Investigation of malformation symptoms in (*Searsia lancea*)

Juan Swanepoel

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Supervisors:
Dr. M. Gryzenhout
Ms. M. Westcott

DECLARATION

(i) I, Juan Swanepoel, declare that the Master's Degree research dissertation that I herewith submit for the Master's Degree qualification Botany and the University of the Free State is my independent work, and that I have not previously submitted it for a qualification at another institution of higher education.

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Juan Swanepoel

07 April 2016

Date

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"Veni vidi amavi"

"We came, we saw, we loved."

SUMMARY

The common karee (*Searsia lancea*, Anacardiaceae) is a common, widely distributed tree in South Africa. Popular as a garden and street ornament, its fruit and foliage serve as a source of food for many animals and humans. It also has applications in the leather tanning industry and phytoremediation. Disease symptoms on *S. lancea* were reported that resemble malformations of the closely related mango (Mangifera *indica*, Anacardiaceae). This disease was named karee malformation disease (KMD). Formal investigation was conducted to determine whether malformation symptoms on the two separate genera of the Anacardiaceae family share a causal agent, namely *Fusarium* spp. A pilot study and review of literature identified other relevant aspects worthy of study including insect associations, and differences in phytohormone and nutrient concentrations between healthy and affected trees. It was determined that *Fusarium* spp., which cause malformation of *M. indica*, does not cause malformation of *S. lancea*. It is also unlikely that the dominant fungal group, *Alternaria alternata*, causes *S. lancea* malformations. However, this study identified interesting fungal and insect associations with healthy and malformed tissues of *S. lancea*. It is possible that the dominant insect group, namely Psyllidae, causes malformations of *S. lancea* directly, or indirectly by acting as a vector of another pathogen. Lower concentrations of the phytohormones gibberelic acid and jasmonic acid, and higher concentrations of salicylic acid were noted in malformed compared to healthy tissues of *S. lancea*. However, only the differences for salicylic acid were significant. Higher concentrations of the mineral nutrients nitrogen and potassium were noted for malformed tissues, while the phosphorus concentration was the same for both conditions of *S. lancea*.

Key terms: *Searsia lancea*, *Rhus lancea*, Anacardiaceae, plant malformations, *Fusarium*, *Alternaria*, Psyllidae, salicylic acid, gibberellic acid, jasmonic acid, endophytes, nitrogen, potassium, phosphorus, common karee, karee malformation disease

OPSOMMING

Die rooi karee (*Searsia lancea*, Anacardiaceae) is ‘n algemene, wyd verspreide boom in Suid Afrika. Dit is gewild as ‘n tuin en straat versiering, en die vrugte en loof dien as voedselbron vir verskeie diere asook mense. Dit het ook toepassings in die leerlooiery industrie en plant-remediëring. Siekte simptome op *S. lancea* was aangemeld en lyk baie soos misvormings van die na verwante mango (*Mangifera indica*, Anacardiaceae). Die siekte is kareemisvorming (KMD) genoem. ‘n Formele ondersoek was geloots om vas te stel of misvorming simptome op hierdie verskillende genera van die Anacardiaceae familie ‘n gemene oorsaak deel, naamlik *Fusarium* spp. ‘n Proefsteek en resensie van literatuur het ander relevante aspekte identifiseer wat die moeite werd is om te ondersoek, insluitend insek assosiasies en verskille in plant hormoon- en voedingstof konsentrasies tussen gesonde en misvormde bome. Dit was bevind dat *Fusarium* spp., wat misvormings van *M. indica* veroorsaak, nie misvormings van *S. lancea* veroorsaak nie. Dit is ook onwaarskynlik dat die dominante swam groep, *Alternaria alternata*, misvormings van *S. lancea* veroorsaak. Nieteenstaande het hierdie studie interessante swam en insek assosiasies met gesonde en misvormde weefsel van *S. lancea* identifiseer. Dit is moontlik dat die dominate insek groep, naamlik Psyllidae, misvormings van *S. lancea* direk, of indirek as ‘n vektor van ‘n ander patogeen, kan veroorsaak. Laer konsentrasies van plant hormone gibberelliensuur en jasmoonsuur, en hoër konsentrasies van salisiensuur was opgemerk in misvormde weefsel in vergelyking met gesonde weefsel van *S. lancea*. Slegs die verskille in salisiensuur was egter beduidend. Hoër konsentrasies van mineraal voedingstowwe stikstof en kalium was opgemerk in misvormde weefsel, terwyl fosfaatkonsentrasies dieselfde was vir beide kondisies van *S. lancea*.

Belangrike terme: *Searsia lancea*, *Rhus lancea*, Anacardiaceae, plant misvorming, *Fusarium*, *Alternaria*, Psyllidae, salisiensuur, gibberelliensuur, jasmoonsuur, endofiete, stikstof, kalium, fosfaat, rooi karee, kareemisvorming

ABBREVIATIONS

AAC: 1-aminocyclopropane-1-carboxylate deaminase
AAS: Atomic absorption spectrum
ABA: Abscisic acid
ARC: Agricultural Research Council
ASGM: Adaptive significance of gall morphology
B: Boron
BAP: N^6-benzyl adenine
BR: Brassinosteroid/brassinolide
$C_2H_2O_2$ or CH_3COOH: Acetic acid
C_2H_3N: Acetonitrile
Ca: Calcium
CAD: Charged aerosol detection
CC: Critical concentration
Cd: Cadmium
CE: Collision energy
CH_2O_2: Formic acid
CiLV: Citrus leprosis virus
CK: Cytokinin
Cl: Chlorine
Co: Cobalt
CPS: Counts per second
Cr: Chromium
CsCl: Caesium chloride
CTAB: Cetyl trimethylammonium bromide
CTHB: Centre of excellence in Tree Health Biotechnology
Cu: Copper
diHZ: Dihydrozeatin
DNA: Deoxyribonucleic acid
DP: Declustering potential
EDTA: Ethylenediamenetetraacetic acid
FABI: Forestry and Agricultural Biotechnology Institute
FBSC: *Fusarium brachygibbosum* species complex
FCCS: *Fusarium chlamydosporum* species complex
FDA: Food and Drug Assurance laboratory
Fe: Iron
FFSC: *Fusarium fujikuroi* species complex
FIESC: *Fusarium incarnatum-equiseti* species complex
FOSC: *Fusarium oxysporum* species complex
FSSC: *Fusarium solani* species complex
FTSC: *Fusarium tricinctum* species complex
$GA_{\#}$: Gibberellic acid
GC-MS: Gas chromatography mass spectrometry
GII: Gall inducing insect
H: Hydrogen
H_2O: Water
HCl: Hydrochloric acid
DST-NRF: Department of Science and Technology National Research Foundation
HR: Hypersensitive response
Hz: Hertz
IAA: Indole-3-acetic acid
IBA: Indole-3-butyric acid
iP: N^6-(Δ^2-isopentenyl) adenine
ISR: Induced systemic resistance
JA: Jasmonic acid
JWB: Jujube witches' broom
K: Potassium
KMD: Karee malformation disease
$La(NO_3)_3.6H_2O$: lanthanum nitrate solution
MEGA: Molecular Evolutionary Genetics Analysis
MeOH: Methanol
Mg: Magnesium
MLST: Multilocus sequence typing
MMD: Mango malformation disease
Mn: Manganese
MRM: Multiple reaction monitoring
MSP: Morphological species/morpho-species
N: Nitrogen
NaCl:Sodium chloride
Ni: Nickel
P: Phosphorus
Pb: Lead
PCR: Polymerase chain reaction
PDA: Potato dextrose agar
Psi: Pounds per square inch
RNA: Ribonucleic acid
RPM: Revolutions per minute
RSNV: Rice stripe necrosis virus
S: Sulphur
SA: Salicylic acid
SANBI: South African National Biodiversity Institute
SAR: Systemic acquired resistance
SDS: Sodium dodecyl sulphate
SEVAG: Chloroform: isoamylalcohol, 24:1, v/v
SNA: Synthetic nutrient-poor agar
SPE: Solid phase extraction
$SrCl_2$: Strontium chloride
TEF: Translation elongation factor
TSWV: Tomato spotted wilt virus
UFS: University of the Free State
ULCV: Urdbean leaf crinkle virus
UV: Ultraviolet
Z: Zeatin
Zn: Zinc
UPLC-MS/MS: Ultra-performance liquid chromatography tandem mass spectrometry

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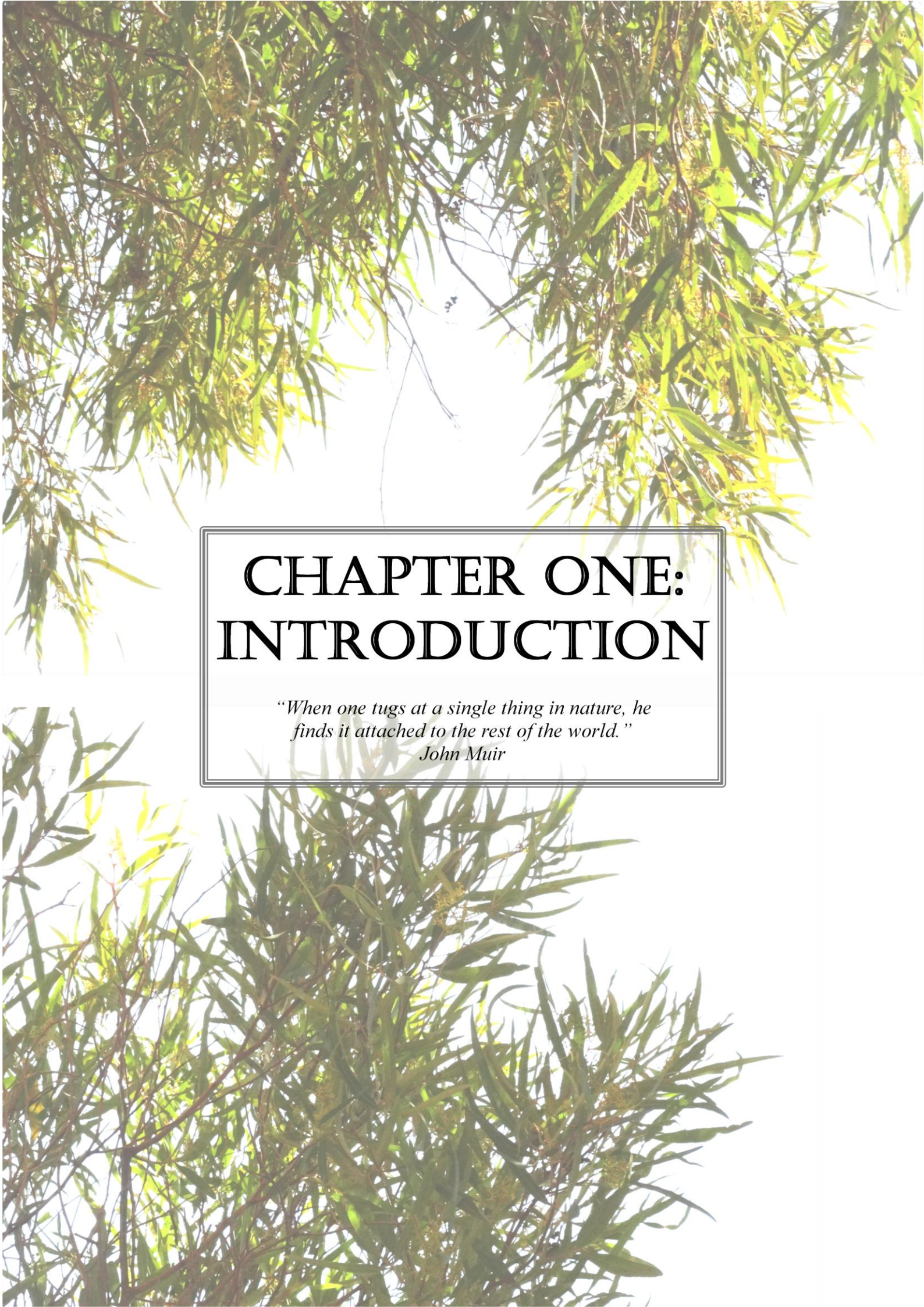

CHAPTER ONE: INTRODUCTION

"When one tugs at a single thing in nature, he finds it attached to the rest of the world."
John Muir

1.1. GENERAL INTRODUCTION

Searsia lancea (Figure 1.1.), previously named *Rhus lancea* (Moffet, 2007), is one of the best known and widely distributed tree species in South Africa (Coates-Palgrave *et al*. 2000). It is known by several common names including common karee, groot karee, hoenderspoor karee, krieboom, red karee, river karee, and mokalabata (Smith, 1966). It is found along river and stream banks, drainage lines, termite mounds and open woodlands, and it is a popular garden feature (Van Wyk, 2001; Coates-Palgrave, 2002) (Figure 1.2.).

The tree is described as evergreen, hardy, frost tolerant, and medium sized (≥ 9 m) with a round crown and trailing branches (Van Wyk, 2001; Venter and Venter, 2009). The bark of *S. lancea* (Figure 1.3.a) is smooth with the trunk and older branches dark brown or dark grey, and younger branchlets are reddish brown in colour (Van Wyk, 2001; Coates-Palgrave, 2002). The leaves are trifoliate, which is characteristic to the Anacardiaceae (Koekemoer *et al*. 2013), and borne on petioles up to 5 cm long (Figure 1.3.b). Leaves are dark olive-green above and a pale yellow-green below with the apex narrowly tapering, base tapering and margins that are usually entire or sometimes slightly serrated (Van Wyk, 2001; Coates-Palgrave, 2002). Narrow, lanceolate leaflets are hairless, leathery, drooping and sometimes resinous exudates are present. Lateral and net venation of leaves is visible from above. Terminal leaflets are usually 2.5 cm – 12 cm x 0.5 cm – 1.2 cm, and lateral leaflets only slightly shorter. Small (3 mm diameter), sweetly scented, yellow-green flowers are borne on clustered sprays (± 9 cm long) at the end of branchlets (Figure 1.3.c). Male and female flowers are borne on different trees during autumn and winter (April/June – September). During spring and early summer (September – January) *S. lancea* bears dull yellow-brown fruit (5 mm diameter) that are spherical and appear slightly flattened (Figure 1.3.d). The wood is an attractive reddish-brown colour, hard, tough, close-grained and heavy with a sweet spicy scent. *Searsia lancea* can typically be distinguished from other *Searsia* spp. based on the lanceolate shape of the leaves and red hues of young branchlets (Figure 1.3.e).

Searsia lancea is an important plant in any habitat and has various uses for humans. The trunk of *S. lancea* is often twisted and contorted, making it unsuitable for timber or furniture production despite the appealing colour, strength and aroma of the wood.

It is, however, often used to make termite-proof fence posts and implement handles, and bow grips from the branches (Coates-Palgrave, 2002; Van Wyk and Gericke, 2007). The bark is used in the leather tanning industry to produce a brown dye. The fruit can be eaten fresh by humans, or soaked in milk or sour milk after rubbing them between the hands to remove the skin. A honey beer or mead can be fashioned by pounding the fruit in water and leaving the mixture to ferment (Cambray, 2005; Van Wyk and Gericke, 2007). This is thought to be the origin of the common name 'karee', derived from the Khoi word 'karri' which means mead. The tree is easy to propagate from seed and cuttings (Coates-Palgrave *et al*. 2000; Van Wyk and Van Wyk, 1997; Coates-Palgrave, 2002; Venter and Venter, 2009) making it a popular choice in gardens and as a street tree. This species is also a suitable candidate for phytoremediation and reforestation efforts of platinum and gold mine tailings when supplemented with certain ameliorants (Lange *et al*. 2012; Olowoyo *et al*. 2013).

The foliage of *S. lancea* is often browsed by game including kudu (*Tragelaphus strepsiceros*), roan (*Hippotragus equines*), sable (*H. niger*), and elephant (*Loxodonta africana*) (Martin, 2003; Woolley *et al*. 2011). Similarly, foliage is used in fodder for livestock such as cows and goats (Van Wyk and Gericke, 2007). The presence of tannins, however, taints the milk of livestock if consumed in large amounts. Birds such as bulbuls (Pycnonotidae) and guinea fowl (Numididae), and vervet monkeys (*Chlorocebus aethiops*) often eat the fruits (McDougall, 2010; Forshaw, 2011).

Despite being a common feature of the natural and urban landscape, *S. lancea* is surprisingly under-represented in research literature. Essential oils derived from *S. lancea* have been proven to have antioxidant and –microbial activity with significant activity against the bacteria *Escherichia coli* and *Clostridium perfringens*, and the fungus *Aspergillus flavus* (Gundidza *et al*. 2008; Mulaudzi *et al*. 2012). Other publications including these and environmental impact assessments only note the presence of *S. lancea* in the particular study area (Oliver, 2007; Erasmus, 2008). The scarcity of research on *S. lancea* is also true for studies on the diseases and pests associated with this common tree. The only disease report on the species is of leaf spot caused by a fungal pathogen *Muribasidiospora indica* (Crous *et al*. 2000; Crous *et al*. 2003).

Potential threats to such a ubiquitous natural resource as represented by *S. lancea*, of which the potential biological and economic benefits are poorly studied, must be identified and neutralized, if necessary. If this is not done *S. lancea* is at risk of disappearing from urban and especially natural landscapes. Effects to this end have been noted for other dominant tree species in response to disease. For example, native American chestnut (*Castanea dentata*) was completely annihilated from North America as a result of the introduced chestnut blight disease caused by the fungus *Cryphonectria parasitica* (Anagnostakis, 1987), and American elm (*Ulmus americana*) populations are in rapid decline as a result of Dutch elm disease caused by the fungus *Ophiostoma ulmi* (Agrios, 2005). Some plant pathogens have also been found able to infect plants related to their known host. An example from South Africa is the discovery of the *Eucalyptus* fungal pathogen *Chrysoporthe austroafricana* on native *Syzygium* spp. (Myrtales) (Heath *et al*. 2007) where it was previously better known to cause severe cankers on non-native *Eucalyptus* and *Tibouchina* spp. (Myrtales).

The Anacardiaceae is generally characterized by resinous bark and fruit; small unisexual green-yellow to white flowers; superior ovaries; and fruit often laterally flattened, borne on drupes (Koekemoer *et al*. 2013). Of the 60 genera and 600 species of Anacardiaceae worldwide, 14 genera and 133 species are indigenous to South Africa. Many Anacardiaceae species are widely cultivated as popular garden ornaments and as shade trees (Van Wyk, 2001; Coates-Palgrave, 2002). This family also contains species valued in the timber (*Astronium* spp., *Myracrodruon* spp. and *Schinus* spp.) and leather tanning industry (*Harpephyllum caffram*, *Heeria argentea* and *Searsia lancea*), as well as species important for food and cooking (*Anacardium occidentale*, *Mangifera indica*, *Pistacia vera*, *Schinus molle*, *Sclerocarya birrea* and *Searsia lancea*) (Van Wyk and Gericke, 2007; Koekemoer *et al*. 2013; Moyo and Van Staden, 2013). Of these mango (*Mangifera indica*), marula (*Sclerocarya birrea*), cashew (*Anacardium occidentale*) and pistachio (*Pistacia vera*) are cultivated or exploited in South Africa (Moyo and Van Staden, 2013).

Important diseases listed for the cultivation of *M. indica* in South Africa include anthracnose, powdery mildew, bacterial black spot and malformations (Anonymous, 2003). Cultivation of *A. occidentale* in South Africa is not considered as threatened by disease as is *M. indica*. However, anthracnose of *A. occidentale* in Brazil is an

important disease (Freire *et al*. 2002). Diseases for *S. birrea* are not well known, but associated pests include marula fruit fly (*Certitis cosyra*), red marula caterpillar (*Mussidia nigrivenella*) and various beetle species (Anonymous, 2010). Pistachio (*P. vera*) is a relatively new crop in South Africa. Similar to *S. birrea* little is known of the diseases effecting *P. vera* cultivation in South Africa (Haddad and Dippenaar-Schoeman, 2004), with only two insect species namely the woolly chafer (*Sparrmannia flava*) and stinkbug (*Atelocera raptoria*) known pests that respectively cause defoliation and leaf damage (Haddad and Dippenaar-Schoeman, 2004). Beyond South Africa disease of *P. vera* include panicle and shoot blight (Michailides and Morgan, 1993), and witches' broom disease caused by a phytoplasma (cell wall-less bacteria) (Zamharir and Mirabolfathi, 2011).

Disease symptoms similar to malformations have been observed on the common karee (*Searsia lancea*) in the past, but have not been formally investigated up to date. These malformations typically occur in inflorescences and leaves. They bear a close resemblance to those associated with malformation disease of *M. indica* (Krishnan *et al*. 2009). Since *M. indica* and *S. lancea* are classified in the Anacardiaceae and both occur in South Africa, it is reasonable to consider whether the malformations on these two tree species could be caused by a similar causal agent, namely *Fusarium* spp. (Marasas *et al*. 2006). This is because it is known that some pathogens are able to co-infect other hosts, or have the ability to shift their host range when new, compatible plants occur closely to their natural host (Slippers *et al*. 2005)

Due to the vast economic impact that mango malformation has on *M. indica* it is a disease of great importance. Mango (*M. indica*) malformation has been reported in Bangladesh, Brazil, Cuba, Egypt, Florida, India, Israel, Malaysia, Mexico, Pakistan and South Africa (Marasas *et al*. 2006; Krishnan *et al*. 2009). The disease has crippling economic effects in India, which according to the United Nations Food and Agricultural Organization 2002 yearbook produces 1564200 metric tonnes of mango, of which 0.3% is exported. South Africa produces 28000 metric tonnes of mango annually, of which 32.5% is exported. Mango malformation disease is characterized by floral and vegetative malformations caused by certain *Fusarium* spp. found in the *Fusarium fujikuroi* species complex (Marasas *et al*. 2006). These are described as an increase in flower number and size, increased number of male flowers, sterility

and abortions of hermaphrodite flowers and generally shortened, branched and thickened inflorescences for floral malformations (Marasas *et al*. 2006). Vegetative malformations are described as bunched, small, scaly leaves and loss of apical dominance which results in a witches' broom-like appearance when vegetative buds develop (Krishnan *et al*. 2009). Management of *M. indica* malformation disease includes preventative methods such as establishing plantations and nurseries away from infected orchards and not using scions from infect orchards for propagation. When infection does occur treatment methods include removing and burning infected tissue from the tree, and integrative pruning and chemical (aracacide and fungicide) treatments (Noriega-Cantú *et al*. 1999; Marasas *et al*. 2006).

Due to the impact of *M. indica* malformation disease, it is important to study the malformation disease of *S. lancea* to ascertain its threat to this keystone native tree and whether it is caused by the same causal agents. Although *S. lancea* is not an export species it is very popular in the South African ornamental garden industry, which may suffer some economic impact. However, what is more concerning is that one of the symptoms of *M. indica* malformation is flower sterility (Marasas *et al*. 2006; Krishnan *et al*. 2009), which may have a significant ecological impact for *S. lancea* (Guimarães *et al*. 2014). The aim of this study was to confirm the hypothesis that the *Fusarium* spp. that cause malformation of *M. indica* in South Africa are also associated with malformation of *S. lancea*. This hypothesis was tested by isolations for *Fusarium* spp. from both healthy and malformed tissues of *S. lancea* to determine if the pathogenic species occur on *S. lancea*. Additional aims were to establish preliminary baseline data of fungal and insect associations with *S. lancea* malformations, since it could be possible that the malformations are caused by other fungal species, or other types of causal agents such as insects. Studies on the physiology of malformation development were initiated by determining differences in concentrations of certain phytohormones and mineral nutrients of both healthy and malformed tissues of *S. lancea*.

During our studies we identified the confusing use of jargon and terminology prevalent to malformation disease within and across different disciplines. These were dissected and re-structured to promote consistent, uniform application in a review of relevant literature. Such a review will be useful for plant ecology, phytopathology,

plant physiology and entomology and research relevant to malformation diseases in general.

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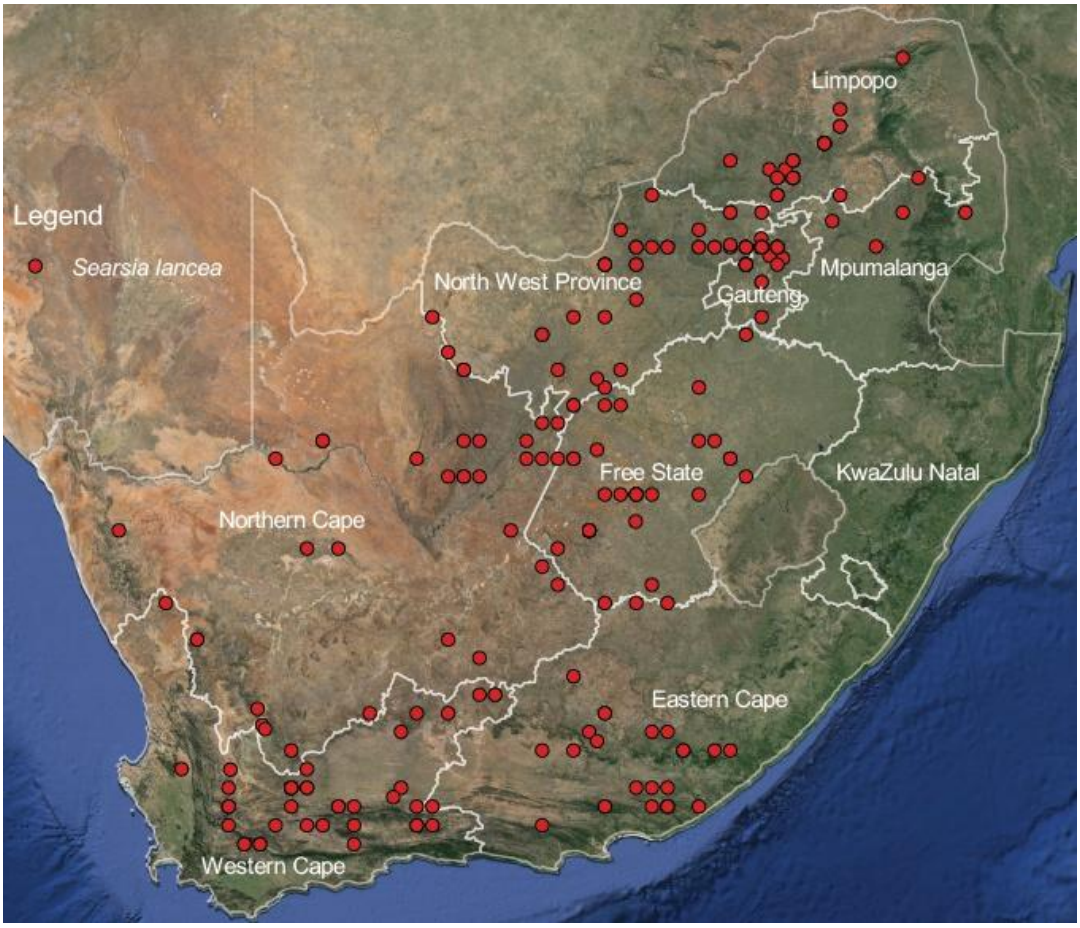
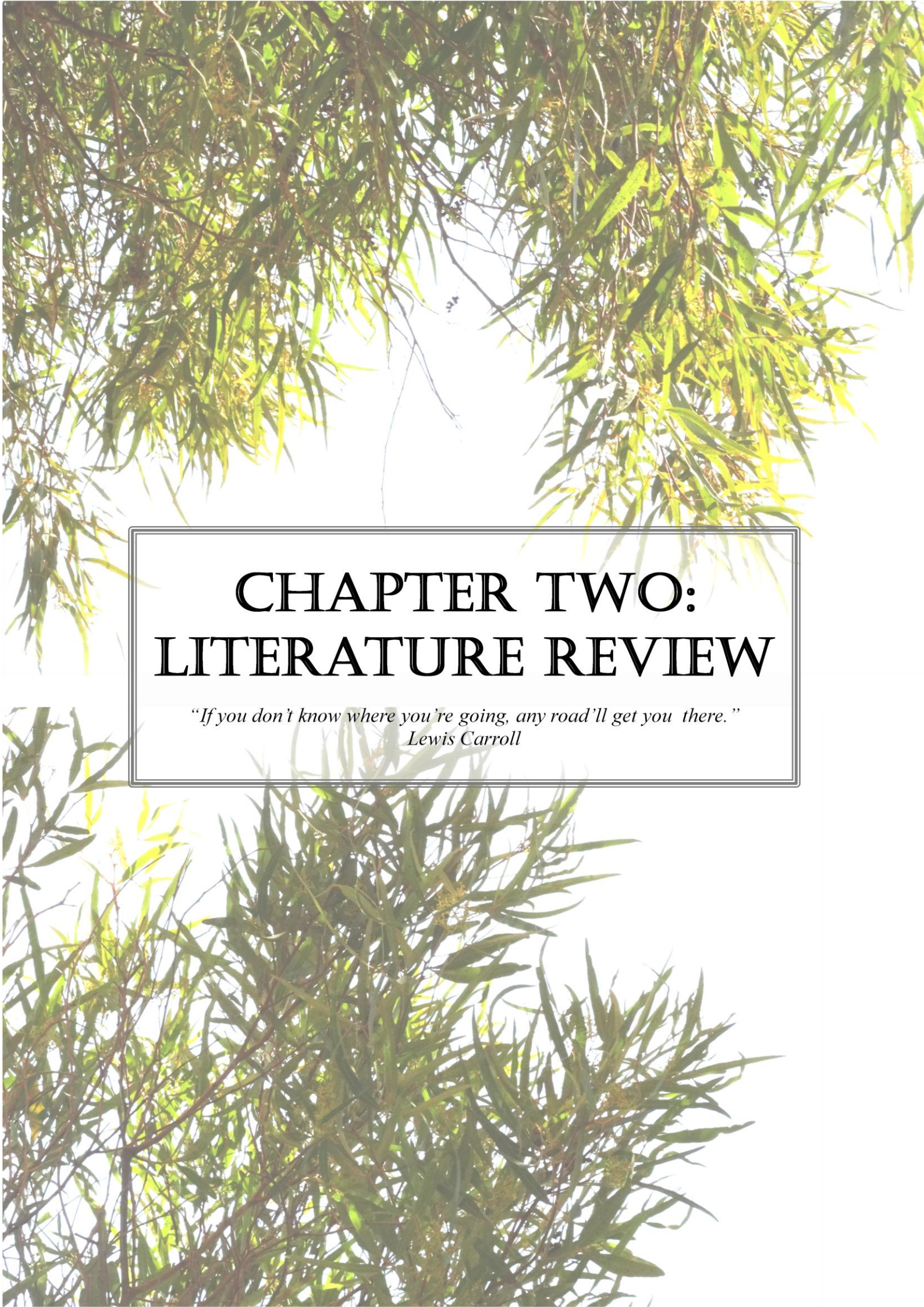


Figure 1.1. Distribution range of the common karee (*Searsia lancea*) according to collection data of the South African National Biodiversity Institute (SANBI, Pretoria, Gauteng Province).

Figure 1.2. Tree shapes of the common karee tree (*Searsia lancea*).

Figure 1.3. Characteristic **a)** bark and wood, **b)** leaves, **c)** flowers, **d)** fruit and **e)** twisted/contorted growth of the common karee (*Searsia lancea*).



CHAPTER TWO: LITERATURE REVIEW

"If you don't know where you're going, any road'll get you there."
Lewis Carroll

2.1. INTRODUCTION

When considering plant malformation diseases they are generally described by scientists as a type of disease where plant tissues, such as leaves, stems and inflorescences typically become deformed and non-functional. The best studied case of plant malformation is that of mango (*Mangifera indica*) (Marasas *et al*. 2006). Floral shoots of *M. indica* that suffer malformation fail to set fruit, resulting in catastrophic losses for this fruit crop. Up to 62 million tonnes, which represents an average of 50% of crops, are lost annually worldwide (Singh and Singh, 1998; Noeriga-Cantú *et al*. 1999; Sarris, 2003; Nafees *et al*. 2010). What was once also considered malformation of *Protea* spp. causes similar economic losses in Australia, California, Hawaii, Israel and South Africa where *Protea* spp. are cultivated as ornamentals by reducing flower exports (Cutting, 1991).

In addition to crop and economic losses, there is reason for concern over the natural survival of affected plant species. Sterile flowers are a symptom of water berry (*Syzygium cordatum*) malformation, a tree native and common to South Africa (Kvas *et al*. 2008). Changes noted in flowers of *M. indica* can also affect reproduction, and thus potentially threaten the survival of the species if not investigated and monitored (Marasas *et al*. 2006; Krishnan *et al*. 2009). It is reasonable to assume that if no fruit is produced to aid in seed dispersal, no flowers are produced in which seeds can develop, or if flowers are sterile the reproductive success and ultimate survival of a plant species can be questioned (Guimarães *et al*. 2014). Once the natural balance of such a disease occurrence is thus disturbed towards a higher incidence of malformation, or should the disease be introduced to areas where there is no resistance, it can have catastrophic implications for the natural occurrence of a species that will also affect the balance of the greater ecosystem (Anagnostakis, 1987; Agrios, 2005).

Plant malformation is not an uncommon or strange reference in natural science, but it is ill defined. For instance the designation of malformation on *Protea* spp. has been replaced by witches' broom of *Protea* spp., a change that highlights problems in defining what exactly the term 'malformation' means (Wieczorek and Wright, 2003). Literature on plant malformation is generally scarce, and that which is available is concerned with aspects of causality, spread, and physiology without very much

description or distinction. This is especially so due to similarities with other types of disease symptoms which result from aberrant growth such as abnormalities, galls or witches' broom. As a result the word 'malformation' is used ambiguously, making review of the concept very difficult.

Concerns on crop and economic losses, diseases that cause deformations in plants, their impact on plant species reproduction and survival, and the unclear distinction between other similar concepts, are significant reasons to promote further research and critical review. This will promote effective control and management regimes. The aim of this review is to attempt to clarify usage of various terms indicating deformed plant organs, and to investigate possible patterns based on causal organism, evolutionary roles, physiological triggers and nutritional changes.

2.2. DISCUSSION

2.2.1. Defining the terms associated with changes in plant shape

Various types of symptoms refer to plant organs that undergo changes in shape contrary to the norm. These are simple types of changes in plant tissue that result in different looking structures that can either be simple or complex. At the cellular level plant cells can divide or grow abnormally. This results in overgrowth due to increased cell division (hyperplasia) or enlarged cells (hypertrophy), or "under-growth" when tissues or organs fail to develop (hypoplasia) or start to degenerate (atrophy) (Table 1). Plant organs can also develop abnormally when cells produce incorrect components, e.g. bracteody, carpellody, petalody, phyllody, sepalody, and staminody (Table 1).

Malformations, abnormalities, witches' brooms and galls are terms that refer to large or complex types of abnormal looking growths. The term 'malformation' lacks clear definition from literature, but can be considered from etymology of the word simply as the 'bad or abnormal formation of cells, organs or tissues of an organism that alter its normal appearance and/or function' (Flanigan *et al*. 2012). Similarly there is no clear definition from literature for the term 'abnormality', but use of this term may have originated from general observations to describe all the concepts that "...*give plants an abnormal look*" (Hernández and Hennen, 2003). Agrios (2005) defines

galls as the hyperplasia and/or hypertrophy of plant stems, leaves, flowers or roots in response to certain microbial pathogens or insect pests. Witches' broom is generally defined as the dense clustering of branches in woody plants, resulting from proliferated growth caused by hyperplasia and/or hypertrophy (Agrios, 2005).

From literature it appears that the term 'abnormality' is used as a general description, whereas the terms 'gall' and 'witches' broom' appear to refer to more distinct morphologies. However, in many publications use of these terms remain ambiguous. The term 'malformation' is poorly defined, sometimes accompanied by varying descriptions to further confuse its definition (Cook, 1923; Quoirin *et al*. 2004; Krishnan *et al*. 2009; Raj *et al*. 2009). These descriptions and latter definitions also fail to clearly distinguish plant malformations from abnormalities, witches' broom, and galls. To exacerbate this problem, these terms (abnormalities, galls, malformations, witches' broom) are used loosely and interchangeably without explaining context. This could be because different scientific communities (e.g. plant pathologists, botanists and entomologists) have different understandings of these concepts.

This may not be obvious, but a critical study on past literature illustrates the confusing use of these terms. For example a very old publication on cotton (*Gossypium* sp.) malformation in Haiti (Cook, 1923) likens malformations (Table 2) to abnormalities, galls and witches' broom without distinguishing the concepts from one another. More recent examples include a publication on the pathology of rust fungi (Hernández and Hennen, 2003) that considers galls, witches' brooms and abnormalities as types of malformations attributed to hypertrophy, hyperplasia and hypoplasia (Table 1). This publication also recognized malformation as a type of abnormality. Earlier, well cited work (Meyer 1966; Meyerowitz *et al*. 1989) recognized irregular spatial development of a specific plant organ and consequent replacement of another (bracteody, carpellody, petalody, phyllody, sepalody, staminody; Table 1) and galls as types of abnormalities. However, in these same publications Goebel (1900) was cited who considered such abnormalities synonymous with malformations without addressing the consequent confusion. One of the symptoms of tomato (*Lycopersicon esculentum*) abnormality is floral malformation (Table 2), suggesting once more that malformation is a type of abnormality (Lozano *et al*. 1998; Pracros *et al*. 2006). Mango (*M. Indica*)

malformation (Table 2) is often described as resembling witches' broom (Singh and Dhillon, 1989; Singh, 1998; Krishnan *et al*. 2009), yet fails to explain why it is not formally considered witches' broom or offer any distinction between malformations and witches' broom.

Some publications use these terms as unifying descriptions and disease designations. This is confusing because they still fail to clearly define and distinguish disease symptoms from one another. For instance 'witches' broom malformation' of *Protea cynaroides*, 'tumorous gall-like malformation' of Mexican giant cardon (*Pachycereus pringlei*), and 'witches' broom' of *Byrsonima sericea* (Table 2) (Cutting, 1991; Dubrovsky and De La Luz, 1996; Guimarães *et al*. 2014) are descriptions incorporating various terms. In these cases, however, it could be that the different terms are used as adjectives attempting to describe the way the particular symptom looks.

The general lack of clear descriptions and definitions of these concepts describing deformed plant tissues, and the variation and overlap of usage obligates the expansion of literature in this review on plant malformation. This expansion includes literature on plant abnormalities, galls and witches' broom. Although this proved time consuming and confusing, it presented an opportunity for critical review to provide clear definitions and distinctions between these terms. However, the collected descriptions (Table 2) alone were not sufficient to do so. We thus investigated if the treatment of different causal agents and aspects of plant physiology that result in producing these changes could aid distinction and definition of the terminology.

2.2.2. Causal agents that induce shape changes in plants

a) *Abiotic factors*

Abiotic factors are capable of inducing physiological stress in plants that manifest as symptoms changing organ morphology. These include changes in organ identity, number of organs, organ size, absence of specific organs in the reproductive whorl, shoot proliferation and cessation of growth and development (Zieslin *et al*. 1979; Lozano *et al*. 1998; Chimonidou-Pavlidou, 2004; Tarchoun *et al*. 2013). Examples of such stresses include deviation of temperature, moisture, salinity, radiation, chemical

exposure, or macro and micronutrient availability beyond levels of tolerance. The induced reactions in a plant can influence proper physiological and morphological development that result in the latter symptoms (Meyer, 1966; Goodman *et al*. 1967; Hopkins and Hüner, 2004; Nabors and González-Barreda, 2004).

Roses (*Rosa hybrida* cv. Madelon) are affected by drought at different developmental stages (Chimonidou-Pavlidou, 2004). During the petal and/or stamen initiation stage, floral buds are aborted or malformed. Malformation consists of cessation of growth and development, the absence of carpels, and tightly packed stamens in the centre of the receptacle (Table 2). Low night temperature induces another type of malformation in *R. hybrida* of the Baccara variety, and is named 'bullhead' malformation (Table 2) (Zieslin *et al*. 1979). This malformation is described as a reduced length to diameter ratio of the floral bud, causing a flattened appearance. Other symptoms associated with this type of malformation include an increase in size and weight of floral buds, an increase in the number of petals and proliferation of secondary florets.

Tomato (*L. esculentum*) plants grown at low temperatures exhibit floral abnormalities (Lozano *et al*. 1998). These are described as homeotic (when genes are involved in early development and differentiation) and meristematic (zones of growth and cell differentiation) transformations during the development of organs, especially in reproductive whorls (Table 2). Homeotic transformation affects stamen and carpel identity and produces organs intermediate between the two, i.e. producing stamens/carpels that resemble the other in form and that are thus not distinct (fusion). Meristematic transformation produces an excess of organs of the reproductive whorl.

Reproductive organs of hot pepper (*Capsicum annuum*) develop abnormally (Table 2) when grown in low night temperature conditions (Tarchoun *et al*. 2013). These abnormalities are cultivar dependant and changes ovary diameter, style length, the number of ovules and locules, and length and diameter of flowers. Changes in fruit set percentage and fruit condition were also observed during different pollination strategies (self-pollination vs. artificial pollination).

b) *Genetics*

Spontaneous abnormalities can occur in conifer cones and consist of vegetative growth from the apex of cones, and the formation of bisexual cones in trees that are normally exclusively male (pollen-bearing) or female (ovule-bearing) (Rudall *et al*. 2011). These changes are hypothesized, as stated by authors, to be the results of spontaneous genetic transformation although no more explanations are given (Table 2). Genetic and epigenetic alterations of date palm (*Phoenix dactylifera*) occur during the *in vitro* process of tissue culture and result in floral abnormalities (Cohen *et al*. 2004). These abnormalities include a higher than usual number of carpels, undescribed distortion of carpels and stigmas, and impaired pollen tube elongation (Table 2). It remains unclear why these genetic changes occur. Without a clear biotic causal agent it is logical to assume changes result from changes in abiotic factors. Alternatively these changes occur spontaneously and require genetic clarification.

c) *Bacteria*

Bacteria cause a range of symptoms in plants such as leaf spots and blights, soft rot of fruits and roots, wilts, overgrowths, scabs and cankers (Agrios, 2005). Some of these include symptoms that involve changes in plant organ shape, for example leafy galls and crown galls (Goethals *et al*. 2001; Escobar and Dandekar, 2003; Quoirin *et al*. 2004; Gelvin, 2009; Păcurar *et al*. 2011; Gohlke and Deeken, 2014; Kado, 2014). Witches' broom of many species, including *Castanea crenata*, *Hibiscus rosa-sinensis* and *Spartium junceum*, are also attributed to bacteria, along with yellowing of leaves (Table 2) (Marcone *et al*. 1996; Montano *et al*. 2001; Jung *et al*. 2002). Some bacteria, in contrast, are able to promote plant growth and suppress disease development (Van Loon, 2007).These are caused by rather distinct groups of bacteria, as discussed below.

Leafy galls are dramatic symptoms caused by *Rhodococcus fascians* on a wide range of plants (Goethals *et al*. 2001; Agrios, 2005). These are usually described as hypertrophied shoots with multiple meristematic centres and suppressed elongation. On blackwattle (*Acacia mearnsii*) leafy galls are described (Quoirin *et al*. 2004) as a malformation (Table 2). Other symptoms that are associated with *R. fascians* infection include leaf deformation, witches' broom, and fasciation (Goethals *et al*.

2001). Witches' broom caused by *R. fascians* (Table 2) is described as misshapen and aborted leaves borne on clusters of fleshy stems on the crown of the infected plant. The age of the plant, bacterial strain, and conditions in which the bacterium will grow determines what symptoms will appear on infected plants. Leafy galls develop exclusively at the site of infection and are described as the local amplification of multiple buds experiencing shoot proliferation and growth inhibition (Agrios, 2005). Secondary leafy gall formation at non-infected sites does not occur (Goethals *et al*. 2001).

Species of *Rhizobium* cause crown gall disease on a number of plant species, which usually consists of galls on lower parts (stems and roots) of the plant (Agrios, 2005; Păcurar *et al*. 2011; Gohlke and Deeken, 2014; Kado, 2014). Examples (Table 2) include crown gall of daisies (*Bellis perennis*) caused by *Agrobacterium tumefaciens*, cane gall disease of *Rubus* spp. such as raspberries and blackberries caused by *A. rubi*, and crown gall of grape (*Vitis vinifera*) by *A. vitis* (Escobar and Dandekar, 2003; Gelvin, 2009; Păcurar *et al*. 2011). Upon infection *Agrobacterium* transfers DNA in the form of plasmids into cells of the affected plant where it is expressed (Agrios, 2005). With this ability *A. tumefasciens* has been instrumental in genetic engineering by incorporating specific, desirable genetic traits, for instance to produce genetically improved crops (Mullins *et al*. 2001; Agrios, 2005).

Mollicutes are a group of bacteria characterized by a lack of cell walls (Agrios, 2005; Gasparich, 2010) and only occur in the vascular bundles of plants. Two genera are associated with plants, namely *Spiroplasma* and *Phytoplasma.* Stunting, leaf yellowing, sterility, reduced fruit size, shortened internodes and floral malformation are usually associated with *Spiroplasma* while virescence, phyllody, sterility, internode elongation, stunting, leaf/shoot discolouration, leaf curling and witches' broom are associated with *Phytoplasma* (Gasparich, 2010). Phytoplasmas have been discovered recently as they cannot be cultured (Christensen *et al*. 2005). Modern molecular advents are responsible for the bulk of information on phytoplasmas (Christensen *et al*. 2005; Pracros *et al*. 2006; Gasparich, 2010). Phytoplasmas are responsible for over 200 diseases affecting several hundred plant species (Gasparich, 2010). These bacteria are only transmitted by insects, usually leafhoppers and psyllids (Agrios, 2005; Gasparich, 2010; Griffiths, 2013).

Phyotoplasma species often are associated with malformation-type symptoms. Colour-breaking and malformed floral spikes of ornamental *Gladiolus* spp. (Table 2) in India have been associated with '*Candidatus* Phytoplasma asteris' (16SrI group) (Raj *et al*. 2009). The malformations are not described, but are associated with other symptoms including leaf stripe, colour-breaking, yellowing, stunted growth, small corms, and an underdeveloped root system. Leaf malformation of plumed cockscomb (*Celosia argentea*) and flamingo feather (*C. spicata*) are associated with another phytoplasma from the 16SrIII-J subgroup (Eckstein *et al*. 2012). The malformation is not described beyond noting that it is associated with the characteristic symptom of phyoplasma infection, i.e. witches' broom (Table 2). Witches' broom of *Protea* spp. is described as the proliferation of young shoots and leaves (Cutting, 1991). This disease threatens cut flower exports from South Africa (Cutting, 1991; Wieczorek and Wright, 2003). This disease is caused by an unknown phytoplasma that is vectored by three species of arthropods, namely Protea witches' broom mite (*Aceria proteae*), *Proctolaelaps* sp., and *Oxycarenus maculatus*.

Non-pathogenic, plant associated and soil borne bacteria known as rhizobacteria generally promote growth and suppress disease in plants (Van Loon, 2007; Lugtenberg and Kamilova, 2009). Rhizobacteria have various benefits for plants and promote growth (Lugtenberg and Kamilova, 2009). Rhizobacteria stimulate plant root growth by producing the growth hormone auxin for plants, they assist in managing physiological stress of plants with enzyme 1-aminocyclopropane-1-carboxylate (AAC) deaminase that reduce levels of the stress hormone ethylene. They play a role in biofertilization by converting N_2 to ammonia for plant use in special structures on roots called nodules, and they facilitate rhizoremediation by the degradation of soil pollutants. Rhizobacteria are able to suppress disease development by antagonizing pathogens through production of antibiotics and lytic enzymes, competing for resources, or by optimizing the general plant defence through a process known as induced systemic resistance (ISR) (Van Loon, 2007).

d) *Fungi*

More than 10000 species of fungi are able to cause disease symptoms in plants (Agrios, 2005). Symptoms include anthracnose, blight, die-back, canker, leaf curling, wilt, as well as deformations (Agrios, 2005; Horst, 2008). Fungi are able to produce

various types of deformation-like symptoms such as galls (Ploetz, 2007), malformations (Marasas et al. 2006) and witches' broom (Guimarães *et al*. 2014).
Galls are typically formed by a group of fungi known as rusts and smuts (Teliomycete, Basidiomycota). Some examples include large fleshy galls (Table 2) at the apex of seed and flower pedicels on *Vachellia karroo* (previously named *Acacia karroo*) caused by the rust *Ravenelia macowaniana* (McGeogh, 1993). The smut fungus *Ustilago esculenta* (Table 2) induces a hypertrophic response in the stems of Manchurian wild rice (*Zizania latifolia*), forming edible galls (Yang and Leu, 1978; Chung and Tzeng, 2004). These galls prevent development of seeds and inflorescences. Infection of rust species in *Gymnosporangium* in their primary Cupressaceae hosts results in galls, stem swelling, witches' broom and dieback of twigs and branches (Dervis *et al*. 2010). For example, gall formation on the primary Cupressaceae host red cedar (*Juniperus virginiana*) caused by *G. juniper-virginiae* and *G. globosum* (Table 2) are described as transformed axillary buds (axillary buds that become galls instead of intended organ e.g. flower) (Stewart, 1915).

Galls can also be formed by fungi in the Ascomycota. Black knot disease of *Prunus* spp. is caused by *Apiosporina morbosa* (Table 2) and consist of rough spindle-shaped galls (black knots) on woody tissues of primary twigs and branches that may result in death of the plant (Fernando *et al*. 2005; Zhang *et al*. 2005). Cushion galls of cacao (*Theobroma cacao*) are produced on flower cushions, leaf nodes and wounded sections of branches and stems, with dieback resulting from interaction with other pathogens (Table 2). The disease is caused by *Fusarium decemcellulare* (Ploetz, 2006; Ploetz, 2007).

Deformation-type symptoms other than galls are caused by various types of fungi. Cacao (*T. cacao*) production is threatened by witches' broom caused by the basidiomycete *Moniliophthora perniciosa* with the most dramatic symptom including hypertrophied shoots (Aime and Phillips-Mora, 2005; Leal *et al*. 2007). Species of *Exobasidium* cause shape changes in leaves of fetterbush (*Pieris formosa*), *Lyonia ovalifolia*, and *Rhododendron* spp. (Hernández and Hennen, 2003; Li and Guo, 2008). Symptoms on *P. formosa* and *L. ovalifolia* are referred to as leaf deformation and consist of hypertrophy and red leaf spots on the upper leaf surface (adaxial) of *P. formosa* while the adaxial and lower leaf surface (abaxial) of *L. ovalifolia* become

concave-convex to subglobose in shape (Li and Guo, 2008). These leaf deformations could be comparable to leaf malformations of *Rhododendron* spp. but requires verification (Hernández and Hennen, 2003; Li and Guo, 2008).

Mango malformation disease (MMD) is described (Table 2) as short, thick and excessively branches inflorescences that bear more and larger flowers than normal (Krishnan *et al*. 2009). The majority of these flowers are male with poor pollen viability, while ovaries in the few bisexual flowers that do appear are often enlarged and non-functional, resulting in sterility or floral bud abortion (Krishnan *et al*. 2009). Vegetative malformation (Table 2) is characterized by a loss of apical dominance that causes stunting, with small, clumped shootlets bearing small scaly leaves that are often described as witches' broom (Marasas *et al*. 2006; Krishnan *et al*. 2009). The disease is caused by species of *Fusarium*, including *F. mangiferae*, *F. mexicanum, F. proliferatum*, *F. pseudocircinatum*, *F. sterilihyphosum, F. subglutinans* and *F. tupiense* (Marasas *et al.* 2006; Liima *et al*. 2009; Otero-Colina *et al*. 2010; Lima *et al*. 2012). Various other *Fusarium* spp. are also associated with malformation disease of water berry (*S. cordatum*) inflorescences (Kvas *et al*. 2008), which are described as larger, excessively branched and sterile flowers (Table 2).

e) *Insects*

Direct mechanical damage on plants as a result of insect behaviour can cause symptoms such as leaf spotting, necrosis, development of lesions and deformations in plant tissues. These deformations appear as leaf curling, stunting, galls and malformations in addition to direct mechanical damage through feeding habits (Carter, 1962; Goodman *et al*. 1967). Peach (*Prunus persica*) fruit 'catfacing' is designated as a malformation (Fenton *et al*. 1944; Fenton and Brett, 1946) and is associated with feeding habits of the tarnished plant bug (*Lygus oblineatus*). It is described as numerous, deep indentations of the fruit of which the tissue may appear corky. Malformation of apples (*Malus domesticus*) is described as irregular fruit development (Table 2) resulting in distortion of the fruit shape (Fryer, 1916; Carter, 1962). This malformation is associated with feeding of capsid bugs. Direct evidence of *L. oblineatus* and capsid bugs causing these respective malformations or acting as vectors for other pathogens remains unclear as it appears there are no publications on these diseases after those referred to.

There are numerous examples of insects causing galls. Over 13000 of these gall-inducing insects (GII's) are currently known, predominantly from the orders Coleoptera, Diptera, Hemiptera, Hymenoptera, Lepidoptera and Thysanoptera (Mani, 1964). The GII provides a specific stimulus to the plant resulting in the formation of galls. Galls induced by different GII species are anatomically and physiologically distinct on overlapping plant hosts, as well as on different hosts, and are often diagnostic to the GII's (Mani, 1964; Shorthouse *et al*. 2005).

Examples of GII's include the stem-galling moth (*Epiblema strenuana*) that causes gall formation on whitetop weed (*Parthenium hysterophorus*). Associated symptoms of *E. strenuana* gall formation on *P. hysterophorus* include a reduction in main shoot length, and flower and leaf production (Florentine *et al*. 2005). The *Eucalyptus* gall wasp (*Leptocybe invasa*) causes what is only described as typical galls and associated swelling of leaf midribs, petioles and stems that can lead to stunted growth on *Eucalyptus* spp. trees (Kim *et al*. 2008). Leaf galls of maize (*Zea mays*) are described as severe swelling in leaves and are caused by maize orange leafhoppers (*Cicadulina bipunctata*) (Tokuda *et al*. 2013). Different species of oak gall wasps (*Andricus* spp.) cause galls on oak trees (*Quercus* spp.) described as plant growths that differ in morphology depending on the species of *Andricus* that induces gall formation (Stone and Cook, 1998). The agamic (asexual) generation of *A. lignicola*, for example, forms woody galls on terminal buds on *Q. ruber* (Hartley and Lawton, 1992).

By acting as vectors insects are indirect causal agents of many disease with deformation-like symptoms such as galls, malformations, abnormalities and witches' broom (Carter, 1962;, Miller, 1993). For example, it is suggested that insects are responsible for dispersal of the unidentified fungus (Table 2) that causes witches' broom galls of *Byrsonima sericea* (Guimarães *et al*. 2014). Phytoplasmas are the most prominent example of vectored pathogens, usually by psyllids and leafhoppers (Garnier *et al*. 2001; Gasparich, 2010). For instance the leafhopper *Orosius argentatus* is the vector of the phytoplasma that causes witches' broom (Table 2) of alfalfa (*Medicago sativa*) (Helson, 1951; Bowyer *et al*. 1969; Khan *et al*. 2002). The leafhopper *Hishimonus sellatus* vectors the JWB phytoplasma associated with witches' broom of jujube (*Zizyphus jujube*) (Jung *et al*. 2003). The leafhopper

Matsumuratettix hiroglyphicus acts as a vector to a phytoplasma that causes sugarcane white leaf (SWL) disease that does not cause plant deformation, but symptoms including complete leaf chlorosis and the proliferation of tillers (Hanboonsong *et al*. 2002).

f) *Mites*

In addition to symptoms of necrosis, chlorosis, virescence, stunting, and shortened internodes, plant feeding mites known as eriophyid mites are able to cause deformations to host plants as a by-product, or through salivary secretions, during feeding (Carter 1962; Pećinar *et al*. 2009; Petanović and Kielkiewicz, 2010). Deformations caused on plants include floral and vegetative malformation, gall formation, and abnormalities such as phyllody and shortening of internodes (Rancic *et al*. 2006; Petanović and Kielkiewicz, 2010). Examples include malformation of cut-leaf teasel (*Dipsacus laciniatus*) caused by the mite *Leipothrix dipsacivagus* (Pećinar *et al*. 2009). It is described as a reduction in size of inflorescences, leaf distortion (wrinkling and rolling), shortening of internodes, reduced growth, and chlorosis and necrosis of leaves (Table 2). Severe leaf curling, and leaf edge enrolling and upfolding (Table 2) constitutes of what is considered leaf malformation of creeping thistle (*Cirsium arvense*) caused by *Aceria anthocoptes* (Rancic *et al*. 2006). Leaf and fruit mosaic, fruit drop and leaf malformation (Table 2) of *Ficus* spp. in Japan is caused by *A. ficus* (Ashihara *et al*. 2004). The leaf malformation on *Ficus* spp. is not described specifically but resemble that of *C. arvense*. Woody galls of *Populus* spp. in North America and Europe is described as a swollen mass causing retarded growth and deformation of twigs (Table 2), and is caused by the eriphyid mites *A. parapopuli* and *A. populi* (Petanović and Kielkiewicz, 2010).

Similar to insects, some mites are indirect causal agents of deformation-like symptoms by acting as vectors of pathogens (Carter, 1962; Agrios, 2005; Petanović and Kielkiewicz, 2010). The mango bud mite (*A. mangiferae*) carries conidia of *Fusarium mangiferae* to the apical buds of *M. indica*, facilitating infection of the tree to cause MMD (Gamliel-Atinsky *et al*. 2009). *Brevipalpus* mites act as vectors for viruses such as the citrus leprosies virus (CiLV) that causes lesions on fruit, leaves and branches of citrus fruit (Rodrigues *et al*. 2003; Bastianel *et al*. 2010; Rodrigues and Childers, 2013). An association between eriophyid mites and the fungus

Sphaerotheca phytoptophila, which causes witches' broom of hackberry (*Celtic australis*), has also been observed (Snetsinger and Himelick, 1957; Carter 1962). Mites from the genera *Sancassania* and *Tyrophagus* introduce spores of aflatoxin-producing *Aspergillus flavus* into peanuts (*Arachis hypogaea*) during feeding (Carter, 1962).

g) *Nematodes*

Nematodes can cause symptoms including necrosis, formation of lesions, rots, yellowing, wilting and reduced growth in addition to other cellular modifications that result in deformations such as root knots, galls and malformations (Goodman *et al*. 1967; Greco *et al*. 1984; Agrios, 2005; Horst, 2008). For instance, feeding by *Ditylenchus dipsaci* on up to 450 plant species (Table 2) involves withdrawal of plant cell content. This causes cells adjacent to feeding wounds to divide and enlarge in a form of hypertrophy and hyperplasia to form malformation in plant stems and bulbs (Jones *et al*. 2013).

According to the title of a publication on dogwood (*Cornus florida*) canker the symptoms, consisting of localized swelling of trunk tissues with bark disruption (Table 2), is comparable to stem malformations (Santamour and McArdle, 1987). The cankers are associated with a nematode allied to the *Aphelenchoides fragariae* complex, and another nematode *Panagrolaimus subelongatus*. These cankers have important secondary effects when additional damage occurs after affected stems and branches break off, leaving the open wound susceptible to infestation by the dogwood borer (*Synanthedon scitula*).It remains to be determined whether both or only one of these nematodes cause the cankers directly, and how the cankers are caused and why.

Similar in concept to insect galls, cyst nematodes manipulate the internal physiology and morphology of host plant roots to form feeding sites known as syncytias (Ithal *et al*. 2007). In the case of the soybean cyst nematode (*Heterodera glycine*), plant hormone levels that regulate genes influencing cell structure and plant defense are altered (Ithal *et al*. 2007). This enables *H. glycine* to fuse plant cells and form syncytia.

h) *Viruses*

Viruses can cause a range of general plant disease symptoms including chlorosis, dwarfing, lesions, mosaics, necrosis, reduced yields, stunting and yellowing in addition to various types of malformations (Agrios, 2005). These symptoms may vary, however, based on environmental conditions that can either favour or discourage expression (Hillocks and Thresh, 2000; Agrios, 2005). Viruses infect plants through mechanical wounds, fertilization by infected pollen, or via vectors such as insects, mites, fungi, nematodes and birds (Agrios, 2005; Horst, 2008).

There are a number of examples of virus diseases that have deformation-type symptoms. The *Tomato spotted wilt virus* (TSWV) affect over 900 plant species, causing general symptoms of chlorosis, necrosis and malformation (Chatzivassiliou *et al*. 2000; Salomone *et al*. 2003; Agrios, 2005). Detailed descriptions of this malformation caused by the TSWV, transmitted by *Frankiniella schultzei* and *Thrips tabaci*, is not noted (Agrios, 2005).

Malformation, distortion and mosaic patterning of leaves are described for Cassava Mosaic Disease (CMD) (Hillocks and Thresh, 2003). On cassava (*Manihot esculenta*) the virus is transmitted by the whitefly (*Bemisia tabaci*). No distinction is made between malformation and distortion (Table 2) in this case and symptoms are described as the unequal expansion of the leaf lamina in reponse to stress induced by chlorotic areas. Distortion, reduction in leaflet size and stunting are secondary effects.

Crinkling is a disease of rice (*Oryza sativa*) caused by the Rice Stripe Necrosis Virus (RSNV) in South America (Morales *et al*. 1999; Johnson *et al*. 2012). Transmitted by a fungal vector (*Polymyxa graminis*) symptoms include leaf striping, severe plant malformation and seedling death (Table 2). However, description of how the malformation appears is lacking.

2.2.3. Ecological advantage and/or function of shape changes in plants

The reason why abnormalities, galls, malformations or witches' broom symptoms are formed by the different causal agents is mostly unclear. Research on the ecology behind abnormality, malformation and witches' broom formation is surprisingly underrepresented. Because of this, they are discussed and related in terms of three well known hypotheses on the adaptive significance for gall morphology (ASGM) induction in insects. These include the nutrition hypothesis, the microenvironment hypothesis, and the enemy hypothesis (Price *et al*. 1987; Stone and Schönrogge, 2003).

The nutritional hypothesis for gall formation suggests that galled tissues represent superior nutritional value as opposed to un-galled tissue. This has been supported by several studies (Abrahamson and Weis, 1987; Price *et al*. 1987; Whitham, 1992; Fay *et al*. 1993; Castro *et al*. 2012) but also rejected by others (Anderson and Mizell, 1987; Brewer *et al*. 1987; Hawkins and Unruh, 1988; Hartley, 1990). A similar hypothesis might be proposed for some nematodes and fungi. The nematode *H. glycine* induces the formation of syncytias in *G. max* (Table 2), which is followed by a redirection of nutrients to syncytia where it is ingested by the nematode (Ithal *et al*. 2007; Jones *et al*. 2013). During parthenocarpy in witches' broom of *T. cacao* caused by *M. perniciosa*, metabolites were redirected to increase and decrease at different developmental stages of the pathogen in affected tissues, compared to unaffected tissues (Scarpari *et al*. 2005; Melnick *et al*. 2012). An increase in sucrose correlates with the biotrophic infection stage of the pathogen, which then decreases during the shift to the necrotrophic stage.

The microenvironment hypothesis of GIIs suggests that galls offer protection from unfavourable abiotic conditions such as dessication (Price *et al*. 1987; Stone and Schönrogge, 2003). It has also been used to explain the significance of plant deformations caused by mites (Petanović and Kielkiewicz, 2010). This hypothesis could be extended to viruses. In this case the induced microenvironment creates an environment within tissue necessary for multiplication and reproduction (Horst, 2008; Pallas and Garcia, 2011).

Witches' brooms represent a new, unique microhabitat or niche that insects may find attractive, offering a sight of protection from abiotic conditions, predators and/or additional nutritional benefit. Witches' broom (Table 2) is commonly produced as result of phytoplasma infection (Montano *et al*. 2001; Khan *et al*. 2002; Jung *et al*. 2003; Wieczorek and Wright, 2003). A vector-phytoplasma-plant host interaction system can hold significant distribution and competitive advantages for different phytoplasmas by attracting the appropriate insect vectors to the specific plants host (Lee *et al*. 2000). The nematode *Ditylenchus* sp. forms galls on *Miconia alibicans* that harbour a higher diversity of arthropods than unaffected tissues (Maruyama *et al*. 2012). It is likely these galls represent nutritional and protective benefits, similar to that of syncytias in feeding activity of *D. dipsaci*, and also explains the higher arthropod diversity on galls (Maruyama *et al*. 2012; Jones *et al*. 2013). A similar strategy to that of phytoplasmas to attract appropriate vectors for dispersal may be employed by viruses that cause shape change in plants.

The enemy hypothesis states that galls can protect inhabitants from natural predators such as birds or other arthropods (Price *et al*. 1987; Stone and Schönrogge, 2003). Similarly eriophyid mites have been considered to induce abnormalities such as galls to use for shelter and protection against predators (Petanović and Kielkiewicz, 2010). Though structurally galls would offer some amount of protection from enemies and predators, it is by no means absolute and therefore the enemy hypothesis has failed to garner as much support or evidence as the former two hypotheses and no analogs to pathogens are known.

The three hypotheses on ASGM formation prove that a suitable basis for initial ecological investigation of shape changes in plants. Deviating from these three hypotheses but recognizing that shape changes in plants have ecological function, is the fact that abiotic conditions and spontaneous genetic alteration are known speciation and evolutionary drivers. Similarly, shape changes in plants caused by abiotic conditions and spontaneous genetic alterations may be considered initial stages of natural selection (Meyerowitz *et al*. 1989). This statement and its relevance, however, are completely dependent on specific experimental and phylogenetic investigation that fall beyond the scope of this review. It could also be that there is some evolutionary value in such changes when they affect and/or are

utilized by organisms other than the causal agent. If research fails to identify any of these factors significant in a case, having no effect on the causal agent and/or its survival on/off the host plant, the abnormality, gall, witches' broom and/or malformation in that case are simply the coincidental response of the host plant.

2.2.4 Physiology

Plant hormones (phytohormones) concentration and their distribution in plants are often pointed out as the reason for morphological changes resulting in abnormalities, galls, malformations and witches' brooms (Zieslin *et al*. 1979; Meyerowitz *et al*. 1989; Singh and Dhillon, 1989; Cutting, 1991; Hartley, 1998; Lozano *et al*. 1998; Singh, 1998; Tanaka *et al*. 2003; Marasas *et al*. 2006; Rudall *et al*. 2011; Melnick *et al*. 2012; Tarchoun *et al*. 2013; Gohlke and Deeken, 2014; Guimarães *et al*. 2014). Phytohormones are signal molecules that have important physiological functions through physiological and biochemical interactions (Stern *et al*. 2003; Kohli *et al*. 2013). These function include directing growth and development of plant cells and co-ordinating growth of various vegetative and floral organs (Hopkins and Hüner, 2004; Nabors and González-Barreda, 2004). By chemically binding to specific receptors phytohormones cause signal transduction cascades that may affect transport of ions across membranes, changes in the complement of enzymes produced in tissues, and turning certain genes on or off (Clarke *et al*. 2000; Knight and Knight, 2001; Stern *et al*. 2003; Guo, 2011). The different phytohormones and their functions are discussed in more detail below.

Changes in mineral nutrient levels of plants have been detected in response to abnormalities, galls, malformations and witches' brooms (Abrahamson and McCrea, 1986; Singh *et al*. 1991; Hartley, 1998; Larson, 1998; Singh and Singh, 1998; Stone and Schönrogge, 2003; Florentine *et al*. 2005; Shah *et al*. 2009; Castro *et al*. 2012; Ashfaq *et al*. 2014). A mineral nutrient is considered essential if it is necessary for a plant to complete a normal lifecycle, or if it is a component of plants and/or their metabolites (Stern *et al*. 2003; Hopkins and Hüner, 2004; Nabors and González-Barreda, 2004). Plants require seventeen essential elements (Table 3), each performing specific functions within plants to maintain physiological homeostasis (Stern *et al*. 2003; Hopkins and Hüner, 2004; Nabors and González-Barreda, 2004).

The optimal amount of each element, known as the critical concentration (CC), is defined as the concentration necessary to achieve maximum growth and to maintain homeostasis (Hopkins and Hüner, 2004; Nabors and González-Barreda, 2004). Below CC plants show deficiency symptoms, and above CC the element becomes toxic to the plant when species specific thresholds are exceeded. Moreover, under these conditions plants may become stressed and thus more susceptible to other abiotic and biotic conditions (Meyer, 1966; Goodman *et al*. 1967; Hopkins and Hüner, 2004; Nabors and González-Barreda, 2004; Agrios, 2005). It has also been suggested that the nutrient status of the host plant may affect growth and development of pathogens (Agrios, 2005; Scarpari *et al*. 2005; De Souza *et al*. 2006; Meinhardt *et al*. 2006; Garcia *et al*. 2007). Measured changes in nutrients in response to agents causing deformation-like symptoms are thus significant to the plant, and may have important implication for the causal agent or possible associated vectors. Examples of changes in nutrient levels in response to abnormality, gall, malformation and witches' broom formation are discussed in Section 2.2.4.c.

a) *Types of phytohormones*

The nomenclature of phytohormones has been the focus of much debate since their discovery (Hanson and Trewavas, 1982; Wyers and Paterson, 2001; Hopkins and Hüner, 2004), with synonyms and alternate definitions summarized by Wyers and Paterson (2001). Following is a brief historic account of the "five classical" phytohormone groups, namely auxins, cytokinins (CK), gibberellins (GA), ethylene and abscisic acid (ABA). The organs from which they have been isolated and associated responses and functions in plants are summarized in Table 4.

Auxins were discovered by Charles Darwin and his son Francis Darwin in 1881, and were formally named by Frits Went in 1926 (Stern *et al*. 2003; Hopkins and Hüner, 2004; Nabors and González-Barreda, 2004). Auxins are chemically characterized as having an acidic side chain on an aromatic ring (Hopkins and Hüner, 2004). Discovery of the first naturally occurring auxin, namely indole-3-acetic acid (IAA) (Figure 2.1.a), sparked the discovery of other phytohormones such as 4-chloroindole-3-acetic acid, phenylacetic acid and indole-3-butyric acid (IBA) (Kende and Zeevaart, 1997; Stern *et al*. 2003; Hopkins and Hüner, 2004; Nabors and

González-Barreda, 2004). Some of the functions derived from plant responses to excess and/or low levels of auxin include cell enlargement and elongation, fruit development, root and shoot growth and vascular differentiation (Table 4).

Substances regulating cell division were first discovered by Gottlied Haberlandt in 1913 and eventually came to be known as cytokinins (CK) (Stern *et al*. 2003). Cytokinins are N^6-substituted adenine derivatives of which the most common naturally occurring member is zeatin (Z) (Figure 2.1.b) (Kende and Zeevaart, 1997; Stern *et al*. 2003; Hopkins and Hüner, 2004; Nabors and González-Barreda, 2004). Other cytokinins include N^6-furfuryl adenine (kinetin), N^6-(Δ^2-isopentenyl) adenine (iP), dihydrozeatin (diHZ) and N^6-(benzyl) adenine (BAP) (Hopkins and Hüner, 2004). Functions of CK include cell division, root and shoot growth and differentiation, leaf expansion and stimulating germination (Table 4).

Gibberellins were discovered in the fungal genus *Gibberella* by Eiichi Kurosawa in 1926, and are characterized by a 20-carbon *ent*-gibberellane structure (Figure 2.1.c) (Stern *et al*. 2003; Hopkins and Hüner, 2004; Nabors and González-Barreda, 2004). There are over 125 gibberellins currently known (Hopkins and Hüner, 2004). Gibberellins that retain the 20-carbon structure are called C_{20}-gibberellins, and include the naturally occurring gibberellins isolated and characterized from plants, gibberellic acid (GA_3). Most gibberellins, however, have lost one carbon atom and are hence known as C_{19}-gibberellins, including the most active naturally occurring gibberellins GA_1 and GA_{20} (Hopkins and Hüner, 2004). Different gibberellins are all designated as GA with the 'A' representing that it has been proven to be naturally occurring and has been described chemically. They are distinguished from one another by simple subscript numbers that correlate with order of discovery (Hopkins and Hüner, 2004). Gibberellins appear to play an important role in seed germination and development (Table 4).

After observing stem elongation, swelling of stems and abnormal horizontal growth of pea seedlings in a laboratory, a Russian student, Dimitry Neljubow found that normal growth resumed once seedlings were placed outside (Stern *et al*. 2003). It was determined that ethylene gas from lamps in the laboratory induced the abnormal growth. In 1934 R. Gane discovered that ethylene (Figure 2.1.d) is also produced naturally by plants as a simple gaseous hydrocarbon (Stern *et al*. 2003; Hopkins and

Hüner, 2004; Nabors and González-Barreda, 2004). Ethylene is able to induce plant resistance in response to infection to pathogens and invasion to pests, as well as inhibit and initiate abnormal growth responses (Table 4).

Abscisic acid (ABA) is a phytohormone group represented by a single member (Figure 2.1.e). It was originally named for its perceived role in leaf abscission, although it was later shown to be driven by ethylene rather than ABA (Stern *et al*. 2003; Hopkins and Hüner, 2004; Nabors and González-Barreda, 2004). Abscisic acid appears to have no specific function that could cause obvious shape changes in plants, but has been associated with growth inhibition and stomatal closure (Table 4).

The five classical phytohormone groups were the first discovered and acknowledged phytohormones. However, recently a group of substances with similar chemical structure to animal steroids was discovered in the plant genus *Brassica*. This group has subsequently been named brassinosteroids (BR). Unlike animal steroids, BRs do not enter cells but bind to receptor proteins found in the plasma membrane (Weyers and Paterson, 2001; Hopkins and Hüner, 2004; Nabors and González-Barreda, 2004). Performing functions related to plant growth and development (Table 5) similar to the “five classical” phytohormones, BRs are now widely considered and accepted as another group of phytohormones. Different BRs have structural variation in the A/B-rings and side chain. Over 40 BRs are recognized to be naturally occurring, although it is still unclear whether brassinolide (Figure 2.2.a) is the only biologically active member (Yokota, 1997).

Jasmonic acid (JA) (Figure 2.2.b) and salicylic acid (SA) (Figure 2.2.c) have recently been associated with aspects of plant growth, qualifying them for phytohormone status (Weyers and Paterson, 2001; Hopkins and Hüner, 2004; Nabors and González-Barreda, 2004). These include JA stimulating flower, fruit and seed formation, and SA in general plant growth and development (Table 5). The majority of research on JA and SA investigates the roles of these compounds in inducing plant defense responses, such as induced systemic resistance (ISR) and systemic acquired resistance (SAR), thus affecting effectiveness of pathogens such as those that induce deformation-like symptoms. Salicylic acid is primarily responsible for defense against biotrophic pathogens, and JA against necrotrophic pathogens

(Nabors and González-Barreda, 2004; Agrios, 2005; Spoel *et al*. 2007; Van Loon, 2007).

b) *Mechanisms that alter phytohormone levels*

Levels of phytohormones are changed once a biological signal is received by a protein detector which triggers a cascade of biochemical processes to determine a specific cellular response (Hopkins and Hüner, 2004; Peleg and Blumwald, 2011; Kohli *et al*. 2013). These processes include interactions between and within different classes of signals, intersection and divergence of biochemical pathways, and secondary messengers such as G-proteins and kinases to co-ordinate developmental signals. As a simplified example it has been found that CK and IAA are involved in a homeostatic feedback loop with one another which serves to maintain appropriate concentrations of the two phytohormones (Peleg and Blumwald, 2011). It has also been demonstrated that ethylene regulates expression of genes associated with biosynthesis, perception and action of auxins (Peleg and Blumwald, 2011). Exogeneous application of ABA is also able to affect CK's (Kohli *et al*. 2013). The ecological implication and exact molecular and chemical mechanics involved in these and other phytohormone crosstalk interactions are summarized in several reviews (Knight and Knight, 2001; Ho *et al*. 2003; Fujita *et al*. 2006; Spoel *et al*. 2007; Peleg and Blumwald, 2011; Kohli *et al*. 2013; Alba *et al*. 2015).

Considering the complexity of phytohormone crosstalk, the alteration in production, concentration or perception of a single phytohormone can upset the balance of other phytohormones, as well as metabolic and other processes in plants (Hopkins and Hüner, 2004; Nabors and González-Barreda, 2004; Fujita *et al*. 2006). These lead to abnormal shape changes in plants (Singh, 1998; Tanaka *et al*. 2003; Goethals *et al*. 2004). The mechanisms of how this is achieved by the causal agent usually involves the causal agent producing and introducing its own phytohormones in the plant, or introducing or affecting genes in the plant that regulate phytohormone production, perception or expression (Quoirin *et al*. 2004, Agrios, 2005).

Leafy galls such as those of *A. mearnsii* caused by *Rhodococcus fascians* (Table 2) is the result of changes in auxin and CK concentration (Goethals *et al*. 2001; Quoirin *et al*. 2004; Agrios, 2005). These phytohormones have been isolated from cultures of

R. fascians that cause leafy gall formation, indicating that the bacterium is able to produce these phytohormones independently. Symptoms of leafy galls have been replicated, though less pronounced, from exogenous application of CK (Goethals *et al*. 2001; Quoirin *et al*. 2004; Agrios, 2005). The fungus *Aciculosporium take* causes witches' broom of bamboo spp. (Table 2). Although the exact mechanism of how these symptoms appear upon *A. take* infection is not clear, proliferated growth is shown to be associated with changes in phytohormone concentrations such as those of IAA. Indole-3-acetic acid has also been isolated from cultures of *A. take* (Tanaka *et al*. 2003; Chung and Tzeng, 2004; Agrios, 2005).

In the case of *Rhizobium radiobacter* (previously known as *Agrobacterium tumefasciens*) that causes crown gall tumours, genes from plasmids of the bacterium are integrated into the host plant genome. These genes encode for enzymes such as those involved in synthesis of auxins and CKs (Quoirin *et al*. 2004; Pãcurar *et al*. 2011). Considering their functions of cell growth and development (Table 4), manipulation of auxin and CK concentrations within a plant ultimately results in the observed gall symptoms.

Viruses alter gene expression in plants. When such alterations affect phytohormone expression, perception, and/or regulation, symptoms corresponding to the function of the affected phytohormone are often observed. These symptoms may vary depending on subsequent effects of changed gene expression on other processes involved in the signal transduction (Pallas and García, 2011; Morales *et al*. 1999; Chatzivassiliou *et al*. 2000; Hillocks and Thresh, 2000; Agrios, 2005). Research of such effects on plants by viruses are absent, but considering discussed examples of spontaneously altered genes for abnormalities of conifer cones (Rudall *et al*. 2011) and flowers of tomato plants (*Lycopersicon esculentum*) grown at low temperatures (Lozano *et al*. 1998), this could be probable. Phytoplasmas also alter gene expression in plants. For example the stolbur phytoplasma (isolate PO) introduces effector proteins in *L. esculentum* that alter genes to result in floral abnormalities (Pracros *et al*. 2006; Sugio *et al*. 2011)

Insect galls are generally accepted to be products of phytohormone imbalances induced by a combination of salivary secretions, physical actions that insert salivary secretions such as feeding, and the stress associated with these actions (Carter,

1926; Rohfritsch *et al*. 1982; Raman, 2007; Stuart et al. 2012; Tokuda et al. 2013). For example catfacing of *P. persica* caused by the tarnished plant bug (*L. oblineatus*) is described as the result of feeding punctures (Fenton *et al*. 1944; Fenton and Brett, 1946). It is more likely the result of a combination of the insects' salivary secretions and/or related stress induced than the feeding punctures alone. Saliva from larvae of the Hessian fly (*Mayetiola destructor*) in the gall midge group, has been found to contain effector proteins that initiate gene-for-gene interactions with host plants. Although *M. destructor* does not cause galls to form on its host plant (*Triticum* spp.), it could be that effector proteins that alter gene expression involved in phytohormone regulation and result in gall formation, is present in other gall forming members of this group (Stuart *et al*. 2012).

Effector proteins have been identified in cyst nematodes that alter phytohormone regulation (Grunewald *et al*. 2009; Haegeman *et al*. 2012; Jones *et al*. 2013). Various aspects of auxin transport are regulated by a group of genes in the plant (*PIN*1, *PIN*2, *PIN*3, *PIN*4, *PIN*7) of which the natural expression is manipulated by effector proteins introduced by nematodes (Grunewald *et al*. 2009). This enables nematodes to initiate formation of syncytias and subsequent expansion of plant tissue. Altered phytohormone level can also occur post induction of abnormalities, galls, malformations or witches' brooms, e.g. water stress observed in galled leaves of *Z. mays* is the proposed reason for increased ABA levels post gall induction (Tokuda *et al*. 2013). This is a reasonable proposal, considering the role of ABA regulating water stress, but requires further investigation.

c) *Changes in nutrient levels in response to shape changes in plants*

Literature on changes in nutrient concentration of misshapen plants is sorely lacking and cited examples require further verification and investigation as to why these changes occur. However some studies have looked at the concentrations of boron (B), calcium (Ca), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), nitrogen (N), nickel (Ni), phosphorus (P), lead (Pb), sulphurs (S) and zinc (Zn) in malformed and healthy tissues of *M. indica* (Singh *et al*. 1991; Singh and Singh, 1998; Shah *et al*. 2009). Macronutrients that generally appear in higher concentration in malformed tissues than in healthy tissues include Ca and N, and at later developmental stages also K

and P (Singh *et al*. 1991). Of the micronutrients investigated it appears that malformed leaves have significantly higher concentrations of Zn, malformed shoots significantly higher concentrations of Cu, Fe, Mn and Zn, and malformed panicles significantly higher concentrations of Mn and significantly lower concentrations of Fe and Zn (Singh *et al*. 1991; Singh and Singh, 1998). Boron, Mg, S, Cd, Co, Cr, and Pb showed no significant difference in concentration between malformed and healthy tissues (Singh *et al*. 1991; Singh and Singh, 1998; Shah *et al*. 2009). The reasons and mechanisms of this have not yet been investigated.

General changes in nutrient uptake, transport, and concentration have been considered a buffer to compensate for loss of biomass to herbivores (Baldwin and Preston, 1999). Furthermore, many pathogenic organisms are able to re-direct and manipulate the transport of nutrients to their advantage (Abrahamson and McCrea, 1986; Singh *et al*. 1991; Hartley, 1998; Larson, 1998; Singh and Singh, 1998; Stone and Schönrogge, 2003; Shah *et al*. 2009; Castro *et al*. 2012; Melnick *et al*. 2012; Jones *et al*. 2013). For example the nematode *Heterodera glycine* forms syncytias which act as a nutrient sink on *Glycine max* (Ithal *et al*. 2007; Jones *et al*. 2013). Nutrient changes have been noted in witches' broom of *T. cacao* caused by the fungus *M. perniciosa* (Scarpari *et al*. 2005; Melnick *et al*. 2012), and galls on *Solidago altissima* caused by larvae of GII *Eurosta solidaginis* and *Gnorimoschema gallaesolidaginis* (Abrahamson and McCrea, 1986). However, whether causal agents derive benefit from these deformations has not been investigated. In the case of the Urdbean leaf crinkle virus (ULCV) it has been shown to cause an increase in Fe, Mg, N and Zn, and decrease in Ca, K, Na and P concentrations in black lentil (*Vigna mungo*), in which it causes crinkling of leaves, stunting of plants and malformation of floral organs. It appears these changes are an indirect result of ULCV infection (Ashfaq *et al*. 2014). Whether this is the case for *Fusarium* spp. that cause MMD remains to be addressed.

Changes in nutrient concentration affect other physiological processes. For instance photosynthesis and transpiration have been shown to change in response to gall formation (Florentine *et al*. 2005; Castro *et al*. 2012). The horn-shaped gall of the Rashed tree (*Copaifera langsdorffii*) caused by an unknown species of Diptera acts as a sink for sugars and amino acids (soluble N) and has been shown to affect

localized photosynthetic output and electron transport rates (Castro *et al*. 2012). Galls induced by *E. strenuana* have been shown to alter mineral (B, Cl, Mg, Z) concentration and flow, as well as to significantly reduce gas exchange, negatively impacts leaf-water potential, stomatal conductance, and rates of photosynthesis and transpiration of *P. hysterophorus* (Florentine *et al*. 2005).

2.3. CONCLUSION

This review broadly summarized observed shape changes in plants as either abnormalities, galls, malformations or witches' broom, as designated by their respected authors (Table 2). Whereas galls and witches' broom appear to have distinct definitions and morphologies, malformation and abnormalities appear to be terms that are used loosely, and may encompass several types of deformations as brought about by terminology such as bracteody, carpellody, distortions, epinasty, petalody, phyllody, sepalody (Table 1). In some cases malformation and abnormality even encompass the more distinct terms of galls and witches' brooms.

Causal agents were shown not necessarily to cause only certain types of symptoms (Table 2). Cases designated as abnormalities are caused by abiotic factors, genetics and phytoplasmas. However examples were too limited to recognize any clear predominance in causality. Witches' brooms appear to be caused predominantly by phytoplasmas with a single reference to fungal causation. Galls are induced by some bacteria, fungi, and mites, but are predominantly caused by insects. From these examples it appears plant galls and witches' brooms appear to show stronger association to specific groups of causal agents. Galls appear to be more associated with insect causal agents, and witches' broom with phytoplasmas. Some nematodes and certain types of bacteria cause very specific types of structures with unique names, namely syncytia and nodules respectively. Most importantly the designation of malformation is associated with examples of all possible causal agents, and is the only deformation associated with viruses.

Studies to understand the ecological function or significance of shape changes in plants is generally lacking. For some types of causal agents and shape changes such as insect galls, more literature and reviews are available. Three proposed

hypotheses on the ecological significance of gall formation in insects have been made, namely that insects may derive nutrition, shelter and/or protection benefits from galls (Price *et al*. 1987; Stone and Schönrogge, 2003). This enables proposed comparison between the other ecological benefits for the other types of biotic causal agents. For instance, similar advantages might be associated with fungal and bacterial galls, but requires further investigation. Phytoplasmas appear to exploit the gall-insect association by producing witches' brooms that to some extent replicate the benefits insects can derive from galls. Furthermore, by attracting perhaps non-galling herbivorous insects to feed on witches' brooms where phytoplasmas are present in the phloem the phytoplasm is sure to find a suitable insect vector (Lee *et al*. 2000). Based on the close evolutionary relationship of phytoplasmas, and some viruses with their hosts and associated vectors (Lee *et al*. 2000) one would expect that there may be more to resultant morphological changes in the plant.

Deformations may simply also just favour the plant. Spontaneous distortion as result of gene mutation or in response to abiotic factors can have a significant evolutionary advantage if it should persist and favour survival of the individual and its progeny (Meyerowitz *et al*. 1989). In cases where no ecological function or significance can be identified change in plant shape can be regarded simply as the plants' non-specific response to a stimulus.

It is widely accepted that phytohormones are involved in formation of abnormalities, galls, malformations and witches' brooms (Zieslin *et al*. 1979; Meyerowitz *et al*. 1989; Singh and Dhillon, 1989a; Cutting, 1991; Hartley, 1998; Lozano *et al*. 1998; Singh, 1998; Tanaka *et al*. 2003; Marasas *et al*. 2006; Rudall *et al*. 2011; Melnick *et al*. 2012; Tarchoun *et al*. 2013; Gohlke and Deeken, 2014; Guimarães *et al*. 2014). The mechanisms how all the different causal agents induce change in phytohormone levels is not fully understood, but some examples indicate that causal agents produce and introduce their own phytohormones into plants (Goethals *et al*. 2001; Tanaka *et al*. 2003; Agrios, 2005), cause genetic alteration in the plant (Morales *et al*. 1999; Chatzivassiliou *et al*. 2000; Hillocks and Thresh, 2000; Quoirin *et al*. 2004; Pãcurar *et al*. 2011; Pallas and Garcia, 2011) or introduce proteins that elicit genetic change (Carter, 1926; Rohfritsch *et al*. 1982; Raman, 2007; Stuart *et al*. 2012; Tokuda *et al*. 2013). Although change in nutrient levels have been associated with

galls, malformations and witches' brooms it does not appear to be causative (Abrahamson and McCrea, 1986; Singh *et al*. 1991; Singh and Singh, 1998; Scarpari *et al*. 2005; Shah *et al*. 2009; Melnick *et al*. 2012). Based on our review, there also does not appear to be a pattern between effects by specific groups of causal agents on phytohormones and nutrients, and the mechanisms used to bring about these changes.

This review has examined the broad range of causal agents, their ecology and mechanisms along with prominent plant physiological components involved in the formation of abnormalities, galls, malformations and witches' brooms. If these aspects are incorporated in an attempt to more clearly distinguish the latter concepts from one another, the following practice is recommended when describing plant disease symptoms related to changes in plant shape as summarized in Figure 2.3. Such an approach will be useful to distinguish the concepts from one another to name and describe a plant disease. In conjunction with this approach, formal definitions of the plant pathology concepts of abnormality, gall, malformation and witches' broom are proposed.

Abnormality *– Plant deformation characterized by symptoms that result in a change in general plant shape of individual organs (change in size, length, placement, fusion of organs, number of a particular organ, absence of a particular organ, distortion, leaf crinkling and/or resetting, tumours, galls and witches' brooms).*

Gall *- Plant abnormality characterized by localized swelling from excessive hyperplasia and/or hypertrophy that positively correlates with interaction of the plant with a pathogen or pest.*

Witches' broom *– Plant abnormality characterized by excessive proliferation and elongation of multiple shoots from a single meristematic area.*

Malformation *– Plant deformation characterized by a complex combination of general plant disease symptoms and plant abnormality symptoms (including galls, swellings, tumours and/or witches' brooms).*

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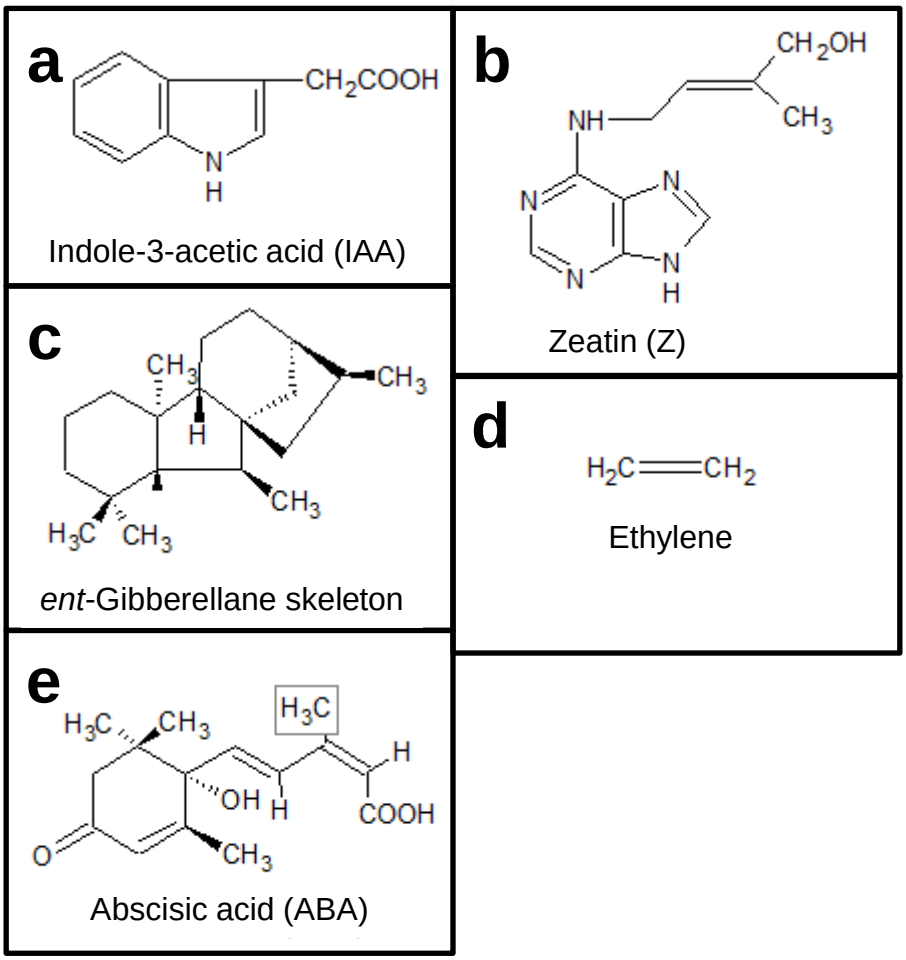


Figure 2.1. Chemical structures of the five classic plant hormones, modified from Kende & Zeevaart (1997) and Hopkins & Hüner (2004) using ChemSketch.Ink Freeware; a) Indole-3-acetic acid (IAA); b) Zeatin, most common naturally occurring auxin; c) *ent*-Gibberellane skeleton; d) Ethylene; e) Abscisic acid (ABA).

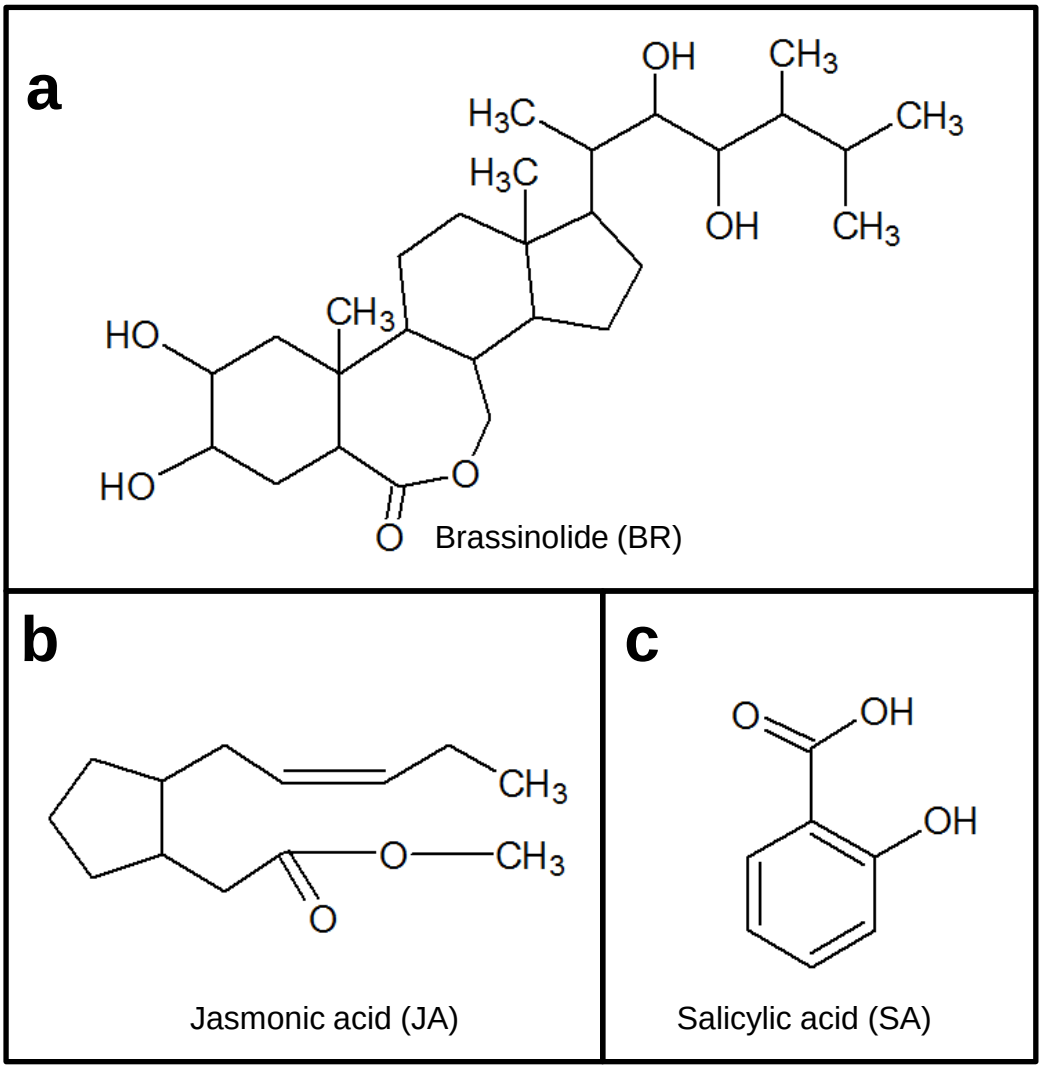


Figure 2.2. Chemical structures of a) brassinilode (BR), b) jasmonic acid (JA) and c) salicylic acid (SA) modified from Hopkins & Hüner (2004) using ChemSketch.Ink Freeware.

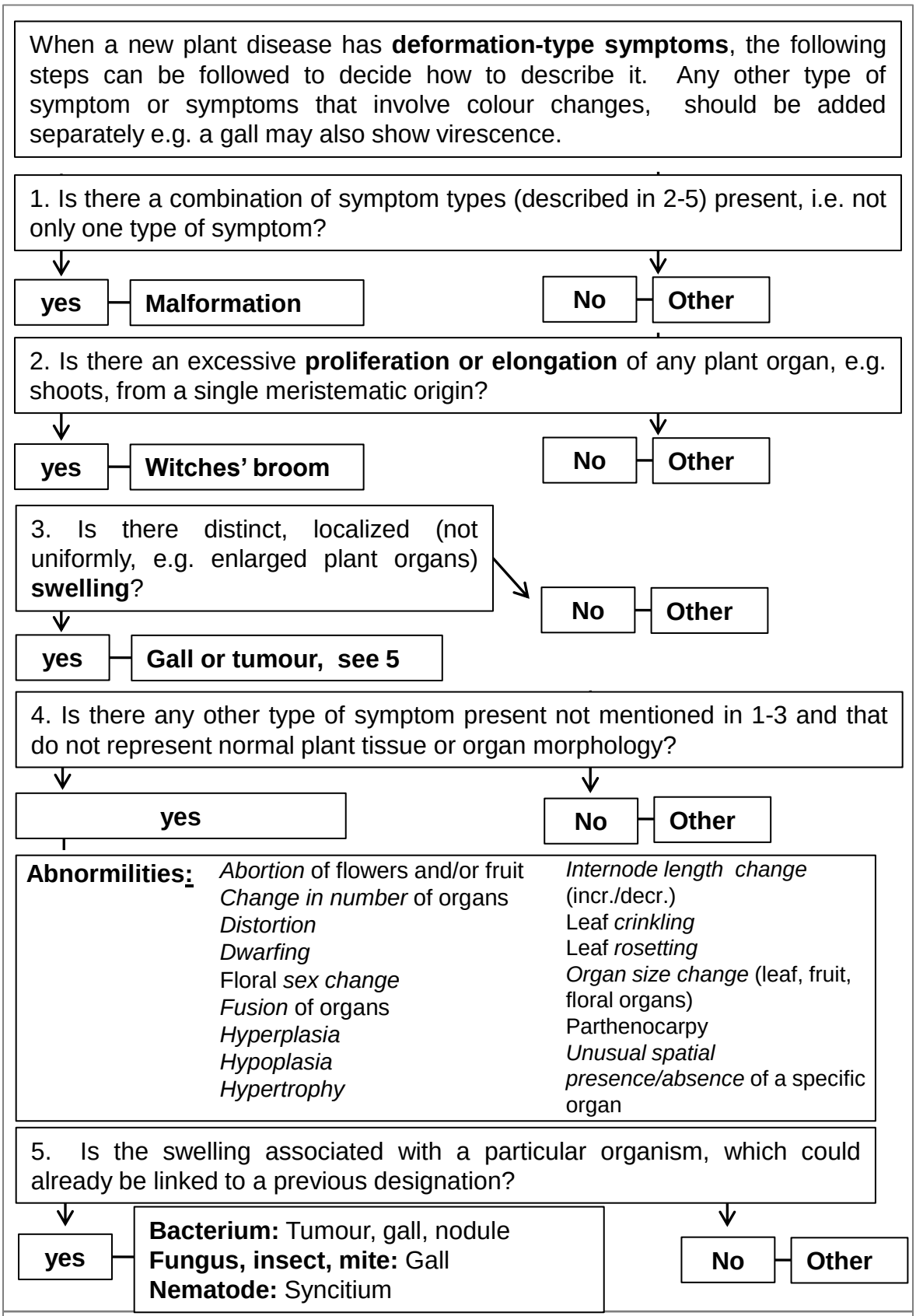


Figure 2.3 Simple dichotomy key to aid distinction between abnormalities: galls, malformations and witches' brooms.

TABLE 1 - GLOSSARY OF TERMS

Term:	Definition:
Abnormality	Growth or development of a plant that differs from normal growth or development primarily as result of genetic deviation, or deviation in response to abiotic conditions.
Anthracnose	Disease characterized by black, sunken lesions.
Atrophy	Degeneration or withering of tissues or organs.
Bracteody	Production of bracts in place of shoots and blossoms.
Carpellody	Production of carpels in place of other floral organs.
Chlorosis	Yellowing of green, photosynthetic tissue.
Distortions	General deformation or change in shape of plant organs.
Epinasty	Downward bending/curving of plant parts e.g. leaves.
Fasciation	Perpendicular elongation in direction of growth of a plants apical meristem.
Galls	A type of plant malformation distinguished by excessive, localized swelling in response to a biotic inducer (primarily insects and fungi) that derives benefit in terms of nutrition, shelter and/or protection from predators.
Hyperplasia	Overgrowth due to increased cell division.
Hypertrophy	Overgrowth due to abnormal cell enlargement.
Hypopolasia	Incomplete or underdevelopment of a tissue or organ.
Malformation	Secondary effect of abnormal growth and development in plants caused by bacteria, phytoplasmas, fungi, insects, nematodes and plant viruses. This includes calls, witches' broom and other uncharacterized abnormal growths and development determined to be caused by biotic agents.
Necrosis	Tissue die-off.
Parthenocarpy	Formation of fruit without pollen fertilization.
Petalody	Production of petals in place of other floral organs.
Phyllody	Production of vegetative leaves in place of other floral organs.
Sepalody	Production of sepals in place of other floral organs.
Staminody	Production of stamens in place of other floral organs.
Virescence	Normally white or coloured tissue that develops chloroplasts and becomes green.
Witches' broom	A type of plant malformation characterized by dwarfed leaves, elongated internodes and proliferation of shoots taking on a broom-like appearance, primarily caused by bacteria (including Phytoplasmas) and fungi.

(Meyer, 1966; Cambridge Learner's Dictionary, 2003; Agrios, 2005; Horst, 2008)

TABLE 2 - EXAMPLES OF ABNORMALITIES, GALLS, MALFORMATIONS AND WITCHES' BROOM FROM LITERATURE INCLUDING DESCRIPTION AND CAUSAL AGENTS WHERE AVAILABLE.

Plants speces:	Description:	Designation:	Causal agent:
>450 Plant species	Increased cell division and enlargement (hyperplasia and hypertrophy) around feeding wound (Jones *et al*. 2013).	Malformation	Nematode - *Ditylenchus dipsaci*
Acacia mearnsii	Multiple mersitematic centres, hypertrophied shoots and suppressed elongation (Quoirin *et al*. 2004)	Leafy gall (fasciation)	Bacteria - *Rhodococcus fascians*
Almond	Early flowering. Stunted growth. Leaf rosetting. Dieback. Off-season growth. Proliferation of shoots. Witches' broom (Abou-Jawdah *et al*. 2002).	Witches' broom	Bacteria - AlmWB Phytoplasma
Asclepias curassavica	Undescribed malformation and necrosis of apical leaves (Thompson & Van Zijl, 1995; Salomone *et al*. 2003).	Malformation	Virus - Tomato Spotted Wilt Virus vectored by *Frankiniella schultzei* and *Thrips tabaci*
Bamboo spp.	Dwarfed leaves, elongated internodes, and bud proliferation (Tanaka *et al*. 2003).	Witches' broom	Fungus - *Aciculosporium take*
Byrsonima sericea	Excessive branching from main axis and floral buds as result of undeveloped gynoecium; phyllody; degeneration of stamens (Guimarães *et al*. 2014).	Witches' broom - Gall	Fungus - Unidentfied
Capsicum annuum	Cultivar dependant changes in ovary diameter, style length, number of ovules and locules, and length and diameter of flowers. Additional variables affecting fruit set percentage and condition (Tarchoun *et al*. 2003).	Abnormality	Abiotic factors - low night temperature
Capsicum annuum	Reduced size and deformation of fruit, stunting, shoot proliferation, and curling and deformation (malformation) of leaves (Saied *et al*. 2014).	Malformation	Bacteria - *Spiroplasma citri*
Castanea crenata	Small leaves. Yellowing of young leaves (Jung *et al*. 2002).	Witches' broom	Bacteria - '*Candidatus* Phytoplasma castaneae'
Celosia argentea *Celosia spicata*	Undescribed leaf malformation associated with witches' broom (Eckstein *et al*. 2012).	Malformation and witches' broom	Bacteria - Phytoplasma of the 16SrIII-J subgroup
Cirsium arvense	Leaf malformation described as severe leaf curling, and leaf edge enrolling and upfolding (Rancic *et al*. 2006).	Malformation	Mite - *Aceria anthocoptes*
Conifers	Vegetative growth from cone apex, and bisexual cones in a normally unisex group (Rudall *et al*. 2011).	Abnormality	Unknown genetic occurrence
Copaifera langsdorffii	Pilous, closed, horn-shaped galls. New growth is red becomiing brown and glabrous when mature. Cell in gall cortex are compact with reduced intercellular spaces as result of hypertrophy and homogenization of parenchyma cells (Castro *et al*. 2012).	Horn-shaped gall	Insect - Unknown Diptera sp.

Cornus florida	Localized trunk swelling and bark disruption (Santamour & McArdle, 1987).	Dogwood canker and malformation	Nematode - *Aphelenchoides fragariae* and *Panagrolaimus subelongatus*
Dipsacus laciniatus	Reduced size of inflorescences, distortion of leaves, shortened internodes, reduced growth, and chlorosis and necrosis of leaves (Pećinar *et al*. 2009).	Malformation	Mite - *Leipothrix dipsacivagus*
Eucalyptus spp.	Typicals galls described as distinct swelling of leaf midribs, petioles and stems that may cause stunting (Kim *et al*. 2008).	Gall	Insect - *Leptocybe invasa*
Ficus spp.	Undescribed leaf malformation in addition to leaf and fruit mosaic, and fruit drop (Ashihara *et al*. 2004).	Malformation	Mite - *Aceria ficus*
Gladiolus spp.	Undescribed floral spike malformation associated with leaf stripe, colour-breaking, yellowing, stunted growth, small corm and underdeveloped root system (Raj *et al*. 2009).	Malformation	Bacteria - '*Candidatus* Phytoplasma asters' (16SrI group)
Glycine max	Fusing together of plant cells (could be considered hyperplasia) to form feeding sites (Ithal *et al*. 2007).	Cysts (syncytia)	Nematode - *Heterodera glycine*
Gossypium sp.	Extreme reduction of leaves, floral buds and other organs, chlorophyll deficiency leading to mosaic appearance, and then variety dependant buckling of leaves, reddening, dieback, shortening of internodes and increasing number of branches, dwarfing, floral bud abortion. Described as plant distortion/crippling/abnormality/aberrant growth, or also generalized gall-formations or similar to witches' broom structures (Cook, 1923).		Unknown
Hibiscus rosa-sinensis	Excessive axillary branching. Small leaves. Deformed flowers (Montano *et al*. 2001).	Witches' broom	Bacteria - '*Candidatus* Phytoplasma brasiliense
Juniperus virginiana	Galls produced from transformed axillary buds (Stewart, 1915; Dervis *et al*. 2010).	Gall	Fungus - *Gymnosporangium juniper-virginia*e and *G. globosum*
Lycopersicon esculentum	Homeotic transformation - affecting stamen and carpel identity producing intermediate organs. Meristematic transformation - producing excess number of organs of the reproductive whorl (Lozano *et al*. 1998).	Abnormality	Abiotic factors - Low temperature
Lycopersicon esculentum	Sepal hypertrophy, virescence, phyllody and aborted reproductive organs (Pracros *et al*. 2006).	Floral abnormalities	Bacteria - Stolbur Phytoplasma (isolate PO)
Lyonia ovalifolia	Leaf malformation described as concave adaxial, and convex abaxial leaf surface of affected areas and resultant subglobose shape (Li & Guo, 2008).	Leaf malformation	Fungus - *Exobasidium ovalifoliae*
Malus domestica	Different parts of the fruit develop at different rates, resulting in distorted fruit shape (Fryer 1916; Carter, 1962).	Malformation	Insect - Capsid bug

Mangifera indica	Floral malformation (short, thick, branched inflorescences. Increased number and size of predominantly male flowers with poor pollen viability. Few bisexual flowers bear enlarged, non-functional ovaries) and vegetative malformation (loss of apical dominance, and small, clumped shootlets with scaly leaves often described as witches' broom) (Marasas *et al*. 2006; Krishnan *et al*. 2009).	Malformation	Fungus - *Fusarium mangiferae, F. proliferatum, F. sterilihyphosum*
Manihot esculenta	Malformation/distortion as result of unequal leaf expansion caused by stress induced by chlorotic patterns on leaves (Hillocks & Thresh, 2003).	Malformation / distortion	Virus - Cassava Mosaic Disease
Medicago sativa	Proliferation of shoots, yellowing of leaves and tillering of stems (Khan *et al*. 2002).	Witches' broom	Bactera - AlfWB Phytoplasma
Not specified	Misshapen and aborted leaves on clusters of fleshy stems at crown of affected plant (Goethals *et al*. 2001).	Witches' broom	Bacteria - *Rhodococcus fascians*
Oryza sativa	Undescribed plant malformation, foliar necrosis and seedling death (Morales *et al*. 1999; Johnson *et al*. 2012).	Malformation	Virus - Rice Stripe Necrosis Virus vectored by *Polymyxa graminis*
Pachycereus pringlei	Tumorous gall-like malformations that vary in shape, surface texture and size - ball-like to fragmented, smooth to rough and cracked, and 1 - 70 cm respectively (Dubrovsky & De La Luz, 1996).	Tumorous gall-like malformation	Unknown
Parthenium hysterophorus	Gall formation with accompanied reduction in main shoot length, and flower and leaf production (Florentine *et al*. 2005).	Gall	Insect - *Epiblema strenuana*
Phoenix dactylifera	Increased number of carpels, undescribed distortion of carpels and stigmas and impaired pollen tube elongation (Cohen *et al*. 2004).	Abnormality	Genetic
Populus spp.	Solid swollen mass (Petranović & Kielkiewicz, 2010).	Galls	Mites - *Aceria parapopuli* & *A. populi*
Protea spp.	Proliferation of young shoots and leaves (Cutting, 1991; Wieczorek & Wright, 2003)	Witches' broom	Bacteria - Phytoplasma
Prunus persica	Numerous, deep indendations in fruit with corky tissue (Fenton *et al*. 1944; Fenton & Brett, 1946).	Malformation 'catfacing'	Insect - *Lygus oblineatus*
Prunus spp.	Rough, spindle-shaped galls on woody tissues (Fernando *et al*. 2005; Zhang *et al*. 2005).	Gall	Fungus - *Apiosporina morbosa*
Quercus ruber	Woody galls on terminal buds (Hartley & Lawton, 1992).	Gall	Insect - *Andricus lignicol*a
Quercus spp.	Described as general plant growths induced by the GII, with slight morphological variation depending on which species of *Andricus* is in question (Stone & Cook, 1998).	Gall	Insect - *Andricus* spp.
Rhododendron sp.	Undescribed leaf malformation (Hernández & Hennen, 2003).	Leaf malformation	Fungus - *Exobasidium* spp.

Rosa hybrida cv. Baccara	Reduced length to diameter ratio of floral bud, causing a flattened appearance with additional increase in size and number of floral buds (and thus weight), increased number of petals and proliferation of secondary shoots (Zieslin *et al*. 1979).	Bullhead malformation	Abiotic factors - low night temperature
Rosa hybrida cv. Madelon	Cessation of growth and development, remaining buds with no carpels and stamens tightly packed in centre of receptacle (Chimonidou-Pavlidou, 2004).	Malformation	Abiotic factors - drought stress
Spartium junceum	Excessive number of shoots with extremely shortened internodes sprout from numerous axillary buds (Marcone *et al*. 1996).	Witches' broom	Bacteria - Unknown Phytoplasma
Syzygium cordatum	Enlarged, excessively branched sterile flowers (Kvas *et al*. 2008)	Malformation	Fungus - *Fusarium* spp.
Theobroma cacao	Large, hemespherical galls on flower cushions, leaf nodes and wounded parts of branches and stems (Ploetz, 2006; Ploetz, 2007).	Cushion- or green back gall	Fungus - *Fusarium decemcellulare*
Theobroma cacao	Disorganized proliferation of new shoots (Aime & Phillips-Mora, 2005; Leal *et al*. 2007).	Witches' broom	Fungus - *Moniliophthora perniciosa*
Vachellia karroo	Swollen, fleshy tissue at the apex of seed and flower pedicels (McGeogh, 1993).	Gall	Fungus - *Ravenelia macowaniana*
Vigna mungo	Extreme crinkling, puckering and rugosity of leaves. Stunting of the plant. Undescribed floral malformation (Ashfaq *et al*. 2014).	Malformation	Virus - Urdebean Leaf Crinkle Virus (ULCV)
Vitis vinifera	Tumor/gall formation on the stem and crown, and necrotic lesions on roots (Escobar & Dandekar, 2003)	Crown gall	Bacteria - *Agrobacterium vitis*
Zea mays	Severe swelling of leaf veins and stunted growth (Tokuda *et al*. 2013).	Gall	Insect - *Cicadulina bipunctata*
Zizania latifolia	Hypertrophy, ceased development of seeds and inflorescneces (Yang & Leu, 1978; Chung & Tzeng, 2004).	Gall	Fungus - *Ustilago esculenta*

TABLE 3 - ESSENTIAL ELEMENTS & THEIR FUNCTION IN PLANTS.

MACRONUTRIENTS

Element:	**Function:**
Calcium (Ca)	Plays a role in cell division; required for physical integrity and function of membranes; regulating certain enzymes; implicated in hormonal and environmental responses.
Carbon (C)	Important component of organic compounds.
Hydrogen (H)	Important component of organic compounds.
Magnesium (Mg)	Component of chlorophyll; stabilizes ribsosome structure; activator of numerous enzymes.
Nitrogen (N)	Incorporated into amino acids, proteins, nucleic acids, hormones and chlorophyll.
Oxygen (O)	Important component of organic compounds.
Phosphorus (P)	Important role in photosynthesis and intermediary metabolism; component of nucleotides and phospholipids.
Potassium (K)	Activator of certain enzymes; plays role in starch and protein synthesis; regulates osmotic potential e.g. opening/closing of stomata.
Sulfur (S)	Important in electron transfer reaction of photosynthesis and N-fixation; Constituent of proteins, vitamins and coenzyme A (important component of respiration and fatty acid metabolism).

MICRONUTRIENTS

Element:	**Function:**
Boron (B)	Role in cell division; structural inegrity of cell walls.
Chlorine (Cl)	Component of oxygen-evolving complez; maitaining charge balance across membranes.
Copper (Cu)	Important co-factor for various oxidative enzymes.
Iron (Fe)	Component of redox enzymes involved in photosynthesis, N-fixation and respiration; necessary for chlorophyll synthesis.
Manganese (Mn)	Co-factor of numerous enzymes and oxygen-evolving complex.
Molybdenum (Mo)	Important role in N metabolism.
Nickel (Ni)	Co-factor for enzymes involved in N metabolism.
Zinc (Zn)	Activator of numerous enzymes; possible association with auxins.

(Hopkins & Hüner, 2004; Nabors & González-Barreda, 2004; Agrios, 2005)

TABLE 4 - THE "FIVE CLASSIC" PHYTOHORMONES, WHERE TO FIND THEM & WHAT THEY DO.

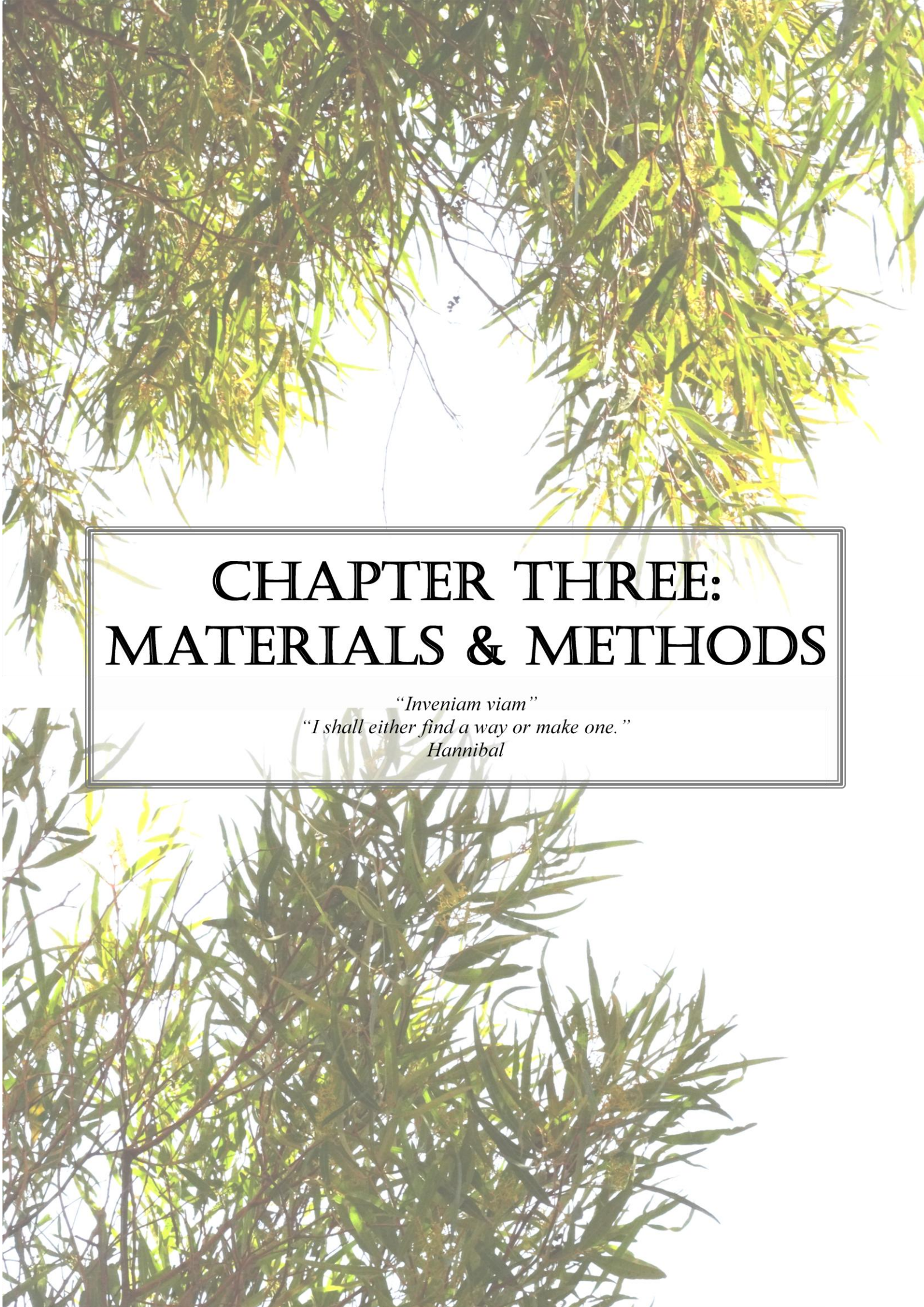
Phytohormone:	Isolated from:	Functions & responses:
Abscisic acid	Fruit	Induces protein storage synthesis
	Leaves	Inhibits growth
	Roots	Modulates water stress (stomatal closure)
	Stems	Promotes dormancy
Auxin	Apical buds	Apical dominance
	Embryos	Cell enlargement and elongation
	Flowers	Gravitropism
	Roots	Permeability of membranes
	Seeds	Phototropism
	Shoots	Regulates development of fruit
	Young leaves	Promotes root and shoot growth
		Vascular diferentiation
Cytokinin	Fruit	Chloroplast development
	Leaves	Growth of lateral buds
	Roots	Leaf expansion
	Seeds	Promotes root growth and differentiation
		Promotes shoot growth
		Retards aging
		Stimulates cell division and growth
		Stimulates germination
Ethylene	Fruit	Permeability of membranes
	Leaves	Chlorosis
	Roots	Induces plant resistance to infection
	Seeds	Inhibitory and abnormal growth responses
	Stems	Leaf abscission
	Tissues undergoing senescence	Promotes ripening of some fruits
	Seeds	Promotes seed germination
		Reduced apical dominance
		Secondary growth of stems and roots
		Stimulates formation of adventitious roots
Gibberellins	Embryos	Promotes bud growth
	Fruit	Promotes leaf growth
	Meristematic regions	Promotes cell division
	Roots	Promotes seed germination
	Seeds	Promotes stem elongation
	Young leaves	Stimulates mobilization of endosperm during embryo growth and for development of flowers and fruits

(Kende & Zeevaart, 1997; Hopkins & Hüner, 2004; Nabors & González-Barreda, 2004; Agrios, 2005)

TABLE 5 - BRASSINOSTEROIDS, JASMONIC ACID & SALICYLIC ACID, WHERE TO FIND THEM AND WHAT THEY DO.

Phytohormone:	Isolated from:	Functions & responses:
Brassinosteroids	Flowers	Stem elongation
	Fruit	Vasacular differentiation
	Leaves	Leaf morphogenesis
	Seeds	Leaf abscission
	Stems	Promotes pollen tube growth
		Retards root growth and development
Jasmonic acid	Throughout plants, highest concentrations in actively growing tissues.	Plant defense responses
		Inhibits seed, pollen and root growth
		Stimulates formation of flowers, fruits and seed
		Promotes protein accumulation during seed development
Salicylic acid	Throughout plants.	General plant growth and development
		Ion uptake
		Inhibits ethylene synthesis
		Photosynthesis
		Seed germination
		Thermogenesis
		Flower phenology

(Creelman & Mullet, 1995; Popova et al. 1997; Yokota, 1997; Hopkins & Hüner, 2004; Nabors & González-Barreda, 2004; Agrios, 2005; Spoel et al. 2007; Van Loon, 2007; Rivas-San Vicente & Plasencia, 2011)



CHAPTER THREE: MATERIALS & METHODS

"Inveniam viam"
"I shall either find a way or make one."
Hannibal

3.1. COLLECTION OF SAMPLES FROM DISEASED TREES

A series of surveys of the common karee (*Searsia lancea*) was done at several localities (Figure 3.1.1.) across the range of *S. lancea* (Figure 1.1., chapter 1) as established by the South African National Biodiversity Institute (SANBI, Pretoria, Gauteng Province) from collection data. This was done to determine the rough geographical range of the malformation symptoms. From these, four localities (Figure 3.1.2) were identified to establish transects recording malformation occurrence per tree as observed percentiles across ten trees. These included Bloemfontein (Free State Province, 29°07'47.89"S; 26°09'53.18"E), Christiana (North West Province, 27°54'44.23"S; 25°09'42.35"E), Kimberley (Northern Cape Province, 28°45'12.44"S; 24°46'17.70"E) and Tshwane (Gauteng Province, 25°45'04.05"S; 28°15'45.93"E).

Sampling was conducted during autumn and winter of 2013 – 2014 corresponding to the temporal range of flowering and fruit-bearing of *S. lancea* (Coates-Palgrave, 2002). Malformed and healthy samples were sampled for comparison from the same tree. Malformed samples consisted of the entire malformed shoot and its extension (petiole/peduncle/branchlet) from the main shoot or branch from which it was borne. Healthy samples were floral (or vegetative shoots if flowers/fruit were absent) of similar size and age to malformed shoots, and the peduncle or petiole on which it was borne. Samples were removed from trees using garden shears, placed in brown paper bags and transported to the laboratory for isolations and the collection of insects.

Field observations were supplemented by closer examination of the morphology of malformed tissues under a stereomicroscope (Olympus SZX10) with a camera (Olympus DP72) and imaging software (Olympus CellSens Standard 1.13).

3.2. FUNGAL DIVERSITY

3.2.1. Isolation of fungal species

Fungi were isolated from up to 20 healthy and malformed samples, respectively of *S. lancea* for each site. Each sample was divided into separate glass petri dishes

containing healthy or malformed leaves, twigs/branches and inflorescences. Each of these was cut into approximately 5 mm x 5 mm sections. Sections were surface sterilized by sequential submergence (0.5 min 96% ethanol; 5 min commercial bleach; 1 min 96% ethanol). Samples were then placed on potato dextrose agar (PDA) (20 g dextrose; 20 g agar; broth from 250 g potatoes made up to 1 litre) and kept in an incubator at 25°C to facilitate fungal growth. Cultures were grouped into morphotypes based on differences in colour, shape and texture of cultures on the primary PDA plates (Leslie and Summerell, 2006). Needle scrapings of each morphotype in a culture were transferred to new, separate PDA plates and grown at 25°C in an incubator until sporulation occurred. Mature cultures were identified under a microscope to genus level where fruiting structures could be found (Leslie and Summerell, 2006).

3.2.2. Identification of *Fusarium* species

Fungal cultures morphologically resembling *Fusarium* spp. were selected from the fungal culture collection. *Fusarium* cultures are usually fluffy and vary between white and various shades of pink, orange, red, yellow and brown, and usually have a typical curved, septate macroconidia and microconidia of various shapes borne on phialides (Leslie and Summerell, 2006). Single spore isolations were conducted to ensure that cultures represent a single individual and species (Leslie and Summerell, 2006). A small scraping from a freshly grown culture was transferred to SNA petriplates that stimulates sporulation. A scraping from the SNA culture was dissolved in a 1 ml droplet of distilled water on a water agar plate and spread with a bent glass rod. Single hyphal extensions (24 – 36 hours) from germinating spores were transferred with a needle to fresh PDA plates. If cultures did not sporulate a single hyphal tip was removed from each isolate and transferred to PDA. Cultures were deposited with the National Collection of Fungi (Agricultural Research Council, Pretoria, South Africa).

a) *Deoxyribonucleic acid (DNA) extraction*

Fusarium isolates were identified to species level by DNA sequence comparison (O'Donnell *et al*. 2009). Extraction of DNA was done according to a modified method by Möller *et al*. (1992). Fungal tissue was obtained by scraping freshly grown

cultures, freeze drying (Virtis, Feezemobile II, SP Scientific, United States) approximately 0.5 g fungal tissue and homogenized (Qiagen's Tissue Lyser, Haan) to a powder. Powdered tissue was placed in an Eppendorf tube with 500 µl TES (100 mM Tris pH 8.0; 10 mM EDTA; 2% SDS), and 200 µl (50 – 100 mg) Proteinase K and vortexed. The solution was incubated for 60 min at 60°C, upon which 140 µl (5M) sodium chloride (NaCl) and 65 µl 10% CTAB were added, vortexed and incubated for 10 min at 60 °C. One volume (450 µl) SEVAG was added and vortexed, and the solution was incubated for 30 min at 0 °C, then centrifuged at 12000 rpm for 20 min at 4 °C. Approximately 1000 µl of the supernatant was transferred to a fresh tube, 440 µl isopropanol added, mixed and left refrigerated overnight at 4°C. The supernatant was discarded, the DNA pellet washed twice with cold 70% ethanol and dried on a heat block for one hour in a laminar flow. The pellet was dissolved in Millipore purified water and frozen overnight to facilitate the DNA to dissolve. RNA-ase (2 µl) was added to the thawed solution and left at 37°C for 120 min to reduce RNA contamination. DNA concentrations were determined with a Nanodrop2000 (Thermo Fisher, South Africa). DNA was aliquoted and diluted to 20-50 µg/ml and the rest was stored at -20 °C.

b) *Polymerase chain reaction (PCR) and sequencing*

Using Primers EF-1 (5'-ATGGGTAAGGG(A/G)GACAAGAC-3') and EF-2(5'-GGA(G/A)GTACCAGT(G/C)ATCATGTT-3') (O'Donnell *et al*. 1998) the Translation Elongation Factor 1-alpha (TEF-1α) gene region was amplified. Accurate distinction of species in current *Fusarium* species complexes has been achieved using this gene region (O'Donnell *et al*. 2010). Following PCR reactions performed at appropriate parameters using the Robust PCR kit (KAPA Biosystems, South Africa) according to the instructions of the manufacturer, the products of amplification were visualized using 1% agarose gels (Cleaver Scientific, AEC-Amersham, South Africa) containing Gelred DNA stain (Biotium, Anatech, South Africa) under UV visualization using a Geldoc XR+ imaging system (Bio-Rad, South Africa). Using the EXO/SAP Amplicon Purification system, PCR amplicons were purified (Werle *et al*. 1994). The purified PCR product was sent to the Microbiology Department of the University of the Free State for sequencing

c) *Phylogenetic identification*

Using Geneious v. 7.0.6 (Biomatters, New Zealand) chromatograms were compiled in contigs and verified manually. These DNA sequences were compared to those of valid existing *Fusarium* species on Genbank (http://www.ncbi.nlm.nih.gov/genbank) the *Fusarium* DNA databases FUSARIUMID v.10 (http://isolate.fusariumdb.org/blast/php) (Geiser *et al*. 2004) and the *Fusarium* Multilocus Sequence Typing (MLST) database (http://www.cbs.knaw.nl/fusarium)to determine the appropriate species complex. DNA datasets for the relevant species complexes were obtained from Dr. Kerry O'Donnell (United States Department of Agriculture, USA) and supplemented where necessary through additional relevant sequences from other publications. Using MEGA v.6.06 (http://www.megasoftware.net/)phylogenetic analyses were performed to accurately place with relevant species. DNA datasets were aligned using the MEGA Muscle function and manual verification of alignments. Maximum likelihood analyses were done after appropriate evolutionary models were determined with MEGA and confidence level of branches was determined using a 1000 replicate bootstrap analysis.

3.3. INSECT DIVERSITY

An insect survey was conducted simultaneously with the survey for fungi. The same samples collected for fungal isolates were screened for the presence of insects prior to fungal isolations. The entire content of each brown paper bag for both the malformed and healthy samples was deposited into a glass petri dish and studied under an Olympus SZX10 stereomicroscope (Wirsam, South Africa). Insects present were collected using a needle and forceps, placed in small, marked vials containing 80% ethanol and stored at 4°C. The samples were then cut into 5 mm x 5 mm sections and different parts (leaves, twigs/branches, inflorescences, malformations) studied separately to collect and store any stray insects. The collected insects were separated into different morphological species groups (MSP) based on obvious morphological characteristics. Identifications to family level were obtained by Mr Jaco Saaiman, Mr Ian Cloete and Mr Delroy Mabunda who were postgraduate students of the Zoology and Entomology Department of the University of the Free

State (Bloemfontein). Two MSP's were identified to genus level and two more to species level by the Biosystematic Division of the Agricultural Research Council (ARC, Pretoria, Gauteng Province) out of the 18 MSP submitted for identification.

3.4. PHYTOHORMONE AND NUTRIENT ANALYSIS OF SAMPLES

3.4.1. Phytohormone analysis by ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS)

Phytohormone analyses samples were collected in Tshwane (Gauteng Province) where a large concentration of symptoms was found close to laboratories where the analyses could be conducted. Samples were collected similar to those for fungal and insect comparisons, but were immediately placed in Falcon tubes containing liquid nitrogen for flash freezing. This was done to cease or stall metabolic activity within the samples as it is known that phytohormone levels change in response to changes in temperature, humidity and radiation (Du *et al*. 2012). Initial sample preparation involved homogenization of samples at the laboratory of Dr. Sanushka Naidoo (University of Pretoria). Following freezing once more with liquid nitrogen homogenized samples were transported to the Food and Drug Assurance Laboratories (FDA, Pretoria, Gauteng Province) for final preparation and analysis.

Due to faulty equipment at the University of the Free State at the time of this study the phytohormone analysis was conducted at the Food and Drug Assurance Laboratories (FDA labs, Pretoria, Gauteng Province). Biological standards of only three phytohormones namely gibberellic acid (GA_3), jasmonic acid (JA) and salicylic acid (SA) were available to quantify the presence of these phytohormones in tissues of *S. lancea*. Changes in GA_3 concentration has been associated with plant deformations (Zieslin *et al*. 1979; Singh and Dhillon, 1989; Lozano *et al*. 1998; Singh, 1998; Tarchoun *et al*. 2013), whereas this study presents a novel comparison of JA and SA between healthy and diseased plant tissues.

a) *Sample preparation*

A weighed amount of 0.2 g of each sample was placed into separate 50 ml polypropylene tubes and suspended in 2 ml Bieleski's solvent [75% methanol (MeOH) + 20% deionised water (H_2O) + 5% formic acid (CH_2O_2)]. The suspension

was sonicated at 50 Hz for 5 min, shaken on a platform at room temperature for 30 min, and centrifuged at 9500 g at 4°C for 10 min. The supernatants were collected and stored. The pellet was re-suspended in 2 ml Bieleski's solvent, shaken on a platform at room temperature for 30 min and again centrifuged at 9500 g at 4 °C for 10 min. The resulting supernatants were mixed, 1ml of each was filtered through nylon Clarinert syringe filters (0.22 µm filter pore size, 13 mm, Agela Technologies, Wilmington, DE 19808, US) and the filtrate was stored in vials at -4°C to be used for JA and SA detection and quantification (Segarra *et al*. 2006).

For GA_3 content determination the remaining supernatants, after 1 ml was removed from each for JA and SA detection, were used. The mixed samples were air dried at 60°C in a TurboVap LV (Biotage) concentration evaporator and the resulting dried extracts were re-suspended in a solution of 90% deionised water (H_2O), 10% MeOH and 0.1% CH_2O_2. Solid phase extraction (SPE) cartridges (C8/SCX, 500 mg, AgelaTechnologies) were conditioned with 5 ml MeOH and 5 ml (1M) CH_2O_2. Samples were eluted using 3 ml MeOH, air dried in the concentration evaporator at 60°C, re-suspended in 200 µl 10% MeOH filtered through nylon Clarinert syringe filters and stored in vials at -4°C.

b) *Chromatography and detection of phytohormones*

Ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) is a rapid, highly sensitive method of quantitative analysis for phytohormones in small tissue samples of any part of any plant species (Chiwocha *et al*. 2003; Izumi *et al*. 2009; Balcke *et al*. 2012). It is superior to conventional gas chromatography mass spectrometry (GC-MS) because it is able to analyse compounds that cannot be volatized well, or are at risk of thermal degradation (Pan and Wang, 2009). In addition this method allows for the analysis of many compounds without derivatization steps.

One millilitre of each sample was transferred to separate 1 ml glass vials (12 mm screw cap, Stargate Scientific, Roodepoort, South Africa), loaded in the auto sampler (Shimadzu, SIL20ACXR) and introduced to mobile phases A [95% H_2O + 5% acetonitrile (C_2H_3N) + 0.05% acetic acid ($C_2H_4O_2$)] and B (5% H_2O + 95% C_2H_3N + 0.05% $C_2H_4O_2$) by a Shimadzu LC-20AD UPLC binary pump. Column conditions were sequentially exposed to mobile phases A and B (4 min at 100% A, gradually

increased for 4 min to 100% B, kept for 6 min at 100% B) and finally returned to the initial gradient conditions. The different components (analytes) of the sample solution/mobile phases mixture were separated at a 300 µl/min flow rate on a Luna 5 µM C_{18}(2) 150 x 2.00 mm column (Phenomenex, USA) (stationary phase) at 40°C. Separation was achieved in a 20 min gradient run. The intensity (counts per second - cps) of each analyte was recorded.

Multiple reaction monitoring (MRM) was used with -4500 V (500°C source temperature). These were the following: MRM1 of 208.920 – 58.900 and MRM2 of 208.920 – 164.900 for JA; MRM1 of 136.771 – 93.000 and MRM2 of 236.771 – 64.900 for SA; and MRM1 of 344.897 – 143.000 and MRM2 of 344.897 – 239.100 for GA_3. The curtain gas was set to 30 pounds per square inch (psi), GS1 to 30 psi, and GS2 to 40 psi. The CAD gas was set to 8 psi, and collision energy (CE) and de-clustering potential (DP) set to compound specific optimization conditions. Standard curves, or spiked concentrations were constructed using the signal area of mass for each analyte to determine unknown concentrations of analytes within the samples, and were presented as µg/kg.

3.4.2. Nutrient quantification

Three mineral nutrients were investigated, namely nitrogen (N), phosphorus (P) and potassium (K). These nutrients were selected based on their general importance for plants, for instance they constitute the majority of commercial fertilizers, and indications that their levels change in response to malformation in *Mangifera indica* (Singh *et al*. 1991). In addition the perception exists that these nutrients are important in phytohormone structure, for instance N, intermediary metabolism for P and activating enzymes for K (Hopkins and Hüner, 2004; Nabors and González-Barreda, 2004; Agrios, 2005). Studies on changes in levels of these are indicative of the cascade of chemical events that accompany appearance of malformations in general, including in *S. lancea*.

Because large amounts of tissues are needed for nutrient analyses samples were collected in Christiana (North-West Province) at a site where particularly large and numerous malformations were found. Samples were placed in brown paper bags and transported directly to the Animal, Wildlife and Grassland Sciences Laboratory

of the University of the Free State (Bloemfontein, Free State Province) for sample preparation and analysis.

a) *Sample preparation*

Samples were dried in an oven at 105°C (±2°C) and approximately 0.22 mg of the dried sample was removed for N quantification (Balthrop *et al*. 2011). For the K and P quantifications additional steps were needed. Approximately 1 g of each sample was measured into a tin cup and placed in a cool furnace with a gradual increase in temperature up to 550 °C (±20 °C) over 90min for P quantification (Balthrop *et al*. 2011). The samples were incinerated for 4 – 16 hours, left to cool after the furnace was opened, dissolved in 10 ml 25 % HCl solution placed over a boiling water bath and covered with watch glasses for 60 min. Once cooled the samples were transferred and made up to the 100 ml mark in volumetric flasks with distilled water, and filtered. Using 5 ml of the filtrates dilutions were made with distilled water up to the 250 ml mark in volumetric flasks. Ten millilitres of each dilution then transferred separately to new volumetric flask and 2.5 ml 3% strontium chloride ($SrCl_2$) solution added to each and made up to the 100 ml mark with distilled water resulting in 500 x dilutions.

For K quantification approximately 5 g of the sample was incinerated similar to the samples for P quantification. The resultant ash was transferred to a 250 ml beaker and moistened with deionised water. Hydrochloric acid (12M) was added drop-wise and agitated until effervescence ceased. The sample was dried by evaporation with occasional stirring using a glass rod. Once dried 15 ml HCl (6M) was added to the residue along with 120 ml deionised water. The solution was stirred with a glass rod that remained in the beaker and was covered by a watch-glass. The solution was gently brought to and maintained at boiling temperature until it appeared that the ash was dissolved to satiation. The solution was filtered through ash free filter paper and filtrate was collected in a 250 ml volumetric flask. The residue on the filter paper was expected to be white or near white (Balthrop *et al*. 2011). If, however, the residue on the filter paper was black the residue was incinerated again between 450 – 475 °C for 3 – 5 hours, or until the ash appeared white or nearly white. It was then dissolved with 2 ml HCl (12M) and evaporated to dryness. An additional 5 ml HCl (6M) was added to the filtrate, filtered through ash free filter paper and collected in a 250 ml

volumetric flask. The solution in the volumetric flask was made up to the 250 ml mark with deionised water (Balthrop *et al*. 2011).

b) *Nutrient quantification*

i. Nitrogen (N) content by combustion

The Dumas principle was used to determine the N content of a sample (Balthrop *et al*. 2011). This requires combustion at 950 °C in the presence of oxygen to produce NO_x gas that is reduced to $N_2$by the instrument and measured in a thermal conductivity cell.

Approximately 0.22 g of sample tissue was weighed to the nearest 0.1 mg in a tin cup. The tin cup containing the sample tissue was closed and placed in the auto sampler of a Dumas apparatus (Leco FP-528 Protein/Nitrogen Determinator, Part number 200-625, Leco, USA) and analysed according to the manufacturer's instructions. The N% was automatically recorded and used to calculate the crude protein of the sample as follows:

% CP = % N x F

Where:
CP = crude protein
N = nitrogen
F = appropriate protein conversion factor of 6.25 (forage, feeds and mixed feeds)

ii. Potassium (K) content by atomic absorption spectrum

An atomic absorption spectrophotometer (GBC SavantAA Series, Part number 01-0996-00, GBC Scientific Equipment, USA) was adjusted for K quantification (Balthrop *et al*. 2011) according to manufacturer's instructions and optimized using an oxidizing air-acetylene flame at 766.5 nm, which is the wavelength appropriate for K quantification. A calibration curve was prepared by adding the following reagents per 100 ml diluted standard solution of K; 5ml of lanthanum nitrate solution [$La(NO_3)_3$.6H2O], caesium chloride (CsCl) solution and hydrochloric acid (HCl) (6M) respectively. The measured absorbance for the lanthanum-caesium blank solution was subtracted from that of the calibration solution to give the corrected absorbance. A calibration curve indicating K content was constructed by plotting corrected absorbance against the K content. To test the K content of samples aliquots of the

test solution and blank solution were diluted with deionised water and 5 ml of $La(NO_3)_3.6H_2O$, CsCl solution and HCl (6M) respectively, that were added to the standard solution as above. The absorbance of the test and blank solution were measured parallel to that of the calibration solution under identical conditions. The absorbance of the test and blank solutions, respectively were then subtracted from that of the calibration absorbance. The concentration of K was determined using the calibration curve with the following calculation:

$$\mathbf{c_s = (A_s - b)/m}$$

Where:
$\mathbf{c_s}$ = concentration of the element in the sample solution
$\mathbf{A_s}$ = absorbance value of the sample solution
b = y-intercept of the regression line
m = slope of the regression line

iii. Phosphorus (P) content by the colorimetric method

The colorimetric principle enables quantification of a given compound by comparing colour intensity of a solution measured as absorbance, to that of a standard solution with known concentration (Fiske and Subarrow, 1925; Gerdel, 1928; Hiskey and Young, 1951; Read, 1984). Following the method described by Fiske and Subbarow (1925) using the reducing agent 1-amino-2-naphthol-4-sulphonic acid, samples and standards were loaded in a colorimeter and concentrations determined (Fiske and Subarrow, 1925; De Waal, 1979; Read, 1984).

3.5. STATISTICAL ANALYSIS OF RESULTS

To test for significant differences two sample t-tests assuming unequal variances were performed using the data analysis tool in Microsoft Excel 2007. This was done to determine whether differences between healthy and malformed tissues of *S. lancea* in number of fungi and insects, and phytohormone and nutrient contents were significant. Resultant t-stats were compared to relevant critical values (t-crit) determined using t-distribution table(s) at 95% confidence levels ($\alpha = 0.05$) (Appendix C). Subsequent manual t-test calculations were performed using the formula:

$$t = \frac{\dot{X}^1 - \dot{X}_2}{\sqrt{\frac{(n_1 - 1){S_1}^2 + (n_2 - 1){S_2}^2}{n_1 + n_2 - 2} \times \frac{n_1 + n_2}{n_1 \times n_2}}}$$

And t-test analysis using R i386 v. 3.2.2 statistic software and input commands:

>setwd("drive:\\folder\\folder")

>name<-read.csv("filename.csv",header=T)

>attach(name)

>t.test(y variable~xvariable,paired=F)

When the t-stat value for a t-test is lower than the corresponding t-crit values obtained from the t-distribution table (Appendix) it means the null hypothesis (H_0: $\dot{X}_1 = \dot{X}_2$) is accepted and there exists no significant difference between samples. When the t-stat value for a t-test is higher than the corresponding t-crit values the null hypothesis is rejected (H_0: $\dot{X}_1 \neq \dot{X}_2$) and there exists a significant difference between samples.

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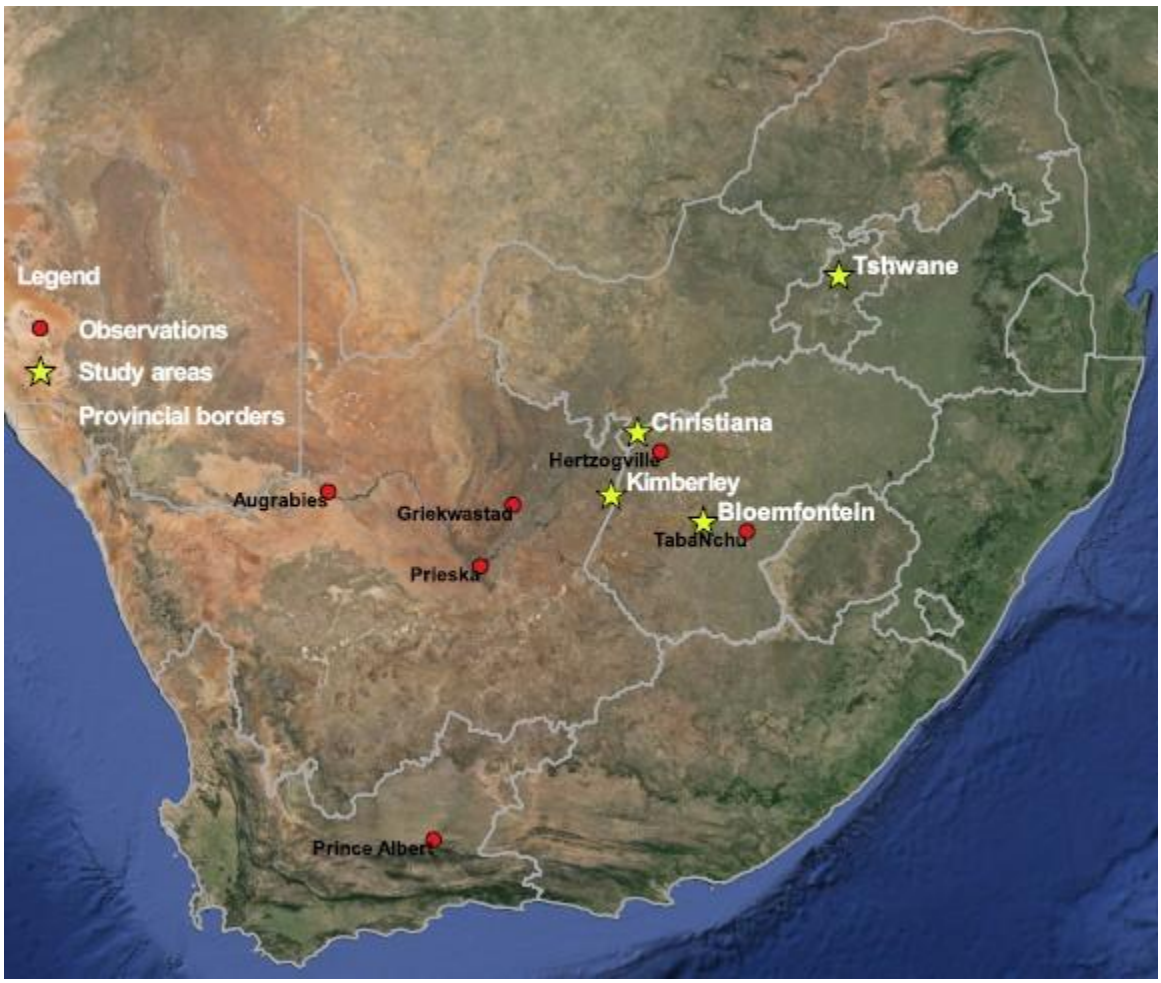

Figure 3.1.1. Map for observations and study areas of common karee (*Searsia lancea*) malformations.

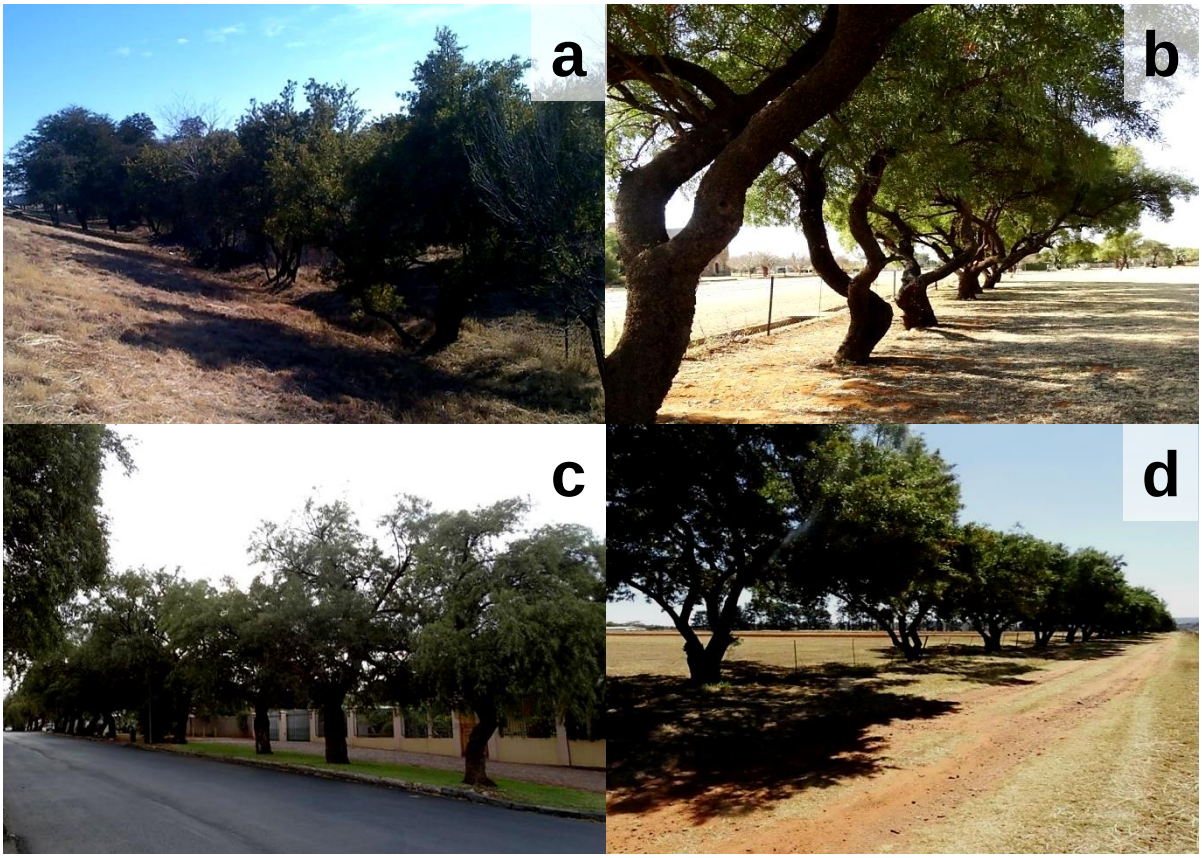

Figure 3.1.2. Transects used for study of malformation of the common karee (*Searsia lancea*) in **a)** Bloemfontein; **b)** Christiana; **c)** Kimberley; **d)** and Tshwane.

CHAPTER FOUR: RESULTS

"What I see in Nature is magnificent structure that we can comprehend only very imperfectly, and that must fill a thinking person with a feeling of humility. This is a genuinely religious feeling that has nothing to do with mysticism."
Albert Einstein

4.1. COLLECTION OF SAMPLES FROM DISEASED TREES

Observations at various locations in South Africa from 2013 – 2015 (Figure 3.1.1., Chapter three) indicated that malformation symptoms of the common karee (*Searsia lancea*) occurred sporadically and were not that widespread. Of the 10 locations investigated only two (Christiana and Tshwane) had a constant load of malformations on trees in all years surveyed. Malformations often grew large in the latter two locations and were commonly present throughout the flowering season (autumn – winter). Some locations such as Bloemfontein only presented a small number of malformations evidenced by dead tissues which indicates that if malformations are present they will not necessarily develop in the following season. Transects in four locations confirmed these observations (Figure 3.1.2, Chapter three). No malformations were observed in Kimberley and Bloemfontein transects, while 5 out of 10 trees in Christiana, and 7 out 10 trees in Tshwane were affected (Table 6).

Field and laboratory observations indicate that malformations occur at meristematic parts of the plant such as axillary buds and terminal growth tips of main shoots (Figure 4.1.1.a). Elongation of affected shoots stops and this section of the affected tissue becomes swollen. Some elongation may still occur but it is inconsistent, leading to bending and contorting of affected branches/twigs to various degrees (Figure 4.1.1.b). Proliferation of the affected meristematic tissue occasionally occurs, causing formation of numerous additional vegetative and floral shoots among malformed tissues (Figure 4.1.1.c). Some flowers borne on such shoots remain unopened or become enlarged. Affected leaves suffer a range of symptoms including chlorosis (yellowing) and enlargement (Figure 4.1.1.b). Vegetative or floral shoots appear to be affected equally and to similar extents. Leaf symptom morphology and degree also varies between trees, and between trifoliate leaves and individual leaflets.

4.2. FUNGAL DIVERSITY

4.2.1. Isolation of fungal species

A total of 576 morphological species (MSP's) were identified from 1328 isolates that were obtained from both healthy and malformed tissues of *S. lancea* (Appendix A). Of these 127 isolates were obtained from healthy and 1201 from malformed samples of *S. lancea*. MSP's that sporulated and that could be identified represented 13 fungal genera namely *Alternaria*, a Basidiomycete genus, *Chaetomium*, *Epicoccum*, *Fusarium*, *Nigrospora*, *Penicilium*, *Pestalotiopsis*, *Phomopsis*, *Trichoderma*, *Tricothecium*, *Xylariaceae* and yeasts. The majority of fungal MSP's occurred at less than 1% of the total number of fungal MSP's. The most dominant group was MSP1 identified as *Alternaria alternata* (Figure 4.2.a).

MSP1 (*A. alternata*) was dominant in both healthy and malformed samples, but especially prominent in malformed samples of *S. lancea* (Figure 4.2.b). Twenty two MSP's were associated with both healthy and malformed samples (Figure 4.2.c). There were 72 MSP's associated (at an average of 0.08%) only with healthy, and 482 MSP's associated (at an average of 0.11%) only with malformed samples of *S. lancea*. The t-stat value for fungal diversity between healthy and malformed tissues of *S. lancea* was higher than the corresponding t-crit values from the t-distribution table and indicated significant difference in number of fungi between healthy and malformed tissues of *S. lancea* (Figure 4.2.d.). Besides *A. alternata* none of the MSP groups in malformed tissues were consistent enough to be considered as a causal agent of the malformations.

4.2.2. Identification of *Fusarium* species

Very few isolates of *Fusarium* spp. (19 out of 1328) were obtained from the isolations. These were represented by MSP60, MSP61, MSP62, MSP63, MSP64, MSP111 and MSP112, and originated only from malformed samples of *S. lancea*. *Fusarium* isolates were grouped in diverse lineages and represented seven species complexes in *Fusarium* (Figures 4.2.1). These included the *Fusarium Fujikuroi* Species Complex (FFSC), *Fusarium Chlamydosporum* Species Complex (FCSC), *Fusarium Incarnatum-Equiseti* Species Complex (FIESC), *Fusarium Tricinctum*

Species Complex (FTSC), *Fusarium Oxysporum* Species Complex (FOSC), *Fusarium Brachygibbosum* Species Complex (FBSC) and the *Fusarium Solani* Species Complex (FSSC).

Only one isolate (P) grouped in the FFSC, where all of the mango malformation pathogens reside, and were identified as the maize pathogen *F. temperatum*, with a 100% bootstrap support (Figure 4.2.1.a). It grouped separately from species known to cause malformation symptoms in mango. Other isolates identified as *F. neocosmosporiellum* in the *Fusarium Solani* Species Complex (FSSC, Ne2, Ne4, Ne9, Figure 4.2.1.b). Isolate L grouped separately from the other described species in the FTSC (Figure 4.2.1.c), and isolate S grouped in the FOSC (Figure 4.2.1.d). In the FIESC (Figure 4.2.1.e) four *S. lancea* isolates (G, I, K, N) formed three genotypes apart from other previously described multi-locus sequence type (MLST) groups (O'Donnell *et al*. 2009). Most of the *S. lancea* isolates (9 in total) grouped in the FCSC (Figure 4.2.1.f) isolates D, E, and C grouped in a clade separate from the rest of the isolates (A, Q, O, T, B, U) (Figure 4.2.1.f) and were genetically diverse (O'Donnell *et al*. 2009). Isolate J was identified as *F. brachygibbosum* with a 100% bootstrap support (Figure 4.2.1.g).

4.3. INSECT DIVERSITY

Eighteen morphological species were identified from the insects collected from *S. lancea* tissue. These belonged to 14 families (Anthocoridae, Aphididae, Cercopidae, Cicadellidae, Coccinellidae, Encyrtidae, Formicidae, Lygaeidae, Psyllidae, Reduviidae, Scarabaeidae, Termitidae, Thripidae and one unknown), and 6 orders (Coleoptera, Hemiptera, Hymenoptera, Isoptera, Lepidoptera and Thysanoptera) (Appendix B). Two MSP's were identified up to genus rank including; MSP8 that was a species of pirate bugs, *Orius* sp. (Anthocoridae, Hemiptera), and MSP17 that represented an parasitic wasp species, *Ooencyrtus* sp. (Encyrtidae, Hemiptera). Morpho-species ten MSP10 was identified as a species of jumping plant lice, *Agonoscena crotalaria* (Psyllidae, Hemiptera), and MSP16 as black flower thrips, *Haplothrips gowdeyi* (Thripidae, Thysanoptera). Only three species have an overall abundance greater than 1% (Figure 4.3.1), namely MSP9 (Psyllidae, Hemiptera) that

was the most prominent at 93.40%, MSP3 (Cercopidae, Hemiptera) at 1.62% and MSP1 (Aphididae, Hemiptera) at 1.45%.

Malformed tissues clearly harboured a higher insect diversity compared to healthy tissues (Figure 4.3.2). Seven MSP's (MSP4, MSP5, MSP6, MSP7, MSP13, MSP14 and MSP16) were associated only with malformed tissues (Figure 4.3.2), and 3 MSP's (MSP2, MSP12, and MSP17) were associated only with healthy tissues (Figure 4.3.2). Eight MSP's (MSP1, MSP3, MSP8, MSP9, MSP10, MSP11, MSP15 and MSP18) were associated with both healthy and malformed tissues (Figure 4.3.3). However the chances that MSP1, MSP3, MSP8, MSP9, MSP10 and MSP15 occurred on malformed rather than healthy tissues of *S. lancea* were higher. The dominant MSP9 (Psyllidae, Hemiptera) occurred on malformed tissues in numbers approximately double those on healthy counterparts. In contrast MSP11 (Reduviidae, Hemiptera MSP1) and MSP18 (Lepidoptera MSP1) occurred on malformed tissues in lower ratios compared to that of healthy tissues. The t-stat value for insect diversity was lower than t-crit values obtained from the t-distribution table, which indicated no significant difference in insect number between healthy and malformed tissues of *S. lancea* (Figure 4.3.4).

4.4. PHYTOHORMONE AND NUTRIENT ANALYSIS OF SAMPLES

4.4.1. Phytohormone analysis by ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS)

The phytohormone content for the four healthy samples of *S. lancea* ranged between 41.5 – 142.0 $\mu g.kg^{-1}$ for GA_3, 90.4 – 120.5 $\mu g.kg^{-1}$ for JA, and 229.1 – 1103.0 $\mu g.kg^{-1}$ for SA (Figure 4.4.1.). The malformed complements range between 37.0 – 67.2 µg.kg for GA_3, 86.5 – 107.1 µg.kg for JA, and 1833.8 – 3094.9 µg.kg for SA. The GA_3 and JA content were generally higher in healthy (80.4 and 105.7 µg.kg average, respectively) than malformed (47.5 and 97.8 µg.kg average, respectively) tissues, while SA content was lower in healthy (771.3 µg.kg average) than malformed (2689.4 µg.kg average) tissues of *S. lancea*. When the data for samples was combined GA_3 and JA levels were generally lower, and SA levels higher in malformed tissues of *S. lancea*. The generally lower levels between healthy and malformed tissues of two phytohormones investigated (GA_3 and JA) were

determined to be non-significant (Figure 4.4.2). However the level of SA was determined to be significantly higher (Figure 4.4.2) in malformed when compared with healthy tissues of *S. lancea*.

4.4.2. Nutrient quantification

No difference in P levels was observed between healthy and malformed tissues of *S. lancea* (Figure 4.4.2). However higher levels of N and K were found in malformed tissues compared to healthy tissues (114.24 g/kg and 9.45 g/kg respectively) compared to those of healthy tissues (79.42 g/kg and 5.51 g/kg respectively) of *S. lancea* (Figure 4.4.2.). Samples were generally too small for single analysis and multiple samples from a single tree had to be pooled. In addition the sample site offered few malformations when sampling for nutrient analysis was conducted. This unfortunately reduced the sample size for nutrient analysis to a single representative for both conditions (healthy and malformed) and variance could not be determined to test for significant differences between healthy and malformed tissues of *S. lancea*.

4.5. REFERENCES

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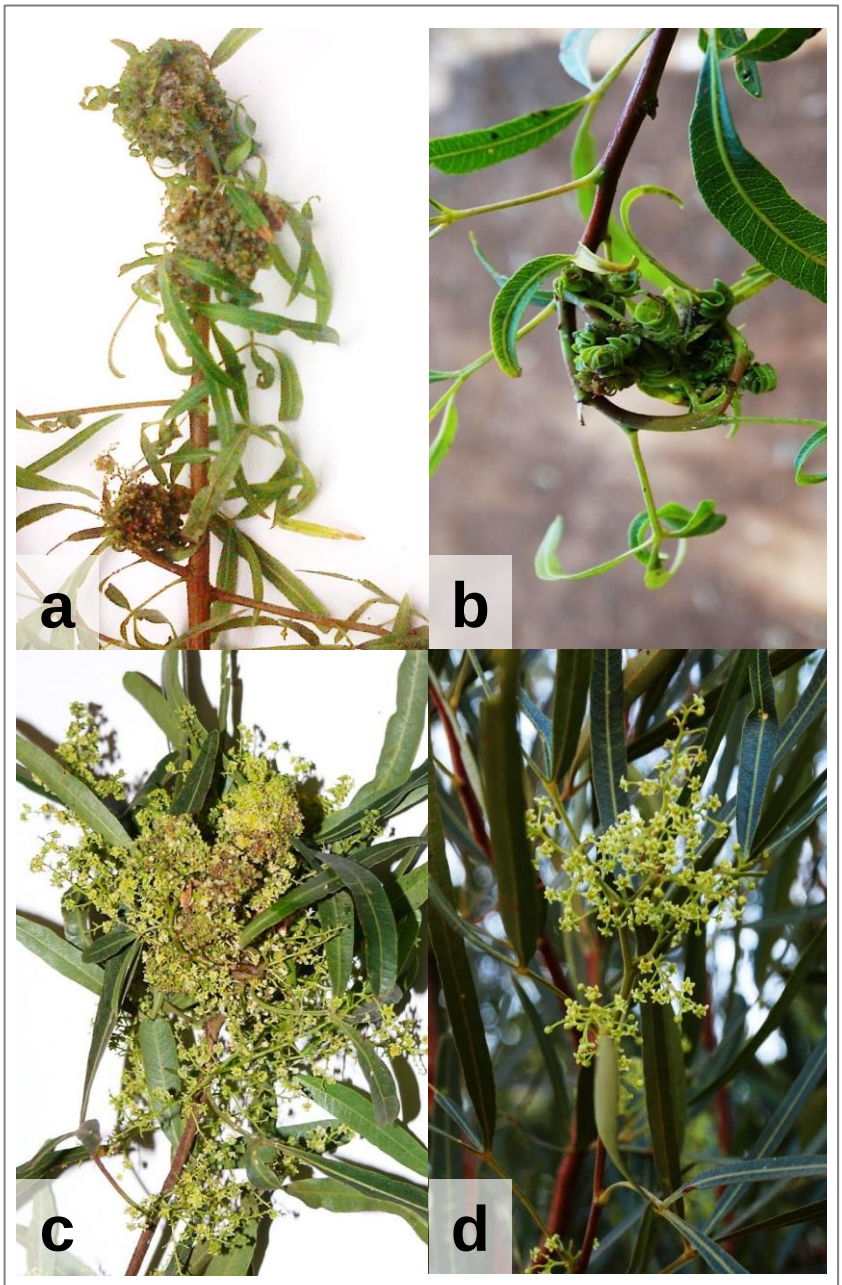

Figure 4.1.1. Malformations of the common karee (*Searsia lancea*) depicting **a)** Growth at meristematic regions; **b)** Bending and reduced leaf size and contortion; **c)** Proliferation and clumping; compared to **d)** healthy floral and vegetative inflorescences.

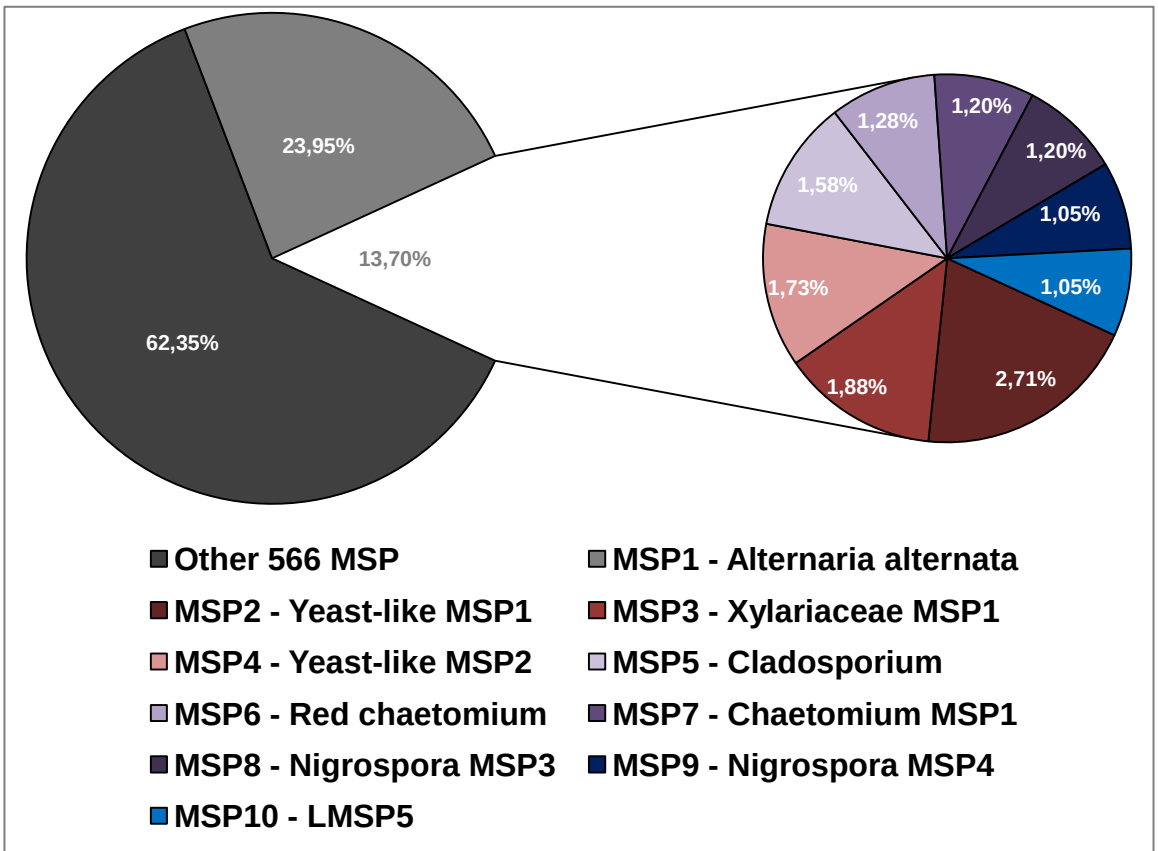

Figure 4.2.a. Proportions of fungal diversity associated with the common karee (*Searsia lancea*).

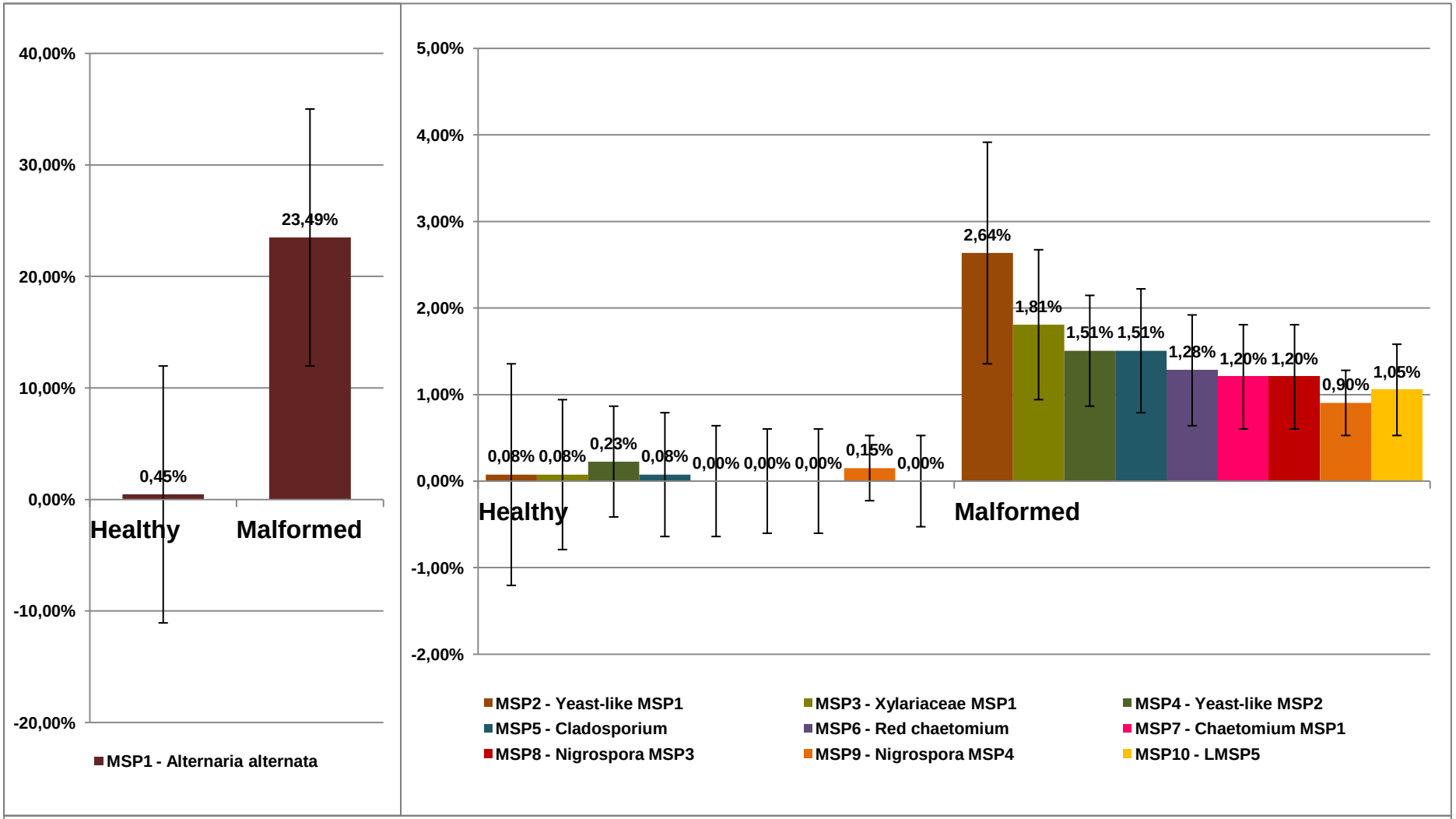

Figure 4.2.b. Comparison of ten dominant fungal species (occurrence > 1%) occurrence between healthy and malformed tissues of the common karee (*Searsia lancea*).

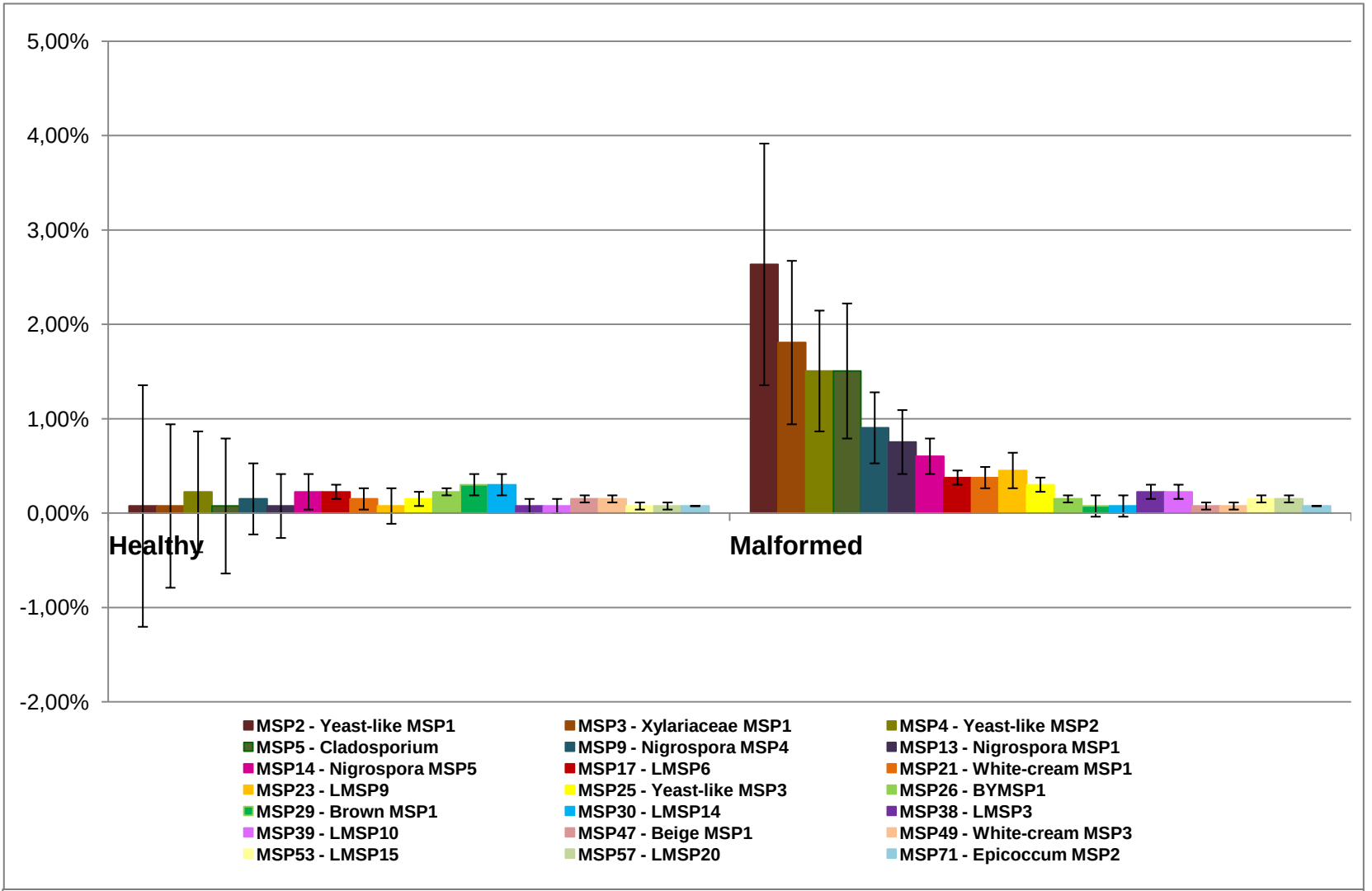

Figure 4.2.c. Comparison of occurrence values for morphological fungal species associated with both healthy and malformed tissues of the common karee (*Searsia lancea*).

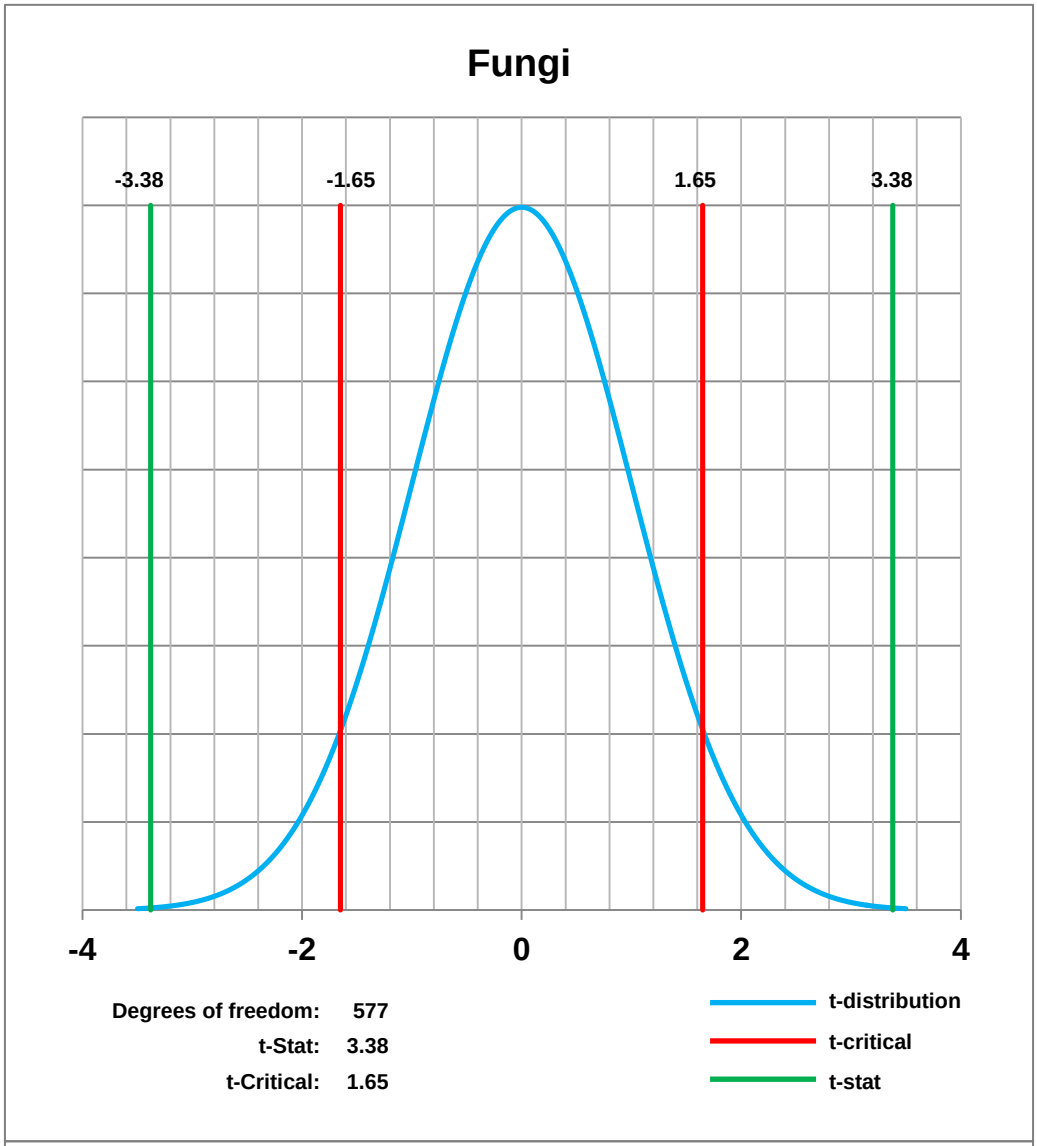

Figure 4.2.d. Normal distribution for significant difference in number of fungi between healthy and malformed tissues of the common karee (*Searsia lancea*).

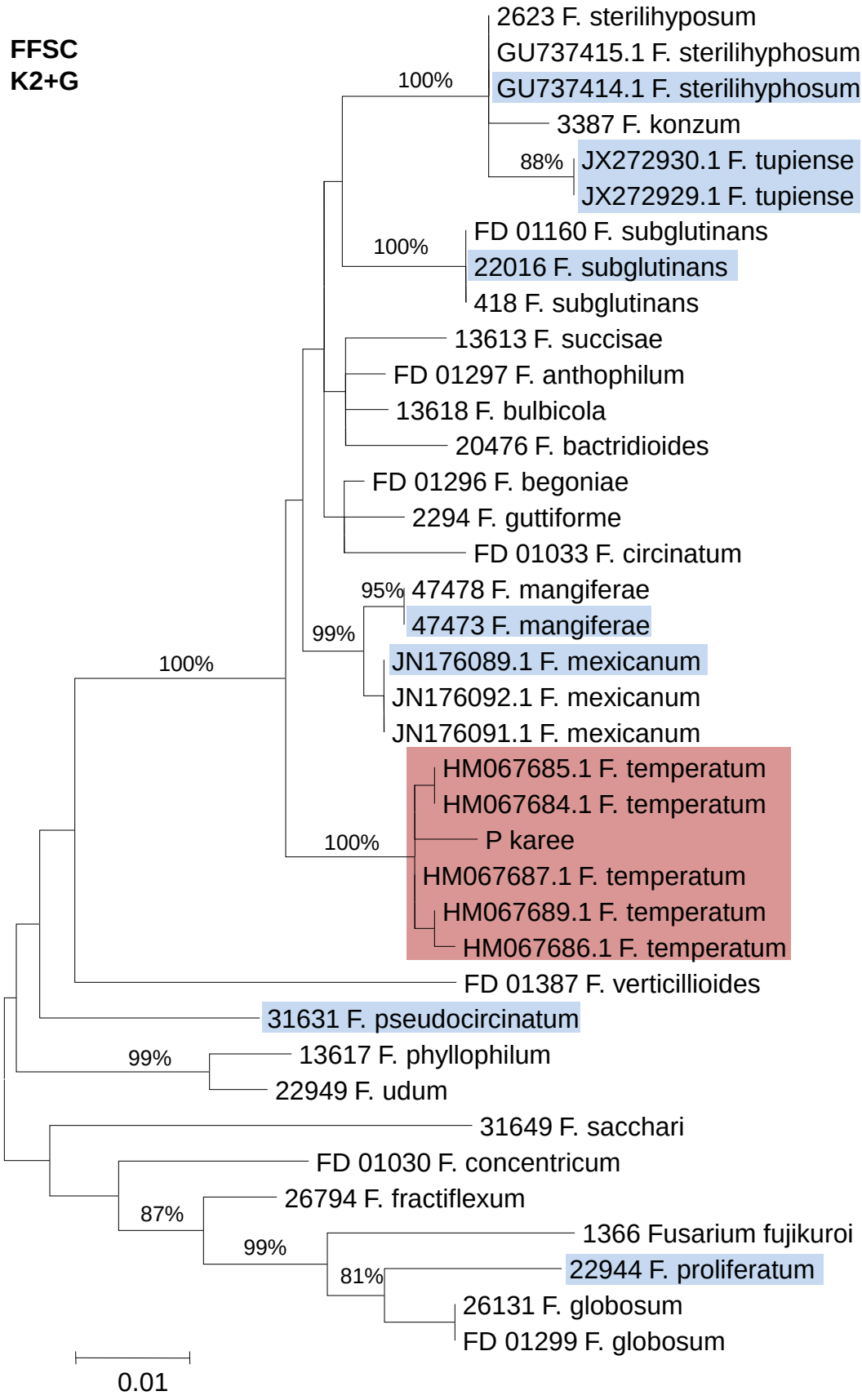

Figure 4.2.1.a. Unrooted Maximum Likelihood phylogram of the *Fusarium fujikuroi* species complex based on translation elongation factor alpha one gene sequences with bootstrap support values. Isolates from *Searsia lancea* in red, and species associated with mango malformation in blue. The appropriate evolutionary model used in the analysis is indicated.

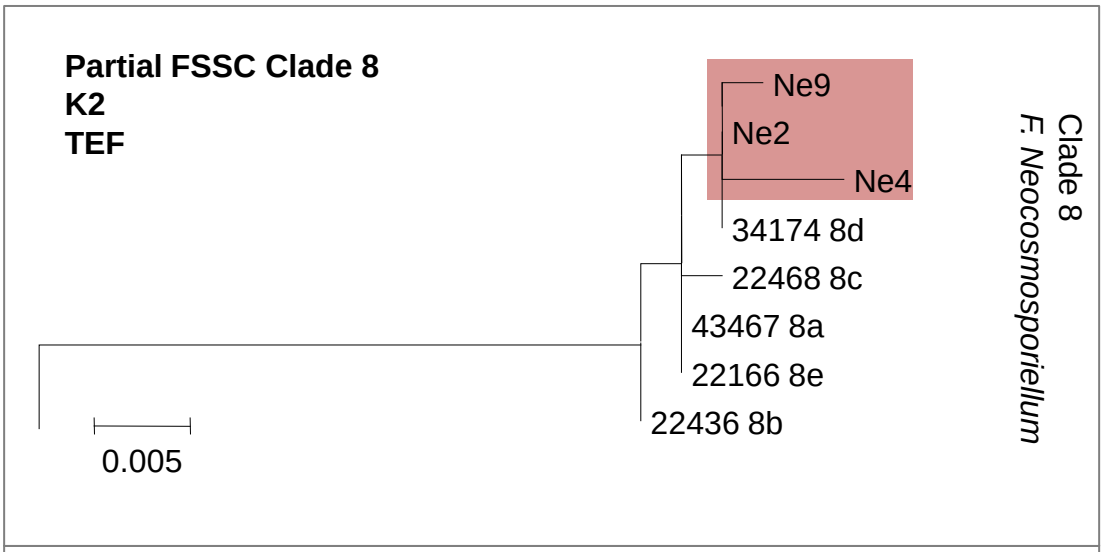

Figure 4.2.1.b. Unrooted Maximum Likelihood phylogram of a portion of the *Fusarium solani* species complex based on translation elongation factor alpha one gene sequences with bootstrap support values. The isolates from this study are included in the box. The appropriate evolutionary model used in the analysis is indicated.

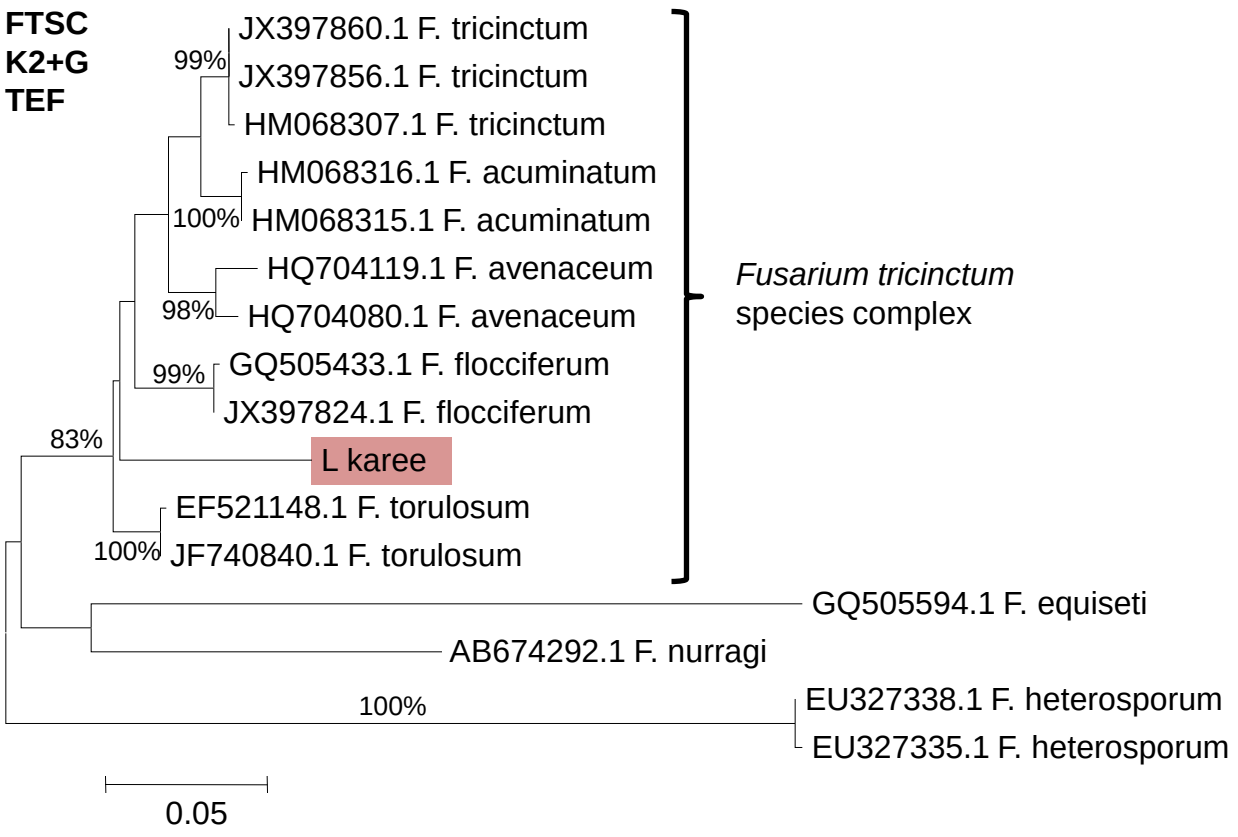

Figure 4.2.1.c. Rooted Maximum Likelihood phylogram of the *Fusarium tricinctum* species complex based on translation elongation factor alpha one gene sequences with bootstrap support values. The isolate from this study is included in the box. *F. equiseti*, *F. nurragi* and *F. heterosporum* represent the outgroups. The appropriate evolutionary model used in the analysis is indicated.

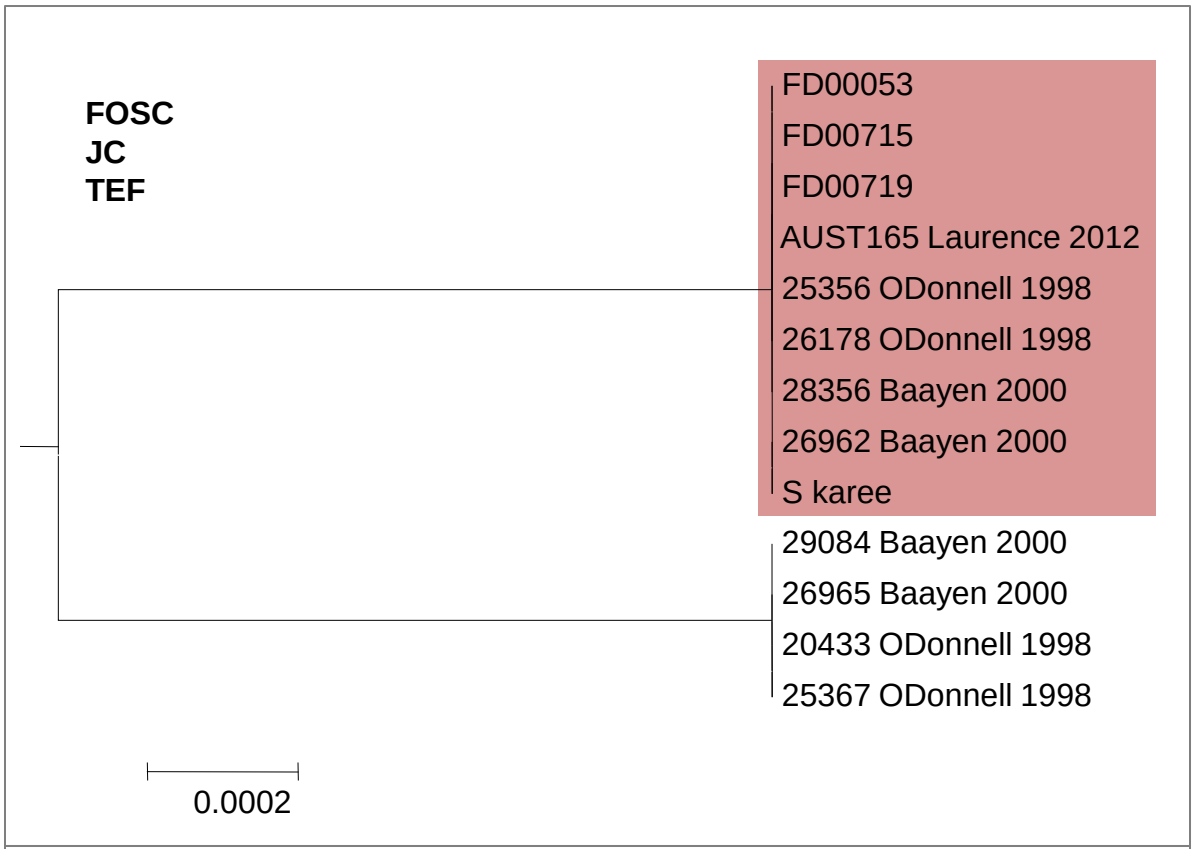

Figure 4.2.1.d. Partial unrooted Maximum Likelihood phylogram of the *Fusarium oxysporum* species complex based on translation elongation factor alpha one gene sequences with bootstrap support values. The isolate from this study is included in the box. The appropriate evolutionary model used in the analysis is indicated.

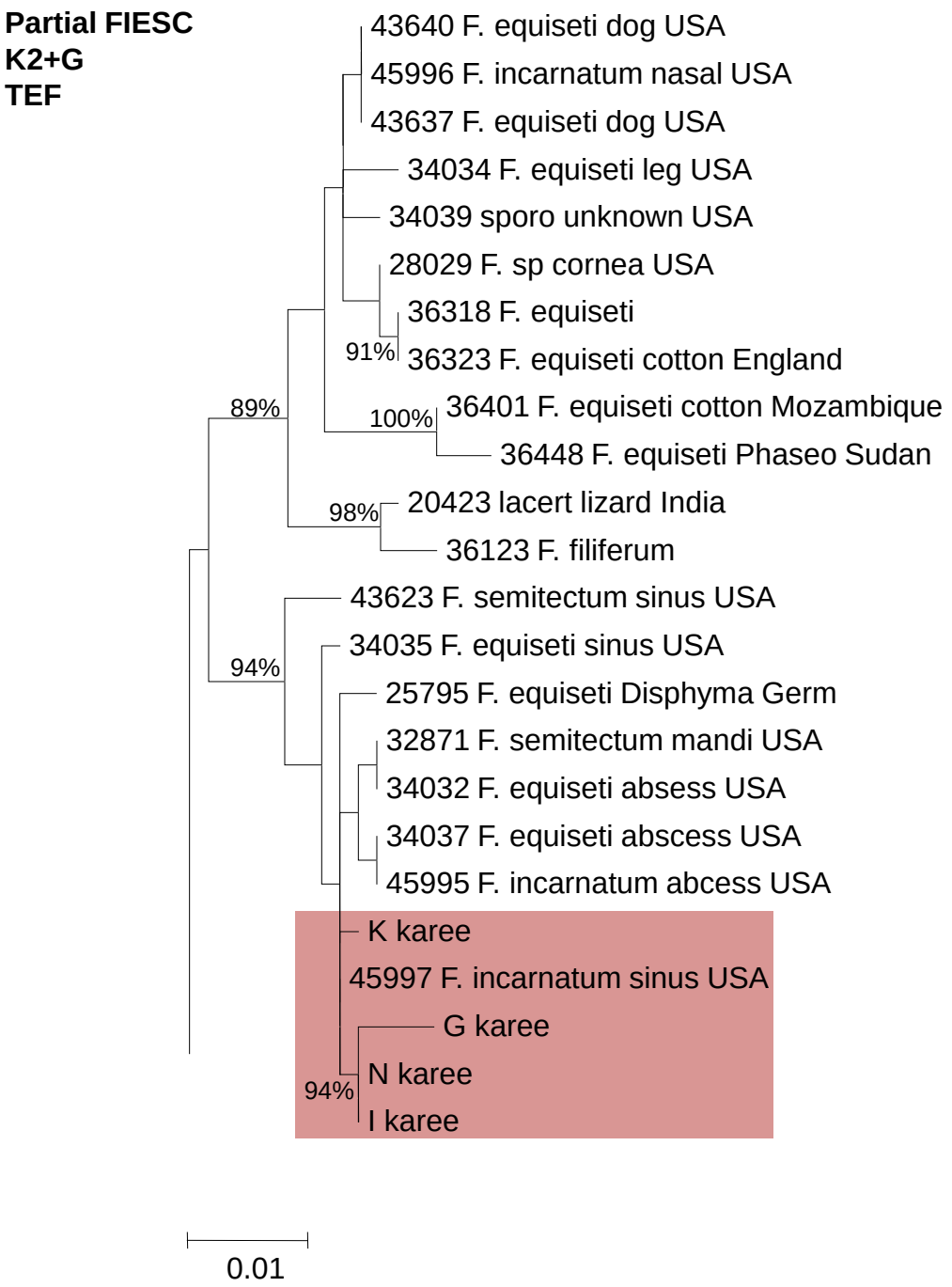

Figure 4.2.1.e. Unrooted Maximum Likelihood phylogram of a portion of the *Fusarium equiseti-incarnatum* species complex based on translation elongation factor alpha one gene sequences with bootstrap support values. The isolates from this study are included in the box. The appropriate evolutionary model used in the analysis is indicated.

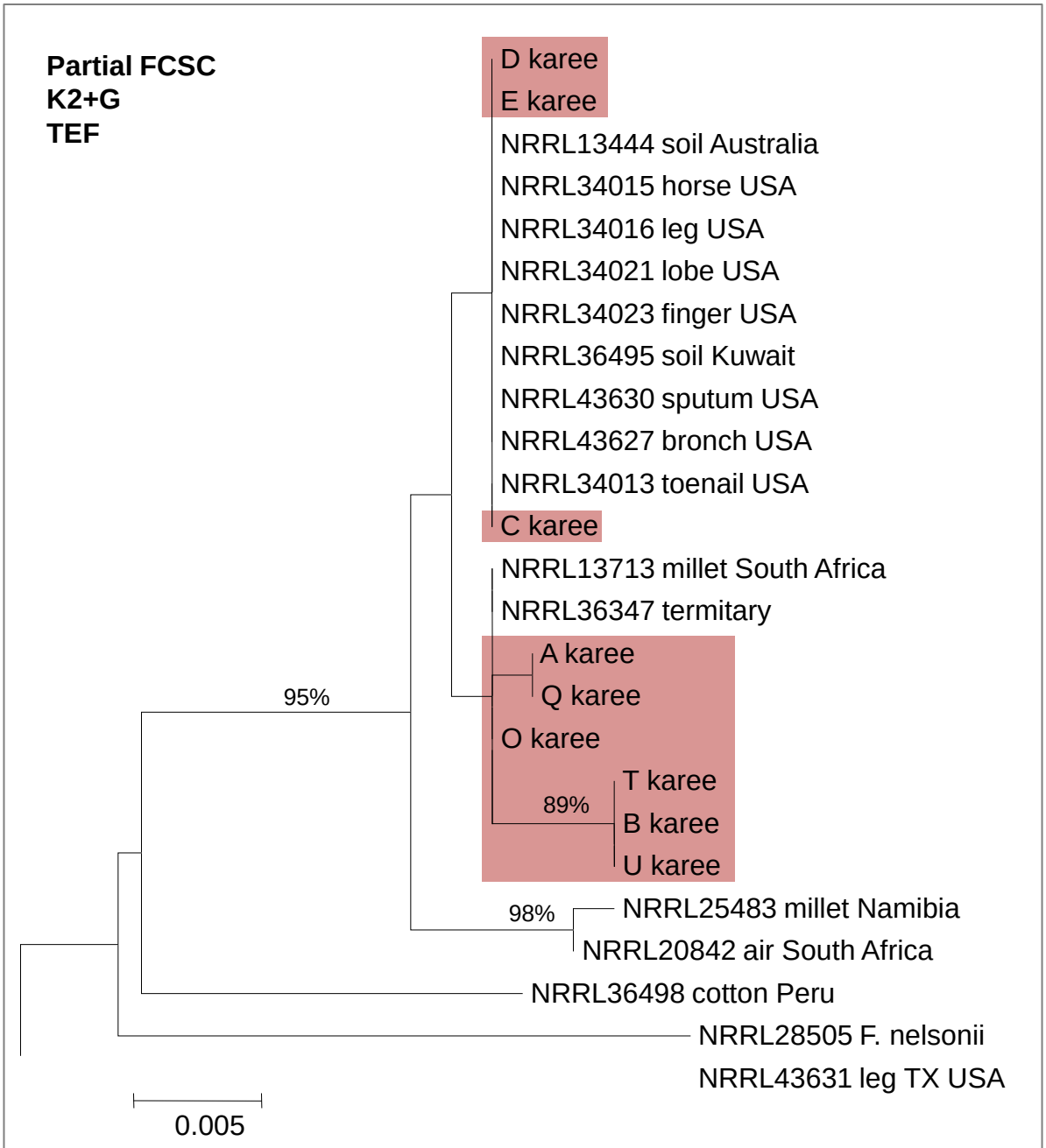

Figure 4.2.1.f. Unrooted Maximum Likelihood phylogram of a portion of the *Fusarium chlamydosporum* species complex based on translation elongation factor alpha one gene sequences with bootstrap support values. The isolates from this study are included in the box. The appropriate evolutionary model used in the analysis is indicated.

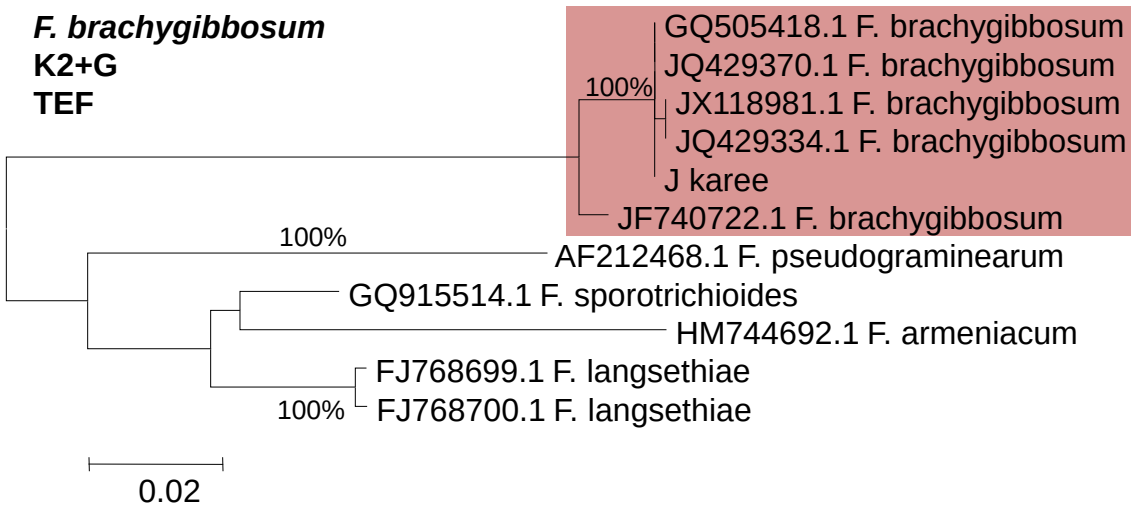

Figure 4.2.1.g. Unrooted Maximum Likelihood phylogram of isolates of *F. brachygibbosum* and closely related species based on translation elongation factor alpha one gene sequences with bootstrap support values. The isolate from this study is included in the box. The appropriate evolutionary model used in the analysis is indicated.

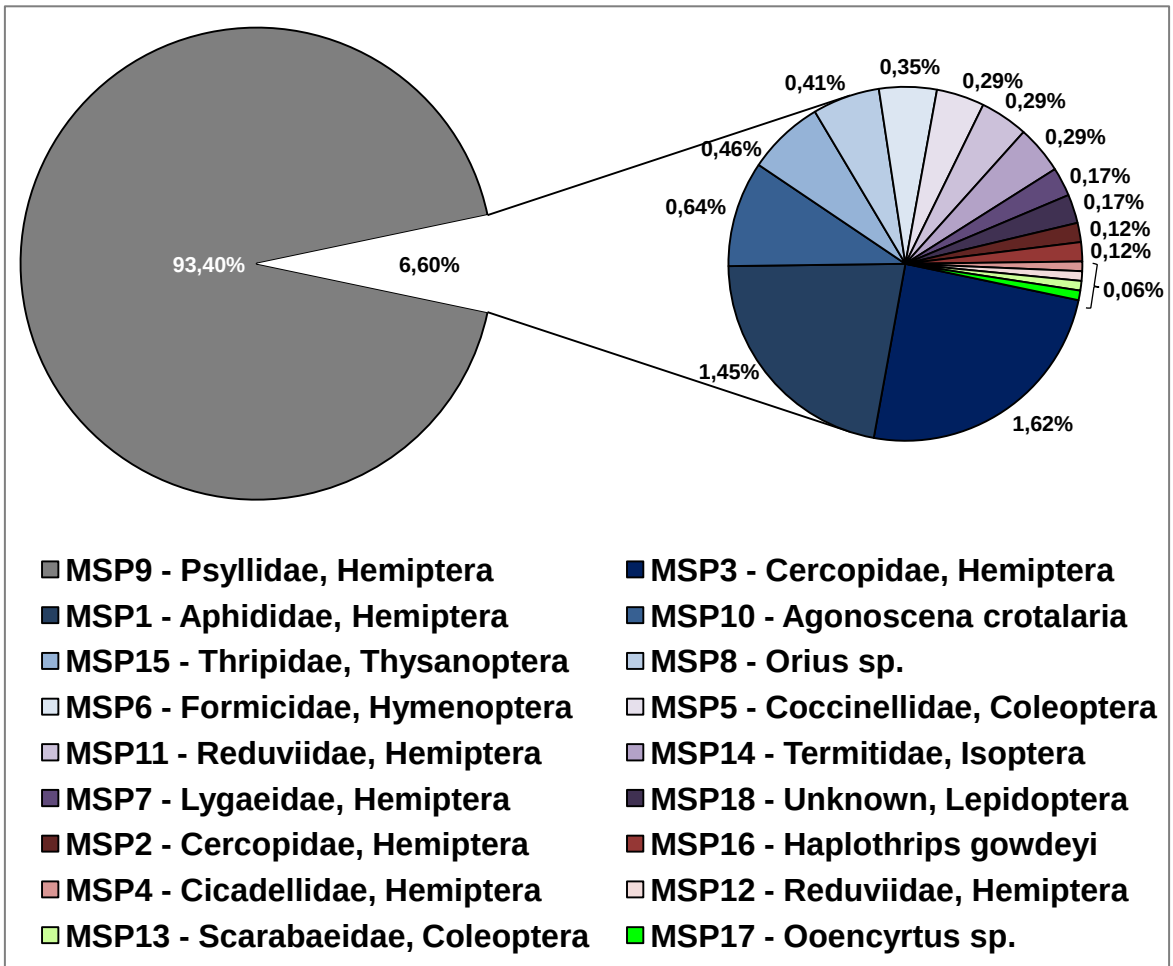

Figure 4.3.1. Insect morphological insect species associated with the common karee (*Searsia lancea*) expressed as percentage of total.

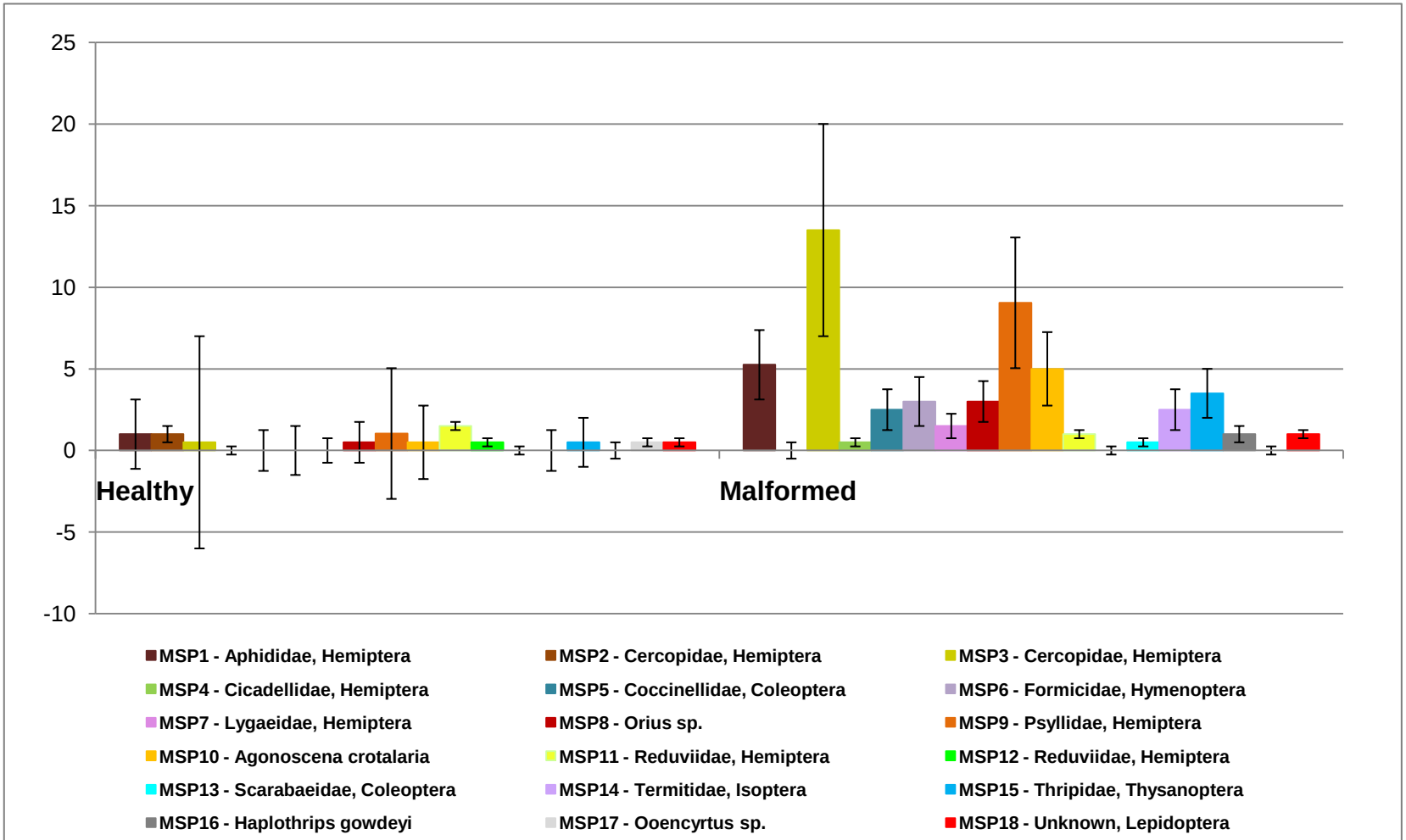

Figure 4.3.2. Comparison of ratios between healthy and malformed tissues of the common karee (*Searsia lancea*) of all associated morphological insect species.

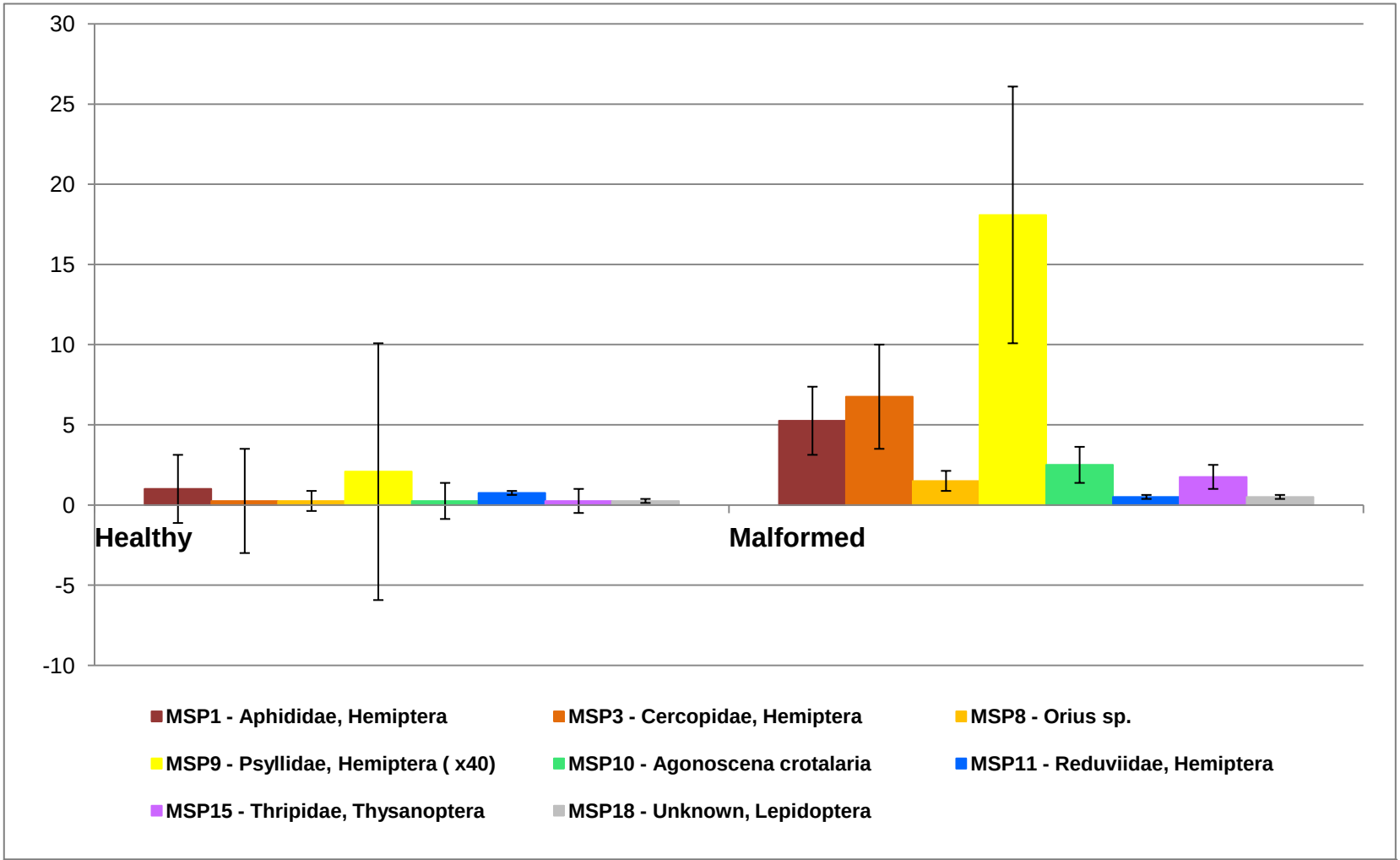

Figure 4.3.3. Comparison of ratios between healthy and malformed tissues of the common karee (*Searsia lancea*) of associated morphological insect species that occur on both tissues.

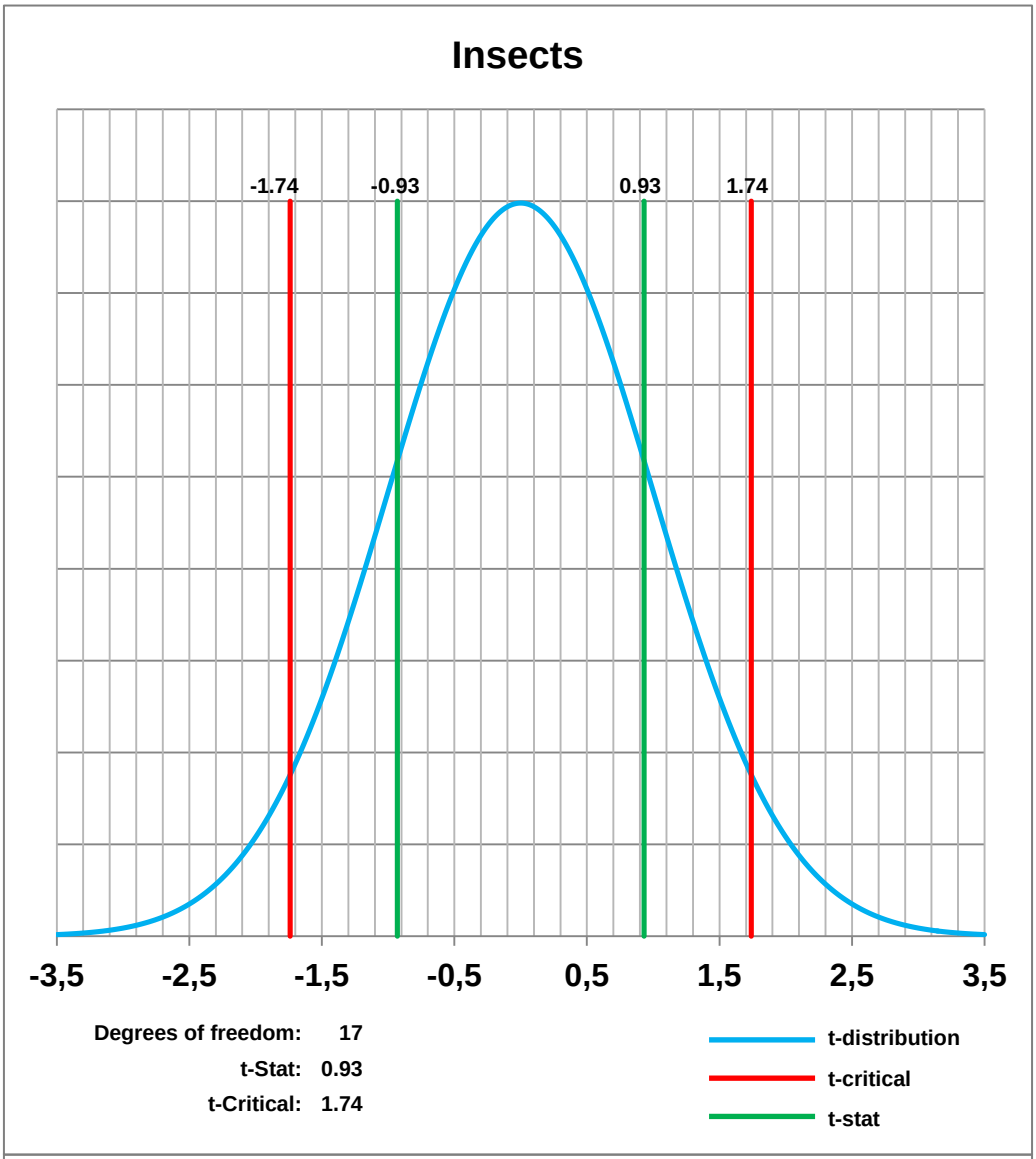

Figure 4.3.4. Normal distribution for significant difference in number of insects between healthy and malformed tissues of the common karee (*Searsia lancea*).

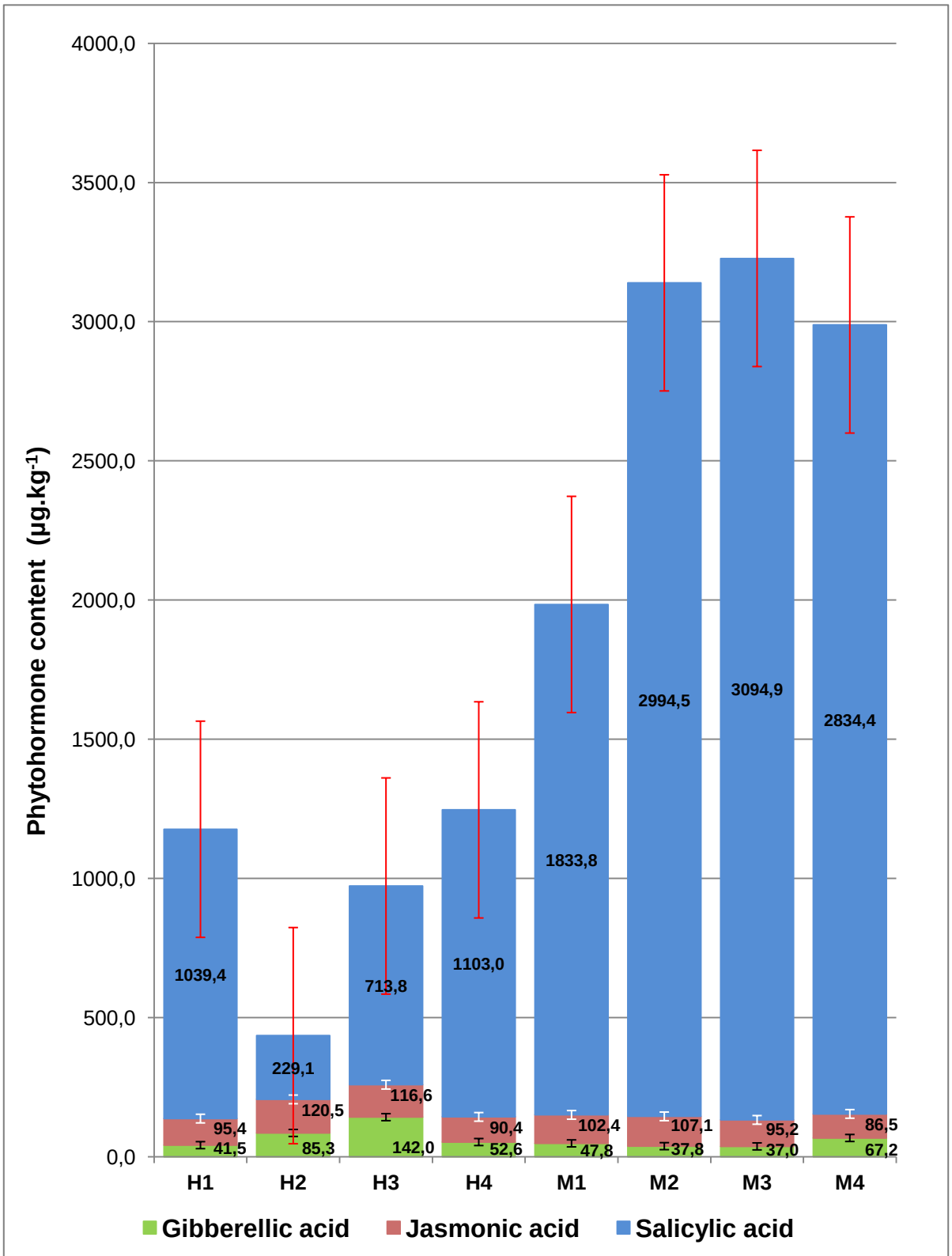

Figure 4.4.1. The gibberellic acid, jasmonic acid and salicylic acid contents of **a)** Healthy and **b)** Malformed tissue of the common karee (*Searsia lancea*).

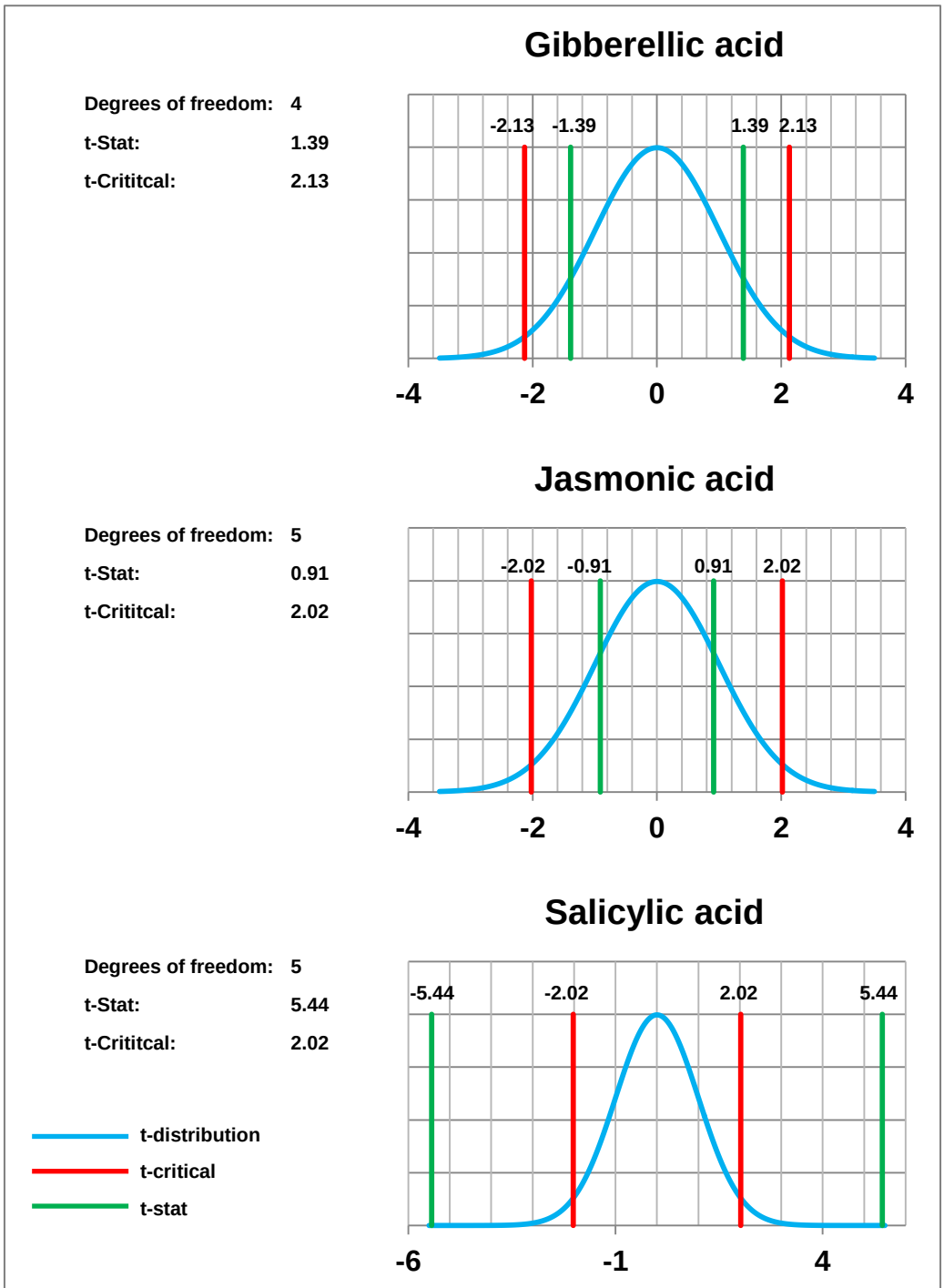

Figure 4.4.2. Normal distribution for non-significant difference in gibberellic acid and jasmonic acid, and significant difference in salisylic acid content between healthy and malformed tissues of the common karee (*Searsia lancea*).

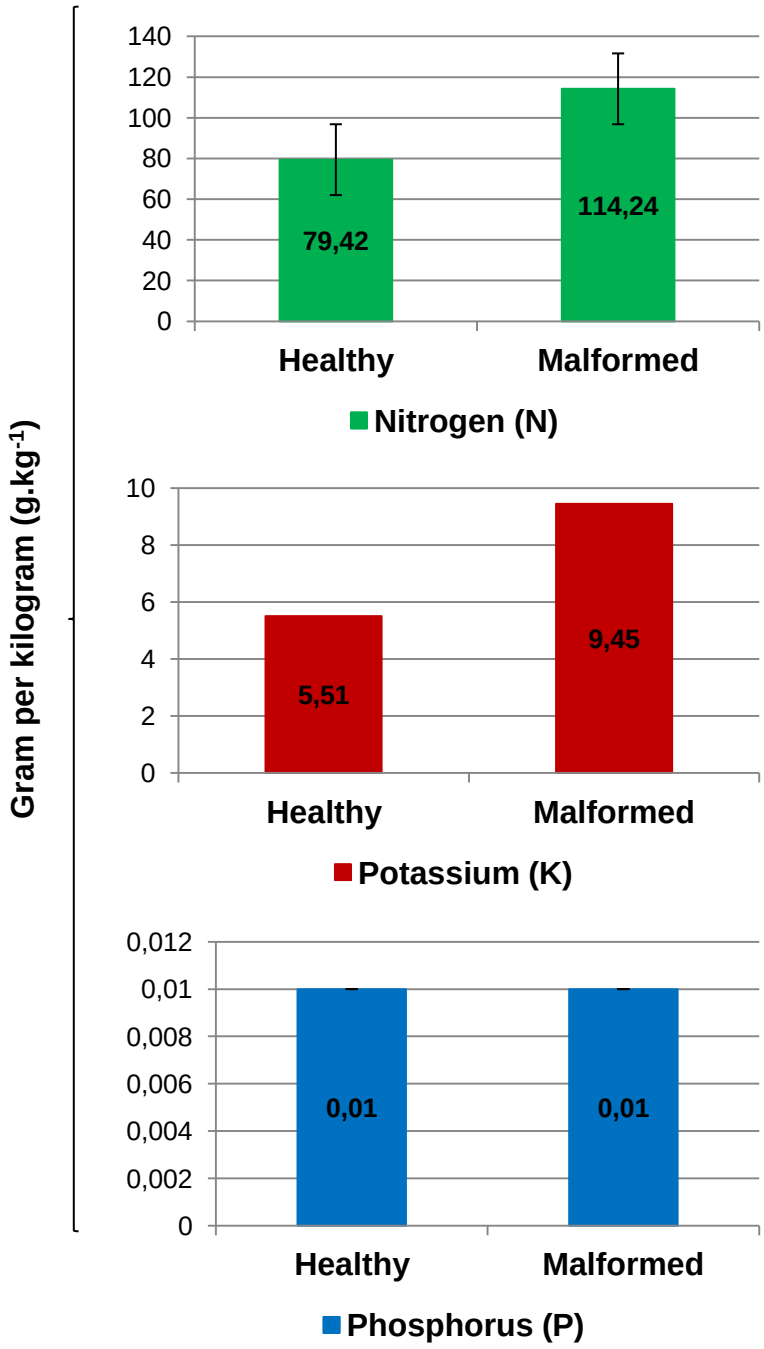

Figure 4.4.3. The nitrogen (N), potassium (K) and phosphorus (P) content of healthy and malformed tissues the common karee (*Searsia lancea*).

TABLE 6 - TRANSECT DATA SHOWING PERCENTILE OF MALFORMATION OCCURRENCE

TREE NO. → TRANSECT ↓	1	2	3	4	5	6	7	8	9	10
Bloemfontein	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
Christiana	0%	10%	10%	0%	5%	0%	0%	20%	0%	15%
Kimberley	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
Tshwane	5%	5%	5%	0%	10%	20%	0%	5%	0%	15%

CHAPTER FIVE: DISCUSSION

"With imagination and daring, the landscape can be sculpted with as much whimsy and inventiveness as a sculptor casting in bronze or carving in wood."
Anthony Archer-Wills

5.1. GENERAL TRENDS

Studies from this MSc dissertation reports on a new disease of the common native tree *Searsia lancea* (common karee). This disease consists of malformation symptoms of leaves and inflorescences. Fungi do not appear to be the direct causal agents of the disease but appear to infect the diseased (malformed) tissues in much larger numbers than healthy tissues. Similarly a great diversity and number of insects were also found in the malformed tissues, although not shown to be significantly different from those occurring on healthy tissues. Some of the collected insects, such as psyllids could possibly be direct or indirect as vectors of plant pathogens or causes of symptoms.

The physiological studies, albeit preliminary, initiated in this study already suggested changes between malformed and healthy tissues. Gibberellic acid (GA_3) and jasmonic acid (JA) levels were found to be generally lower, and salicylic acid (SA) higher in malformed than healthy tissues. The higher concentration of SA in malformed tissues was the only phytohormone concentration that differed significantly. No difference in phosphorus (P) levels was observed between healthy and malformed conditions, although levels of nitrogen (N) and potassium (K) were determined to be generally higher in malformed tissues. More detailed discussions on the results obtained from this study follows below.

5.2 DISCUSSION

5.2.1. First report of a new disease of the common karee (*Searsia lancea*) in South Africa

This is the first formal report of malformation symptoms on the common karee (*Searsia lancea*). Symptoms include chlorosis, gall-like swelling of leaves and parts of inflorescences, smaller and larger than normal leaf size, leaf contortion, enlarged flowers, unopened flower buds and witches' broom-like proliferation of shoots and leaves. The disease reported for *S. lancea* has been encountered before and were considered to have little to no impact by gardeners and amateur botanists (Venter and Venter, 2009). To the best of our knowledge the disease has not yet been seen on other *Searsia* spp.

Detailed studies on the literature related to different types of diseases where the plant tissues are deformed, showed that there is a level of confusion as to the appropriate terminology to use. A process was developed in Chapter 2 of this thesis (Figure 2.3) to facilitate the correct usage of such terminology. Swellings were not consistent and could not yet be positively associated with a causal organism, and proliferation of shoots and leaves was also not consistent and severe enough between the observed symptoms for the disease to be considered a traditional tumour or witches' broom. Naming the new disease on *S. lancea* as a malformation thus appeared to be the most logical, and it was thus named "karee (*S. lancea*) malformation disease" (KMD).

With deformation-type disease, a thorough summary of relevant literature indicated that several types of abiotic and biotic causal agents, such as microbes, insects, nematodes and mites, can be involved. Description of the new disease on *S. lancea* is the first step in studying and possibly eliminating some of the many possible causal agents, when compared with similar diseases of other closely related plant species. However, it is clear that from our results that it is still unknown what the specific causal agent or other possible contributors to the disease is. More future studies on this disease will thus be necessary.

5.2.2. *Fusarium* spp. associated with the common karee (*Searsia lancea*) and KMD

The symptoms of KMD resemble that of mango (*Mangifera indica*) malformation disease (MMD) (Krishnan *et al*. 2009). Similarity of the symptoms must still be determined through proper anatomical and morphological review and comparison. Due to these similarities between KMD and MMD and the fact that the two tree species are closely related (Both belong to the Anacardiaceae), the hypothesis stated by our study was that KMD could be caused by similar causal agents than MMD, i.e. species of the fungal genus *Fusarium*. However, our studies clearly showed that these two diseases do not share a common causal agent. None of the *Fusarium* spp. known to cause MMD across the world (*F. sterilihyphosum; F. tupiense; F. subglutinans; F. mangiferae; F. mexicanum; F. pseudocircinatum; F. proliferatum*) (Marasas *et al*. 2006; Lima *et al*. 2009; Otero-Colina *et al*. 2010; Lima

et al. 2012) were isolated from *S. lancea*. In fact, the only species isolated from KMD symptoms that grouped within the *Fusarium fujikuroi* species complex (FFSC), which contains the MMD causing *Fusarium* spp. was *F. temperatum*. Since *Fusarium* spp. are implicated in other deformation-like type diseases as well, such as malformation of waterberry (*Syzygium cordatum*) inflorescences (Kvas *et al*. 2008), some of the isolated *Fusarium* spp. could possibly cause KMD. However, too few *Fusarium* spp. were isolated from KMD symptoms in general to be considered causative.

Although consisting of only a small number, the obtained *Fusarium* isolates represented diverse *Fusarium* species complexes, of which some are known as important plant pathogens. *Fusarium temperatum* is a known maize (*Zea mays*) pathogen that causes stalk rot and seedling malformation which is not described further (Scauflaire *et al*. 2011). Only three isolates of *F. temperatum* were, however, obtained and it is thus unlikely that this species is involved in KMD. Other *Fusarium* isolates from *S. lancea* grouped within the *Fusarium tricinctum* species complex (FTSC), *Fusarium neocosmosporiellum* species complex (FOSC), *Fusarium chlamydosporum* species complex (FCSC), *Fusarium incarnatum/equiseti* species complex (FIESC), *Fusarium brachygibbosum* species complex (FBSC) and *Fusarium solani* species complex (FSSC), which are fairly widespread and common fungi (Leslie and Summerell, 2006). Members of the FFTSC, FOSC, and FIESC cause diseases in cereal grains, nonspecific vascular wilt diseases, and cankers and dieback in some tropical trees, respectively (Gordon and Martyn, 1997; Botallico, 1998; Mullins *et al*. 2001; Yli-Mattila *et al*. 2002; Leslie and Summerell, 2006; Niessen *et al.* 2012). Our study reports the presence of these species in *S. lancea* for the first time. This could have important epidemiological consequences where these phytopathogenic species could occur in this unsuspected host and be unknowingly transported to other areas where diseases can spread. The tree can also be an important plant where additional genetic diversity can be developed that will be detrimental to the occurrence of plant disease (Aoki *et al*. 2012).

5.2.3. Distribution and ecology of karee (*Searsia lancea*) malformation disease

Based on our survey, only two of the ten sites visited in our study, namely Christiana (North West Province) and Tshwane (Gauteng Province), had sufficient and

consistent malformation occurrence on *S. lancea* trees across the two years sampled to be noteworthy. The disease was either not observed in the other sites, or only a small number of symptoms from the previous season was seen. The disease thus appears not to be that widespread, and to occur sporadically. The impact of KMD on naturally occurring and planted trees thus appear not to be great, although should be confirmed with more extended future surveys.

The variation in frequency and severity of KMD symptoms determined from transects implies that the disease is not caused by abiotic conditions. Abiotic conditions would have resulted in more uniformly distributed symptoms. In addition, superficial recognition of the variation in climatic conditions based on the different biomes of South Africa (Mucina and Rutherford, 2006) where surveys were conducted, and where the disease was found to occur, suggest that malformation distribution is not limited by climatic factors. A more complete spatial distribution of KMD in South Africa, and corresponding frequency and severity transect surveys are, however, required to scientifically confirm the latter.

5.2.4. Fungal associates of *Searsia lancea* and KMD symptoms

This study has shown that *Searsia lancea* harbours a considerable diversity of endophytic fungi. However, the majority of these (464 out of 577 morphospecies) were represented by single isolates. Some of the groups identified include yeasts, common endophyte genera such as *Alternaria*, *Cladosporium* and *Nigrospora* (El-Morsy, 2000; Heuchert *et al*. 2005; Schubert and Braun, 2005; Bensche *et al*. 2012; Zhao *et al*. 2012; Armitage *et al*. 2015), saprophytic *Chaetomium* species (Leen and Hanlil, 1999), and members of the Xylariaceae family. None of these, except the *Alternaria* group, occurred in numbers sufficient to suspect a link with the observed symptoms.

a) *Comparisons of fungal endophytes between healthy and malformed tissues of Searsia lancea*

The fact that the majority (1201 out of 1328 morphospecies) of isolates were obtained from malformed tissues of *S. lancea*, represents an interesting ecological trend. It would have been expected that malformed tissues harbour relatively the same number and diversity than equivalent healthy tissues that are of the same age or that it would be dominantly infected with particular fungi, in the case where fungi could be the causal agent of the malformation. This significant difference in number and prevalence of isolates between healthy and malformed tissues could possibly be explained using hypotheses related to the adaptive significance of gall morphology (ASGM) formation for insects (Price *et al*. 1987; Stone and Schönrogge, 2003). This suggests that morphological and physiological changes associated with malformations create optimal conditions for fungal invasion and survival. Alternatively, these changes could have resulted in reduced plant defence and resistance, making the plant tissues more susceptible to secondary invasions by fungi present in the environment. The increased size of the plant tissues and changed morphology of the tissues resulting in uneven surfaces and enclosed spaces, also increase the surfaces for infection and changes the microclimatic conditions in the malformations that could trap fungal propagules and be more conducive to fungal growth.

The significant difference in number and prevalence of fungi between healthy and malformed tissues of *S. lancea* warrants further investigation for its ecological meaning. More sampling and identification of these and other isolated endophytes to species level will provide a more complete baseline of natural *S. lancea* – endophyte associations, which will be necessary to distinguish between natural, normal associations of endophytes with the tree, and those which are novel as a result of secondary invasion of malformation symptoms. It is also necessary to understand the ecology of *S. lancea* with endophytes during disease development, since it could be possible that such fungal successions could be conducive to secondary disease development which may result in secondary disease symptoms such as die-back.

b) *The dominant fungal morphospecies group, Alternaria alternata*

Alternaria alternata (MSP1) was by far the most dominant fungal group obtained from the malformation symptoms. This species is a known pathogen of various plants, causing stem and leaf spots, and post-harvest rots of over 100 plant species (Akimitsu *et al*. 2003; Woudenberg *et al*. 2013; Armitage *et al*. 2015). Examples include citrus brown spot of tangerines characterized by brown or black lesions of leaves, twigs and fruit surrounded by a yellow halo, and post-harvest black rot of strawberries (Huang *et al*. 2015; Zhang *et al*. 2015). However, neither *A. alternata* nor any other species of *Alternaria*, are known to cause malformation symptoms and it is unlikely that this fungal group could be responsible for the malformation symptoms. The ability of *A. alternata* to occur as a common endophyte or an important secondary pathogen in numerous hosts (Woudenberg *et al*. 2013), could be important in complications resulting from the disease and these aspects could be investigated further. Numerous malformations die off, probably because of of *A. alternata* infection.

5.2.5. Insect associations of *Searsia lancea* and KMD symptoms

Similar to the number and diversity of fungi, the diversity of insects associated with malformations on *S. lancea* is equally great. These include members of the families Aphididae (MSP1), Anthocoridae (MSP8), Cercopidae (MSP2,3), Cicadellidae (MSP4), Coccinellidae (MSP5), Encyrtidae (MSP17), Formicidae (MSP6), Lygaeidae (MSP7), Psyllidae (MSP9,10), Reduviidae (MSP11,12), Scarabaeidae (MSP13), Termitidae (MSP14), Thripidae (MSP15,16), and an unknown Lepidopteran (MSP18). Only five or less insects belonging to the Cicadellidae, Cocinellidae, Encyrtidae Lygaeidae, Scarabaeidae, Termitidae, and Lepidopteran groups were collected, and their occurrence can be considered as chance. The remaining MSP's included groups generally considered to be plant feeders (Aphididae, Cercopidae, Formicidae, Psyllidae including jumping plant lice namely *Agonoscnena crotalaria*, and Thripidae including black flower thrips namely *Haplothrips gowdeyi*), and predatory groups (Anthocoridae including the pirate bug or *Orius* sp., and Reduviidae) (Hanna 1970; Picker *et al*. 2002). Of these the plant feeders are of special interest since these include known pathogen vectors and gall formers (Lattin,

1999; Grové et al. 2001; Koyoma *et al*. 2004; Tokuda *et al*. 2013) and future studies could focus on these to investigate possible causality.

a) *Insect comparisons between healthy and malformed tissues of Searsia lancea*

Similarly to what was observed for the fungi, the majority of insects (1545 out of 1727 morphospecies) were collected from malformed tissues of *S. lancea*. The ASGM (Price *et al*. 1987; Stone and Schönrogge, 2003) could again possibly be useful for understanding this trend. However, differently from the fungal colonization patterns, the differences in insect numbers between the healthy and malformed tissues were not significant. This most likely is due to the fact that the insects are not as immobile as fungi once the plant tissues were infected or colonized, and they could still move between different parts of the tree, i.e. between healthy and malformed tissues. In this sense, the patterns observed for the fungi could be more indicative of different patterns between healthy and malformed tissues. This is because both healthy and malformed tissues would be exposed to the same inoculum load of fungal propagules in the air, but only those able to differentially infect healthy or affected plant tissues would be detected in surface sterilized plant tissues.

Black flower thrips (*H. gowdeyi*, MSP16) and MSP6 (Formicidae) were the only groups of which more than five individuals were collected only from malformed tissues of *S. lancea*. Both of these MSP's are plant feeders (Picker *et al*. 2002). Their presence on only malformed tissues of *S. lancea* could be interpreted ecologically to indicate increased nutritional value of malformed tissue, or that they were exploiting the new morphological characteristics of *S. lancea* malformations to take shelter from the elements or hide from predators (Price *et al*. 1987; Stone and Schönrogge, 2003). Since thrips are known to be able to induce galls or to vector pathogens that can deform plant tissues, thrips could possibly contribute to malformations and should be studied in future.

Morphological species collected from both healthy and malformed tissues of *S. lancea* included plant feeding groups MSP1 (Aphididae), MSP3 (Cercopidae), MSP9 (Psyllidae), jumping plant lice (*A. crotalaria* in the Psyllidae, MSP10) and MSP15 (Thripidae). Predatory *Orius* sp. (Anthocoridae, MSP8) and MSP11 (Reduviidae)

were also found from both tissue types (Picker *et al*. 2002). Of these, all except MSP11 were collected in higher numbers from malformed than healthy tissues of *S. lancea*. In relation to the ASGM (Price *et al*. 1987; Stone and Schönrogge, 2003) these might all be seen as better able to exploit and compete for resources provided by malformations. In the case of MSP11, only a single individual were found occurring more on healthy than malformed tissues and could be a chance occurrence, or else it is simply not able to hunt as effectively on malformed tissues by being a weaker competitor or unable to access sheltered prey.

The only groups of which more than five individuals were collected only from healthy tissues of *S. lancea* were MSP2 (Cercopidae) and MSP12 (Reduviidae). Morphological species 2 is a plant feeder (Hanna, 1970), and its absence on malformed tissues could be explained as this species is possibly a weaker resource competitor and may be unable to exploit shelter from predators, making it an easy prey. The absence of predatory MSP12 from malformed tissues might also be explained as being a weaker competitor in such crowded habitats.

More comprehensive, targeted and repeated insect surveys are necessary to determine the complete range of insect species (including all life stages), definite identifications and the degree of their association with diseased tissues of *S. lancea*. This will also provide more insight if the differences observed between healthy and malformed tissues are significant and will confirm the possible ecological explanations discussed above. This will be a significant contribution to unravelling what appears to be a complex biological system.

Results from the insect surveys in this study indicate that there is possibly no straight answer if insects, or which insect group, could be the causal agent for the malformation symptoms. The most direct method to test such hypotheses will be to target insect groups with significant occurrence that are known to cause deformations or vector phytopathogens, such as the psyllids. These will have to be reared and inoculated to *S. lancea* trees that show no symptoms of KMD to determine whether they are causal. In the case of insects, vectors of pathogens, pathogen-free and pathogen-infected insects will have to be used. Methods that can screen for such pathogens in the insects i.e. phytoplasmas or viruses, will have to be

used. If these are unsuccessful other causal agents such as mites must be considered and searched for in future.

b) *Dominant insect group, namely the Psyllidae*

The Phyllidae (MSP9) was by far the dominant group of insects associated with *S. lancea*. Psyllids (MSP9 and MSP10) are known vectors of bacterial, phytoplasma and viral plant diseases, including those causing deformation-type symptoms (Hodkinson, 1974; Tedeschi *et al*. 2006; Griffiths, 2013). Psyllid feeding alone can also cause damage including localized necrosis, galling of leaves and stems, and undescribed malformation of meristematic tissues (Hodkinson, 1974; Wright and Samways, 1998; Picker *et al*. 2002). This family occurs mostly on dichotomous plants such as *S. lancea* but divergence to closely related and even distantly related plant hosts has also been observed (Hodkinson, 1974; Picker *et al*. 2002). Due to their prominence and the fact that alone or as a vector, these insects could cause deformation-type symptoms, they would be a logical starting point of future studies on possible causal agents of KMD. Morphological species 9 was represented by larvae that could not be identified to species level. It is, however, possible that rearing and DNA-sequence based comparisons will reveal that MSP9 and MSP10 (identified from adults as jumping plant lice or *A. crotalaria*) are the same species.

5.2.6. Host jump possibilities of fungi and insects from *Searsia lancea* to other plants

Occurrence data shows that mango (*M. indica*) producing areas in South Africa overlap with the natural and ornamental range of *S. lancea*. Main areas of *M. indica* cultivation in South Africa include Letsitele Valley, Hoedspruit and Trichardtsdal in the Limpopo Province, and Onderberg in the Mpumalanga Province (Anonymous, 2003). Considering collection data for *S. lancea* from the South African National Biodiversity Institute (SANBI, Pretoria, Gauteng Province) (Figure 1.1, Chapter 1), and its popularity in gardens and streets (Coates-Palgrave *et al*. 2000), there is overlap in the distribution of the species that can facilitate host jumps of pathogens between *S. lancea* and *M. indica*. However, current surveys did not yet cover *S. lancea* trees in those areas. Because South Africa is one of the largest global

producers of *M. indica* (Krishnan *et al*. 2009), of which 32.5% is exported annually, the potential that the causal agent of KMD can jump to *M. indica* should be investigated. Such a jump could have grave consequences on the mango industry should the disease prove to be serious on mango, and if the disease could potentially spread across the globe affecting other mango producing regions. Similarly, the causal agent of KMD can also infect related cashew and pistachio trees. On the other hand, the potential of *Fusarium* species causing MMD to equally infect native *S. lancea* populations, should also be tested through inoculations.

The dominant fungus, *Alternaria alternata* and dominant insect, *Agonoscena crotalaria*, were already not exclusively found on *S. lancea*. *Alternaria alternata* is a common endophyte and pathogen of many plant species (Woudenberg *et al*. 2013; Armitage *et al*. 2015). *Agonoscena crotalaria* displays a degree of host plasticity between related dichotomous species (Hodkinson, 1974; Picker *et al*. 2002). If it is found to be, or to vector, the causal agent of KMD it could possibly jump to important, related species such as *M. indica.*

Once a causal agent for KMD has been identified, its host jump ability can be tested by inoculating plant species of concern such as commercial mango, cashew and pistachio and determine whether similar symptoms develop. This should also be done to determine whether MMD causing *Fusarium* spp. are able to affect *S. lancea*. Developing such a technique would also prove invaluable in studies to further understanding the ecology of KMD and its possible impact on other members of the Anacardiaceae.

5.2.7. Phytohormone analysis of KMD symptoms in *Searsia lancea*

The ultimate aim of investigating phytohormone differences between healthy and malformed tissues of *S. lancea*, is to better understand how the morphological changes are brought about, how it changes phytohormone levels with possible secondary effects, and to compare the mechanisms of malformation production in *S. lancea* to other plant-phytohormone systems. Once the causal agent is determined, understanding of the action of the pathogen and how it is able to bring about such morphological changes in *S. lancea* will be invaluable. For example, it will be

important to determine if the pathogen independently produces additional phytohormones, or whether it introduces effector proteins that suppress or antagonize phytohormone expression (Quoirin *et al*. 2004; Agrios, 2005).

However, in this study it was important to first establish the methodology for phytohormone determination. For these purposes only three phytohormones were chosen. It will be the aim of future studies to do full comparisons of more phytohormones that will yield more complete data towards understanding of the physiological changes affected in the case of KMD.

The methods used for phytohormone quantification prove efficient but will benefit with further development and optimization to delineate a suitable timeframe. The phytohormones salicylic acid (SA), gibberellic acid (GA_3) and jasmonic acid (JA) were compared between healthy and malformed tissues as standards were readily available. Differences in GA_3 has been noted between healthy and malformed tissues of *M. indica* (Singh and Dhillon, 1989), whereas such comparison of JA and SA is novel.

a) *Salicylic acid, gibberellic acid and jasmonic acid*

Of the three phytohormones tested, only salicylic acid (SA) levels were significantly higher in malformed than healthy tissues of *S. lancea*. Known effects of higher than normal levels of SA include effects on seed germination and increased plant defence responses (Popova *et al*. 1997). SA has a defence related role in the hypersensitive response (HR) and systemic acquired resistance (SAR) (Popova *et al*. 1997; Clarke *et al*. 2000) that could play a role in the complex biology of the various communities present in the malformations, including that of the causal agent. SA has also been shown to influence levels of nutrients, e.g. the inhibition of K^+ absorption (Harper and Balke, 1981; Popova *et al*. 1997). It is thus possible that the role of SA in plants, namely general plant growth and development, ion uptake, ethylene synthesis, photosynthesis, thermogenesis and flower phenology (summarized in Chapter 2, Table 5), is more pronounced in malformed than healthy tissues of *S. lancea*. However, based on the paucity on examples of possible effects of higher levels of SA in plant tissues, more detailed studies will be needed to understand the implications of these changes in KMD.

General observations indicate that GA_3 and JA levels are lower in healthy than malformed tissues of *S. lancea*. These differences were, however, not significant, and could be attributed to low variance and sample size of the data set and should be re-assessed following analysis of an appropriate number of samples. If a re-assessment supports the generally observed differences in GA_3 and JA levels between healthy and malformed tissues, it is reasonable to accept that these phytohormones could be part of the interactions present in malformation development (summarized in Chapter 2, Table 4; Table 5). For instance reduced GA_3 concentration could result in reduced or unusual flower and bud development, along with ceased elongation, and altered leaf development (reduced size, contortion) (Kende and Zeevaart, 1997; Hopkins and Hüner, 2004; Nabors and González-Barreda, 2004). Other roles such as leaf abscission and senescence have been attributed to lower levels of JA (Creelman and Mullet, 1995; Nabors and González-Barreda, 2004). Symptoms that can result from lower levels of JA include reduced involvement in plant defence responses, inhibition of seed germination, pollen and root growth, protein accumulation during seed development, and tendril coiling (Creelman and Mullet, 1995; Clarke *et al*. 2000; Hopkins and Hüner, 2004; Nabors and González-Barreda, 2004; Agrios, 2005; Spoel *et al*. 2007; Smart *et al.* 2013). Careful experimental design is necessary to determine the true significance of our observations and to correlate these with possible effects.

5.2.8. Comparisons of nutrients in malformed and healthy tissues of *Searsia lancea*

Our results generally show no change in phosphorus (P) levels, and higher levels of nitrogen (N) and potassium (K) in malformed tissues. Unfortunately due to the nutrient quantification method that required us to pool samples into a single sample for each respective nutrient quantification of both conditions, significance for variance could not be determined. The changed levels of N and K most likely will be adequate for physiological function and it is unlikely that levels will be sufficiently high to become toxic (Hopkins and Hüner, 2004). Normally an increase in N results in enhanced growth of shoots and delayed flowering (Hopkins and Hüner, 2004),

which could explain observed symptoms of proliferation and unopened buds, but this would require further investigation.

An increased content of K contradicts the effects of an increase in SA (Figure 4.4.1) (Harper and Balke, 1981; Popova *et al*. 1997). This suggests the K level, upon analysis of a larger data set will most likely be significantly lower as opposed to higher in malformed tissues than healthy tissues of *S. lancea*. Higher levels of N and K could also correspond to the nutrition hypothesis for the adaptive significance of galls (Price *et al*. 1987; Hartley and Lawton, 1992; Koyama *et al*. 2004). More studies on how these are possibly exploited are however needed.

5.3. CONCLUSION

KMD represents an example of a natural disease on a native tree in its native environment. Besides the usual questions that needs to asked, such as what causes the disease and how commonly occurring it is, this system also provides other opportunities. For instance, the differences in diversity and abundance for fungal and insect species between healthy and malformed tissues, indicates that *S.lancea* malformations present a habitat distinct from the rest of the tree that should be characterized more. It is also imperative to understand the adaptive significance of these malformations for the causal agent.

Other interesting questions include how the disease affects *S. lancea* performance as a species used in phytoremediation (Lange *et al*. 2012). It could be that trees persist but their role in remediation is reduced. Alternatively trees may die as a result of overwhelming physiological stress from a combination of disease and the unfavourable abiotic conditions present in the site to be remediated. Understanding the underlying ecological implications will contribute to understanding of this new disease of the ubiquitous *S. lancea*, and influence disease management, control and related research.

Relatively little is known about physiological changes and their purposes and interactions associated with deformation-type symptoms. There is also not a well-studied model system on which to model the processes associated in particular with KMD. Because the causal agent of KMD is not a fungus like MMD, the associated

physiological changes will not necessarily be similar. In addition methods are not always well described or even uniform across physiological studies of other species. This is more difficult until the correct causal agent for KMD has been identified and it can be used to induce disease symptoms in the laboratory. However, it is clear that the processes could be complex and careful planning would be necessary to answer appropriate questions. For instance, particular effects measured in the induced malformations could be induced by changes in levels of phytohormones and/or nutrients, or those processes can themselves cause the changes in phytohormones and nutrient levels.

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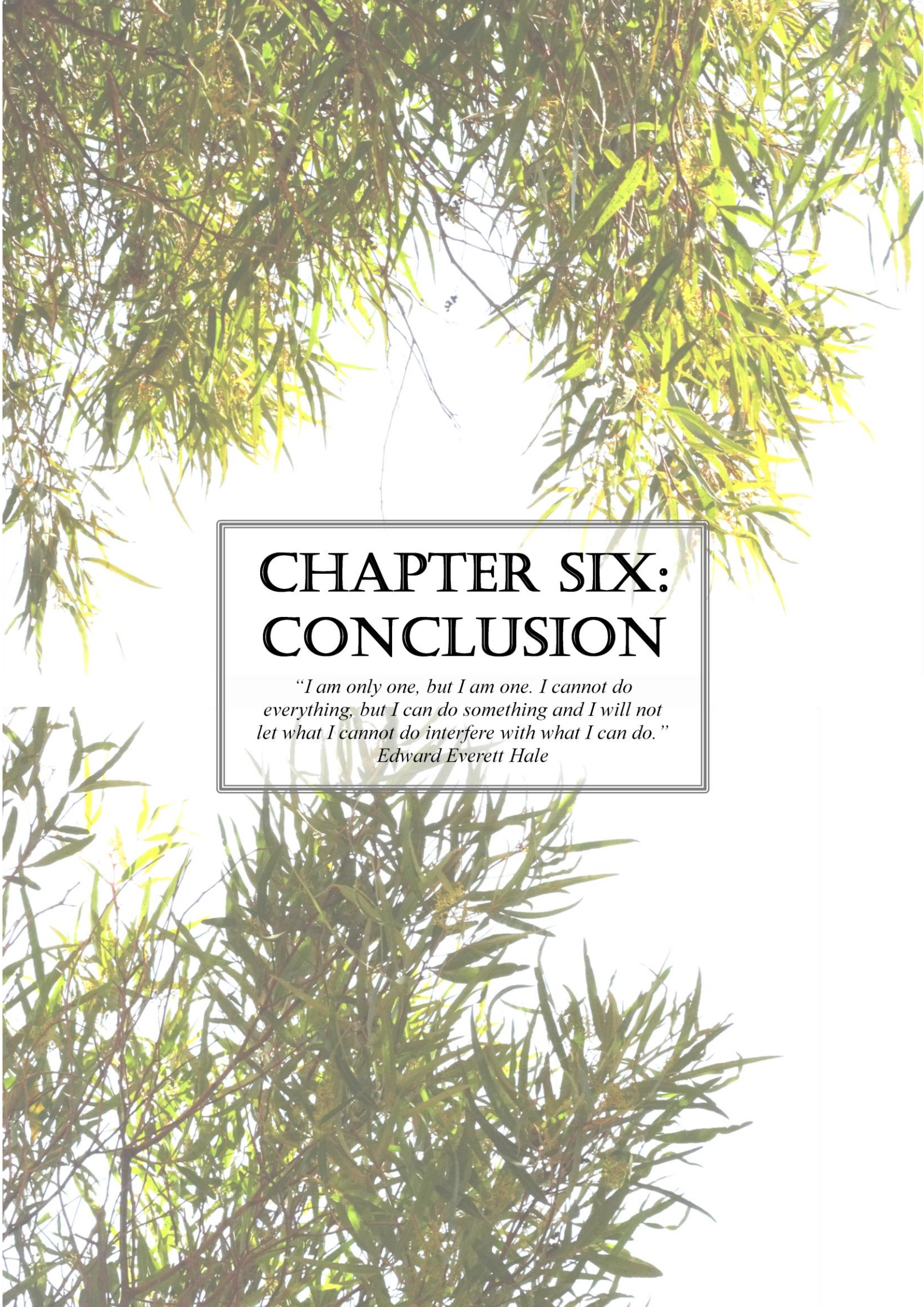

CHAPTER SIX: CONCLUSION

"I am only one, but I am one. I cannot do everything, but I can do something and I will not let what I cannot do interfere with what I can do."
Edward Everett Hale

6.1. CONCLUSION

This study reports on a new disease of the common karee (*Searsia lancea*). Disease symptoms include chlorosis, gall-like swelling, smaller and larger than normal leaf size, leaf contortion, enlarged flowers, unopened flower buds and proliferation of shoots and leaves (witches' broom-like). The disease is not commonly occurring, despite the widespread occurrence of this keystone native tree. The disease was named karee malformation disease and exhibits symptoms similar to that of mango malformation disease. This is also significant because mango (*Mangifera indica*) and *S. lancea* belong to the same plant family, namely the Anacardiaceae.

Unlike MMD, results showed that KMD is not caused by species in the fungal genus *Fusarium*. Extensive fungal isolations from malformed disease symptoms, compared to those from healthy tissues, also showed no other likely fungal causal agent. It is thus suggested that other possible causal agents, such as insects, mites, or insect borne pathogens such as phytoplasmas or viruses, be investigated in future.

During surveys conducted in this study, insects associated with malformations were sampled concurrently with fungal isolations. A particular morphological group of psyllids were found to occur very dominantly in malformed tissues. Since this group of insects can cause galling or vector plant pathogens that can cause disease symptoms such as malformations, it is suggested that these insects should be studied for the potential role they may play in the formation of the disease. Other possible insect candidates such as thrips and aphids should also be considered.

Comparisons of the insect communities between healthy and malformed tissues indicated community differences and high diversity. The malformations thus appear to be a unique niché within the tree, possibly attracting its own suite of organisms. The interactions between the various organisms, as well as those with the tree itself, most likely are complex.

In this study a foundation was established for future work in studying possible changes in phytohormone and nutrient levels in malformed tissues. This is important because such physiological changes may be the reasons for the malformations to occur. It could also be side-effects due to other processes. Nonetheless, these all play a role in the formation of the malformations and the maintenance of the complex

communities linked to the malformations. Future studies on the ecological reasons and impact of malformations in this native system will be interesting, and will add to the understanding of those on more commercial host systems.

A very important aspect related to KMD is the threat it may pose for the mango industry. Future studies should survey if there could be a possibility in geographic overlap that may predispose mango trees to this disease. Similarly, it is not yet known whether the *Fusarium* spp. causing MMD could potentially infect native *S. lancea* trees.

Clearly more research needs to be done on KMD to determine the causal agents, the numerous interactions occurring in the malformations and to ascertain the correct geographical spread and impact of the disease. Due to numerous types of possible causal agents, the research will be multidisciplinary in nature and therefore challenging. It is also challenging due to the confusing usage of terminology making proper literature searches difficult. Literature reviews also revealed that relatively little has been done on all of the aspects involved with deformation-type diseases, making comparisons difficult. However, through this thesis the basic work had been completed and interactions were determined by a process of elimination. Important recommendations for future research could already be made.

APPENDIX A – FUNGI ISOLATED

MSP	DESIGNATION	TOT.	H.	M.	MSP	DESIGNATION	TOT.	H.	M.
1	Alternaria alternata	318	6	312	51	LMSP2	3	3	0
2	Yeast-like MSP1	36	1	35	52	LMSP12	3	3	0
3	Xylariaceae MSP1	25	1	24	53	LMSP15	3	1	2
4	Yeast-like MSP2	23	3	20	54	LMSP16	3	0	3
5	Cladosporium	21	1	20	55	LMSP18	3	0	3
6	Red chaetomium	17	0	17	56	LMSP19	3	0	3
7	Chaetomium MSP1	16	0	16	57	LMSP20	3	1	2
8	Nigrospora MSP3	16	0	16	58	LMSP21	3	0	3
9	Nigrospora MSP4	14	2	12	59	Nigrospora MSP2	3	0	3
10	LMSP5	14	0	14	60	Fus MSP1	3	0	3
11	Epic MSP1	12	0	12	61	Fus MSP2	3	0	3
12	Chaetomium MSP2	12	0	12	62	Fus MSP4	3	0	3
13	Nigrospora MSP1	11	1	10	63	Fus MSP6	3	0	3
14	Nigrospora MSP5	11	3	8	64	Fus MSP7	3	0	3
15	LMSP1	10	0	10	65	Yeast-like MSP4	2	0	2
16	LMSP7	9	0	9	66	Yeast-like MSP5	2	2	0
17	LMSP6	8	3	5	67	Yeast-like MSP6	2	0	2
18	Phamopsis MSP1	8	0	8	68	Dark Green MSP5	2	0	2
19	BYMSP3	7	0	7	69	Dark Green MSP6	2	0	2
20	Chaetomium MSP3	7	0	7	70	Pestalotriops	2	0	2
21	White-cream MSP1	7	2	5	71	Epic MSP2	2	1	1
22	LMSP4	7	0	7	72	Chaetomium MSP10	2	0	2
23	LMSP9	7	1	6	73	Chaetomium MSP11	2	0	2
24	Chaetomium MSP4	6	0	6	74	Chaetomium MSP12	2	0	2
25	Yeast-like MSP3	6	2	4	75	Chaetomium MSP13	2	0	2
26	BYMSP1	5	3	2	76	Brown MSP2	2	0	2
27	Chaetomium MSP5	5	0	5	77	Beige MSP2	2	0	2
28	Chaetomium MSP6	5	0	5	78	White-cream MSP5	2	0	2
29	Brown MSP1	5	4	1	79	White-cream MSP6	2	0	2
30	LMSP14	5	4	1	80	White-cream MSP7	2	0	2
31	LMSP8	5	0	5	81	White-cream MSP8	2	0	2
32	Chaetomium MSP7	4	0	4	82	White-cream MSP9	2	0	2
33	BYMSP2	4	0	4	83	LMSP22	2	0	2
34	Dark Green MSP1	4	0	4	84	LMSP23	2	0	2
35	Dark Green MSP2	4	0	4	85	LMSP24	2	0	2
36	Trichoderma	4	0	4	86	LMSP25	2	0	2
37	Basidiomycete	4	0	4	87	LMSP26	2	0	2
38	LMSP3	4	1	3	88	LMSP27	2	2	0
39	LMSP10	4	1	3	89	LMSP28	2	0	2
40	LMSP11	4	0	4	90	LMSP29	2	2	0
41	LMSP13	4	0	4	91	LMSP30	2	0	2
42	LMSP17	4	0	4	92	LMSP31	2	0	2
43	Dark Green MSP3	3	0	3	93	LMSP32	2	0	2
44	Dark Green MSP4	3	0	3	94	LMSP33	2	0	2
45	Chaetomium MSP8	3	0	3	95	LMSP34	2	0	2
46	Chaetomium MSP9	3	0	3	96	LMSP35	2	0	2
47	Beige MSP1	3	2	1	97	LMSP36	2	0	2
48	White-cream MSP2	3	0	3	98	LMSP37	2	0	2
49	White-cream MSP3	3	2	1	99	LMSP38	2	0	2
50	White-cream MSP4	3	0	3	100	LMSP39	2	0	2

101	LMSP40	2	0	2	156	Penicillium	1	1	0
102	LMSP41	2	0	2	157	Phamopsis MSP2	1	1	0
103	LMSP42	2	2	0	158	Brown MSP3	1	0	1
104	LMSP43	2	0	2	159	Brown MSP4	1	0	1
105	LMSP44	2	0	2	160	Brown MSP5	1	0	1
106	LMSP45	2	0	2	161	Brown MSP6	1	0	1
107	LMSP46	2	0	2	162	Brown MSP7	1	0	1
108	BYMSP7	2	2	0	163	Brown MSP8	1	0	1
109	Yeast-like MSP36	2	0	2	164	Brown MSP9	1	0	1
110	Yeast-like MSP37	2	0	2	165	Brown MSP10	1	0	1
111	Fus MSP3 MAL	2	0	2	166	Brown MSP11	1	0	1
112	Fus MSP5	2	0	2	167	Brown MSP12	1	0	1
113	BYMSP4	1	0	1	168	Brown MSP13	1	0	1
114	BYMSP5	1	0	1	169	Brown MSP14	1	0	1
115	BYMSP6	1	0	1	170	Brown MSP15	1	0	1
116	Yeast-like MSP7	1	0	1	171	Brown MSP16	1	0	1
117	Yeast-like MSP8	1	0	1	172	Brown MSP17	1	0	1
118	Yeast-like MSP9	1	1	0	173	Brown MSP18	1	0	1
119	Yeast-like MSP10	1	0	1	174	Brown MSP19	1	0	1
120	Yeast-like MSP11	1	0	1	175	Brown MSP20	1	0	1
121	Yeast-like MSP12	1	0	1	176	Brown MSP21	1	0	1
122	Yeast-like MSP13	1	0	1	177	Brown MSP22	1	0	1
123	Yeast-like MSP14	1	0	1	178	Brown MSP23	1	0	1
124	Yeast-like MSP15	1	0	1	179	Brown MSP24	1	0	1
125	Yeast-like MSP16	1	0	1	180	Brown MSP25	1	1	0
126	Yeast-like MSP17	1	0	1	181	Brown MSP26	1	0	1
127	Yeast-like MSP18	1	0	1	182	Brown MSP27	1	0	1
128	Yeast-like MSP19	1	0	1	183	Brown MSP28	1	0	1
129	Yeast-like MSP20	1	1	0	184	Brown MSP29	1	0	1
130	Yeast-like MSP21	1	0	1	185	Beige MSP3	1	0	1
131	Yeast-like MSP22	1	0	1	186	Beige MSP4	1	0	1
132	Yeast-like MSP23	1	0	1	187	Beige MSP5	1	0	1
133	Yeast-like MSP24	1	0	1	188	Beige MSP6	1	0	1
134	Yeast-like MSP25	1	0	1	189	Beige MSP7	1	0	1
135	Yeast-like MSP26	1	0	1	190	Beige MSP8	1	0	1
136	Yeast-like MSP27	1	0	1	191	Beige MSP9	1	0	1
137	Yeast-like MSP28	1	0	1	192	Beige MSP10	1	0	1
138	Yeast-like MSP29	1	0	1	193	Beige MSP11	1	0	1
139	Yeast-like MSP30	1	0	1	194	Beige MSP12	1	0	1
140	Yeast-like MSP31	1	0	1	195	Beige MSP13	1	0	1
141	Yeast-like MSP32	1	0	1	196	Beige MSP14	1	1	0
142	Yeast-like MSP33	1	0	1	197	Beige MSP15	1	0	1
143	Yeast-like MSP34	1	0	1	198	Beige MSP16	1	1	0
144	Yeast-like MSP35	1	0	1	199	Beige MSP17	1	0	1
145	Chaetomium MSP14	1	0	1	200	Beige MSP18	1	1	0
146	Chaetomium MSP15	1	0	1	201	Beige MSP19	1	0	1
147	Chaetomium MSP16	1	0	1	202	Beige MSP20	1	0	1
148	Chaetomium MSP17	1	0	1	203	Beige MSP21	1	0	1
149	Chaetomium MSP18	1	0	1	204	Beige MSP22	1	0	1
150	Chaetomium MSP19	1	0	1	205	Beige MSP23	1	0	1
151	Chaetomium MSP20	1	0	1	206	Beige MSP24	1	0	1
152	Chaetomium MSP21	1	0	1	207	Beige MSP25	1	1	0
153	Chaetomium MSP22	1	0	1	208	Beige MSP26	1	1	0
154	Chaetomium MSP23	1	0	1	209	Beige MSP27	1	0	1
155	Epic MSP3	1	1	0	210	Beige MSP28	1	1	0

211	Beige MSP29	1	0	1	266	Dark Green MSP45	1	1	0
212	Beige MSP30	1	0	1	267	White-cream MSP10	1	0	1
213	Beige MSP31	1	0	1	268	White-cream MSP11	1	0	1
214	Beige MSP32	1	0	1	269	White-cream MSP12	1	0	1
215	Beige MSP33	1	1	0	270	White-cream MSP13	1	0	1
216	Beige MSP34	1	0	1	271	White-cream MSP14	1	0	1
217	Beige MSP35	1	0	1	272	White-cream MSP15	1	0	1
218	Beige MSP36	1	0	1	273	White-cream MSP16	1	0	1
219	Beige MSP37	1	1	0	274	White-cream MSP17	1	0	1
220	Beige MSP38	1	0	1	275	White-cream MSP18	1	0	1
221	Beige MSP39	1	0	1	276	White-cream MSP19	1	1	0
222	Beige MSP40	1	0	1	277	White-cream MSP20	1	0	1
223	Beige MSP41	1	0	1	278	White-cream MSP21	1	0	1
224	Beige MSP42	1	1	0	279	White-cream MSP22	1	1	0
225	Beige MSP43	1	0	1	280	White-cream MSP23	1	0	1
226	Beige MSP44	1	0	1	281	White-cream MSP24	1	0	1
227	Beige MSP45	1	0	1	282	White-cream MSP25	1	0	1
228	Dark Green MSP7	1	0	1	283	White-cream MSP26	1	0	1
229	Dark Green MSP8	1	0	1	284	White-cream MSP27	1	0	1
230	Dark Green MSP9	1	0	1	285	White-cream MSP28	1	0	1
231	Dark Green MSP10	1	0	1	286	White-cream MSP29	1	0	1
232	Dark Green MSP11	1	0	1	287	White-cream MSP30	1	0	1
233	Dark Green MSP12	1	1	0	288	White-cream MSP31	1	0	1
234	Dark Green MSP13	1	0	1	289	White-cream MSP32	1	0	1
235	Dark Green MSP14	1	0	1	290	White-cream MSP33	1	1	0
236	Dark Green MSP15	1	0	1	291	White-cream MSP34	1	0	1
237	Dark Green MSP16	1	0	1	292	White-cream MSP35	1	0	1
238	Dark Green MSP17	1	0	1	293	White-cream MSP36	1	0	1
239	Dark Green MSP18	1	0	1	294	White-cream MSP37	1	0	1
240	Dark Green MSP19	1	0	1	295	White-cream MSP38	1	0	1
241	Dark Green MSP20	1	0	1	296	White-cream MSP39	1	0	1
242	Dark Green MSP21	1	0	1	297	White-cream MSP40	1	0	1
243	Dark Green MSP22	1	0	1	298	White-cream MSP41	1	0	1
244	Dark Green MSP23	1	0	1	299	White-cream MSP42	1	0	1
245	Dark Green MSP24	1	0	1	300	White-cream MSP43	1	0	1
246	Dark Green MSP25	1	0	1	301	White-cream MSP44	1	0	1
247	Dark Green MSP26	1	0	1	302	White-cream MSP45	1	1	0
248	Dark Green MSP27	1	0	1	303	White-cream MSP46	1	0	1
249	Dark Green MSP28	1	0	1	304	White-cream MSP47	1	0	1
250	Dark Green MSP29	1	0	1	305	White-cream MSP48	1	0	1
251	Dark Green MSP30	1	0	1	306	White-cream MSP49	1	0	1
252	Dark Green MSP31	1	0	1	307	White-cream MSP50	1	0	1
253	Dark Green MSP32	1	0	1	308	White-cream MSP51	1	0	1
254	Dark Green MSP33	1	1	0	309	White-cream MSP52	1	0	1
255	Dark Green MSP34	1	0	1	310	White-cream MSP53	1	0	1
256	Dark Green MSP35	1	0	1	311	White-cream MSP54	1	0	1
257	Dark Green MSP36	1	0	1	312	White-cream MSP55	1	1	0
258	Dark Green MSP37	1	0	1	313	White-cream MSP56	1	0	1
259	Dark Green MSP38	1	0	1	314	White-cream MSP57	1	1	0
260	Dark Green MSP39	1	1	0	315	White-cream MSP58	1	0	1
261	Dark Green MSP40	1	0	1	316	White-cream MSP59	1	0	1
262	Dark Green MSP41	1	0	1	317	White-cream MSP60	1	0	1
263	Dark Green MSP42	1	1	0	318	White-cream MSP61	1	0	1
264	Dark Green MSP43	1	0	1	319	White-cream MSP62	1	0	1
265	Dark Green MSP44	1	1	0	320	White-cream MSP63	1	1	0

321	White-cream MSP64	1	0	1	376	LMSP90	1	0	1
322	White-cream MSP65	1	1	0	377	LMSP91	1	0	1
323	White-cream MSP66	1	1	0	378	LMSP92	1	0	1
324	White-cream MSP67	1	0	1	379	LMSP93	1	0	1
325	White-cream MSP68	1	0	1	380	LMSP94	1	0	1
326	White-cream MSP69	1	1	0	381	LMSP95	1	0	1
327	White-cream MSP70	1	0	1	382	LMSP96	1	0	1
328	White-cream MSP71	1	0	1	383	LMSP97	1	0	1
329	White-cream MSP72	1	0	1	384	LMSP98	1	0	1
330	White-cream MSP73	1	0	1	385	LMSP99	1	1	0
331	White-cream MSP74	1	0	1	386	LMSP100	1	0	1
332	Trichothecium	1	0	1	387	LMSP101	1	0	1
333	LMSP47	1	0	1	388	LMSP102	1	0	1
334	LMSP48	1	0	1	389	LMSP103	1	0	1
335	LMSP49	1	0	1	390	LMSP104	1	1	0
336	LMSP50	1	0	1	391	LMSP105	1	0	1
337	LMSP51	1	0	1	392	LMSP106	1	1	0
338	LMSP52	1	0	1	393	LMSP107	1	0	1
339	LMSP53	1	0	1	394	LMSP108	1	0	1
340	LMSP54	1	0	1	395	LMSP109	1	0	1
341	LMSP55	1	0	1	396	LMSP110	1	0	1
342	LMSP56	1	1	0	397	LMSP111	1	0	1
343	LMSP57	1	0	1	398	LMSP112	1	0	1
344	LMSP58	1	0	1	399	LMSP113	1	0	1
345	LMSP59	1	0	1	400	LMSP114	1	1	0
346	LMSP60	1	0	1	401	LMSP115	1	0	1
347	LMSP61	1	0	1	402	LMSP116	1	1	0
348	LMSP62	1	1	0	403	LMSP117	1	0	1
349	LMSP63	1	0	1	404	LMSP118	1	0	1
350	LMSP64	1	0	1	405	LMSP119	1	0	1
351	LMSP65	1	0	1	406	LMSP120	1	0	1
352	LMSP66	1	1	0	407	LMSP121	1	0	1
353	LMSP67	1	0	1	408	LMSP122	1	1	0
354	LMSP68	1	0	1	409	LMSP123	1	0	1
355	LMSP69	1	0	1	410	LMSP124	1	0	1
356	LMSP70	1	0	1	411	LMSP125	1	0	1
357	LMSP71	1	0	1	412	LMSP126	1	0	1
358	LMSP72	1	0	1	413	LMSP127	1	0	1
359	LMSP73	1	0	1	414	LMSP128	1	0	1
360	LMSP74	1	0	1	415	LMSP129	1	0	1
361	LMSP75	1	0	1	416	LMSP130	1	1	0
362	LMSP76	1	0	1	417	LMSP131	1	0	1
363	LMSP77	1	0	1	418	LMSP132	1	0	1
364	LMSP78	1	0	1	419	LMSP133	1	0	1
365	LMSP79	1	0	1	420	LMSP134	1	0	1
366	LMSP80	1	0	1	421	LMSP135	1	0	1
367	LMSP81	1	0	1	422	LMSP136	1	1	0
368	LMSP82	1	0	1	423	LMSP137	1	1	0
369	LMSP83	1	0	1	424	LMSP138	1	0	1
370	LMSP84	1	0	1	425	LMSP139	1	0	1
371	LMSP85	1	1	0	426	LMSP140	1	0	1
372	LMSP86	1	1	0	427	LMSP141	1	0	1
373	LMSP87	1	0	1	428	LMSP142	1	0	1
374	LMSP88	1	0	1	429	LMSP143	1	0	1
375	LMSP89	1	1	0	430	LMSP144	1	0	1

431	LMSP145	1	0	1	486	LMSP200	1	0	1
432	LMSP146	1	0	1	487	LMSP201	1	0	1
433	LMSP147	1	0	1	488	LMSP202	1	0	1
434	LMSP148	1	0	1	489	LMSP203	1	0	1
435	LMSP149	1	1	0	490	LMSP204	1	0	1
436	LMSP150	1	0	1	491	LMSP205	1	0	1
437	LMSP151	1	0	1	492	LMSP206	1	0	1
438	LMSP152	1	0	1	493	LMSP207	1	0	1
439	LMSP153	1	1	0	494	LMSP208	1	1	0
440	LMSP154	1	0	1	495	LMSP209	1	0	1
441	LMSP155	1	0	1	496	LMSP210	1	0	1
442	LMSP156	1	0	1	497	LMSP211	1	0	1
443	LMSP157	1	0	1	498	LMSP212	1	0	1
444	LMSP158	1	0	1	499	LMSP213	1	0	1
445	LMSP159	1	0	1	500	LMSP214	1	0	1
446	LMSP160	1	0	1	501	LMSP215	1	1	0
447	LMSP161	1	0	1	502	LMSP216	1	0	1
448	LMSP162	1	0	1	503	LMSP217	1	0	1
449	LMSP163	1	0	1	504	LMSP218	1	0	1
450	LMSP164	1	0	1	505	LMSP219	1	0	1
451	LMSP165	1	0	1	506	LMSP220	1	0	1
452	LMSP166	1	0	1	507	LMSP221	1	0	1
453	LMSP167	1	0	1	508	LMSP222	1	0	1
454	LMSP168	1	0	1	509	LMSP223	1	1	0
455	LMSP169	1	0	1	510	LMSP224	1	0	1
456	LMSP170	1	0	1	511	LMSP225	1	0	1
457	LMSP171	1	1	0	512	LMSP226	1	0	1
458	LMSP172	1	0	1	513	LMSP227	1	0	1
459	LMSP173	1	0	1	514	LMSP228	1	0	1
460	LMSP174	1	0	1	515	LMSP229	1	0	1
461	LMSP175	1	0	1	516	LMSP230	1	0	1
462	LMSP176	1	0	1	517	LMSP231	1	0	1
463	LMSP177	1	0	1	518	LMSP232	1	0	1
464	LMSP178	1	1	0	519	LMSP233	1	1	0
465	LMSP179	1	0	1	520	LMSP234	1	0	1
466	LMSP180	1	0	1	521	LMSP235	1	0	1
467	LMSP181	1	0	1	522	LMSP236	1	0	1
468	LMSP182	1	0	1	523	LMSP237	1	0	1
469	LMSP183	1	0	1	524	LMSP238	1	0	1
470	LMSP184	1	0	1	525	LMSP239	1	0	1
471	LMSP185	1	1	0	526	LMSP240	1	0	1
472	LMSP186	1	0	1	527	LMSP241	1	0	1
473	LMSP187	1	0	1	528	LMSP242	1	0	1
474	LMSP188	1	0	1	529	LMSP243	1	0	1
475	LMSP189	1	1	0	530	LMSP244	1	0	1
476	LMSP190	1	0	1	531	LMSP245	1	0	1
477	LMSP191	1	0	1	532	LMSP246	1	0	1
478	LMSP192	1	1	0	533	LMSP247	1	0	1
479	LMSP193	1	0	1	534	LMSP248	1	0	1
480	LMSP194	1	0	1	535	LMSP249	1	0	1
481	LMSP195	1	0	1	536	LMSP250	1	0	1
482	LMSP196	1	1	0	537	LMSP251	1	0	1
483	LMSP197	1	0	1	538	LMSP252	1	0	1
484	LMSP198	1	0	1	539	LMSP253	1	0	1
485	LMSP199	1	0	1	540	LMSP254	1	0	1

541	LMSP255	1	1	0
542	LMSP256	1	0	1
543	LMSP257	1	0	1
544	LMSP258	1	0	1
545	LMSP259	1	0	1
546	LMSP260	1	0	1
547	LMSP261	1	0	1
548	LMSP262	1	0	1
549	LMSP263	1	0	1
550	LMSP264	1	0	1
551	LMSP265	1	0	1
552	LMSP266	1	0	1
553	LMSP267	1	1	0
554	LMSP268	1	0	1
555	LMSP269	1	0	1
556	LMSP270	1	0	1
557	LMSP271	1	0	1
558	LMSP272	1	0	1
559	LMSP273	1	0	1
560	LMSP274	1	0	1
561	LMSP275	1	0	1
562	LMSP276	1	0	1
563	LMSP277	1	0	1
564	BYMSP8	1	0	1
565	BYMSP9	1	1	0
566	Yeast-like MSP38	1	0	1
567	Yeast-like MSP39	1	1	0
568	Yeast-like MSP40	1	1	0
569	Yeast-like MSP41	1	1	0
570	Yeast-like MSP42	1	1	0
571	Yeast-like MSP43	1	0	1
572	Yeast-like MSP44	1	0	1
573	Yeast-like MSP45	1	0	1
574	Yeast-like MSP46	1	0	1
575	Yeast-like MSP47	1	0	1
576	Yeast-like MSP48	1	0	1

Total number of fungal isolates:

1328

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Total isolated from healthy tissues:

127

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Total isolated from malformed tissues:

1201

APPENDIX B – INSECTS COLLECTED

MSP	DESIGNATION	TOTAL	HEALTHY	MALFORMED
1	Aphididae, Hemiptera (MSP1)	25	4	21
2	Cercopidae, Hemiptera (MSP1)	2	2	0
3	Cercopidae, Hemiptera (MSP2)	28	1	27
4	Cicadellidae, Hemiptera (MSP1)	1	0	1
5	Coccinellidae, Coleoptera (MSP1)	5	0	5
6	Formicidae, Hymenoptera (MSP1)	6	0	6
7	Lygaeidae, Hemiptera (MSP1)	3	0	3
8	Orius sp., Anthocoridae, Hemiptera (MSP1)	7	1	6
9	Psyllidae, Hemiptera (MSP1)	1613	166	1447
10	Agonoscena crotalaria, Psyllidae, Hemiptera (MSP2)	11	1	10
11	Reduviidae, Hemiptera (MSP1)	5	3	2
12	Reduviidae, Hemiptera (MSP2)	1	1	0
13	Scarabaeidae, Coleoptera (MSP1)	1	0	1
14	Termitidae, Isoptera (MSP1)	5	0	5
15	Thripidae, Thysanoptera (MSP1)	8	1	7
16	Haplothrips gowdeyi, Thripidae, Thysanoptera (MSP2)	2	0	2
17	Ooencyrtus sp., Encyrtidae, Hemiptera (MSP1)	1	1	0
18	Lepidoptera (MSP1)	3	1	2
	TOTAL:	1727	182	1545

APPENDIX C – t-DISTRIBUTION TABLE

TABLE of CRITICAL VALUES for STUDENT'S *t* DISTRIBUTIONS

Column headings denote probabilities (α) ***above*** tabulated values.

d.f.	0.40	0.25	0.10	0.05	0.04	0.025	0.02	0.01	0.005	0.0025	0.001	0.0005
1	0.325	1.000	3.078	6.314	7.916	12.706	15.894	31.821	63.656	127.321	318.289	636.578
2	0.289	0.816	1.886	2.920	3.320	4.303	4.849	6.965	9.925	14.089	22.328	31.600
3	0.277	0.765	1.638	2.353	2.605	3.182	3.482	4.541	5.841	7.453	10.214	12.924
4	0.271	0.741	1.533	2.132	2.333	2.776	2.999	3.747	4.604	5.598	7.173	8.610
5	0.267	0.727	1.476	2.015	2.191	2.571	2.757	3.365	4.032	4.773	5.894	6.869
6	0.265	0.718	1.440	1.943	2.104	2.447	2.612	3.143	3.707	4.317	5.208	5.959
7	0.263	0.711	1.415	1.895	2.046	2.365	2.517	2.998	3.499	4.029	4.785	5.408
8	0.262	0.706	1.397	1.860	2.004	2.306	2.449	2.896	3.355	3.833	4.501	5.041
9	0.261	0.703	1.383	1.833	1.973	2.262	2.398	2.821	3.250	3.690	4.297	4.781
10	0.260	0.700	1.372	1.812	1.948	2.228	2.359	2.764	3.169	3.581	4.144	4.587
11	0.260	0.697	1.363	1.796	1.928	2.201	2.328	2.718	3.106	3.497	4.025	4.437
12	0.259	0.695	1.356	1.782	1.912	2.179	2.303	2.681	3.055	3.428	3.930	4.318
13	0.259	0.694	1.350	1.771	1.899	2.160	2.282	2.650	3.012	3.372	3.852	4.221
14	0.258	0.692	1.345	1.761	1.887	2.145	2.264	2.624	2.977	3.326	3.787	4.140
15	0.258	0.691	1.341	1.753	1.878	2.131	2.249	2.602	2.947	3.286	3.733	4.073
16	0.258	0.690	1.337	1.746	1.869	2.120	2.235	2.583	2.921	3.252	3.686	4.015
17	0.257	0.689	1.333	1.740	1.862	2.110	2.224	2.567	2.898	3.222	3.646	3.965
18	0.257	0.688	1.330	1.734	1.855	2.101	2.214	2.552	2.878	3.197	3.610	3.922
19	0.257	0.688	1.328	1.729	1.850	2.093	2.205	2.539	2.861	3.174	3.579	3.883
20	0.257	0.687	1.325	1.725	1.844	2.086	2.197	2.528	2.845	3.153	3.552	3.850
21	0.257	0.686	1.323	1.721	1.840	2.080	2.189	2.518	2.831	3.135	3.527	3.819
22	0.256	0.686	1.321	1.717	1.835	2.074	2.183	2.508	2.819	3.119	3.505	3.792
23	0.256	0.685	1.319	1.714	1.832	2.069	2.177	2.500	2.807	3.104	3.485	3.768
24	0.256	0.685	1.318	1.711	1.828	2.064	2.172	2.492	2.797	3.091	3.467	3.745
25	0.256	0.684	1.316	1.708	1.825	2.060	2.167	2.485	2.787	3.078	3.450	3.725
26	0.256	0.684	1.315	1.706	1.822	2.056	2.162	2.479	2.779	3.067	3.435	3.707
27	0.256	0.684	1.314	1.703	1.819	2.052	2.158	2.473	2.771	3.057	3.421	3.689
28	0.256	0.683	1.313	1.701	1.817	2.048	2.154	2.467	2.763	3.047	3.408	3.674
29	0.256	0.683	1.311	1.699	1.814	2.045	2.150	2.462	2.756	3.038	3.396	3.660
30	0.256	0.683	1.310	1.697	1.812	2.042	2.147	2.457	2.750	3.030	3.385	3.646
31	0.256	0.682	1.309	1.696	1.810	2.040	2.144	2.453	2.744	3.022	3.375	3.633
32	0.255	0.682	1.309	1.694	1.808	2.037	2.141	2.449	2.738	3.015	3.365	3.622
33	0.255	0.682	1.308	1.692	1.806	2.035	2.138	2.445	2.733	3.008	3.356	3.611
34	0.255	0.682	1.307	1.691	1.805	2.032	2.136	2.441	2.728	3.002	3.348	3.601
35	0.255	0.682	1.306	1.690	1.803	2.030	2.133	2.438	2.724	2.996	3.340	3.591
36	0.255	0.681	1.306	1.688	1.802	2.028	2.131	2.434	2.719	2.990	3.333	3.582
37	0.255	0.681	1.305	1.687	1.800	2.026	2.129	2.431	2.715	2.985	3.326	3.574
38	0.255	0.681	1.304	1.686	1.799	2.024	2.127	2.429	2.712	2.980	3.319	3.566
39	0.255	0.681	1.304	1.685	1.798	2.023	2.125	2.426	2.708	2.976	3.313	3.558
40	0.255	0.681	1.303	1.684	1.796	2.021	2.123	2.423	2.704	2.971	3.307	3.551
60	0.254	0.679	1.296	1.671	1.781	2.000	2.099	2.390	2.660	2.915	3.232	3.460
80	0.254	0.678	1.292	1.664	1.773	1.990	2.088	2.374	2.639	2.887	3.195	3.416
100	0.254	0.677	1.290	1.660	1.769	1.984	2.081	2.364	2.626	2.871	3.174	3.390
120	0.254	0.677	1.289	1.658	1.766	1.980	2.076	2.358	2.617	2.860	3.160	3.373
140	0.254	0.676	1.288	1.656	1.763	1.977	2.073	2.353	2.611	2.852	3.149	3.361
160	0.254	0.676	1.287	1.654	1.762	1.975	2.071	2.350	2.607	2.847	3.142	3.352
180	0.254	0.676	1.286	1.653	1.761	1.973	2.069	2.347	2.603	2.842	3.136	3.345
200	0.254	0.676	1.286	1.653	1.760	1.972	2.067	2.345	2.601	2.838	3.131	3.340
250	0.254	0.675	1.285	1.651	1.758	1.969	2.065	2.341	2.596	2.832	3.123	3.330
inf	0.253	0.674	1.282	1.645	1.751	1.960	2.054	2.326	2.576	2.807	3.090	3.290