FOSC	MZ966220
CMW 55307	2016	KwaZulu Natal	Mtubatuba	MI	*F. oxysporum*	99,7	*F. oxysporum*	FOSC	MZ966239
CMW 55322	2016	KwaZulu Natal	Mzingazi	MI	*F. oxysporum*	98,05	*F. oxysporum*	FOSC	MZ966224

Table 1 (continued)

CMW isolate number	Collection date	Province	Location	Tissue[a]	FID Based ID[b]	%	Phylogeny Based ID	Species complex[c]	GenBank Accession Numbers (TEF1α)
CMW 55764	2016	KwaZulu Natal	Mzingazi	MI	*F. oxysporum*	98,48	*F. oxysporum*	FOSC	MZ966240
CMWF 1019	2008	KwaZulu Natal	Kwambonambi	MI	*F. oxysporum*	98,72	*F. oxysporum*	FOSC	MZ966223
CMWF 1057	2008	KwaZulu Natal	Kwambonambi	MI	*F. oxysporum*	97,73	*F. oxysporum*	FOSC	MZ966230
CMWF 1046	2008	Western Cape	Belville	MI	*F. oxysporum*	98,67	*F. oxysporum*	FOSC	MZ966225
CMW 55768	2016	KwaZulu Natal	Kwambonambi	MI	*F. oxysporum*	92,57	*F. oxysporum*	FOSC	MZ966241
CMW 55777	2016	KwaZulu Natal	Nyalazi	MI	*F. oxysporum*	99,85	*F. oxysporum*	FOSC	MZ966237
CMW 55320	2016	KwaZulu Natal	Mzingazi	MI	*F. oxysporum*	93,47	*F. oxysporum*	FOSC	MZ966238
CMW 55771	2016	KwaZulu Natal	Mtubatuba	MI	*F. oxysporum*	97,33	*F. oxysporum*	FOSC	MZ966246
CMW 55309	2016	KwaZulu Natal	Mzingazi	HI	*F. oxysporum*	99,4	*F. oxysporum*	FOSC	MZ966234
CMW 55774	2016	KwaZulu Natal	Mtubatuba	MI	*F. oxysporum*	93,04	*F. oxysporum*	FOSC	MZ966242
CMW 55310	2016	KwaZulu Natal	Kwambonambi	MI	*F. oxysporum*	93,2	*F. oxysporum*	FOSC	MZ966235
CMW 55306	2016	KwaZulu Natal	Mzingazi	HI	*F. oxysporum*	93,67	*F. oxysporum*	FOSC	MZ966219
CMW 55738	2016	KwaZulu Natal	Kwambonambi	MI	*F. equiseti*	93,8	*Fusarium sp. nov.* 3	FIESC	MZ966178
CMW 55737	2016	KwaZulu Natal	Kwambonambi	MI	*F. scirpi*	88	*Fusarium sp. nov.* 3	FIESC	MZ966177
CMW 55736	2016	KwaZulu Natal	Kwambonambi	MI	*F. camptocera*	92,67	*Fusarium sp. nov.* 3	FIESC	MZ966174
CMW 55742	2016	KwaZulu Natal	Kwambonambi	HI	*F. equiseti*	92,22	*Fusarium sp. nov.* 3	FIESC	MZ966175
CMW 55743	2016	KwaZulu Natal	Kwambonambi	HI	*F. caatingaense*	90,8	*Fusarium sp. nov.* 3	FIESC	MZ966176
CMW 55735	2016	KwaZulu Natal	Kwambonambi	MI	*F. caatingaense*	96,502	*Fusarium sp. nov.* 4	FIESC	MZ966155
CMW 55739	2016	KwaZulu Natal	Kwambonambi	MI	*F. caatingaense*	96,05	*Fusarium sp. nov.* 4	FIESC	MZ966156
CMWF 1056	2008	Western Cape	Belville	MI	*F. guilinense*	95,95	*Fusarium sp. nov.* 5	FIESC	MZ966153
CMW 55747	2016	KwaZulu Natal	Kwambonambi	MI	*F. guilinense*	96,75	*Fusarium sp. nov.* 5	FIESC	MZ966152
CMWF 1031	2008	Western Cape	Hartenbos	MI	*F. guilinense*	98,98	*Fusarium sp. nov.* 5	FIESC	MZ966150
CMWF 1017	2008	KwaZulu Natal	Kwambonambi	MI	*F. guilinense*	98,67	*Fusarium sp. nov.* 5	FIESC	MZ966151
CMW 55745	2016	KwaZulu Natal	Mtubatuba	HI	*F. guilinense*	91,49	*Fusarium sp. nov.* 5	FIESC	MZ966149

Table 1 (continued)

CMW isolate number	Collection date	Province	Location	Tissue[a]	FID Based ID[b]	%	Phylogeny Based ID	Species complex[c]	GenBank Accession Numbers (TEF1α)
CMW 55329	2016	KwaZulu Natal	Kwambonambi	HI	*F. caatingaense*	97,01	*F. caatingaense*	FIESC	MZ966148
CMW 55328	2016	KwaZulu Natal	Kwambonambi	MI	*F. caatingaense*	98,88	*F. caatingaense*	FIESC	MZ966146
CMWF 1007	2008	KwaZulu Natal	Kwambonambi	MI	*F. caatingaense*	97,64	*F. caatingaense*	FIESC	MZ966154
CMWF 1028	2008	Western Cape	Hartenbos	MI	*F. caatingaense*	99,2	*F. caatingaense*	FIESC	MZ966147
CMWF 1182	2008	Western Cape	Belville	MI	*F. scirpi*	98,32	*F. scirpi*	FIESC	MZ966173
CMW 55730	2016	KwaZulu Natal	Nyalazi	MI	*F. lacertarum*	95,66	*F. lacertarum*	FIESC	MZ966159
CMW 55744	2016	KwaZulu Natal	Mtubatuba	HI	*F. lacertarum*	94,94	*F. lacertarum*	FIESC	MZ966158
CMW 55734	2016	KwaZulu Natal	Kwambonambi	MI	*F. lacertarum*	97,24	*F. lacertarum*	FIESC	MZ966157
CMW 55330	2016	KwaZulu Natal	Kwambonambi	MI	*F. clavum*	97,4	*F. clavum*	FIESC	MZ966170
CMW 55761	2016	KwaZulu Natal	Kwambonambi	HI	*F. clavum*	95,61	*F. clavum*	FIESC	MZ966163
CMW 55327	2016	KwaZulu Natal	Mtubatuba	HI	*F. clavum*	97,43	*F. clavum*	FIESC	MZ966166
CMW 55333	2016	KwaZulu Natal	Mzingazi	MI	*F. clavum*	97,92	*F. clavum*	FIESC	MZ966169
CMW 55750	2016	KwaZulu Natal	Nyalazi	MI	*F. clavum*	98,56	*F. clavum*	FIESC	MZ966168
CMW 55775	2016	KwaZulu Natal	Kwambonambi	HI	*F. clavum*	97,83	*F. clavum*	FIESC	MZ966165
CMW 55752	2016	KwaZulu Natal	Mzingazi	MI	*F. clavum*	97,75	*F. clavum*	FIESC	MZ966162
CMW 55754	2016	KwaZulu Natal	Kwambonambi	HI	*F. clavum*	97,98	*F. clavum*	FIESC	MZ966167
CMW 55326	2016	KwaZulu Natal	Mzingazi	MI	*F. clavum*	95,15	*F. clavum*	FIESC	MZ966161
CMW 55334	2016	KwaZulu Natal	Mtubatuba	MI	*F. clavum*	93,63	*F. clavum*	FIESC	MZ966164
CMW 55325	2016	KwaZulu Natal	Kwambonambi	HI	*F. clavum*	98,44	*F. clavum*	FIESC	MZ966160
CMW 55332	2016	KwaZulu Natal	Mzingazi	MI	*F. clavum*	84,11	*F. clavum*	FIESC	MZ966172
CMW 55331	2016	KwaZulu Natal	Mzingazi	MI	*F. clavum*	97,64	*F. clavum*	FIESC	MZ966171
CMWF 1051	2008	KwaZulu Natal	Kwambonambi	HI	*F. chlamydosporum*	998,3	*F. sporodochiale*	FCSC	MZ966264

[a]Tissue indicates whether the inflorescence was malformed or healthy (MI= malformed inflorescence, HI = healthy inflorescence)

[b] FID Based ID represent the top BLAST hit for the *TEF1*α sequence searches against the *Fusarium* ID database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Geiser et al. 2004).

[c] FFSC denotes the *F. fujikuroi* species complex, FOSC denotes the *F. oxysporum* species complex, FIESC denotes the *F. incarnatum-equiseti* species complex and FCSC denotes the *F. chylamydosporum* species complex.

DNA extraction, PCR amplification and sequencing

DNA was extracted from 7-day-old cultures using a modified CTAB (hexadecyltrimethylammonium bromide) method (Steenkamp et al., 1999). Briefly, fungal tissue was scraped from the growth medium and homogenized in 500µl DNA extraction buffer containing 100 mM Tris-HCl (pH 8.0), 10mM ethylenediaminetetra-acetic acid di-sodium salt (EDTA; pH 8.0), 2% (w/v) sodium dodecyl sulphate and 0.2 µg/µl proteinaise K (Sigma-Aldrich, St Loius, Missouri, USA). This was followed by freezing for 10 min at -70 °C, defrosting and incubation at 60 °C for 5 min, after which the sample was subjected to a standard phenol-chloroform extraction procedure (Maniatis et al.,1982; Sambrook et al., 1989). Extracted nucleic acids were precipitated using absolute ethanol and incubation at -20 °C overnight, and then harvested by centrifugation at 14,000 rpm for 30 min at 4 °C. Precipitates were washed in 500 µl 70% ethanol, centrifuged at 14, 000 rpm for 10 min at 4 °C, air dried and resuspended in 50µl of sterile distilled water (Sambrook et al., 1989). Extracted DNA was visualized and quantified using agarose gel electrophoresis and a nanodrop spectrophotometer (nanodrop, Wilmington, USA), respectively. PCR of the gene encoding the translation elongation factor 1-alpha region (*TEF1*α) was carried out using the primer pairs and amplification protocols as described in Yilmaz et al. (2021). Each 25 µl reaction mixture contained 20 ng of DNA, 200µM dNTPs, (Fermentas, Nunningen, Germany), 10µM of each primer and 2.5 mM $MgCl_2$, as well as 0.1 µM *Taq* polymerase and reaction buffer (Roche, Molecular Biochemicals, Manheim, Germany).

PCR products were purified using ExoSAP-IT (Affymetrix Inc., Santa Clara, California, USA) and electrophoresed to assess product integrity and to estimate the DNA concentration. Cleaned products were sequenced in both directions with the original primers using the ABI 377 automated sequencer and the BigDye Terminator v 3.1 sequencer cycle sequencing kit (Applied Biosystems, Foster City, CA). Electropherograms were visualized and consensus sequences were generated from forward and reverse sequences using BioEdit version 7.0.9 (Hall, 1999). Newly generated sequences were submitted to GenBank, and accession numbers are provided in Table 1.

Sequence analysis

All *TEF1*α sequences generated in this study were compared, using BLAST searches, with those in the Fusarium-ID database (http://isolate.fusariumdb.org/index.php) maintatined by Geiser et al. (2004). This was done to obtain putative identifications for the isolates examined. These comparisons also revealed the species complexes to which the respective isolates belonged. For the different species complexes, *TEF1*α sequences generated in this study were added to reference sequence datasets built around the ex-type sequences published by O'Donnell et al. (2000, 2009), Sandoval-Denis et al. (2018a, 2018b), Maryani et al. (2019), Lombard et al. (2019a, 2019b) and Yilmaz et al. (2021).

All sequence data sets were aligned with MAFFT v. 1.764b (Katoh and Standley; 2013), using the G-INS-i option, and manually trimmed in Geneious Primer (11.0.5) where needed. The respective datasets were then subjected to Maximum Likelihood (ML) analysis with IQtree v. 1.6.8 (Nguyen et al., 2015) using the most suitable substitute model parameters, as indicated by Modelfinder (Kalyaanamoorthy et al., 2017). Branch support was estimated in IQtree using the same model parameters and 1000 pseudoreplicates using of UFBoot (Minh et al., 2013). Trees were visualized in Figtree v. 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree) and edited for presentation in Affinity Designer v 1.6.1 (Serif (Europe) Ltd, Nottingham, UK).

Results

Fungal isolates

A total of 118 *Fusarium* isolates were obtained from *S. cordatum* inflorescences collected in this study (Table 1; Figure 1). In 2008, 47 *Fusarium* isolates were obtained from malformed *S. cordatum* in Gauteng (3 isolates), Mpumalanga (1 isolate), Western Cape (12 isolates) and KwaZulu-Natal (31 isolates). The higher number of samples obtained from the KwaZulu-Natal province, prompted the subsequent collection in 2016 where 15 *S. cordatum* trees were sampled from different locations, covering a larger area including Dukuduku, Mtubutaba, Nyalazi and Mzingazi in addition to Kwambonambi. During this

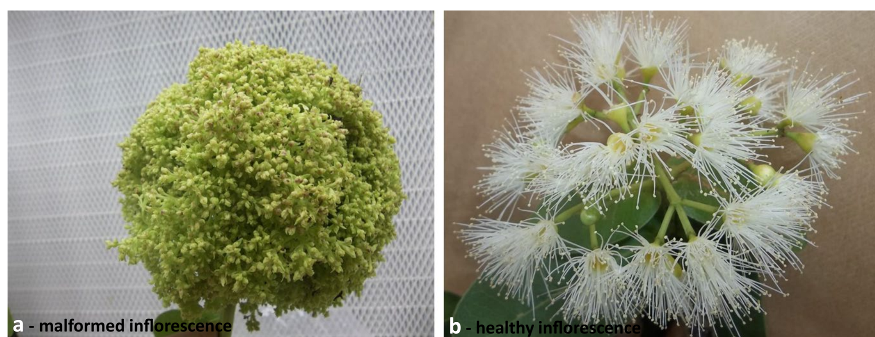


Fig. 1 Malformed (a) and Healthy (b) inflorescences of *Syzygium cordatum* trees in South Africa

latter collection, healthy inflorescences were also examined. From the second collection, a total of 71 *Fusarium* isolates were obtained, 53 of which were isolated from malformed inflorescences and 18 from healthy inflorescences.

Identification of Fusarium *species*

BLAST searches of the *TEF1*α sequences determined in this study against those in the Fusarium-ID database revealed that the 118 isolates examined represented four different species complexes in the genus *Fusarium* (Table 1). These were as follows: *F. fujikuroi* species complex (FFSC; 39 isolates), *F. oxysporum* species complex (FOSC; 45 isolates), *F. incarnatum-equiseti* species complex (FIESC; 33 isolates) and *F. chylamydosporum* species complex (FCSC; 1 isolate). The majority of the isolates (38%) were therefore members of FOSC, followed by FFSC (33%) and the FIESC (28%).

In order to assign the isolates to species, phylogenetic analyses of *TEF1*α sequences were performed. Dataset characteristics and substitution models applied during the analysis are summarized in Table 2. In all cases, datasets were made up of sequences generated in this study and those for known species and reference strains in the particular species complexes.

The *TEF1*α based phylogeny separated the 39 FFSC isolates examined into six well-supported and exclusive groups representing existing or new species residing in the so-called African, Asian, and American clades of the complex (O'Donnell, et al., 1998) (Fig. 2). Of the 39 isolates, 33 isolates formed part of the Asian clade where they resided in clades representing *F. annulatum* (16 isolates), *F. fujikuroi* (12 isolates), and *F. sacchari* (1 isolate). *Fusarium annulatum* was re-introduced by Yilmaz et al. (2021) as the name for some isolates previously identified as "*F. proliferatum*". Previous studies (including whole genome sequence analysis) showed that the ex-type of *F. annulatum* (CBS 258.54^{T}) resolved in the same clade with isolates named "*F. proliferatum*" (O'Donnell et al., 1998; Brankovics et al., 2020). The ex-type specimen for *F. proliferatum* was however never preserved. Yilmaz et al. (2021) therefore used CBS 480.96 as an epitype of *F. proliferatum*, which was isolated from the same substrate (soil) and location (Papua New Guinea) and resembled the original drawing of *F. proliferatum*. The 16 isolates examined in this study clustered together with the ex-type of *F. annulatum,* although our BLAST results matched those of *F. proliferatum*. Of the seven African clade isolates, one isolate grouped with *F. verticillioides*. The remaining six isolates formed a unique cluster, which was designated as *Fusarium* sp. nov. 2. The three American clade isolates also formed a unique cluster including NRRL 25807 and they are designated as *Fusarium* sp. nov. 1.

Phylogenetic analysis of the FOSC based on *TEF1*α (Fig. 3) did not provide sufficient resolution to robustly delineate groups representative of species. Species delineation, based on *TEF1*α alone, was also not clear for species obtained from the Maryani et al. (2019) and Lombard et al. (2019a) datasets. The generalized name *Fusarium oxysporum sensu lato* was therefore used for the isolates obtained in this study.

The *TEF1*α phylogeny separated the 33 FIESC isolates into seven well-supported and exclusive groups representing existing or new species in this complex (Fig. 4). Of the seven groups, three and four formed respectively part of the so-called *F. equiseti* and *F. incarnatum* clades of the complex (O'Donnell et al., 2009; O'Donnell et al., 2012). In

Table 2 Model parameters for the maximum likelihood phylogenetic analyses performed for the respective datasets analyzed in this study

Dataset [a]	Number of taxa	Alignment length (nucleotides)	Model parameters [b]	References
FFSC				
*TEF1*α	82	668	TNe+G4 Unequal transition/transversion rates with unequal purine/pyrimidine rates and equal base frequencies (TNe) and 4 discrete gamma (G) rate categories to account for rate heterogeneity across sites	Tamura and Nei, 1993
FOSC				
*TEF1*α	107	574	TNe+G4	Tamura and Nei, 1993
FIESC				
*TEF1*α	79	622	TNe+G4	Tamura and Nei, 1993
FCSC				
*TEF1*α	12	696	TIM2e+G4 Transition model with equal base frequencies (TIM2e) and 4 discrete gamma (G) rate categories to account for rate heterogeneity across sites	Yang, 1994

[a] Individual datasets were constructed for *TEF1*α gene region of the respective species complexes of *Fusarium*, where FFSC denotes the *F. fujikuroi* species complex, FOSC denotes the *F. oxysporum* species complex, FIESC denotes the *F. incarnatum-equiseti* species complex and FCSC denotes the *F. chylamydosporum* species complex.

[b] Model parameters implemented in IQtree v. 1.6.8 (Nguyen et al. 2015) and determined for each dataset using Modelfinder (Kalyaanamoorthy et al. 2017).

the *F. incarnatum* clade, four isolates arising from this study grouped wit*h F. caatingaense* previously referred to as the FIESC20 clade (Xia et al., 2019), as well as three unique clusters that likely represent new species (i.e., *Fusarium* sp. nov. 3 represented by five isolates, Fusarium sp. nov. 4 represented by two isolates and *Fusarium* sp. nov. 5 represented by five isolates). Of the *F. equiseti* clade isolates, 13 isolates resolved as *F. clavum* (previously referred to as FIESC5) (O'Donnell et al., 2009), one isolate was likely conspecific with *F. scirpi*, while three isolates formed a group with *F. lacertarum*, previously known as FIESC 4 (Xia et al., 2019).

Phylogenetic analysis for the single isolate residing in the FCSC grouped with *F. sporodochiale* CBS 199.63, MRC 117 and MRC 35 (Figure 5). *Fusarium sporodochiale* was described by Lombard et al. (2019b) and the ex-type was isolated from soil in South Africa, however the ecology of the species is not well studied.

Overall, the phylogenetic analyses revealed that the 118 isolates collected from both healthy and malformed *S. cordatum* inflorescences represented at least 15 species and/or lineages of which five represent new species. Four species (*F. annulatum*, *F. oxysporum*, *F. caatingaense*, *Fusarium* sp. nov. 5) was isolated from trees sampled from at least three or all four sampling regions and multiple isolates were obtained for each species. With regards to sampling area, the greatest number of species was obtained from KwaZulu-Natal with seven species isolated from malformed and one from healthy inflorescences, with six of these species also found in both tissue types (Table 3). Species residing in the FOSC and FIESC were isolated from both healthy and malformed inflorescences, whereas species residing in the FFSC were isolated only from malformed inflorescences. Finally, the single species in FCSP was isolated from healthy inflorescences.

Discussion

Results of this study showed that *S. cordatum* inflorescences represent a remarkable reservoir of *Fusarium* species diversity. Overall, the 118 *Fusarium*

isolates collected, represented 15 species residing in four different species complexes and five of these species are new to science. The number of species and the number of novel species probably also represents an underestimate of the diversity from this unusual niche. This is particularly notable when considering that samples were not collected from all areas of South Africa where these trees are found.

The most abundant *Fusarium* isolates arising from this study were members of the FOSC. Species residing in this complex are typically grouped in *formae speciales* and races that represent plant pathogenic and host cultivar associations (Summerell, 2019). Many of these groups have, however, evolved multiple times independently and numerous *formae speciales* or groups of these are polyphyletic (Summerell, 2019). To resolve some of the taxonomic questions linked to the polyphyletic nature of these groups, a number of new *F. oxysporum* species have been described (Maryani et al., 2019; Lombard et al., 2019b) although providing names for *formae speciales* is not broadly supported (Summerell, 2019). Our analyses, however, did not provide robust resolution for the species in this complex (Fig. 3), we therefore kept the broader naming (*sensu lato*) for the *Fusarium oxysporum* isolates.

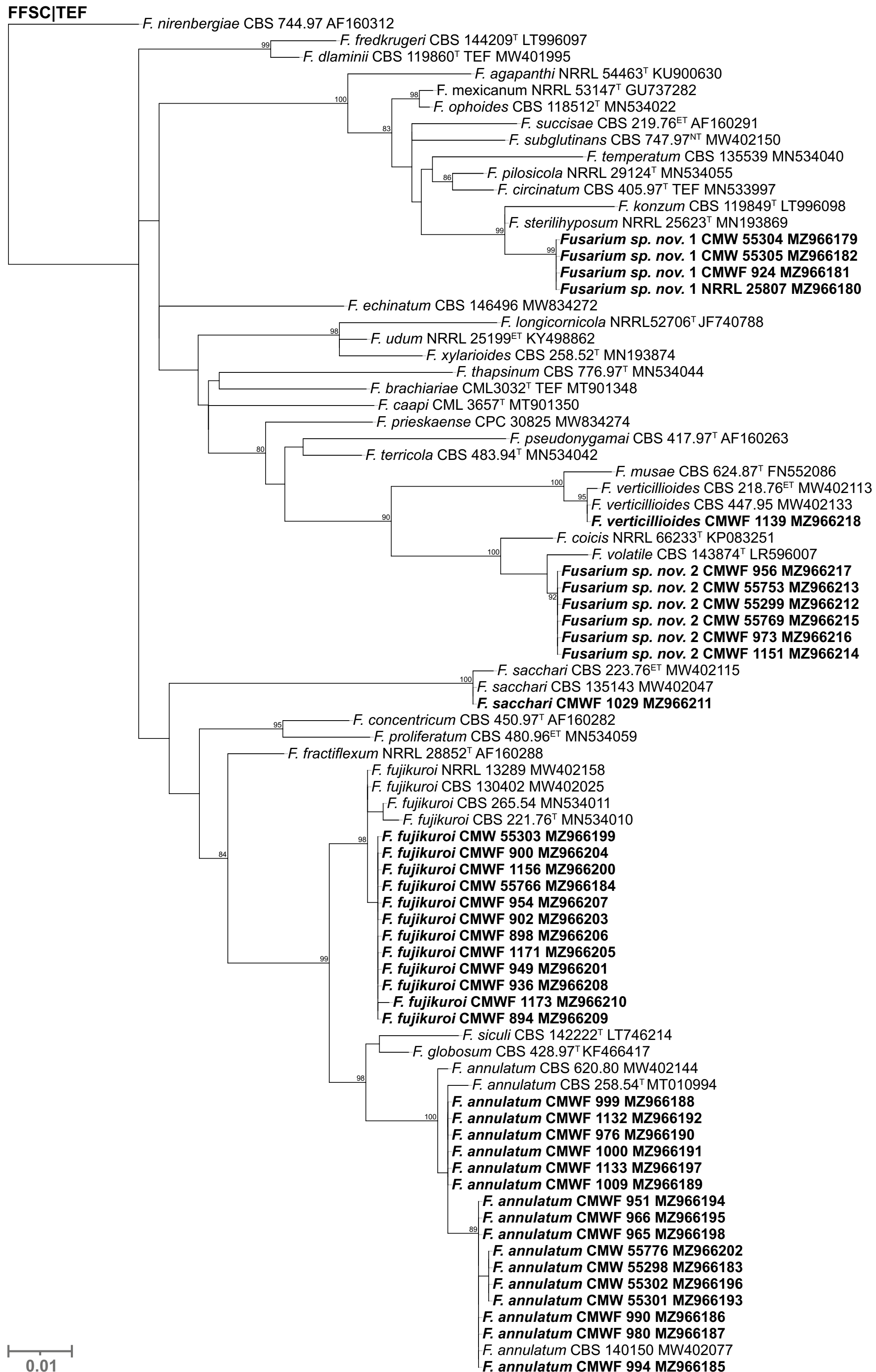
The larger number of FOSC isolates obtained in this study was not surprising since species in this complex has wide geographic distributions and are found in both cultivated and uncultivated soils as well as under different climatic conditions (Edel-Hermann and Lecomte, 2019). In addition, species in this group have wide host ranges that include both monocotyledon and dicotyledon plants (Edel-Hermann and Lecomte, 2019). As pathogens, they cause wilts, root, stem, and crown rots as well as vascular diseases on economically important plants (Booth, 1971; Leslie and Summerell, 2006a; Summerell, 2019; Edel-Hermann and Lecomte, 2019). Some isolates residing in the FOSC were obtained from both malformed and healthy tissues. The role of FOSC taxa in *S. cordatum* malformation is however not yet clear, but it is relevant that members of this complex have previously been associated with malformed inflorescences of *Cicer arietinum* (Chickpea) (Arunodhayam et al., 2014; Haseeb et al., 2014) and *M. indica* (Liew et al., 2016).

The second highest number of *Fusarium* isolates obtained from the *S. cordatum* inflorescences resided in the FFSC. Species in this complex include numerous plant pathogens and important mycotoxin producers (Leslie and Summerell, 2006b; Windels, 2000; Yilmaz et al., 2021). Most FFSC isolates represented *F. annulatum* and *F. fujikuroi*, which were obtained from all sampling sites and from both the 2008 and 2016 KwaZulu-Natal surveys. *Fusarium annulatum* (as *F. proliferatum*) has been reported as the causal agent of MMD in China (Zhan et al., 2010; Lv et al. ,2010), Egypt (Wafaa et al., 2010; Haggag, 2010) and Malaysia (Nor et al., 2013). Likewise, *Fusarium fujikuroi* has been associated with MMD in Australia (Liew et al., 2016), although its role in malformation is currently unknown. Species in this complex were also the only collections found exclusively on malformed inflorescences. The possibility that they or either of the two new species delineated here are involved in the development of malformed *S. cordatum* inflorescences needs to be considered in future studies.

Several members in the FIESC were recovered from healthy and malformed inflorescences. Some species in this complex are associated with diseases in agriculturally important crops causing cankers and rots (Villani et al., 2016), while others are ubiquitous, cosmopolitan, and soil-inhabiting as plant saprotrophs and some are endophytes found in healthy plant tissues (Burgess and Summerell, 1992, Leslie and Summerell, 2006a). Interestingly, *Fusarium scirpi,* has been isolated from malformed *M. indica* inflorescence tissue, but was not linked to the epidemiology of MMD disease and is rather considered as an environmental species (Liew et al., 2016). Three novel species residing in this complex arose from this study and their possible role in malformation disease needs to be determined.

One isolate residing in the FCSC was obtained in this study. Little is known regarding the biology of *F. sporodochiale*, which is a relatively newly discovered species that have previously been isolated from soil and termite mounds (Lombard et al., 2019b).

Overall, the results from this study serve as the first important step towards the characterization of floral malformation on *S. cordatum*. Malformation appears to be associated with a number of species including at least five novel *Fusarium* species residing in four different species complexes. The new species should be formally described in future studies. However, as a priority, the species residing in the FFSC should be


FFSC|TEF
F. nirenbergiae CBS 744.97 AF160312
F. fredkrugeri CBS 144209T LT996097
F. dlaminii CBS 119860T TEF MW401995
F. agapanthi NRRL 54463T KU900630
F. mexicanum NRRL 53147T GU737282
F. ophoides CBS 118512T MN534022
F. succisae CBS 219.76ET AF160291
F. subglutinans CBS 747.97NT MW402150
F. temperatum CBS 135539 MN534040
F. pilosicola NRRL 29124T MN534055
F. circinatum CBS 405.97T TEF MN533997
F. konzum CBS 119849T LT996098
F. sterilihyposum NRRL 25623T MN193869
Fusarium sp. nov. 1 CMW 55304 MZ966179
Fusarium sp. nov. 1 CMW 55305 MZ966182
Fusarium sp. nov. 1 CMWF 924 MZ966181
Fusarium sp. nov. 1 NRRL 25807 MZ966180
F. echinatum CBS 146496 MW834272
F. longicornicola NRRL52706T JF740788
F. udum NRRL 25199ET KY498862
F. xylarioides CBS 258.52T MN193874
F. thapsinum CBS 776.97T MN534044
F. brachiariae CML3032T TEF MT901348
F. caapi CML 3657T MT901350
F. prieskaense CPC 30825 MW834274
F. pseudonygamai CBS 417.97T AF160263
F. terricola CBS 483.94T MN534042
F. musae CBS 624.87T FN552086
F. verticillioides CBS 218.76ET MW402113
F. verticillioides CBS 447.95 MW402133
F. verticillioides CMWF 1139 MZ966218
F. coicis NRRL 66233T KP083251
F. volatile CBS 143874T LR596007
Fusarium sp. nov. 2 CMWF 956 MZ966217
Fusarium sp. nov. 2 CMW 55753 MZ966213
Fusarium sp. nov. 2 CMW 55299 MZ966212
Fusarium sp. nov. 2 CMW 55769 MZ966215
Fusarium sp. nov. 2 CMWF 973 MZ966216
Fusarium sp. nov. 2 CMWF 1151 MZ966214
F. sacchari CBS 223.76ET MW402115
F. sacchari CBS 135143 MW402047
F. sacchari CMWF 1029 MZ966211
F. concentricum CBS 450.97T AF160282
F. proliferatum CBS 480.96ET MN534059
F. fractiflexum NRRL 28852T AF160288
F. fujikuroi NRRL 13289 MW402158
F. fujikuroi CBS 130402 MW402025
F. fujikuroi CBS 265.54 MN534011
F. fujikuroi CBS 221.76T MN534010
F. fujikuroi CMW 55303 MZ966199
F. fujikuroi CMWF 900 MZ966204
F. fujikuroi CMWF 1156 MZ966200
F. fujikuroi CMW 55766 MZ966184
F. fujikuroi CMWF 954 MZ966207
F. fujikuroi CMWF 902 MZ966203
F. fujikuroi CMWF 898 MZ966206
F. fujikuroi CMWF 1171 MZ966205
F. fujikuroi CMWF 949 MZ966201
F. fujikuroi CMWF 936 MZ966208
F. fujikuroi CMWF 1173 MZ966210
F. fujikuroi CMWF 894 MZ966209
F. siculi CBS 142222T LT746214
F. globosum CBS 428.97T KF466417
F. annulatum CBS 620.80 MW402144
F. annulatum CBS 258.54T MT010994
F. annulatum CMWF 999 MZ966188
F. annulatum CMWF 1132 MZ966192
F. annulatum CMWF 976 MZ966190
F. annulatum CMWF 1000 MZ966191
F. annulatum CMWF 1133 MZ966197
F. annulatum CMWF 1009 MZ966189
F. annulatum CMWF 951 MZ966194
F. annulatum CMWF 966 MZ966195
F. annulatum CMWF 965 MZ966198
F. annulatum CMW 55776 MZ966202
F. annulatum CMW 55298 MZ966183
F. annulatum CMW 55302 MZ966196
F. annulatum CMW 55301 MZ966193
F. annulatum CMWF 990 MZ966186
F. annulatum CMWF 980 MZ966187
F. annulatum CBS 140150 MW402077
F. annulatum CMWF 994 MZ966185
99
100
98
83
86
99
99
98
80
100
95
90
100
92
100
95
84
98
99
98
100
89
0.01

◂**Fig. 2** ML tree based on *TEF1*α showing identities and diversity of *Fusarium* species in FFSC associated with *Syzgium cordatum*. Bootstrap values ≥80% are shown above branches. Sequences obtained from ex-type cultures are indicated by ^{T}, epi-type cultures indicated by ^{ET}, neo-type cultures indicated by ^{NT}. GenBank accession numbers are shown next to the culture number with the accession numbers. The sequences created during this study are indicated by bold text. The tree was rooted to *F. nirenbergiae* (CBS 744.97)

characterized in more detail and subjected to pathogenicity studies since they were the taxa found only in malformed inflorescences. An important component of future studies must also be to consider other biotic and/or abiotic factors associated with a floral malformation in *S. cordatum* including the effects of climate change as temperature and drought stress have been linked to malformation previously (Chimonidou-Pavlidou, 2004). It will also be important to consider the possible role of other biotic factors such as insects (Takei et al., 2015; Wool, 2004), mites (Jeppson et al., 1975; Lindquist et al., 1996) nematodes (Todd and Atkins, 1958; Khan et al., 2012), phytoplasmas (Weisburg et al., 1989) and viruses (Qazi et al., 2007) that have previously been linked to malformations in inflorescences in other species. In terms of biotic factors, the priority focus should be on *Eriophyoid* mite species that were commonly observed on malformed inflorescences. Their presence could increase disease severity, similarly to what has been reported for *Fusarium mangiferae* and floral and bud malformations in mangoes (Gamliel-Atinsky et al., 2009; Gamliel-Atinsky et al., 2010).

Fig. 3 ML tree based on *TEF1*α showing identities and diversity of *Fusarium* species in FOSC associated with *Syzgium cordatum*. Bootstrap values ≥80% are shown above branches. Sequences obtained from ex-type cultures are indicated by ^{T}, epi-type cultures indicated by ^{ET}. Sequences obtained from strains during this study are indicated by bold text. The tree was rooted to *F. foetens* (CBS 120665)

FOSC|TEF

F. foetens CBS 120665 MH485009
F. triseptatum CBS 130302 MH485015
F. triseptatum CBS 116619 MH485001
F. triseptatum CBS 258.50 MH484964
"*Fusarium oxysporum*" CMW 55321 MZ966263
F. triseptatum CBS 119665 MH485007
F. odoratissimum CBS 130310 MH485013
F. purpurascens InaCCF 886^{T} LS479827
F. phialophorum InaCCF 971^{T} LS479741
F.tardicrescens NRRL 36113^{T} LS479665
F. odoratissimum InaCCF 817^{T} LS479753
F. trachichlamydosporum CBS 102028 MH484988
F. tardichlamydosporum InaCCF 958^{T} LS479729
F. sangayamense InaCCF 960^{T} LS479732
F. oxysporum CBS 144135 MH485045
F. oxysporum CBS 144134^{ET} MH485044
F. kalimantanense InaCCF 917^{T} LS479690
F. hoodiae CBS 132474^{T} MH485020
F. libertatis CBS 144749^{T} MH485035
F. languescens CBS 645.78 MH484971
F. curvatum CBS 238.94^{T} MH484984
F. contaminatum CBS 114899^{T} MH484992
F. veterinarium CBS 109898 MH484990
F. pharetrum CBS 144751 MH485043
F. nirenbergiae CBS 115424 MH484997
F. nirenbergiae CPC 30807 MH485041
"*Fusarium oxysporum*" CMWF 1048 MZ966247
F. nirenbergiae CBS 129.24 MH484955
F. nirenbergiae CBS 130301 MH485017
F. nirenbergiae CBS 130303 MH485014
"*Fusarium oxysporum*" CMW 55765 MZ966254
F. nirenbergiae CBS 181.32 MH484958
"*Fusarium oxysporum*" CMW 55767 MZ966261
"*Fusarium oxysporum*" CMWF 1055 MZ966252
"*Fusarium oxysporum*" CMWF 938 MZ966257
F.nirenbergiae CBS 196.87 MH484977
F. nirenbergiae CBS 129.81 MH484976
"*Fusarium oxysporum*" CMWF 979 MZ966258
F. nirenbergiae CBS 115419 MH484995
"*Fusarium oxysporum*" CMWF 1036 MZ966251
F. nirenbergiae CBS 840.88^{T} MH484978
F. nirenbergiae CBS 123062 MH485010
F. nirenbergiae CBS 127.81 MH484974
"*Fusarium oxysporum*" CMWF 1043 MZ966259
"*Fusarium oxysporum*" CMWF 955 MZ966255
"*Fusarium oxysporum*" CMW 55316 MZ966253
F. nirenbergiae CBS 115417 MH484994
F. nirenbergiae CBS 149.25 MH484956
F. nirenbergiae CBS 115416 MH484993
"*Fusarium oxysporum*" CMWF 985 MZ966262
F. nirenbergiae CBS 130300 MH485016
F. nirenbergiae CBS 758.68 MH484968
"*Fusarium oxysporum*" CMW 55313 MZ966260
F. nirenbergiae CBS 744.79 MH484973
"*Fusarium oxysporum*" CMW 55314 MZ966249
"*Fusarium oxysporum*" CMW 55759 MZ966256
"*Fusarium oxysporum*" CMW 55311 MZ966248
"*Fusarium oxysporum*" CMW 55319 MZ966250
F. duoseptatum InaCCF 916^{T} LS479688
F. carminascens CBS 144738^{T} MH485028
F. callistephi CBS 115423 MH484996
"*Fusarium oxysporum*" CMWF 1058 MZ966232
F. hexaseptatum InaCCF 866^{T} LS479805
F. oxysporum NRRL 25598 AF008496
F. cugenangense InaCCF 984^{T} LS479757
F. cugenangense CBS 130308 MH485011
Fusarium sp. CBS130323 MH485018
CBS 680.89 *Fusarium* sp. MH484980
CBS 128.81 *Fusarium* sp. MH484975
F. grossmichelii InaCCF 833^{T} LS479744
CBS 217.49 *F. elaeidis* MH484961
"*Fusarium oxysporum*" CMW 55312 MZ966231
"*Fusarium oxysporum*" CMW 55773 MZ966243
"*Fusarium oxysporum*" CMW 55318 MZ966233
"*Fusarium oxysporum*" CMW 55324 MZ966244
F. glycines CBS144746^{T} MH485033
F. gossypinum CBS 116613^{T} MH485000
"*Fusarium oxysporum*" CMW 55778 MZ966222
F. fabacearum CBS 144742 MH485029
"*Fusarium oxysporum*" CMW 55317 MZ966228
"*Fusarium oxysporum*" CMW 55758 MZ966227
F. callistephi CBS 187.53^{T} MH484966
"*Fusarium oxysporum*" CMW 55756 MZ966221
"*Fusarium oxysporum*" CMW 55323 MZ966245
"*Fusarium oxysporum*" CMW 55763 MZ966229
"*Fusarium oxysporum*" CMW 55760 MZ966236
F. tardicrescens NRRL 54005 LS479674
"*Fusarium oxysporum*" CMW 55757 MZ966226
F. tardicrescens NRRL 54008 LS479673
"*Fusarium oxysporum*" CMW 55315 MZ966220
"*Fusarium oxysporum*" CMW 55307 MZ966239
"*Fusarium oxysporum*" CMW 55322 MZ966224
"*Fusarium oxysporum*" CMW 55764 MZ966240
"*Fusarium oxysporum*" CMWF 1019 MZ966223
"*Fusarium oxysporum*" CMWF 1057 MZ966230
"*Fusarium oxysporum*" CMWF 1046 MZ966225
F. tardicrescens NRRL 37622 LS479649
"*Fusarium oxysporum*" CMW 55768 MZ966241
"*Fusarium oxysporum*" CMW 55777 MZ966237
"*Fusarium oxysporum*" CMW 55320 MZ966238
"*Fusarium oxysporum*" CMW 55771 MZ966246
F. fabacearum CBS144744 MH485031
F. fabacearum CBS144743^{T} MH485030
"*Fusarium oxysporum*" CMW 55309 MZ966234
"*Fusarium oxysporum*" CMW 55310 MZ966235
"*Fusarium oxysporum*" CMW 55774 MZ966242
"*Fusarium oxysporum*" CMW 55306 MZ966219

0.01

Fig. 4 ML tree based on *TEF1*α showing identities and diversity of *Fusarium* species in FIESC associated with *Syzgium cordatum*. Bootstrap values ≥80% are shown above branches. Sequences obtained from ex-type cultures are indicated by ^{T}, epi-type cultures indicated by ^{ET}, neo-type cultures indicated by ^{NT}. GenBank accession numbers are shown next to the culture number with the accession numbers. The sequences created during this study are indicated by bold text. The tree was rooted to *F. concolor* (NRRL 13459)

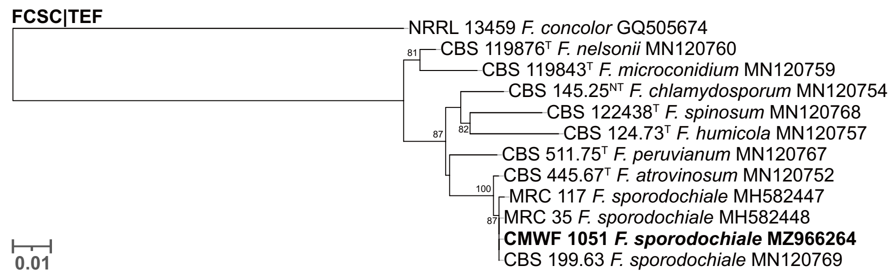


Fig. 5 ML tree based on *TEF1*α showing identities and diversity of *Fusarium* species in FCSC associated with *Syzgium cordatum*. Bootstrap values ≥70% are shown above branches. Sequences obtained from ex-type cultures are indicated by ^{T}, neo-type cultures indicated by ^{NT}. GenBank accession numbers are shown next to the culture number with the accession numbers. The sequences created during this study are indicated by bold text. The tree was rooted to *F. concolor* (NRRL 1345)

Table 3 Number of isolates from malformed and healthy inflorescences

Species Complex[a]	Species Identity[b]	COLLECTION SITES[c]				
		KZN (HI)	KZN (MI)	M (MI)	WC(MI)	Gauteng (MI)
FFSC	*F. fujikuroi*	0	11	0	0	1
	F. annulatum	0	13	1	1	1
	F. verticillioides	0	1	0	0	0
	F. sacchari	0	1	0	0	0
	Fusarium sp. nov. 1	0	3	0	0	0
	Fusarium sp. nov. 2	0	5	0	0	1
FOSC	*F. oxysporum*	8	30	0	7	0
FIESC	*F. clavum*	5	8	0	0	0
	F. caatingaense	1	2	0	1	0
	F. scirpi	0	0	0	1	0
	F. lacertarum	1	2	0	0	0
	Fusarium sp. nov. 3	2	3	0	0	0
	Fusarium sp. nov. 4	0	2	0	0	0
	Fusarium sp. nov. 5	1	2	0	2	0
FCSC	*F. sporodochiale*	1	0	0	0	0
	Total	**19**	**83**	**1**	**12**	**3**

[a] FFSC denotes the *F. fujikuroi* species complex, FOSC denotes the *F. oxysporum* species complex, FIESC denotes the *F. incarnatum-equiseti* species complex and FCSC denotes the *F. chylamydosporum* species complex.

[b] Species were assigned according to their phylogenetic placement based on *TEF1*α

[c] Collection sites across South Africa with KZN representing the KwaZulu-Natal isolates from 2008 and 2016 and M and WC representing isolates sampled from Mpumalanga and the Western Cape during the 2008 survey, respectively. Tissue indicates whether the inflorescence was malformed or healthy (HI = healthy inflorescence, MI= malformed inflorescence).

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Authors' contributions All authors conceived research.
R.I.M. conducted experiments.
All authors contributed material.
R.I.M., N.Y. and G.F. analyzed data and conducted statistical analyses.
R.I.M. wrote the manuscript.
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Declarations

Conflicts of interest/Competing interests To the best of our knowledge, the named authors have no conflict of interest, financial or otherwise.

Data availability The data that support the findings of this study are available from the corresponding author, upon reasonable request. The sequence data generated was submitted to GenBank

Code availability Not applicable

Ethics approval Not applicable

Consent to participate All the authors have participated in the manuscript and have agreed to the submission policies.

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Chapter 3: Description of a novel *Fusarium* species from *Syzygium cordatum* inflorescences

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Abstract

Syzygium cordatum (Myrtaceae) is an important indigenous and widely distributed tree in South Africa. Symptoms resembling those of mango malformation disease that are assocaiated with infections by *Fusarium* (*Ascomycota*, *Hypocreales*, *Nectriaceae*) commonly occur on the inflorescences of this plant. This study describes a novel *Fusarium* species isolated from *S. cordatum* inflorescences collected in the KwaZulu Natal Province of South Africa. Morphological data and the results of genealogical concordance analysis based on sequences for the genes encoding translation elongation factor (*tef1α*), the second largest subunit of RNA polymerase (*rpb2*), calmodulin (*cmdA*) showed that it represents a distinct species in the *Fusarium incarnatum-equiseti* species complex. This new taxon was named *F. syzygium* sp. nov. and represents the first novel *Fusarium* species to be described from *S. cordatum* inflorescences.

1 Introduction

The genus *Fusarium* Link (*Hypocreales*, *Nectriaceae*) represents one of the most economically important and diverse genera in the *Sordariomycetes* (*Ascomycota*). The many species in this genus are adapted to diverse climatic conditions in multiple biomes including tropical and temperate grasslands, shrubs, and forests as well as harsh desert and alpine environments (Leslie and Summerell, 2006). Some species are important plant pathogens responsible for causing a wide range of diseases including blight, wilt, cankers and rots (Ploetz, 1994; Parry et al., 1995; Windels, 2000; Backhouse and Burgess, 2002; Ploetz and Freeman, 2009; Summerell et al., 2011). Many *Fusarium* species produce secondary metabolites such as fumonisins, trichothecenes, and zearalenone (Van Asselt et al., 2012; De Boevre et al., 2014; Pleadin et al., 2017; O'Donnell et al., 2018; Avila et al., 2019) that cause various disease symptoms in animals (Logrieco et al., 2002; Desjardins, 2006; Leslie and Summerell, 2006; Freire and Sant'Ana, 2018; Mishra et al., 2022; Munkvold et al., 2021). In humans, they are often considered as emerging pathogens and are among the most prevalent in immune-compromised individuals (Al-Maqtoofi and Thornton, 2016; Paterson and Lima, 2017; Claeys et al., 2020; Vartivarian et al., 1993). In humans, these fungi are also responsible for infections such as keratitis, onychomycosis, and other allergic infections (Nucci and Anaissie, 2002; Doczi et al., 2004; Thomas et al., 2010; Uemura et al., 2022).

Fusarium diversity studies have primarily focused on economically important agricultural and forestry ecosystems with little attention given to natural ecosystems (Walsh et al., 2010). However, due to increasing interest in fungal biodiversity in indigenous ecosystems (Crous et al., 2006), *Fusarium* species associated with indigenous flora is increasingly receiving attention. In addition, the application of molecular tools, particularly those employing DNA sequence information, has provided insights into differentiating morphologically similar or cryptic species that could not previously be resolved, thus, leading to the discovery of new species and cryptic species.

Syzygium cordatum (Myrtaceae) is an indigenous South African tree species (Palgrave, 1977). It has a wide distribution and commonly occurs along water courses and in swampy areas (Palgrave, 1977). The plant has many functional uses including in traditional medicine (Van Wyk et al., 1997) and as a source of firewood and timber (Dlamini, 1981). Despite representing a prominent part of many South African landscapes and gardens, little is known regarding the

floral malformations commonly observed on these trees. These result in abnormal development of vegetative shoots and floral panicles exhibiting phyllody and hypertrophy (Kumar et al., 1993; Ploetz, 1994). This is similar to the malformations observed in *Mangifera indica* (mango), *Tabebuia rosea* (rosy trumpet) and *Paullinia cupana* (guarana plants), where the symptoms are known to be caused by *Fusarium* species from different *Fusarium* species complexes. In the case of mango malformation, *F. annulatum*, *F. proliferatum* (Haggag et al. 2010; Zhan et al. 2010), *F. sterilihyphosum* (Steenkamp et al., 1999; Steenkamp et al., 2000), *F. mangiferae* (Britz et al., 2002), *F. subglutinans* (Steenkamp et al., 2002), *F. mexicanum* (Otero-Colina et al., 2010) and. *F. tupiense* (Lima et al., 2012) are associated with the disease. In the case of malformation of *P. cupana* and *T. rosea*, *F. decemcellulare* (de Queiroz et al., 2020) and *F. pseudocircinatum* (Montoya-Martínez et al., 2021) are the respective causal agents.

A previous survey (Mkandawire et al., 2022) assembled a collection of 118 *Fusarium* strains from both healthy and malformed inflorescences from *S. cordatum*. Based on the analysis of nucleotide sequences for the gene encoding translation elongation factor 1α (*tef1α*), these fungi represented 18 *Fusarium* species of which five were considered to be novel and residing in species complexes of *F. fujikuroi* (FFSC) and *F. incarnatum-equiseti* (FIESC). Two of those species residing in FFSC have since been described as *F. curculicola* (Vermeulen et al., 2021) and *F. babinda* (Summerell et al., 1995, Crous et al. 2021). *F. babinda* was first described by Summerell et al (1985) and it was later recognized as a separate species complex (Jacobs-Venter et al., 2018; Sandoval-Denis et al., 2018a). However, based on the ex-type isolate of *F. babinda* (BBA 69872 = F11217 = NRRL 25807) designated in Summerell et al. (1995), Crous et al. (2021) confirmed that *F. babinda* clustered with species within the FFSC. The aim of the present study was to describe a novel FIESC associated with *S. cordatum* inflorescences by utilizing morphological characteristics and multigene phylogenetic analysis to allow Genealogical Concordance Phylogenetic Species Recognition (GCPSR) (Taylor et al., 2000).

2 Materials and Methods

Sample collection and fungal isolations

In 2008 and 2016, malformed and healthy inflorescences were collected from *S. cordatum* trees in different places in South Africa, including Gauteng, Mpumalanga, the Western Cape, and KwaZulu-Natal (Table 1; Mkandawire et al., 2022). Collected material was subsequently

surface disinfested by submerging small pieces of an inflorescence in 1% (v/v) sodium hypochlorite solution and then 70% (v/v) ethanol, for 1 min each. Samples were then rinsed in sterile distilled water and plated directly onto half strength Potato Dextrose Agar (PDA; 20g/L PDA, 5g/L Agar; Biolab Diagnostics, Wadeville South Africa) medium and onto *Fusarium* selective medium (FSM) (Nash and Snyder, 1962) for 7 days at 25 ºC. Isolates resembling *Fusarium* were transferred to fresh PDA plates, incubated at 25 ºC for 7 days, and used to prepare spore suspension in order to establish pure cultures. For each isolate, a spore suspension was then prepared by flooding the plate with sterile water and used to inoculate water agar medium (WA; 20 g/L Agar; Biolab Diagnostics). Single germinating conidia were then used to establish pure cultures for the respective isolates. These cultures were subsequently stored in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa (Table 1).

DNA extraction, PCR amplification and sequencing

Seven-day-old cultures were used to extract DNA using a modified CTAB (hexadecyltrimethylammonium bromide) procedure (Steenkamp et al., 1999). A 500 ml DNA extraction buffer comprising 100 mM Tris-HCl (pH 8.0), 10 mM ethylenediaminetetra-acetic acid di-sodium salt (EDTA; pH 8.0), 2% (w/v) sodium dodecyl sulphate, and 0.2 g/l proteinase K was used to quickly homogenize fungal tissue that had been scraped from the half PDA (Sigma-Aldrich, St Louis, Missouri, USA). This was followed by 10 minutes of freezing at -70 ºC, followed by 5 minutes of defrosting and incubation at 60 ºC before the sample underwent a standard phenol-chloroform extraction method (Maniatis et al.,1982; Sambrook et al., 1989). Absolute ethanol was used to precipitate the extracted nucleic acids, which were subsequently harvested by centrifugation at 14,000 rpm for 30 min at 4 ºC. Precipitates were washed in 500 l of 70% ethanol, centrifuged for 10 min. at 14, 000 rpm, dried by air drying, and then resuspended in 50µl of sterile distilled water (Sambrook et al., 1989). Agarose gel electrophoresis and a nanodrop spectrophotometer (nanodrop, Wilmington, USA) were used respectively to estimate the quality and quantity of the extracted DNA.

By making use of the primer pairs and amplification procedures described by Yilmaz et al. (2021), portions of the genes encoding translation elongation factor 1-alpha region (*tef1α*), calmodulin (*cmdA*), and the second largest subunit of RNA polymerase (*rpb2*) were amplified by PCR. Each 25µl reaction mixture contained 20 ng of DNA, 200 µM dNTPs, (Fermentas,

Nunningen, Germany), 10 µM of each primer, 2.5 mM $MgCl_2$, 0.1 µM Taq polymerase, and reaction buffer (Roche, Molecular Biochemicals, Manheim, Germany). Amplicons were purified using ExoSAP-IT (Affymetrix Inc., Santa Clara, California, USA), and then electrophoresed to determine product integrity and concentration. Using the BigDye Terminator v3.1 sequencer cycle sequencing kit and the ABI 377 automated sequencer, cleaned products were sequenced in both directions using the original primers (Applied Biosystems, Foster City, CA). Using BioEdit version 7.0.9, electropherograms were viewed and consensus sequences were created from forward and reverse sequences (Hall, 1999).

Phylogenetic Analysis

Geneious Prime v. 2022.0.2 (BioMatters Ltd., Auckland, New Zealand) was used to assemble the newly created sequence contigs. From the earlier FIESC-published work, a sequencing dataset was created (Table 1). All alignments were carried out using Geneious Prime v. 2022.0.2 and MAFFT v. 1.764b (Katoh and Standley, 2013), choosing the G-INS-i option. Single-gene trees were inferred for the *tef1α*, *cmdA*, and *rpb2* datasets, as well as trees representing concatenated datasets. This was done using Maximum Likelihood (ML) analysis in IQtree v. 2.1.2. (Nguyen et al. 2015). The latter was based on the optimal model parameters for each gene/partition according to Modelfinder (Kalyaanamoorthy et al., 2017) implemented in IQtree v. 2.1.2. Branch support was estimated using ultrafast bootstrapping using UFBoot2 (Hoang et al. 2018), also as implemented in IQtree v. 2.1.2. Bayesian Inference analyses were performed using MrBayes v. 3.2.7 (Ronquist et al. 2012). The most suitable model for each dataset or partition was selected based on the Akaike information criterion (Akaike 1974) using MrModeltest v. 2.4 (Nylander 2004) All trees were visualized using TreeViewer 2.0.1 165 (https://treeviewer.org/) and edited in Affinity Publisher 1.10.4 (Serif (Europe), Nottingham, UK).

Morphological characterization

Morphology of the *Fusarium* isolates was characterized using features of macro and micro conidia of cultures grown on PDA, Synthetic nutrient-poor agar (SNA) (Nirenberg, 1976), and carnation leaf agar (CLA) (Fisher et al., 1982). This was done following the protocols described previously (Leslie and Summerell, 2006; Lombard et al., 2019a; Sandoval-Denis et al., 2018a, b, 2019). Color notations of Rayner, (1970) were utilized.

After incubation for 7 days at 25 °C in darkness, under continuous fluorescent light, and with a 12/12 h fluorescent light/dark cycle, colony morphology, pigment production, and odor generation were recorded on PDA. Colony growth rates were calculated by inoculating 5 mm agar blocks taken from 7-day-old cultures developing on SNA and placing these at the centers of Petri dishes containing full strength PDA (15 g/L potato destrose agar, 20 g/L agar). The plates were replicated 4 times and were incubated at temperatures ranging from 10-35 °C at 5 °C intervals in complete darkness. Over a 7-day period, two measurements of colony diameter perpendicular to each other were made.

A Sony NEX-5N camera was used to record the morphological characteristics of colonies. All micromorphological observations, unless otherwise stated, were made using water as the mounting media from fungal structures grown on carnation leaf agar (CLA) (Fisher et al., 1982), incubated at 25 °C under a 12/12 h near-ultraviolet light (nuv)/dark cycle (Fisher et al., 1982; Leslie and Summerell, 2006). Following Kornerup and Wanscher's protocols, colony color codes were established (1967). A Nikon Eclipse Ni compound and SMZ18 dissecting microscopes (Nikon, Japan), each outfitted with a Nikon DS-Ri camera and the NIS-Elements BR imaging software, were used to capture all measurements and images. Where available, up to 50 measurements were taken for all characteristic morphological structures, and maximum and minimum values with averages were calculated.

3 Results

Phylogenetic analyses

The previous *tef1α* phylogeny of Mkandawire et al. (2022; Figure 2) resolved 9 FIESC isolates into three well-supported and exclusive groups. To determine the phylogenetic placement of these potential novel isolates, a multigene phylogenetic tree for *tef1α*, *cmdA*, and *rpb2* was generated. The alignment contained 69 taxa and was 3 037 bp long including the gaps (*tef1α*: 1 – 668; *rpb2*: 669 – 2 486; *cmdA*: 2 487 – 3 037). This yielded a tree displaying the overall topology shown in previous studies (Xia et al., 2019; Wang et al., 2021; other articles from Table 1). In addition, individual gene phylogenies were generated to assess the genealogical concordance of the novel species of FIESC. The most appropriate substitution model for each partition was TNe+G4. All trees were rooted to *F. concolor* (CBS 961.87) (Fig. 1).

Taken together, these phylogenies separated the nine strains into three unique clusters which likely represented new species and were marked as "*Fusarium* sp. nov. 3" (n=5), "*Fusarium* sp. nov. 4" (n=2), and "*Fusarium* sp. nov. 5" (n=2) (Mkandawire et al. 2022, Figure 2). The species labelled as "*Fusarium* sp. nov. 3" by Mkandawire et al. (2022) is undisguisable from the type of *F. citri* (LC6896) based on *cmdA* and *rpb2* phylogenetic trees and therefore labelled as "*F. citri*" in Fig. 1. And the concatenated tree resulted in the same topology as the *rpb2* phylogeny. The additional *F. citri* isolates obtained from previous studies resolved in different clades. Because the type strain of *F. citri* ($LC6896^T$) is not available, it was not possible to determine whether this is a new species or due a sequence assembly error. For this reason, it was decided to name "*Fusarium* sp. nov. 3" as *F. citri* until it can be more clearly resolved. (Fig. 1).

"*Fusarium* sp. nov. 4" in the study of Mkandawire et al. (2022) resolved as a unique clade based on *tef1α* phylogeny. However, the isolates of this species (CMW 55735 and CMW 55739) grouped in the *F. incarnatum* clade on *rpb2*. For *cmdA*, it was closely associated with the neotype strain of *F. incarnatum* (CBS 132.73). The additional *F. incarnatum* isolates obtained from previous studies were resolved in different clades based on *cmdA*. The concatenated tree resulted in the same topology as the *rpb2* phylogeny. Since the phylogenetic analysis for this species and *F. incarnatum* were unresolved, it was again decided to treat "*Fusarium* sp. nov. 4" as *F. incarnatum* until such time as more data are available for testing this species hypothesis (Fig. 1).

"*Fusarium* sp. nov. 5" in the study of Mkandawire et al. (2022) separated into a unique and novel clade based on ML analysis of all genes, as well combined 3-gene data set. Within the FIESC, this new taxon was most closely related to *F. guilinense*. A description of the species is provided below.

Taxonomy

Fusarium syzygium Mkandawire, Fourie, & Yilmaz **prov. nom.** Fig. 2
Etymology: Name refers to the host, *Syzygium cordatum.*
Typus: South Africa, KwaZulu Natal, *Syzygium cordatum* malformed inflorescence, December 2008, M. Kvas, (**holotype** designated here permanently preserved in a metabolically inactive state, a dried culture of ex-type CMWF1056).

Conidiophores borne on aerial mycelium 35 – 94 µm tall, unbranched, sympodial or irregularly branched, bearing terminal or lateral phialides, often reduced to single phialides; aerial phialides mono- and polyphialidic, subulate to subcylindrical, sometimes proliferating percurrently, smooth- and thin-walled, 6 – 50 µm, with inconspicuous periclinal thickening; aerial conidia hyaline, falcate, curved dorsiventrally, tapering towards both ends, with a blunt and straight to slightly curved apical cell and blunt to barely notched basal cell, smooth- and thin-walled, 1– 4 septate; 1– septate conidia: 13.5 – 23.9 × 2.38 – 4.53 µm (av. 17.84 x 3.56 µm, n = 34); 2 – septate conidia: 16.46 – 21.63 × 2.66 – 4.86 µm (av. 19.10 x 3.73 µm, n = 43); 3 – septate conidia: (16.91 – 30.44) x (0.94 – 4.93) µm (av. 23.95 × 3.94 µm); 4 – septate conidia: 26.47 – 33.09 × 2.91 – 4.13 µm (av. 29.41 × 3.67µm n =11). *Sporodochia* cream white to pale brown, formed abundantly on surface of medium. *Sporodochial conidiophores* densely and irregularly branched, bearing apical whorls of 2 – 4 phialides; sporodochial phialides monophialidic, subulate to subcylindrical, 3.29 – 47.72 × 2.12 –5.44 µm, smooth, thin-walled, with inconspicuous periclinal thickening; sporodochial conidia falcate, slightly curved dorsiventrally to almost straight, tapering towards both ends, with a slightly papillate, curved apical cell and a notched to foot-like basal cell, (1– 5) septate, hyaline, smooth- and thin-walled; 1 – septate conidia: 12.66 – 28.78 × 2.02 – 4.63 µm (av. 18.39 × 3.48 µm, n = 50); 2 – septate conidia:4.53 – 24.16 × 3.1 – 4.48 µm (av. 13.84 × 3.80 µm, n = 15); 3– septate conidia: 20.03– 37.07 × 2.86× 6.23 µm (av. 27.12 × 4.33 µm). 4 – septate conidia: 29.82 .03 – 41.63 × 3.46 × 5.62µm (av. 33.4 × 4.5 µm, n = 12); 5 – septate conidia: 33.86 – 43.17 × 4.27 – 5.04 µm (av. 37.38 × 4.73 µm, n = 5). *Mesoconidia* observed. *Aerial conidia* present but very rare, aseptate, single septate, oval to obovoid, ellipsoid, short clavate or fusiform. 5.8 – 10.4 x 2.3 – 3.7 µm (av. 6.7 x 2.8 µm n = 6). *Chlamydospores* not observed.

Culture characteristics — Colonies on PDA incubated at 25 °C in the dark with an average radial growth rate of 3.4 × 4 mm/d and occupying an entire 90 mm Petri dish in 5 d; raised, aerial mycelia dense, colony margin round, radially striated, white to light orange, reverse is salmon orange to yellow. Odour absent. Reverse pale yellow to yellow with yellow diffusible pigments visible in the medium. On OA in the dark occupying an entire 90 mm Petri dish in 7 d; surface white, floccose, radiate, with abundant aerial mycelium, margin irregular, lobate, serrate or filiform. Reverse straw to pale luteous, without diffusible pigments. On the ML tree, isolates of this novels species also clusters close to *F. guilinense*, however the aerial mycelia in *F. guilinense* are reduced to monophialides unlike in *F. syzygium* where both mono phialides

and poly phialides are observed. While there are some similarities in the shape and curve of the macroconidia, *F. syzygium* can also be distinguished from *F. guilinense* by the number of septation which are more than 3 and the presence of a distinct basal foot cell. In addition, *F. syzygium* has sporodochia conidia which are absent in *F. guilinense* (Wang et al., 2019). It has macroconidia that are similar to those of *F. incarnatum* which are falcate, curved dorsiventrally, tapering towards both ends, with blunt apical cell and blunt to barely notched basal cell.

Additional material examined: South Africa, Kwambonambi, collected and isolated from *Syzygium cordatum* malformed inflorescence, R. Mkandawire, December, 2016, CMW55747. Even though these isolates exhibit similar culture and morphological characteristics, this isolate took longer to produce sporodochia than the ex-type (CMWF1056).

4 Discussion

This is the first study to describe a novel *Fusarium* species from malformed *S. cordatum* inflorescences. GCPSR-based analyses of three individual gene partitions and the combined 3-gene data strongly supported recognition of the novel species, *F. syzygium*, residing in the *F. incarnatum* clade of the FIESC.

This study used a polyphasic approach involving multi-locus GCSPR (Taylor et al., 2000) and morphological characteristics to classify species in the FIESC. The GCPSR was based on sequences for three nuclear genes (i.e., *tef1α, cmdA*, and *rpb2*) which are known to be suitable for resolving species and improving the understanding of the systematics within the *Fusarium* genus (Geiser et al., 2004: O'Donnell et al., 2009; O'Donnell et al., 2012). However, these selected marker genes still have limitations such as incongruent topologies and the lack of resolution needed to distinguish species boundaries. For example, the molecular phylogeny based on *tef1α* was incongruent with those for *rpb2*, *cmdA* and the concatenated dataset for species previously named as "*Fusarium* sp. nov. 3" and "*Fusarium* sp. nov. 4" (Mkandawire et al. 2022). Strains previously identified as "*Fusarium* sp. nov. 3" and "*Fusarium* sp. nov. 4" only based on *tef1α* are intermingled with *F. citri* and *F. incarnatum* species based on *cmdA* and *rpb2* and, respectively. The concatenated phylogenies resembled *rpb2* phylogenies, which could not be used for distinguishing the species as well. Since the sequences apart from *tef1α* could not give strong enough phylogenetic evidence for a definitive conclusion, the isolates

were set aside until a more robust set of traits become available to determine if these groups warranted separation as different species.

Here, the standardised set of morphological methods of Gerlach and Nirenberg (1982), Leslie and Summerell (2006) and Sandoval-Denis et al., (2018a) were used to define key characteristics of *F. syzygium*. However, species of *Fusarium* and FIESC are difficult to identify based solely on morphology due to the limited diagnostic morphological characters for some species (O'Donnell et al., 1998; O'Donnell et al., 2000; Wang et al., 2019). This was also true in the present study where in our study, *F. syzygium* did not have distinct morphological characteristics separating it from the common features in species of the FIESC.

The isolates of *F. syzygium*, originated from malformed inflorescence of *S. cordatum* in Kwambonambi and Belville. Even though this study has not provided a direct link between malformation disease and *Fusarium* species, the presence of these isolates only in malformed inflorescences presents an interesting dimension in malformation diseases and that needs to be studied in detail. *Fusarium* species have been known to cause floral malformation diseases in Mangoes (Steenkamp et al., 2000; Britz et al., 2002; Liew et al., 2016). With regards to *Fusarium* species within the FIESC, even though their aetiology is not clear, they have been associated with mango malformation disease in Australia (Liew et al., 2016). There is a possibility that these species reside in the plants as endophytes and only cause malformations when the plant is stressed from other biotic factors. As such, future studies should consider pathogenicity tests to determine the exact role that this species plays in *S. cordatum* malformation.

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Table 1: Details of *Fusarium* Strains included in the Phylogenetic Analysis

Species	Culture collection	Genbank accession number			Substrate	Country	Reference
		tef1	*cmdA*	*rpb2*			
F. aberrans	CBS131385^{T}	MN170445	MN170311	MN170378	*Oryza australiensis*	Australia	Xia et al., 2019
F. arcuatisporum	LC12147^{T} = CGMCC3.19493^{T}	MK289584	MK289697	MK289739	Unknown	Unknown	Wang et al., 2019
F. brevicaudatum	NRRL43638T = UTHSC_R-3500	GQ505665	GQ505576	GQ505843	*Trichechus* sp	USA	Xia et al., 2019
F. caatingaense	MUM1859 = URM6779^{T}	LS398466		LS398495	*Dactylopius opuntiae*	Brazil	Santos et al., 2019
F. cateniforme	CBS150.25^{T} = ATCC11853	MN170451	MN170317	MN170384	Unknown	Unknown	Xia et al., 2019
F. citri	CN008I3 = MRC1642	MH582446		MH582090	Unknown	unknown	O'Donnell et al., 2018
F. citri	NRRL25084 = ARSEF1641	JF740715			*Adelphocoris sp*	Austria	O'Donnel et al., 2012
F. citri	NRRL52765 = ARSEF2304	JF740839		JF741165	*Heteropsylla cubana*	Papa New Guinea	O'Donnel et al., 2012
F. citri	LC4879	MK289615	MK289665	MK289768	*Amygdalus trioba*	China	Wang et al., 2019
F. citri	LC6896^{T} = CGMCC3.19467^{T}	MK289617	MK289668	MK289771	*Citrus reticulata*	China	Wang et al., 2019
F. citri	LC7922	MK289634	MK289687	MK289788	Capsicum	China	Wang et al., 2019
F. citri	LC7937	MK289640	MK289693	MK289794	Capsicum	China	Wang et al., 2019
F. citri	CBS621.87	MN170452	MN170318	MN170385	*Medicago sativa*	Denmark	Xia et al., 2019, O'Donnel et al., 2009
F. citri	CBS678.77	MN170453	MN170319	MN170386	Soil	Japan	Xia et al., 2019
F. citri	CBS130905	MN170454	MN170320	MN170387	*Triticum*	Iran	Xia et al., 2019
F. citri	CPC35143 = CCF1881	MN170455	MN170321	MN170388	*Lactuca sativa*	Czech Republic	Xia et al., 2019
F. clavus	CBS126202^{T}	MN170456	MN170322	MN170389	Soil	Namibia	Xia et al., 2019
F. clavus	NL19-041003	MZ921827	MZ921515	MZ921695	Soil	Netherlands	Crous et al., 2021
F. coffeatum	CBS635.76 = BBA62053 = NRRL20841^{T}	MN120755	MN120696	MN120736	*Cynodon lemfuensis*	New Zealand	Lombard et al., 2019
F. compactum	NRRL36323=CBS186.31E^{T}	GQ505648	GQ505560	GQ505826	Cotton yarn	England	O'Donnell et al., 2009
F. concolor_OUT	FCONSCNRRL13459 = ATCC60096= CBS961.87 = FRCM-2405 = IMI296456^{T}	GQ505674	GQ505585	GQ505852	Plant debris	South Africa	O'Donnell et al., 2009
F. croceum	CBS131777^{T}	MN170463	MN170329	MN170396	*Triticum* sp.	Iran	Xia et al., 2019
F. croceum	NL19-059006	MZ921831	MZ921519	MZ921699	Soil	Netherlands	Crous et al., 2021

F. duofalcatisporum	NRRL36448 = CBS384.92^{T}	GQ505652	GQ505564	GQ505830	*Phaseolus vulgaris*	Sudan	O'Donnell et al., 2009
F. equiseti	NRRL26419^{NT}=CBS307.94E^{T}=BBA68556^{NT}	GQ505599	GQ505511	GQ505777	Soil	Germany	O'Donnell et al., 2009
F. equiseti	CBS148218 = NL19-25004	MZ921833	MZ921521	MZ921701	Soil	Netherlands	Crous et al., 2021
F. fasciculatum	CBS131382^{T}	MN170473	MN170339	MN170406	*Oryza australiensis*	Australia	Xia et al., 2019
F. flagelliforme	NRRL36269^{T} = CBS162.57^{T}	GQ505645	GQ505557	GQ505823	*Pinus nigra*	Croatia	O'Donnell et al., 2009
F. flagelliforme	NL19-041004	MZ921839	MZ921527	MZ921707	Soil	Netherlands	Crous et al., 2021
F. flagelliforme	NL19-041004	MZ921839	MZ921527	MZ921707	Soil	Netherlands	Crous et al., 2021
F. gracilipes	NRRL43635^{T} = UTHSC_06-638	GQ505662	GQ505573	GQ505840	Horse	USA	O'Donnell et al., 2009
F. guilinense	NRRL13335 = FRCR-2138	GQ505590	GQ505502	GQ505768	Alfafa	Australia	O'Donnell et al., 2009
F. guilinense	NRRL32865 = FRCR-8480	GQ505614	GQ505526	GQ505792	Human endocarditis	Brazil	O'Donnell et al., 2009
F. hainanense	LC11638^{T} = CGMCC3.19478^{T}	MK289581	MK289657	MK289735	*Oryza sp*	China	Wang et al., 2019
F. humuli	CQ1039 = CGMCC3.19374^{T}	MK289570	MK289712	MK289724	*Humulus scandens*	China	Wang et al., 2019
F. incarnatum	NRRL13379 = FRCR-5198 = BBA62200	GQ505591	GQ505503	GQ505769	*Oryza sativa*	India	O'Donnell et al., 2009
F. incarnatum	NRRL32866 = FRCR-8822	GQ505615	GQ505527	GQ505793	Human	USA	O'Donnell et al., 2009
F. incarnatum	NRRL32867 = FRCR-8837	GQ505616	GQ505528	GQ505794	Human	USA	O'Donnell et al., 2009
F. incarnatum	CBS132.73^{NT} = ATCC2438 7 =IMI128222= NRRL25478^{NT}	MN170476	MN170342	MN170409	*Trichosanthes dioica*	Malawi	Xia et al., 2019
F. incarnatum	CBS132907	MN170477	MN170343	MN170410	*Triticum*	Iran	Xia et al., 2019
F. incarnatum	CBS 132.73 = ATCC 24387	MN170476	MN170342	MN170409			Xia et al., 2019
F. ipomoeae	LC12165^{T} = CGMCC3.19496^{T}	MK289599	MK289704	MK289752	*Ipomea aquatica*	China	Wang et al., 2019
F. irregulare	LC7188^{T} = CGMCC3.19489^{T}	MK289629	MK289680	MK289783	Bamboo	China	Wang et al., 2019
F. lacertarum	NRRL20423^{T}=IMI300797^{T}=CBS130185=ATCC42771	GQ505593	GQ505505	GQ505771	Unknown	Unknown	O'Donnell et al., 2009
F. longicaudatum	CBS123.73^{T}=ATCC24370=IMI160825=NRRL25477^{T}	MN170481	MN170347	MN170414	Unknown	Tanzania	Xia et al., 2019
F. longifundum	CBS235.79^{T} = NRRL36372^{T}	GQ505649	GQ505561	GQ505827	Air	Curaçao	O'Donnell et al., 2009
F. luffae	LC12167^{T} = CGMCC3.19497^{T}	MK289601	MK289698	MK289754	*Luffa aegyptiaca*	China	Wang et al., 2019
F. mucidum	CBS102395^{T}	MN170485	MN170351	MN170418	*Anacardium occidentale*	El Salvador	Xia et al., 2019

F. multiceps	NRRL43639^{T} = CBS130386^{T} = UTHSC_04-135	GQ505666	GQ505577	GQ505844	*Trichechus sp.*	USA	O'Donnell et al., 2009
F. nanum	LC12168^{T} = CGMCC3.19498	MK289602	MK289651	MK289755	*Musa nana*	China	Wang et al., 2019
F. neoscirpi	CBS610.95^{T} = NRRL26861^{T} = NRRL26922^{T}	GQ505601	GQ505513	GQ505779	Soil	France	O'Donnell et al., 2009
F. persicinum	CBS479.83^{T}	MN170495	MN170361	MN170428	unknown	Unknown	Torbati et al., 2019
F. scirpi	NRRL36478^{NT} = CBS447.84^{NT} = FRCR-6252	GQ505654	GQ505566	GQ505832	Soil	Australia	O'Donnell et al., 2009
F. serpentinum	CBS119880^{T}=BBA62209=MRC1813	MN170499	MN170365	MN170432	Unknown	Unknown	Xia et al., 2019
F. sulawesiense	LC12178E^{T}	MK289610	MK289708	MK289763	*Syngonium auritum*	China	Wang et al., 2019
F. tanahbumbuense	InaCCF965^{T}	LS479448	LS479432	LS479863	Musa sp	Indonesia	Maryani et al., 2019
F. toxicum	CBS406.86^{T} = FRCR-8507 = IMI309347=NRRL25796^{T}	MN170508	MN170374	MN170441	Soil	Germany	Xia et al., 2019
F. toxicum	NL19-041005	MZ921845	MZ921533	MZ921713	Soil	Netherlands	Crous et al., 2021
F. wereldwijsianum	CBS148244^{T} = NL19-94009	MZ921850	MZ921538	MZ921718	Soil	Netherlands	Crous et al., 2021
F. wereldwijsianum	CBS148219 = NL19-99002	MZ921848	MZ921536	MZ921716	Soil	Netherlands	Crous et al., 2021
CMW55736[a]	CMW55736[a]				*Syzygium cordatum*	South Africa	in this study
CMW55737[a]	CMW55737[a]				*Syzygium cordatum*	South Africa	in this study
CMW55738[a]	CMW55738[a]				*Syzygium cordatum*	South Africa	in this study
CMW55742[a]	CMW55742[a]				*Syzygium cordatum*	South Africa	in this study
CMW55743[a]	CMW55743[a]				*Syzygium cordatum*	South Africa	in this study
CMW55735[b]	CMW55735[b]				*Syzygium cordatum*	South Africa	in this study
CMW55739[b]	CMW55739[b]				*Syzygium cordatum*	South Africa	in this study
F. syzygium prov nom	CMW55747				*Syzygium cordatum*	South Africa	in this study
F. syzygium prov nom	CMWF1056^{T}				*Syzygium cordatum*	South Africa	in this study

Figure 1. ML tree based on tef1 *tef1α*, *cmdA*, and *rpb2* showing identities and diversity of *Fusarium* in FIESC isolates associated *Syzygium cordarum*. Bootstrap values ≥ 80% are shown above branches. Sequences obtained from ex-type, neo-type and iso-type cultures are indicated by T, NT and IT. Sequences obtained from strains during this study are indicated by bold text, while the new species, *F. syzygium* prov. nom. , is in bold blue text. Cultures marked with a and b were identified as "*Fusarium* sp. nov. 3" and "*Fusarium* sp. nov. 4" in Mkandawire et al. (2022), respectively. All trees were rooted to *F. concolor*.

tef1α

F. cateniforme CBS150.25[T]
F. scirpi CBS447.84[NT]
F. serpentinum CBS119880[T]
100 F. wereldwijsianum CBS148219
F. wereldwijsianum CBS148244[T]
F. neoscirpi CBS610.95[T]
91 F. arcuatisporum LC12147[T]
93 F. longicaudatum CBS123.73[T]
F. brevicaudatum NRRL43638[T]
F. gracilipes NRRL43635[T]
F. longifundum CBS235.79[T]
100 F. flagelliforme NL19-041004
F. flagelliforme CBS162.57[T]
96 F. equiseti CBS148218
100 F. equiseti CBS307.94[ET]
99 F. toxicum CBS406.86[T]
F. toxicum NL19-041005
F. duofalcatisporum CBS384.92[T]
F. ipomoeae LC12165[T]
93 F. compactum CBS186.31[ET]
F. lacertarum CBS130185[T]
100 F. clavus CBS126202[T]
F. clavus NL19-041003
96 F. croceum CBS131777[T]
F. croceum NL19-059006
"F. citri" CMW55738[a]
"F. citri" CMW55743[a]
"F. citri" CMW55736[a]
100 **"F. citri" CMW55742[a]**
"F. citri" CMW55737[a]
F. citri LC6896[T]
F. citri NRRL52765
F. citri LC7922
F. citri LC7937
F. citri CBS130905
F. citri CBS678.77
100 F. citri LC4879
F. citri CBS621.87
F. citri CPC35143
F. citri MRC1642
F. citri NRRL25084
F. humuli CQ1039[T]
F. mucidum CBS102395[T]
F. fasciculatum CBS131382[T]
90 F. incarnatum NRRL32866
F. incarnatum NRRL32867
100 F. incarnatum CBS132907
F. incarnatum LC13705
F. incarnatum NRRL13379
99 F. incarnatum CBS132.73[NT]
F. guilinensis_LC12160[T]
F. tanahbumbuense InaCCF965[T]
82 100 **"F. incarnatum" CMW55735[b]**
"F. incarnatum" CMW55739[b]
F. coffeatum CBS635.76[T]
F. irregulare LC7188[T]
91 F. sulawesiense LC12178[ET]
F. luffae LC12167[T]
F. multiceps CBS130386[T]
F. caatingaense MUM1859[T]
100 F. guilinense NRRL13335
87 F. guilinense NRRL32865
100 **F. syzygium prov. novm CMW55747**
F. syzygium prov. nom. CMWF1056[T]
F. nanum LC12168[T]
99 F. persicinum CBS479.83[T]
F. hainanense LC11638[T]
F. aberrans CBS131385[T]
F. concolor CBS961.87
x4
0.038

rpb2

F. incarnatum NRRL32866
F. incarnatum NRRL32867
F. incarnatum CBS132907
F. incarnatum NRRL13379
87 F. incarnatum LC13705
88 **"F. incarnatum" CMW55735[b]**
"F. incarnatum" CMW55739[b]
F. incarnatum CBS132.73[NT]
F. irregulare LC7188[T]
F. multiceps NRRL43639[T]
F. caatingaense MUM1859[T]
F. luffae LC12167[T]
F. sulawesiense LC12178[ET]
F. tanahbumbuense InaCCF965[T]
F. guilinensis LC12160[T]
88 F. nanum LC12168[T]
F. persicinum CBS479.83[T]
100 **F. syzygium prov. nom. CMW55747**
F. syzygium prov. nom. CMWF1056[T]
98 F. guilinense NRRL13335
F. guilinense NRRL32865
F. hainanense LC11638[T]
F. aberrans CBS131385[T]
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F. citri CBS678.77
F. citri CPC35143
F. citri CBS130905
100 F. citri CBS621.87
F. citri LC4879
F. citri LC7922
F. citri LC7937
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81 **"F. citri" CMW55736[a]**
"F. citri" CMW55738[a]
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F. citri NRRL52765
F. humuli CQ1039T
F. fasciculatum CBS131382[T]
F. compactum CBS186.31[ET]
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F. lacertarum NRRL20423[T]
95 F. ipomoeae LC12165[T]
91 99 F. clavus CBS126202[T]
F. clavus NL19-041003
F. longifundum CBS235.79[T]
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83 F. equiseti CBS307.94[ET]
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90 F. brevicaudatum NRRL43638[T]
F. longicaudatum CBS123.73[T]
x4
F. concolor CBS961.87
0.03

cmdA

F. arcuatisporum LC12147[T]
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F. serpentinum CBS119880[T]
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F. equiseti CBS148218
86 F. toxicum NL19-041005
F. equiseti CBS307.94[ET]
F. toxicum CBS406.86[T]
F. gracilipes NRRL43635[T]
F. mucidum CBS102395[T]
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F. lacertarum CBS130185[T]
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98 F. croceum CBS131777[T]
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81 F. flagelliforme NL19-041004
92 F. flagelliforme CBS162.57[T]
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F. citri CBS621.87
F. citri CBS678.77
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F. citri LC7922
F. citri LC7937
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F. guilinense NRRL32865
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"F. incarnatum" CMW55739[b]
89 **F. syzygium sp. nov. CMW55747**
F. syzygium sp. nov. CMWF1056[T]
F. irregulare LC7188[T]
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F. incarnatum CBS132907
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100 F. incarnatum NRRL13379
F. incarnatum NRRL32867
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95 F. luffae LC12167[T]
F. sulawesiense LC12178[ET]
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F. persicinum CBS479.83[T]
84 F. hainanense LC11638[T]
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F. citri LC6896[T]
F. fasciculatum CBS131382[T]
x4
F. concolor CBS961.87
0.042

concat

98 F. incarnatum NRRL32866
F. incarnatum NRRL32867
100 F. incarnatum CBS132907
F. incarnatum NRRL13379
F. incarnatum LC13705
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"F. incarnatum" CMW55739[b]
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F. guilinensis LC12160[T]
F. irregulare LC7188[T]
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F. guilinense NRRL32865
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F. syzygium prov. nom. CMWF1056T
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100 F. nanum LC12168[T]
86 F. persicinum CBS479.83[T]
99 F. hainanense LC11638[T]
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F. citri CBS130905
F. citri CBS678.77
F. citri CPC35143
F. citri MRC1642
F. citri NRRL25084
100 F. citri CBS621.87
F. citri LC4879
F. citri LC7922
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"F. citri" CMW55742[a]
"F. citri" CMW55743[a]
94 96 **"F. citri" CMW55736[a]**
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F. citri NRRL52765
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F. fasciculatum CBS131382[T]
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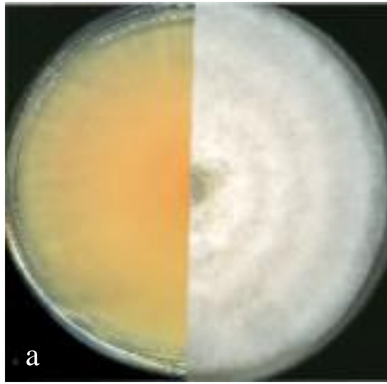
F. wereldwijsianum CBS148244[T]
F. scirpi CBS447.84[NT]
F. neoscirpi CBS610.95[T]
F. serpentinum CBS119880[T]
F. arcuatisporum LC12147[T]
87 F. brevicaudatum NRRL43638[T]
F. longicaudatum CBS123.73[T]
F. cateniforme CBS150.25[T]
F. compactum CBS186.31[ET]
100 F. lacertarum CBS130185[T]
F. duofalcatisporum CBS384.92[T]
95 F. ipomoeae LC12165[T]
100 F. clavus CBS126202[T]
F. clavus NL19-041003
F. longifundum CBS235.79[T]
98 F. equiseti CBS148218
100 F. equiseti CBS307.94[ET]
100 F. toxicum CBS406.86[T]
F. toxicum NL19-041005
F. gracilipes NRRL43635[T]
82 100 F. flagelliforme NL19-041004
F. flagelliforme CBS162.57[T]
100 F. croceum CBS131777[T]
F. croceum NL19-059006
F. mucidum CBS102395[T]
x4
F. concolor CBS961.87
0.041

Figure 2. Reverse of colony and colony of PDA on a). normal light b). ultra-violet c). dark; d.-e). sporodochia on media surface, f-g). aerial conidia; h-i). sporodochia conidiophores; i) aerial conidiation; k-i) conidiophores on aerial mycelim; m-n). polyphialidic phialides; o). lateral phialides; p-q), aerial phialides; r). area conidia; s). sporodochia conidia. Scale bars: = 10 μm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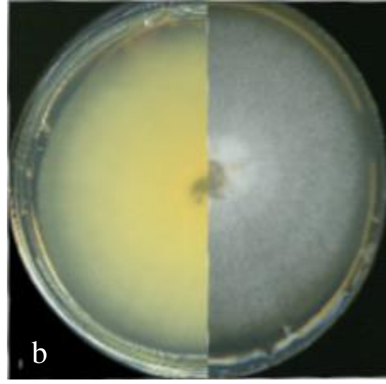
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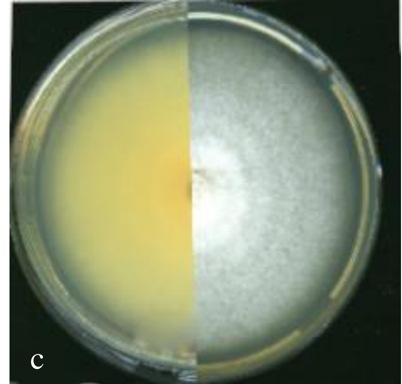
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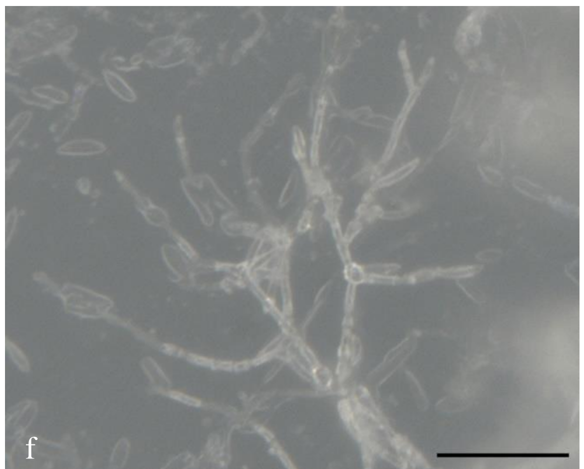
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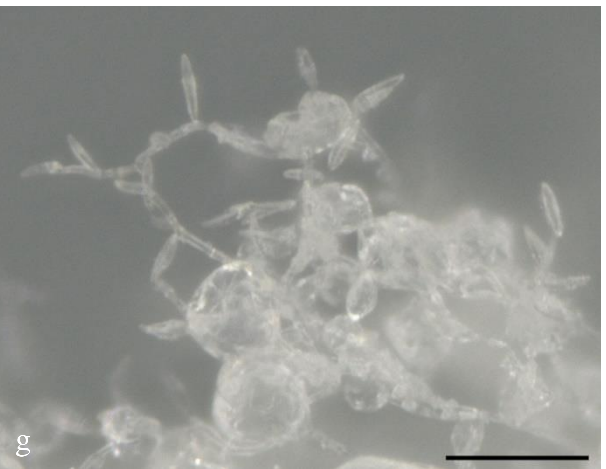
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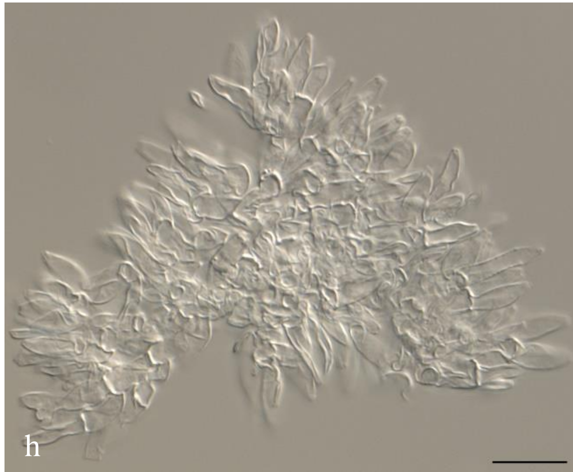
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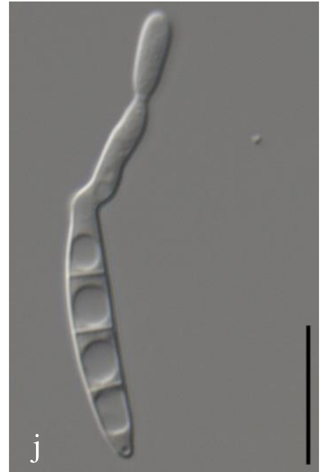
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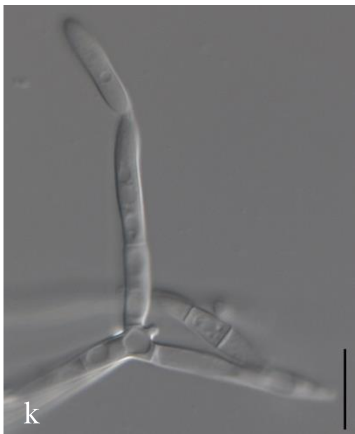
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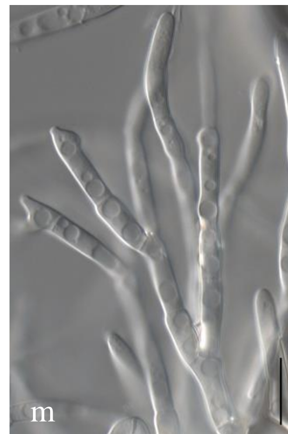
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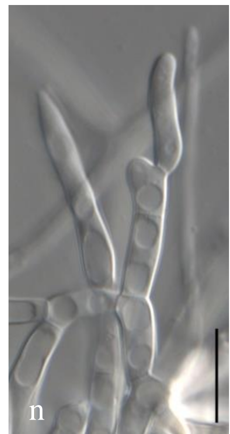
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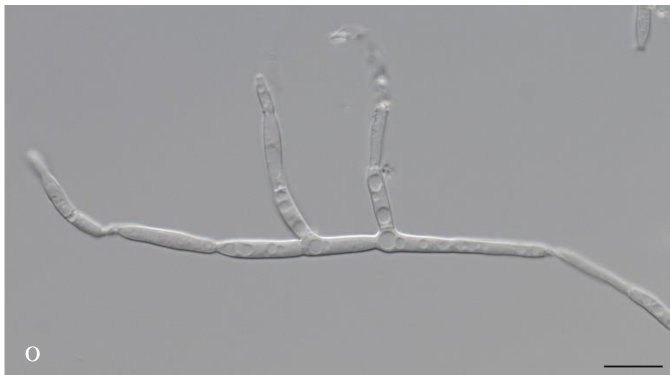
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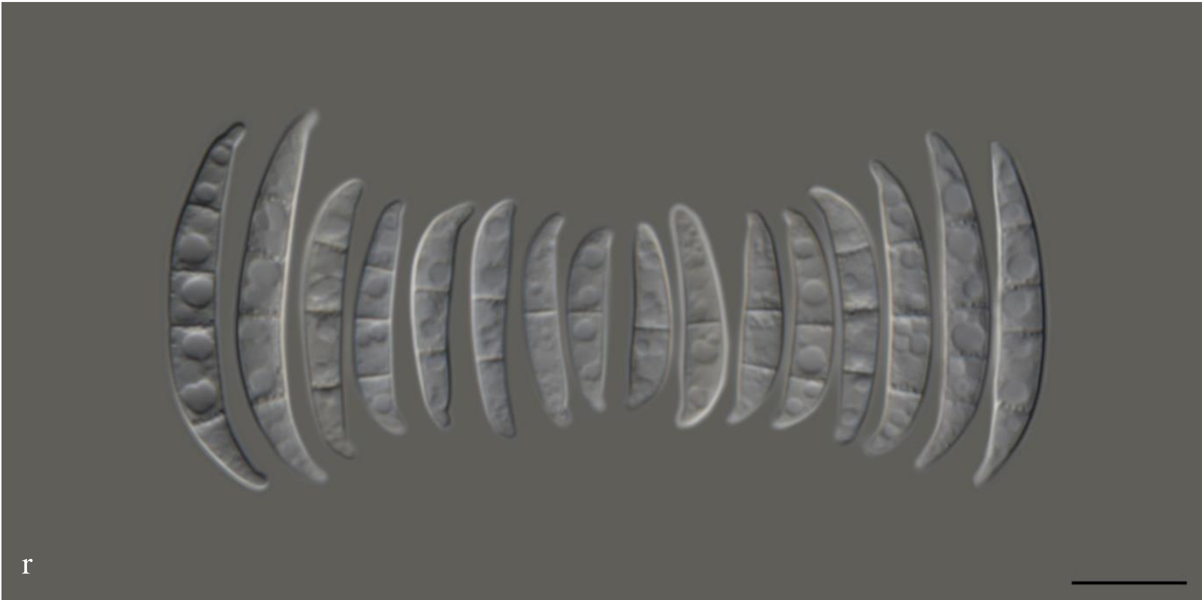
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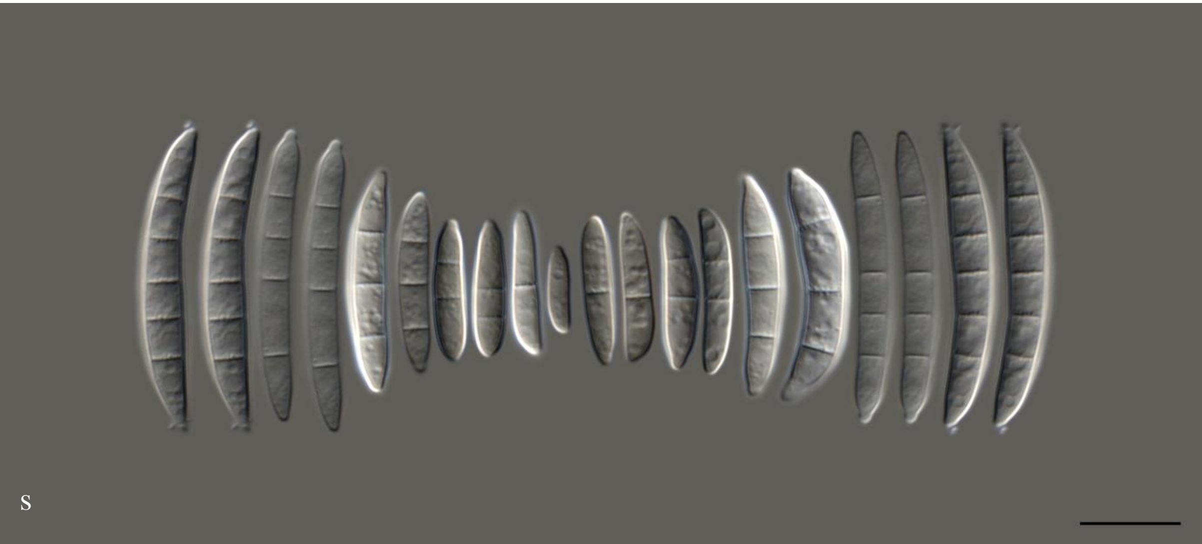
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Chapter 4: Diversity of mites associated with malformed inflorescences of *Syzygium cordatum* in South Africa.

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Abstract

Mites are considered serious pests in agriculture and forestry ecosystems where they cause damage through direct feeding and indirect damage via transmission of plant pathogens. Despite their economic importance, knowledge of their presence and diversity in indigenous ecosystems and their impact is limited. The aim of this study was to collect and identify acaroid species associated with healthy and malformed *Syzygium cordatum* inflorescences collected from various regions of KwaZulu Natal, South Africa. Results revealed the presence of mites only in malformed inflorescences. These mites were identified based on their morphological characteristics. Thereafter, the *cytochrome c oxidase subunit 1* gene region of representative mites was sequenced to establish a DNA database for future identification of these species. Morphological identification revealed a dominant group belonging to *Eriophyoidae* (gall forming mites), close to *Eriophyes afroensis* although variation within this group was observed. A second group resided in the *Stigmaeidae*, close to *Agistemus collyerae*. The phylogenetic analyses supported the morphological identifications showing the presence of two closely related or cryptic *Eriophyes* species. The exact role of the mites identified in this study could not be established in this study. However, the consistent presence of eriophyoid mites in the malformed tissues and not in healthy inflorescences could suggest a possible role in malformation as their feeding cause direct damage to their host plants that result in the production of galls or other abnormalities and/or distortions. The *Stimaeidae*, on the other hand, could possibility play a predatory role in the niche similar to many other mite species in this genus.

1. Introduction

Phytophagous mites are economically important pests of plants in agricultural, forestry and natural ecosystems (Bolland et al., 1998; Jeppson et al., 1975). These arthropods reside in the subclass Acariformes of Arachnida (Chelicarata), in the order Trombidiformes and suborder Prostigmata. Species belonging to the families *Tetranychidae* (spider mites), *Tenuipalpidae* (false spider mites/flat mites), *Tarsonemidae* (broad mites) and *Eriophyoidae* (gall forming mites) are particularly important (Jeppson et al., 1975; Bolland et al. 1998; Moraes and Flechtmann, 2008). They cause economic damage to plants through direct feeding and can also cause indirect damage by transmitting plant pathogens such as viruses, phytoplasmas (prokaryotes without a cell wall) and fungi (Jeppson et al., 1975; Lindquist and Amrine 1996; Van Leeuwen et al., 2010).

The *Eriophyoidae* mites include more than 4000 obligatory phytophagous species. They are considered the second most economically important family of mite pests after spider mites (*Tetranychidae*) (Lindquist and Amrine, 1996). Many of these species are highly host specific and reported from a single host species or genus, with non-host specific species feeding on closely related host plants (Skoracka et al., 2009). The species of this superfamily mostly cause direct damage to their host plants that result in the production of galls or other abnormalities and/or distortions. They can infest different plant parts such as the stems, leaves and floral parts. Symptoms on stems include galls and on leaves include erinea, leaf deformation, discoloration and russeting (Westphal and Manson, 1996). In the case of floral parts, malformations manifest in symptoms such as shriveling and russeting, flower clustering, distortion of apical shoots, phyllody and galling of flowers (Craemer and Neser, 1990; Craemer, 1996). In addition, eriophyoid species can facilitate infection by vectoring pathogens such as viruses (Agrios, 1980; Hatcher and Paul, 2001), phytoplasmas (Hoat et al., 2016) and fungal spores (Gamliel-Atinsky et al., 2009a) and their direct feeding creates wound sites in plants for easy pathogen penetration (Gamliel-Atinsky et al., 2009b).

Syzygium cordatum (water berry) is an important native species in the South African landscape. It has multiple uses and locally, it is a source of ethno-herbal medicine, a source of food as its fruits are edible, a source of fuel wood and charcoal, timber, building materials and fences, and for landscaping purposes as an ornamental plant (Dlamini, 1981; Dlamini and Geldenhuys, 2009). Floral malformations, associated with abnormal development of vegetative shoots and

floral panicles resulting in phyllody and hypertrophy have been observed among trees in various regions in South African where the species occur naturally (Mkandawire et al., 2022). Different species in the genus *Fusarium,* as well as eriophyoid mites could be of particular interest linked to the similarity of symptoms observed in these indigenous *S. cordatum* (Mkandawire et al*.,* 2022) in comparison to mango bud and Floral Malformation Disease (MMD) (Gamliel-Atinsky et al., 2009).

Recently, Mkandawire et al. (2022) recorded a high diversity of *Fusarium* species, comprising of 22 lineages residing in four different *Fusarium* species complexes, from both healthy and malformed inflorescences of *S. cordatum*. The presence of mites, and their potential role in the occurrence of *S. cordatum* floral malformation disease, were however not studied. The aim of this study was to consider the presence of mites associated with healthy and malformed *S. cordatum* inflorescences. To achieve this goal, mites were collected from different regions in KwaZulu Natal in South Africa and identification was done based on morphological descriptions. DNA sequence data were used to confirm their differences and to establish a data base for future studies.

2. Materials and Methods

2.1 Field survey and mite collection

In 2016, malformed and healthy inflorescences of *S. cordatum* were sampled from five locations across the KwaZulu-Natal province of South Africa, namely Dukuduku, Mtubatuba, Nyalazi, Mzingazi and Kwambonambi. A total of 15 trees were sampled, and from each tree 100 grams of each of the malformed and healthy inflorescences were examined for the presences of mites. For this purpose, inflorescences were cut into 1 cm length pieces, using a surfaced sterilized pair of scissors, and placed into collection plastic bags. Thereafter, 50 ml of 70% (V/V) ethanol was added to the bag and shaken for approximately 30 seconds. A small hole was cut in the corner of the bag and the rinsate was decanted into petri dishes. Specimens of mites were then preserved in absolute alcohol in 15ml vials for subsequent DNA extraction and morphological descriptions. From each 15ml vial, 3ml was examined was examined under a microscope to differentiate the species into different morpho groups.

2.2 Morphological identification

For morphological identification, mite samples were mounted on slides in Hoyer's medium according to a modified procedure of Gutierrez (1985), and then observed using a high-power phase-contrast microscope (Olympus, Toyko, Japan). Morphological description terminology followed that of Lindquist (1996) and measurements were made following the approaches of Amrine and Manson (1996) and de Lillo et al. (2010). Legs were measured from the distal margin of the tarsus to the proximal margin of the trochanter; empodia were measured from the distal apex to their junction with the margin of the tarsal segment. Other measurements included: ventral opisthosomal annuli were counted from the posterolateral corner of coxal field; the length of the prodorsal shield, that includes the frontal lobe, were measured; the position of some leg setae was measured from the proximal margin of the segment bearing the seta; the number of microtubercles between setae *sc* were counted as the microtubercles on the first complete annulus (the first one is often incomplete) behind the prodorsal shield. Mites were also sent to a taxonomist at the Agriculture Research Council (ARC) Biosystematics division for confirmation of the groupings recovered from the inflorescences.

External morphology of the collected mites was further studied using scanning electron microscopy (SEM) (Philips XL30), at the Scanning Electron Facility of the University of Pretoria (AAFC). Specimens already stored in absolute alcohol were transferred with a pipette into a microporous capsule (30-µm pores) partly immersed in absolute alcohol in a small Petri dish, for 15 min. Thereafter, specimens immersed in absolute alcohol were transferred to a Petri dish with absolute alcohol for another 15 min to ensure that specimens were effectively submerged in undiluted absolute alcohol before undergoing critical-point drying. Specimens were then mounted on SEM specimen stubs using a small paint brush with a few remaining hairs, and sputter-coated with gold before examination under SEM.

2.3 DNA Sequence Comparisons

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from a group of 45 adult mites collected from 50g of malformed inflorescences of *S. cordatum* using the NucleoSpin ® Tissue Kit single columns (Macherey-Nagel) according to manufacturer's recommendations. The DNA

concentration was quantified using a Nanodrop spectrophotometer. Thereafter, a 700 bp region of the mitochondrial cytochrome *c* oxidase subunit I (CO)I gene region was amplified using the primer pair - LCO1490: 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3' and HCO2198: 5'- TAA ACT TCA GGG TGA CCA AAA AAT CA-3' (Folmer et al., 1994). The polymerase chain reaction (PCR) was carried out using a reaction mixture of total volume 25 µL containing 17.75 µL of water, 5 mM Mytaq buffer containing $MgCl_2$ and dNTPs (BioLine, South Africa), 0.5 µM of each primer, 0.25 U Mytaq polymerase and 1µL (50-100 ng) of mite DNA. The thermal cycling profile was as follows: one cycle of 5 min at 95°C, 35 cycles of 60 s at 95°C, 60 s at 50°C, 60 s at 72°C, and a final cycle of 5 min at 72°C. Amplification products were analysed by 1.5% agarose gel electrophoresis. PCR products were purified using ExoSAP-IT (Affymetrix Inc., Santa Clara, California, USA) according to manufacturer's protocol and electrophoresed to assess product integrity. Cleaned products were sequenced in both directions with the original primers using the ABI 377 automated sequencer and the BigDye Terminator v 3.1 sequencer cycle sequencing kit (Applied Biosystems, Foster City, CA). Electropherograms were visualized and consensus sequences were generated from forward and reverse sequences using BioEdit version 7.0.9 (Hall, 1999).

Phylogenetic analyses

The DNA sequences were analyzed using BioEdit version 7.0.9 (Hall, 1999). Sequences were translated into amino acids to screen for the presence of premature stop codons as part of sequence quality check. The sequences were then searched against the GenBank database of the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST) algorithm (Schäffer et al., 2001). Nucleotide sequences of mite species in the genera *Aceria* and *Agistemus* were downloaded and included in the analysis. Sequences were aligned using MAFFT version 7 (https://mafft.cbrc.jp/alignment/software) (Katoh et al., 2019) set on the L-INS-i option and trimmed to a length of 550 bp to remove misleading data and remain with informative data. A phylogenetic tree was constructed by the neighbor-joining method using MEGA 6 program (Tamura et al. 2013). Bootstrap analysis was performed with 1000 replicates at 70% confident limit (Felsenstein, 1985).

Sequence divergence analysis

Intraspecific divergence was determined to evaluate specimen groupings based on morphology and/or to determine the possible presence of cryptic species. Nucleotide diversity (π), average number of nucleotide substitutions (D_{xy}), and net nucleotide substitutions between species and/or populations (D_a) were calculated using DNA Sequence Polymorphism (DnaSP) version 5.10.01 (Librado and Rozas, 2009). For comparative purposes π, D_{xy} and D_a values were converted to percentages.

3. Results

3.1 Morphology

Mites were only found in malformed inflorescences (Fig 1), and they were never present in the healthy structures. Morphological observations revealed mites at various developmental stages within the bracts and panicles of malformed inflorescences belonging to the *Eriophyoidae* and *Stigmaeidae*. Mites in the *Eriophydae* were dominant in all malformed inflorescences. For every 1ml of preserved rinsate, there were between 1300-1600 *Eriophyoidae* mites for every mite in the *Stigmaidae* family. Morphological details of the eriophyoid mite species is described below.

3.2 Species descriptions

The body of adult mites was creamy white and curved.

Family Eriophyoidae Nalepa
Genus *Eriophyes* Von Siebold

Diagnosis: Prodorsal shield pattern with a short median line at posterior quarter, complete undulated admedian line, sternal microtubercles oval and slightly pointed; no accessory setae present, coxae granulate and 1st setiferous coxal tubercles further apart than 2nd pair; empodial featherclaw 5- or 6-rayed.

Female: (Figs. 2) – Body vermiform (n = 20), 280-330 µm, 58 (51-64) thick. Gnathosoma 23 (20-27), rostrum about 24 µm, projecting slightly forward and curved ventrad; body is subsemicircular with a small triangular anterior lobe; ornamentation consisting of a short median line at posterior quarter of shield; admedian lines, gently undulate; 2 submedian lines and a lateral line; laterally shield with a band of granules; dorsal tubercles slightly ahead of rear shield margin; dorsal setae 114-18 µm long, directed upwards and anteriad; abdomen with about 58-63 rings, microtuberculate with oval microtubercles, touching ring margins, fading dorsally from about 15th ring towards posterior end and becoming progressively more elongate; lateral pair of setae on about 13th ring, about 23-27 µm long; ventral setae: 1st pair on about 24th ring, about 40-46 µm long; 2nd pair on about 39th ring, about 13-1714 µm long; 3rd pair on about 6th ring from rear, about 19 µm long; accessory caudal setae absent; genital coverflap with cross lines of granules and about 9 longitudinal markings; genital setae about 12-14 µm long; internal genital apodeme.

Female. Dimensions (n=20):
Male – unknown
Host plant – *Syzygium cordatum* Inflorescence
Habitus – Abundant population in bracts of malformed inflorescences
Type locality – Kwambonambi, KwaZulu natal, South Africa

The morphological identification was in agreement with results obtained from the ARC Biosystematics division that confirmed the dominant group was in the genus *Eriophyes* most likely *Eriophyes afroensis.* Morphological variation was observed amongst the specimens suggesting that they could represent more than one species of *Eriophyes*. The other specimens belonging to Stigmaidea were identified as *Agistemus collyerae*

3.3 Molecular identification

Phylogenetic analysis

The COI sequences for 45 mite samples, obtained from malformed inflorescences, were compared to corresponding sequences from GenBank. The BLAST searches of the COI revealed that the isolates examined represented two different families i.e., *Eriophyoidae* and

Stigmaidae. A total of 31 sequences represented mites from the *Eriophyoidae* with 14 mite sequences from *Stigmaidae.*

The COI-based phylogeny separated the 31 eriophyoid specimens examined into 2 well-supported and exclusive groups, closely related to a larger lineage that represent multiple species from a number of different genera. (Fig. 3). Phylogenetic analysis of the 14 *Stigmaidae* isolates based on COI datasets (Fig. 4) also grouped the isolates into a well-supported and exclusive group that is closely related to *Agistemus terminalis*.

Sequence divergence analysis

Phylogenetic analysis of the 31 eriophyoids specimens divided the mites into two exclusive groups. The genetic divergence between these groups, i.e., average number of nucleotide substitutions per site (D_{xy}) and the net nucleotide substitutions per site (D_a) between groups (populations) (Nei, 1987) were estimated as 19.8% (0.198) and 19.4% (0.194), respectively. The genetic divergence between these groups therefore not only supports the separation of the specimens into two exclusive groups, it suggests that the groups are in fact separate species since genetic distances between 6.6 to 26.5% usually suggests interspecific variation (Laska et al., 2018; Young et al., 2019).

4. Discussion

The results of this study showed that mites are commonly present in malformed inflorescence of *S. cordatum* but not in healthy structures. These mites were found to represent two taxa belonging in the families of *Eriophyoidae* and in the *Stigmaeidae*. This study could not determine the exact roles of the mites in the malformation. However, the presence and dominance of the eriophyoid mites within the malformed parts could potentially suggest their involvement in the aetiology of the disease. Species within the *Eriophyoidae* would be of particular importance since they belong to the gall forming family that are responsible for malformation either directly by causing deformations through feeding (Oldfield and Proeseler, 1996) or indirectly through their interactions with *Fusarium* (Gamliel-Atinsky et al., 2009). The *Stimaeidae*, on the other hand, could possibility play a predatory role in the niche similar to many other mite species in the genus (Leiva et al., 2013).

This study represents the first molecular analysis of *Eriophyoidae* mites associated with inflorescence malformation in *S. cordatum.* Molecular approaches for species identification such as the use of DNA barcoding has the potential to delineate species within the acari (Hebert et al., 2003) which are often difficult when only based on morphology as a result of its tiny size and structural simplicity (Knowlton, 1993; Lindquist and Amrine, 1996). In this study, phylogenetic analysis of species within the eriophyoid mites revealed two well-supported groups. These findings were also supported by the sequence divergence data which suggested the presence of cryptic species as well as morphological identification that revealed differences between the mite specimens. Cryptic speciation in eriophyoid mites is uncommon due to their intimate association with their hosts, their lack for long-range host seeking ability, and their fecundity rates (Sabelis and Bruin, 1996; Magalhães et al., 2007). However, some examples of complexes of morphologically similar species or cryptic species are known within the *Eriophyoidae*, and may occur on the same host species, or on closely related hosts (Skoracka et al., 2015). It is therefore possible that *Eriophyes afroensis*, currently the only valid eriophyoid species recorded from the genus *Syzygium* (Ueckerman, 1993; Oldfield, 1996) and another closely related species both occur within the niche.

The other less dominant group of mites within the *Stigmaedae* family was confirmed to be *Agistemus collyerae*. The mites in this genus are the second most abundant mites found in plants as free living and in some cases as predators of phytophagous mites of the families Eriophyoidae, Tetranychidae and Tenuipalpidae and other small arthropods (Santos and Laing 1985; Gerson and Smiley 1990). Different species of the *Agistemus* are known to predate on eriophyoid species such as *Aceria oleae* and *Oxycenus maxwelli* in *Olea europaea* (olive) (Leiva et al., 2013) and *Eriophyes vitis* in *V. vinifera* (grapes) (Vermaak, 2019). While their active role is not clear in this dynamic interaction, there is a possibility that they play a predatory role in this niche as part of the ecosystem (Santos and Laing, 1985; Gerson and Smiley 1990).

Since the causal agent in malformation disease in *S. cordatum* is still unknown, there is a need to conduct pathogenicity studies by looking at all parameters such as the interaction of biotic and abiotic factors that could be responsible for causing malformation and how these influence development of malformation diseases. In addition, understanding the interaction of these

organisms and the environment could bring new insights into understanding malformation disease dynamics under this ever-changing climate (Chimonidou-Pavlidou, 2004).

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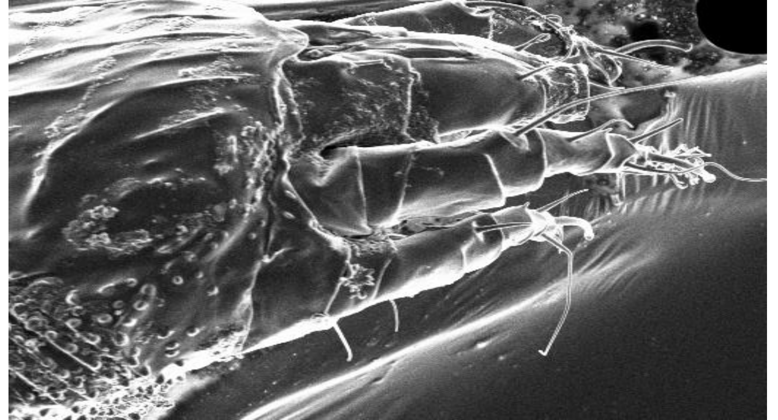
Figure 1. Eriophyoid mites in *S. cordatum* malformed inflorescence

Figure 2. A. Female body of Eriophyes species *Eriophyes afroensis.* B and C. Prodorsal shield showing rostrum (R), gnathosoma (G), admedian line (ML), lateral line (LL), submedium line (SL).



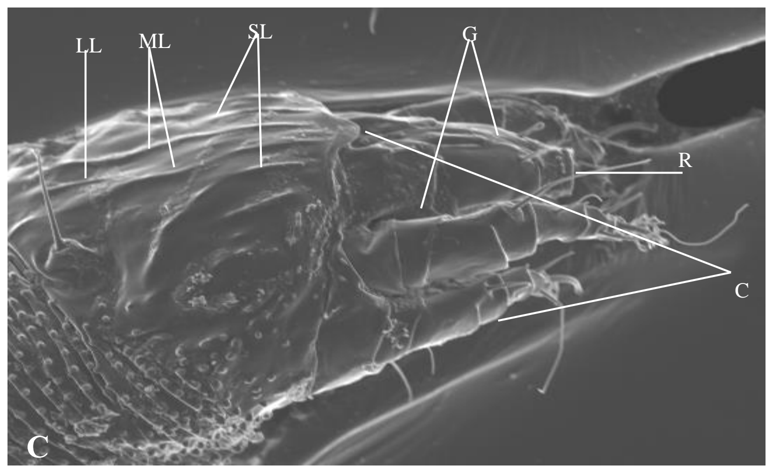


Figure 2. female coxigenital region (CG), genital line (GL), ventral setae (VS).

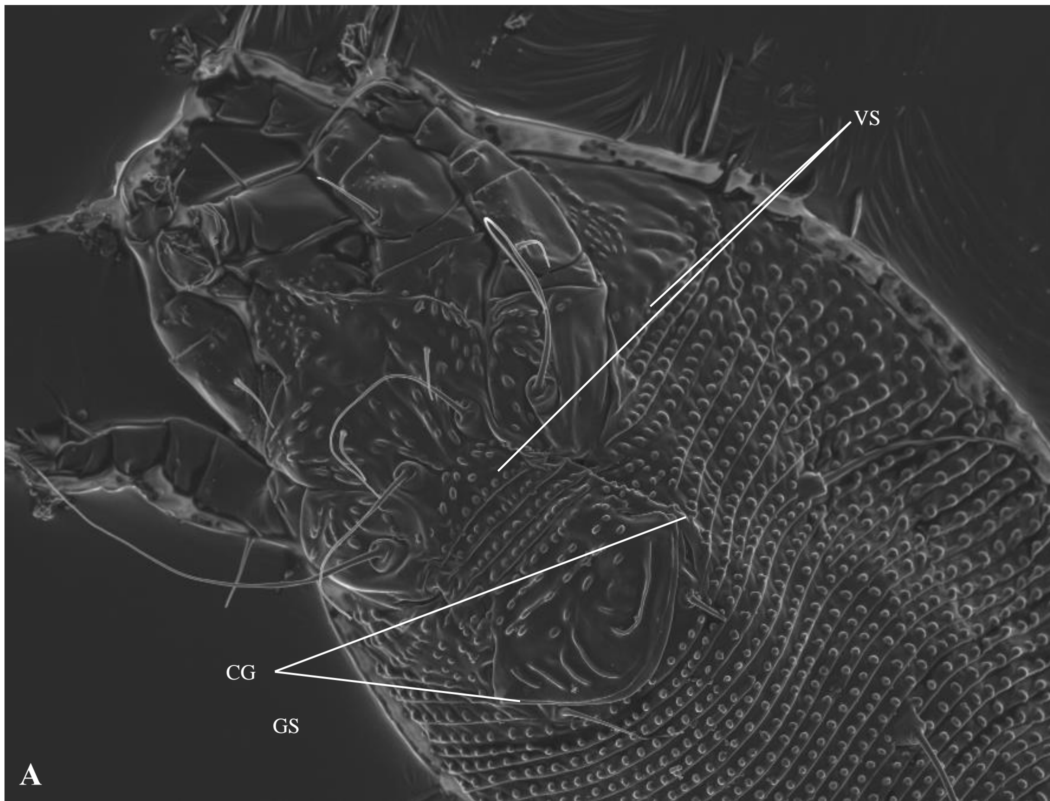
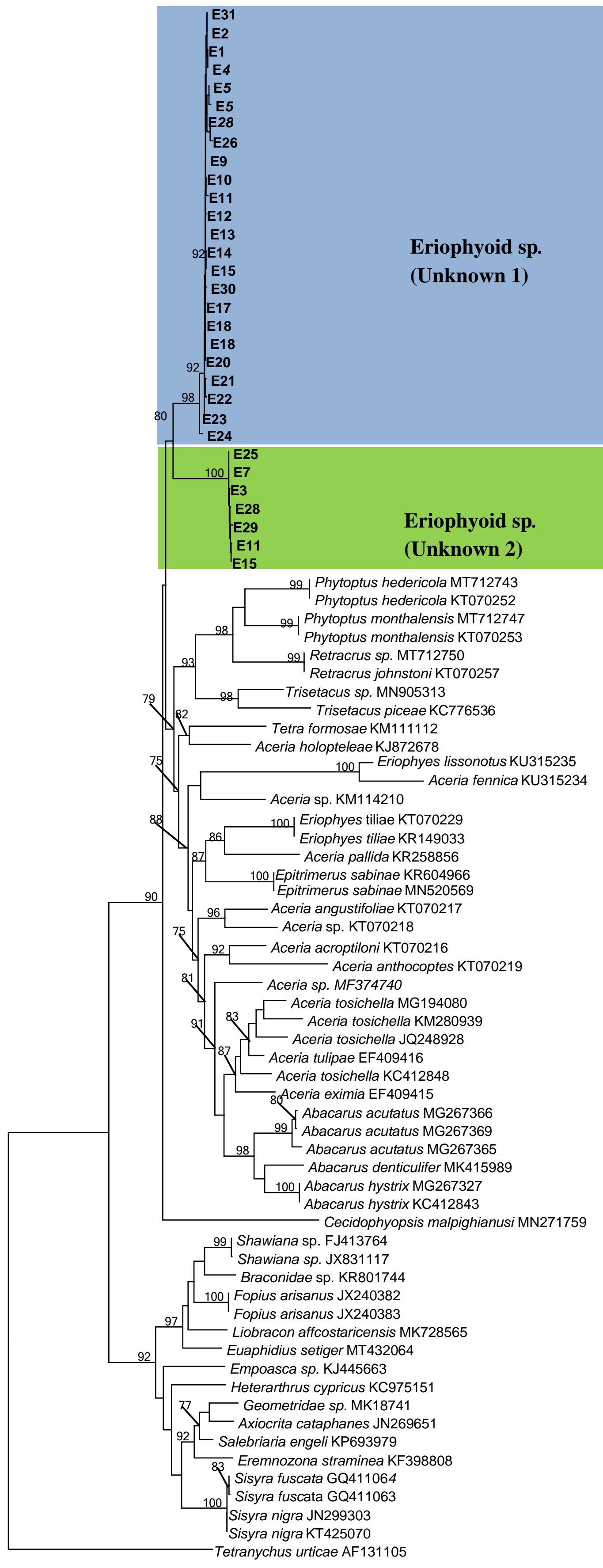
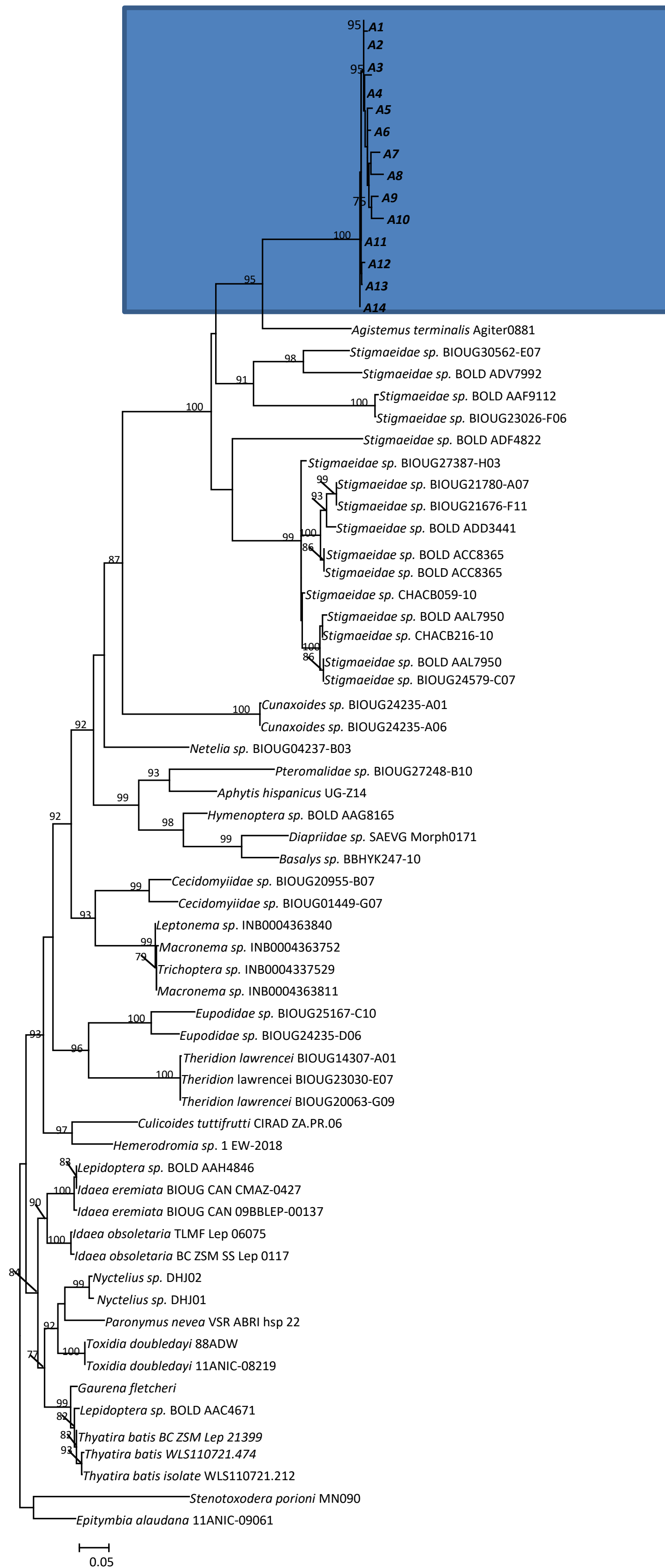


Figure 3. ML tree based on mitochondrial COI sequences showing phylogenetic relationship among species within the *Aceria* genus. *Tetranychus urticae* was included as an outgroup. Nucleotide sequences were obtained from the GenBank database and this study *Aceria sp.* nov 1 *and Aceria sp.* nov. 2 identified in this study.



E31
E2
E1
E4
E5
E5
E28
E26
E9
E10
E11
E12
E13
E14
E15
E30
E17
E18
E18
E20
E21
E22
E23
E24
Eriophyoid sp.
(Unknown 1)
E25
E7
E3
E28
E29
E11
E15
Eriophyoid sp.
(Unknown 2)
Phytoptus hedericola MT712743
Phytoptus hedericola KT070252
Phytoptus monthalensis MT712747
Phytoptus monthalensis KT070253
Retracrus sp. MT712750
Retracrus johnstoni KT070257
Trisetacus sp. MN905313
Trisetacus piceae KC776536
Tetra formosae KM111112
Aceria holopteleae KJ872678
Eriophyes lissonotus KU315235
Aceria fennica KU315234
Aceria sp. KM114210
Eriophyes tiliae KT070229
Eriophyes tiliae KR149033
Aceria pallida KR258856
Epitrimerus sabinae KR604966
Epitrimerus sabinae MN520569
Aceria angustifoliae KT070217
Aceria sp. KT070218
Aceria acroptiloni KT070216
Aceria anthocoptes KT070219
Aceria sp. MF374740
Aceria tosichella MG194080
Aceria tosichella KM280939
Aceria tosichella JQ248928
Aceria tulipae EF409416
Aceria tosichella KC412848
Aceria eximia EF409415
Abacarus acutatus MG267366
Abacarus acutatus MG267369
Abacarus acutatus MG267365
Abacarus denticulifer MK415989
Abacarus hystrix MG267327
Abacarus hystrix KC412843
Cecidophyopsis malpighianusi MN271759
Shawiana sp. FJ413764
Shawiana sp. JX831117
Braconidae sp. KR801744
Fopius arisanus JX240382
Fopius arisanus JX240383
Liobracon affcostaricensis MK728565
Euaphidius setiger MT432064
Empoasca sp. KJ445663
Heterarthrus cypricus KC975151
Geometridae sp. MK18741
Axiocrita cataphanes JN269651
Salebriaria engeli KP693979
Eremnozona straminea KF398808
Sisyra fuscata GQ411064
Sisyra fuscata GQ411063
Sisyra nigra JN299303
Sisyra nigra KT425070
Tetranychus urticae AF131105

Figure 3. ML tree is based on mitochondrial COI sequences showing phylogenetic relationship among species within the *Stigmaideae* family. *Epitymbia alaudana* and *Stenotoxodera porioni* were included as an outgroup.


A1
A2
A3
A4
A5
A6
A7
A8
A9
A10
A11
A12
A13
A14
Agistemus terminalis Agiter0881
Stigmaeidae sp. BIOUG30562-E07
Stigmaeidae sp. BOLD ADV7992
Stigmaeidae sp. BOLD AAF9112
Stigmaeidae sp. BIOUG23026-F06
Stigmaeidae sp. BOLD ADF4822
Stigmaeidae sp. BIOUG27387-H03
Stigmaeidae sp. BIOUG21780-A07
Stigmaeidae sp. BIOUG21676-F11
Stigmaeidae sp. BOLD ADD3441
Stigmaeidae sp. BOLD ACC8365
Stigmaeidae sp. BOLD ACC8365
Stigmaeidae sp. CHACB059-10
Stigmaeidae sp. BOLD AAL7950
Stigmaeidae sp. CHACB216-10
Stigmaeidae sp. BOLD AAL7950
Stigmaeidae sp. BIOUG24579-C07
Cunaxoides sp. BIOUG24235-A01
Cunaxoides sp. BIOUG24235-A06
Netelia sp. BIOUG04237-B03
Pteromalidae sp. BIOUG27248-B10
Aphytis hispanicus UG-Z14
Hymenoptera sp. BOLD AAG8165
Diapriidae sp. SAEVG Morph0171
Basalys sp. BBHYK247-10
Cecidomyiidae sp. BIOUG20955-B07
Cecidomyiidae sp. BIOUG01449-G07
Leptonema sp. INB0004363840
Macronema sp. INB0004363752
Trichoptera sp. INB0004337529
Macronema sp. INB0004363811
Eupodidae sp. BIOUG25167-C10
Eupodidae sp. BIOUG24235-D06
Theridion lawrencei BIOUG14307-A01
Theridion lawrencei BIOUG23030-E07
Theridion lawrencei BIOUG20063-G09
Culicoides tuttifrutti CIRAD ZA.PR.06
Hemerodromia sp. 1 EW-2018
Lepidoptera sp. BOLD AAH4846
Idaea eremiata BIOUG CAN CMAZ-0427
Idaea eremiata BIOUG CAN 09BBLEP-00137
Idaea obsoletaria TLMF Lep 06075
Idaea obsoletaria BC ZSM SS Lep 0117
Nyctelius sp. DHJ02
Nyctelius sp. DHJ01
Paronymus nevea VSR ABRI hsp 22
Toxidia doubledayi 88ADW
Toxidia doubledayi 11ANIC-08219
Gaurena fletcheri
Lepidoptera sp. BOLD AAC4671
Thyatira batis BC ZSM Lep 21399
Thyatira batis WLS110721.474
Thyatira batis isolate WLS110721.212
Stenotoxodera porioni MN090
Epitymbia alaudana 11ANIC-09061
0.05

Chapter 5: General Discussion and conclusion

This doctoral thesis reports on the floral malformation disease of *Syzygium cordatum* (waterberry), and represents a detailed follow-up of the work previously reported by Kvas et al., (2008). Waterberry Malformation Disease (WMD) is characterised by inflorescences with short, thickened, enlarged, or hypertrophied rachises. In addition, the panicles are greener, heavier with increased branching, which causes panicles to be compact and overcrowded by the unusually enlarged flowers (Schlosser, 1971; Hiffny et al., 1978). Such panicles have numerous flowers that remain unopened and are predominantly male and rarely bisexual. These flowers are sterile and do not bear fruit (Kumar et al., 1993; Ploetz, 1994; Marasas et al., 2006). Even though this has been observed in mangoes, it is equally similar in *S. cordatum.*

In general, floral malformation can be caused by biotic agents such as fungi, phytoplasmas, viruses, insects and nematodes (Bos, 1999; Takei et al., 2015; Bertaccini, 2007). Malformation may also result from various abiotic factors such as drought, temperature, nutrient stress and hormonal imbalance (Engin and Gokbayrak, 2010). In terms of symptoms, WMD is similar to Mango Malformation Disease (MMD). In MMD, various *Fusarium* species have been associated with and/or are known to cause this disease (Leslie and Summerell, 2006; Marasas et al., 2006; Steenkamp et al., 2000; Iqbal et al., 2006). *Fusarium*-associated floral malformation have also been reported in plant species such as *Tabebuia rosea* (rosy trumpet) (Montoya-Martínez et al., 2021), *Averrhoa bilimbi* (bilimbi fruit) (Bastos and Santos 2001), *Anacardium occidentale* (cashew) (Matos et al., 2016) and *Theobroma cacao* (cacao) (del Castillo et al., 2016; Vicente et al., 2012). Even though the aetiology of WMD is not clearly known, different *Fusarium* species have been recovered from malformed inflorescences suggesting a possible association with the disease (Mkandawire et al., 2022).

Another similarity between MMD and WMD is the presence of mites, which are generally known to exacerbate the symptoms of malformation diseases. Even though the mechanisms are not clear, mites have been reported to act as vectors by carrying fungal spores on their bodies from one point to the other, thereby increasing the dispersal of the fungus. In addition, the feeding activity of mites can create wound sites through which the fungus can gain access for colonising the plant tissue (Gamliel-Atinsky et al., 2009a). However, in WMD, the actual role of the eriophyoid mites recovered in this niche is not clear.

Against this background, the aims of the studies making up this thesis were to: (1) Characterise the diversity of *Fusarium* species obtained from healthy and malformed inflorescences in *S.*

cordatum in South Africa; (2) Describe novel *Fusarium* species associated with WMD; (3) Identify and compare mites species composition collected from healthy and malformed *S. cordatum* inflorescences in South Africa.

***Fusarium* spp. associated with *S. cordatum* (waterberry) malformation diseases**

This study recovered 118 *Fusarium* isolates residing in four different *Fusarium* species complexes i.e., *Fusarium fujikuroi* (FFSC), *Fusarium oxysporum*, *Fusarium incarnatum-equisetti* and *Fusarium chlamydosporum* species complexes. Of these complexes and as shown for MMD, *Fusarium* species in the FFSC are considered important associates of WMD. Using Koch's postulate a number of different FFSC species (e.g., *F. mangiferae*, *F. sterilihyphosum, F. mexicanum*, *F. pseudocircinatum, F. proliferatum* and *F. tupiense*) have been shown to cause MMD in different countries (Freeman et al., 1999; Otero-Colina et al., 2010; Freeman et al., 2014a; Haggag et al., 2010; Zhan et al., 2010; Lima et al., 2012). Other *Fusarium*-associated floral malformation with FFSC members have also been reported in *Swietenia macrophylla* (big-leaf mahogany malformation disease) (Santillán-Mendoza et al., 2018) and *Tabebuia rosea* (rosy trumpet) in Mexico (Montoya-Martínez et al., 2021). In terms of the species identified in the present study, members belonging to the FFSC were recovered only from malformed inflorescences as compared to other complexes that were detected on both healthy and malformed structures.

A striking similarity between WMD and MMD is the presence of the FFSC species *F. proliferatum* (*F. annulatum*) that also represented the dominant taxon isolated from malformed *S. cordatum* inflorescences. This fungus is a causal agent of MMD in Malaysia (Leslie, 1995), Egypt (Haggag et al., 2010) and China (Zhan et al., 2010). In addition, it has been reported to cause floral malformation disease in *Cocos nucifera* (coconut) and *Cannabis sativa* (Cannabis) (Punja, 2020). The fungus has a wide host range, and is present in different geographic regions (Leslie and Summerell, 2006) as both a pathogen and an endophyte (Dame et al., 2016). In *S. cordatum*, the role of *F. proliferatum* in causing disease is not clear. Pathogenicity studies combined with an in-depth understanding of the genetic differences between pathogenic and endophytic strains of *F. proliferatum* would need to be undertaken to answer this question. This is of importance as it has been observed previously that some fungal species reside within plant tissues as endophytes but can become pathogenic when the plant is stressed (Swart and

Wingfield, 1991; Slippers and Wingfield, 2007) such as would be the case due to mite infestation.

The second most dominant species of *Fusarium* isolated in this study, from WMD-affected tissue was *F. fujikuroi sensu stricto*. This fungus is the causal agent of bakanae disease in rice and it is widely distributed in Asia, Africa, and Europe (Ou, 1985; Prà et al., 2010). Despite being isolated from malformed mango tissues in Australia, its role in disease epidemiology on MMD is not known as inoculation trials (Koch's Postulates) did not show any causal relationship with the disease (Liew et al., 2016). The fact that this species has a wide distribution might explain its presence and association with *S. cordatum* malformed inflorescenses. However, considering that *F. fujikuori* is a pathogen of rice, suggests that there is need for research to understand its possible role in WMD.

The recovery of *Fusarium* strains representing *F. sacchari* and *F. verticilloides* was not unexpected. This is because *F. sacchari* is a well-known pathogen of sugarcane and it is responsible for pokkah boeng disease (Leslie and Summerell, 2006). The presence and impact of *F. sacchari* in South African sugarcane plantations has been well documented due to its role in causing stem rot and wilting of sugarcane, while also increasing the severity of infestation by the African sugar-cane borer (*Eldana saccharina*) (McFarlane et al., 2009; Govender et al., 2010). The isolation of *F. sacchari* from malformed inflorescences of *S. cordatum* could also be expected considering that the area from which most of the samples were collected is a sugarcane producing area. *Fusarium verticillioides* is a common pathogen of maize and also has a wide distribution predominantly in humid tropical regions, including South Africa (Boutigny et al., 2012; Marasas et al., 1984; Leslie and Summerell, 2006). It has previously been isolated from malformation mango flowers but was not shown to cause MMD (Liew et al., 2016). The presence of *F. verticillioides* in the area where the sampling for the present study was conducted can be attributed to the weather conditions that favour proliferation and spread of the fungus and the production of maize in the region which is the staple food of many people (Alberts et al., 2019).

Based on phylogenetic analysis of the gene encoding translation elongation factor (*TEF-1α*), at least five novel species were isolated from WMD symptomatic tissue. Two of these reside in the FFSC and three in the FIESC. During the time when the present study was conducted, the two species in the FFSC were described as *F. curcunicola* (Vermeulen et al., 2021) and *F. babinda* (Summerell et al., 1995, Leslie and Summerell, 2006). The former was originally

isolated from *Oryza sativa* seed from Nigeria and has also been found in South Africa from weevils and their associated galleries on *Amaranthus cruentus* (Vermeulen et al., 2021). The isolation of *F. curcunicola* on WMD symptoms is thus relevant due to its arthropod association, which resembles the important mite-*Fusarium* association observed for MMD. The second species, *F. babinda* was originally recovered from forest soils from Australia and was described by Summerell et al., (1995).

While the involvement of the above mentioned *Fusarium* species in WMD is not clear, their recovery in this niche could also be associated with the agricultural activities in the areas where sampling took place. However, the recovery of these species might also point towards possible host jumps from agricultural crops to indigenous plants such as *S. cordatum* trees. Malformation diseases caused by *Fusarium* species that have undergone possible host jumps have previously been suggested for the involvement of *F. mexicanum* in MMD (Montoya-Martínez et al., 2021) and *F. mexicanum* and *F. pseudocircinatum* causing malformation disease in big-leaf mahogany (Santillán-Mendoza et al., 2018). Host jumps are not rare phenomena in the emergence of fungal pathogens, and they are more likely to occur when native and agricultural systems are in close proximity to one another (Burgess and Wingfield 2002; Stenlid et al.2011), as might have been the case in the current study. Host range studies would provide a greater understanding of the roles played by such alternative hosts in the establishment of a given disease, including WMD (Dinoor, 1974).

Mites associated with Waterberry (*Syzygium cordatum*) malformation diseases

The striking similarity between WMD and MMD also includes the association of Eriophyoid mites with these diseases. In the case of MMD, *Aceria mangiferae* was considered a putative causal agent as eriophyoid mites are known to cause bud proliferation, “witches’ broom”, and gall symptoms of inflorescences in other plants (Westphal et al., 1996). Eriophyoid mites cause these abnormalities through salivary secretions containing lipophobic substances that elicit different biochemical changes, ultimately causing morphological changes (de Lillo and Mofreda, 2004).

In mite-floral malformation interactions, *Aculus anthobius* is known to cause floral malformations *Galium* species (lady's bedstraw), result in greening of blossoms with the whole inflorescence appearing to be deformed and compact with green and leaflike flowers with

shortened stalks (Mifsud et al., 2006). Another member of this mite genus, *A. minutus* is also known to induce galling in *Asperula cynanchica* (squinancywort), resulting in floral malformations characterised by greenish and leaf-like flowers with short stalks on a shortened and compact inflorescence (Mifsud et al., 2006). Some other well-known mites that cause flower abnormalities include *Aceria lantana* in *Lantana camara* (common lantana) (Craemer and Neser 1990; Urban et al., 2003), *Eriophyes prosopidis* in *Prosopis cineraria* (scree bean) (Bhatnagar et al., 2019), *Eriophyes fraxinivus* (*Aceria fraxinivora*) in *Fraxinus excelsior* (European ash) (Jeppson et al., 1975) and *Aceria rhodiolae* in *R. rosea* (Roivainen, 1950). In the present study, it is thus likely that the eriophyid mite *Eriophyes afroensis* or a closely related cryptic species is involved in WMD as these mites were found only on the bracts of all malformed inflorescences. However, to resolve this question equivocally, the Leaches rules of proof (Leach, 1940) and pathogenicity studies with the associated fungi would be needed to understand their possible role in WMD.

The present study recorded a significant number of predatory mites from the species *Agistemus collyerae* on WMD-affected inflorescences. Despite their significant numbers, the results led us to hypothesise that they do not have any epidemiological role in the malformation disease. This is due to the fact that mites in this family are predatory in nature and they feed on small arthropods such as spider mites and other mites, as well as on small insects such as crawlers of scale insects. For this reason, they present a potential opportunity to be explored as biological control agent in management and control of eriophyoid mites. For example, *Agistemus aimogastaensis* is known to be a predator of eriophyid mites such as *Aceria oleae* and *Oxycenus maxwelli* (Leiva et al. 2013).

Mites-*Fusarium* Species association in Waterberry (*Syzygium cordatum*) malformation diseases

Research conducted as part of this thesis led to the recovery of *Fusarium* species in both malformed and healthy inflorescences on *S. cordatum.* However, *Fusarium* species in the FFSC were found only in malformed inflorescences. Considering that species within the FFSC have been linked to different types of floral malformations, with Koch's postulates in some cases confirming their causal role, it is reasonable to at least hypothesis that they could be responsible for causing WMD. However, their presence within the malformed tissues represents an

interesting epidemiological and ecological situation that deserves to be to be studied in greater detail than was possible during the time allocated to the present undertaking.
Broadly, the results of the studies conducted lead to the hypothesis that gall formation results in morphological and physiological changes that provide a conducive environment for fungal invasion and colonisation. For instance, gall formation creates a micro-environment which increases nutrients important for fungal and other pathogen invasions. Several past studies have shown increased concentration of nutrients (e.g., nitrogen and potassium) in the galled area, which could be advantageous for the proliferation of *Fusarium* (Stone and Schönrogge, 2003; Swanepoel, 2016). In addition, there are reduced chemical defence mechanisms in the galled areas due to a reduction of phenolic compounds during gall formation (Stone and Schönrogge, 2003), which could have also made those parts susceptible to fungal colonisation.

Studies on other arthropod-*Fusarium* pathosystems have shown to favour proliferation of *Fusarium* species. For example, advanced infestation by English grain aphids, *Sitobion avenae* and *Frankliniella occidentalis* increases colonisation of *F. graminearum* (Drakulic et al., 2015) and *F. verticillioides* (Parsons and Munkvold, 2010, 2012) as infection of these *Fusarium* species were greatly assisted by arthropod activity. This is due to the creation of wound sites which makes fungal penetration and colonisation easy. This is also as a result of the suppressed plant defence mechanism by altering the salicylic acid (SA) pathway (Makandar et al., 2012) and tricking the host into disabling defence responses (Thompson and Goggin, 2006; Walling, 2008), subsequently leaving the plant susceptible to secondary attack by the fungus. Under such circumstances, it could be be hypothesised that *Fusarium* species found associated with WMD came in after the infestation of malformation of floral parts.

Overall, there is a higher likelihood that WMD is caused by mite infestation for the following reasons: (1) the underside of all the malformed floral bracts had mites, (2) no mites were observed from healthy inflorescences, and (3) *Fusarium* species were not isolated from all malformed floral parts. However, the interaction between mites and *Fusarium* species in disease development and particularly malformation is likely as this interaction is known to enhance disease severity in other similar situations. In disease development, mites can either vector the pathogen or create wounds to allow pathogen penetration (Agrios 1980; Hatcher and Paul 2001). For example, in MMD, despite the disease being caused by *Fusarium* species, the presence of *Aceria mangiferae* is believed to increase disease severity through its role in vectoring the fungal spores and aiding in conidia penetration and colonisation (Gamliel-

Atinsky et al., 2009b). Not only has this vectoring been observed in fungal pathogens, but it is also prevalent in mite-viral interactions (Slykhuis, 1955, Seifers et al., 2009 and Stephan et al., 2008) and mite-phytoplasma (Wieczorek et al., 2003). While these references to mite-pathogen interactions may not share the same symptoms as those observed in WMD, there are some marked similarities. Future research on eriophyoid related plant disorders, especially those without typical symptoms should be considered and additional pathogens as causal agents should be evaluated.

Conclusions and directions for future research

This study reports on inflorescence malformation disease of *Syzygium cordatum*, which commonly occurs in areas where the plant occurs naturally and in other areas of South Africa where it is grown as an ornamental plant. The disease symptoms are similar to those of MMD and understanding its dynamics on *S. cordatum* is important because of the plant's significance in the South African landscape. The results of this study have provided some insights into possible cause in of WMD.

The investigations presented in this thesis were not able to ascertain the specific cause of WMD. However, based on the isolation of mites only from malformed diseases, we speculate that their presence is essential for the development of the disease. In addition, the interaction of mites with *Fusarium* species, especially those within FFSC, during disease development seem likely to be involved in the development of WMD. This is an area that requires consideration in future studies as it will provide insights into WMD aetiology and the epidemiology of the agents associated with the disease.

The absence of pathogenicity trials with the organisms associated with WMD remains a limitation of the research presented in this thesis. Such trials were attempted many times but these failed. For example, seeds of *S. cordatum* were planted on three different occasions, but most of the seedlings died within three years, and those that survived did not flower. Although the possibility of conducting the pathogenicity trials *in situ* were considered, it was regarded as a biosecurity issue as it would have involved introducing biological organisms for which the epidemiological implications were not fully understood. In addition, the flowering *S. cordatum* plants that could have been used in this study were already showing signs of WMD. It is however hoped that future research including pathogenicity trials with the *Fusarium* spp. and

trials including Leaches Rules of Proof (Leach, 1940) for the mites will be possible. Only then will it be possible to fully understand the cause of WMD.

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