Diversity and nature of microbial endophytes associated with southern African *Oxalis*

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Declaration

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Michelle Jooste

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SUMMARY

Symbioses between plants and micro-organisms have profound influences on biodiversity, ecosystem structure and functioning and patterns of evolution. The Greater Cape Floristic Region (Cape) of southern Africa is global biodiversity hotspot, and is renowned for its diverse and extremely rich flora. At least some of this remarkable diversity has been attributed to abiotic factors such as palaeoclimatic stability, reliable seasonal water availability, geographical gradients and diverse soil types. Cape soils contain some of the lowest nitrogen and phosphorus levels measured globally though, and these may be limiting factors in plant growth. Despite the obvious importance of plant microbial endophytes, the role of such associations in generating and maintaining plant diversity has largely been neglected to date.

The Cape is also renowned for the most diverse geophyte flora in the world, including 2100 species from 20 families. Despite this, the role of plant-microbial interactions has not yet been confirmed in any Cape geophyte lineage. Cape *Oxalis* (Oxalidaceae) is recognised as the sixth largest plant lineage and the largest geophytic genus in the Cape. Although widespread in southern Africa, *Oxalis* has undergone extensive radiation in the Cape and currently includes more than 200 known species. Members occur across a vast range of environments, but are well-represented in the nutrient poor and/or drought prone habitats of the Cape. The evolutionary success of this genus in the Cape may partly be attributed to various unique life history traits (geophytic habit, winter flowering, variable seed strategies), but this is still poorly understood.

Cape *Oxalis* species are highly unusual in terms of their seed germination strategies. Although dormant seeds represent the ancestral state in *Oxalis*, approximately 60% of the lineage has exendospermous seeds that lack a dormancy period, a mechanism known as recalcitrance. The morphological gulf between these strategies (and potential intermediate morphologies) has been poorly quantified, with questions regarding their ecological function and evolution still unanswered. I hypothesized that the binary classification of seed germination strategies (dormancy and recalcitrance) oversimplifies seed physiology and morphology of Cape *Oxalis*. Here I identified three physiological germination strategies (supported by morphology and phenology), in a system where the ancestral dormant state has evolved towards a maximally recalcitrant peak among Cape *Oxalis*. Additionally, a mosaic of intermediate character states is reflected in extant taxa. Recalcitrance and intermediate

germination strategies are rare among angiosperms (11% of species). Insights gained from studying Cape *Oxalis* as an ideal model system has promoted our understanding of the evolution of the recalcitrant germination strategy, among *Oxalis* and angiosperms in general.

Recalcitrant and some intermediate *Oxalis* seeds are metabolically active when shed, which enables them to germinate, establish and reach maturity much more rapidly than dormant seeds. The majority of these recalcitrant species also display a strategy of inverse germination relative to other angiosperm seedlings, where cotyledons and the first foliar leaf develop rapidly, relative to the hypocotyl, root hairs and roots that subsequently emerge. This is a remarkable phenomenon where seedlings are capable of rapid growth and development temporarily, without well-established roots to supply the seedling with nutrients. Furthermore, 70% of the recalcitrant *Oxalis* species (and a few intermediate species) produce large amounts of (often acidic) mucilage around the base of the hypocotyl of seedlings. The mucilage secreted by developing recalcitrant seedlings could both include growth promoting endophytes and serve as a potential attractant to plant growth promoting micro-organisms from the soil environment.

As a first step towards exploring the inter-organismal associations of Cape *Oxalis*, I thus studied intra-plant, intra- and inter-species, and inter-site microbe richness and community composition of rhizosphere and endosphere microbes associated with *Oxalis* hosts. Overall, 46 culturable bacterial and 39 culturable fungal morphotypes were associated with host plants (regardless of seed germination strategy). The endophytic microbial richness and composition changed according to the surrounding environment. The most common and frequently encountered bacterial endophytes included members from the genus *Bacillus* Cohn - a group well-known for various plant-growth promoting properties. A surprisingly diverse collection of bacterial and fungal endophytes was also commonly found in the reproductive and vegetative propagules of all hosts.

Next culture-independent 16S metabarcoding was conducted to document non-culturable bacterial endophytes associated with *Oxalis*. Despite various caveats associated with this approach, significant insights into the diversity of bacterial endosymbionts associated with Cape *Oxalis* host plants were gained. Putative genus-level identification revealed bacterial taxa from 118 genera, as well as various uncultured bacteria, which collectively belong to 79 families, 39 orders and 19 classes from eight bacterial phyla. Metabarcoding results confirmed the presence of six out of nine bacterial genera identified with culture-dependent

techniques. Even though bacterial endophyte species identities could not yet be confirmed, the majority of these genera include various well-known plant endophytes with strong growth promoting and nitrogen-fixing abilities.

Filter exclusion experiments were conducted to determine if endophytes were vertically-transmitted to seeds, determine if mucilage plays a role to actively attract microbes from the soil and to assess microbial richness isolated from the mucilage of *Oxalis* seedlings. Fluorescent microscopy was implemented in order to visualize endophytic bacteria in cryo-sectioned seeds. I report evidence for a novel, vertically transmitted symbiosis between communities of nitrogen-fixing and plant growth-promoting *Bacillus* endophytes and selected *Oxalis* hosts. Three common nitrogen-fixing *Bacillus* species have known oxalotrophic properties and appear to be housed inside specialised cavities (containing oxalates) within the plant body and seeds.

The discovery of vertical transmission and potential benefits to both host and endophyte suggest a particularly tight mutualism in the *Oxalis*-endophyte system. This discovery suggests unexpected ways in which these geophytes might avoid nitrogen deficiency, and suggest that vertically-inherited mutualisms could be impacting plant survival in nutrient-depleted environments such as the Cape. Knowledge of vertical transmission of nitrogen-fixing and oxalotrophic bacteria among angiosperms may have far-reaching conservation, agricultural and economic applications.

OPSOMMING

Simbiose tussen plante en mikro-organismes het verrykende effekte op biodiversiteit, ekosisteem-struktuur en -funksionering en patrone van evolusie. Die Groter Kaapse Floristies Streek (Kaap) van suider Afrika is ‘n globale biodiversiteit brandpunt, en is bekend vir sy diverse en besonder ryk flora. Ten minste sommige van hierdie merkwaardige diversteit is al toegeskryf aan abiotiese faktore soos paleoklimatologiese stabiliteit, betroubare seisoenale beskikbaarheid van water, geografiese gradiënte en diverse grondtipes. Kaapse gronde bevat egter van die laagste globaal-gemete stikstof- en fosfaat-vlakke, en dit mag groeibeperkend wees. Ten spyte van die duidelike belang van plantmikrobiese endofiete, is die rol wat sulke assosiasies speel in die generasie en behoud van plant diversiteit steeds swak ondersoek.

Die Kaap is bekend vir die mees diverse geofiet-flora in die wêreld, en sluit 2100 spesies in 20 families in. Ten spyte hiervan is die rol van plantmikrobiese interaksies nog nie bevestig vir enige Kaapse geofiet-ontwikkelingslyn nie. *Oxalis* (Oxalidaceae) word gereken as die sesde grootse plantontwikkelingslyn en die grootste geofietiese genus in die Kaap. Alhoewel wydverspreid in suider Afrika, het *Oxalis* ekstensief gediversifiseer in die Kaap, en sluit tans meer as 200 bekende spesies in. Lede kom in ’n diverse reeks omgewings voor, maar is goed verteenwoordig in die voedingstof arm en/of droogte geteisterde habitatte in die Kaap. Die evolusionêre sukses van die genus in die Kaap mag deels toegeskryf word aan verkeie unieke lewens kenmerke (geofitiese groeivorm, winter blomtyd, varierende saadstrategieë), maar dit word nog swak verstaan.

Kaapse *Oxalis* is besonder ongewoon in terme van hulle saadontkiemingsstrategieë. Alhoewel rustende sade die voorouerlike staat in *Oxalis* verteenwoordig, produseer 60% van die ontwikkelinglyn eksendosperme sade sonder ’n rusperiode, ’n meganisme bekend as nie-rustende sade. Die morfologiese gaping tussen strategieë (en potensiële intermediêre morfologiese vorms) is swak gekwantifiseer, met vrae rakende die ekologiese funksie en evolusie steeds onbeantwoord. Ek stel die hipotese dat die binêre klassifikasie van saadontkiemings-strategieë (rustend en nie-rustend) saadfisiologie en -morfologie van Kaapse *Oxalis* oorvereenvoudig. Ek het drie fisiologiese ontkiemingsstrategieë geïdentifiseer (gerugsteun deur morfologie en fenologie), in ‘n sisteem waar die voorouerlike rustende staat geëvolueer het na ‘n maksimaal nie-rustende piek binne Kaapse *Oxalis*. Addisioneel word ’n mosaïek van intermediere kenmerkstate in bestaande taksa gereflekteer. Nie-rustende en intermediêre ontkiemingsstrategieë is skaars tussen angiosperme (11% van spesies). Insigte

verkry deur die studie van Kaapse *Oxalis* is ’n ideale modelsisteem wat ons begrip van die evolusie van die nie-rustende ontkiemingstrategie van *Oxalis* en angiosperme in die algemeen, versterk.

Nie-rustende en sommige intermediêre *Oxalis* sade is metabolies aktief wanneer hulle vrygestel word, wat hulle instaat stel om baie vinniger as rustende sade te ontkiem, vestig en volwassenheid te bereik. Die meerderheid van hierdie nie-rustende spesies het ook ‘n omgekeerde onkiemingsvolgorde relatief tot ander angiosperm saailinge, waar die saadlobbe en die eerste loofblare vinnig ontwikkel relatief tot die hipokotiel, wortelhare en wortels wat later eers verskyn. Hierdie is ‘n merkwaardige verskynsel waar saailinge instaat is om vinnig te groei en ontwikkel, terwyl hulle tydelik sonder goed ontwikkelde wortels bestaan wat hulle van voedingstowwe kan voorsien. Verder produseer 70% van die nie-rustende *Oxalis* species (en ‘n paar intermediêre spesies) groot hoeveelhede (dikwels suur) slym aan die basis van die hipokotiel van hulle saailinge. Die slym wat deur die onwikkelende nie-rustende saailinge afgeskei word kan groei-bevorderende endofiete insluit, en kan ook as ‘n potensiële aantrekking dien vir plantgroei-bevorderinde mikro-organismes uit die grond omgewing.

As ‘n eerste stap in die ondersoek van inter-organismiese assosiasies van Kaapse *Oxalis* het ek die intra-plant, intra- en inter-spesie en inter-lokaliteit mikrobe rykheid en gemeenskapssamestelling van risosfeer en endosfeer mikrobe wat met *Oxalis* gashere geassosieer is bestudeer. In totaal is 46 kweekbare bakterië en 39 kweekbare fungus morfotipes met die gasheerplante geassosieer (ongeag die ontkiemingstrategie). Die endofitiese mikrobiese rykheid en samestelling het verander saam met die omringende omgewing. Die mees algemene en mees dikwels geisoleerde bakteriese endofiete het lede van die genus *Bacillus* Cohn ingesluit – ‘n groep wat goed bekend is vir sy groei-bevorderlike eienskappe. ‘n Verrassend diverse groep bakteriële en fungus endofiete is ook dikwels in die reproduktiewe en vegetatiewe plantdele van alle gashere aangetref.

Volgende is kultuur-onafhanklike 16S-strepieskode tegnieke uitgevoer om die nie-kweekbare bakteriese endofiete wat met *Oxalis* geassosieer word, te dokumenteer. Ten spyte van verskeie voorbehoude wat met hierdie benadering gepaard gaan, is beduidende insigte in die diversiteit van bakteriese endosimbionte van *Oxalis* gashere aan die lig gebring. Voorlopige genusvlak-identifikasie het bakteriële taxa van 118 genera, sowel as verskeie onbekende bakterieë, wat gesamentlik aan 79 families, 39 ordes en 19 klasse van agt bakterieë-filums behoort. Hierdie resultate bevestig die teenwoordigheid van ses van die nege bakteriese

genera wat met kultuurafhanklike tegnieke geïdentifiseer is. Alhoewel identifikasies van bakteriese endofiet spesies nog nie bevestig kon word nie, sluit die meerderheid van hierdie genera verskeie bekende plantendofiete in, met sterk groei-bevorderende en stikstofvasleggingssvermoëns.

Filter-uitsluitingseksperimente is uitgevoer om te bepaal of die endofiete vertikaal na sade toe oorgedra word, om vas te stel of die slym ‘n aktiewe rol speel om mikrobe aan te trek uit die grond en om die mikrobiese rykheid binne die slym van *Oxalis* saailinge te bepaal. Fluorisensie mikroskopie is gebruik om die endofitiese bakterië in sade te visualiseer wat met krioseksie gesny is. Ek rapporteer bewyse vir ‘n nuwe, vertikaal-oorgedraagde simbiose tussen gemeenskappe van stikstofbindende en plantgroei-bevorderende *Bacillus* endofiete en geselekteerde *Oxalis* gasheer spesies. Die algemene stikstofbindende *Bacillus* spesies het bekende oksalotrofiese eienskappe en dit lyk asof hulle gehuisves word binne gespesialiseerd holtes (wat oksalate bevat) binne die plantliggaam en sade.

Die ontdekking van vertikale oordrag en potensiële voordele vir beide gasheer en endofiet suggureer ‘n besonder nou mutualisme in die *Oxalis*-endofiet sisteem. Die ontdekking stel onverwagte wyses voor waarop hierdie geofiete stikstof-tekorte mag vermy, en suggureer dat vertikaal oorgeerfde mutualismes mag impakteer op plantoorlewing in voedingstof-arm omgewings soos die Kaap. Kennis van vertikale oordrag van stikstofbindende en oxalotrofiese bakterië tussen angiosperme mag verrykende gevolge vir bewaring, landbou en ekonomiese toepassings hê.

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Chapter 1 Introduction

Background and motivation for the study

Symbioses between plants and micro-organisms have profound influences on biodiversity (Nannipieri *et al.*, 2003), ecosystem structure and functioning (Loreaul *et al.*, 2001) and patterns of evolution (Poole *et al.*, 2003). Micro-organisms such as fungi, bacteria and viruses are often found in the rhizosphere, the soil environment surrounding plant roots (O'Gara *et al.*, 1994; Garate and Bonilla, 2000). As these micro-organisms affect the chemical, physical and biological properties of the soil environment, they influence the development of roots and therefore the growth of the whole plant (Martin *et al.*, 2007). In the case of beneficial interactions between plants and micro-organisms, the level of intimacy of these associations may be escalated to a long-term or permanent endosymbiosis, where the micro-organisms (the endosymbiont) reside within the tissue of the host plant (Schulz and Boyle, 2005; Ryan *et al.*, 2008). Symbioses with beneficial microbes offer host plants the opportunity to acquire or utilize sources of biologically active metabolites, biofunctional chemicals, phytohormones, nutrients and minerals that can help overcome various biotic and abiotic stressors (Schulz *et al.*, 2002; Khan *et al.*, 2012; Kong *et al.*, 2017; Nelson, 2018).

Endophytic colonization of host plants

Rhizosphere micro-organisms have been described as the critical intermediary link between the plant and the soil environment, as these micro-organisms occur at the site of water and nutrient acquisition (Lynch, 1990). The soil often contains nutrients that plants need in inaccessible or un-absorbable forms (Lynch, 1990). Many of the beneficial micro-organisms that occupy the rhizosphere play a vital role in the turnover of carbon, nitrogen and other important nutrients by making them available to the plants in easily-consumable forms (Klemedtsson *et al.*, 1987; Merckx *et al.*, 1987; Robinson *et al.*, 1987). Plant roots exude organic (especially sugars and amino acids) and inorganic compounds and are an important source of energy and carbon to such micro-organisms living in the rhizosphere, and root exudates often stimulate microbial growth (Marschner, 1995; Brimecombe *et al.*, 2007). The root cap cells of many plants secrete mucilage that covers the root tips. This mucilage consists of organic compounds such as polysaccharides and polygalacturonic acids, which is known to attract and increase bacterial diversity and fungal biomass around plant roots (Baudoin *et al.*, 2003).

Many of the fungi and bacteria that live in the soil are able to colonize the inter-cellular spaces of plant roots, but only a small number of these micro-organisms are able to colonize intra-cellular spaces of epidermal and cortical cells (Bazin *et al*., 1990; Sieber and Grunig, 2013). Root tissues form a microcosm that is morphologically, chemically and physically unique and provide protected habitats for micro-organisms such as bacteria, actinomycetes, protozoa, nematodes, microalgae and fungi. The diversity, frequency and population density of endophytic species depend on the edaphic and climatic conditions of the rhizosphere, the diversity of niches available within the host tissue and the level of competition between endophytic micro-organisms (Sieber and Grunig, 2013).

Colonization of bacteria from the rhizosphere into the host root tissue happens due to stochastic and deterministic factors through a series of active and/or passive colonization events. Some bacteria (called passenger endophytes) can enter the root tissue at sites where lateral roots develop or where tissue has torn, but are typically restricted to the root cortex (Hardoim *et al*., 2008). Competent endophytes refer to those bacteria with the ability to colonise specific plant tissues (such as the vascular tissue) once they have entered the root cortex, spread throughout the organs of the host and manipulate the host's metabolism without any detrimental effects (Hardoim *et al*., 2008).

Endophytic fungi may occupy the root, stem or leaf tissue of host plants in the form of microscopic hyphae (Stone *et al*., 2000). Most terrestrial plants (at least 80%) form mutualistic relationships with endophytic fungi (Petrini, 1986), termed arbuscular mycorrhizal symbioses. These endophytic fungi colonize the roots of host plants by penetrating the inter-cellular spaces as well as the intra-cellular root tissue and form beneficial associations with hosts by enhancing host growth capacity, survival and reproduction (Safir, 1987; Smith and Read, 1997).

Leaves and leaf surfaces (phyllosphere) serve as another interface for closely associated plant-microbial interactions (Kinkel, 1997; Beattie and Lindow, 1999; Andrews and Harris, 2000; Lindow and Leveau, 2002). Bacteria and fungi found on leaf surfaces are sometimes able to penetrate the surface (often via stomata), enter the plant tissue and live within the host plant as endophytes (Lindow and Leveau, 2002; Lambais *et al*., 2006). Bacteria are the most common long-term inhabitants of the phyllosphere and the diversity of bacterial species within these environments is comparable to soil or ocean environments (Yang *et al*., 2001; Lambais *et al*., 2006). Under certain conditions, fungal inhabitants within host leaves can be

mutualistic towards the plant hosts (Andrews and Harris, 2000) or work synergistically with other fungal endophytes to increase the host plant's tolerance to external stress, enhance plant growth and increase reproductive success (Rodriguez *et al*., 2009).

Vertical inheritance of endophytes

Among angiosperms the majority of plant-endophyte symbioses are restricted to plant vegetative tissues, however, a few microbes are capable of inhabiting reproductive tissues and are vertically transmitted (Compant *et al*., 2011; Truyens *et al*., 2015). Plants are known to exert stronger selection pressures on endophytes that are passed on to their reproductive propagules, relative to other vegetative organs (Hallmann, 2001; Stamp, 2003). To date the most comprehensive reviews have indicated that the majority of all fruits and seeds of angiosperms do not contain any endophytes (Hallmann, 2001; Compant *et al*., 2010; Truyens *et al*., 2015). A recent study that set out to investigate the primary symbiont hypothesis assessed culturable seed endophytes from 98 host species, representing 39 families (Newcombe *et al*., 2018). These authors showed that each individual seed either contained no endophytes (70% of seeds) or a single primary bacterial or fungal symbiont per seed (27%). This means that only 3% of seeds hosted more than two culturable endophytes (Newcombe *et al*., 2018).

Endophytes that are capable of passing through these selection barriers have to possess highly specialized physiological traits, in order to ensure successful colonization and establishment in reproductive organs (Compant *et al*., 2010). Due to these strong selection pressures, the majority of known seed endophytes have various beneficial traits to their hosts (Truyens *et al*., 2015). They represent a small subset of rhizosphere and plant endophytes (Mundt and Hinkle, 1976; Massol-Deya *et al*., 1995; Cankar *et al*., 2005; Compant *et al*., 2008, 2010; López-López *et al*., 2010). To date, very few studies have assessed the biodiversity of seed endophytes, but the majority of known seed endophytes includes members from the bacterial genera *Bacillus* Cohn, *Pseudomonas* Migula and *Rahnella* Izard (Mandt and Hinkle, 1976; Misaghi and Donndelinger, 1990; Barac *et al*., 2004; Cankar *et al*, 2005; Okunishi *et al*., 2005).

Newcombe *et al*. (2018) stated that our current understanding of seed endophytes and vertical transmission is fragmented, as available information comes from three different ‘schools’, namely the long standing disciplines of seed pathology, grass endophyte ecology and more recently high throughput sequencing. These authors stated that grass endophyte ecologists

and seed pathologists generally focus their research efforts on a small fraction of terrestrial plants. Reportedly, the majority of high throughput sequencing research has focused on the total host plant microbiome, which mostly targets vegetative plant organs or the rhizosphere rather than the seeds (Newcombe *et al*., 2018, Shazad *et al*., 2018).

Methods of endophyte isolation and identification

Surface sterilization

Regardless of the technique implemented to extract endophytes, the first hurdle is to establish a surface sterilization protocol to eliminate epiphytes and surface contaminants. There are countless studies and available protocols to achieve this, and they most frequently include the use of sterile, de-ionised water, high concentrations of ethanol and low concentrations of household bleach (sodium hypochlorite) (Schulz *et al*., 1993).

In addition to the surface sterilization techniques, there are two other proposed protocols to detect contamination. Some studies report sequencing the plant-washing fluid, usually sterile de-ionised water used during the last wash step in a given protocol to establish if all possible epiphytes and contaminant have been removed or destroyed during preceding washing steps (Schulz *et al*., 1993). The other method involves the inclusion of sterilization controls, where additional plant samples are surface sterilized with a given protocol and then 'dabbed' onto agar plates to determine if any bacteria or fungi survived the sterilization. If no colony formation is observed, then the protocol is deemed successful (Schultz *et al*., 1998, Sánchez-Márquez *et al*., 2007). In the relatively few studies on seed endophytes, some authors have reported dissecting fruits in a laminar flow cabinet after surface sterilization and removing seeds under aseptic conditions. This is another way to prevent the exposure of the studied seeds to airborne contamination (Newcombe *et al*., 2018).

Classical culture-dependant techniques

Since the 19th century, the majority of studies have implemented agar culture-based techniques to isolate bacterial and/or fungal endophytes (as reviewed in Hardoim *et al*., (2015)). In order to release endophytes from plant tissues, plant organs are typically macerated in sterile, de-ionised water. These samples are then plated onto agar, incubated for a number of days (typically 5 to 10 days), and colonies of interest are isolated and sub-cultured until pure culture colonies are obtained (Schultz *et al*., 1998). Some colonies are morphologically distinct and can be identified, but the most common practice involves the

use of Sanger sequencing to identify samples of isolated plant endophytes to genus or species level (Truyens *et al*., 2015).

As the majority of published literature on plant endophytes implemented these techniques, there is a wealth of available protocols on how to create optimal growing conditions for various plant endophytes (in terms of agar type, pH, incubation temperature and light or dark conditions). A plethora of bacterial and fungal growth media are available, and these media can relatively easily be amended to re-create specific growing conditions. Culture-dependant techniques are regarded as a relatively affordable approach, as laboratory consumables can be purchased and Sanger sequencing implemented at a relatively low cost. There are, however, also many known limitation of agar culture methods. It is reported that these techniques yield only 1-5% of the true endophytic diversity among plants (Guo *et al*., 2001; Duong *et al*., 2006; Hyde and Soytong, 2007). Some bacteria and fungi are known to be fastidious, and do not successfully grow on some agar media, while other endophytes may thrive and dominate agar plates (Guo *et al*., 2001; Duong *et al*., 2007; Zhu *et al*., 2008). Therefore, researchers typically include multiple agar media, in order to 'capture' as much endophytic diversity as possible.

Modern culture-independent techniques

Recent technological developments in the field of high-throughput DNA metabarcoding have revolutionized our current understanding of endosphere microbiomes (Hardoim *et al*., 2015). These culture-independent techniques allow the investigation of complex microbiomes and are valuable tools for exploring, identifying and characterizing genetic and/or metabolic components involved in plant-endophytic interactions (Hardoim *et al*., 2015). These methods allow much larger sample sizes and support deeper analyses and investigation of endophytic microbial communities (Knief, 2014).

Generally metabarcoding analyses are much more expensive than culture-dependent techniques. Reportedly, many laboratories have difficulties in optimizing microbiome DNA samples from surface sterilized plant tissues, as the ratio of plant DNA is much greater than endophytic microbial DNA (Dos-Santos *et al*., 2017). Depending on the primer sets implemented, metabarcoding analyses typically yield accurate identifications to family or genus level, while culture-dependant techniques are encouraged to successfully identify endosymbionts to species level (Duong *et al*., 2006). The interpretations of metabarcoding results are also highly dependent on comprehensive and up-to-date databases. As this is still a

developing field, future technologies will likely overcome the majority of these obstacles (Hardoim *et al.*, 2015; Shahzad *et al*., 2018)

Visualization of plant endophytes

Exact locations of endophytic bacterial and fungal colonization are visualised with histological sections, green fluorescent protein (gfp) tagging or fluorescence *in situ* hybridization (FISH) (Compant *et al*., 2010; Hardoim *et al*., 2015; Truyens *et al*., 2015). These techniques allow detailed visualization of microbial colonization within plant tissues. However, most often these techniques indicate the presence or absence of stained microbes, and do not aid with the identification of endophytes or help discern between true endophytes and/or contaminants (Pohjanen *et al*., 2014; Hardoim *et al*., 2015). The application of gfp-tagging often involves liquid dyes, which could cause stained endophytic bacteria or fungal spores to move in and around histological sections of plant tissues. FISH often involves the tagging of microbial isolates and the inoculation of living plant material. This method is very effective to assess the distribution of microbes throughout a host plant, but is not very useful to identify unknown natural endophytes (Pohjanen *et al*., 2014).

Frequently studied plant-endophyte systems

Plant-microbial interactions may be beneficial, neutral or harmful towards the host plant (Dobbelaere *et al*., 2003). Beneficial interactions includes interactions that are responsible for mineral and nutrient uptake and supply to the plant (such as free living or nitrogen-fixing (diazotrophic) bacteria), interactions that prevent the growth or activity of plant pathogens and therefore indirectly stimulate plant growth (such as biocontrol agents) and interactions that directly influence plant growth through the production of phytohormones (such as plant growth promoting rhizobacteria or mycorrhizal fungi) (Azcon and Ocampo, 1981; Whipps, 1990; Brimecombe *et al*., 2007). Neutral interactions refer to interactions where the presence of micro-organisms in the rhizosphere or plant tissue neither benefit nor harm the plant (Schroth and Hildebrand, 1964; Hawes, 1990; Brimecombe *et al*., 2007). Harmful interactions include the presence and activities of endophytic plant pathogens and deleterious rhizobacteria from the rhizosphere.

Due to these distinct beneficial and harmful interactions between plants and the microbiome, understandably most research has focused on plants with economical, agricultural or medicinal importance, as well as model plant species (Bulgarelli *et al.*, 2012; Lundberg *et al*.,

2012; Peiffer *et al.*, 2013). The extensive research focused on a relatively limited group of host plants has created a biased view on endophyte diversity and community compositions among all terrestrial plants. There is growing recognition that microbial associations are ubiquitous across all terrestrial plants (Dastogeer and Wylie, 2017), and the list of known plant hosts increases yearly (Rodriguez *et al*., 2009; Nair and Padmavathy, 2014). It has been predicted that all seed plants accommodate at least one endophytic species (Lewis, 1985; Rodriguez *et al*., 2009; Nair and Padmavathy, 2014), but the diversity of endophytic communities among natural ecosystems remains largely unexplored (Hardoim *et al*., 2012; Carrell and Frank, 2014; Miyambo *et al*., 2016).

Disparities in our current understanding of plant-endophyte interactions

The most recent and comprehensive reviews of the plant microbiome repeatedly mention four major themes in which our current scientific understanding is lacking and that are priorities for future research. These include: 1) studying interactions between endophytic bacteria and fungi within a single host plant, 2) understanding mechanisms of infection and inoculation in adult plant and seeds/seedlings, 3) studying the seed microbiome (particularly in non-grass hosts) and 4) investigation of ecologically relevant host species growing under natural and field conditions (Hardoim *et al*., 2015; Truyens *et al*., 2015; Liu *et al*., 2017; Martin *et al*., 2017; Newcombe *et al*., 2018; Shahzad *et al*., 2018).

Endophytic bacterial and fungal communities are mostly investigated separately, while interactions between these endophytes in a single host are rarely investigated (Hardoim *et al*., 2015; Truyens *et al*., 2015) and have prospects of being a "fascinating" research area (Frey-Klett *et al*., 2011). A few studies have shown that interactions between some microbial endophytes can cause the shift of entire microbiome populations of a host (Elasri *et al*., 2001; Andreote *et al*., 2009). Understanding the mechanisms of endophyte infection and inoculation of both seeds/seedlings and adult plants is another area that requires more research. Authors have suggested that the combination of techniques or future technologies will aid the understanding of such systems (Liu *et al*., 2017; Shahzad *et al*., 2018). Martin *et al.* (2017) mentioned that the interaction between plant endophytes and the establishment of a rhizosphere and/or the acquisition of additional endophytes are also still poorly understood.

In a recent review, Newcombe *et al.* (2018) stated that despite the recent and increasing focus on plant microbiome studies, seeds are rarely considered for analyses. The advancement of

metabarcoding has enabled the exploration of plant microbiomes, and to date the majority of studies have reported results of plant-associated microbiomes from the rhizosphere, endophere and phyllosphere (Redford and Fierer, 2009; Bulgarelli *et al*., 2012; Bodenhausen *et al*., 2013; Shakya *et al*., 2013; Lebeis, 2014), with surprisingly few reports of seed microbiomes (Nelson *et al.*, 2018). This is, despite predictions that metabarcoding techniques will help to reveal the true diversity of bacterial and fungal population present in seeds (Truyens *et al*., 2015). Before we can understand the possible roles of seed endophytes in germination, seedling development and establishment and subsequent plant growth, we first need to affirm their diversity (Truyens *et al*., 2015). Busby *et al*. (2017) proposed that vertically transmitted seed endophytes may be the best place to begin searching for the core plant microbiome.

The majority of studies on plant-endophyte interactions are commonly based on plants grown under controlled and optimized conditions (such as greenhouse conditions), which do not represent realistic field conditions (Hardoim *et al*., 2015). Furthermore, most of our current insights into plant-endophyte interactions are based on economically important crops grown under 'experimental' or greenhouse conditions. Various authors have proposed that future research should aim to investigate bacterial and fungal endophytes from ecologically relevant host species growing under natural and field conditions (Hardoim *et al*., 2015; Martin *et al*., 2017; Shahzad *et al*., 2018).

Here we contribute to the study of endophytes associated to native, non-crop plant species sampled in the wild. We studied both fungal and bacterial endophytic associations using culture-based methods, and considered and compared endophytic diversity between different plant organs (vegetative and reproductive, including seeds) and the rhizosphere. Culture-independent metabarcoding and green fluorescent protein labelling techniques are also employed to study and visualize bacterial endophytes, and we provide a critique on the use of metabarcoding techniques in the study of plant bacterial endophytes.

Study system

South Africa is a global biodiversity hotspot and ranked as the third most biologically diverse country in the world (Government of South Africa, 2015). The Greater Cape Floristic Region (Cape) of southern Africa is globally renowned for its diverse and extremely rich flora (Manning and Goldblatt, 2012; Snijman *et al*., 2013). To date this remarkable diversity has

been attributed to abiotic factors such as palaeoclimatic stability, reliable seasonal water availability, geographical gradients and diverse soil types (Jansson, 2003; Linder, 2003). Despite the obvious importance of endophytes (Teste *et al*., 2017), the role of such associations in generating and maintaining plant diversity has largely been neglected. There is growing evidence that many species harbour unique rhizospheric and endophytic micro-organisms (Cowan *et al*., 2013; Miyambo *et al*., 2016).

The sandstone derivation and low pH of most Cape soils, together with predictable winter rainfall and relatively frequent wildfires, all contribute to dystrophic conditions (Linder, 2003). This leads to severe nutrient deficiencies, to the extent that the Cape contains some of the lowest nitrogen and phosphorus levels measured globally (Specht and Moll, 1983). Nitrogen and phosphorus are essential for plant development, but are often limiting in plant growth (Olson and Kurtz, 1982; Raven, 1988; Rustad *et al*., 2001). Positive effects of diverse assemblages of microbes found in the soil are most noticeable in habitats or ecosystems with severe nutrient deficiencies (van der Heijden *et al*., 2008). Beneficial interactions with microbes may increase the host plants' tolerance to biotic and abiotic stress, by enabling better mineral and nutrient uptake and supply to the plant (such as nitrogen-fixing or phosphate-solubilizing bacteria), prevent the growth or activity of plant pathogens and directly influence plant growth through the production of phytohormones (such as plant growth promoting rhizobacteria or mycorrhizal fungi) (Azcon and Ocampo, 1981; Whipps, 1990; Haas and Defago, 2005; Brimecombe *et al*., 2007).

The Cape is also renowned for the most diverse geophyte flora in the world, including 2100 species from 20 families (Procheş *et al*., 2006; Cowling *et al*., 2009). Although the factors driving this remarkable Cape geophyte diversity are still poorly understood, geographical distribution, climatic factors (rainfall quantity and reliability) and plant growth form (storage organ size) have been suggested (Cowling *et al*., 2009). The role of plant-microbial interactions has, however, not yet been explored in any Cape geophyte lineage (Procheş *et al*., 2005).

Cape *Oxalis* (Oxalidaceae) is recognised as the sixth largest plant lineage and the largest geophytic genus in the Cape (Manning and Goldblatt, 2012). Although widespread in southern Africa, Cape *Oxalis* has undergone extensive radiation and currently includes more than 200 known species (Salter, 1944; Oberlander *et al*., 2011). Members occur across a vast range of environments, but are well-represented in the nutrient poor and/or drought prone

habitats of the Cape (Mucina and Wardell-Johnson, 2011). The evolutionary success of this genus in the Cape may partly be attributed to various unique life history traits (geophytic habit, winter flowering, variable seed strategies), but this is still poorly understood. All southern African *Oxalis* are characterized by the presence of a subterranean bulb, from which seasonal above-ground stems emerge annually. Unlike most other Cape lineages, *Oxalis* species emerge, flower and fruit during the predictably wet winter months, senescing to the bulb during dry summers.

Cape *Oxalis* is highly unusual in terms of their seed germination strategies, as approximately 60% of the lineage has exendospermous seeds (Salter, 1944) that lack a dormancy period, a mechanism known as recalcitrance. Recalcitrant *Oxalis* seeds have a fully developed embryo, no or highly reduced endosperm at seed release and germinate readily and immediately upon release from the seed capsule (Hildebrand, 1884; Salter, 1944; Brink, 2017). Germination commences with cotyledon opening, and the initiation of first foliar leaf development occurs within 24 hours of release from the testa. During this time interval the radicle remains underdeveloped, but in at least some species (e.g.*Oxalis hirta* Jacq.) a mucilaginous substance is secreted around the base of the hypocotyl, which extends to cover the root tip (Davey, 1946). Radicle development only commences days later, upon full establishment of the first foliar leaf (Dreyer, *pers. comm*).

Seed dormancy is regarded as the ancestral state in southern African *Oxalis* (all non-southern African species have dormant seeds) and recalcitrance has independently evolved at least three times in the group (Oberlander *et al*., 2011; Brink, 2017). This inverse sequence of germination could be regarded as a potentially risky strategy, which is highly unusual among angiosperms (Kawano and Nagai, 1975; Mogie, 1990; Finch-Savage and Leubner-Metzger, 2006).These seedlings develop and grow without established roots to acquire nutrients that are essential to the development of any seedling. Yet, recalcitrant *Oxalis* seems to be physiologically and evolutionary successful in the Cape. Are these seedlings utilizing stored starch and nutrients from their cotyledons? Or are their nutrient requirements satisfied through associations with seed endophytic bacteria given the unfavourable abiotic conditions associated with the Cape? Pilot studies have revealed the presence of bacteria in their seedling mucilage (Dreyer, *pers. comm*.), but nothing is known about the origin, identity or diversity of the microbes associated with Cape *Oxalis* hosts or their seeds.

Preliminary $\delta^{15}N$ isotope profiles of *Oxalis* leaf material indicated that they have unusually wide ranges and some samples had relatively `light' nitrogen-values (Jooste, 2015). Isotope profiles of dormant and recalcitrant *Oxalis* species spanned a wide range (dormant: $\delta^{15}N$ from 5.04 to 16.51, recalcitrant: $\delta^{15}N$ from 2.26 to 15.78). These 'light' $\delta^{15}N$ values are close to atmospheric levels and may reflect values associated with symbiotic nitrogen fixing micro-organisms (Shearer and Kohl, 1986; Sprent *et al*., 1996; Högberg, 1997; Tjepkema *et al*., 2000). Unfortunately, no reference plant or soil samples were measured (Jooste, 2015), so these data are preliminary indications only. These observations did, however, further support the question of whether *Oxalis* species have the ability to form associations with beneficial nitrogen-fixing organisms.

Associations between arbuscular mycorrhizal fungi and seven non-southern African *Oxalis* species have been described: *O. acetosella* L., *O. corniculata* L., *O. corymbosa* Knuth., *O. europaea* Jord., *O. exilis* Cunn., *O. stricta* L.and *O. valdiviensis* Barneoud (Harley and Harley, 1987; Fontenla *et al*., 1998; Yamato, 2004). The well-known endophytic fungus, *Glomus tenuis* Greenall, has been found to colonise the roots of *O. acetosella*, enabling better root proliferation and an increased ability to absorb phosphates from the soil (Farley and Fitter, 1999; Turnau *et al*., 1999). To our knowledge, no beneficial fungal association with any southern African *Oxalis* has been described, while some of the most common plant-associated endophytic bacteria, including genera such as *Bacillus*, *Pantoea* Gavini, *Pseudomonas* and *Rahnella*, have been detected within leaves and roots of *O. corniculata* in China (Peng *et al*., 2013). Another study assessed endophytes present in *O. corniculata* from Pakistan, and isolated two distinct bacterial strains that were either closely related to *Pantoea agglomerans* Ewing & Fife or *Agrobacterium tumefaciens* Smith & Townsend, and *Bacillus pumilus* Cohn or *Kocuria rhizophila* Stackebrandt from roots, stems and leaves (Mufti *et al*., 2015). All of these bacterial species are known plant endophytes with various plant growth promoting properties (Mcinroy and Kloepper, 1995; Xing *et al*., 2006), and the authors proposed that the identified endophytes would enhance the solubilization of phosphates and the production of ammonia (Mufti *et al*., 2015).

The most notable report was the discovery of a novel strain of nitrogen-fixing, oxalate-oxidising *Azospirillum* Tarrand in the roots of the southern African species, *O. pes-caprae* L. that had successfully invaded in Turkey (Sahin, 2005). Several plant genera, including *Oxalis* (Hulme, 2004), are known to accumulate large amounts of oxalate crystals within the plant

tissue (Lodewyckx *et al*., 2002; Sahin, 2005). A limited number of nitrogen fixing bacterial strains have been reported that can utilize oxalate produced by the host plants as their only carbon source. These oxalotrophic bacteria include strains of *Azospirillum, Bacillus, Beijerinckia* Derx, *Burkholderia* Yabuuchi, *Mesorhizobium* Jarvis and *Xanthobacter* Wiegel (Sahin, 2003). Sahin (2005) suggested that the endophytic *Azospirillum* strain found in *Oxalis* may be important for soil fertility and cycling of elements necessary for the growth of the host plant. Five other oxalotrophic endophytic bacteria have been isolated from *O. spiralis* G. Don and *O. tuberosa* Molina from from Colombia, namely *Serratia fonticola* Bizio, *Bacillus amyloliquefaciens* Priest, *B. cereus* Frankland & Frankland, *B. subtilis* Cohn and *B. vallismortis* Roberts.

Given the known suite of beneficial and specialized endophytes associated with global *Oxalis* species and the documented range of plant-associated symbionts across angiosperms in general (Beattie, 1995; Hallmann *et al*., 1997), it is very likely that Cape *Oxalis* host many important, but undocumented microbial associates. Our research is the first attempt to explore and document this interesting phenomenon. Results may help explain how *Oxalis* is able to persist and thrive in the challenging environments of the Cape. *Oxalis* and their endophytes offer an ideal model system to study the diversity and nature of host-microbe relationships, and explore possible implications for physiological survival, bulb formation and seedling establishment. Understanding these interactions may be vital to the long-term conservation of this large and variable angiosperm genus that constitutes such a major component of the Cape flora.

Aims of the study

The main aim of this study was to investigate the diversity and nature of endophytic bacterial and fungal communities associated with the large, native Cape *Oxalis* radiation. At one level results will feed into the growing international literature on plant endophytic associations. Strengths of our proposed research include a focus on native, non-crop plants, exploration of both fungal and bacterial associates, and implementation of different diversity assessment techniques. Insights gained through this may help understand unique biological attributes displayed by our study taxa, such as their unique reproductive biology, germination success, seedling establishment and rapid bulb formation in the Cape, despite the challenging nutrient poor and drought-prone environments they occupy.

Before we could even attempt to study the potential benefits offered by endophytic associates in Cape *Oxalis* species, we needed to expand our general knowledge of their biology. We focussed specifically on documenting the diverse seed germination strategies, as we suspected that endophytic associations may aid germination and seedling establishment of Cape *Oxalis*. Specific objectives included:

- Re-assess the binary classification of Cape *Oxalis* germination strategies, with an emphasis on species with potential intermediate strategies in order to better understand their ecological function and evolution
- Quantify the morphological and phenological characters associated with each of the germination strategies displayed by *Oxalis*
- Document and describe the inverse germination and other unique biological traits displayed by recalcitrant species

Once we understood the reproductive biological attributes of *Oxalis* seedlings better, this study shifted focus towards a search for endophytic associations that may help explain the unusual reproductive strategies we observed, specifically within the context of the hostile Cape environment. Specific objectives for this part of the study were to:

- Determine if *Oxalis* harbours bacterial and fungal endophytes in vegetative and/or reproductive plant organs using culture-dependent techniques for six phylogenetically representative *Oxalis* hosts from multiple localities
- Identify bacterial and fungal endophytes to genus or species level, in order to better understand the role of associated endophytes based on descriptions in the literature
- Investigate endophyte richness and community compositions among various vegetative and reproductive plant organs relative to the microbes found in the rhizosphere of plant roots
- Investigate richness and community compositions among various plant organs, host species and different sampling locations
- Expand on the documentation of associated endophytes through the use of culture-independent metabarcoding techniques
- Assess the culture-independent metabarcoding approach to studying bacterial endophytes in *Oxalis* by exploring potential caveats associated with this approach, and comparing endophytic diversity revealed through plating versus barcoding techniques
- Test whether microbial communities are attracted to and inhabit mucilage secreted around the base of the hypocotyl of germinating recalcitrant seedlings in order to determine if endophytes are vertically transmitted from parent plants to their offspring
- Explore the nature of endophytic associations with hosts, with particular emphasis on endophytic *Bacillus* species with known oxalotrophic metabolisms and nitrogen fixation properties

Structure of thesis

The editorial style and formatting of all data chapters of this thesis (Chapters 2 to 5) have been prepared as scientific papers to be submitted for publication in various international journals, as described below.

Chapter 2: **A dormancy spectrum in *Oxalis* seeds from the Cape Flora**
Seed physiological traits were used to assign germination strategies to 64 *Oxalis* species. We tested for morphological/phenological signals corresponding to defined strategies with cluster, principal component, K-means clustering and discriminant analyses. Here we show that dormant, intermediate and recalcitrant germination strategies exist among Cape *Oxalis*, with two possible morphological groups within each strategy. These could reflect a continuum of germination states, where an ancestral dormant strategy evolved towards a maximally recalcitrant peak, with a mosaic of intermediate states reflected in extant taxa. Environmental factors may affect germination strategy and distribution throughout the Cape, as recalcitrant and intermediate species are confined to the winter rainfall region. They occupy specialized niches and may face adverse impacts under predictions of climate change (hotter and drier winters), meriting focused future conservation.

This chapter has been submitted to the American Journal of Botany and is currently under review.

Chapter 2 Notes: Observations and descriptions of various morphological and phenological traits associated with the different seed germination strategies were included as supplementary notes to this publication. Details of some of these findings were less relevant to the paper itself, but highly relevant to the collective focus of this dissertation. As a notes section at the end of Chapter 2, I thus included a brief section on seed and seedling morphological traits relevant to the inverse germination, as well as other unique biological traits displayed by recalcitrant species.

Chapter 3: **Choosing your companions: endophytes in vegetative and reproductive organs of Cape *Oxalis***
I studied the bacterial and fungal endophytes associated within six Cape *Oxalis* species (three dormant and three recalcitrant species). Their selection was based on their co-occurrence at three well-separated sampling localities, and to be as phylogenetically representative as possible. This enabled comparisons between intra-plant, inter-species and inter-site microbial

richness, isolation frequencies and community compositions. Culturable endophytes isolated from sterilized vegetative and reproductive plant organs were studied. Colonies of microbes on various artificial media were morphotyped, enumerated and identified using sequence data obtained from the 16S and ITS regions. Overall, 46 bacterial and 39 fungal morphotypes were isolated from *Oxalis* host plants. Endophytic communities associated with *Oxalis* host species were an order of magnitude higher than typically reported for other plant hosts, using similar culture-dependant techniques. Our results indicated that location, host species and plant organ type, as well as interactions between these variables, influenced the richness, isolation frequency and the structure of bacterial and fungal communities associated with *Oxalis* plants. However, seed germination strategies did not significantly affect endophytic community compositions.We also determined that assemblages of various plant growth promoting and nitrogen-fixing *Bacillus* were ubiquitous from all host plants, including vegetative and reproductive propagules.

This chapter has been prepared for submission to the 'Microbiome Biology' special edition of BMC Microbiology.

Chapter 4: **Metabarcoding of *Oxalis* bacterial endophytes: caveats associated with sequencing and interpretation of data**

We have shown that diverse communities of culturable endophytic bacteria and fungi, many with known plant growth promoting traits, are associated with Cape *Oxalis* hosts. Given the known limitation of agar culture methods that yield only 1-5% of true endophytic diversity, we aimed to assess more accurate levels of endophytic diversity using a metabarcoding approach. We studied bacterial endophytes from various surface sterilized and macerated plant organs using six universal 16S bacterial markers and the IonTorrent metabarcoding platform. Sequence results revealed that between 65 and 95% of all sequence reads (contigs) were plant chloroplasts and/or mitochondria. Due to the high number of host plant DNA reads, potential bacterial endophyte sequences reads were inconsistent. Furthermore, the recommended 16S Reference Library and GreenGenes microbial databases for the IonReporter metabarcoding platform could not accurately distinguish between plastids and true endophyte contigs. Sequence identities were therefore verified using three other databeses (namely NCBI, SILVA and RDP). Based on consistent results and sequence identities, we have putatively identified 97 bacterial genera that belong to 68 families. Despite various caveats, these methods offered a glimpse into the rich treasure trove of

bacterial endosymbionts associated with *Oxalis* host plants, and methods need to be refined in furture studies.

Chapter 5: **Nitrogen-fixing bacteria and *Oxalis* – evidence for a vertically inherited bacterial symbiosis**

Both plating and metabarcoding results emphasized the common presence of *Bacillus* endophytic bacteria in most organs of both dormant and recalcitrant species tested. Here we report evidence for a novel, vertically transmitted symbiosis between communities of nitrogen-fixing and plant growth-promoting *Bacillus* endophytic bacteria (EB) and selected *Oxalis* hosts from nitrogen-deficient environments of the Cape. EB are ubiquitous and diverse across species and plant bodies, and are prominent in seeds. Three common nitrogen-fixing EB have known oxalotrophic properties and appear to be housed inside specialised cavities (containing oxalates) within the plant body and seeds. The discovery of vertical transmission and potential benefits to both host and endophyte suggest a particularly tight mutualism in the *Oxalis*-EB system. This discovery suggests unexpected ways in which geophytes might avoid nitrogen deficiency, and suggest that such symbioses are more common than previously expected.

This chapter has been submitted to BMC Plant Biology, and is currently under review.

Chapter 6: **General discussion and conclusion**

This chapter provides an overview of the role of endophytic bacterial and fungal communities inhabiting vegetative and reproductive tissues of *Oxalis* hosts. The biological and evolutionary importance of these associations with *Oxalis* in the Cape region are discussed. Future avenues of relevant research are suggested with particular emphasis on the vertical transmission of nitrogen fixing bacterial endophytes and implementing of metabarcoding techniques. Potential applications and recommendations for future conservation are also provided.

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Declaration by the candidate (for chapter currently under review at American Journal of Botany)

With regards to Chapter 2 (A dormancy spectrum in *Oxalis* seeds from the Cape Flora), the nature and scope of my contribution were as follows:

Nature of contribution	Extent of contribution (%)
Assessment of seed physiological traits and seed and seedling morphological traits to reconsider the current binary classification of *Oxalis* seeds	100
Data analysis, interpretation and manuscript preparation	80

The following co-authors have contributed to Chapter 2:

Name	e-mail address	Nature of contribution	Extent of contribution (%)
Guy F. Midgley	Copyright	Provided guidance and edited the manuscript	5
Kenneth C. Oberlander	Copyright	Provided guidance, especially in terms of data analysis, and edited the manuscript	7.5
Léanne L. Dreyer	Copyright	Provided guidance, funding and edited the manuscript	7.5

Signature of candidate: .....……

Declaration by co-authors:

The undersigned hereby confirm that

1. The declaration above accurately reflects the nature and extent of the contributions of the candidate and the co-authors to Chapter 2.
2. No other authors contributed to Chapter 2 than those specified above.
3. There are no conflicts of interest relevant to Chapter 2 of this dissertation.

Signature	Institutional affiliation	Date
Guy F. Midgley	Stellenbosch University	December 2018
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Chapter 2 A dormancy spectrum in *Oxalis* seeds from the Cape Flora

ABSTRACT

Rationale: Seed germination strategy has profound ecological and evolutionary consequences, with transitions between germination strategies receiving renewed recent attention. *Oxalis* from the Cape Flora, South Africa, is known to exhibit two contrasting germination strategies: dormancy and recalcitrance. The morphological gulf between these strategies (and potential intermediate morphologies) has been poorly quantified, with questions regarding their ecological function and evolution remaining unanswered. We reconsidered this binary classification, emphasizing potential intermediate states.

Methods: Seed physiological traits were used to assign strategies to 64 *Oxalis* species. We tested for seed and seedling morphological/phenological signal corresponding to defined strategies with cluster, principal component, K-means clustering and discriminant analyses.

Key results: We show that dormant, intermediate and recalcitrant germination strategies exist among Cape *Oxalis*, with two possible morphological groups within each strategy. These could reflect a continuum of germination states, where an ancestral dormant strategy evolved towards a maximally recalcitrant peak, with a mosaic of intermediate states reflected in extant taxa.

Main conclusions: Environmental factors may affect germination strategy and distribution throughout the Cape, as recalcitrant and intermediate species are confined to the winter rainfall region. They occupy specialized niches and may face adverse impacts under predictions of climate change (hotter and drier winters), meriting focused future conservation.

Key words: dormant, endosperm, germination strategy, intermediate, recalcitrant, seedlings

INTRODUCTION

Across all life, many species produce dormant eggs or seeds that do not hatch or germinate immediately after release, despite being fully mature and exposed to favourable environmental conditions (Evans and Dennehy, 2005). Instead, they hatch or germinate in intervals over a long period of time, allowing bet-hedging against future catastrophes. Despite the numerous benefits associated with dormancy, many species reduce or eliminate the dormant stage due to trade-offs associated with population growth (Ellner, 1985; MacArthur and Wilson, 1996). Venable (2007) elegantly stated that "the best a non-germinating seed can do is survive, while a germinating seed may either die without leaving any descendants or make 100's or even 1000's of new seeds".

All angiosperms depend on seeds to ensure the dispersal of their progeny in space and time and consequently germination strategies and post-germination traits are subjected to strong selection pressures (Donohue *et al*., 2010; Huang *et al*., 2010; Dayrell *et al*., 2016). Many plants have elaborate mechanisms to control seed germination, so that seed dormancy is broken only during periods of favourable environmental conditions for factors such as light, temperature, moisture, oxygen and nutrients (Nikolaeva, 1969, 1977; Baskin and Baskin, 1989). A selection of unrelated angiosperms has done away with a dormancy period altogether, in a strategy known as recalcitrance. Recalcitrant seeds are desiccation sensitive, and have high water content and well-developed embryos when shed from capsules (Crocker, 1916; Martin, 1946; Grushvitzky, 1967; Roberts, 1973; Pammenter and Berjak, 1999; Floyd and Friedman, 2000; Forbis *et al*., 2002).

Further study proved this binary classification to be too narrow, as some seeds display substantial variation in terms of their morphology and physiological responses to desiccation (Normah *et al*., 1986; Farrant *et al*., 1989; Connor *et al*., 1996; Hong and Ellis, 1996; Daws *et al*., 2004, 2006). Consequently, a third category of intermediate seeds was introduced (Ellis *et al*. 1990). These seeds are shed with high water contents and are capable of withstanding considerable desiccation, although not to the same extent as dormant seeds (Ellis *et al*., 1990; Hong and Ellis, 1996). It is currently estimated that 89% of angiosperms have dormant seeds, 5% have recalcitrant seeds and only 1% have intermediate seeds, while the strategies of the remainder are unknown (Tweddle *et al*., 2003; Gold and Hay, 2008; Wyse and Dickie, 2017).

Many authors have suggested that a continuum between two states, namely extreme dormancy and maximal recalcitrance, may be favoured above two or three discreet strategies

(Berjak and Pammenter, 1997; Sun 1998; Pammenter and Berjak, 1999; Kermode and Finch-Savage, 2002; Berjak *et al*., 2004; Berjak and Pammenter, 2008). The continuum concept accommodates the documented within- and between-species physiological and morphological variation, as well as effects of seed provenance on seed desiccation tolerance (Daws *et al*., 2004, 2006). Plasticity of seed morphological, phenological and physiological traits is regarded as an important source of variation influencing the shift between different germination strategies (Clauss and Venable, 2000; Venable, 2007).

A strong association between seed germination strategies and habitat preference has been documented (Roberts and King, 1980; Von Teichman and Van Wyk, 1994; Berjak *et al*., 2004), consistent with theoretical expectations favouring recalcitrance in less changeable environments (Dayrell *et al*., 2016). On seasonal time scales, plants with dormant and intermediate seeds are characteristic of temperate or arid habitats with strong seasonality (Jurado and Flores, 2005; Baskin and Baskin, 2014), but also occur (albeit at very low frequency) in all other habitat types (Hong and Ellis, 1996). Germination of recalcitrant seeds is usually initiated immediately or soon after shedding (Farrant *et al*., 1985, 1986), therefore these seeds are commonly associated with aseasonal and moist environments, such as tropical, sub-tropical and wetland habitats (Tweddle *et al*., 2003). Due to the primary benefits of dormancy being linked to bet-hedging in stochastic environments, it has also been proposed that, over longer time scales, geologically and climatically stable environments would be expected to have relatively greater proportions of recalcitrant species (Dayrell *et al*., 2016).

One such example of a climatically stable system is the botanically rich Greater Cape region of southern Africa (Cowling *et al*., 2014). Climatic conditions for the Greater Cape range from seasonal winter-rainfall in the southwest, aseasonal rainfall in the east, and semi-arid conditions with scant winter rainfall to the north-west (Manning and Goldblatt, 2012; Snijman, 2013). Within the predominantly winter rainfall region, plants rely on the seasonal availability of water for their dormant seeds to germinate (Keeley and Bond, 1997). In contrast to what is known for other climatically stable systems (Dayrell *et al*., 2016), only two known Cape lineages have species with recalcitrant seeds, namely members from the monocotyledonous tribe Amaryllideae (Amaryllidaceae) (Snijman and Linder, 1996; Berjak *et al*., 2004) and many species from the eudicot genus *Oxalis* (Oxalidaceae) (Hildebrand, 1884; Salter, 1944; Brink, 2017).

Oxalis is the largest geophytic genus in the Cape (*ca*. 210 species) (Proches *et al*., 2006; Manning and Goldblatt, 2012) and therefore contributes a large diversity component to the Cape Flora. *Oxalis* seeds have been defined as endospermous (dormant) and exendospermous (recalcitrant) (Hildebrand, 1884; Salter, 1944). Recalcitrant-seeded taxa represent the majority of *Oxalis* in southern Africa (approx. 60% (Salter, 1944)). Although mostly treated as a binary variable (presence/absence of endosperm), Salter (1944) noted a 'tendency to a transition' towards the exendospermous seed type among a few endospermous-seeded *Oxalis* species. Reportedly, eight endospermous species displayed various intermediate structural and behavioural traits, which suggests that a strictly dormant/recalcitrant classification may not adequately reflect germination strategies among Cape *Oxalis* (Salter, 1944; Brink, 2017).

The high species diversity and intriguing seed biology of the Cape *Oxalis* provide an ideal model system to study and explore the diversity of germination strategies. We aimed to re-assess the binary classification of germination strategies for Cape *Oxalis* and to investigate putative intermediate states using seed physiological traits. We also aimed to determine which seed and seedling morphological and developmental traits could be associated with each of the germination strategies. Information on traits that consistently distinguish between germination strategies might shed light on different selective regimes driving the evolution of seed germination strategies within Cape *Oxalis*.

MATERIALS AND METHODS

Sample collection

Sixty-four Cape *Oxalis* species with a wide taxonomic distribution (Salter, 1944; Oberlander *et al*., 2011) were selected based on phylogenetic placement and availability (Appendix S1; see Supplemental Data with this article). Seeds were collected from various field localities throughout the Cape region of South Africa (Cape Nature Conservation Board Permit No. 0028-AAA088-00243) and from plants in the Stellenbosch University Botanical Garden *Oxalis* research collection. Due to the need for large numbers of seeds that could not be generated in a research collection context, we could not explicitly account for maternal effects and so variation in wild- vs. garden-collected seed at least partly reflects this. However, there is no obvious pattern of bias or change in variance between the wild- vs. garden-collected seeds in our analyses (data not shown), and so we consider variation due to maternally-inherited effects minor compared to natural between-species variation. Mature

seeds were harvested from capsules, and seeds were exposed to experimental treatments starting on the day of harvest.

Seed physiology

We used three different physiological measures to place seeds on a dormancy/recalcitrance spectrum. The 'Kew 100-seed test for desiccation tolerance' (adapted from Pritchard *et al*. (2004)) and the 'seed storage category screening' (adapted from Hong and Ellis (1996)) were used to determine seed desiccation tolerance, to assess seed moisture content at shedding and to assess critical moisture contents of all desiccation-tolerant species. Samples of 100 seeds per species were used and subdivided into smaller batches depending on experimental treatment. An initial (control) germination test was conducted on 26 seeds per species, where the rumination success (% germination) of seeds was assessed when germinated at ambient air temperature on moist filter paper in petri-dishes. A desiccation treatment was conducted by drying 32 seeds with 5g silica crystals in sealed plastic bags. A moisture-stored treatment was conducted by placing another 32 seeds on wet filter paper in sealed plastic bags to maintain high humidity (bags were opened every second day for aeration). The seeds of both experimental treatments were then incubated with an ecologically representative day/night light cycle (13 hours light, 11 hours dark) at 25°C for a two-week period.

After incubation the germination success from each experimental treatment was assessed by placing 13 seeds per species on moist filter paper in petri-dishes at ambient air temperatures. One petri-dish (90 mm x 15 mm) was used per species, but seeds were equally dispersed with at least 1 cm space between seeds. Germination was defined as the splitting of the tegmen (lignified tegmens split into multiple (usually five) segments, while un-lignified tegmens split into 2 segments). The germination success of all treatments (initial control, desiccated and moisture-stored) was recorded daily for a period of five weeks. All data were plotted as germination progress curves. As none of the exendospermous species could withstand desiccation, germination progress curves alone could not be used to define strategies among *Oxalis,* and were not further explored. Consequently we included an assessment of initial moisture content and critical moisture content of seeds.

Ten untreated seeds were used to determine initial moisture content at shedding by calculating the weight difference between fresh (day 1) and oven-dried (2 weeks at 40°C) seeds (Equation 1 from Reeb *et al*. (1999)). A sample of six seeds per experimental treatment was used to determine seed moisture contents after each treatment.

In order to assess the critical moisture content of all desiccation-tolerant species, 13 seeds per desiccated experimental treatment were dried for two additional weeks (under the same desiccation treatment conditions as described above). After the incubation period, a germination test was conducted using seven seeds, while the remaining sample of six seeds was used to determine seed moisture contents. As we worked with a very limited supply of seeds, we did not assess ability of seeds to survive freezing and we reduced the number of desiccation treatments to two treatments, as suggested by Hong and Ellis (1996). These authors suggested that seeds should be dried to about 12% and 5% moisture content, which we were able to achieve after two and four weeks of desiccation. According to the literature, dormant seeds typically have critical moisture contents below 7% and intermediate seeds above 8%, while recalcitrant seeds span a wide range (20 to 96%) depending on the oil content (Ellis *et al*., 1989; Probert and Longley, 1989; Pritchard, 1991; Hong and Ellis, 1996; Pammenter and Berjak, 1999).

Seed and seedling morphology

We aimed to determine if morphological signal among seed and seedling data correlated with assigned germination strategies (using physiological traits). In order to test this, an independent morphological and developmental *Oxalis* seed and seedling dataset was compiled for all studied species. A daily digital image record was taken to document the sequence of development until seedlings reached maturity, using five seeds per species from the initial control germination treatment. Morphological data were collected for all of these individual seeds from the day that seeds were shed and harvested, until seedlings reached maturity. Seedling maturity was defined as one day after the leaflets of the first foliar leaf of the seedling had fully emerged and unfolded. A total of 71 morphological seed and seedling traits were studied (Appendix S1), which included 32 qualitative (discrete, unordered), three qualitative (discrete, ordered) and 36 quantitative (continuous) traits. Continuous traits were measured to scale from images imported to ImageJ (Abràmoff *et al*., 2004).

The seeds of 20 dormant *Oxalis* species did not germinate throughout the duration of this study (the first growing season after shedding), however, these seeds were viable as they successfully germinated within the following growing season (Brink, 2017). Germination data and seedling morphological traits from these species were not included in our study, as these seeds were used in another study. Based on the available morphological and developmental data for the studied species, three separate datasets were constructed, namely a

seed, seedling, and combined seed-and-seedling dataset. Due to the lack of germination, the seed morphological traits of the 20 above-mentioned species were included in the seed dataset (which included data for all 64 *Oxalis* species), but excluded from the seedling and combined seed-and-seedling datasets (which included data for only 44 *Oxalis* species). These three separate datasets were used to assess our assignment of pre-defined germination strategies based on physiological traits and in order to compare our results to previous studies on the seed (Obone, 2005) and seedling (Brink, 2017) morphology of Cape *Oxalis*. Additionally, embryo development (relative size and pigmentation) and presence of endosperm was assessed by sectioning fresh seeds lengthwise. A Leica M125 stereo microscope, Leica MC 170 HD camera and LAS CORE software (Leica, Switzerland) were used to document these seed sections. As we often had a limited sample of seeds, it was not possible to assess these traits for all species.

Cluster, Principal Component and K-means clustering Analyses

All data were analysed using the R statistical environment, version 3.4.1 (R-Core-Team, 2014). CA's and PCA's were implemented to assess major sources of variation in discrete and continuous seed (13 traits), seedling (58 traits) and combined (71 traits) datasets. These analyses were conducted to determine if species or strategies cluster together based on morphological and developmental traits. Data for five replicates per species were included in all analyses (Appendix S2 and Appendix S3). The Gower's method was used to calculate distances to centre and scale data for the CA's and data for the PCA's were centred and scaled with the built-in scale function of the FactoMineR package ("PCA" function (Lê *et al*., 2008)). The mean clustering method was applied for the CA's that were conducted with the Dendextend (Galili, 2015) package. The FactoMineR ("PCA" function (Lê *et al*., 2008)) and Factoextra ("fviz_pca_ind" function (Kassambara and Mundt, 2016)) packages were used for PCA's.

The NbClust (Charrad *et al*., 2014) package was used for K-means clustering (Ward, Silhouette and Gap statistic methods) in order to determine the optimal number of clusters in the data (the majority rule was used to determine the best number of clusters based on the results from the three methods). Predefined physiological germination strategies (as determined in the previous section) were mapped onto these clusters for each dataset. Additionally, sub-optimal clusters in the data were explored, but this did not aid in elucidating patterns among groups.

Discriminant Analyses

Discriminant analyses (DA) were implemented to test if morphological and developmental traits were predictive of membership to each of our assigned physiological germination strategies. Continuous and ordered discrete data of the seed (8 traits), seedling (31 traits) and combined (39 traits) datasets were centred and scaled with the inbuilt scale function (dudi.pca from the ade4 package (Dray and Dufour, 2007)) for DA's. Categories proposed in the seed physiology section of this work were used as *a priori* grouping variables in DA's. Statistical analyses to test support for groups were done with the use of one-way multivariate analysis of variance (MANOVA) and Pillai's tests and Monte-Carlo Permutation tests with 9999 replicates (Dray and Dufour, 2007).

RESULTS

Seed physiology

Many of the endospermous *Oxalis* seeds (24.2% of our initial sample) did not germinate during the time period of this study. These species included *O. ambigua* Jacq., *O. convexula* Jacq., *O. crispula* Sond., *O. fenestrata* Dreyer, Roets and Oberl., *O. lichenoides* T.M.Salter, *O. luteola* Jacq., *O. melanosticta* Sond., *O. obtusa* Jacq., *O. obtusa* var. *atrata* T.M.Salter, *O.* cf. *pes-caprae* L. (project number MO1632), *O. pulchella* Jacq., *O. purpurea* L., *O. zeekoevleyensis* R.Knuth. Failure to germinate within the same year as collection has been recorded for some of these species by Brink (2017). Morphological or phenological data of seedlings could therefore not be documented for these species. However, the seeds of all species were viable, as they successfully germinated in the subsequent growing season (Brink (2017) and *pers obs*.). We suggest that these seeds have a longer and possibly mandatory delay in germination.

Among the remaining species, 22.6% had desiccation-tolerant seeds and 53.2% had desiccation-sensitive seeds. The majority of desiccation-tolerant species' seeds germinated within 1 to 2 days after shedding, while three species had a dormancy period of at least 4 to 9 days before germination (Fig. 1A-a). All desiccation-sensitive seeds germinated within one or two days after shedding. Unexpectedly, seeds of some typical exendospermous, recalcitrant species (*sensu* Salter, 1944) proved to be desiccation-tolerant (including *O. commutata*, *O. eckloniana* C.Presl, *O. phloxidiflora* Schltr., *O. stenopetala* T.M.Salter, *O. suteroides* T.M.Salter and *O. zeyheri* Sond).

Seed moisture contents (mc) at shedding showed substantial overlap between desiccation-tolerant (22.2 to 86.3% mc) and desiccation-sensitive (50 to 96.2 % mc) species (Fig. 1A-b). The critical seed moisture contents (cmc) of desiccation-sensitive seeds could not be determined, as all seeds lost viability after a two week desiccation period. This indicated that recalcitrant seeds cannot tolerate substantial water-loss and we would expect that these seeds have very high cmc values. Cmc of desiccation-tolerant seeds showed a distinctive divide between species that were able to survive only one desiccation treatment (8 to 19% cmc) and species that were able to survive both desiccation treatments (2 to 5% cmc) (Fig. 1A-c).

Defining seed germination strategies based on seed physiology

Here we used a composite of three physiological traits to classify seeds into three categories: dormant, recalcitrant and intermediate (Appendix S4). These traits included seed desiccation-tolerance, time from shedding to germination and lowest critical seed moisture contents for desiccation-tolerant species (Fig. 1A and 1B). Consequently, dormant *Oxalis* seeds were defined as seeds that could survive a desiccation period of four weeks without loss of viability, had critical moisture contents between 2 and 5% and a minimum dormancy period of at least four days (up to a year) before germination. Recalcitrant seeds were defined as seeds that could not survive a desiccation period of two weeks and were therefore unable to tolerate water loss and germinated within one to two days after shedding. Seeds lying between these two categories were defined as intermediate.

Among the studied species, 28.6% had dormant seeds, 53.9% had recalcitrant seeds and 17.5% had intermediate seeds. All dormant species were endospermous and all recalcitrant species lacked endosperm (Salter, 1944). The new intermediate group included five species previously described as endospermous (*O. depressa* Eckl. and Zeyh., *O. dilatata* L.Bolus, *O. imbricata*, *O. stellata* Eckl. and Zeyh., *O. virginea* Jacq.) and six species described as exendospermous (*O. commutata*, *O. eckloniana*, *O. phloxidiflora*, *O. stenopetala*, *O. suteroides*, *O. zeyheri*) (Salter, 1944) (Fig. 1B).

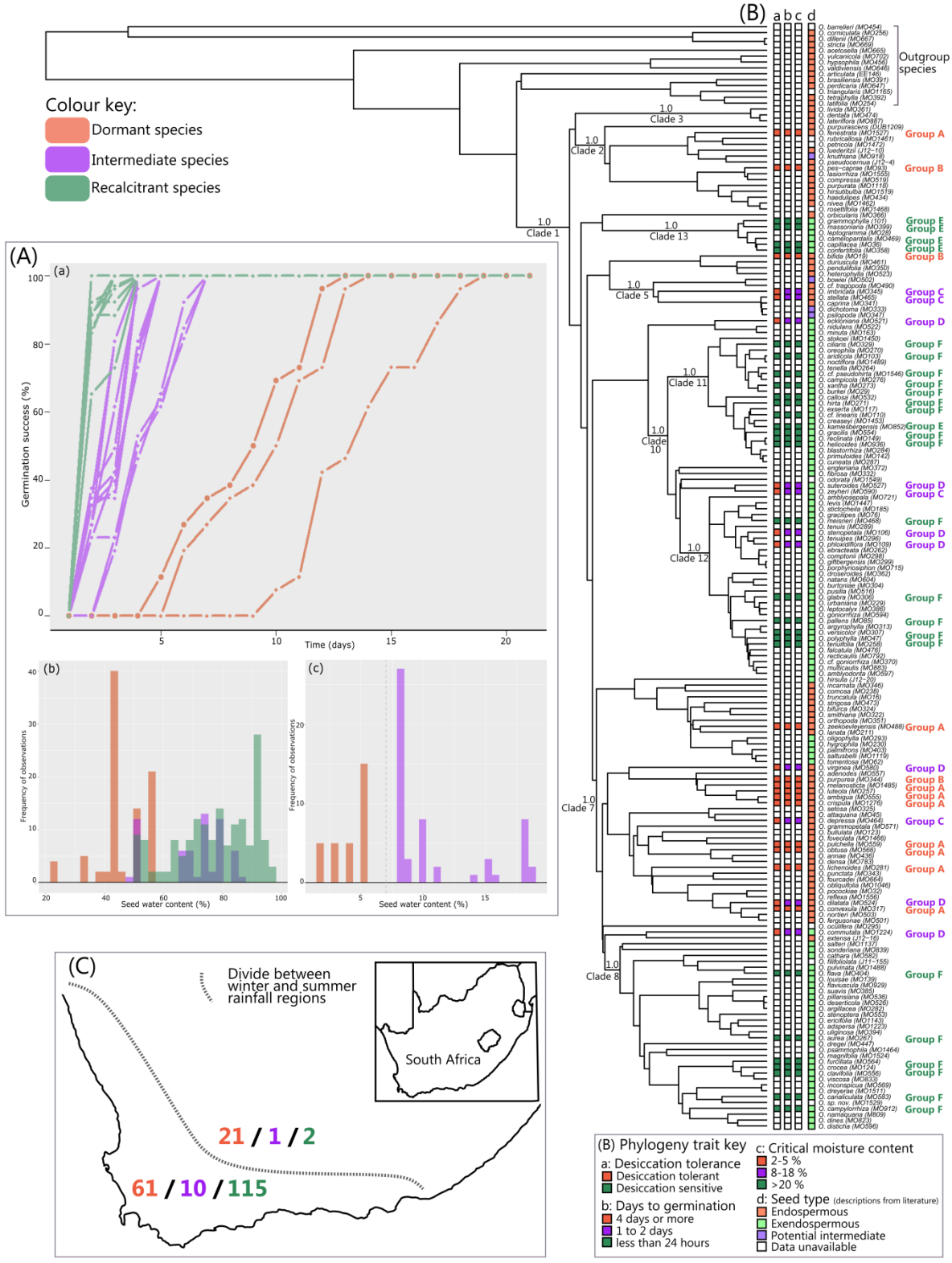

Figure 1: The phylogenetic, physiological and geographic distribution of dormant, recalcitrant and intermediate seed germination strategies in southern African *Oxalis* (A) Physiological traits of seed germination strategies. (a) Germination progress curves of dormant, intermediate and recalcitrant seeds (seeds exposed to the initial germination test from the 'Kew 100-seed test'). (b) Comparative percentages of seed moisture content at

shedding between dormant, intermediate and recalcitrant seeds. (c) Critical seed moisture contents for desiccation-tolerant dormant and intermediate seeds. The vertical dashed line represents the critical moisture content classically used to distinguish between dormant and intermediate seeds (Ellis *et al*., 1989; Probert and Longley, 1989; Pritchard, 1991; Hong and Ellis, 1996; Pammenter and Berjak, 1999). (B) Phylogenetic distribution of characters/germination strategies. (a) Desiccation tolerance. (b) Number of days to germination. (c) Critical seed moisture content. (d) Presence/absence of endosperm in *Oxalis* seeds, as described in the literature (Salter, 1944). Posterior probability values and major clade names corresponding to Jooste *et al.* (2016) are indicated at relevant nodes. Groups labelled at right correspond to groups identified in the text and following figures. Details of phylogeny reconstruction are available in Jooste *et al*. (2016). (C) Geographic distribution of germination strategies, where the number of species from each strategy is indicated with representative colours. The divide between the winter and summer rainfall regions is indicated with a dashed line (Snijman, 2013).

Exploring morphological groupings among germination strategies

Cluster analyses

The majority of within-species replicates formed distinct clusters. Analysis of seed morphology indicated at least four clusters that loosely corresponded to the three seed germination strategies as defined above (Appendix S2 A). However, there were a few odd placements (*e.g. O. bifida, O. virginea* and *O. zeyheri*) and overlap among strategies. This unclear pattern may be due to the relatively few traits included in the seed dataset. Seedling morphology data revealed three clusters that corresponded to our predefined strategies, with one odd placement (*O. cf. pallens*) (Appendix S2 B). Analysis of the combined seed-and-seedling dataset clearly separated our predefined categories, with one cluster corresponding to dormant species, one to recalcitrant species and two clusters with intermediate species (Appendix S2 C).

Principal Component Analyses (PCA) with K-means optimal clustering

The five within-species replicates formed distinct clusters in PCA's (Appendix S3), with a few notable exceptions. PCA and K-means clustering showed that within each of the seed, seedling and combined seed-and-seedling morphological datasets there were at least three clusters corresponding to germination strategies.

In the seed dataset, the first two principal components explained 69.2% and 11.2% of the variation in the data. K-means cluster analyses identified five optimal clusters (Fig. 2A). The dormant species with seeds that did not germinate formed the first coherent cluster

(subsequently referred to as Group A, Fig. 3A). The second cluster included dormant species that germinated within the first growing season and a few intermediate species that appear to be morphologically most similar to other dormant taxa. The third cluster included intermediate and two recalcitrant species (*O. glabra* and *O. cf. pallens*), indicating overlap among our predefined strategies. The fourth cluster included recalcitrant species and one intermediate species (*O. stenopetala*) in the region of overlap, while the last cluster included recalcitrant species only.

In the seedling dataset, clusters corresponded to germination strategies and the first two principal components explained 24.5% and 11.3% of the variation in the data respectively. K-means cluster analyses revealed four optimal clusters (Fig. 2B). The first cluster included only two of the three dormant species that germinated (*O. bifida, O. purpurea*). The second cluster included the other dormant species (*O. pes-caprae*) all intermediate species and an overlap with one recalcitrant species (*O. cf. pallens*), again indicating some overlap among our predefined strategies. The two remaining clusters included recalcitrant species only.

In the combined dataset clusters the first two principal components explained 30.4% and 11.2% of the variation in the data. K-means cluster analyses showed four distinct clusters (Fig. 2C). The combined seed and seedling dataset was deemed the most robust and representative, as it included the most traits and species (even though the dormant species that did not germinate had to be excluded). Descriptions and interpretations of all traits important in explaining distribution of data from the seed, seedling and combined datasets are included as Chapter 2 additional notes A.

The first cluster included distinct groups of dormant (hereafter Group B, Fig. 3B) and intermediate (Group C endospermous intermediate, Fig. 3C) species, and corresponded to a similar cluster found in the seed data set. Although K-means clustering placed these taxa in one cluster, the clear gap between dormant and intermediate morphologies in this cluster supports its division into two groups. All Group B and C seeds have lignified tegmens, are endospermous at seed release and have green-pigmented embryos, but display different physiological responses to desiccation. The second cluster (Group D exendospermous intermediate, Fig. 3D) held the remaining intermediate and one recalcitrant species (*O. cf. pallens*). The two remaining clusters included recalcitrant species only, namely one small cluster that consisted of species from one subsection (Section *Angustatae* subsection *Pardales sensu* Salter (1944), except for *O. kamiesbergensis* T.M.Salter, subsequently

referred to as Group E, Fig. 3E), while the other included the remaining recalcitrant species (subsequently referred to as Group F, Fig. 3F). All three data sets agreed on separating recalcitrant species from the other clusters, although precise clustering among recalcitrant taxa differed across data sets.

The number of seeds per capsule and cotyledon shape determined the spread of data across the first principal component and cotyledon petiole length and hypocotyl width were the most important traits across the second principal component. The most important traits separating clusters included morphological traits such as tegmen surface texture, tegmen lignification, presence or absence of endosperm and embryo pigmentation, and phenological traits such as number of days until cotyledons opened, until root hair development and until the first foliar leaf became visible (Fig. 2C). The traits that determined the spread of clusters for this dataset were largely the same as those identified among the separate seed and seedling datasets.

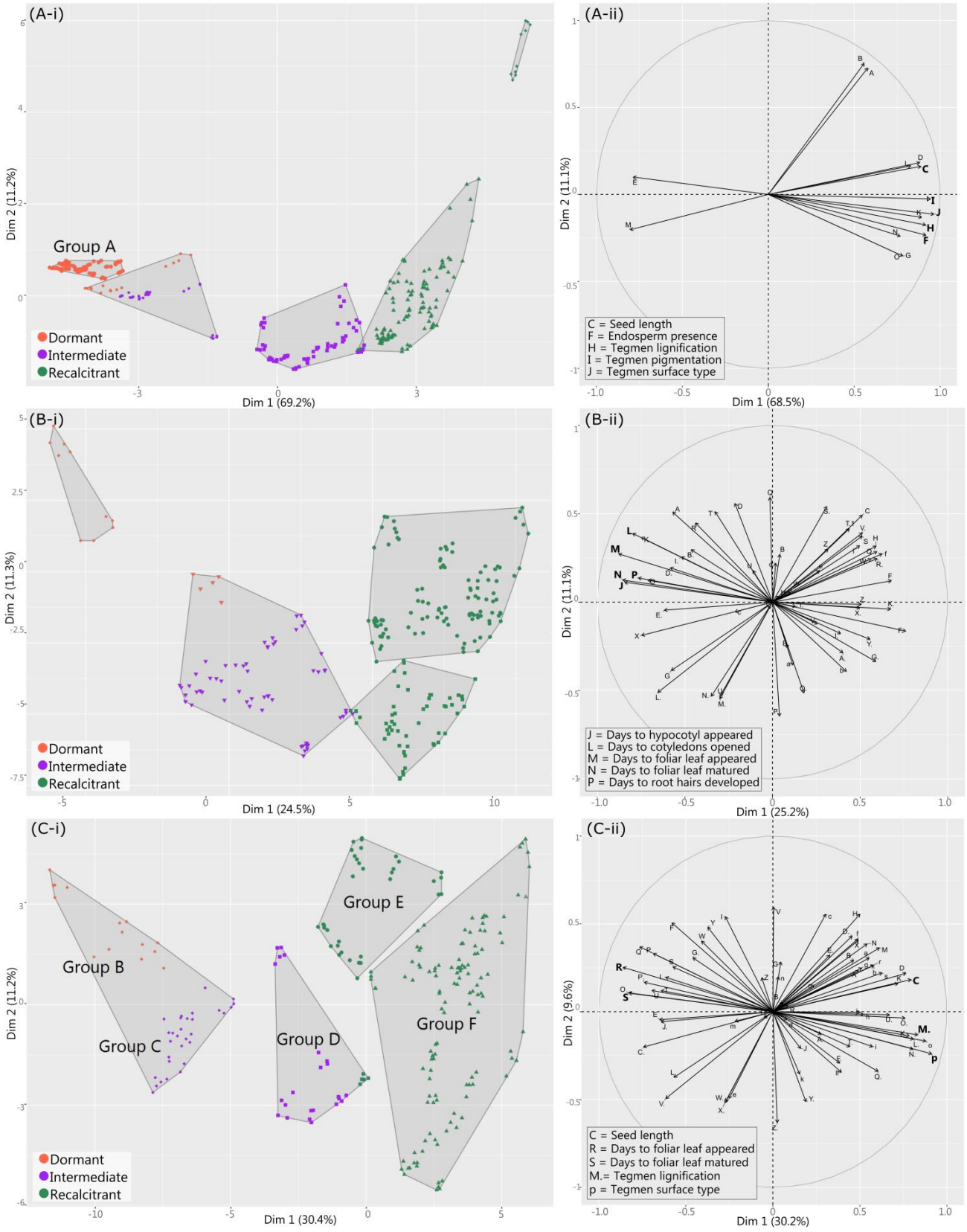

Figure 2: PCA with K-means clustering with the optimal number of clusters among discrete and continuous *Oxalis* seed and seedling morphological and developmental data. Support for three main germination strategies is evident among the seed (A), seedling (B) and combined

seed and seedling (C) datasets. Individual factor maps with optimal clusters (i) and variable factor maps (ii). Five traits with the strongest grouping effects of each of the respective datasets are indicated in bold text with a key to these selected variable names provided. A key to all variable factor names is provided in Appendix S5 a-c.

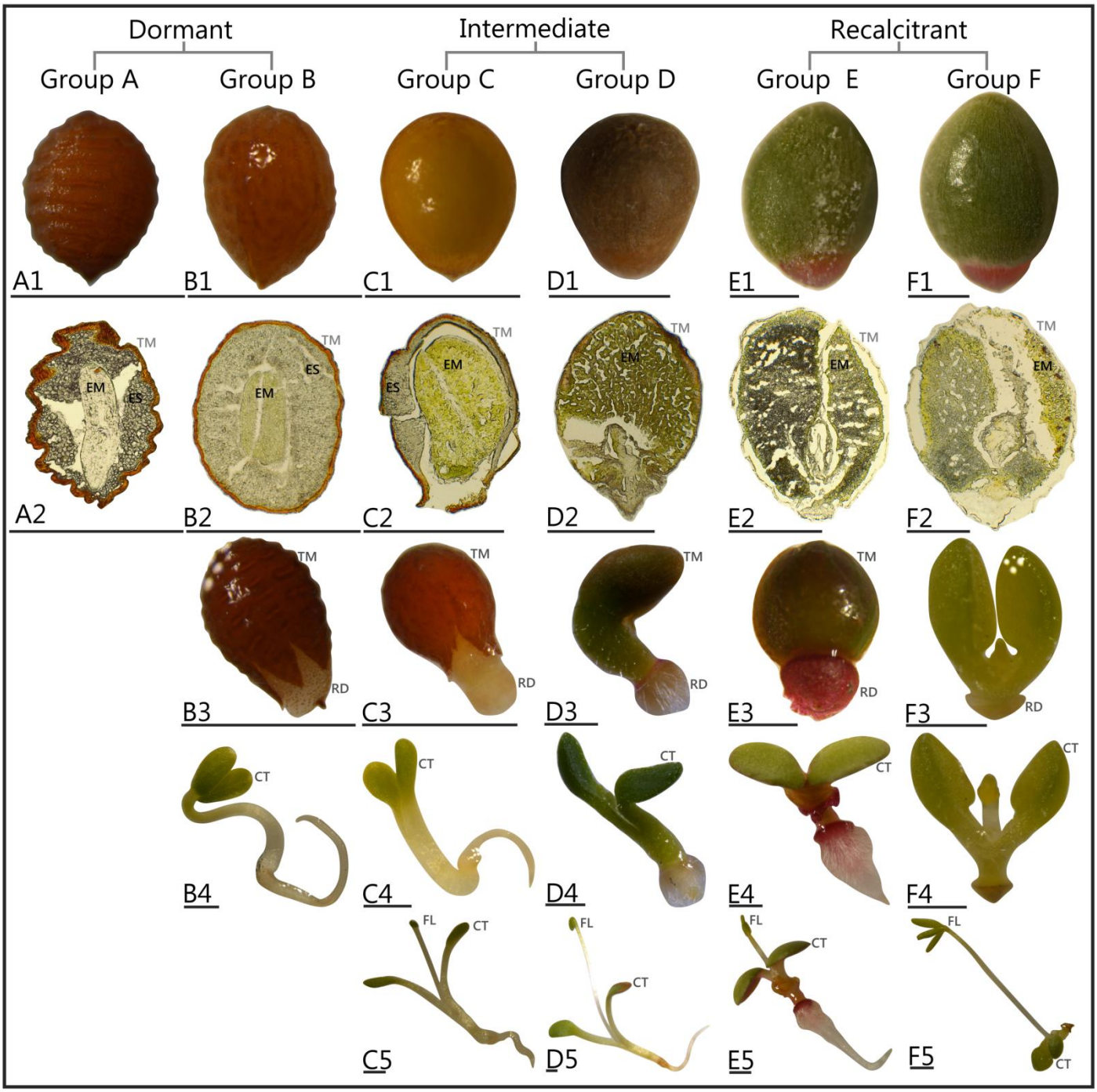

Figure 3: Seed morphology corresponding to six morphological groups (Groups A to F) from three germination strategies among Cape *Oxalis*. Each column contains photos of representative seeds and seedlings from each group. (1) Seed with tegmen, (2) seed cross section (TM=tegmen, ES=endosperm, EM=embryo), (3) germinating seed (RD=radicle), (4) seedling with opening cotyledons (CT=cotyledons), (5) seedling with emerging first foliar

leaf (FL=foliar leaf). All scale bars represent 1 mm. All seeds/seedlings oriented with radicle pointing to bottom (rows 1-2) or bottom right (rows 3-6). A key to all species names is provided in Appendix S6.

Confirming germination strategies with Discriminant Analyses

DA's were implemented to determine if morphological and developmental seed and seedling traits were predictive of our three proposed seed germination strategies. Results of the discriminant analysis (DA) of the seed dataset showed that species primarily clustered according to seed germination strategies (MANOVA and Pillai's test ($F_{2, 1.156}$=52.352, p<0.0001), Monte-Carlo permutation test (mean obs=0.1444564, p<0.0001), Fig. 4A). However, there was a detectable overlap between the dormant and intermediate groups, while the recalcitrant seeds formed a separate cluster. DA of the seedling dataset also indicated that species clearly clustered according to seed germination strategies (MANOVA and Pillai's test ($F_{2, 1.883}$=107.58, p<0.0001), Monte-Carlo permutation test (mean obs=0.06072856, p<0.0001), Fig. 4B), this time without any overlap between any of the three groups.

Clustering according to germination strategies were also evident from the DA using the combined seed-and-seedling dataset (MANOVA and Pillai's test ($F_{2, 1.912}$=111.55, p<0.0001), Monte-Carlo permutation test (mean obs=0.04902814, p<0.0001), Fig. 4C), again without overlap between groups. Among the combined dataset traits important in separating strategies along the first axis were tegmen surface type, tegmen permeability, the number of days until the first foliar leaf appears. The separation of recalcitrant and intermediate strategies was strongly influenced by seed dry weight, number of days until the first foliar leaf matured and cotyledon petiole length (measured after cotyledons opened and after the first foliar leaf matured). Dormant and intermediate strategies were strongly influenced by tegmen surface type and the number of days until the roothairs developed.

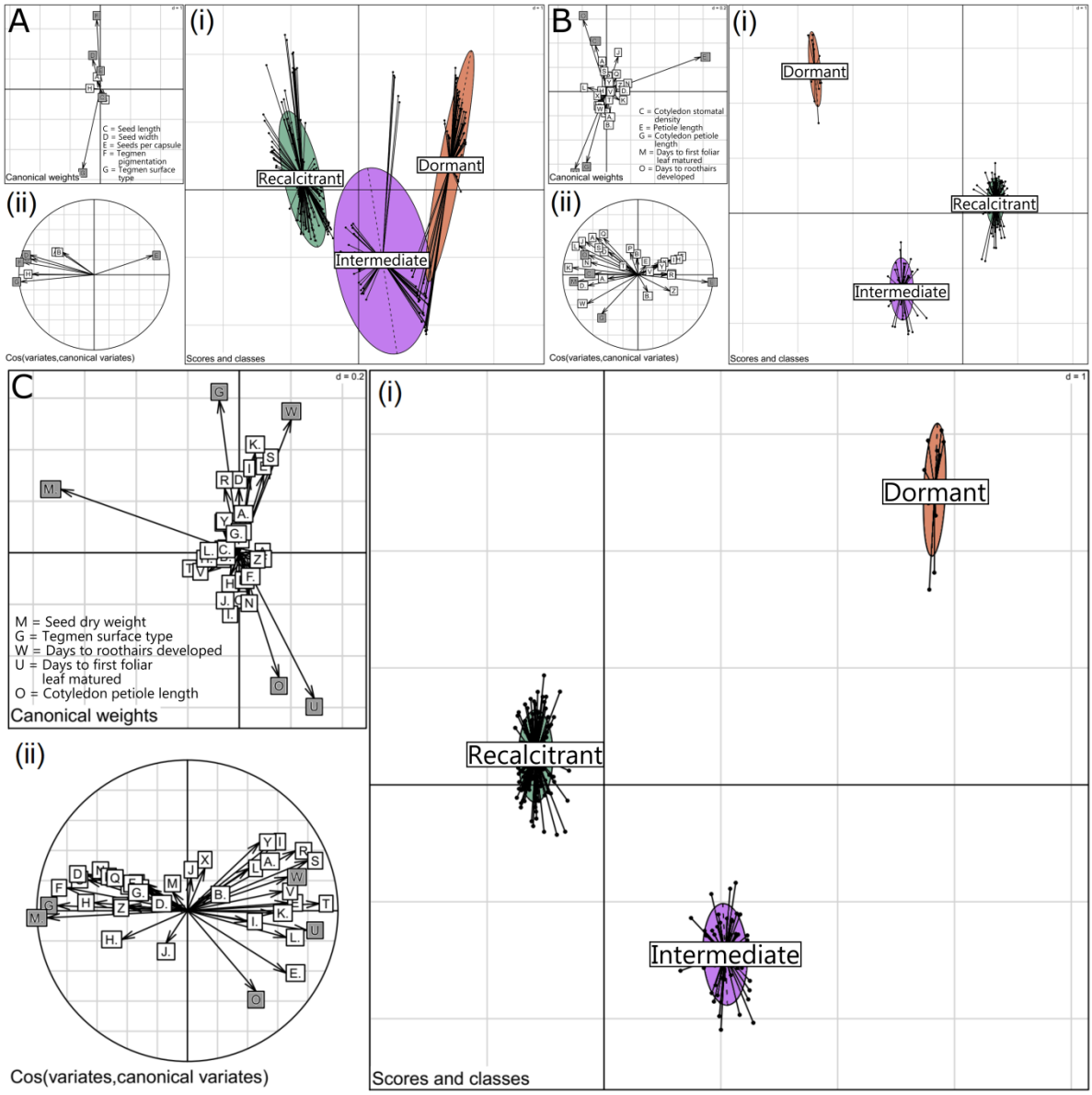

Figure 4: Discriminant analyses indicating that morphological and developmental *Oxalis* seed and seedling traits were predictive of membership to each of our assigned physiological germination strategies. Support for three germination strategies is evident among the seed (A), seedling (B) and combined seed and seedling (C) datasets. Individual factor maps (i) are used to visualise the spread of data and variable factor maps (ii) are used to assess the specific continuous and ordered discrete traits that explain these groupings. Five traits with the strongest grouping effects of each of the respective datasets are shaded in grey with a key to these selected variable names.

Our results for seed data are mostly consistent with Obone (2005) and Brink (2017). For seeds, both Obone (2005) and our results support a primary split between dormant and mostly recalcitrant taxa. Although our results include some typically dormant taxa in the recalcitrant cluster, these could be due to broader taxonomic sampling, or partially non-overlapping sets of seed characters. For seedling data, in contrast to a clear pattern separating dormant from

recalcitrant/intermediate taxa in our study, Brink (2017) found a somewhat more complex pattern. Cluster analyses showed two distinct recalcitrant clusters and two dormant clusters, one consisting of *O. bifida* and the other containing all other dormant taxa. However, PCA's showed a close similarity between all dormant taxa and there was clear morphological support separating a dormant/recalcitrant strategy, consistent with our results.

In summary, all datasets showed that germination strategies were associated with suites of seed and seedling morphological and developmental traits. The majority of traits identified in the separate seed and seedling datasets were also important in separating strategies in the combined dataset. These data and analyses provide strong, independent evidence in support of the recognition of three germination strategies among Cape *Oxalis*, as well as sub-groups among each strategy.

DISCUSSION

The division of *Oxalis* germination strategies into two discrete types, namely dormancy and recalcitrance, is an oversimplification. Many species occupy positions intermediate between these two states. We have identified three germination strategies, defined according to three seed physiological traits, namely desiccation tolerance, time from shedding to germination and lowest seed critical moisture content. We have also shown that there is morphological and phenological support underpinning these physiologically-defined strategies. Analyses of morphological data revealed two potential subgroups among each of the three seed germination strategies (Groups A to F). Even though we have identified six groupings among the three germination strategies, it is important to note that we have sampled approximately 27% of the *ca*. 230 *Oxalis* taxa in southern Africa, and that increased sampling might blur the boundaries between these defined strategies and groups. Thus, the data could just as likely represent a continuum of states.

It is of interest that there is very little pattern corresponding to the current taxonomy (Salter 1944) or to phylogeny (Fig. 1B) among the three strategies or six groups. A recent study that assessed seedling morphological traits of Cape *Oxalis* showed that dormant species of widespread taxonomic affinity clustered together regardless of relatedness (Brink, 2017). This was interpreted as a possible signal of stabilising selection (Brink, 2017). The same study found possible morphological signals for convergent evolution of recalcitrance in unrelated lineages. However, our findings indicated that all three strategies included species from multiple, unrelated lineages. The balance of evidence thus suggests a complex pattern of

convergence on germination strategy within Cape *Oxalis*. No paper has examined the evolution of germination strategy in Cape *Oxalis* in an explicit phylogenetic context – such a study will have to await germination data on the *ca.* 73% of remaining unsampled *Oxalis* taxa.

Potential adaptive significance of morphological traits associated with strategies

Several seed traits show distinct variation between the three germination strategies indicative of classic quantity/quality trade-offs. Dormant species (Groups A and B) produce capsules with many seeds, where each seed is very small (low seed length and mass) and endosperm is present. This may be a low risk strategy for dormant species, as seeds have the ability to maintain dormancy, survive desiccation and can consequently be effectively dispersed through space and time until they encounter favourable germination conditions (Evans and Dennehy, 2005). Recalcitrant species (Groups E and F) seem to follow the exact opposite strategy, where species produce few seeds per capsule, but with high seed mass and containing no endosperm upon release from the capsule. The embryos of recalcitrant seeds are large and ready to germinate upon release, which coincides with the fact that these seeds are dispersed and germinate in the wettest season of the Cape. Species with intermediate seeds (Groups C and D) are intermediate in number, size and amount of endosperm relative to the dormant and recalcitrant strategies.

Dormant species (Group B) have seedlings that germinate with their hypocotyl emerging from the seed, followed by substantial root growth and then the development of root-hairs. These species form long roots with a long and thin hypocotyl. Cotyledons only unfold once the root and root-hairs are well-established. The first foliar leaf develops and matures at a much later stage. This sequence of development is similar to the classical sequence of development documented among the majority of angiosperm seedlings (Esau, 1960). Intermediate species (Groups C and D) display various sequences of development where either the hypocotyl or root hairs emerge from the seed, but all seedlings reach maturity much more rapidly than dormant species. Recalcitrant species (Group E) display root-first germination. The hypocotyl and root hairs emerge and develop, followed by cotyledons unfolding and the development and maturation of the first foliar leaf. The majority of recalcitrant species (Group F) displays a strategy of inverse germination, where cotyledons and the first foliar leaf develop rapidly and appear to sustain rapid growth of the seedling,

until the hypocotyl, root-hairs and roots subsequently emerge. This is a remarkable phenomenon where seedlings are capable of rapid growth and development temporarily without well-established roots to supply the seedling with nutrients. Many of these recalcitrant species produce large amounts of mucilage upon germination. Preliminary investigation of microbes within the mucilage (under sterile control and various experimental conditions) revealed the presence of both bacteria and fungi. Subsequent research is aimed at investigating potential associations between recalcitrant *Oxalis* species and mucilage-dwelling microbes.

Potential evolutionary trends among germination strategies

Dormancy is regarded as the ancestral state among Oxalidaceae and *Oxalis* species, while recalcitrance appears to have had multiple independent origins within Cape *Oxalis* (Salter, 1944; Oberlander *et al*., 2011, Fig. 1B). Our results suggest that dormant and recalcitrant strategies may be viewed as two extreme states, connected physiologically through the intermediate strategy and morphologically through at least two intermediate groups. We suggest that seed germination has evolved from the ancestral dormant state towards a derived, maximally recalcitrant peak. Even though we have opted for discrete groups in explaining the variety of intermediate morphologies evident among our sampled taxa, the dormant (Group B), intermediate (Groups C and D) and recalcitrant (Groups E and F) groups could represent an over-simplification of the reality. It is possible that all of the above-mentioned groups could represent a continuum of states, bounded by typical dormancy and maximal recalcitrance, and connected evolutionarily by taxa containing a mosaic of morphologically and physiologically intermediate seed/seedling characters, some of which are still represented among extant Cape *Oxalis* species.

Many authors have proposed a general evolutionary trend of increasing embryo size (development) and decreasing endosperm, in order to acquire recalcitrant seeds among angiosperms (Martin, 1946; Berjak and Pammenter, 1997; Sun 1998; Forbis *et al*., 2002; Kermode and Finch-Savage, 2002; Berjak *et al*., 2004). Based on this hypothesis, one would expect that a transition from dormancy to maximal recalcitrance among Cape *Oxalis* species would be a step-by-step assembly of traits associated with recalcitrance, where certain physiological and morphological traits (such as desiccation tolerance, and consequently increased embryo size and decreased endosperm) are lost or acquired. As possible descendants of the various steps in this process, taxa in the intermediate strategy (Groups C

and D) might provide information on the assembly of the recalcitrant strategy, and the selective pressures leading to its establishment.

Absence of a strong phylogenetic signal of recalcitrance (and associated morphological and phenological traits) among angiosperm clades has been attributed to convergent evolution of traits in response to environmental conditions (Lord *et al*., 1995; Rees, 1996; Forbis *et al*., 2002). Berjak and Pammenter (2008) stated that if desiccation sensitivity is a derived trait, there must be selective advantages to losing desiccation tolerance, even though it is such an important functional trait. These selective advantages would include either direct fitness advantages, such as competition avoidance or higher population growth rates (discussed in more detail below), or ecological trade-offs within a niche under specific environmental conditions (*sensu* Grubb (1977)). Fitness advantages could benefit seeds at the stage of shedding, dispersal, germination and/or seedling establishment and growth.

Environmental factors affecting germination strategies

Tweddle *et al*. (2003) proposed that seeds shed in seasonal environments will be highly influenced by two important environmental factors, namely temperature and water availability. Using the presence or absence of endosperm as a rough proxy for dormancy/recalcitrance, the vast majority of exendospermous *Oxalis* species (121out of 123) are restricted to the winter rainfall region of the Cape (Salter, 1944, Fig. 1C). Exendospermous *Oxalis* species flower and set seed during the early winter months (May to June) (Dreyer *et al*., 2006), therefore ensuring that seeds can take advantage of high seasonal water availability and presumably maximising the amount of time for growth and establishment. Endospermous *i.e*. mostly dormant *Oxalis* species are distributed more evenly in both the winter (63 species) and summer (28 species) rainfall regions of southern Africa (Salter, 1944, Fig. 1C). African *Oxalis* most likely originated in the Cape region (Oberlander *et al*., 2011) and recalcitrance is also likely to have evolved there, given the almost complete confinement of exendospermous taxa to the Cape region. Given this, it is likely that the decrease in reliable winter rainfall moving east might create a significant barrier to the establishment of recalcitrant seedlings, and thus of recalcitrant taxa, outside of the Cape. At the least, this difference in geographic distribution of germination strategies strongly implies a close linkage of recalcitrance to the winter rainfall Cape region.

The summer rainfall region often experiences below zero temperatures (especially during the winter months), with relatively low humidity compared to the winter rainfall region

(Manning and Goldblatt, 2012; Snijman, 2013). Desiccation tolerant seeds that are capable of surviving below-zero temperatures would be favoured in these habitats (Tweddle *et al*., 2003) or would be forced to adopt a desiccation avoidance strategy (Pammenter and Berjak, 2000). Recalcitrant and intermediate *Oxalis* seeds have high moisture contents, indicating that these seeds would not be able to survive freezing, due to ice formation in their embryos (Ellis *et al*., 1990). Even though we did not assess freezing ability directly, we would predict that dormant *Oxalis* seeds would remain viable under sub-zero temperatures, as is typical for all dormant seeds (Ellis *et al*., 1990). These environmental factors would prevent the successful establishment of recalcitrant and intermediate species in summer rainfall regions of the Cape, consequently limiting species with these strategies to the winter rainfall region. We therefore predict that both reliable seasonal rainfall (available moisture) and minimum air temperature would influence and/or determine the distribution of *Oxalis* species throughout the two rainfall regions of the Cape. Dussert *et al*. (2000) proposed that dispersal methods, fruiting phenology and habitat-related descriptions are required in order to fully understand factors affecting or determining germination strategies.

Fitness advantages associated with different germination strategies

Seed dormancy is regarded as a bet-hedging strategy to spread the risk of unsuccessful reproduction in unpredictable or stochastic environments (Cohen, 1966; Venable 2007; Poisot *et al*., 2011; Moreira and Pausas, 2012). Due to the ability of dormant seeds to survive desiccation, these seeds would be capable of avoiding unfavourable conditions, and would be able to establish large seeds banks. Seeds may remain viable for long periods of time (species-specific responses), allowing species to 'select' optimal timing to initiate germination (Linkies *et al*., 2010; Baskin and Baskin, 2014). Desiccation tolerant seeds are more likely to be dispersed in space and in time, and are therefore able to minimize competition between siblings (Cheplick, 1992). Dreyer *et al*. (2006) reported that dormant *Oxalis* species have a relatively long flowering period. Dormant *Oxalis* seeds consequently experience less climatic constraint, as seeds that are shed late in the season are able to remain dormant (and viable) until the following growing season (Dreyer *et al*., 2006).

Recalcitrant seeds have the ability to germinate immediately or soon after shedding, which may be advantageous in particular scenarios. If the seeds are shed under predictably favourable environmental conditions, such as the wet winter months of the Cape, germination success immediately after seed dispersal will be highly likely and loss of dormancy may

become favourable (Kermode and Finch-Savage, 2002). These seeds are metabolically active when shed, which enables them to germinate, establish and reach maturity much more rapidly than dormant seeds (Kermode and Finch-Savage, 2002). Rapid germination of recalcitrant seeds decreases the period of time that seeds are exposed to post-shedding predation or microbial decay, and increases the amount of growth (seedling biomass) before seedlings are exposed to unfavourable environmental conditions (Tweddle *et al*., 2003). Recalcitrant seeds do not produce large amounts of endosperm or lignified tegmens, possibly indicating a more efficient utilization of resources in comparison with dormant seeds (Berjak and Pammenter, 2008). Recalcitrance could, however, come with the cost of decreased growth rates or high mortality rates if seeds are shed during periods of unfavourable environmental conditions (low humidity and low available moisture) (Farnsworth, 2000; Tweddle *et al*., 2003). Given the short flowering period and early flowering peak of exendospermous species (Dreyer *et al*., 2006), suboptimal environmental conditions during the flowering period might have major repercussions for recalcitrant *Oxalis* recruitment.

Intermediate seeds are capable of both desiccation tolerance and rapid germination due to their well-developed embryos upon release. The investment in lignified tegmens and endosperm may be costly, but these structures ensure that the well-developed and metabolically active embryos are able to survive periods of desiccation (Ellis *et al*., 1990). These seeds have the benefit of immediate germination if environmental conditions are favourable, or delay germination until conditions become favourable. This view is, however, challenged by the comparative rarity of species with an intermediate germination strategy.

Future climate change and conservation

More than a third of Cape *Oxalis* species are listed as rare or endangered (Raimondo *et al*., 2009). As recalcitrant (and possibly some intermediate) seeds cannot be stored under typical seed bank conditions (desiccation or cryo-storage), the value of conservation of these species in the wild becomes apparent. Under predicted models of future climate change, the winter rainfall region of the Cape may experience warmer temperatures and lower rainfall, especially during the winter months (Cowling and Pressey, 2001; Tyson *et al*., 2002; Cowling *et al*., 2003; Midgley *et al*., 2003; Rouget *et al*., 2003; Thomas *et al*., 2004). If the establishment and success of recalcitrance in this system are indeed linked to reliable seasonal water availability, then such future conditions would be detrimental to seed germination and seedling establishment of recalcitrant and intermediate species, making them

more vulnerable to extinction. This emphasises the need for conservation in this diverse and remarkable Cape genus.

CONCLUSIONS

Here we have shown that the division of *Oxalis* germination strategies into two discrete types, namely dormancy and recalcitrance, is an oversimplification. Many species occupy positions intermediate between these two states and an intermediate germination strategy does exist among Cape *Oxalis*, with two possible morphological groups within each strategy. These could reflect a continuum of germination states, where an ancestral dormant strategy evolved towards a maximally recalcitrant peak, with a mosaic of intermediate states reflected in extant taxa. Environmental factors may affect germination strategy and distribution throughout the Cape, as recalcitrant and intermediate species are confined to the winter rainfall region. They occupy specialized niches and may face adverse impacts under predictions of climate change (hotter and drier winters), meriting focused future conservation.

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SUPPLEMENTAL MATERIALS

Appendix S1: *Oxalis* species with corresponding physiological, morphological and phenological data. The seed, seedling and combined seed-and-seedling datasets are recorded on three separate sheets. (Provided electronically: Excel workbook)

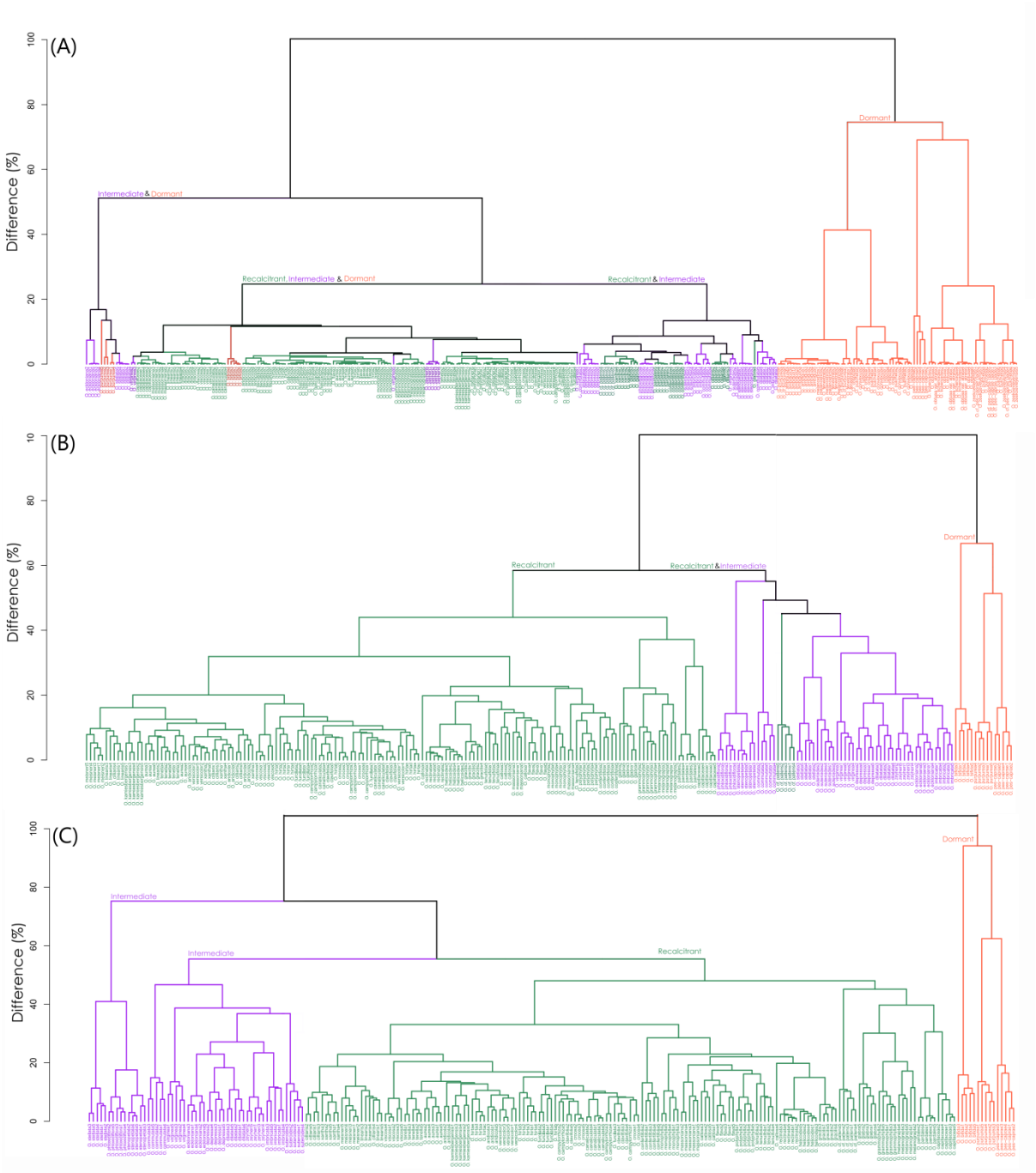
Appendix S2: Cluster dendrograms indicating the percentage difference between *Oxalis* species, based on discrete and continuous seed and seedling morphological and developmental data. Data from the seed (a), seedling (b) and combined seed-and-seedling (c) dataset were analysed. The majority of replicates per species cluster together coherently. Cluster analyses revealed coherent clustering of data into at least three (or four) clusters, supporting three germination strategies. (Included below)

Appendix S3: Individual factor maps of principal component analyses to visualize discrete and continuous *Oxalis* seed (a), seedling (b) and combined seed-and-seedling (c) morphological and developmental data for five replicates per species. (Included below)

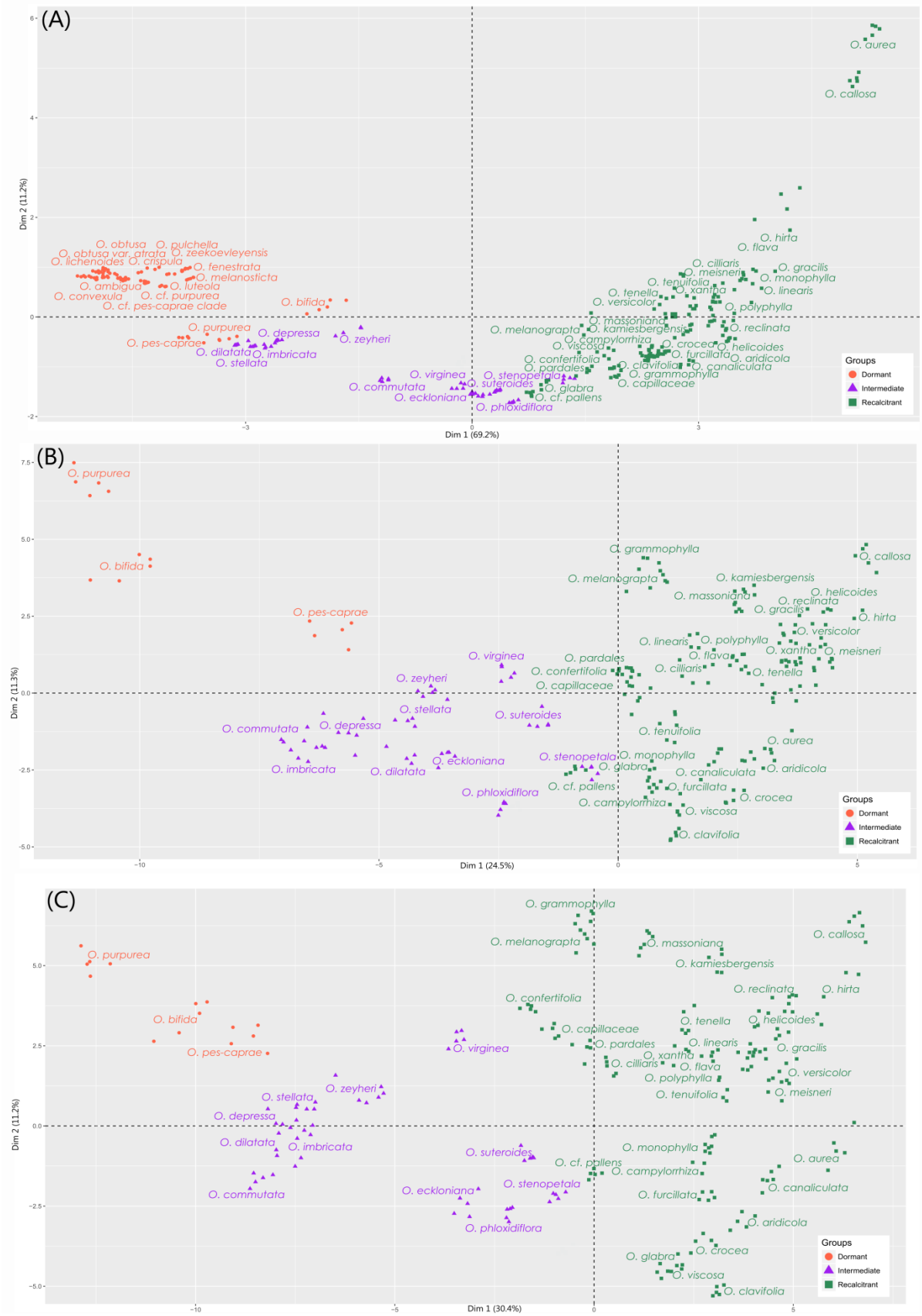
Appendix S4: A composite of three seed physiological traits used to define germination strategies among Cape *Oxalis*. (Included below)

Appendix S5: A key to variable factor names used in Figure 2a-c. Abbreviations correspond to descriptions of seed, seedling and combined seed-and-seedling datasets as indicated on three separate sheets. (Provided electronically: Excel workbook)

Appendix S6: A key to *Oxalis* species names from Figure 3. Group names and codes correspond to abbreviations used in this figure. (Included below)



Appendix S2 Cluster dendrograms indicating the percentage difference between *Oxalis* species, based on discrete and continuous seed and seedling morphological and developmental data. Data from the seed (a), seedling (b) and combined seed-and-seedling (c) dataset were analysed. The majority of replicates per species cluster together coherently. Cluster analyses revealed coherent clustering of data into at least three (or four) clusters, supporting three germination strategies.



Appendix S3 Individual factor maps of principal component analyses to visualize discrete and continuous *Oxalis* seed (a), seedling (b) and combined seed-and-seedling (c) morphological and developmental data for five replicates per species.

Appendix S4: A composite of three seed physiological traits used to define germination strategies among Cape *Oxalis*.

***Oxalis* species**	**Trait 1 Desiccation tolerance or sensitivity**	**Trait 2 Number of days to germination**	**Trait 3 Critical seed moisture content (%)**	**Germination strategy**
O. convexula	NA	NA	NA	Dormant
O. ambigua 1	NA	NA	NA	Dormant
O. ambigua 2	NA	NA	NA	Dormant
O. cf. pes-capae clade	NA	NA	NA	Dormant
O. cf. purpurea	NA	NA	NA	Dormant
O. crispula	NA	NA	NA	Dormant
O. fenestrata	NA	NA	NA	Dormant
O. lichenoides	NA	NA	NA	Dormant
O. luteola	NA	NA	NA	Dormant
O. melanosticta	NA	NA	NA	Dormant
O. obtusa	NA	NA	NA	Dormant
O. obtusa var. atrata	NA	NA	NA	Dormant
O. pulchella 1	NA	NA	NA	Dormant
O. pulchella 2	NA	NA	NA	Dormant
O. zeekoevleyensis	NA	NA	NA	Dormant
O. bifida	Desiccation tolerant	9 days	5	Dormant
O. pes-caprae	Desiccation tolerant	5 days	5	Dormant
O. purpurea	Desiccation tolerant	4 days	5	Dormant
O. commutata	Desiccation tolerant	1 day	8	Intermediate
O. depressa	Desiccation tolerant	1 day	8	Intermediate
O. dilatata	Desiccation tolerant	1 day	8	Intermediate
O. eckloniana	Desiccation tolerant	1 day	18	Intermediate
O. imbricata	Desiccation tolerant	1 day	8	Intermediate
O. phloxidiflora	Desiccation tolerant	1 day	8	Intermediate
O. stellata	Desiccation tolerant	2 days	8	Intermediate
O. stenopetala	Desiccation tolerant	1 day	15	Intermediate
O. suteroides	Desiccation tolerant	1 day	10	Intermediate
O. virginea	Desiccation tolerant	1 day	10	Intermediate
O. zeyheri	Desiccation tolerant	2 days	18	Intermediate
O. aridicola	Desiccation sensitive	1 day	NA	Recalcitrant
O. aurea	Desiccation sensitive	1 day	NA	Recalcitrant
O. callosa	Desiccation sensitive	1 day	NA	Recalcitrant
O. campylorrhiza	Desiccation sensitive	1 day	NA	Recalcitrant
O. canaliculata	Desiccation sensitive	1 day	NA	Recalcitrant
O. capillaceae	Desiccation sensitive	1 day	NA	Recalcitrant
O. cf. pallens	Desiccation sensitive	1 day	NA	Recalcitrant
O. cilliaris	Desiccation sensitive	1 day	NA	Recalcitrant
O. clavifolia	Desiccation sensitive	1 day	NA	Recalcitrant
O. confertifolia	Desiccation sensitive	1 day	NA	Recalcitrant

O. crocea	Desiccation sensitive	1 day	NA	Recalcitrant
O. flava	Desiccation sensitive	1 day	NA	Recalcitrant
O. furcilata	Desiccation sensitive	1 day	NA	Recalcitrant
O. glabra	Desiccation sensitive	1 day	NA	Recalcitrant
O. gracilis	Desiccation sensitive	1 day	NA	Recalcitrant
O. grammophylla	Desiccation sensitive	1 day	NA	Recalcitrant
O. helicoides	Desiccation sensitive	1 day	NA	Recalcitrant
O. hirta 1	Desiccation sensitive	1 day	NA	Recalcitrant
O. hirta 2	Desiccation sensitive	1 day	NA	Recalcitrant
O. kamiesbergensis	Desiccation sensitive	1 day	NA	Recalcitrant
O. linearis	Desiccation sensitive	1 day	NA	Recalcitrant
O. massoniana	Desiccation sensitive	1 day	NA	Recalcitrant
O. meisneri	Desiccation sensitive	1 day	NA	Recalcitrant
O. melanograpta	Desiccation sensitive	1 day	NA	Recalcitrant
O. monophylla	Desiccation sensitive	1 day	NA	Recalcitrant
O. pardales	Desiccation sensitive	1 day	NA	Recalcitrant
O. polyphylla	Desiccation sensitive	1 day	NA	Recalcitrant
O. reclinata	Desiccation sensitive	1 day	NA	Recalcitrant
O. tenella	Desiccation sensitive	1 day	NA	Recalcitrant
O. tenuifolia	Desiccation sensitive	1 day	NA	Recalcitrant
O. versicolor	Desiccation sensitive	1 day	NA	Recalcitrant
O. viscosa	Desiccation sensitive	1 day	NA	Recalcitrant
O. xantha	Desiccation sensitive	1 day	NA	Recalcitrant

Appendix S6: A key to *Oxalis* species names from Figure 3. Group names and codes correspond to abbreviations used in this figure.

Group	**Code**	**Species**
Group A	A1	*O. obtusa*
	A2	*O. obtusa*
Group B	B1	*O. purpurea*
	B2	*O. bifida*
	B3	*O. pes-caprae*
	B4	*O. pes-caprae*
Group C	C1	*O. commutata*
	C2	*O. commutata*
	C3	*O. dilatata*
	C4	*O. dilatata*
	C5	*O. dilatata*
Group D	D1	*O. virginea*
	D2	*O. suteroides*
	D3	*O. suteroides*
	D4	*O. suteroides*
	D5	*O. suteroides*

Group E	E1	*O. capillaceae*
	E2	*O. capillaceae*
	E3	*O. melanograpta*
	E4	*O. melanograpta*
	E5	*O. melanograpta*
Group F	F1	*O. reclinata*
	F2	*O. helicoides*
	F3	*O. glabra*
	F4	*O. glabra*
	F5	*O. glabra*

Chapter 2 additional notes A: Seed and seedling traits associated *Oxalis* Groups A to F

Group A - Dormant species that possibly require a mandatory dormancy period before germination

This dormant group included species that did not germinate within the first season after shedding. Included species produced capsules containing 25 to 100 seeds per capsule and dried seeds were light (0.03 and 0.07 mg per seed) and small (from 0.59 x 0.65 mm to 1.08 x 1.76 mm). Seeds had lignified and pigmented tegmens with an irregular surface type (clearly visible depressions and crests) and no visible hypocotyl protrusion. All of these seeds contained endosperm and mature seeds had un-pigmented embryos (Figure 3A).

Group B - Dormant species that germinated soon after shedding

These seeds had capsules with 5 to 90 seeds per capsule and dried seeds weighed between 0.03 and 0.91 mg per seed. Seeds had lignified and pigmented tegmens with an irregular surface type (‘deep grooves’ or ‘small bumps’). Seeds had tegmens that were relatively impermeable to water in comparison to intermediate seeds (desiccation or moisture stored conditions did not have any effect on the timing of germination). They contained endosperm, but had green-pigmented embryos (Figure 3B). The hypocotyl emerged within 4 to 22 days, root hairs developed within 5 to 23 days, cotyledons opened within 8 to 30 days and the first foliar leaf emerged within 10 to 29 days after germination. Germination sequence in this group followed the sequence typical of most angiosperms, by which the radicle develops before the plumule (Esau, 1960; Bewley & Black, 1994). These dormant seedlings had relatively narrow hypocotyl widths that ranged from 0.34 to 0.55 mm, relatively long root lengths that ranged from 3.33 to 14.5 mm (measured after cotyledons opened) and relatively long hypocotyl lengths that ranged from 0.58 to 6.12 mm (measured after the foliar leaf matured). All species had elliptical cotyledons, with no or short cotyledon petioles (0 to 1.47 mm) and relatively high abaxial (AB) stomatal densities (11 to 51 stomata/mm ^{2}) on cotyledons that were amphistomatic or hypostomatic.

Group C - Endospermous intermediate seeds

Species from the first sub-cluster of intermediate seeds typically produced 5 to 50 seeds per capsule and dried seeds weighed 0.03 to 0.10 mg per seed. Seeds from this cluster had

lignified and pigmented tegmens with an irregular surface with small crests or smooth surface with a papery texture. Seeds had tegmens that were relatively permeable to water in comparison to dormant seeds. All seeds contained endosperm at shedding and had green-pigmented embryos (Figure 3C). Root hairs developed within 3 to 13 days, cotyledons unfolded within 6 to 10 days and the first foliar leaf emerged within 8 to 19 days after germination. They also followed the classical angiosperm sequence of seed germination. Seedlings had relatively short root lengths (0.54 to 5.15 mm) after the cotyledons opened, and hypocotyl widths (0.26 to 0.64 mm) and lengths (0.12 to 0.59 mm) after the first foliar leaf matured. All species had elliptical cotyledons, relatively short (0.41 to 1.99 mm) or long (2.87 to 3.67 mm) cotyledon petioles and relatively low abaxial (AB) stomatal densities (14 to 28 stomata/mm^2) on cotyledons that were all amphistomatic.

Group D - Exendospermous intermediate seeds

Species from this intermediate sub-cluster had capsules with 20 to 45 seeds per capsule and dried seed weights ranged from 0.10 to 0.12 mg per seed. All species from this group had green-pigmented and non-lignified tegmens with a velvety or smooth texture. Seeds had tegmens that were much more permeable to water in comparison to intermediate Group C seeds. At shedding, all seeds lacked endosperm and had green-pigmented embryos (Figure 3D). Root hairs developed within 2 to 6 days, cotyledons unfolded within 2 to 5 days and the first foliar leaf emerged within 3 to 18 days after germination. The root and first foliar leaf developed simultaneously. Seedlings had relatively long roots (0.42 to 12.041 mm) after the cotyledons opened, and large hypocotyl widths (0.43 to 1.04 mm) and lengths (0.17 to 0.96 mm) after the first foliar leaf matured. All species had elliptical cotyledons, relatively short (0.87 to 2.92 mm) or long (2.99 to 5.23 mm) cotyledon petioles and relatively low abaxial (AB) stomatal densities (14 to 28 stomata/mm^2) on cotyledons that were amphistomatic, except for two species (*O. phloxidiflora* and *O. stenopetala*) that had epistomatic cotyledons.

Group E - Pardales recalcitrant clade

Species produced 5 to 30 seeds per capsule, dried seeds were relatively heavy (1.78 to 3.54 mg per seed), but similar in size (from 1.27 x 1.67 mm to 1.80 x 2.72 mm) to seeds from Group F. Seeds had un-lignified tegmens with a smooth, papery texture. The majority of these seeds had semi-transparent pigmented tegmens (except *O. kamiesbergensis*, which is only very distantly related to the rest of this group (Oberlander et al., 2011) with a transparent tegmen). Seeds contained no endosperm and had green-pigmented embryos (Figure 3E). Root

hairs developed within 2 to 8 days, cotyledons unfolded within 1 to 4 days and the first foliar leaf emerged within 1 to 3 days after germination. As with species from Group D, the root and first foliar leaf developed simultaneously. Seedlings had hypocotyl widths ranging from 0.43 to 1.65 mm. All species had ovate cotyledons that were either sessile or had very short (0 to 0.65 mm) petioles. Four of these species had epistomatic cotyledons, while the remainder had amphistomatic (17 to 27 stomata/mm^2 on AB surface) cotyledons.

Group F - Main recalcitrant group

Species produced capsules with 5 to 25 seeds per capsule, dried seeds were relatively light (0.10 to 0.82 mg per seed), but similar in size (1.11 x 1.41 mm to 2.44 x 3.95 mm) to members of Group E. Seeds from this cluster had un-lignified and un-pigmented tegmens with a smooth surface texture. Seeds contained no endosperm and had green-pigmented embryos (Figure 3F). The sequence of germination was inverted relative to most angiosperms, where the first foliar leaf developed before the root and hypocotyl. Seedlings had hypocotyl widths ranging from 0.52 to 1.44 mm. All species had ovate cotyledons that were either sessile or on short (0 to 1.92 mm) petioles. Fifty per cent of these species had epistomatic cotyledons, while the remainder had amphistomatic (7 to 28 stomata/mm^2 on AB surface) cotyledons.

Interpretation of morphological and phenological traits associated with germination strategies

Seed traits show distinct variation between the three germination strategies. Dormant species produce capsules with many seeds, where each seed is very small (low seed mass). This may be a low risk strategy for dormant species, as seeds have the ability to survive desiccation and can consequently be effectively dispersed through space and time until they encounter favourable germination conditions (Evans & Dennehy, 2005). Recalcitrant species seem to follow the exact opposite strategy, where species produce few seeds per capsule, but with high seed mass, potentially indicating relatively higher investment of resources in these seeds. Typically, large seeds reduce the rate of water loss as they have a lower surface to volume ratio (Berjak & Pammenter, 2008). This would be a beneficial trait, as recalcitrant seeds do not have tegmens or endosperm to help prevent water loss. However, recalcitrant seeds are dispersed and germinate in the wettest time of the year. Species with intermediate seeds are intermediate in number and mass between these two strategies.

Endosperm is the storage tissue that feeds the developing seed and releases hormones to cue germination (Forbis *et al*., 2002). Seeds that are shed with underdeveloped embryos would require endosperm, as observed among the dormant *Oxalis* seeds that did not germinate within the first season after shedding (Group A). Unlike these seeds, all other desiccation tolerant dormant (Group B) and intermediate (Group C) seeds that contained endosperm at shedding had green-pigmented embryos. These embryos would be metabolically active and have the potential of relatively rapid germination under favourable conditions. These seeds also have endosperm storage tissue, which can release ABA hormones to suppress germination (Koornneef *et al*., 2002; Finch-Savage & LeubnerMetzger, 2006). However, it may be that dormant species (Group B) must germinate within a few days to weeks, or they will lose viability. Exendospermous intermediate (Group D) and all recalcitrant (Groups E and F) seeds were shed with well developed, green-pigmented embryos that filled the entire volume of the seeds and no visible endosperm. As these embryos are ready to germinate upon release, these species do not have to invest resources into the production of endosperm tissue.

Dormant and endospermous intermediate seeds (Groups A to C) are capable of desiccation tolerance and are able to undergo periods of dormancy, during which they need to be protected from unfavourable environmental conditions. These seeds typically have lignified and pigmented tegmens with an irregular surface with depressions and crests. Tegmen lignification would prevent water loss (low water permeability) and protect seeds against predators or microbial decay. Tegmen pigmentation is most likely a by-product of lignification (Kannenberg & Allard, 1964), but could also prevent UV-radiation damage to the seed. UV-light exposure could cause seeds to germinate prematurely or more rapidly than normal, which would be detrimental to developing seedlings (Noble, 2002). It is possible that the irregular tegmen surface (with crests and depressions) enlarge the seed surface area. This could aid with water uptake before germination, to break dormancy. Alternatively this could aid with rapid seed dehydration (if seeds are shed during unfavourable conditions). Often dormant seeds are much more likely to survive long periods of desiccation if they are stored at lower seed moisture contents. Recalcitrant species are shed with metabolically active embryos, which have cotyledons that are capable of photosynthesis (pre- and post-shedding from the capsule) (Maciejewska *et al*., 1974; Ry¢ *et al*., 1980; Ruuska *et al*., 2004). Recalcitrant species have thin and transparent tegmens, would allow UV-light to reach the embryos while seeds are still in their capsules, allowing embryos to photosynthesise and develop rapidly. Thin and transparent tegmens with stomata have previously been described

among angiosperm seeds (Macloskie, 1884; Corner, 1976; Berjak & Pammenter, 2008), which would allow gas exchange of the metabolically active seed to take place. Again, the well-developed embryos of intermediate seeds (Group D) are protected by a semi-lignified and pigmented tegmen. We found that these semi-lignified tegmens were relatively more water permeable than the lignified tegmens of Groups B and C. We would also expect to find that these tegmens (especially the 'velvet' type) would allow gas exchange of the metabolically active embryo while it is still in its capsule. We also suggest that the pigmented tegmens associated with recalcitrant species from Group E would provide desiccation resistance, protection from predation and could aid in UV-protection of the developing embryo.

Dormant and intermediate species (Groups B to D) had elliptical cotyledons. The cotyledon petioles of dormant species' seedlings are often shorter that those of most of the intermediate seedlings. Often these species (Groups B to D) had stomata on both cotyledon surfaces, and it is possible that these cotyledons are adapted to maximize their photosynthetic capacity by ensuring that cotyledons are positioned in well-ventilated areas, where both surfaces are exposed to the atmosphere – allowing high rates of gas exchange that promotes rapid uptake of nutrients and rapid vegetative growth. Group E recalcitrant species had elliptical, sessile cotyledons. The majority of recalcitrant species (Group F) had ovate and fleshy cotyledons with stomata on the adaxial (AD) surface only. It seems as if a relatively large proportion of seed resources had been invested to 'build' such large cotyledons and we suspect that these cotyledons are capable of more rapid photosynthetic rates (especially with AD located stomata for recalcitrant species that are growing in the wet winter months). Higher photosynthetic rates would enable more rapid seedling establishment and growth. Cotyledon petioles of recalcitrant seedlings are either short or absent. Often species with sessile cotyledons have AD located stomata, which would allow sufficient exposure of the photosynthetic surface (AB located stomata would be too close to the soil surface to allow efficient gas exchange). Brink (2017) suggested that cotyledon shape aided with the shedding of the tegmen. It is also possible that the cotyledons shape is non-adaptive, so a developmental artefact determined by the embryo structure. Embryos of dormant *Oxalis* seeds have a larger proportion of root tissue relative to the undeveloped cotyledons, while approximately 90% of the recalcitrant seed volumes are filled with cotyledon tissue. The constraints formed by the tegmen and testa during seed development would then force ovate-shaped cotyledons.

Dormant species (Group B) have seedlings that germinate with their hypocotyl emerging from the seed, followed by substantial root growth and then the development of root-hairs. These species form long roots with a long and thin hypocotyl. Cotyledons only unfold once the root and root-hairs are well-established. The first foliar leaf develops and matures at a much later stage. This sequence of development is similar to the classical sequence of development documented among the majority of angiosperm species (Esau, 1960). Intermediate species (Groups C and D) display various sequences of development where either the hypocotyl or root hairs emerge from the seed. The cotyledons and foliar leaves of species from Group D unfold more rapidly than in members from Group C. Overall, species from Group C follow a very similar pattern of germination to Group B, except that seedlings reach maturity much sooner. Species from Group D seem to display a simultaneous sequence of development where the roots and foliar leaves develop at the same rate. These species have relatively shorter hypocotyls and roots than dormant species. Group E recalcitrant species display root-first germination. The hypocotyl and root hairs emerge and develop, followed by cotyledons unfolding and the development and maturation of the first foliar leaf. The majority of recalcitrant species (Group F) display a strategy of inverse germination, where cotyledons and the first foliar leaf develop rapidly and appear to sustain rapid growth of the seedling, until the hypocotyl, root-hairs and roots subsequently emerge. This is a remarkable phenomenon where seedlings are capable of rapid growth and development without well-established roots to supply the seedling with nutrients. Many of these recalcitrant species produce large amounts of mucilage upon germination. Preliminary investigation of microbes within the mucilage (under sterile control and various experimental conditions) revealed the presence of both bacteria and fungi. Subsequent research is aimed at investigating potential associations between recalcitrant *Oxalis* species and mucilage-dwelling microbes.

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Chapter 2 additional notes B: Descriptions of inverse (foliar-leaf first) germination among recalcitrant *Oxalis*

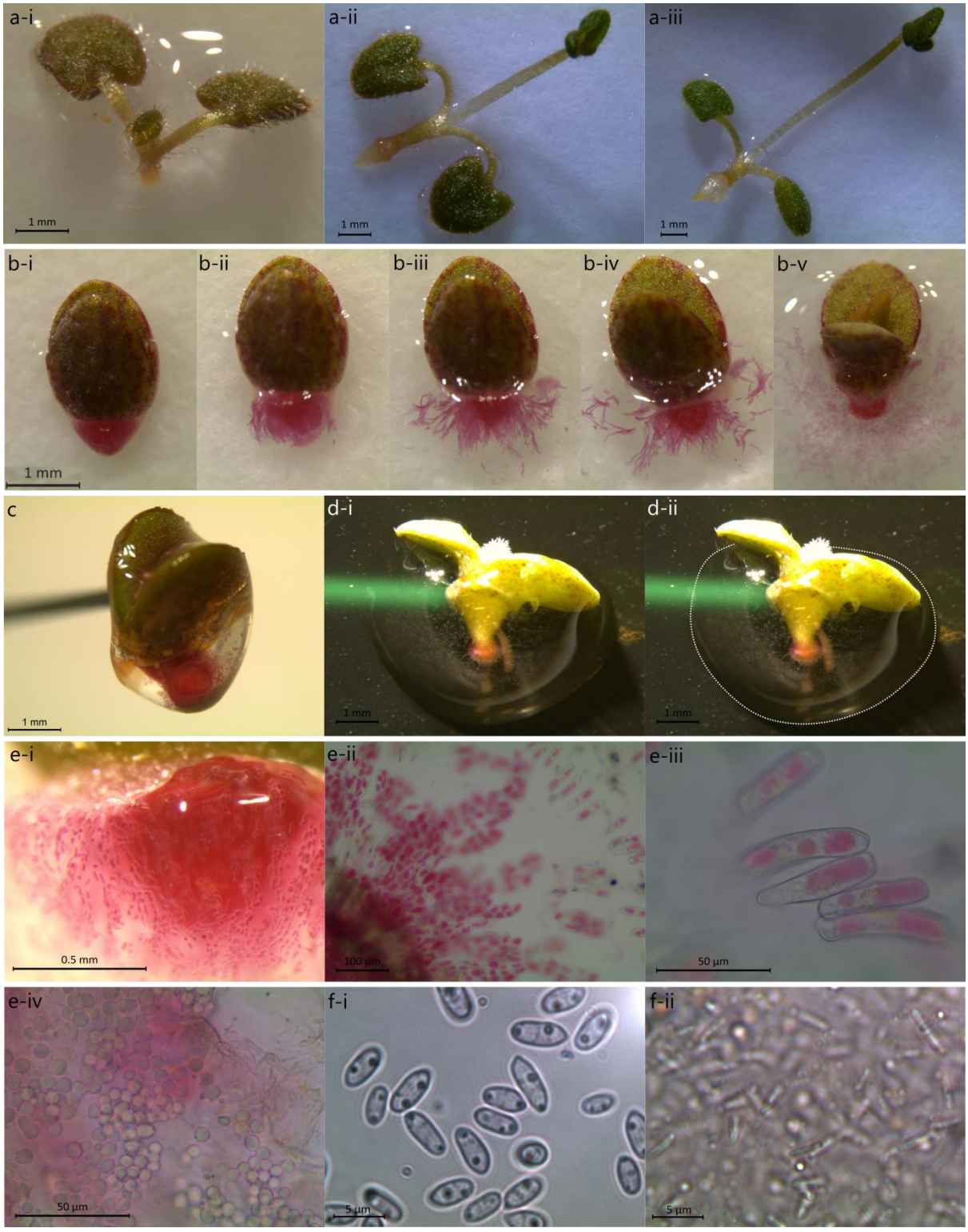
Recalcitrant *Oxalis* L. seeds do not contain any visible endosperm upon shedding and embryos are well-developed and green-pigmented, filling the entire volume of each seed. These seeds are metabolically active when shed, which enables them to germinate, establish and reach maturity much more rapidly than dormant seeds (Kermode and Finch-Savage, 2002).

We have documented various morphological and phenological traits associated with the germination and development of seedlings for 28 recalcitrant *Oxalis* species (Group F, as defined in Chapter 2). Cotyledons unfolded within 1 to 5 days after germination, root hairs developed after 2 to 6 days, the first foliar leaf emerged after 2 to 5 days and roots emerged within 2 to 15 days after germination. The majority of these recalcitrant species displayed a strategy of inverse germination relative to other angiosperm seedlings, where cotyledons and the first foliar leaf develop rapidly, relative to the hypocotyl, root hairs and roots that subsequently emerge (Additional Figure 1a). This is a remarkable phenomenon where seedlings are capable of rapid growth and development temporarily without well-established roots to supply the seedling with nutrients.

Another interesting observation was that 70% of these recalcitrant *Oxalis* species (and a few intermediate species) has seedlings that produced large amounts of mucilage around the base of the hypocotyl, usually within 30 minutes to 24 hours after germination (Additional Figure 1b-d). Many of these species had a bright red or pink hypocotyl that appeared to shed its cells (single cells or clusters of cells) into the mucilage (Additional Figure 1e). Pilot studies revealed that this seedling-secreted mucilage had a pH that ranged from neutral (pH 7) to highly acidic (pH 3) (Dreyer *pers comm., pers obs*.). The production of mucilage, and acidic mucilage in particular, would be regarded as a costly carbon sink to young *Oxalis* seedlings during this vulnerable stage of their development. We suspect that the mucilage secreted by developing recalcitrant seedlings could either include growth promoting endophytes and/or serve as a potential attractant to plant growth promoting micro-organisms from the soil environment.

Pilot studies revealed the presence of culturable bacteria and fungi isolated from the mucilage of recalcitrant seedlings that were surface sterilized and germinated under sterile laboratory

conditions (Additional Figure 1f). We suspect that the mucilage and associated microbes have a vital role to play in nutrient acquisition, seedling establishment and unusual inversed sequence of germination associated with recalcitrant *Oxalis*. Given these intriguing observations and preliminary findings, the focus of this dissertation thus now shifts towards documenting and understanding the diversity and role of microbial associations with Cape *Oxalis*.



Additional figure 1: Inverse germination and unique biological traits associated with recalcitrant *Oxalis* seeds and seedlings. a) Inverse (foliar-leaf first) germination of *O. clavifolia* Sond. one (i), three (ii) and five (iii) days after germination. b) *O. kamiesbergensis* T.M. Salter producing mucilage, photographed at 20 minute intervals after release from the seed capsule. Mucilage produced within one hour of germination of *O. callosa* (c) and *O.*

helicoides T.M. Salter (d-i). A replicate photo of *O. helicoides* (d-i) was included, with a white dotted-line to indicate the large amount of mucilage, relative to the size of the seedling (d-ii). (e) The red hypocotyl (i) and hypocotyl cells shed into the mucilage (ii, iii) of *O. reclinata* Jacq., as well as unidentified structures observed in the mucilage (iv). (f) Unidentified microbial organisms sampled from the seedling-mucilage produced by *O. recticaulis* Sond. (i, ii).

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Declaration by the candidate

With regards to Chapter 3 (Choosing your companions: endophytes in vegetative and reproductive organs of Cape *Oxalis*), the nature and scope of my contribution were as follows:

Nature of contribution	Extent of contribution (%)
Isolation, culturing and identification of bacterial and fungal endophytes associated with *Oxalis* host plants	100
Data analysis, interpretation and manuscript preparation	80

The following co-authors have contributed to Chapter 3:

Name	e-mail address	Nature of contribution	Extent of contribution (%)
Francois Roets	Copyright	Provided guidance, especially in terms of data analysis, and edited the manuscript	5
Guy F. Midgley	Copyright	Provided guidance and edited the manuscript	5
Kenneth C. Oberlander	[redacted]	Provided guidance, especially in terms of data analysis, and edited the manuscript	5
Léanne L. Dreyer	Copyright	Provided guidance, funding and edited the manuscript	5

Signature of candidate: .....……

Declaration by co-authors:

The undersigned hereby confirm that

1. The declaration above accurately reflects the nature and extent of the contributions of the candidate and the co-authors to Chapter 3.
2. No other authors contributed to Chapter 3 than those specified above.
3. There are no conflicts of interest relevant to Chapter 3 of this dissertation.

Signature	Institutional affiliation	Date
Francois Roets	Stellenbosch University	December 2018
Guy F. Midgley	Stellenbosch University	December 2018
Kenneth C. Oberlander	University of Pretoria	December 2018
Léanne L. Dreyer	Stellenbosch University	December 2018

Chapter 3 Choosing your companions: endophytes in vegetative and reproductive organs of Cape *Oxalis*

ABSTRACT

Rationale: Plant-endophytic microbial interactions often enable host plants to overcome various biotic and abiotic stressors. The Cape Floristic Region is globally renowned for its extremely diverse flora that flourishes on severely nutrient-depleted soils in a strongly seasonal climate. It is likely that many Cape plants depend on microbes for survival, but very little is currently known about the microbiome associated with Cape plants. We aimed to study intra-plant, intra- and inter-species, and inter-site microbe richness and community composition in this understudied system.

Methods: We studied the bacterial and fungal endophytes associated with *Oxalis,* the most species-rich geophytic Cape genus. Culturable endophytes were isolated from surface-sterilized vegetative and reproductive plant organs for six host species at three locations. Colonies of microbes on various artificial media were morphotyped, enumerated and identified using sequence data. Putative endophyte identities were used to research possible functional roles of known plant endophytes as described in the literature.

Key results: Overall, 46 bacterial and 39 fungal morphotypes were associated with *Oxalis* host plants. Our results confirm that location, host species and plant organ, as well as interactions between these variables, influence the richness, isolation frequency and the structure of bacterial and fungal communities associated with *Oxalis* plants. The most common bacterial endophytes included members from the genus *Bacillus* - a group well-known for various plant-growth promoting properties. A surprisingly diverse collection of bacterial and fungal endophytes were present in *Oxalis* bulbs and seeds, which was fairly consistent between different locations and host species.

Main conclusions: *Oxalis* hosts a large suite of endophytic microbes whose numbers and composition change according to the surrounding environment. However, assemblages of putative plant growth-promoting and nitrogen-fixing *Bacillus* were ubiquitous from all host plants, regardless of sampling locations, *Oxalis* host or organ. They were also commonly found in the reproductive and vegetative propagules of all hosts. If communities of these *Bacillus* endophytes offer benefits to *Oxalis* hosts, these data suggests that vertically-

inherited mutualisms could be impacting plant survival in nutrient-depleted environments such as the Cape.

Keywords: *Bacillus*, endophytic bacteria, endophytic fungi, Cape Flora, endosphere, microbiome, *Oxalis*, rhizosphere

INTRODUCTION

A great diversity of plant-microbial symbioses has been identified, and these interactions profoundly influence plant biology and physiology (Martin *et al*., 2007), biodiversity (Nannipieri *et al*., 2003), ecosystem functioning and structure (Loreaul *et al*., 2001; Miyambo *et al*., 2016) and patterns of evolution (Poole *et al.*, 2003). The majority of soil micro-organisms live in the environment directly surrounding plant roots (rhizosphere) (Vogel *et al*., 2009). Some of these rhizosphere micro-organisms are in associative relationships, where they closely interact with plant roots, while other micro-organisms have the ability to colonize the root and spread throughout the host organs via the xylem. These can reside in the intercellular spaces of the plant tissue (most often bacteria) or penetrate individual cells (most often endomycorrhizal fungi) (Rodriguez *et al*., 2009).

Plant-microbial interactions are typically classified as being beneficial, neutral or harmful towards the host plant (Dobbelaere *et al*., 2003). Beneficial interactions can further be divided into three categories, namely interactions that are responsible for mineral and nutrient uptake and supply to the plant (such as free-living or endophytic nitrogen-fixing bacteria), interactions that prevent the growth or activity of plant pathogens and therefore indirectly sustain plant growth (such as biocontrol agents) and interactions that directly influence plant growth through the production of phytohormones (such as plant growth promoting rhizobacteria or mycorrhizal fungi) (Azcon and Ocampo, 1981; Whipps, 1990; Brimecombe *et al*., 2007). Depending on micro-organismal life strategy, associations can be classified as being either obligate or facultative (Hardoim *et al.*, 2008).

Plant endophytes are generally thought to constitute a subset of the micro-organisms present in the rhizosphere (Bulgarelli *et al*., 2013). The majority of terrestrial plants (at least 80%) are known to form mutualistic mycorrhizal symbioses with fungi (Petrini, 1986). Due to nutrient exchanges between plants and fungi and the interaction between fungi and other micro-organisms present in the rhizosphere, these interactions improve plant growth and development. These interactions increase the host plants' tolerance to biotic and abiotic stress.

The fungi, in return, mostly acquire access to a carbohydrate source and a protected environment in which to complete their life cycle (Smith and Read, 1997).

The diversity, frequency and population density of endophytic species depend on the edaphic and climatic conditions of the rhizosphere, the diversity of niches available within the host tissue and the amount of competition between endophytic micro-organisms (Sieber and Grunig, 2013). To date, most studies have focused on microbial communities from the rhizosphere and plant roots (due to the strong influence of/association with the rhizosphere). Colonization of bacteria from the rhizosphere into the host root tissue happens due to stochastic and deterministic factors through a series of colonization events. A small portion of bacteria that colonise plant root cortex have the ability to spread via the apoplast of xylem vessels and colonise the specific plant tissues or organs (Chi *et al.*, 2005; Hardoim *et al.*, 2008). The phyllosphere (leaf surfaces) is another common entry point for fungal spores and endophytic bacteria (Hallman *et al*., 1997). In a few rare cases, interactions between the bacteria and host plant may lead to permanent associations where bacterial gene expression occurs in the host plant, where beneficial proteins/enzymes are transferred to the host (Hardoim *et al*., 2008) or where endophytes are vertically transmitted (Compant *et al*., 2011; Truyens *et al*., 2015).

Plants are known to exert strong selection pressures on which endophytes are passed on to their reproductive and vegetative propagules (Hallmann, 2001). To date, the most comprehensive reviews have indicated the majority of all fruits and seeds of angiosperms not to contain any endophytes (Hallmann, 2001; Compant *et al.*, 2010; Truyens *et al*., 2015). Endophytes capable of passing through these selection barriers have to possess highly specialized physiological traits, in order to ensure successful colonization and establishment in reproductive organs (Compant *et al.*, 2010). These endophytes often require cell motility in order to move throughout host tissue to reach seeds, require the ability to form endospores and/or have amylase activity to use stored starch in seed endosperm (Mano *et al*., 2006; Compant *et al*., 2011). As seeds mature, endophytes are exposed to rapidly changing physiological conditions; therefore endospores would be a suitable form of protection (Mano *et al*., 2006; Compant *et al.*, 2011; Kane, 2011). To date very few studies have assessed the biodiversity of seed endophytes, but the majority of known seed endophytes includes members from the bacterial genera *Bacillus* Cohn, *Pseudomonas* Migula and *Rahnella* (Mandt and Hinkle, 1976; Misaghi and Donndelinger, 1990; Barac *et al*., 2004; Cankar *et al*., 2005; Okunishi *et al*., 2005). Most of these endophytes are a small subset of rhizosphere and

plant endophytes (Mundt and Hinkle, 1976; Massol-Deya *et al*., 1995; Cankar *et al.*, 2005; Compant *et al.*, 2008, 2010; López-López *et al*., 2010), however, not much information is currently available on which specific endophytic species are able to colonise plant reproductive organs (Compant *et al.*, 2011). A limited number of studies have focussed on microbes of plant clonal propagules such as bulbs, including pathogenic bacteria and fungi from garlic bulbs (Cui *et al*., 2008, Deng *et al.*, 2012), fungal contaminants *Fusarium* Link, *Penicillium* Link and *Alternaria* Nees from lily bulbs (Altan *et al*., 2010) and two saprophytic strains *Rahnella* M.L. and *Erwina* Winslow *et al*. from hyacinth bulbs (Jafra *et al*., 2008). However, to our knowledge there are no reports of any beneficial endophytic bacteria or fungi isolated from bulbs.

The overwhelming majority of published research on plant endophytes has focused on agricultural or model plant species (Bulgarelli *et al.*, 2012; Lundberg *et al*., 2012; Peiffer *et al.*, 2013), despite growing recognition that microbial associations are ubiquitous across all terrestrial plants (Dastogeer and Wylie, 2017). The list of known plant hosts increases yearly and it has been predicted that all seed plants accommodate at least one endophytic species (Lewis, 1985; Rodriguez *et al*., 2009; Nair and Padmavathy, 2014). However, the diversity of endophytic communities in natural ecosystems remains largely unexplored (Hardoim *et al*., 2012; Carrell and Frank, 2014; Miyambo *et al*., 2016).

The unique biogeographic region located at the south western tip of the African continent, the Greater Cape Floristic Region (Cape), is globally renowned for its extremely species-rich and diverse flora (Myers *et al.*, 2000; Manning and Goldblatt, 2012). This region is also characterised by nutrient-depleted soils, with some of the lowest nitrogen and phosphorus levels measured globally (Specht and Moll, 1983). These elements limit plant productivity, but symbiotic plant–microbe interactions can help overcome these limitations (Hardoim *et al.*, 2008). There is growing evidence that many Cape plant species harbour unique rhizospheric and endophytic micro-organisms (Slabbert *et al*., 2010; Cowan *et al*., 2013), although very little is known about micro-organismal community composition within and between these plant species in general (Waterman *et al.*, 2011). Symbiotic associations between Cape legumes and nitrogen-fixing rhizobia and various Cape plants and arbuscular mycorrhizal fungi have been studied (Serpent, 2001). To date AM fungal associations have been documented in several Cape plant families, including geophytes of the families

Hyacinthaceae, Iridaceae, Orchidaceae and Oxalidaceae, but endophytic fungi were isolated from roots only (Allsopp and Stock, 1994, Waterman *et al*., 2011).

The genus *Oxalis* is species-rich in southern Africa (*ca*. 230 species (Dreyer *et al*., 2017)) and occur across a vast range of environments, but is concentrated in the nutrient poor and/or drought prone habitats of the Cape (Mucina and Wardell-Johnson, 2011). All southern African *Oxalis* are characterized by the presence of a subterranean bulb, from which seasonal above-ground stems emerge annually (Oberlander *et al*., 2009). Unlike most other Cape lineages, *Oxalis* species emerge, flower and fruit during the predictably wet winter months, senescing entirely to a new bulb during dry summers (Salter, 1944).

Globally, a total of 14 bacterial and two fungal endophytes associated with 11 *Oxalis* species have been reported. Associations between arbuscular mycorrhizal fungi, *Glomus tenuis* Greenall, and seven global *Oxalis* species from China and Europe have been described (Harley and Harley, 1987; Fontenla *et al*., 1998; Yamato, 2004). Reportedly these associations enable better root proliferation and an increased ability to absorb phosphates from the soil (Farley and Fitter, 1999; Turnau *et al*., 1999). Another study identified bacterial endophytes with known plant growth promoting properties in *O. corniculata* L. from Pakistan (Mcinroy and Kloepper, 1995; Xing *et al*., 2006). The authors proposed that the identified endophytes would enhance the solubilization of phosphates and the production of ammonia (Mufti *et al*., 2015). The only report of a bacterial endophyte associated with the roots of a southern African species, *O. pes-caprae* L. (a well-known invasive species), was sampled in its invaded range in Turkey (Sahin, 2005).

The range of plant-associated symbionts documented across angiosperms is increasing rapidly, and their importance in symbiotic interactions with their hosts is becoming more evident (Beattie, 1995; Hallmann *et al*., 1997). It is therefore likely that this may hold true for the southern African *Oxalis* radiation as well. This study aimed to survey the diversity of bacterial and fungal endophytes associated with phylogenetically representative Cape *Oxalis* species in order to assess intra-plant, inter-species and inter-site richness, isolation frequency and community composition. A subsidiary aim was to document endophyte diversity in vegetative and reproductive propagules, as potential vertically-transmitted symbionts. We also aimed to identify ubiquitous endophytes in order to begin to understand their influences on host plants, as described and documented in available literature.

MATERIALS AND METHODS

Sampling design and sterilization protocol

Endophyte richness was assessed by implementing culture-dependent approaches. Six phylogenetically representative *Oxalis* species (*O. glabra* Thunb., *O. hirta* L., *O. obtusa* Jacq., *O. pes-caprae* L., *O. purpurea* L. and *O. tenuifolia* Jacq.) were sampled from three locations (Malmesbury [-33.481121, 18.753625], Stellenbosch [-33.932358, 18.874571] and Tulbagh [-33.311688, 19.096747]) in the Western Cape Province, South Africa (Cape Nature Conservation Board Permit No. 0028-AAA088-00243). *Oxalis pes-caprae*, *O. purpurea* and *O. obtusa* are dormant-seeded species (Salter 1944), while *O. glabra*, *O. hirta* and *O. tenuifolia* are recalcitrant-seeded species that display inverted germination where seedling foliar leaf development precedes root development (Jooste *et al*., 2018; Chapter 2). Five individuals of each species (at least 10m apart), with no external signs of microbial infections (asymptomatic), were collected at each location during May-June of 2016 and 2017. Plants were dug out with minimal disturbance to the below-ground organs and all excess soil was shaken off, until no soil was visible on roots and bulbs. Before samples were processed for endophyte isolation, plant roots were gently washed in 5 ml sterile water in order to obtain rhizosphere samples. Hereafter roots, bulbs, stems/rhizomes (depending on the above-ground growth forms of species: *O. glabra*, *O. hirta* and *O. tenuifolia* have above ground stems; *O. pes-caprae*, *O. purpurea* and the sampled populations of *O. obtusa* do not), leaves and seeds of all plants were aseptically separated using a scalpel and individually surface sterilized. For surface sterilization, samples were washed in a 33% dilution of household bleach (±5% sodium hypochlorite) for 1 minute and 75% ethanol for 1 minute, interspersed with three one-minute washes in sterile water. As sterilization controls, a few additional samples of each of the plant organs were dabbed onto bacterial plate count agar (Biolab, Merck) and malt extract agar (Sigma-Aldrich), and incubated for 7 days at 28°C in the dark - no colonies were detected.

Isolation of bacterial and fungal colonies

Plant organs were manually cut into small segments using a scalpel and material transferred to Eppendorf tubes (to 0.5 mL), with five sterile glass beads and sterile water to 1.5 mL, under sterile conditions. Samples were macerated using a TissueLyser (Qiagen TissueLyser, Retsch MM301) at the Central Analytical Facility at Stellenbosch University. 200 µL each of 1:4 sterile-water-diluted macerate was plated onto three agar media: bacterial plate count agar

(Biolab, Merck), nutrient broth agar (Sigma-Aldrich) emended with 4 g potassium oxalate (BMS Education) per litre of agar and malt extract agar (Sigma-Aldrich). Potassium oxalate was added to nutrient broth agar to re-create the high oxalate content of the host plant (as described by Sahin (2005)). After five days of incubation at 28°C in the dark, all morphologically different colonies (in terms of colour, shape, size and/or texture) were sub-cultured onto fresh plates. This process was repeated after another five days of incubation for each identified morphotype until pure cultures were obtained. All bacterial and fungal morphotypes were recorded and photographed. Three individuals of each bacterial and fungal morphotype per *Oxalis* species from each location were kept to test accuracy of morphotype identification. Each representative morphotype was divided, with 50% used for DNA extraction and sequencing, and the remainder stored in 50% glycerol in cryostorage (-80°C).

DNA extraction, amplification and sequencing

To assess accuracy of endophyte morphotyping, three replicates from 25 different bacterial and 15 different fungal morphotypes were sequenced, with the expectation that the DNA sequences of each morphotype triplet would be identical. Bacterial and fungal colonies isolated from seeds and bulbs (reproductive propagules) were prioritized for sequencing in this study. DNA was extracted following a modified 2X CTAB protocol (Doyle and Doyle, 1987). DNA quality was assessed and quantified using a NanoDrop^{TM} (ThermoFisher Scientific). Amplification and sequencing of the 16S rRNA region for bacteria used universal bacterial primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-ACCTTGTTACGACTT-3') (Weisburg *et al*. 1991). PCR amplification reactions were performed using 25 µL reaction mixtures consisting of 9 µL dH_2O, 2.5 µL $MgCl_2$ (25mM), 0.25 µL of each primer (10µM), 12 µL KapaTAQ (KM1000, Kapa Biosystems) and 1 µL DNA (300 to 500 ng/µL). PCR thermal cycling conditions were: 94°C (5 min), 30 cycles of 94°C (1 min), 49°C (1 min) and 72°C (2 min), with final extension at 72°C (10 min). Amplification and sequencing of the ITS rRNA region for fungi used universal fungal primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al*., 1990), with thermal cycling conditions: 95°C (3 mins), 40 cycles of 94°C (30 sec), 50°C (1 min) and 72°C (90 sec), with final extension at 72°C (7 mins). PCR products were sequenced at the Central Analytical Facility at Stellenbosch University. Confirmation of base calling in sequence chromatograms was conducted manually in Chromas v. 2.6.5 (http://www.technelysium.com.au). Obtained sequences were compared to GenBank (NCBI) submissions using online BLAST searches.

We referenced our collection of photographs for all sequenced bacterial morphotypes, in order to identify bacterial morphotypes that were not sequenced throughout the remainder of plant organs (roots, stems and leaves) and rhizosphere samples. If morphologically distinct bacterial isolates were encountered (30.5% of our samples were unique to non-bulb or non-seed organs), isolates were labelled as 'unknown bacterium' with a unique morphotype number.

Phylogenetic analyses

The 16S and ITS datasets for endophytic bacteria and fungi sequenced in this study (excluding all duplicate sequences) were supplemented by sequences downloaded from Genbank (three representative BLAST results per *Oxalis* endophyte sequence) as well as three outgroup species per alignment. Sequences were aligned using the embedded ClustalW function in BioEdit v. 7.2.6 (Hall *et al*., 1999) with subsequent manual alignment. Tree samples were generated in MrBayes v. 3.2 (Ronquist and Huelsenbeck, 2003) using the nst=mixed command to average over GTR submodels and a gamma rate correction, for 2 X 10^7 generations under otherwise default settings. Convergence of parameter values and estimated sample sizes were checked in Tracer (v.1.6) (Rambaut *et al.*, 2014) using a 25% burnin. The consensus trees were used to aid species identification (Supplementary Figures 1a-b).

Endophyte richness and composition analyses

In order to assess sampling effort for bacterial and fungal morphotypes, non-parametric species richness (total number of morphotypes detected per sample) was estimated using EstimateS v.9.1.0 (Colwell, 2009). The Incidence-based Coverage Estimator (ICE) (Chazdon *et al.*, 1998) was used as a robust estimator for species richness, and the non-parametric Chao2 and Jacknife2 (Hortal *et al.*, 2006) were used as the least-biased estimators for small sample sizes. The classic method for Chao2 was implemented, as the estimated incidence distribution was less than 0.5.

Generalized Linear Models (GLM's) with Poisson distribution errors were used to assess the effects of three variables (sampling location, host plant species and organ type/rhizosphere sample) on bacterial and fungal richness. GLM's with a binomial error distribution were used to assess the effects of these variables on bacterial and fungal isolation frequency (percentage of plant replicates where one or more morphotypes were detected). All analyses for bacterial

and fungal communities were done separately. Data were analysed in the R statistical environment, version 3.4.1 (R-Core-Team, 2014). AIC criteria were used to select best fitting GLM models with the lowest values, as indicated in the results section. Residual plots did not reveal deviations from normality or homoscedasticity for any of the best fitting models. *Post-hoc* Tukey tests were performed to compare the estimated values for all significant factors using the "lsmeans()" function in the lsmeans package in R (Lenth *et al.*, 2016).

In order to analyse variation in bacterial and fungal community compositions (presence-absence data for bacterial and fungal morphotypes on a per-organ/plant basis), latent variable modelling with Bayesian ordination and regression analyses were conducted in the boral package (Hui, 2016). This model-based approach explains community composition through underlying latent variables that account for residual correlation among species (Warton *et al.*, 2012). It allows visualization of the similarity of communities between samples, by making use of unconstrained ordination of pure latent variable models and constrained ordination with the influence of variables (*e.g.* categorical variables such as sampling location, host plant species, host germination strategy and organ type/rhizosphere sample) (Hui, 2016). Both unconstrained ordination and constrained ordination (with the influence of variables) analyses were performed using binomial distribution and residual plots did not reveal deviations from normality or homoscedasticity for bacterial or fungal datasets (Supplementary Figures 2a-d).

In order to assess the potential roles of variables (and interactions between variables) on bacterial and fungal community compositions (measured as presence/absence data on a per-organ/plant basis), multivariate GLM's were constructed in the mvabund package in R (Wang *et al.*, 2012). Analyses of bacterial and fungal communities were done separately, and all models were fitted using a binomial distribution with a default link function. Explanatory variables included sampling location, *Oxalis* host species and plant organ/rhizosphere. All two-way and three-way interactions between relevant variables were incorporated into models. The anova() function in mvabund was used for multivariate hypothesis testing for all models, in order to determine if bacterial and fungal community compositions varied across locations, *Oxalis* host species and organ types. P-values were calculated using 999 resampling iterations to account for correlation among data (Wang *et al.*, 2012). In order to determine if certain bacterial or fungal morphotypes are more likely to be associated with specific locations, *Oxalis* hosts or organs, the "p.uni=adjusted" argument in the anova

function was implemented. This resampling method allows the computation of adjusted p-values that takes the correlation between response variables into account (Wang *et al.*, 2012).

RESULTS

Observed and estimated richness

Sequencing revealed that 92% of bacterial and 86.7% of fungal replicates were consistent with morphotyping, with all three replicates showing identical 16S/ITS sequences. For the remaining bacterial and fungal replicate sets, one sequence differed by more than 5bp from the other two. This morphotyping error margin (2.6% of bacterial morphotypes and 4.4% of fungal morphotypes incorrectly assigned) indicates high morphotyping accuracy, and is small enough not to substantially affect our conclusions. Overall, 46 bacterial morphotypes (522 individuals) and 39 fungal morphotypes (249 individuals) were isolated from six *Oxalis* host species sampled from 3 locations (Table 1). The highest bacterial and fungal richness was recorded and estimated for sampling Site 3 – Stellenbosch (bacteria Chao2 = 38.49±2.59, Jacknife2 = 41.97; fungi Chao2 = 53.39±14.68, Jacknife2 = 53.39±14.68). The highest bacterial richness was estimated for *O. hirta* (Chao2 = 33.42±8.1, Jacknife2 = 36.83) and highest fungal morphotype richness was estimated for *O. pes-caprae* (Chao2 = 30.52±2.58, Jacknife2 = 34.07).

Table 1: Total bacterial and fungal morphotype richness estimates for microbial assemblages isolated from three sampling locations, six *Oxalis* hosts species and various plant organs and the rhizosphere. Obs spp – Observed species numbers; Chao2 – second order Chao estimator; ICE – Incidence-based Coverage Estimator; Jack2 – second order Jack estimator; SD – Standard Deviation.

	Bacterial morphotype richness				**Fungal morphotype richness**			
Sample	**Obs spp**	**ICE**	**Chao2±SD**	**Jack2**	**Obs spp**	**ICE**	**Chao2±SD**	**Jack2**
Overall	46	46.85	46.25±0.74	47.01	39	44.31	44.59±5.34	50.98
Sampling location:								
Malmesbury	25	25.82	25.5±1.29	27.98	25	26.79	25.85±1.39	27.03
Stellenbosch	37	37.96	38.49±2.59	41.97	34	45.56	53.39±14.68	56.83
Tulbagh	27	28.31	28±0.12	27.03	18	18.83	18.2±0.62	18.03
Oxalis host species:								
O. glabra	29	29.87	29.12±0.44	26.17	14	31.73	31.8±16	30.73
O. hirta	25	29.77	33.42±8.1	36.83	18	25.33	19.65±2.03	22.07
O. obtusa	21	20	20±0.63	18.07	16	20.82	18.97±3.39	23.93
O. pes-caprae	22	23.3	24.97±4.53	27.9	28	33.92	30.52±2.58	34.07
O. purpurea	20	20	20±0.48	19.03	27	20.08	20.94±5.49	25.87
O. tenuifolia	20	22	22±0.43	22	16	20.08	20.94±5.49	25.87
Oxalis glabra organs:								
Rhizosphere	23	32.9	37±12.04	39.39	7	17.89	16.33±9.44	16
Roots	20	34.74	32.83±10.48	37.39	4	6	4.31±0.88	5.99
Bulbs	14	40.33	22.4±7.55	27.79	1	1	1±0.47	2.8

Stems	20	39.02	30.27±8.33	36.58	6	12	7.87±2.72	11.59
Leaves	8	13.53	10.8±3.93	14.4	5	14.33	14.33±9.38	14
Seeds	9	27.91	16±7.67	19	0	0	0	0
Oxalis hirta organs:								
Rhizosphere	22	29.84	24.9±2.93	29.96	12	21.38	14.8±3.22	19.58
Roots	19	82.59	61.47±32.39	43.4	6	1	1±0.47	2.8
Bulbs	12	31.19	25.07±12.4	25.6	2	2.93	2.93±1.91	5.6
Stems	10	17.79	13.11±3.88	17.39	4	11.6	6.8±4.07	9.4
Leaves	9	19.06	12.11±3.88	16.39	7	26.6	26.6±16.05	19.6
Seeds	4	4.6	4±0.23	5	1	1	1±0.47	2.8
Oxalis obtusa rgans:								
Rhizosphere	20	21.85	20.62±1.09	20.76	11	15.23	12.56±2.08	15.98
Roots	17	32.03	21.36±4.23	27.38	6	21	10.67±5.67	14.2
Bulbs	16	38.73	37±18.11	33.2	5	14.33	14.33±9.38	14
Stems	15	32.68	26.2±10.1	29.59	6	22.25	10.67±5.67	14.2
Leaves	10	13.6	10.93±1.5	13.18	6	20	20±12.57	16.8
Seeds	6	7.82	6.47±1.23	8.8	1	1	1±0.47	2.85
Oxalis pes-caprae organs:								
Rhizosphere	18	20.56	19.47±0.97	20.38	16	20.63	14.92±4.08	20.38
Roots	14	16.24	14.93±1.72	17.79	11	20.63	14.92±4.08	20.38
Bulbs	12	15.53	13.4±2.11	16.79	9	42.6	42.6±23.8	25.2
Stems	16	25.58	20.9±5.05	26.19	10	56.93	26.8±15.13	25.4
Leaves	11	13.86	11.7±1.35	13.99	10	21.58	16.53±6.68	20.99
Seeds	9	10.49	9.31±0.88	10.99	7	28.5	14±7.67	17
Oxalis purpurea organs:								
Rhizosphere	17	18.31	18±0.09	16.58	19	39.07	33.56±10.92	39.18
Roots	14	15.88	14.93±1.72	17.79	4	10	5.4±2.44	8.6
Bulbs	12	20.59	16.67±5.2	21.19	6	12	7.87±2.72	11.59
Stems	17	26.11	20.8±3	25.77	11	25.67	17.53±6.25	22.99
Leaves	12	14.44	13.4±2.11	16.79	9	24.75	15.53±6.68	19.99
Seeds	9	16.41	10.4±2.11	13.79	6	23.5	10.67±5.67	14.2
Oxalis tenuifolia organs:								
Rhizosphere	18	21.72	19.87±2.44	23.78	7	9.63	7.56±1.12	9.18
Roots	15	22.11	18.5±3.96	23.39	4	9.6	9.6±6.52	11.2
Bulbs	14	20.68	18.9±5.05	24.19	5	2.93	2.93±1.91	5.6
Stems	14	21.48	16.33±2.74	20.78	2	2.93	2.93±1.91	5.6
Leaves	11	19.5	15.67±5.2	20.19	5	18	7.8±3.93	11.4
Seeds	13	24.21	22.8±9.91	24.8	5	15.5	7.8±3.93	11.4

Bacterial morphotype richness

Bacterial species richness estimators were similar to the observed species richness among samples. This indicated that adequate sampling of bacterial communities was done, so that downstream analyses could be deemed reliable. The best fitting models for bacterial morphotype richness included all tested variables, an interaction between location and host species and an interaction between location and organ type as factors (Table 2). With regards to sampling site effects, the sampling location at Stellenbosch had significantly higher bacterial morphotype richness per host plant (mean=2.01±1.47, z=-1.29, p>0.001) relative to the other two locations (Figure 1-i). In general, host species had similar bacterial richness, while *O. obtusa* and *O. hirta* had significantly lower richness (mean=1.51±1.42, z=-3.04, p<0.001) than to *O. pes-caprae* (mean=2.72±1.38, z=0.08, p<0.001; Figure 1-ii). Bacterial

morphotype richness per plant was low in bulbs, leaves and seeds, significantly higher in roots and stems (mean=2.84±1.39 to 3.53±1.38, z=1.69, p<0.001), and highest among rhizosphere samples (mean=3.64±1.36, z=2.06, p<0.05, Figure 1-iii).

When considering interaction effects between sampling location and *Oxalis* host species on bacterial morphotype richness, *O. hirta*, *O. pes-caprae* and *O. purpurea* had the lowest richness in Tulbagh and the highest richness in Stellenbosch (Figure 1-iv). *Oxalis tenuifolia*, however, displayed an inverse pattern with the lowest richness in Malmesbury and highest richness in Tulbagh. Overall, *O. tenuifolia* from Tulbagh and *O. obtusa*, *O. pes-caprae* and *O. purpurea* from Stellenbosch had significantly higher bacterial richness (mean=3.30±1.46 to 6.54±1.49, z=0.96, p<0.05) relative to other sampled host species from other locations (Figure 1-iv). With regards to location-organ interactions, bacterial diversity in the rhizosphere, roots and stems was lowest in Tulbagh and highest in Stellenbosch (Figure 1-v). Bacterial richness in leaves was highest and richness in bulbs was lowest in Malmesbury. However, bacterial richness in seeds did not differ between sampling locations (Figure 1-v). Overall, bacterial richness was significantly higher among rhizosphere and root samples from Malmesbury and Stellenbosch (mean=3.32±1.48 to 6.85±1.46, z=4.23, p<0.05).

Frequency of bacterial isolation

Bacteria (regardless of morphotype identities) were isolated from the rhizosphere samples of 83 out of 90 samples and endophytes were isolated from various organs from all 90 plants. In order to assess the frequency of bacterial isolation from host plants, each organ type/rhizosphere sample from a host plant was treated as a sampling unit. The best fitting models for bacterial morphotype isolation frequency included all tested variables, an interaction between location and host species and an interaction between location and organ type as factors (Table 2). Without considering interaction effects, the frequency with which bacteria were isolated did not differ between three sampling locations (Figure 2-i), *Oxalis* host species (Figure 2-ii) or organ types (Figure 2-iii). These data indicated that 40 to 100% of sampled host plants (regardless of species or organ types) from all sampling locations hosted bacteria, but plants samples from Stellenbosch had the most consistent isolation frequencies (Figure 2-i). Host species (regardless of location or organ types) with the most consistently high isolation frequencies included *O. glabra*, *O. pes-caprae* and *O. purpurea*, while the greatest variation in bacterial isolation frequency was observed in *O. obtusa* (Figure 2-ii). Root and stem samples (regardless of location or host species) had the most

consistently high isolation frequencies, while the greatest variation was observed among leaf samples (Figure 2-iii).

When considering interaction effects between sampling location and *Oxalis* host species on bacterial isolation frequency, *O. glabra* and *O. pes-caprae* had the lowest frequencies in Tulbagh and higher values (mean=100%) at the other locations (Figure 2-iv). *Oxalis purpurea* had the lowest frequencies in Tulbagh and highest values in Stellenbosch. *Oxalis tenuifolia* had the highest frequencies at Tulbagh (mean=80%). The highest isolation frequency for *O. hirta* was observed in Malmesbury, while the lowest frequency for *O. obtusa* was observed at this location. Bacterial isolation frequencies among plant organs/rhizosphere samples also differed between the three sampling locations (Figure 2-v). Isolation frequencies among seed samples were highest in Malmesbury, relative to the other sampling locations. Isolation frequencies among rhizosphere, stem and leaf samples were lowest in Tulbagh and highest in Stellenbosch, while frequencies among bulbs were lowest in Stellenbosch and highest in Tulbagh.

Table 2: Best fitting Generalised Linear Models for bacterial and fungal morphotype richness and isolation frequency. Predictor variables included sampling location (Location), host plant species (*Oxalis*) and organ type/rhizosphere sample (Organ). Only predictors and interactions included in the best-fitting model at $p<0.05$ are listed.

	Fixed variables	Interacting variables	AIC	Null deviance	Residual deviance
Richness					
Bacterial morphotypes	Location *Oxalis* Organ	Location:*Oxalis* Location:Organ	1831.8	752.5 on 539 df	408.4 on 507 df
Fungal morphotypes	Location *Oxalis* Organ	Location:*Oxalis*	1029.8	651.1 on 539 df	498 on 517 df
Isolation frequency					
Bacterial morphotypes	Location *Oxalis* Organ	Location:*Oxalis* Location:Organ	168.1	144.3 on 107 df	40.2 on 75 df
Fungal morphotypes	Location *Oxalis* Organ	Location:*Oxalis*	327.9	232 on 107 df	117.8 on 85 df

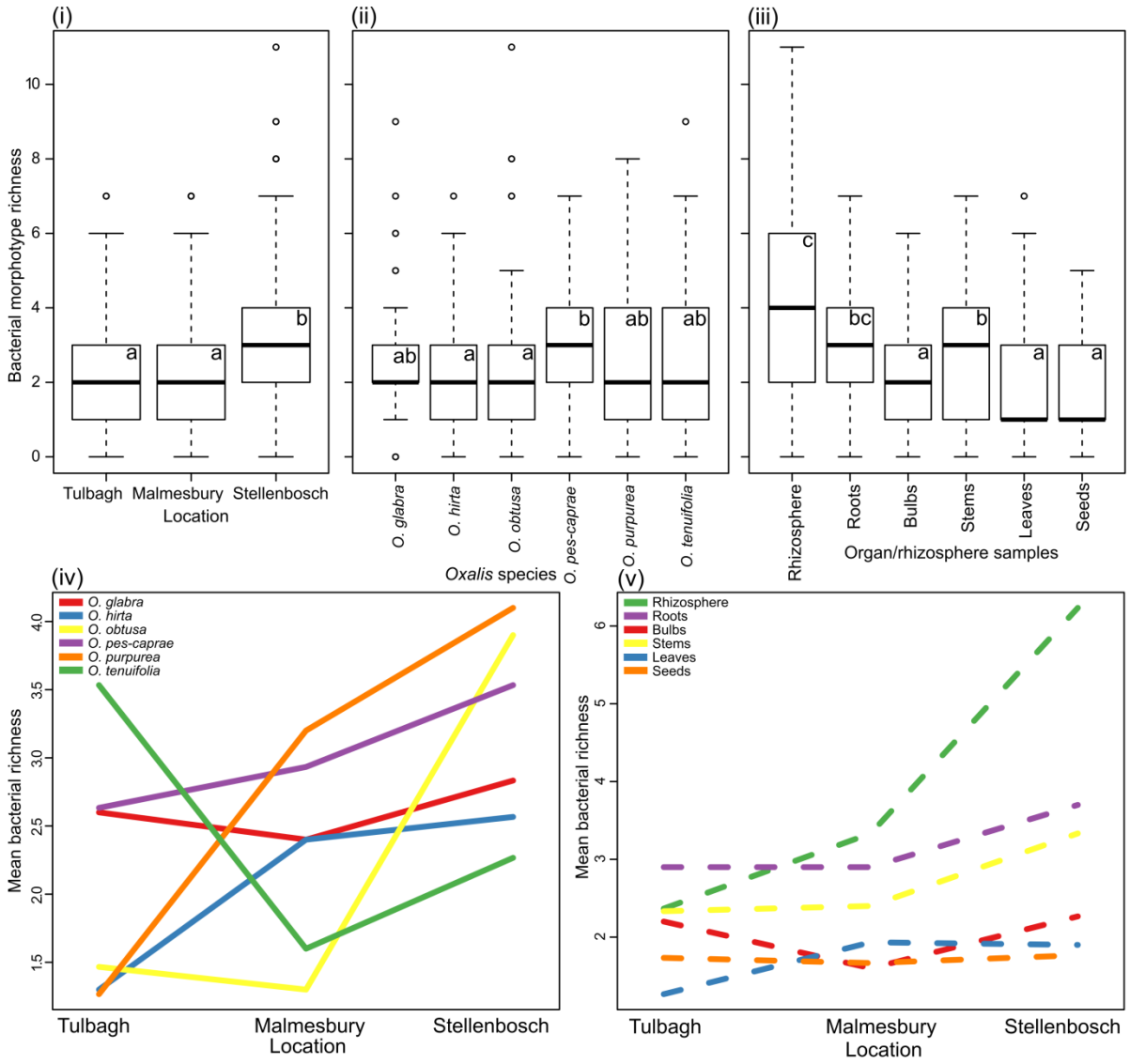

Figure 1: Bacterial morphotype richness associated with *Oxalis* plants. Bacterial richness for sampling location (i), *Oxalis* host species (ii) and organ/rhizosphere sample (iii). Different symbols are significantly different at $p<0.001$. Interaction plots with mean bacterial richness for location and host species (iv) and location and organ/rhizosphere (v).

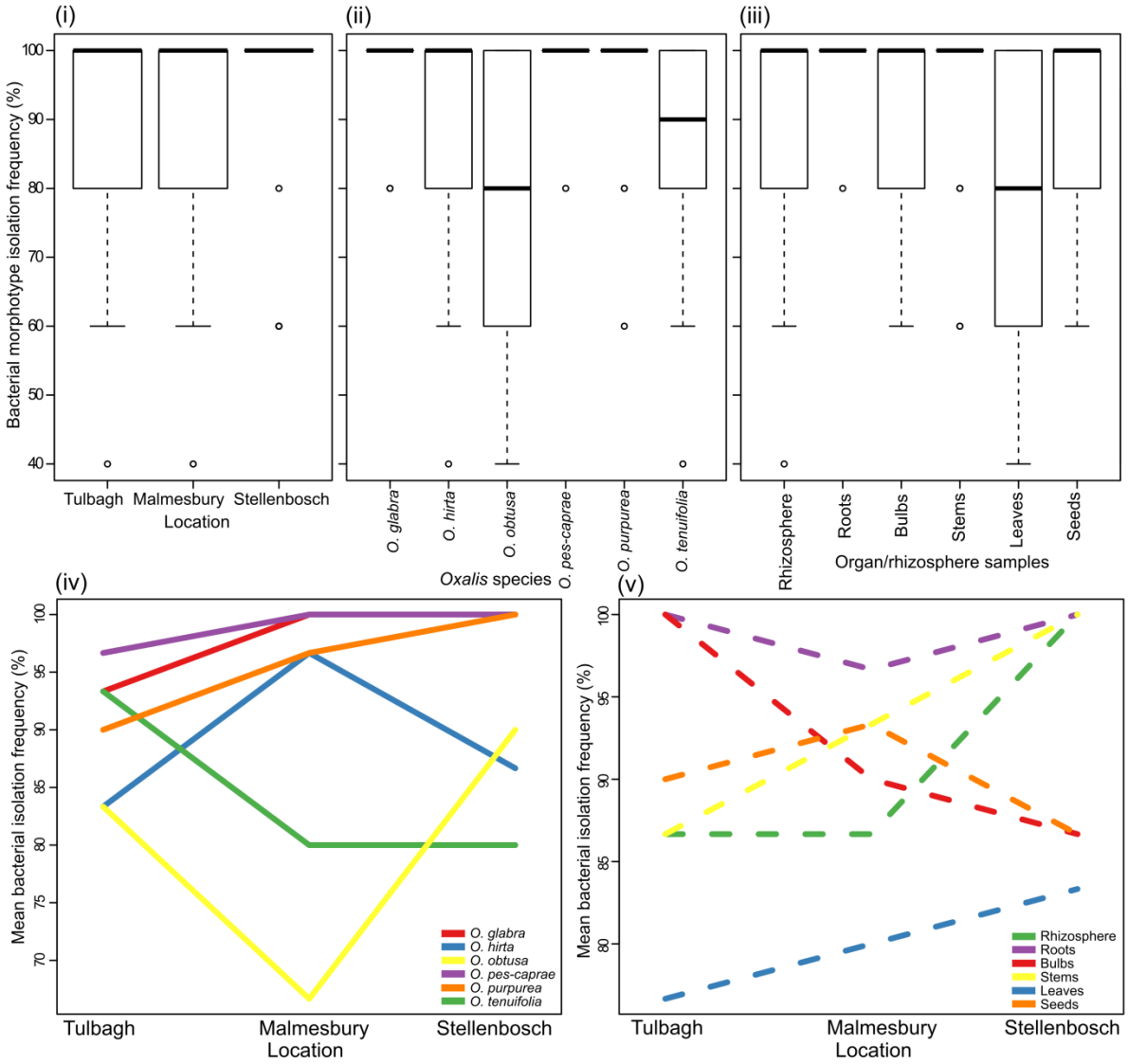

Figure 2: Bacterial isolation frequency associated with *Oxalis* plants. Isolation frequency for sampling location (i), *Oxalis* host species (ii) and organ/rhizosphere sample (iii). Interaction plots with mean isolation frequency for location and host species (iv) and location and organ/rhizosphere sample (v).

Fungal morphotype richness

Fungal species richness estimators were slightly higher than the observed species richness among samples. This indicated potential under-sampling of fungal communities and that the true fungal diversity may be higher. The best fitting models for fungal morphotype richness included all tested variables and an interaction between location and host species (Table 2). Without considering interaction effects, the majority of *Oxalis* host species had similar low levels of fungal richness, relative to *O. pes-caprae* that had significantly higher richness (mean=5.71 ±2.74, z= 0.83, $p<0.05$; Figure 3a-i). Fungal morphotype richness per plant was low in roots, bulbs and seeds, significantly higher in stems and leaves (mean= 0.88±2.20 to

0.99±2.20, z=1.69, p<0.05), and the highest among rhizosphere samples (mean=3.61±1.58, z=2.82, p<0.05, Figure 3a-ii). No significant differences in fungal morphotype richness were detected among sampling locations (Figure 3a-iii). When considering interaction effects between sampling location and *Oxalis* host species on fungal morphotype richness, *O. pes-caprae* and *O. obtusa* had the lowest richness in Tulbagh and the highest richness in Stellenbosch (Figure 3a-iv). *O. hirta* had the lowest richness in Malmesbury and the highest richness at Stellenbosch. *Oxalis tenuifolia* displayed the highest richness in Tulbagh, while the lowest richness among *O. purpurea* was observed at this site. These results indicated fungal richness differed among sampling locations for some *Oxalis* host species. Overall, *O. pes-caprae* from Stellenbosch were the only plants with significantly higher fungal richness (mean=1.32±2.29, z=2.52, p<0.05; Figure 3a-iv) relative to other samples.

Frequency of fungal isolation

Across a total of 90 sampled host plants, fungi (regardless of their morphotype identities) were isolated from the rhizosphere samples of 74.4% of plants and endophytic fungi were isolated from 82.2% of studied plants (therefore 16 host plants did not contain any culturable endophytic fungi). The best fitting models for fungal isolation frequency included all tested variables and an interaction between location and *Oxalis* host species (Table 2). Without considering interaction effects, the majority of *Oxalis* host species had similar fungal isolation frequencies, relative to *O. pes-caprae* with significantly higher values (p<0.05; Figure 3b-i). Fungal isolation frequencies per plant were low in roots, bulbs and seeds, significantly higher in stems and leaves, and highest among rhizosphere samples (p<0.05, Figure 3b-ii). No significant differences in fungal morphotype richness were detected among sampling locations (Figure 3a-iv). These data indicated that 0-100% of sampled host plants (regardless of location or organ types) hosted fungal endophytes, with the highest isolation frequencies observed among *O. pes-caprae* (Figure 3b-i). Rhizosphere samples (regardless of location or host species) had the most consistently high isolation frequencies, while the lowest frequencies were observed among seed samples (mean=10%; Figure 3b-ii). Host plants (regardless of species or organ types) from all sampling locations had consistent isolation frequencies (mean=40%; Figure 3b-iii).

When considering interaction effects between sampling location and *Oxalis* host species on fungal isolation frequency, *O. hirta* and *O. pes-caprae* had the lowest frequencies in Malmesbury and the highest values in Stellenbosch (Figure 3b-iv). *Oxalis obtusa* had the

lowest frequencies in Tulbagh and highest values in Stellenbosch. *Oxalis purpurea* also had the lowest frequencies in Tulbagh, but the highest values were observed in Malmesbury. The highest isolation frequencies for *O. glabra* and *O. tenuifolia* were observed in Tulbagh, while the lowest frequency for *O. glabra* was observed at Stellenbosch and the lowest frequency for *O. tenuifolia* was observed in Tulbagh. This indicated that that sampling location had an effect on fungal isolation frequency in various *Oxalis* host species. Overall, *O. pes-caprae* from Stellenbosch were the only plants with significantly higher fungal isolation frequencies (mean=53.62±3.82%, z=3.05, p<0.05; Figure 3b-iv) relative to all other samples.

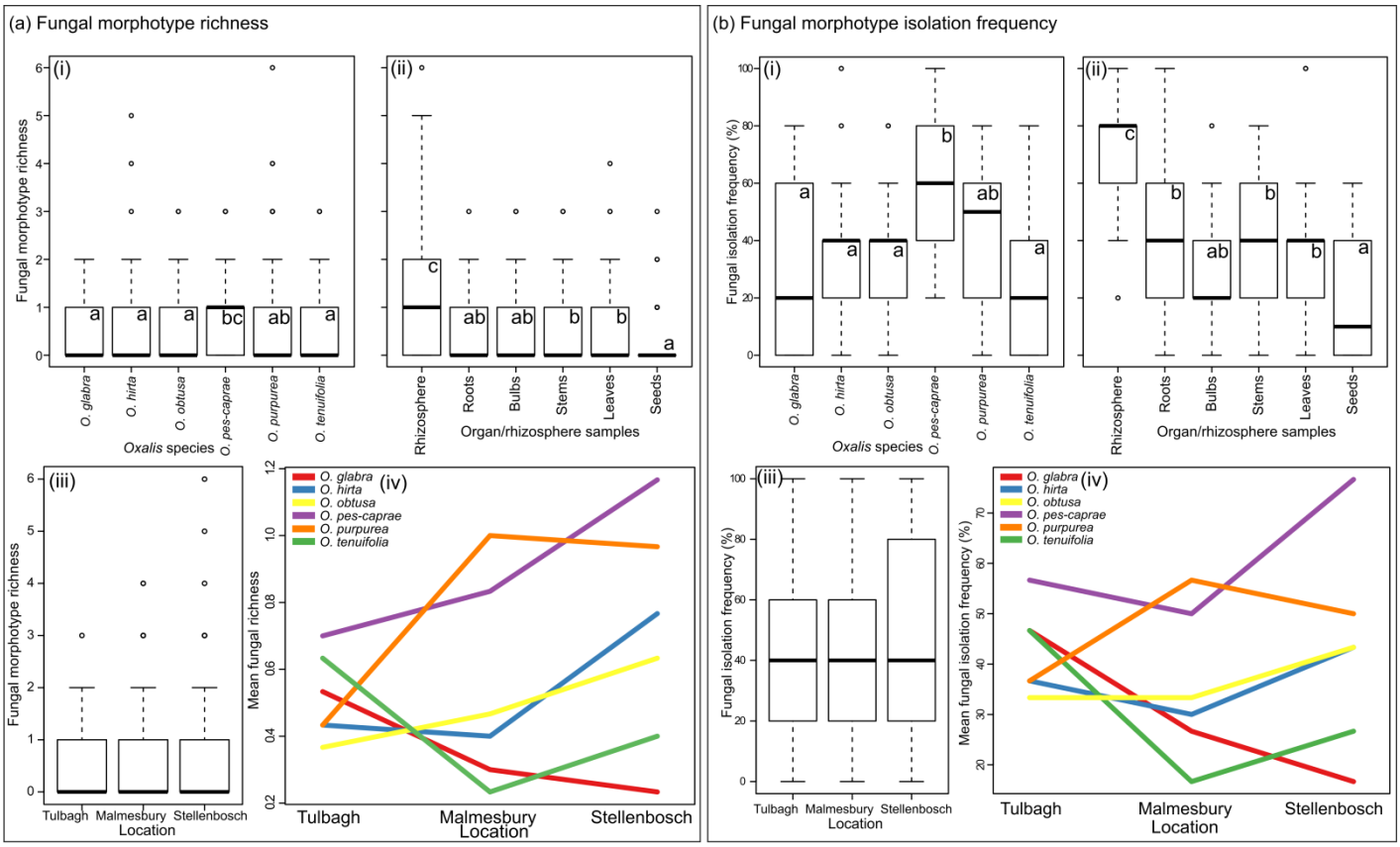

Figure 3: Fungal morphotype richness and fungal isolation frequency associated with *Oxalis* plants. Richness (a) and isolation frequency (b) from sampling location (i), *Oxalis* host species (ii) and organ/rhizosphere sample (iii). All different symbols are significantly different at p<0.05. Interaction plots for mean richness (a) and isolation frequency (b) for location and host species (iv).

Bacterial community ordinations

The unconstrained pure latent variable model for bacterial communities indicated a separation of the ordination into at least two clusters. However, the variables considered in this study (location, host species and organ/rhizosphere sample), showed no pattern - clusters

did not clearly correspond to any of these variables or interactions between these variables (Figure 4a-c). Nevertheless, when these variables were incorporated into the constrained ordination model for bacterial communities, no clustering was seen (Figure 4d), indicating that the variables included have a strong influence on community assemblages**.**

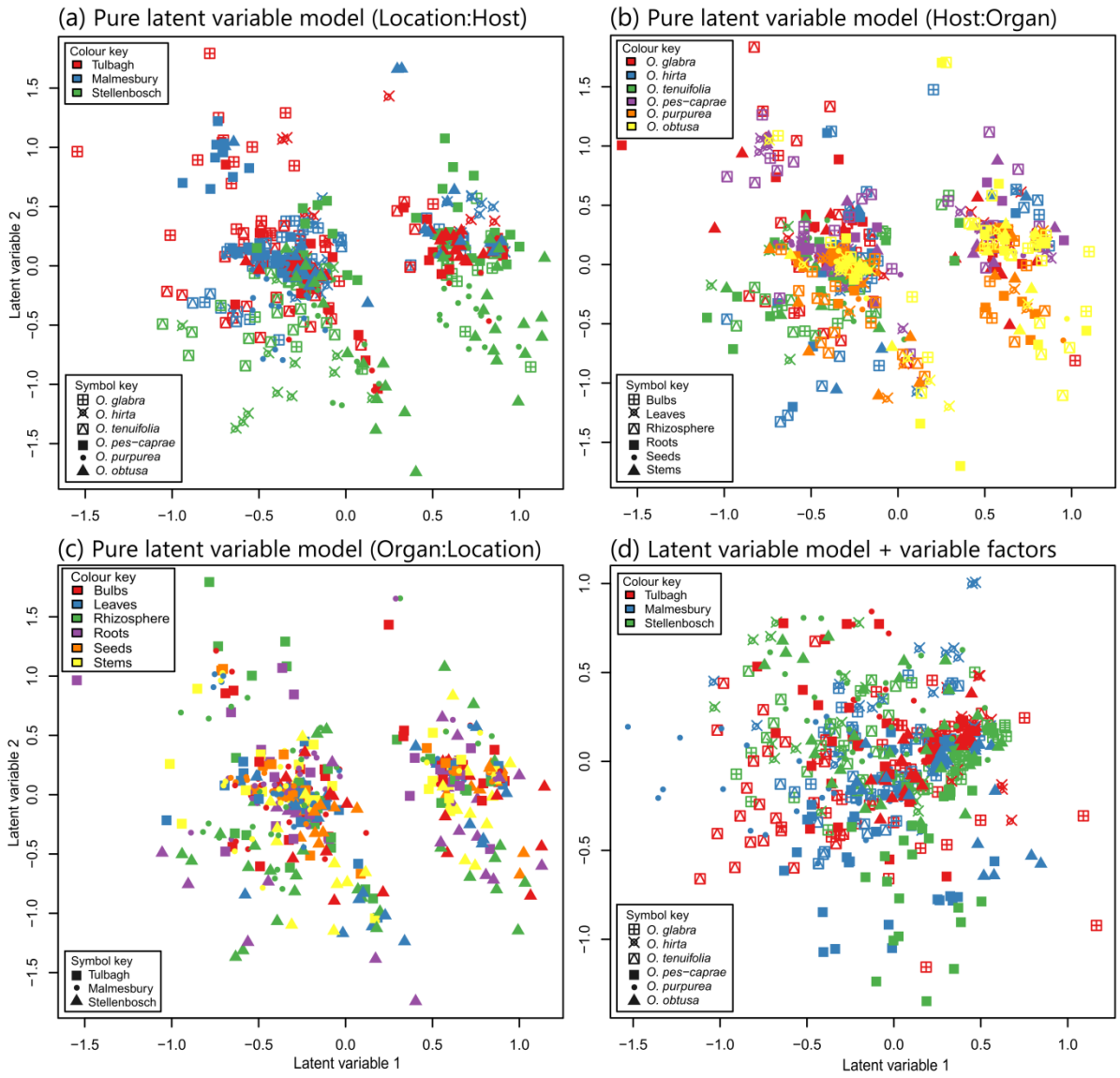

Figure 4: Bayesian ordination and regression analysis of bacterial community compositions associated with *Oxalis* host plants. Community assemblages according to unconstrained pure latent variable models (a, b, c) and constrained latent variable models with predictor variables included (d). Different combinations of predictor variables are coded according to keys in (a) – (c).

Fungal community ordinations

The unconstrained pure latent variable model for fungal communities indicated multiple indistinct clusters. However, the variables considered in this study showed no pattern -

clusters did not clearly correspond to any of these variables or interactions between these variables (Figure 5a-c). When these variables were incorporated into the constrained ordination model for fungal communities, no clustering was seen (Figure 5d), indicating that the variables included have a strong influence on community assemblages**.**

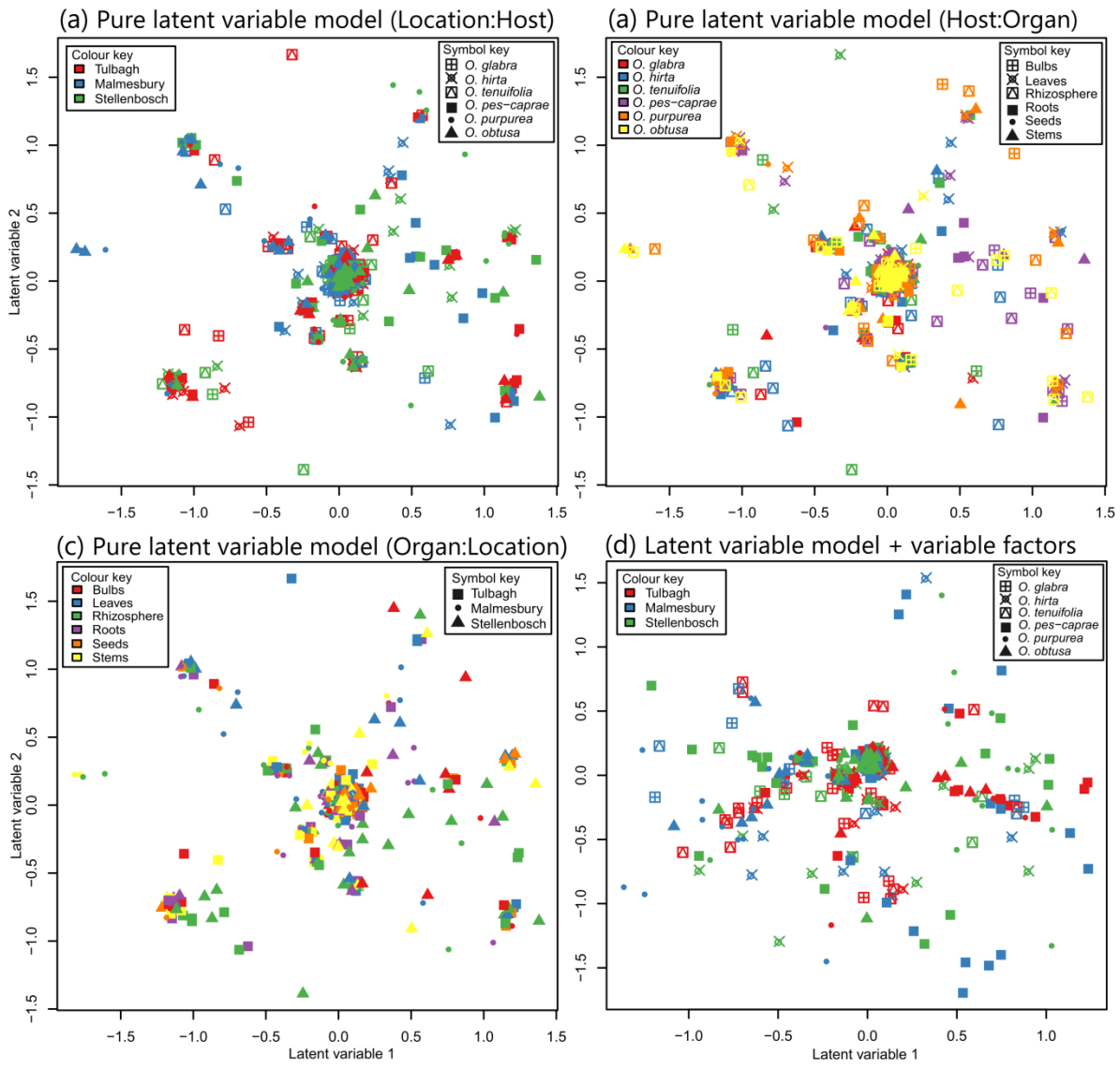

Figure 5: Bayesian ordination and regression analysis of fungal community compositions associated with *Oxalis* host plants. Community assemblages viewed according to unconstrained pure latent variable models (a, b, c) and constrained latent variable models with predictor variables included (d). Different combinations of predictor variables are coded according to keys in (a) – (c).

Factors influencing bacterial community composition

Multivariate generalized linear models revealed that sampling location, *Oxalis* host species and plant organ/rhizosphere sample all had a significant effect on bacterial community

ordinations (Table 3a). All three variables also had significant second order interaction effects. Interactions between variables indicate that bacterial communities respond to specific host species at a given location; organ/rhizosphere samples of a given host species; and organ/rhizosphere samples at a given location. These significant variable effects could describe why no clear clustering was detected in the unconstrained pure latent variable models. Univariate tests revealed that various combinations of the three tested variables, second- and/or third-order interaction effects between these variables had significant effects on community ordinations for individual bacterial morphotypes (Supplementary Table 1a). The majority (95%) of individual bacterial morphotypes were significantly influenced by *Oxalis* host species and/or sampling location. These results indicate that some individual bacterial morphotypes associated with specific *Oxalis* host plants from various locations.

Factors influencing fungal community composition

Similar to results for bacterial communities, multivariate analyses revealed that sampling location, *Oxalis* host species and plant organ/rhizosphere sample all had a significant effect on fungal community ordinations in the dataset including all organs/rhizosphere samples (Table 3b). All three variables also had significant second order interaction effects. Interactions between variables indicate that fungal communities respond to specific host species at a given location; organ/rhizosphere samples of a given host species; and organ/rhizosphere samples at a given location. As observed among the bacterial communities, these significant variable effects could describe why no clear clustering in the unconstrained pure latent variable models was detected. Similarly, univariate tests also revealed that various combinations of the three tested variables, second- and/or third-order interaction effects between these variables had significant effects on community ordinations of some individual fungal morphotypes (Supplementary Table 1b). However, the majority (82%) of individual fungal morphotypes were not significantly influenced by any of these tested variables, indicating that fungal endophytes could associate with various niches among hosts.

Table 3: Multivariate generalised linear modelling assessing the effects of tested variables on bacterial (a) and fungal (a) communities associated with *Oxalis* host plants. Variables included location (Loc), *Oxalis* host species (Host) and plant organ or rhizosphere samples (O/R), and all second- and third-order interaction effects between these variables. Significance levels indicated with asterisks (***p<0.001). Res.df = residual degrees of freedom, Dev = Deviation.

Communities	Variables	Res.Df	Dev	P
(a) Bacteria				
	Sampling location (Loc)	535	650.4	0.001 ***
	Oxalis host species (Host)	530	1235.5	0.001 ***
	Plant organ/rhizosphere (O/R)	525	578.0	0.001 ***
	Loc x Host	514	803.7	0.001 ***
	Loc x O/R	504	303.6	0.001 ***
	Host x O/R	479	489.3	0.001 ***
	Loc x Host x O/R	430	87.9	>0.05
(b) Fungi				
	Sampling location (Loc)	535	650.4	0.001 ***
	Oxalis host species (Host)	530	1235.5	0.001 ***
	Plant organ/rhizosphere (O/R)	525	578.0	0.001 ***
	Loc x Host	514	803.7	0.001 ***
	Loc x O/R	504	303.6	0.001 ***
	Host x O/R	479	489.3	0.001 ***
	Loc x Host x O/R	430	87.9	>0.05

Unique and shared bacteria and fungi

Venn diagrams were used to illustrate similarities and differences between microbial communities associated with sampling locations, *Oxalis* hosts, vegetative and reproductive plant organs. Among the 46 bacterial and 39 fungal morphotypes identified from all hosts sampled at all locations, the majority were present in both the rhizosphere and endosphere of plants (44 bacterial (95.7 %) and 36 fungal morphotypes (92.3%)). These patterns were consistent across sampling locations, with the majority of microbes present in both the rhizosphere and plant endosphere (Figure 6a). Across all sampled host plants from all locations, two bacterial and three fungal morphotypes were isolated from the rhizosphere only, while one bacterial and eight fungal morphotypes were absent from the rhizosphere and isolated from sterilized host plant tissues only.

Comparison of microbes associated with the rhizosphere, vegetative (roots, stems and leaves) and reproductive organs (bulbs and seeds) of all sampled host plants from all locations, indicated that the majority of microbes were shared among the rhizosphere and vegetative organs only (12 bacterial and 9 fungal morphotypes) or shared among the rhizosphere, vegetative and reproductive organs (18 bacterial and 8 fungal morphotypes) (Figure 6b-i). A

few bacterial and fungal morphotypes were unique to the rhizosphere (as described above) or vegetative organs only (2 bacterial and 3 fungal morphotypes). Three fungal morphotypes were unique to bulbs only. A substantial number of bacteria (9 morphotypes) and fungi (5 morphotypes) were isolated from the rhizosphere, bulbs and vegetative organs, but were absent from seeds. No bacteria or fungi were unique to seeds only, but one fungal morphotype was shared among seeds and the rhizosphere, and another fungal morphotype was shared among seeds and vegetative plant organs (Figure 6b-i).

Among the 44 bacterial and 36 fungal endophytic morphotypes, 17 bacteria and 10 fungi were shared among all three locations (Figure 6b-ii). Between two and four bacterial morphotypes and two to seven fungal morphotypes were shared among two of the sampling locations. Unique endophytes were isolated from all sampling locations, with the highest number of unique bacteria and fungi from Stellenbosch (11 bacterial and 7 fungal morphotypes) and lowest number of unique endophytes from Malmesbury (1 bacterial and 5 fungal morphotypes) (Figure 6b-ii). All *Oxalis* species hosted diverse assemblages of bacterial and fungal endophytes, where each species had both unique and shared endophytes in all sampled locations (Figure 6c).

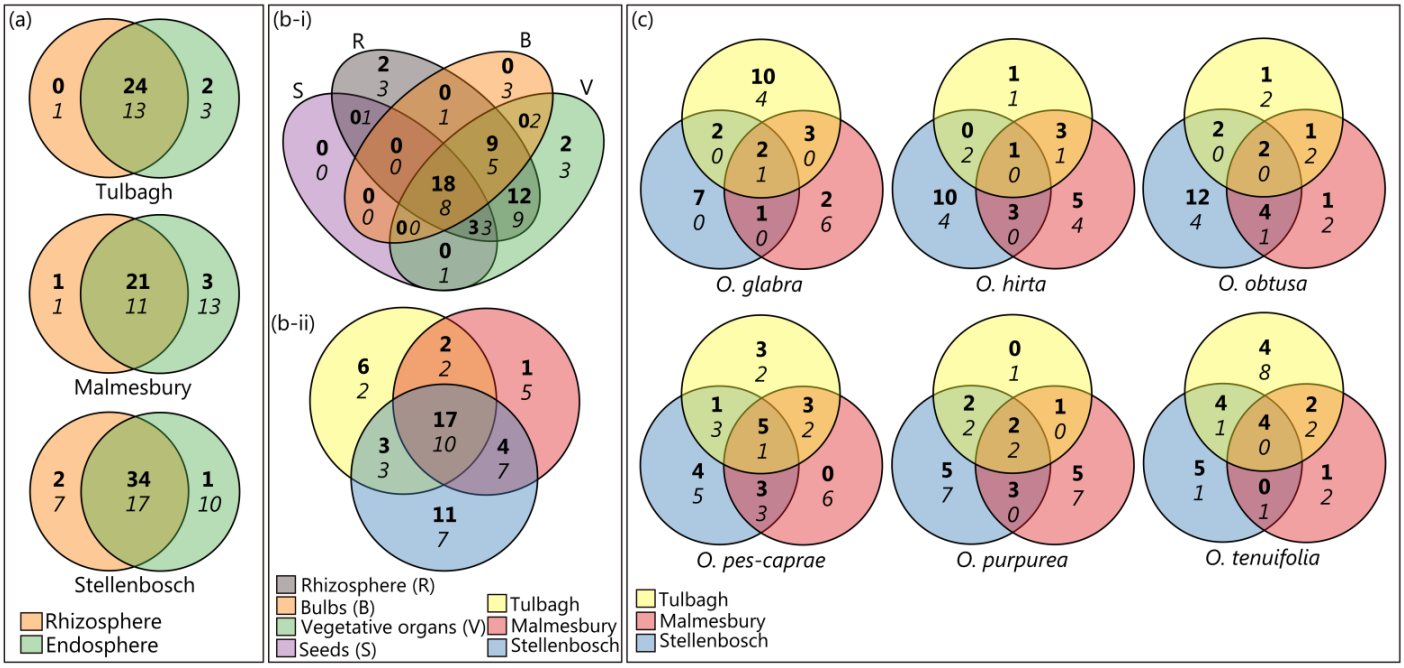

Figure 6: Venn diagrams showing the number of unique and shared bacterial (bold text) and fungal (italic text) morphotypes associated with the rhizosphere and endosphere of *Oxalis* host plants. (a) Microbial communities associated with the rhizosphere and endosphere (all sterilized vegetative and reproductive plant organs) of all host species from three locations. (b-i) Microbial communities associated with the rhizosphere, bulbs, vegetative organs (roots,

stems and leaves) and seeds of all host species from all locations, (b-ii) Total endophytic communities associated with all six *Oxalis* host species from three locations. (c) Endophytic communities associated with individual *Oxalis* host species sampled from three locations. Colours correspond to keys as indicated in each sub-section of the figure.

Frequently encountered bacterial endophytes

The most frequently encountered bacterial endophytes across all locations, host species and vegetative and reproductive plant organs included members of the genus *Bacillus* (Figure 7a). Sequencing identified the most common bacterial morphotypes as *B. aryabhattai* Shivaji, *B. cereus/thuringiensis* Frankland and Frankland/Berliner, *B. licheniformis* Weigmann, *B. megaterium* de Bary, *B. mycoides* Flügge, *B. safensis/pumilus* Satomi *et al.*/Meyer and Gottheil, *B. simplex* Priest *et al.* emend. Heyrman *et al.*/Sumpavapol *et al*. and *B. subtilus/siamensis* Cohn. Various assemblages of these *Bacillus* species were the most frequently encountered species from all individual sampling locations and *Oxalis* host species, and were present among all individual host plants (Figure 7a). A noteworthy observation was that the majority (>75% to 100% of isolates) of 9 out of the 11 *Bacillus* morphotypes were isolated from the bacterial agar enriched with potassium oxalate. This could indicate that isolated *Bacillus* endophytes prefer the growing conditions that most closely represent the high oxalate content of the host plant (Sahin, 2005). All identified *Bacillus* species are known plant endophytes with various associated plant growth promoting effects, as reported in Supplementary Table 2a.

Assessment of univariate community ordination test results for these eight *Bacillus* morphotypes, revealed that all them were significantly influenced by *Oxalis* host species and six morphotypes were also significantly influenced by sampling location (Table 4). Impotantly, these results indicated that some of the individual *Bacillus* endophyte communities were associated with specific *Oxalis* host plants and/or specific sampling locations. The majority of *Bacillus* species were isolated from all three locations, but *B. licheniformis* and *B. simplex* were isolated from host plants sampled at Tulbagh and Malmesbury only. Four of these endophytes (*B. cereus/ thuriengensis*, *B. megaterium*, *B. mycoides* and *B. safensis/pumilus*) were isolated from all *Oxalis* host species, while the other four *Bacillus* endophytes were isolated from four or five hosts. However, no *Bacillus* species showed a host-specific association. All *Bacillus* species had a ubiquitous distribution across rhizosphere, bulb, vegetative (roots, stems and leaves combined) and seed samples. Other well-represented genera included 6 strains from *Paenibacillus* Ash and 5 strains from

Pseudomonas Migula, both genera known to include many species capable of nitrogen fixation (Desnoues *et al*., 2003; Seldin, 2011; Grady *et al*., 2016).

Table 4: Results of univariate generalised linear modelling that assess the effects of three tested variables on presence/absence of endophytic *Bacillus* species associated with *Oxalis* host plants. Variables considered included location (Loc), *Oxalis* host species (Host) and plant organ or rhizosphere samples (O/R) and all second- and third-order interaction effects between these variables. Significance levels indicated with asterisks (*p<0.05 **, p<0.01, ***p<0.001).

Bacillus endophyte	Sampling location (Loc)	*Oxalis* host (Host)	Organ/ rhizosphere (O/R)	Loc x Host	Host x O/R	Loc x O/R	Loc x Host x O/R
B. aryabhattai	p<0.001***	p<0.001***	-	p<0.001***	-	-	-
B. cereus/ thuriengensis	p<0.01**	p<0.001***	-	-	-	-	-
B. licheniformis	p<0.001***	p<0.01**	-	-	-	-	-
B. megaterium	-	p<0.001***	-	p<0.01**	p<0.05*	-	p<0.05*
B. mycoides	p<0.001***	p<0.001***	p<0.001***	0	-	-	-
B. safensis/pumilus	p<0.001***	p<0.001***	-	p<0.001***	-	-	-
B. simplex	p<0.001***	p<0.001***	-	0	-	-	-
B. subtilus/siamensis	-	p<0.001***	-	p<0.001***	-	-	-

Other less common endophytic bacterial species included members from the genera: *Arthrobacter* Conn and Dimmick, *Burkholderia* Yabuuchi *et al*., a few other *Bacillus* species, *Luteibacter* Johansen *et al*., *Lysinibacillus* Ahmed *et al*., *Paenibacillus* Ash *et al*., *Pantoea* Gavini *et al*., *Pseudomonas* Migula and *Xanthomonas* Dowson (Figure 7a).

Frequently encountered fungal endophytes

The most frequently isolated fungal endophytes across all locations, host species and vegetative and reproductive plant organs included members from the genus *Aspergillus* Micheli, *Chaetomium* Kunze, *Fusarium* Link, *Schizophyllum* Fries, *Talaromyces* C.R. Benji and *Thielavia* Zopf (Figure 7b). The most common fungal species were identified as *Aspergillus chevalieri/amstelodami* Thom & Church, *Chaetomium aureum* Chivers, *C. funicola* Cooke, *Fusarium oxysporum* Schlecht, *Schizophyllum cf. commune* Fries, *Talaromyces pinophilus/verruculosus* Samson, *Thielavia hyrcaniae/terricola* Nicot and *Trichoderma longibrachiatum/saturnisporum* Rifai. Most of these species were also the most frequently encountered species from all individual sampling locations and *Oxalis* host species. Significant associations between these fungal taxa and any of the three predictors were sporadic (Supplementary Table 1b). Other endophytic species included members of the

fungal genera: *Acremonium* Link, *Alternaria* Nees, *Aspergillus*, *Chaetomium*, *Cladosporium* Link, *Epicoccum* Link, *Fusarium*, *Humicola* Bunce, *Lecythophora* Beyma, *Mortierella* Coem 1863, *Mucor* Fresen., *Nirospora* Mason, *Oidiodendron* Robak, *Paecilomyces* Samson, *Penicillium* Link, *Pilidium* Höhn., *Pleurostomophora* Vijaykr., *Schizophyllum*, *Sporormiella* Ellis & Everh., *Talaromyces*, *Trametes* Fr. and *Trichoderma*.

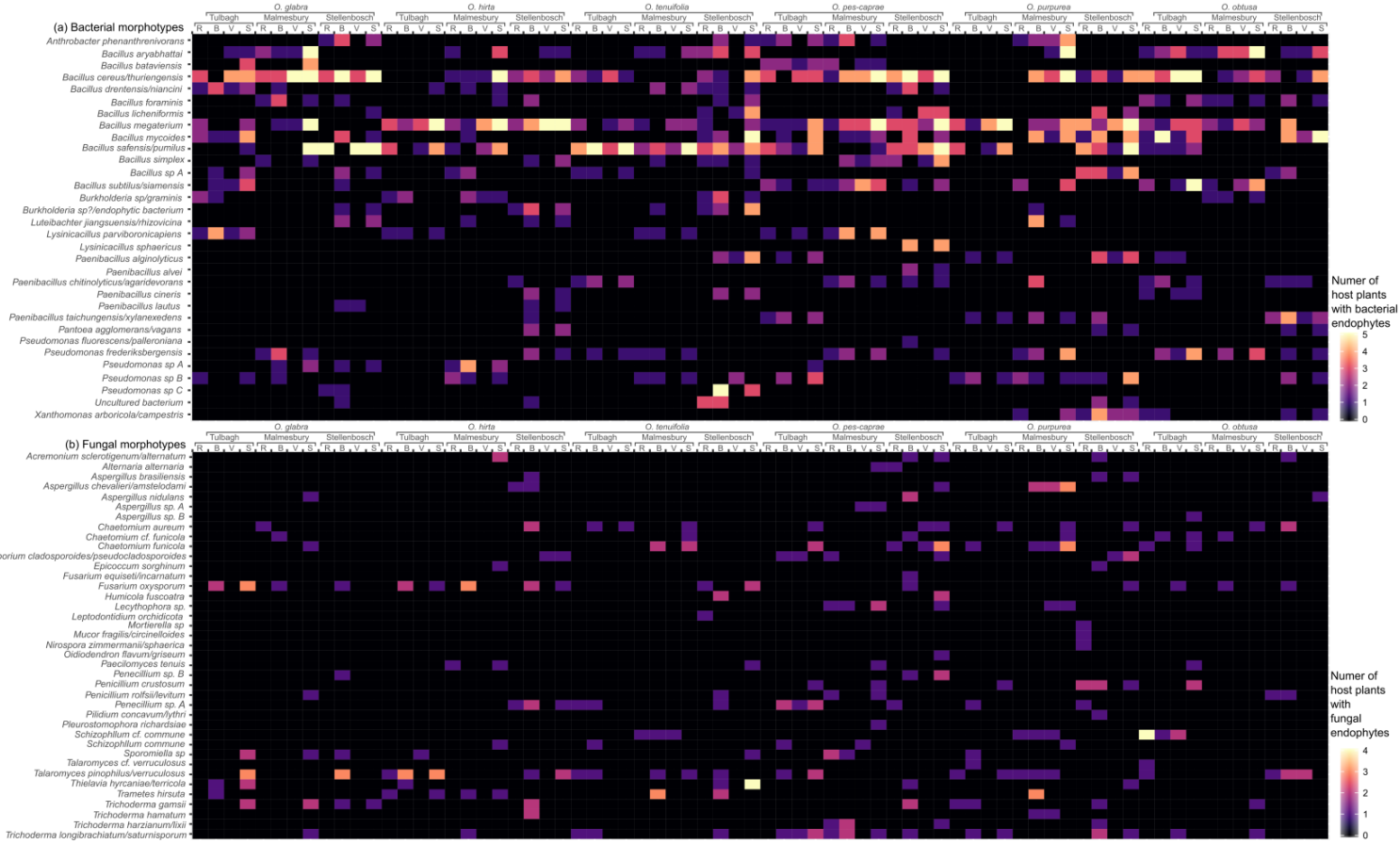

Figure 7: Heat-map showing the relative abundances of sequenced and identified bacterial (a) and fungal (b) morphotyes isolated from various sampled organs, *Oxalis* host plants and sampling locations. Colour tones range from highest (light yellow = isolated from all 5 replicates) to lowest (dark purple = isolated from none of the five replicates) isolation frequencies. R - rhizosphere, B - bulbs, V - vegetative organs (roots, stems and leaves), S - seeds. Each column represents one organ type of five host plants of the same host species sampled from one location.

DISCUSSION

We assessed how sampling location, host species and plant organs affect the richness, frequency and community structure of microbial endophytes associated with Cape *Oxalis*. Our results indicated the complex role of location, host species and plant organs, and interactions between these variables, in influencing the richness and isolation frequency of

both bacteria and fungi. Similarly, location, host species and plant organs, and interactions between these variables, were important factors in determining bacterial and fungal community structure in hosts. *Oxalis* plants are capable of forming associations with diverse assemblages of bacteria and fungi, but the most common and consistent endophytes included plant growth-promoting members of the genus *Bacillus*. By studying the same number of plant replicates, host species and plant organs from three sampling locations, this study offers an equal effort sampling approach, allowing in-depth comparisons to future studies on *Oxalis* endophytes or any other plant-endophyte communities.

Current predictions indicate that all terrestrial plants have associations with at least one endophytic bacterial or fungal species (Smith *et al*., 2008; Rodriguez *et al*., 2009; Nair and Padmavathy, 2014). On average, we recovered 23 culturable endophytic bacterial and 20 culturable endophytic fungal species per host species, which is an order of magnitude higher than commonly reported for endophytes isolated and cultured from plant species (Petrini, 1986; Smith *et al*., 2008; Rodriguez *et al*., 2009; Nair and Padmavathy, 2014). Our richness estimations indicate that these values may be even higher for some *Oxalis* host species and plant organs. Bacterial species richness estimators were similar to the observed species richness among samples, but fungal estimators predict that the true fungal richness may in fact be higher than reported here. This indicates that Cape *Oxalis* harbour incredibly high endophytic diversity relative to other terrestrial plants. There are, however, a few reports of crop plants with equally high numbers of bacterial endophytes (Compant *et al*., 2011; Mashiane *et al*., 2018). The numbers of endophyte associates of angiosperms in general may increase as more studies, using advanced techniques, are published.

Endophytic fungi from non-grass terrestrial plants can be divided into different functional groups based on their life history and ecological significance (Rodriguez *et al*., 2009). Based on this system, the fungi isolated from *Oxalis* hosts are Class 2 endophytes, generally defined as fungi from plant roots, rhizomes and shoots that are capable of being horizontally and vertically transmitted (Petrini, 1996, Rodriguez *et al*., 2009). Class 2 fungal endophytes generally have low within-plant diversity, but authors of the most recent review on this group of fungal endophytes described the assessment of Class 2 species richness and diversity as a "daunting task" as these endophytes may include countless unknown species (Rodriguez *et al*., 2008). Most other studies describe single Class 2 endophytes from host plants, but it is possible that the true endophytic communities are much more diverse (Rodriguez *et al*.,

2008). Given these descriptions, fungal diversity found within Cape *Oxalis* appears to be relatively high, but this view may change as future studies reveal greater diversity of fungal endophytes across terrestrial plants.

Microbial diversity of the rhizosphere

We found that the rhizosphere had higher bacterial and fungal morphotype richness than individual surface-sterilized plant organs, as expected. It is well-known that the soil environment is home to an incredibly diverse community of micro-organisms, representing the greatest reservoir of known (and unknown) biological diversity (Curtis *et al*., 2002; Torsvik *et al*., 2002). Many of these microbes are attracted to the plant rhizosphere and live in close association with plant hosts. Typically only a small subset of rhizosphere microbes is known to inhabit living plant tissue without causing disease symptoms (Linderman, 1988; Hardoim *et al*., 2008; Berendsen *et al*., 2012). Here we have shown that the majority of bacteria and fungi isolated from the rhizosphere were also isolated from the endosphere of *Oxalis* host plants, suggesting that endophytic communities in *Oxalis* are a surprisingly substantial subset of rhizosphere microbes.

Endophytic diversity among plant organs

Endophytic bacterial richness was highest in roots and stems, while endophytic fungal richness was highest in stems and leaves. Roots and leaves are the most common entry point for microbial colonization events (Curtis et al., 2002; Vorholt, 2012), therefore high richness in these two organs would be expected. Many bacterial endophytes are also capable of colonising plant xylem and being spread throughout stems and leaves of their hosts (Hardoim *et al*. 2008). Bulbs, leaves and seeds had the lowest bacterial richness and seeds had the lowest fungal richness. Low microbial diversity in seeds was expected, as it is known that plants exercise selection so that beneficial instead of pathogenic microbes is passed on to their progeny (Santoyo *et al*., 2016; Shahzad *et al*., 2017). The bulbs of *Oxalis* are also reproductive propagules, serving as agents of clonal reproduction and dispersal (Salter, 1944), so bulb-specific endophyte selection may also be operational.

Bacterial and fungal richness varied between host species at different sampling locations, with few consistent patterns associated with particular host species or locations. *Oxalis pes-caprae* was the only host species with significantly higher endophytic bacterial and fungal richness, relative to all other host species. This species is widely distributed throughout the

Cape region and it seems possible that diverse associations with endophytic microbes could aid in its success. *Oxalis pes-caprae* is also a prominent global weed (Castro *et al*., 2007; Sala *et al*., 2007) and it is possible that a large and diverse endophytic community increases the invasive potential of this species. Significant interaction effects were detected between sampling location and organ types, where significantly higher bacterial richness was observed in rhizosphere samples from Stellenbosch than from the other two sites. These results could indicate that *Oxalis* plants have the ability to associate with single or multiple bacterial and/or fungal endosymbionts and that these associations may be influenced by other biotic and/or abiotic conditions. These findings confirm results from many previous studies that the diversity of plant endophytes is generally influenced by edaphic and climatic conditions of the rhizosphere, the diversity of niches available within the host plant, as well as potential competition between endophytes (as reviewed by Sieber and Grunig, 2013).

Frequency of endophytes isolated

At least one bacterial endophyte was isolated from all studied *Oxalis* hosts (90 plants), regardless of location, host species or organ type studied. The consistent isolation of certain bacterial endophytes from most locations, *Oxalis* hosts and organs suggest very close associations between hosts and some bacterial endophytes. Importantly, bacterial endophytes were frequently isolated from bulbs (83 out of 90) and seeds (81 out of 90). This could indicate that some endophytes are capable of being transmitted in vegetative organs across growing seasons, as well as being vertically transmitted across generations (Cope-Selby *et al*., 2017; Shade *et al*., 2017). The frequency of fungal endophyte isolations was less consistent than bacteria, but with no significant differences between hosts or organs. These lower fungal isolation frequencies may indicate facultative associations between *Oxalis* hosts and some fungal endosymbionts.

Rhizosphere versus endophytic community compositions

Bacterial and fungal community compositions associated with *Oxalis* plants were significantly influenced by sampling location, *Oxalis* host species and plant organs, but with complex interactions between these three variables. There is a clear interplay between site-specific factors *i.e*. geographically influenced microbial source communities, host-specific factors *i.e*. the impact in part of phylogenetic history, and organ-level factors *i.e*. the cross-talk between microbial organ preference and movement rates and plant screening ability (Garbeva *et al*., 2008). The microbial communities associated with *Oxalis* hosts respond to

various biotic and abiotic variables, shaping diverse assemblages of bacteria and fungi present in the rhizosphere and endosphere of Cape *Oxalis* hosts.

Plants are known to actively shape the community composition of their rhizosphere microbiome. Recent studies have shown that different plant species grown in the same soil associated with different communities of rhizosphere micro-organisms (Garbeva *et al*., 2008; Berg and Smalla, 2009, Weinert, 2011), while some plant species can create similar communities when grown in different soils (Fierer and Jackson, 2006). These interactions, in turn, affect the diversity of microbes that form endophytic associations with host plants (Hardoim *et al*., 2008; Berendsen *et al*., 2012). The diversity of plant endophytic communities is known to be influenced by plant species, plant genotype, growth stage, organ types and different colonization pathways (Strobel *et al*., 2004; Vieira *et al*., 2011). Given that 95.7 % of bacteria and 92.3% of fungi identified were present in both the rhizosphere and endosphere, this implies that the majority of *Oxalis* endophytes are soil derived. The few species isolated exclusively from the endosphere could epiphytically colonize through leaves or above-ground stems. Alternatively they could be present in the rhizosphere, but were not sampled. Finally, they may represent endophytes that reached the host plant through vertical transmission.

Even though these three variables and interactions had significant effects on community assemblages, certain consistent patterns of location-, species- or organ-specific association were detected, such as the ubiquitous *Bacillus* endophytes. Most isolated endophytic bacteria and fungi were present in all sampled locations or were shared amongst two of the three locations. A number of unique endophytes were also associated with each location. This could indicate that *Oxalis* is capable of hosting a wide range of microbial associates present within a given environment. This may be because *Oxalis* plants have no need to substantially invest in long-term plant defences against microbial colonization (except among bulbs and seeds), as the above-ground organs die off at the end of the winter growing season. Given our latent-variable results, it is also likely that there are other biotic and abiotic factors that were not included in this study, which influenced the observed community compositions. These could include host genotype, developmental stage, soil type and host plant nutrient status (as reviewed by Liu *et al*. (2017) and all references therein). The majority of these variables remain un-tested and should be pursued in future studies on *Oxalis* endophytic associations.

Distribution of endophytes across plant organs – implications for endophyte community persistence and transmission

In total 31.8% (19 bacterial and 8 fungal morphotypes) of all identified microbes were isolated at least once from all organs (including seeds) and the rhizosphere. Notably, more than half (54%) of all endophytes encountered were present in *Oxalis* bulbs. This is noteworthy as persistence of endophytic microbes in *Oxalis* hosts across seasons can only occur through successive, yearly colonisation of new bulb tissue. As all non-bulbous organs of *Oxalis* plants senesce at the end of each growing season, endophytic fungi and bacteria must translocate to the new bulb and basal stem therein, in order to be transferred across yearly growth and re-infect the subsequent year's seasonal growth. This would allow new above-ground organs produced in the subsequent growing seasons to be re-infected from the bulb endophytic reservoir.

The frequent isolation of bacteria from *Oxalis* bulbs and seeds was an important discovery. Plants usually exert strong selection pressures on which endophytes are passed on to their reproductive propagules (Hallmann, 2001). To our knowledge there are no reports of beneficial endophytic bacteria or fungi isolated from bulbs (Cui *et al*., 2008; Altan *et al*., 2010; Deng *et al*., 2012), which makes our isolation of diverse assemblages of bacterial and fungal endophytes from *Oxalis* bulbs interesting. Authors such as Mandt and Hinkle (1976), Misaghi and Donndelinger (1990), Barac *et al.*, (2004), Cankar *et al*., (2005) and Okunishi *et al*., (2005) reported on the endophytic presence of the bacterial genera *Bacillus*, *Pseudomonas* and *Rahnella* in seed. The isolation of seed endophytes in *Oxalis* was thus rather novel, especially as they included these three genera, but many others bacterial genera and fungi as well.

Oxalis seeds were found to harbour a rather diverse collection (21 bacterial and 12 fungal morphotypes) of endophytes. Most seed endophytes included bacteria (19 out of 21 morphotypes) and fungi (8 out of 12 morphotypes) that were shared with the rhizosphere and other plant organs, so seed endophytes represent a subset of the rhizosphere and vegetative endosphere microbiome. Seed endophytes were found in all host species and across all locations, indicating that this is a widespread phenomenon. *Oxalis* is thus capable of allowing various endophytes access to their seeds, although true evidence of vertical transmission (*i.e.* re-isolation of same microbes in established seedlings) was not pursued in this study. Interestingly, most of the seed endophytes are bacteria with many well-documented plant

growth-promoting properties. This could indicate that the suite of seed endophytes is not random, and that *Oxalis* has some ability to select beneficial microbes to reach their progeny.

Many *Oxalis* species occupy very narrow geographic ranges and have very specific habitat requirements (Zietsman *et al.*, 2008), which may dictate possible interactions with endophytic microbes. To date only a handful of studies on *Oxalis* endophytes have been published, none of them on Cape species in their native range. A total of 14 bacterial and two fungal endophytes associated with 11 global *Oxalis* species have been reported. These findings also have more general implications to the invasive biology of *Oxalis* as this genus includes many persistent global weeds (Marshall, 1987; Castro *et al*., 2007). These invasive species are likely to be distributed with a suite of endophtes, or acquire beneficial endophytes from their new habitats. As we have established that hosts have the ability to select beneficial microbes to reach their progeny, this could enhance, and help understand, the invasive success of *Oxalis*.

Potential functional roles of fungal endophytes

Of the isolated endophytic fungi, only a few *Cladosporium*, *Talaromyces* and *Trichoderma* species have known plant-growth promoting traits (summarised in Supplementary Table 2b, Bae *et al*., 2009; Cong *et al*., 2015; Azad and Kaminskyj, 2016).The majority of fungal species isolated from *Oxalis* hosts were, however, common soil microbes (therefore most likely opportunistic colonizers) or known plant pathogens such as *Aspergillus*, *Chaetomium* and *Fusarium* (Rodriguez-Galvez and Mendgen, 1995; Schulz *et al*., 1999; Vu *et al*., 2006). As our richness estimates suggest that fungal richness is under-sampled, there may be other endophytes present in *Oxalis* hosts, but that these fungi are fastidious, slow growing or were out-competed by other fungi when grown on agar (Guo *et al.*, 2001; Duong *et al*., 2007; Zhu *et al*., 2008). Significant associations between individual fungal taxa and any of our three predictors were sporadic (Supplementary Table 1b). We suggest that the majority of culturable fungal endophytes isolated from *Oxalis* may be opportunistic colonizers taking advantage of an ephemeral niche, as there are no apparent or consistent patterns of associations with beneficial fungal species.

None of the fungal endophytes detected in this study have previously been reported from *Oxalis* hosts, but associations with arbuscular mycorrhizal (AM) fungi (genus *Glomus*) have been reported from eight *Oxalis* species (Harley and Harley, 1987; Fontenla *et al.*, 1998; Turnau *et al*., 1999; Yamato, 2004; Becerra *et al*., 2009; Kooren *et al*., 2012). These authors

reported various beneficial plant growth-promoting traits associated with these AM fungi. As AM fungi cannot be identified *in planta* or isolated through conventional plating techniques, their identities are usually confirmed using sequence data obtained from universal or genus-specific DNA regions (Van Tuinen *et al.*, 1998). In a small pilot study we confirmed the presence of AM fungal associates from two of our six studied *Oxalis* hosts (*O. hirta* and *O. purpurea*). However, these observations were not quantified or identified, and remain to be explored in future research.

Potential functional roles of bacterial endophytes

The most common bacterial endophytes isolated from all sampling locations, *Oxalis* host species and various vegetative and reproductive (bulbs and seeds) plant organs included members of the genus *Bacillus*. This well-known genus includes many plant-growth promoting endophytes associated with many angiosperms globally (summarised in Supplementary Table 2b, West *et al*., 2010; Miliute and Buzaite, 2011; Chen *et al.*, 2012). *Bacillus* species found to associate with Cape *Oxalis* have been shown to benefit other host plants through phosphate solubilisation, atmospheric nitrogen-fixation/or exerting and anti-fungal properties (Xie *et al*., 1998; Ding *et al*., 2005; Arvind *et al*., 2011; Kang *et al*., 2014; Ramesh *et al*. 2014). *B. aryabhattai* is known to improve the mobilization of zinc (Ramesh *et al*., 2014) and *B. licheniformis* and *B. megaterium* improve the solubilisation of phosphates (Rojas *et al*.; 2001; Kang *et al*., 2014). Bacterial endophytes with documented nitrogen fixing properties include: *B. cereus, B. licheniformis, B. megaterium, B. safensis, B. subtilus* (Xie *et al*., 1998; Ding *et al*., 2005; Arvind *et al*., 2011; Castillo-Arteaga *et al*., 2016). Many of these bacteria also have well known anti-fungal properties (including *B. siamensis, B. simplex, B. subtilus and B. thuringiensis*) (Downing *et al*., 2000; Rashid *et al*., 2012; Schwartz *et al*., 2013; Nongkhlaw *et al*., 2014). It is likely that some of these documented beneficial properties are extended to their *Oxalis* hosts.

To date, various beneficial bacterial endophytes have been reported from only 11 *Oxalis* species worldwide, including 14 species from the genera *Azospirillum*, *Bacillus* and *Pseudomonas* (Peng *et al*., 2013; Mufti *et al*., 2015; Castillo-Arteaga *et al*., 2016). Two of the bacterial endophytes isolated among Cape *Oxalis*, *B. cereus* and *B. subtilus,* were previously isolated from the rhizosphere and roots of *O. spiralis* G. Don and *O. tuberosa* Molina from Columbia (Castillo-Arteaga *et al*., 2016). All documented bacterial endophytes were isolated from the rhizosphere, roots and leaves of *Oxalis* hosts, but never from *Oxalis* seeds.

Importantly, all of the beneficial *Bacillus* species we found in seeds were isolated from surface-sterilized Cape *Oxalis* seeds, suggesting vertical transmission of selected mutualist endophytes.

The most recent review on seed endophytes includes *Bacillus* and *Pseudomons* among common seed-associated bacterial genera (Truyens *et al*., 2014). *Bacillus cereus, B. megaterium, B. pumilus, B. simplex, B. subtilus* and *B. thuringiensis* have been reported from the seeds of many crop species, including grapevine, maize, peanuts, rice and tomatoes (Mano *et al*., 2006; Kaga *et al*., 2009; Compant *et al*., 2011; Rosenblueth *et al*., 2012; Sobolev *et al*., 2013; Xu *et al*., 2014; Mashiane *et al*., 2018). The presence of these species in a non-model, non-crop geophyte suggests that transmission of *Bacillus* via seed may be a far more common process than previously thought in angiosperms.

Given the known plant growth-promoting traits associated with these bacterial endophytes, and their ubiquitous association with Cape *Oxalis* host species from various sampling locations, it seems reasonable to expect that these bacteria were favoured for endophytic symbioses and vertical transmission. If true, these interactions could be regarded as an overlooked, possibly key mechanism that allows *Oxalis* to thrive in such nutrient-depleted environments as those present in the Cape. Associations with these plant growth promoting bacterial endophytes could help to support and maintain the unique and diverse seed and seedling biology of Cape *Oxalis*. The discovery of these interactions could provide significant insights to our current understanding of the distribution, establishment and evolution of *Oxalis* within the Cape Flora.

CONCLUSION

Cape *Oxalis* hosts diverse communities of bacterial and fungal endosymbionts, greatly exceeding expectations of endophyte diversity for most plant taxa. We have established that sampling locations, host species and host plant organ are variables that significantly influence endophytic microbial communities within host plants, but in idiosyncratic ways. We found assemblages of various plant growth promoting and nitrogen-fixing *Bacillus* taxa to be ubiquitous across all sampled plants, and they were distributed across vegetative organs, as well as propagules such as bulbs and seeds. It seems plausible that this bacterial and fungal symbiont-pool, if shown to have beneficial properties, could be key to the mechanism that allows this genus to flourish in the nutrient-deprived environments of the Cape. The

unexpected widespread occurrence of seed-associated *Bacillus* in a non-crop plant suggests that transmission of this bacterium via seed may be a far more common process than previously thought in angiosperms. This study provides a thorough baseline for future studies on endophytic associations with *Oxalis* or other ecologically and agriculturally important angiosperms from the Cape Flora.

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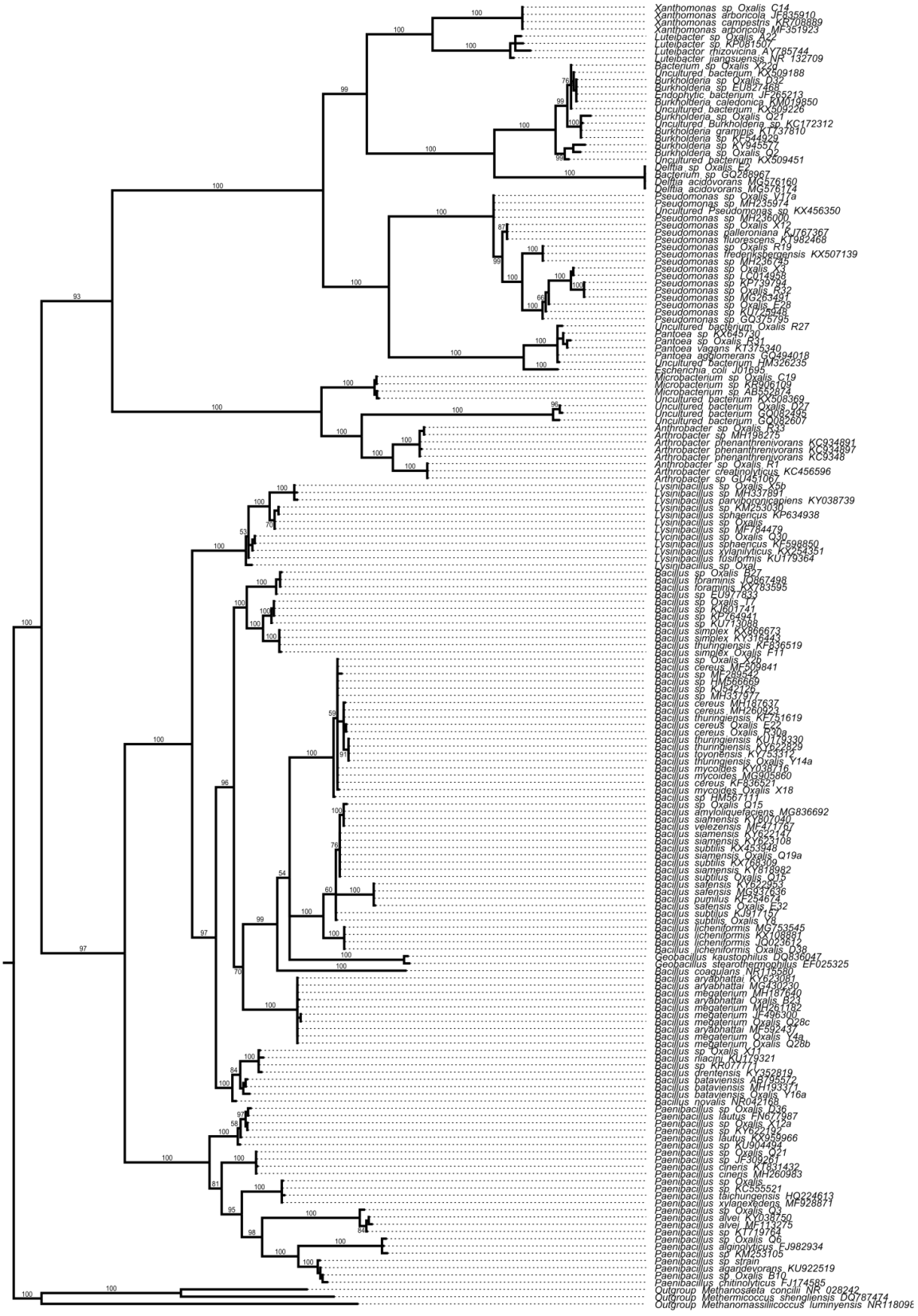
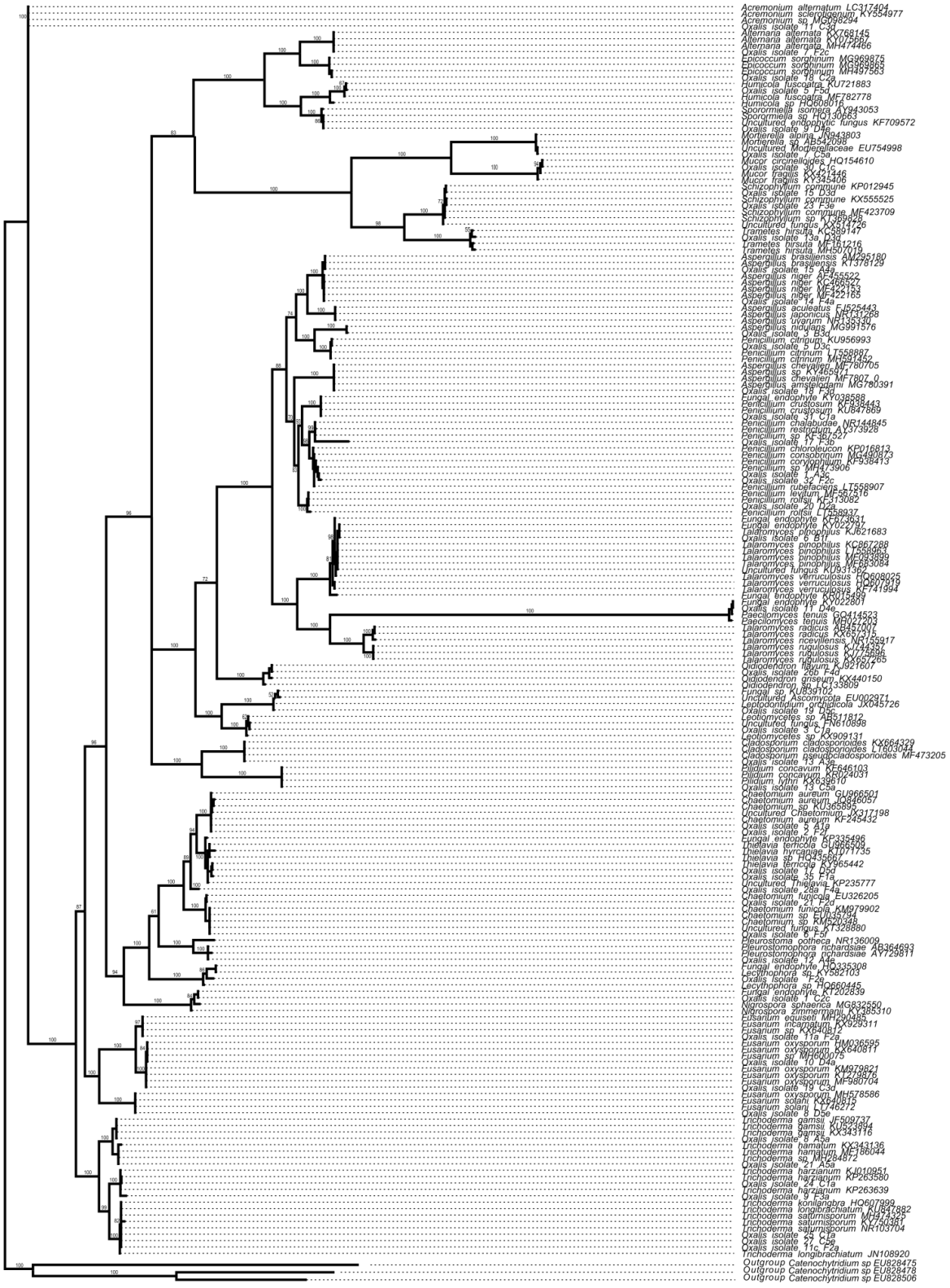
SUPPLEMENTARY MATERIALS

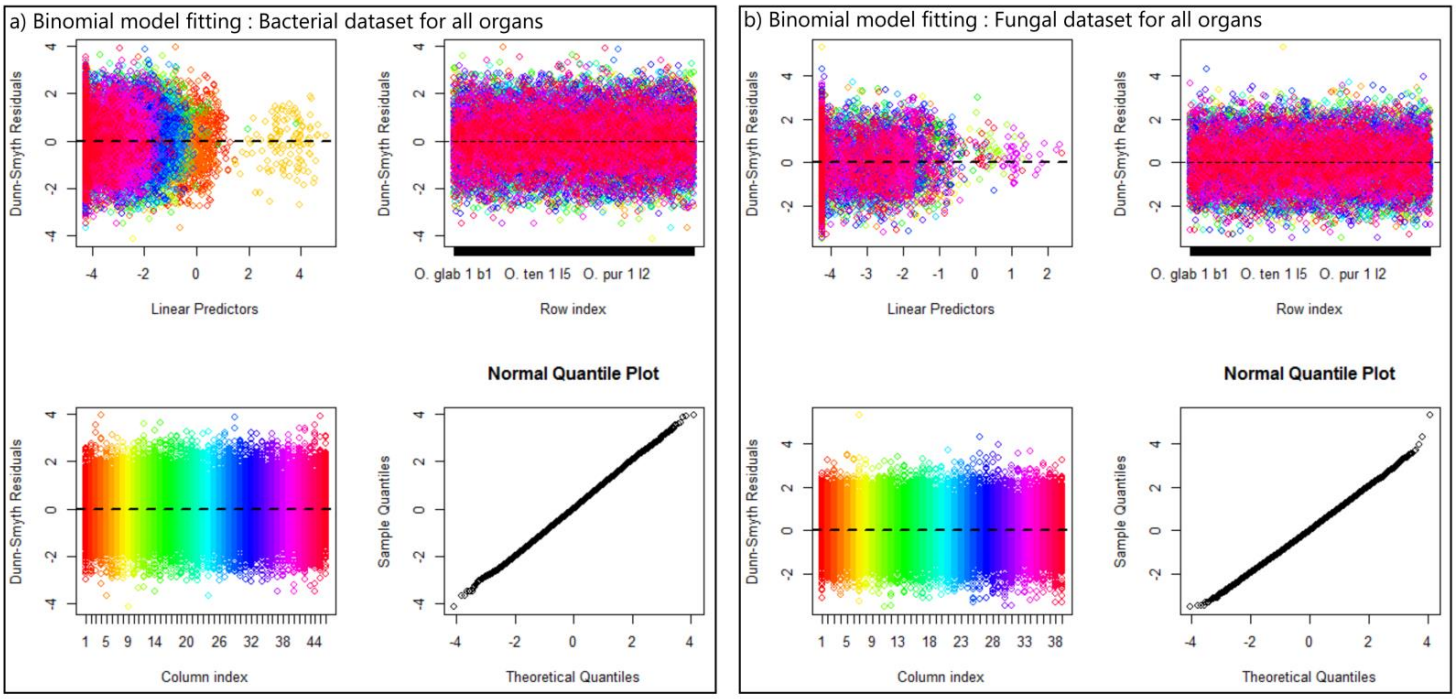


Supplementary figure 1a: A 16S consensus phylogenetic tree from the BEAST posterior

distribution for bacterial morphotypes associated from six studied southern African *Oxalis* species. Posterior probability values for relevant clades are indicated above nodes.



Supplementary figure 1b: An ITS consensus phylogenetic tree from the BEAST posterior distribution for fungal species morphotypes with six studied southern African *Oxalis* species. Posterior probability values for relevant clades are indicated above nodes.



Supplementary figure 2: Binomial model fitting plots for bacterial (a) and fungal (b) community composition presence-absence datasets, returned from 'plot(fit.LVMP)'. Each colour represents a different bacterial or fungal morphotype. In each plot (a and b): Top left: Dunn–Smyth residuals vs. linear predictors; Top right: Dunn–Smyth residuals vs. row index; Bottom left: Dunn–Smyth residuals vs. column index; Bottom right: normal quantile plot of Dunn–Smyth residuals. The normal quantile plots indicate that Binomial model fitting is appropriate for both datasets.

Supplementary Table 1a: Univariate generalised linear modelling results that assess the effects of three tested variables on bacterial communities associated with *Oxalis* host plants. Variables considered included location (Loc), *Oxalis* host species (Host) and plant organ or rhizosphere samples (O/R) and all second- and third-order interaction effects between these variables. Significance levels indicated with asterisks (*p<0.05 **, p<0.01, ***p<0.001).

Bacterial morphotype	Loc	Host	O/R	Loc x Host	Host x O/R	Loc x O/R	Loc x Host x O/R
Bacillus aryabhattai	p<0.001***	p<0.001***	0	p<0.001***	0	0	0
Bacillus cereus/thuriengensis	p<0.01**	p<0.001***	0	0	0	0	0
Bacillus bataviensis		p<0.001***	0	0	0	0	0
Bacillus megaterium	0	p<0.001***	0	p<0.01**	p<0.05*	0	p<0.05*
Bacillus mycoides	p<0.001***	p<0.001***	p<0.001***	0	0	0	0
Bacillus subtilus/siamensis	0	p<0.001***	0	p<0.001***	0	0	0
Bacillus safensis/pumilus	p<0.001***	p<0.001***	0	p<0.001***	0	0	0
Bacillus simplex	p<0.001***	p<0.001***	0	0	0	0	0
Bacillus licheniformis	p<0.001***	p<0.01**	0	0	0	0	0
Bacillus foraminis	0	p<0.001***	p<0.05*	0	0	0	0
Anthrobacter phenanthrenivorans	0	p<0.01**	0	p<0.001***	0	0	0
Bacillus drentensis/niancini	0	p<0.01**	0	p<0.001***	0	0	0
Bacillus sp. A	p<0.001***	p<0.01**	0	0	0	0	0
Burkholderia sp./graminis	0	p<0.01**	0	p<0.001***	0	0	0
Burkholderia sp./endophytic bacterium	p<0.001***	p<0.001***	p<0.05*	0	0	0	0
Luteibachter jiangsuensis/rhizovicina	0	p<0.05*	p<0.01**	0	0	0	0
Lysinicacillus parviboronicapiens	p<0.001***	p<0.001***	0	0	0	0	0
Lysinicacillus sphaericus	p<0.001***	p<0.001***	0	0	0	0	0
Paenibacillus alginolyticus	p<0.001***	p<0.01**	0	0	0	0	0
Paenibacillus alvei	0	0	0	0	0	0	0
Paenibacillus chitinolyticus/agaridevorans	0	p<0.05*	p<0.001***	p<0.01**	0	0	0
Paenibacillus cineris	0	p<0.05*	0	0	0	0	0
Paenibacillus lautus	0	0	0	0	0	0	0
Paenibacillus taichungensis/xylanexedens	0	p<0.001***	p<0.05*	p<0.01**	0	0	0
Pantoea agglomerans/vagans	0	0	0	0	0	0	0
Pseudomonas fluorescens/palleroniana	0	0	0	0	0	0	0
Pseudomonas frederiksbergensis	p<0.01**	0	p<0.01**	0	0	0	0
Pseudomonas sp. A	p<0.01**	p<0.001***	0	0	0	0	0
Pseudomonas sp. B	0	p<0.01**	0	p<0.001***	0	0	0
Pseudomonas sp. C	p<0.001***	p<0.001***	0	0	0	0	0
Uncultured bacterium	p<0.001***	p<0.05*	p<0.05*	0	0	0	0
Xanthomonas arboricola/campestris	0	p<0.001***	0	0	0	0	0
Unknown sp. A (*O. glabra* Site 1)	0	0	0	0	0	0	0
Unknown sp. B (*O. glabra* Site 1)	0	0	0	0	0	0	0
Unknown sp. C	0	0	0	0	0	0	0

(*O. glabra* Site 1)							
Unknown sp. D (*O. glabra* Site 1)	0	0	0	0	0	0	0
Unknown sp. A (*O. tenuifolia* Site 1)	0	p<0.01**	0	0	0	0	0
Unknown sp. B (*O. tenuifolia* Site 1)	p<0.01**	p<0.01**	0	0	0	0	0
Unknown sp. A (*O. pes-caprae* Site 1)	0	p<0.05*	0	0	0	0	0
Unknown sp. A (*O. purpurea* Site 2)	0	0	0	0	0	0	0
Unknown sp. A (*O. glabra* Site 3)	0	p<0.05*	0	0	0	0	0
Unknown sp. A (*O. tenuifolia* Site 3)	0	p<0.05*	0	0	0	0	0
Unknown sp. A (*O. hirta* Site 3)	0	0	0	0	0	0	0
Unknown sp. B (*O. hirta* Site 3)	0	0	0	0	0	0	0
Unknown sp. A (*O. pes-caprae* Site 3)	0	0	0	0	0	0	0
Unknown sp. A (*O. obtusa* Site 3)	0	p<0.05*	0	0	0	0	0

Supplementary Table 1b: Univariate generalised linear modelling results to assess the effects of three tested variables on fungal communities associated with *Oxalis* host plants. Variables considered included location (Loc), *Oxalis* host species (Host) and plant organ or rhizosphere samples (O/R) and all second- and third-order interaction effects between these variables. Significance levels indicated with asterisks (*p<0.05 **, p<0.01, ***p<0.001).

Fungal morphotype	Loc	Host	O/R	Loc x Host	Host x O/R	Loc x O/R	Loc x Host x O/R
Acremonium sclerotigenum/alternatum	0	0	0	0	0	0	0
Alternaria alternaria	0	0	0	0	0	0	0
Aspergillus brasiliensis	0	0	0	0	0	0	0
Aspergillus chevalieri/amstelodami	0	0	0	0	0	0	0
Aspergillus nidulans	0	0	0	0	0	0	0
Aspergillus sp. A	0	0	0	0	0	0	0
Aspergillus sp. B	0	0	0	0	0	0	0
Chaetomium aureum	0	0	0	0	0	0	0
Chaetomium cf. funicola	0	0	0	0	0	0	0
Chaetomium funicola	0	0	0	p<0.01**	0	0	0
Cladosporium cladosporoides/pseudocladosporoides	0	0	0	0	0	0	0
Epicoccum sorghinum	0	0	0	0	0	0	0
Fusarium equiseti/incarnatum	0	0	0	0	0	0	0
Fusarium oxysporum	0	0	p<0.05*	0	0	0	0
Humicola fuscoatra	0	0	0	0	0	0	0
Lecythophora sp.	0	0	0	0	0	0	0
Leptodontidium orchidicota	0	0	0	0	0	0	0
Mortierella sp.	0	0	0	0	0	0	0
Mucor fragilis/circinelloides	0	0	0	0	0	0	0
Nirospora zimmermanii/sphaerica	0	0	0	0	0	0	0
Oidiodendron flavum/griseum	0	0	0	0	0	0	0
Paecilomyces tenuis	0	0	0	0	0	0	0
Penicillium sp. A	0	0	0	0	0	0	p<0.05*

Penecillium sp. B	p<0.05*	p<0.01**	0	0	0	0	0
Penicillium crustosum	0	0	0	0	0	0	0
Penicillium rolfsii/levitum	0	0	0	0	0	0	0
Pilidium concavum/lythri	0	0	0	0	0	0	0
Pleurostomophora richardsiae	0	0	0	0	0	0	0
Schizophllum cf. commune	0	p<0.05*	0	0	0	0	0
Schizophllum commune	0	0	0	0	0	0	0
Sporomiella sp.	0	0	0	0	0	0	0
Talaromyces cf. verruculosus	0	0	0	0	0	0	0
Talaromyces pinophilus/verruculosus	0	0	0	p<0.01**	0	0	p<0.01**
Thielavia hyrcaniae/terricola	p<0.01**	0	0	0	0	0	0
Trametes hirsuta	0	0	0	0	0	0	0
Trichoderma gamsii	0	0	0	0	0	0	0
Trichoderma hamatum	0	0	0	0	0	0	0
Trichoderma harzianum/lixii	0	0	0	0	0	0	0
Trichoderma longibrachiatum/saturnisporum	0	0	0	0	0	0	0

Supplementary Table 2a: Summary of available literature for putative endophytic bacterial identities (16S region), reporting if species are known plant endophytes as well as potential interactions and effects on host plants.

Most likely bacterial identification (16S)	Known plant endophyte	Interaction with host plant	Effects on host plant as documented in available literature	Literature sources
Genus: *Anthrobacter*				
Anthrobacter phenanthrenivorans	No	No	NA	Kallimanis *et al*., 2011
Genus: *Bacillus*				
Bacillus aryabhattai	Yes	Beneficial	Nitrogen (N)-fixation Mobilization of zinc, Iron (Fe)-acquisition, Phosphate (P)-solubilisation	Gulati *et al*., 2011 Ramesh *et al*., 2014 Pereira *et al*., 2016
Bacillus bataviensis	Yes	Beneficial	N-fixation, Improved availability of N	Arvind *et al*., 2011 Gulati *et al*., 2011
Bacillus cereus/thuriengensis	Yes Yes	Beneficial Beneficial	*B. cereus:* N-fixation, Various plant growth promoting mechanisms, Fe-acquisition, P-solubilisation, Anti-pathogenic fungal activity *B. thuriengensis:* Insecticidal activity, plant growth promotion, improved availability of nitrogen, Fe-acquisition	Handelsman *et al*., 1999; Downing *et al*., 2000; Bai *et al*., 2003; Cho *et al*., 2007; Arvind *et al*., 2011; Hussie *et al*., 2011; Hertlein *et al*., 2014; Ibrahim *et al*., 2014; Castillo-Arteaga *et al*., 2016 Sauka *et al*., 2017
Bacillus drentensis/niancini	Yes Unknown	Beneficial Unknown	*B. drentensis:* Improved availability of N, Fe-acquisition, P- solubilisation, *B. niancini:* Unknown	Pereira *et al*., 2016
Bacillus foraminis	No	NA	NA	Arora *et al*., 2014
Bacillus licheniformis	Yes	Beneficial	N-fixation P-solubilization	Xie *et al*., 1998; Probanza *et al*., 2001; Rojas *et al*., 2001; Nongkhlaw *et al*., 2014
Bacillus megaterium	Yes	Beneficial	N-fixation	Ding *et al*., 2005;

			P-solubilization	López-Bucio *et al*., 2007; Kang *et al*., 2014
Bacillus mycoides				
Bacillus safensis/pumilus	Yes Yes	Beneficial Beneficial	*B. safensis* : Possible N-fixation, Improved availability of N *B. pumilus:* Plant growth promoting properties	Mclnroy and Kloepper, 1995; Malfanova *et al*., 2011; Mufti *et al*., 2015
Bacillus simplex	Yes	Beneficial	P-solubilization Anti-fungal activity Co-inoculation strategy to improve N-fixation	Rashid *et al*., 2012; Schwartz *et al*., 2013
Bacillus sp A	Unknown	Unknown	NA	NA
Bacillus subtilus/siamensis	Yes Yes	Beneficial	*B. subtilus*: P- solubilisation, Fe-acquisition *B. siamensis*: N-fixation, Phosphate solubilisation, Anti-fungal activity	Zhang *et al*., 2008; Zhang *et al*., 2010; Hertlein *et al*., 2014; Nongkhlaw *et al*., 2014; Xu *et al*., 2014
Genus: *Burkholderia*				
Burkholderia sp/graminis?	Unknown No	Unknown Beneficial	*B. graminis:* Tolerance to salt and drought stress	Viallard *et al*., 1998; Pereyra *et al*., 2005;
Burkholderia sp?/endophytic bacterium	Yes	Unknown	NA	NA
Genus: *Luteibachter*				
Luteibachter jiangsuensis /rhizovicina	No No	No Beneficial	*L. jiangsuensis:* NA *L. rhizovicina:* PCB-degrading properties associated with plant roots	Wang *et al*., 2011 Cardinale *et al*., 2006
Genus: *Lysinicacillus*				
Lysinibacillus parviboronicapiens	No	No	NA	Miwa *et al*., 2009
Lysinicacillus sphaericus	No	No	NA	Ahmed *et al*., 2007
Genus: *Paenibacillus*				
Paenibacillus alginolyticus	Yes	Unknown	Unknown	Kobayashi *et al*., 2015
Paenibacillus alvei	Yes	Beneficial	Biocontrol properties against phytopathogens and insect herbivores	Antonopoulos *et al*., 2008; Phi *et al*., 2010;
Paenibacillus chitinolyticus/agaridevorans	No	NA	NA	NA
Paenibacillus cineris	Yes	Beneficial	Anti-fungal activity	Paul *et al*., 2013
Paenibacillus lautus	Yes	Beneficial	N-fixation, plant growth promoting effects	Gulati *et al*., 2011, Seldin, 2011,Grady *et al*., 2016,
Paenibacillus taichungensis/xylanexedens	Yes	Beneficial	*P. taichungensis:* N-fixation *P. xylanexedens:*	Gulati *et al*., 2011
Genus: *Pantoea*				
Pantoea agglomerans/vagans	Yes Yes	Beneficial Beneficial	*P. agglomerans:* Possible N-fixation, plant growth promoting effects *P. vagans*: Possible N-fixation, P- solubilisation	Verma *et al*., 2001 Feng *et al*., 2006
Genus: *Pseudomonas*				
Pseudomonas frederiksbergensis	Yes	Beneficial	Phosphate solubilisation, Fe-acquisition, Improved availability of N	Pereira *et al*., 2016

Pseudomonas sp A	Unknown	Unknown	NA	NA
Pseudomonas sp B	Unknown	Unknown	NA	NA
Pseudomonas sp C	Unknown	Unknown	NA	NA
Genus: *Xanthomonas*				
Xanthomonas arboricola/campestris	Yes Yes	Pathogenic Pathogenic	*X. arboricola*: Bacteria spot disease in fruit trees, *X. campestris:* Known soybean pathogen	Ritchie, 1995; Pagani, 2005; Mingma *et al.*, 2014
Uncultured bacterium	Unknown	Unknown	NA	NA

Supplementary Table 2b: Summary of available literature for putative endophytic fungal identities (ITS region), reporting if species are known plant endophytes as well as potential interactions and effects on host plants.

Putative fungal identification (ITS)	Known plant endophyte	Interaction with host plant	Effects on host plant as documented in available literature	Literature sources
Genus: *Acremonium*				
Acremonium sclerotigenum/alternatum	No No	Pathogenic Pathogenic	*A. sclerotigenum:* Known apple fruit pathogen *A. alternatum:* Powdery mildew pathogen	Li *et al.*, 2014; Malathrakis *et al.*, 1985
Genus: *Alternaria*				
Alternaria alternaria	Yes	Pathogenic	Leaf spot disease	Kamble *et al.*, 2000
Genus: *Aspergillus*				
Aspergillus brasiliensis	No	None	Black mold	Samson *et al.*, 2001
Aspergillus chevalieri/amstelodami	No No	None Unknown	*A. chevalieri:* Mold *A. amstelodami:* Unknown	Chen *et al.*, 2017
Aspergillus nidulans	No	Unknown	NA	NA
Aspergillus sp.A	Unknown	Unknown	NA	NA
Aspergillus sp. B	Unknown	Unknown	NA	NA
Genus: *Chaetomium*				
Chaetomium aureum	Yes	Beneficial	Reduces damages caused by pathogenic *Fusarium*	Martínez-Álvarez *et al.*, 2016
Chaetomium cf. funicola	Unknown	Unknown	NA	NA
Chaetomium funicola	No	Unknown	NA	NA
Genus: *Cladosporium*				
Cladosporium cladosporoides/pseudocladosporoides	No No	None None	*C. cladosporoides:* Mold *C. pseudocladosporoides:* Mold	Bensch *et al.*, 2010 de Sangre *et al.*, 2014
Genus: *Epicoccum*				
Epicoccum sorghinum	No	None	Grain mold	Oliveira *et al.*, 2017
Genus: *Fusarium*				
Fusarium oxysporum	Yes	Pathogenic	Rot-causing pathogen	Lagopodi *et al.*, 2002
Genus: *Humicola*				
Humicola fuscoatra	No	Beneficial	Anti-fungal activity	Wicklow *et al.*, 1998
Genus: *Lecythophora*				
Lecythophora sp.	Unknown	Unknown	NA	NA
Genus: *Leptodontidium*				
Leptodontidium cf. orchidicota	Unknown	Unknown	NA	NA
Genus: *Mucor*				
Mucor fragilis/circinelloides	Yes	Unknown	*M. fragilis:* Podophyllotoxin	Huang *et al.*, 2014

	No	None	production *M. circinelloides:* None	Sun *et al*., 2017
Genus: *Nirospora*				
Nirospora zimmermanii/sphaerica	Unknown No	Unknown Beneficial	*N. zimmermanii:* Unknown *N. sphaerica:* Anti-fungal activity	Kim *et al*., 2001
Genus: *Oidiodendron*				
Oidiodendron flavum/griseum	No Yes	None Unknown	*O. flavum:* None *O. griseum:* Endophyte of ericoid mycorrhiza	Adhikari *et al*., 2014 Couture *et al*., 1983
Genus: *Paecilomyces*				
Paecilomyces tenuis	No	No	NA	Han *et al*., 2007
Genus: *Penicillium*				
Penicillium sp A	Unknown	Unknown	NA	NA
Penecillium sp. B	Unknown	Unknown	NA	NA
Penicillium crustosum	No	None	Blue-grey mold	De Jesus *et al*., 1981
Penicillium rolfsii/levitum	No Yes	None Unknown	*P. rolfsii:*mold *P. levitum:* Unknown	Peterson *et al*., 2004 El-Morsy, 2000
Genus: *Pleurostomophora*				
Pleurostomophora richardsiae	Yes	Pathogenic	Branch dieback and collar rot	Ivic and Tomic, 2018
Genus: *Schizophllum*				
Schizophllum cf. commune	Unknown	Unknown	NA	NA
Schizophllum commune	No	Saprophyte	Found on decaying trees	Taylor *et al*., 2006
Genus: *Sporomiella*				
Sporomiella sp	Unknown	Unknown	NA	NA
Genus: *Talaromyces*				
Talaromyces cf. verruculosus	Unknown	Unknown	NA	NA
Talaromyces pinophilus/verruculosus	Yes No	Beneficial None	*T. pinophilus:* Insecticidal activity *T. verruculosus:* None	Vinale *et al*., 2017
Genus: *Thielavia*				
Thielavia hyrcaniae/terricola	Unknown Unknown	Unknown Unknown	*T. hyrcaniae:* Unknown *T. terricola:* Unknown	NA
Genus: *Trametes*				
Trametes hirsuta				
Genus: *Trichoderma*				
Trichoderma gamsii	Yes	Beneficial	P-solubilization, plant growth promotion, biocontrol properties	Rinu *et al*., 2014
Trichoderma hamatum	Yes	Beneficial	Plant growth promotion, biocontrol properties	Mora´n-Diez *et al*., 2009 Studholme *et al*., 2013
Trichoderma harzianum/lixii	Yes Yes	Beneficial Beneficial	*T. harzianum:* Anti-pathenogenic fungal activity *T. lixii:* Seed-biopriming agent	Yedidia *et al*., 1999 Pehlivan *et al*., 2017
Trichoderma longibrachiatum/saturnisporum	Yes Yes	Beneficial Beneficial	*T. longibrachiatum:* Anti-pathenogenic fungal activity *T. saturnisporum:* Biological control agent	Martı´nez *et al*., 2001 Martı´nez *et al*., 2005

Declaration by the candidate

With regards to Chapter 4 (Metabarcoding of *Oxalis* bacterial endophytes: caveats associated with sequencing and data interpretation), the nature and scope of my contribution were as follows:

Nature of contribution	Extent of contribution (%)
Sampling and preparation of *Oxalis* host plant tissues for metabarcoding conducted at CAF (Stellenbosch University)	100
Data analysis, interpretation and manuscript preparation	80

The following co-authors have contributed to Chapter 4:

Name	e-mail address	Nature of contribution	Extent of contribution (%)
Francois Roets	Copyright	Provided guidance, especially in terms of data analysis, and edited the manuscript	5
Guy F. Midgley	Copyright	Provided guidance and edited the manuscript	5
Kenneth C. Oberlander	[redacted]	Provided guidance, especially in terms of data analysis, and edited the manuscript	5
Léanne L. Dreyer	Copyright	Provided guidance, funding and edited the manuscript	5

Signature of candidate: .....……

Declaration by co-authors:

The undersigned hereby confirm that

1. The declaration above accurately reflects the nature and extent of the contributions of the candidate and the co-authors to Chapter 4.
2. No other authors contributed to Chapter 4 than those specified above.
3. There are no conflicts of interest relevant to Chapter 4 of this dissertation.

Signature	Institutional affiliation	Date
Francois Roets	Stellenbosch University	December 2018
Guy F. Midgley	Stellenbosch University	December 2018
Kenneth C. Oberlander	University of Pretoria	December 2018
Léanne L. Dreyer	Stellenbosch University	December 2018

Chapter 4 Metabarcoding of *Oxalis* bacterial endophytes: caveats associated with sequencing and interpretation of data

ABSTRACT

A previous study on Cape *Oxalis* plants has established that diverse communities of culturable endophytic bacteria and fungi, many with known plant growth-promoting traits, are associated with these hosts. Given the known limitation of agar culture methods, estimated to yield only 1-5% of true endophytic diversity, we aimed to assess more accurate endophytic diversity levels through a16S bacterial metabarcoding approach. We studied bacterial endophytes from various surface-sterilized and macerated plant organs using six universal 16S bacterial markers and the Ion Torrent™ metabarcoding platform. Sequence results revealed that 65-95% of all sequence reads (contigs) were plant plastids or mitochondria. As a result, potential bacterial endophyte reads were often low. Furthermore, the recommended 16S curated GreenGenes microbial databases for the Ion Reporter™ metabarcoding platform could not accurately distinguish between plastids and potential endophytic bacterial contigs. Sequence identities were therefore verified using three other databases (namely NCBI, SILVA and RDP). Based on consistent results and sequence identities, we have preliminarily identified taxa from 118 genera, as well sequence matches with various uncultured bacteria, from 79 families, 39 orders and 19 classes that belong to eight bacterial phyla. Despite various caveats associated with this approach, it did help us gain significant insight into the rich treasure trove of bacterial endosymbionts associated with Cape *Oxalis* host plants.

Keywords: GreenGenes, Ion Reporter ™, Ion Torrent™ metabarcoding, microbiome, NCBI, non-culturable endophytes, *Oxalis* symbionts, RDP, SILVA, universal 16S

INTRODUCTION

All terrestrial plants are believed to have associations with at least one endophytic bacterial or fungal species (Smith *et al*., 2008; Rodriguez *et al*., 2009; Nair and Padmavathy, 2014). The geophytic Cape *Oxalis* lineage is known to host a large diversity of culturable bacterial and fungal endosymbionts, greatly exceeding average endophyte diversity observed among most plant taxa (Jooste *et al*., 2018; Chapter 3). At least one bacterial endophyte was isolated from all studied *Oxalis* hosts (90 plants), regardless of location, host species or organ type studied.

On average, we recovered 23 culturable endophytic bacterial and 20 culturable endophytic fungal species per *Oxalis* host species, which is an order of magnitude higher than commonly reported for endophytes isolated and cultured from plant species (Petrini, 1986; Smith *et al*., 2008; Rodriguez *et al*., 2009; Nair and Padmavathy, 2014). Our richness estimates indicated that these values may be even higher for some *Oxalis* host species and plant organs, especially in terms of fungal endophytes.

Studying the total endophyte microbiome of Cape *Oxalis* is regarded as an ecologically important pursuit. Many of the endophyte species revealed by culture-dependent techniques have metabolic and physiological properties that may result in complementary or antagonistic relationships among some members of the *Oxalis* microbiome. Bacterial endophytes (including *Bacillus* Cohn, *Paenibacillus* Ash *et al*., *Pantoea* Gavini *et al*. and *Pseudomonas* Migula species) have various documented phosphate solubilisation, atmospheric nitrogen-fixation and anti-fungal properties (Ding *et al*., 2005; Feng *et al*., 2006; Gulati *et al*., 2011; Paul *et al*., 2013; Ramesh *et al*., 2014; Pereira *et al*., 2016). Fungal endophytes (including *Cladosporium* Link, *Talaromyces* C.R. Benji and *Thielavia* Zopf and *Trichoderma* Rifai species) have known beneficial plant growth-promoting, phosphate -solubilization, biocontrol and anti-fungal properties to reduce damage caused by pathogenic fungi (Wicklow *et al*., 1998; Martínez-Álvarez *et al*., 2016). However, the majority of fungal species isolated from *Oxalis* hosts were most likely opportunistic colonizers from the soil or known plant pathogens (such *as Aspergillus* Micheli, *Chaetomium* Kunze and *Fusarium* Link) (Rodriguez-Galvez and Mendgen, 1995; Schulz *et al*., 1999; Vu *et al*., 2006). It is highly likely that these bacterial and fungal endophytes (whether they are mutualistic, commensalistic or parasitic symbionts) interact and influence one another. In order to fully understand the complexity of the *Oxalis*-endosymbiont system, it is crucial to identify as many participants as possible.

Historically, most studies focused on bacterial and/or fungal plant endophytes used agar culture-based techniques (as reviewed in Hardoim *et al.*, (2015)). However, there are many known limitations associated with these techniques, principally that they provide a limited and highly biased snapshot of true endophyte diversity. Reportedly a mere 1–5% of true plant endophytic diversity is revealed through culture-dependent techniques (Guo *et al*., 2001; Duong *et al*., 2006; Hyde and Soytong, 2008). Some bacteria and fungi are known to be fastidious, and do not successfully grow on agar media, while other endophytes may thrive and dominate agar plates (Guo *et al*., 2001; Duong *et al*., 2007; Zhu *et al*., 2008). Recent

technological developments in the field of high-throughput DNA metabarcoding (culture-independent techniques) have revolutionized our current understanding of plant endosphere microbiomes (Shendure and Ji, 2008; Metzker, 2010; Caporaso *et al*., 2011; Hardoim *et al*., 2015). 16S DNA metabarcoding has fuelled large microbial community studies in various fields, including plant microbiomes (Gottel *et al*., 2011; Mendes *et al*., 2011; Bulgarelli *et a*l., 2012; Lundberg *et al*., 2012; Peiffer *et al*., 2013; Shakya *et al*., 2013; Edwards *et al*., 2015). Metabarcoding allows unprecedented resolution and investigation of complex microbiomes by exploring, identifying and characterizing genetic and/or metabolic components involved in plant-endophytic interactions (Hardoim *et al*., 2015; Bengtsson-Palme *et al*., 2018). These methods allow much larger sample sizes and support deeper analyses and investigation of endophytic microbial communities (Knief, 2014).

A broad criticism of studies on plant-endophyte interactions is that they are mostly based on plants grown under controlled and optimized conditions, which often do not represent realistic field conditions (Hardoim *et al*., 2015). Here we aimed to study culture-independent bacterial endophytes associated with non-crop plant species sampled in the wild. *Oxalis* plants are adapted to surviving various biotic and abiotic stressors associated with the Cape climate and lithology. *Oxalis* and their endophytes therefore offer an ideal model system to study the diversity and nature of host-microbe relationships, and explore possible implications of such associations for their physiological survival in these habitats. Understanding these interactions may be vital to the long-term conservation of this large and variable angiosperm genus that constitutes a major component of the Cape Flora (Manning and Goldblatt, 2012).

The *Oxalis*-endophyte system also provides a useful basis to explore the potential advantages and pitfalls of metabarcoding compared to culture-based sampling methods (Jooste *et al*., 2018; Chapter 3). Given the wealth of culturable microbial endophytes associated with Cape *Oxalis* hosts, and the known underestimation of culture-based techniques, it is reasonable to expect that culture-independent metabarcoding techniques may reveal a larger and more diverse endophytic community. This study aimed to investigate the bacterial endophyte richness among vegetative and reproductive plant organs from representative Cape *Oxalis* hosts sampled from three locations, by implementing culture-independent Ion Torrent™ metabarcoding techniques. *Oxalis* plants are also known to host diverse communities of fungal endophytes, which may also be of critical importance to host plants, but they were not

the focus of this study. The objectives of this study were to characterise the taxonomic richness of bacterial endophytes obtained by metabarcoding techniques, and compare this to the richness of culturable bacterial endophytes associated with the same six host species, as determined in a previous study on Cape *Oxalis* (Jooste *et al*., 2018; Chapter 3). Other than the study by Miyambo *et al*. (2016), to the best of our knowledge our study is only the second assessment of culture-independent bacterial endophyte richness among angiosperm host plants from the Cape Flora.

MATERIALS AND METHODS

Sampling design and sterilization protocol

Endophytic bacterial communities were studied from two representative *Oxalis* species (one recalcitrant species, *O. hirta* L., and one dormant species, *O. pes-caprae* L.) sampled from three locations (Malmesbury [-33.481121, 18.753625], Stellenbosch [-33.932358, 18.874571] and Tulbagh [-33.311688, 19.096747]) in the Western Cape Province, South Africa (Cape Nature Conservation Board Permit No. 0028-AAA088-00243). Four additional *Oxalis* species were sampled from Stellenbosch: two recalcitrant species, *O. glabra* Thunb. and *O. tenuifolia* Jacq., and two dormant species, *O. obtusa* Jacq. and *O. purpurea* L. Three individuals of each species (at least 10 m apart), with no external signs of microbial infections (asymptomatic), were collected during May-June 2017. Plants were collected and processed following the same protocol as described in Chapter 3, (Jooste *et al.*, 2018). Plant organs from Malmesbury and Tulbagh were divided into two batches: seeds separately and all other organs combined (including roots, bulbs, stems and leaves). This design enabled the detection of potentially laterally transferred non-culturable bacteria. Plants from Stellenbosch were divided into three batches: seeds separately, bulbs separately and all other organs combined (including roots, stems and leaves) (Table 1). This sample design allowed valuable, yet cost effective, comparisons of vegetative organs relative to vegetative reproductive (bulbs) and sexually reproductive (seed) organs. Insights gained from such comparisons enabled an assessment of transfer of endophytic associations across years (bulbs) and generations (seeds). After surface sterilization (as described in Jooste *et al.*, 2018; Chapter 3) all plant material was placed in sterile Eppendorf tubes (with lids ajar and tissue paper on top), and oven dried at 50°C for two weeks. This extended drying of plant material attempted to minimize the effects of oxalates interfering with and degrading DNA.

Table 1: Study sample design including 78 samples from six host species, three sampling locations and all vegetative and reproductive plant organs.

Site 1: Tulbagh	Site 2: Malmesbury	Site 3: Stellenbosch
O. hirta	***O. hirta***	***O. hirta***
x 3 plants (organs + seeds)	x 3 plants (organs + seeds)	x 3 plants (non-bulb organs + bulbs + seeds)
O. pes-caprae	***O. pes-caprae***	***O. pes-caprae***
x 3 plants (organs + seeds)	x 3 plants (organs + seeds)	x 3 plants (non-bulb organs + bulbs + seeds)
		O. glabra
		x 3 plants (non-bulb organs + bulbs + seeds)
		O. obtusa
		x 3 plants (non-bulb organs + bulbs + seeds)
		O. purpurea
		x 3 plants (non-bulb organs + bulbs + seeds)
		O. tenuifolia
		x 3 plants (non-bulb organs + bulbs + seeds)
Total: 12 samples	**12 samples**	**54 samples**

Sample processing - Central Analytical Facility, Stellenbosch University

All samples were submitted to the Central Analytical Facility (CAF) at Stellenbosch University. All DNA extractions, quality control, library preparation, enrichment and sequencing of samples for this project were conducted by CAF. Total DNA was extracted from each prepared sample using the Ion 16S™ Metagenomics Kit (ThermoFisher Scientific). DNA extractions were done in triplicate and the three sub-samples were pooled. Purity and concentration of DNA was assessed with a NanoDrop 2000 (NanoDrop, ThermoFisher Scientific). Various hypervariable regions of the 16S bacterial metagenomic DNA were amplified using two primer sets (Primer set V2-4-8 and Primer set V3-6, 7-9 from ThermoFisher Scientific), which allow sequence-based identification of a broad range of bacteria from environmental samples (Ion 16S™ Metagenomics Kit, ThermoFisher Scientific). The PCR reaction mixture consisted of 2 µL extracted DNA, 15 µL 2X Environmental Master Mix (ThermoFisher Scientific), 3 µL 16S Primer Set (10X) and 10 µL water. PCR proceeded using the following program: 95°C for 10 min, followed by 25 cycles of 95°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec, followed by an extension step at 72°C for 7 min. The amplification products were purified and DNA input for library preparation was calculated.

For library preparation, the Ion Plus Fragment Library Kit (ThermoFisher Scientific) was used for end repair, ligation and nick-repair and amplicon purification. The Ion Universal

Library Quantitation Kit (ThermoFisher Scientific) was used to determine library concentration and the Ion PGM™ Hi-Q™ OT2 Kit (ThermoFisher Scientific) was used for template preparation. The Ion PGM™ Hi-Q™ Sequencing Kit (ThermoFisher Scientific) was used for sequencing. A total of 78 samples were run on two S5 530 chips, capable of yielding 8 million reads per chip.

All generated sequence reads were imported as VCF files into the Ion Reporter™ 5.0 Software by CAF. The 16S metagenomics workflow for multiple samples from the Ion Reporter bioinformatics pipeline was used for sequence processing. Primer sequences were removed and the curated GreenGenes reference database (McDonald *et al*., 2012) was used for sequence identification with 98% sequence similarity. Suitable sequence reads (contigs) were classified into OTU's (Operational Taxonomic Units). Krona Tools (http://krona.sourceforge.net) were used to construct krona plots that provide an ordered diagrammatic view of taxonomic richness.

RESULTS

Cyanobacteria vs. *Oxalis* host plastids and/or mitochondria

On average, between 4.9 and 632.4 contig sequence reads were obtained from six variable regions per plant sample. Variable regions V2, V3, V67 and V8 consistently had relatively high read numbers (average 411.9 to 632.4 reads), while V4 (average 67.9 reads) and V9 (average 4.9 reads) had much lower read numbers per sample (Figure 1).

The majority (63.4% to 96.7%) of these sequence reads for five of the six variable regions were identified as cyanobacteria by the Ion Reporter 16S workflow and GreenGenes database. The comparison of these 'cyanobacteria' sequences to *Oxalis* chloroplast sequences and other databases (NCBI, SILVA and RDP) revealed that these contigs were in fact plastids or mitochondria of the host plant (96-100% similarity) and not cyanobacteria (only 81 to 86% similarity). These plastid/mitochondrial 16S reads (hereafter called host reads) dominated the output across most regions (V2=96.7% of all reads, V3=93.9%, V4=63.4%, V67=95.5%, V8=95.2%). V9 was the only region that did not yield host 16S reads, but this region had very low reads in total (average 4.9 reads per sample). Upon excluding host reads, remaining reads per sample were much reduced (average values 4.9-37.8). This pattern was consistent across all samples, including all combined vegetative organ (roots, bulbs, stems and leaves),

bulbs only and seeds only samples (Figure 1a-c), except for V4 reads from bulbs only that yielded relatively higher bacterial endophyte reads than host reads.

It is possible that the high number of host 16S sequences in the original samples may have overwhelmed any endophyte signal. If so, we would expect a negative correlation between number of host and non-host reads. A negative correlation was detected for the total number of reads across all variable regions ($r=-0.654$, $t=-7.38$, $df=73$, $p<0.001$; Figure 2a), which indicated that samples with high numbers of host reads had low bacterial reads and *vice-versa*. Similarly, negative correlations were detected among sequences from V2, V4, V67 and V8 when considered individually (Figure 2b-f). No relationship was detected among reads from V3 ($r=0.02$, $t=0.17$, $df=73$, $p>0.05$; Figure 2c) and relationships for V9 were not assessed as this variable region did not yield any host reads.

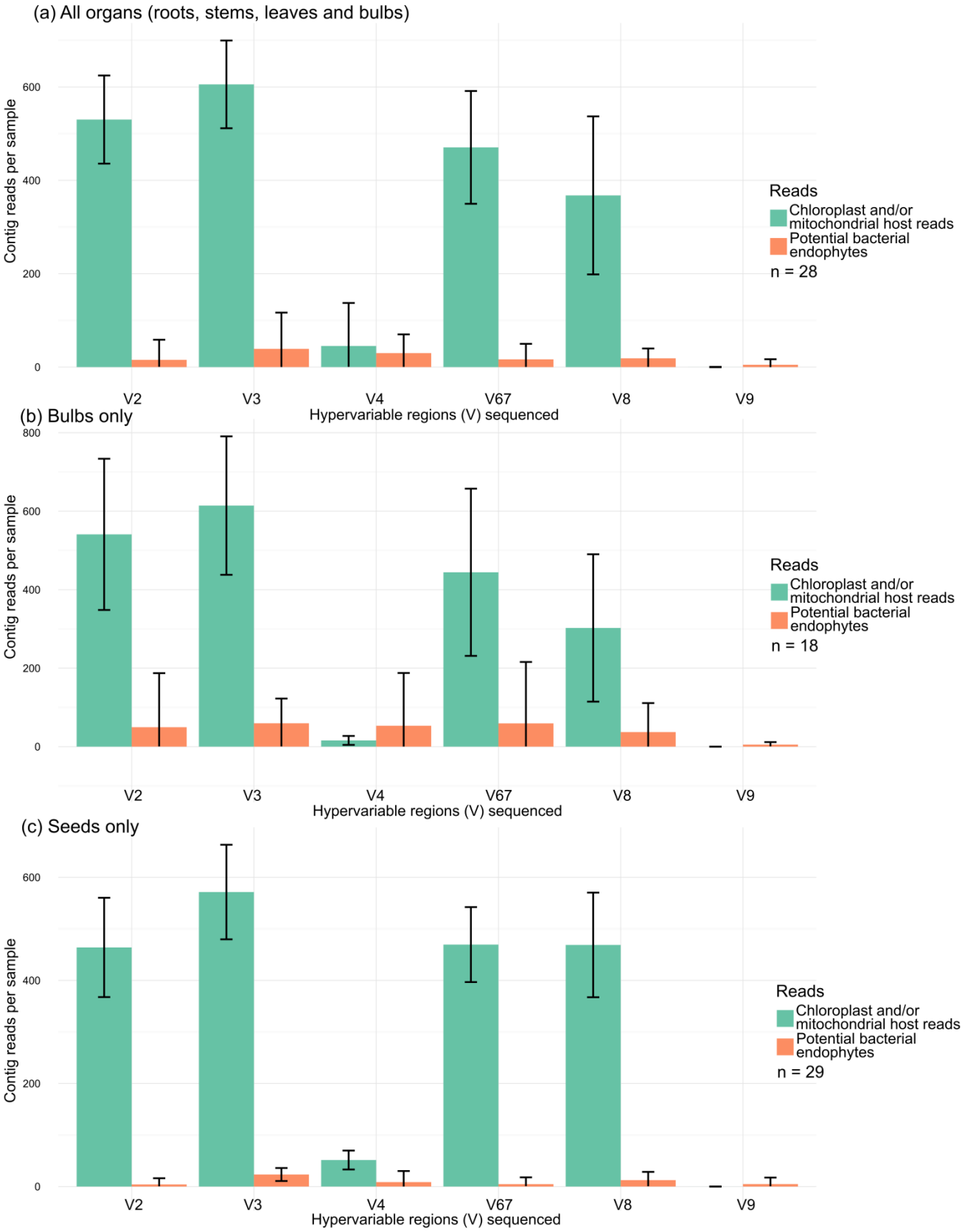


Figure 1: Figure 1: Number of host and non-host (*i.e* potential endophytic bacterial) reads per sample, for each sequenced region. (a) All vegetative plant organs (samples containing roots, bulbs, stems and leaves), (b) samples from bulbs only and (c) samples from seeds only. The number of plant samples considered is indicated below each colour key.

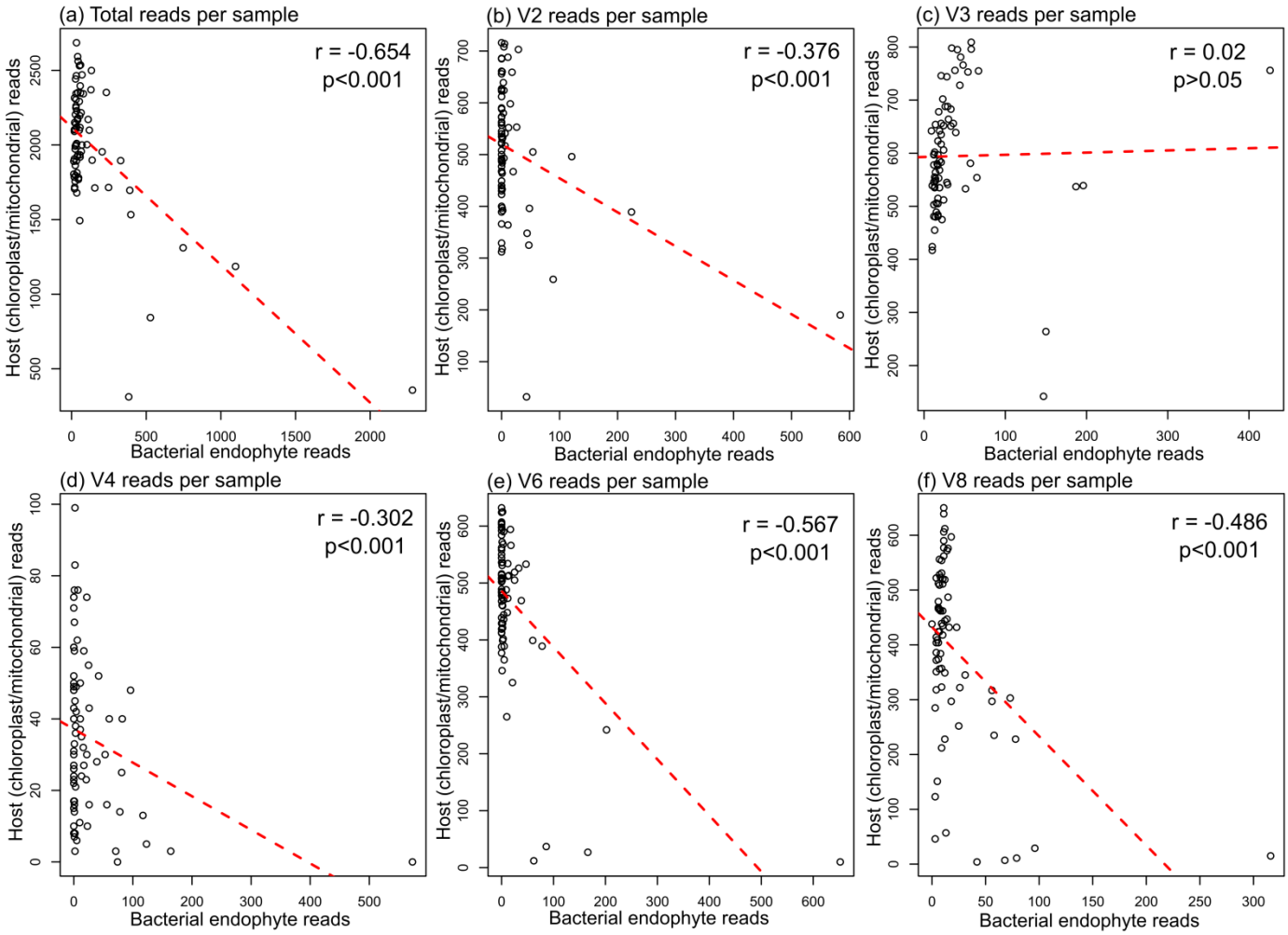


Figure 2: Correlations between number of host and bacterial endophyte contig sequence reads per sample. (a) Total reads per sample (across all variable regions), (b) V2, (c) V3, (d) V4, (e) V67, (f) V8. Pearson's correlation statistics (r) and linear regression lines of best fit (red dashes) are indicated for each plot.

Number of samples per chip

A total of 78 plant samples were submitted for sequencing on two chips (Ion S5 530 chips can accommodate up to 96 samples (Ion 530 Kit-Chef, ThermoFisher Scientific)), therefore only 39 samples were run per chip to ensure that enough wells per chip were available to sequence bacterial DNA (not flooded with host reads only). Due to a technical error three samples (*O. purpurea* [Site 3, plant 1, all organs]; *O. hirta* [Site 3, plant 1, all organs]; *O. hirta* [Site 3, plant 2, seeds]) did not yield any reads, and had to be-run on another chip, which allowed a unique opportunity to assess the number of host and bacterial reads generated from the relatively 'full' and 'empty' chips.

The three re-run samples yielded an average of 10-3385 contig sequence reads per variable region across all plant samples. Variable regions V2, V3, V67 and V8 consistently had relatively high numbers of reads (average 1873 to 3385), while V4 (average 733 reads) and V9 (average 10 reads) had relatively few reads per sample (Figure 3). These three re-run samples yielded proportionately similar numbers of reads per variable region relative to the original samples. The three re-run samples similarly also yielded high numbers of host reads from most variable regions (average V2=85.5%, V3=93.6%, V4=74.9%, V67=96.1%, V8=95.7%), but again none were obtained from the V9 region. The remaining numbers of potential endophytic bacterial contig reads per samples were still relatively low compared to the number of host reads (average 10-216 reads per variable region across all samples), but with notably higher number of bacterial reads than in the original samples. These results indicate that higher number of potential bacterial endophyte reads could have been obtained by decreasing the initial number of samples run per chip. Once all host reads were confirmed, all of the host sequences were excluded from subsequent analyses.

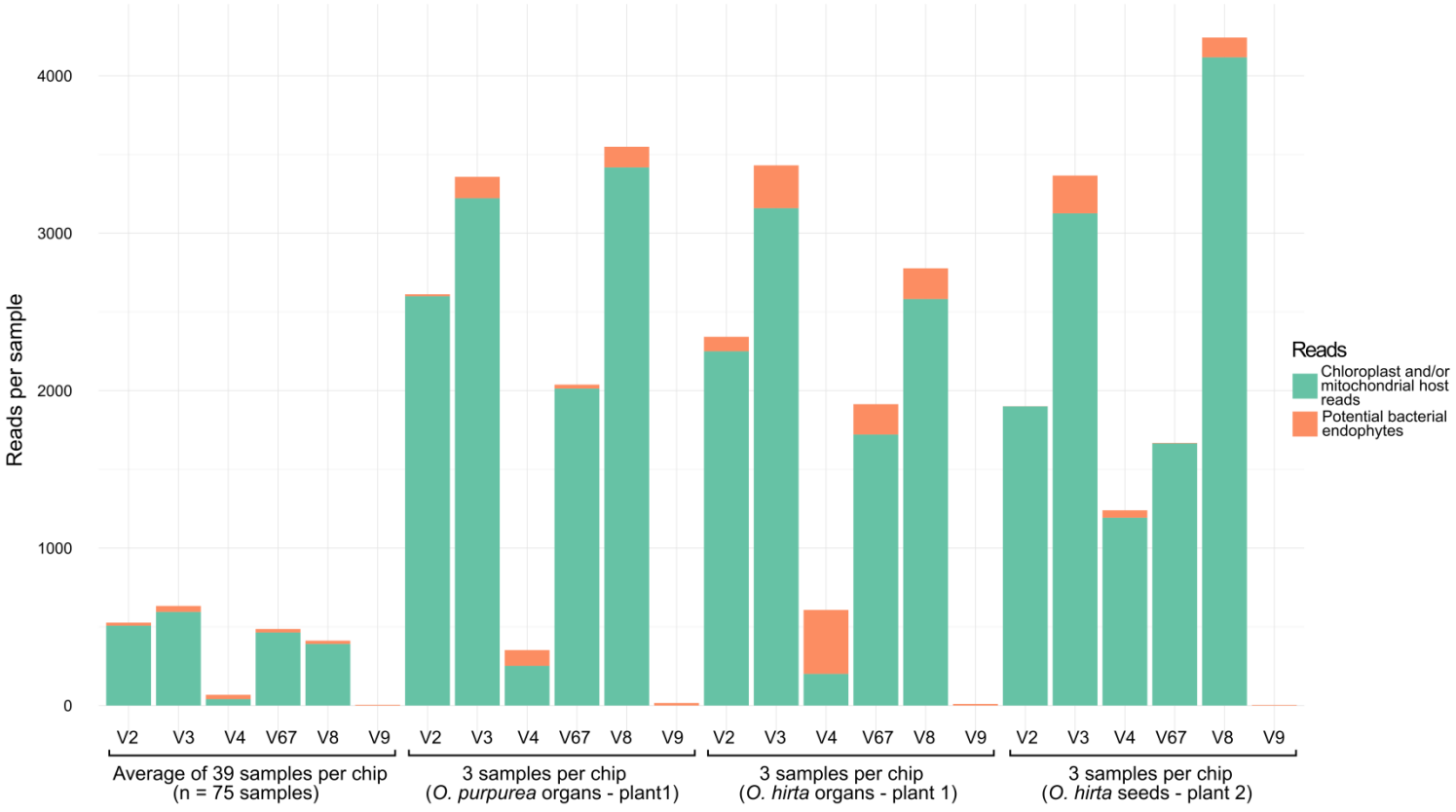


Figure 3: Number of contig sequence reads from three re-run samples (three samples per chip) relative to all original samples (39 samples per chip), indicating the number of host (chloroplast and/or mitochondrial) reads relative to potential endophytic bacterial contigs. The number of sequence reads (±SD) is indicated for the six variable regions.

Phylum-level identification of endophytic bacteria

We observed frequent single base insertions and deletions among the plastid reads generated. These are almost certainly sequencing error as *Oxalis* chloroplasts 16S is highly conserved (Oberlander, unpublished data). Sequence reading errors such as these have previously been described by authors using the Ion Torrent platform (Merriman *et al*., 2012, Quail *et al*., 2012). Due to this variability, the sequence reads from V67 (typically used for 16S bacterial species-level identifications) could not be used for species-level identifications in our study. Single base insertions or deletions may artificially inflate sequence diversity and consequently inflate bacterial endophyte richness among our samples. In order to prevent this artificial inflation, a criterion of 2% variation was used when assigning OTU's (Huse *et al*., 2010) and sequences generated by V4 were used for 16S phylum-level identification (Jovel *et al*., 2016) and sequences generated by V3 were used for 16S genus-level identification (Yang *et al*., 2016).

In order to determine the most likely and reliable endophytic bacterial richness, sequence identification of three databases (NCBI [https://blast.ncbi.nlm.nih.gov/Blast.cgi], SILVA [https://www.arb-silva.de/aligner/] and RDP [https://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp]) was assessed, in addition to the original Ion Reporter GreenGenes identifications. Phylum-level identification of V4 reads (primers designed for phylum-level identification (Jovel *et al*., 2016)) was compared to phylum-level identification of V3 reads (primers designed for genus-level identification and phylum identities were inferred to allow comparison). Total richness among original and re-run samples were compared across all databases and both V3 and V4 regions for all samples studied. Overall, all databases produced similar phylum-level identification results (Figure 4).

The GreenGenes database could not successfully detect additional plant host DNA that were not identified as cyanobacteria (which turned out to be chloroplasts), as mentioned previously. Instead the GreenGenes database assigned bacterial identities to these mitochondria (most likely due to the 16S workflow selected from Ion Reporter). The NCBI, SILVA and RDP databases could, however, successfully identify mitochondria and we could therefore remove these sequences from relevant datasets. Consequently, the number of OTU's from the GreenGenes database (Figure 4a) was higher than the number identified by the other three databases (Figure 4b-c). Between five and 23 mitochondrial OTU's were removed from relevant datasets (Figure 4).

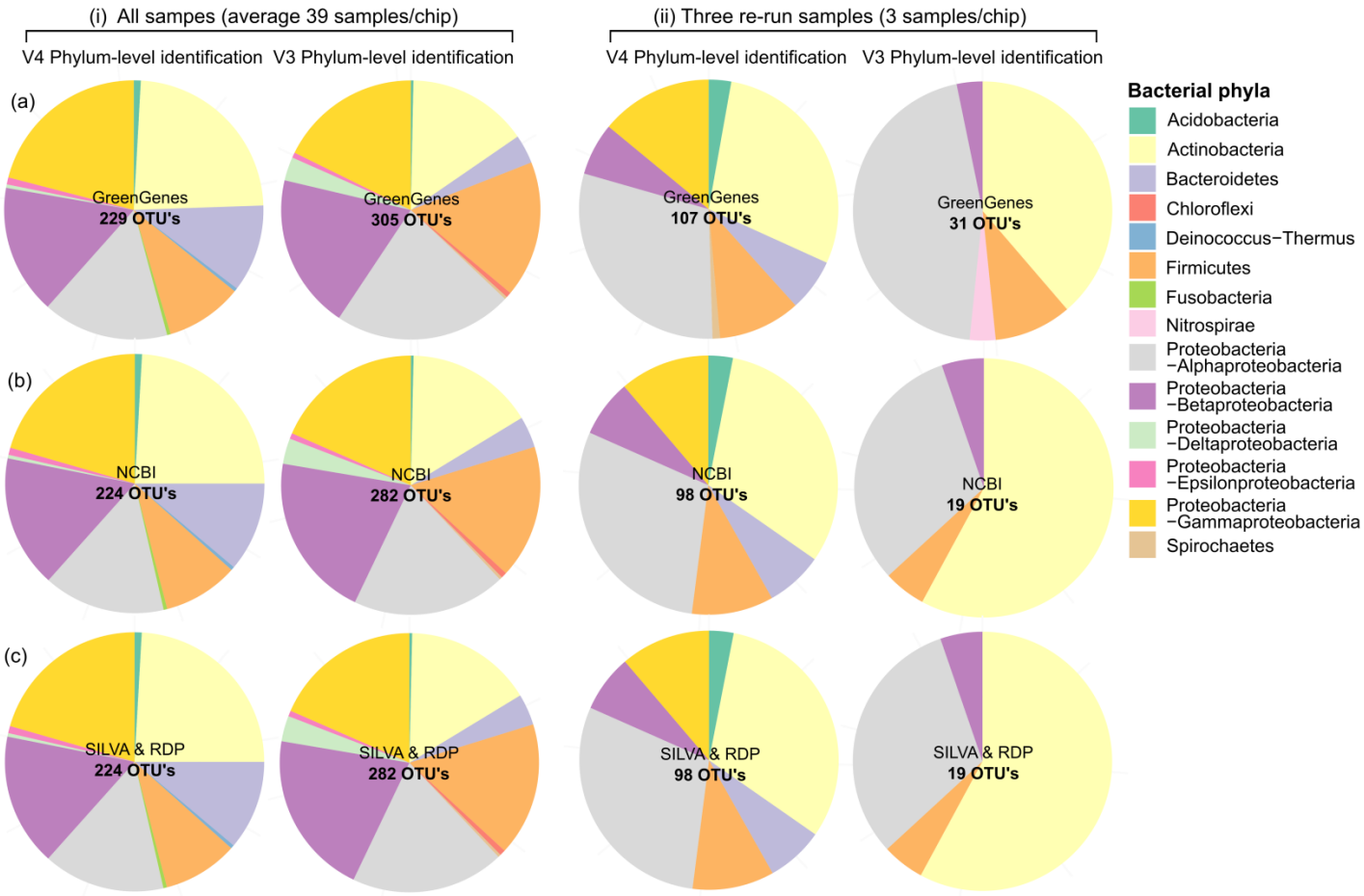


Figure 4: Pie-charts that indicate the relative abundance of unique operational taxonomic units (OTU's) identified to phylum level, based on sequences generated from two 16S variable regions (V4 and V3) from all samples. Sequence identities were established using four databases including GreenGenes (a), NCBI (b), SILVA and RDP (identical results obtained from these two databases, therefore both displayed in c). Phylum-level richness indicated for original samples sequenced with 39 samples per chip (i) and re-run samples with 3 samples per chip (ii). The number of unique OTU's is indicated in the centre of each chart.

As expected, phylum-level identifications of V4 and V3 reads were quite similar among the original samples (X^2=0.272, df=2, p>0.05) (Figure 4i). The relative abundance of OTU's from various bacterial phyla and identities assigned by the four databases were proportionately similar between the two variable regions. V4 reads (224 OTU's in total) were lower than V3 reads (282 OTU's in total), but this was expected as the V3 region of 16S is more variable and therefore more likely to detect sequence variation than V4 (Jover *et al.*, 2016). NCBI often failed to assign a genus-level identity to a bacterial sequence from the V3 reads and matched these sequences with 'uncultured bacteria'. Both SILVA and RDP databases could successfully assign genus-level to the majority (95%) of these sequences (as

both of these databases are specifically curated for 16S microbial identification). The remaining sequences were also identified as 'uncultured bacteria'.

Rather surprisingly, the phylum-level identification of the re-run samples was considerably different, with much higher richness from V4 reads (98 OTU's) (Figure 4ii) relative to the V3 richness (19 OTU's). Due to the striking difference between numbers of OTU's among the three re-run samples, we cautiously assessed the V67 sequence reads (despite the possible single base insertions and deletions) to get an approximation of the most likely OTU counts among the re-run samples. Potential assessment of the V67 reads revealed the same genus-level, and consequently the same phylum-level identities. It therefore seems possible that V4 reads incorrectly inflated sequence diversity. In order to gain the most reliable estimate of bacterial endophyte diversity, we have therefore considered genus-level identities (and inferred phylum-level) of OTU's based on contigs generated from V3 reads.

Despite the higher number of V3 reads generated by the three re-run samples (as seen in Figure 2), these samples did not considerably contribute to higher sequence diversity. The majority of the high number of reads generated was due to duplicates of the same OTU's, which indicated that the number of samples per chip (three vs. 39 samples) did not affect the bacterial endophyte richness data generated. Original and re-run samples could therefore be combined for subsequent analyses of bacterial endophyte richness.

Potential genus-level identification of endophytic bacteria

Despite the various caveats described above, cautious assessment of V3 reads and genus-level identification of OTU's with SILVA and RDP allowed preliminary assessment of bacterial endophyte diversity among samples. All unique OTU sequences could therefore be identified, compared to one another and sequence identities could be verified with SILVA and RDP. The majority of OTU's were successfully identified to genus-level (or closest family-, order- or phylum-level for 'uncultured bacteria') and between 75 to 80% of OTU's could be identified to species level (with less than 2% different from the best match). Assigning species identities based on V3 reads seemed unreliable, but we could use these preliminary identities to gain insights of sequence diversity within given bacterial genera (*e.g.* identify 8 possible unique taxa from the genus *Bacillus* relative to only two unique taxa from the genus *Rhizobium*).

Krona plots were used to visualize the total genus-level endophyte richness and potential relative abundance of genera present among all samples (all host species, sampling location and organ types from original and re-run samples) (Figure 5). The total bacterial endophyte richness associated with *Oxalis* hosts included taxa from 118 identified genera and various uncultured bacteria from 79 families, 39 orders, 19 classes that belong to eight phyla (including Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Firmicutes, Proteobacteria, Spirochaetes and Tenericutes). Richness among Acidobacteria (1 genus and uncultured bacteria from 2 orders and 1 class), Bacteroidetes (7 genera from 4 families, 4 orders and 5 classes), Chloroflexi (2 uncultured bacteria from 2 families, 2 orders and 2 classes), Spirochaetes (1 uncultured bacterium) and Tenericutes (1 uncultured bacterium) were relatively low. Richness among Actinobacteria (19 genera and uncultured bacteria from 17 families, 3 orders and 1 class) and Firmicutes (19 genera and uncultured bacteria from 17 families, 5 orders and 4 classes) were relatively higher, with the highest total taxonomic richness observed among Proteobacteria (72 genera and uncultured bacteria from 37 families, 22 orders and 5 classes).

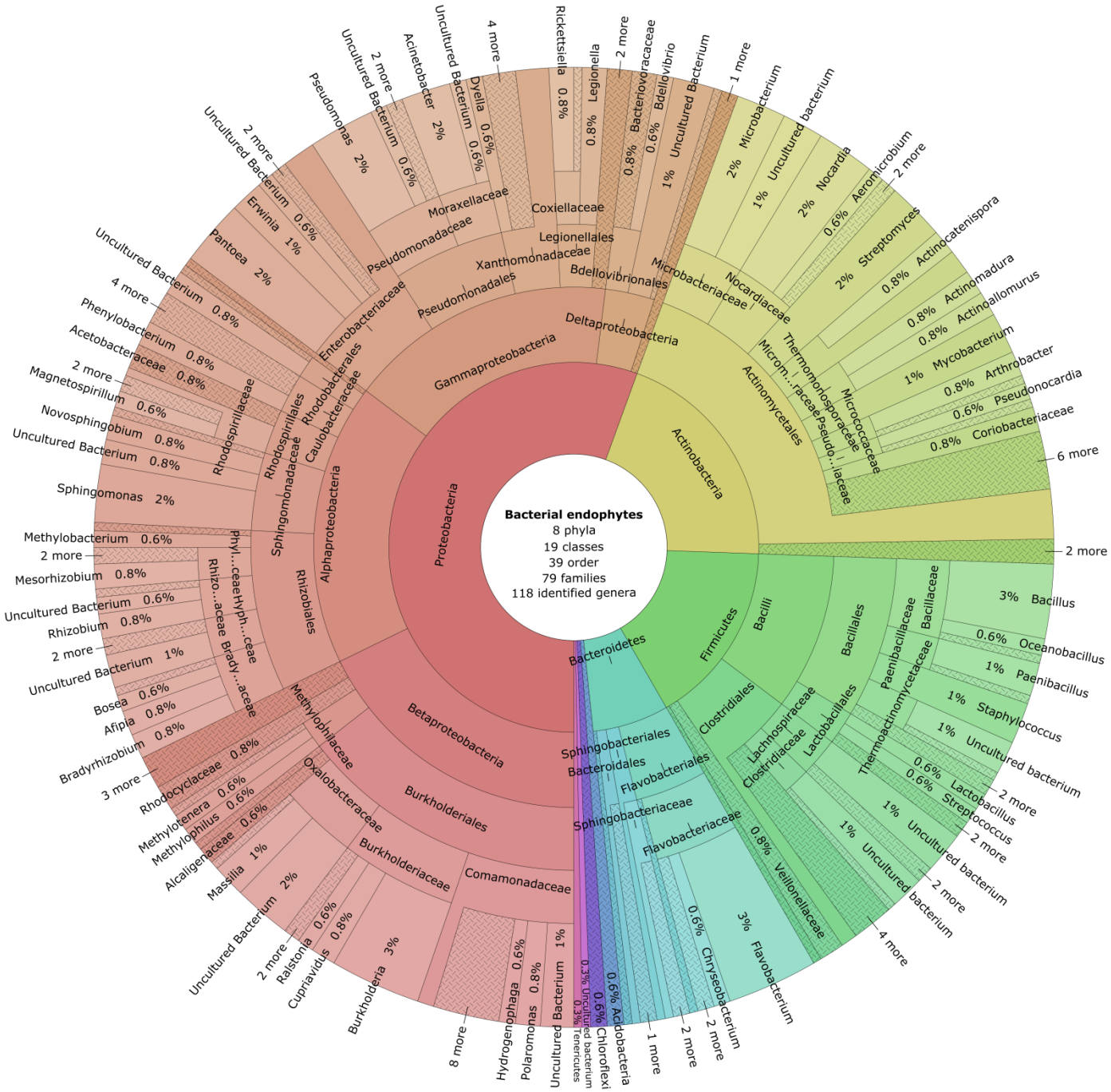


Figure 5: Krona plots of total bacterial endophyte taxonomic richness from *Oxalis* host plants, including six host species, three sampling locations and vegetative and reproductive organ types from original and re-run samples). Taxonomic identities based on V3 reads and OTU's were identified to genus-level (or closest family-, order- or phylum-level) using SILVA and RDP.

In order to gain a preliminary understanding of the richness and relative abundance of bacterial endophytes associated with individual host plants (per host species and organ type),

samples from Stellenbosch (Site 3) were analysed, as the sample design from this site allowed the most comprehensive comparisons. Plant samples from Stellenbosch were separated according to vegetative organs (roots, stems and leaves), vegetative reproductive propagules (bulbs only) and sexual reproductive propagules (seeds only) for each of the six studied host species (Figure 6).

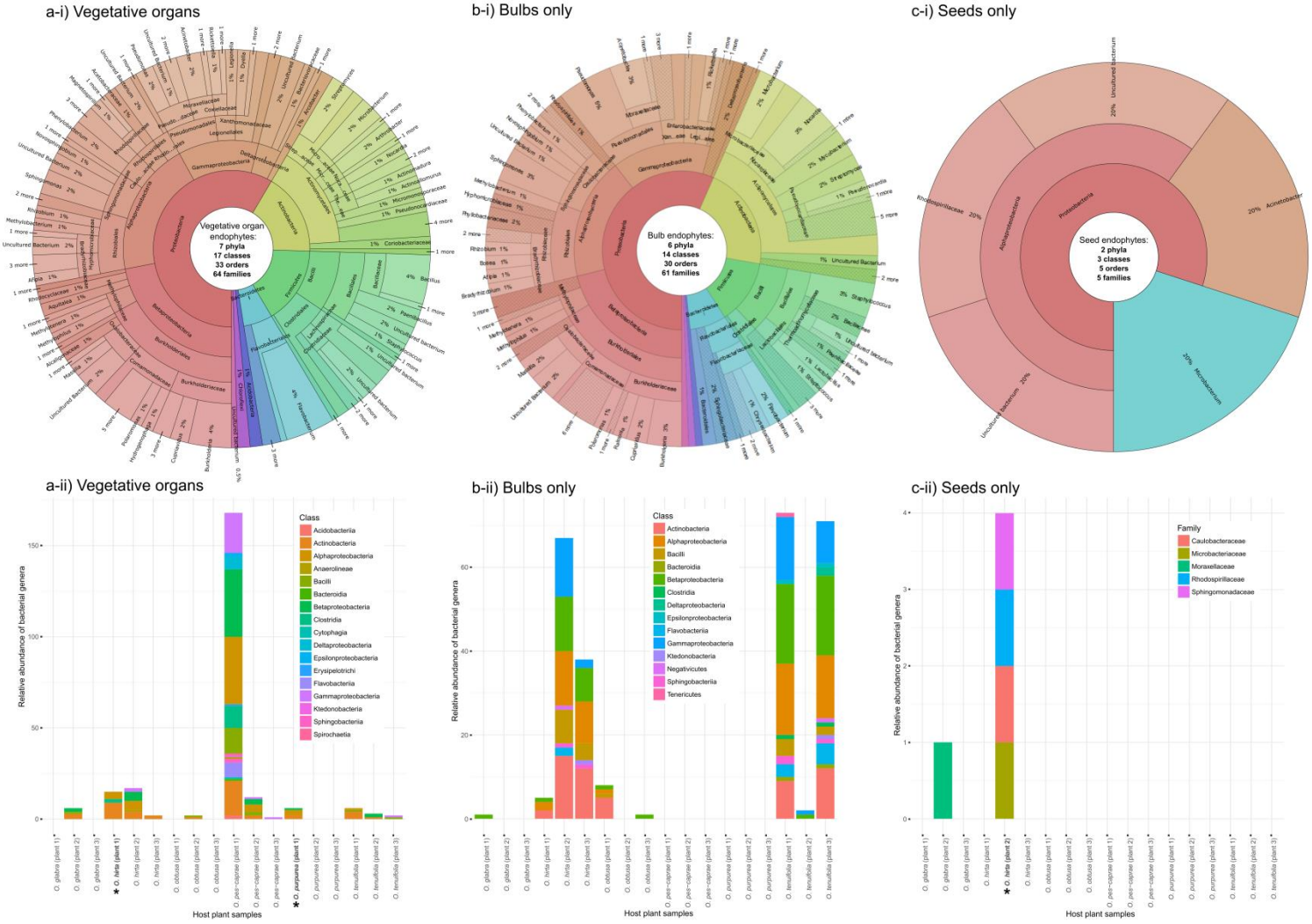


Figure 6: Genus-level taxonomic richness of bacterial endophytes associated with vegetative and reproductive organs of six *Oxalis* hosts samples from Stellenbosch. Samples grouped according to vegetative tissues, including roots, stems and leaves (a), bulbs samples only (b) and seed samples only (c). Total taxonomic richness from all six hosts (i) and taxonomic richness within individual plant samples (i) are indicated. The three re-run samples (3 samples per chip) are indicated with an asterisk (*).

These host species-level and organ-level assessments revealed a number of interesting observations. Total taxonomic richness from the vegetative organs of all host species contained 71 genera and uncultured bacteria from 64 families, 33 orders, 17 classes and 7

phyla (Figure 6a-i). This total taxonomic diversity was high, but did not include all taxa as described in Figure 5. This preliminarily indicated that sampling location (the two other sampling localities, Tulbagh and Malmesbury) also contributed to and affected the *Oxalis* endophytic diversity. Bulb tissues from all host species also contained high total taxonomic richness, including 71 genera and uncultured bacteria from 61 families, 30 orders, 14 classes and 6 phyla (Figure 6b-i). Samples with high taxonomic richness are unevenly distributed, and four samples (*O. hirta* – plants 2 and 3 and *O. tenuifolia* - plants 1 and 3) seem to contain the vast majority of endophyte taxonomic richness. This may be a sequencing and/or analysis artefact, or it may represent true variation in bulb endophyte diversity in two of the three recalcitrant species included in the study. The total diversity among bulbs was relatively lower than other vegetative organs, indicating that some selection of endophytes that inhabit vegetative propagules of *Oxalis* hosts, may be taking place. However, pooled samples including multiple other organs (roots, stems and leaves) were compared to a single organ (bulbs). One would expect total richness in the pooled samples to naturally be higher. The vegetative non-bulb diversity was also driven by a single outlier sample (*O. pes-caprae* (plant 1)), so if these are excluded taxonomic richness in bulbs may be higher than other vegetative organs. Seeds contained considerably fewer bacterial endophytes, with only two genera and uncultured bacteria from 5 families, 5 orders, 3 classes and 2 phyla (Figure 6c-i). This considerably lower diversity in seeds, preliminarily indicated that hosts select which endophytes inhabit their seeds and are consequently passed on to their reproductive propagules. As seeds are formed last, after the other organs have developed, it is also possible that there might not have been sufficient time for colonisation at the time of sampling. Host plants are known to favour mutualistic bacterial endophytes for vertical transmission, and consequently pass along a small subset of total endophyte populations to their seeds (Edwald, 1987).

Endophytic bacterial richness within individual host plants was variable, ranging from zero to 168 taxa. On average, vegetative samples hosted 13.33 bacterial taxa, bulbs hosted 14.78 taxa and seeds hosted 0.28 taxa, from all *Oxalis* host species sampled at Stellenbosch. Rather surprisingly, 31 out of 54 of these studied plant samples (57.41%) contained no bacterial endophytes, while one sample, the vegetative organs of *O. pes-caprae* (plant 1), contained a staggering number of 168 bacterial taxa from 17 classes (Figure 6 ii). It is possible that this particular *O. pes-caprae* plant sample may contain contaminants (due to unsuccessful surface sterilization), but we also suspect that many of the samples with no bacterial endophytes may

be false zero's. It seems unlikely that the number of endophyte taxa could differ so drastically within one host plant, *e.g. O. tenuifolia* (plant 3) with 2 endophyte taxa in vegetative organs, 71 taxa in bulbs and no endophytes in the seeds or *O. glabra* (plant 2) with 6 endophyte taxa in vegetative organs, and no endophytes in the bulbs or seeds (Figure 6 ii). We have established that 95 to 98% of studied *Oxalis* plants host culturable bacterial endophytes (Jooste *et al*., 2018; Chapter 4), therefore it seems highly unlikely that more than half of the samples from Stellenbosch submitted for sequencing contained no bacterial endophytes. On a per-plant basis (combining vegetative organs, bulb and seed sample reads), two out of 18 plants did not yield any bacterial endophyte reads. The three re-run samples (3 samples per chip) provided an additional verification of the true endophytic diversity within vegetative (*O. hirta*: 15 taxa from 3 classes and *O. purpurea*: 6 taxa from 3 classes) and reproductive (*O. hirta*: 4 taxa from 4 families) samples. Due to these irregularities in number of endophytes among samples, we did not pursue further assessment of species-specific, organ-specific or site-specific associations between *Oxalis* hosts and bacterial endophytes.

Despite the potential false zero reads among many of the bulb and seeds samples from Stellenbosch, another noteworthy observation was the distribution of bacterial endophytes between *Oxalis* host species. Collectively, *O. glabra*, *O. hirta* and *O. tenuifolia* from Stellenbosch hosted more bacterial endophytes in their bulbs than the other three species, while *O. glabra* and *O. hirta* hosted more bacterial endophytes in their seeds than the other four species. The majority of these endophytes included bacterial genera with well-know, plant growth-promoting and nitrogen-fixing traits (Ding *et al*., 2005; Feng *et al.*, 2006; Gulati *et al*., 2011; Paul *et al*., 2013; Ramesh *et al*. 2014; Pereira *et al*., 2016). Interestingly, these are the three recalcitrant-seeded (true for *ca*. 60% of the Cape radiation) Cape *Oxalis* species, while the other three *Oxalis* species are dormant-seeded. Recalcitrant seeds are metabolically active when shed, which enables them to germinate, establish and reach maturity much more rapidly than dormant seeds (Kermode and Finch-Savage, 2002). This preliminary information suggests that recalcitrant-seeded species may harbour unique additional bulb and seed bacterial endophytes which may explain the unique anatomical and physiological traits associated with recalcitrant seedlings. This merits in depth further investigation.

Comparison of culture-dependant and culture-independent endophytic richness

Overall, culture-independent techniques (16S metabarcoding) allowed the identification of 118 bacterial genera (Figure 5), of which only six genera (*Arthrobacter* Conn & Dimmick, 1947, *Bacillus*, *Burkholderia* Yabuuchi *et al*. 1993, *Paenibacillus*, *Pantoea* and *Pseudomonas*) were obtained by culture-dependent techniques (agar) from all studied host species, plant organs and sampling locations. A total of nine bacterial genera were identified by culture-dependent techniques, of which three genera (*Luteibachter* Johansen *et al*., 2005, *Lysinibacillus* Ahmed *et al*., 2007 and *Xanthomonas* Dowson, 1939) were identified by culture-dependent techniques only. Based on these preliminary genus-level identities, we have confirmed the presence of various bacterial endophytes documented in the previous chapter (Jooste *et al*., 2018; Chapter 4). However, it is evident that metabarcoding techniques have contributed a wealth of new bacterial genus-level endophyte richness data.

The Stellenbosch sampling scheme allowed more in-depth assessment of the number of unique and shared taxa between culture-dependent and -independent techniques (based on potential genus-level identities) (Figure 7). Due to the high taxonomic richness of bacterial endophytes detected among these *Oxalis* host plants, comparisons were made at the level of bacterial genera, families or classes. Among vegetative organs (roots, stems and leaves) all four bacterial classes identified with culture-dependent techniques (Actinobacteria, Bacilli, Betaproteobacteria and Gammaproteobacteria) were also detected with culture-independent techniques. Metabarcoding revealed the presence of 13 additional bacterial classes (including the outlier sample – *O. pes-caprae* plant 1) associated with *Oxalis* hosts from Stellenbosch (Figure 7a-i). At the genus-level, five genera were detected with both techniques, including members from *Arthrobacter*, *Bacillus*, *Burkholderia*, *Paenibacillus* and *Pseudomonas* (5 families from 4 orders, 4 classes and 3 phyla) (Figure 7a-ii). Culture-dependent techniques revealed the presence of 4 unique genera (*Xanthomonas*, *Pantoea*, *Luteibachter*, *Lysinibacillus* from 4 families, 3 orders, 2 classes and 2 phyla), while culture-independent techniques revealed unique taxa from 67 genera (41 families) and 43 uncultured bacteria (41 different families) (Figure 7a-ii).

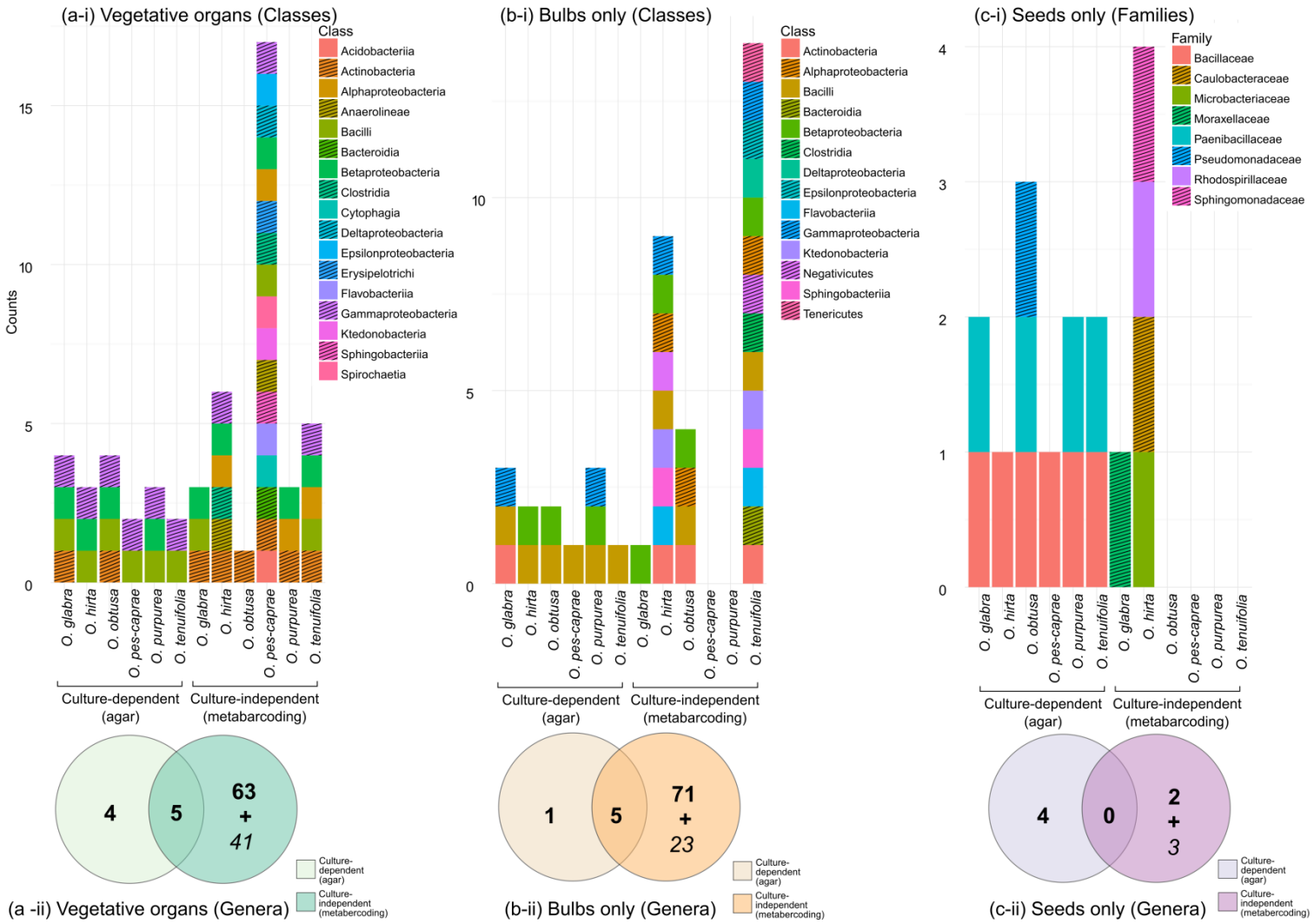


Figure 7: Taxonomic richness of culture-dependent (agar) and culture-independent (16S metabarcoding) bacterial endophytes associated with six *Oxalis* hosts from Stellenbosch. Samples grouped according to vegetative tissues (including roots, stems and leaves) (a), bulb samples only (b) and seed samples only (c). Due to high taxonomic richness, bacterial classes are indicated for bar-plots of vegetative organs (a-i) and bulb samples (b-i), while bacterial families are indicated for seed samples (c-i). The total number of distinct and shared bacterial genera identified from culture-dependent and culture-independent techniques is indicated for vegetative organs (a-ii), bulbs (b-ii) and seeds (c-ii) from all host species sampled at Stellenbosch. The number of identified genera is indicated in bold, while the number of 'uncultured bacteria' from distinct families is indicated in italics.

Among the Stellenbosch bulb samples, all four bacterial classes identified with culture-dependent techniques (Actinobacteria, Bacilli, Betaproteobacteria and Gammaproteobacteria) were also detected with culture-independent techniques. Metabarcoding revealed the presence

of 10 additional bacterial classes (Figure 7b-i). Five bacterial genera were detected with both techniques, including members from *Arthrobacter*, *Bacillus*, *Burkholderia*, *Paenibacillus* and *Pseudomonas* (5 families from 4 orders, 4 classes and 3 phyla) (Figure 7b-ii). Culture-dependent techniques revealed the presence of one distinct genus (*Xanthomonas*), while culture-independent techniques revealed distinct taxa from 71 genera (44 families) and 29 uncultured bacteria (23 different families) (Figure 7b-ii).

Among the seed samples of all host species sampled at Stellenbosch, metabarcoding techniques revealed the presence of two distinct bacterial classes (Actinobacteria and Alphaproteobacteria), while agar culture techniques revealed the presence of one distinct bacterial class (Bacilli) (Figure 7c-i). One bacterial class (Gammaproteobacteria) was identified with both culture-dependent and -independent techniques. However, no bacterial genera were detected with both techniques (Figure 7c-ii). Culture-dependent techniques revealed the presence of four distinct genera in seeds (*Bacillus*, *Paenibacillus*, *Pseudomonas* and *Xanthomonas* from 4 families, 3 orders, 2 classes and 2 phyla), while culture-independent techniques revealed distinct taxa from 2 genera (*Acinetobacter* and *Microbacterium*) and 3 uncultured bacteria from 3 different families (Caulobacteraceae, Rhodospirillaceae and Sphingomonadaceae) (Figure 7c-ii).

Due to the various caveats associated with the metabarcoding results obtained in this study, we cannot confidently assess host species or sampling location effects and interactions of bacterial endophyte richness. However, we can use these preliminary results to supplement initial findings based on culture-dependent techniques (Jooste *et al*., 2018; Chapter 3) and further our understanding of the potential magnitude of taxonomic richness of bacterial endophytes associated with Cape *Oxalis* hosts, among both vegetative tissues, as well as vegetative and reproductive propagules.

DISCUSSION

Historically bacterial endophytes have been isolated from plants using culture-dependent techniques (Torsvik *et al*., 2002; Oliver, 2010; Visagie *et al*., 2014), but recent advances of metabarcoding technologies have enabled the investigation of total culturable and non-culturable endophytic diversity (Schmalenberger and Tebbe, 2003; Leininger *et al*., 2006; Hibbett *et al*., 2011). Despite various caveats associated with the 16S metabarcoding using Ion Torrent and Ion Reporter platforms, this study has still generated valuable data on the total bacterial endophyte diversity among Cape *Oxalis* hosts.

As mentioned though, there were numerous caveats associated with this approach. Briefly, they included a high number of plant host reads, misidentification of host 16S as bacteria or cyanobacteria, inconsistent number of bacterial reads among samples and a discrepancy in taxonomic richness based on different variable regions analysed.

It is a standard practice to study plant endophytes by extracting microbial DNA from surface-sterilized plant organs (Bhojwani and Razdan, 1986). As a result, host DNA will unavoidably be present in any raw extracts. Compounding this issue, attempts to amplify microbial 16S DNA from raw extracts will also co-amplify host plastid and mitochondrial DNA, due to the homology between bacterial and plant plastid and mitochondrial 16S DNA (Dams *et al*., 1988; Hanshew *et al*., 2013). Consequently it is standard practice to exclude the host reads from plant endophyte metabarcoding datasets. Most authors do not report the percentage of host reads (De Souza *et al*., 2017), while some authors automatically exclude host reads without mention. Previous studies have reported between 10 to 60% host reads from various metabarcoding platforms (Dams *et al*., 1988; Beckers *et al*., 2016; Cheng *et al*., 2018). Our results yielded relatively higher numbers of host reads (between 63.4 and 96.7% reads per variable region). This high host read number, may suggest that the DNA extractions, library preparation/builds and/or primer sets used in this study were not correctly optimised to study plant endophytes in *Oxalis*. It is also possible that *Oxalis* plants have proportionately more chloroplasts per cell than most other angiosperms, possibly due to their biology of rapid growth during a shorth growing season (Dreyer, *pers. obs*.) and/or documented variation of ploidy levels (Suda *et al*., 2006; Castro *et al*., 2013).

Specialized primer-sets (799F2, 783R, 783Rabc) have been developed to minimize the binding to and amplification of maize chloroplasts in plant endophyte studies (Chelius and Triplett, 2001), but these specialized primers add significant costs to an already expensive technique. These primers have been successful in some plant-endophyte systems (Edwards *et al*., 2007; Rastogi *et al*., 2010; Sakai *et al*., 2004; Sun *et al*., 2008), while they were not entirely effective in others (Sagaram *et al*., 2009; Bulgarelli *et al*., 2012; Bodenhausen *et al*., 2013; Shade *et al*., 2013). Bacterial enrichment protocols have also been developed to increase bacterial cell counts and decrease the number of plant DNA in order to optimize the bacterial endophyte yield from maize and soya beans (Ikeda *et al*., 2009; Dos-Santos *et al*., 2017). In our *Oxalis* dataset, samples with relatively high host reads had relatively low bacterial endophyte reads, and *vice-versa*. This could be regarded as a positive indication that

bacterial enrichment techniques to reduce host DNA amount, possibly in combination with specialized primer sets, could aid higher bacterial endophyte yields in future studies.

Dos-Santos *et al*. (2017) reported that a high degree of 'interfering factors' such as polysaccharides, phenolic compounds, nucleases and fibres in maize effected the DNA yield of plant endophytes. *Oxalis* species contain large amounts of oxalates (often in the form of oxalic acid or calcium oxalate crystals) (Seal and Sen, 1970; Bahadur *et al*., 1983; Sahin, 2005; Peng *et al*., 2013). All *Oxalis* plant material used in this study were oven-dried after surface sterilization, in order to prevent or minimize any negative effects of oxalates or other compounds of DNA quality. It is, however, still possible that oxalates could have caused degradation of bacterial endophyte DNA during DNA extractions. *Oxalis* oxalate content varies between species (Jooste *et al*., 2016), so future studies could perhaps include plant samples with low oxalate contents as control.

We had the unique opportunity to assess the number and diversity of contig reads from relatively full chips with all original samples (39 samples per chip), relative to three re-run samples run on their own relatively empty chip. The re-run samples yielded significantly higher numbers of contig reads for all variable regions relative to the other original samples, but overall did not contribute to significantly higher taxonomic diversity (based on preliminary V3 genus-level identities). Instead, the three re-run samples yielded repeats of the same taxa (even though identities were not yet confirmed, we could see that various contig reads were identical to one another). This suggests that we did not lose bacterial endophyte richness data in our original samples and that these chips (39 samples per chip) were not overcrowded.

Recent studies showed that data analyses are becoming the most important step in the metabarcoding approach and Massart *et al*. (2015) elegantly stated that "it does not matter how much data you have if you cannot make sense out of it". It is also well known that correct taxonomic identification of DNA sequences is integral to studies of biodiversity (Massart *et al*., 2015; Bengtsson-Palme *et al*., 2018). Identification of metabarcoding sequences largely depend on available/assessable software and curated databases, which may be cost-prohibitive (Massart *et al*., 2015). Public records may have differing levels of taxonomic annotations and issues with contamination or incorrectly annotated records have been reported (Porter and Hajibabaei, 2018).

The Ion Reporter platform offers a recommended 16S metabarcoding workflow to identify contigs and identify 16S bacterial sequences using two curated databases. We have found that this approach was successful for identification of contigs, but that the 16S workflow failed to identify host reads (understandably so as the workflow excluded eukaryote DNA and only allowed 16S microbial database matches). Consequently this workflow falsely inflated potential endophyte diversity among our samples. *Oxalis* chloroplasts were falsely identified as cyanobacteria, which were easy enough to identify and exclude from our dataset. However, *Oxalis* mitochondria were falsely identified as bacteria that were not so easy to detect (possible only once verified with other databases). It should also be noted that the Ion Torrent and Ion Reporter databases are known to be biased towards clinical and medicinal research (Lih *et al*., 2016; Misyura *et al*., 2016; Williams *et al*., 2018), therefore sequence identities were verified using three alternative and reliable databases, including NCBI, SILVA and RDP.

The NCBI database (biased towards eukaryotic organisms) was used to verify host sequence identities, so that these sequences could be excluded from our dataset with relative confidence. We searched this database for additional verification of bacterial identities, but often found that bacterial endophyte sequences were matched with 'uncultured bacteria'. Other authors have reported similar findings, stating that the use of high-throughput metabarcoding is resulting in an ever increasing amount of unnamed, anonymous sequences on NCBI (Nilsson *et al*., 2005; Hibbett *et al*., 2011). SILVA and RDP are two of the leading 16S microbial references databases for metabarcoding data (Cole *et al*., 2008; Quast *et al*., 2012), and were consequently implemented for the most reliable taxonomic identifications (to the nearest available genus- or family-level classification). Both of these databases yielded the same taxonomic identities of all contigs, so genus-level identities of *Oxalis* endophytes could be assigned with relative confidence.

We also found that different variable regions yielded considerably different taxonomic richness data, but decided that the V3 region (genus level identifications) offered the most reliable preliminary estimates of bacterial endophyte diversity within our dataset. The Ion Torrent 16S metabarcoding approach implemented in this study yielded relatively short 16S fragments (between 200 to 250 base pairs in length). Authors have reported potential sequencing artefacts and taxonomic identification errors associated with short DNA fragments and the biodiversity of environmental samples (Degnan and Ochman, 2011; Shokralla *et al*., 2012). Taking these various caveats into consideration, we cautiously and

conservatively attempted to analyse sequence identities from the V3 region to genus-level identities.

Potential genus-level identifications revealed unexpectedly high total endophytic bacterial richness associated with six *Oxalis* host species from three sampling locations throughout the Cape. A preliminary total of 118 bacterial genera were identified, as well as various uncultured bacteria, which collectively belong to 79 families, 39 orders and 19 classes from eight phyla (including Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Firmicutes, Proteobacteria, Spirochaetes and Tenericutes). These are conservative genus-level identities, and we suspect that the true species-level taxonomic richness may be even greater. Final estimates may be revealed by future studies able to overcome the various caveats discussed above. One vegetative organ sample (*O. pes-caprae* from Stellenbosch), contained exceptionally high bacterial richness, and we suspect that this sample may include contaminants (most likely soil contamination due to unsuccessful surface sterilization). This single sample may be contributing to an inflation of the true endophytic diversity among *Oxalis* hosts. However, many bacterial taxa detected in this sample were also found among other *Oxalis* samples (from various host species and sampling locations), possibly indicating that not all taxa were contaminants.

Overall, the taxonomic diversity of endophytes in vegetative organs (combined roots, stems and leaves) and bulbs samples was high. These findings were consistent with the results of our study implementing culture dependent (agar) techniques to identify Cape *Oxalis* endophytes (Jooste *et al*., 2018; Chapter 3). We found that *Oxalis* were capable of hosting a relatively diverse selection of rhizosphere microbes, and that many endophytes were shared among various vegetative and reproductive plant organs (including bulbs and seeds). Culture dependant techniques revealed 46 bacterial endophytes among six *Oxalis* hosts species sampled from three locations (Jooste *et al*. 2018; Chapter 3). Preliminary bacterial endophyte diversity revealed by metabarcoding strengthens our previous suggestions that *Oxalis* hosts a very rich endophyte microbiome. These results highlight the importance of metabarcoding technologies to identify and understand the true richness of plant endophyte communities. *Oxalis* plants also do not seem to invest many resources into plant defences and consequently may host many opportunistic colonizers taking advantage of an ephemeral niche. The true extent of the physiological advantage the plant gained through these diverse associations merits in depth future studies.

The taxonomic richness of seed endophytes was low, relative to the diversity in other plant organs. Importantly, many seed samples (>57% of seed samples from Stellenbosch) did not yield any bacterial endophyte reads. It is well known that plants exert strong selection pressures on the endophytes passed onto their vegetative and reproductive propagules (Hallmann, 2001; Stamp, 2003). Comprehensive reviews have indicated that very few angiosperms host endophytes in their fruit and seeds (Hallmann, 2001; Compant *et al*., 2010; Truyens *et al*., 2015). However, we regarded these 'zero' reads as rather surprising results, as our culture-dependent techniques revealed that 95 to 98% of these same host species from Stellenbosch contained at least one bacterial endophyte in their seeds (Jooste *et al*., 2018; Chapter 3). The taxonomic classification of metabarcoding data indicated a completely unrelated and unique set of genera associated with *Oxalis* seeds (*Acinetobacter* and *Microbacterium*, as well as 3 uncultured bacteria from Caulobacteraceae, Rhodospirillaceae and Sphingomonadaceae), relative to the previously identified culturable bacterial genera (*Bacillus*, *Paenibacillus*, *Pseudomonas* and *Xanthomonas*). Given the documented diversity of culturable endophytes associated with these host species and the various caveats associated with the metabarcoding techniques implemented in this study, we suggest that there were inconsistencies with the yield of endophytes among samples and that some of the missing data may be false zeros. Future studies on *Oxalis* seeds and vertical transmission of bacterial endophytes may shed substantial light on this matter.

To-date, culture-dependent and culture-independent studies have collectively revealed that of the most dominant plant bacterial endophytes include Proteobacteria, Firmicutes, Actinobacteria, and Bacteriodetes and to a lesser extent, Acidobacteria, and Chloroflexi (Sheng *et al*., 2011; Bulgari *et al*., 2014; Akinsanya *et al*., 2015). The most commonly documented culturable bacterial endophytic genera include *Bacillus*, *Streptomyces*, *Pseudomonas* and *Lysinibacillus* (Ulrich *et al*., 2008; Carrell and Frank, 2015). Our results largely agree with these reported findings. Many of the genera documented among Cape *Oxalis* hosts are well-known plant endophytes, and some include members with documented plant growth-promoting and nitrogen fixing properties (Desnoues *et al*., 2003; Liba *et al*., 2006; Zakhia *et al*., 2006, Hara *et al*., 2009; Grady *et al*., 2016). Our findings are also comparable to the taxonomic richness described by another study on 16S metabarcoding of Cape plant bacterial endophyte communities (Miyambo *et al*., 2016). These authors revealed that three fynbos species (*Erepsia anceps* (Haw.) Schwantes, *Phaenocoma prolifera* (L.) D.Don and *Leucadendron laureolum* (Lam.) Fourc.) hosted various communities of

Alphaproteobacteria, Acidobacteria, Betaproteobacteria, Firmicutes and Deltaproteobacteria. Miyambo *et al*. (2016) indicated that these three Cape host species harboured many putative plant growth-promoting bacteria that have the potential to influence host growth and health.

Metabarcoding has provided preliminary support for the presence of plant growth-promoting and nitrogen fixing bacterial endophytes from *Oxalis* hosts, including *Arthrobacter*, *Bacillus*, *Burkholderia*, *Paenibacillus* and *Pseudomonas*. Unfortunately the species-level identities could not yet be confirmed, but must be confirmed by future studies. An interesting observation was the presence of two bacterial genera, *Acinetobacter* and *Microbacterium*, which were common bulb and seed endophytes associated with recalcitrant *Oxalis* species. Both of these genera include species with growth-promoting and nitrogen fixing properties (Zakhia *et al*., 2006). These are preliminary observations, but could suggest that recalcitrant seeds are likely to form associations with known nitrogen-fixing genera, which may support their rapid germination and growth (Jooste *et al*., 2018; Chapters 2 and 3).

Given the preliminary identification of this vast bacterial endophyte richness associated with Cape *Oxalis* host plants and our previous demonstration of substantial culturable fungal endophyte diversity, we expect metabarcoding techniques to yield even higher levels of fungal diversity as well. We suggest that future studies should also explore the true fungal endophytic diversity and focus on the conservation of the wealth of inter-organismal biological diversity. Conservation studies could explore potential applications of beneficial plant endophytes to ameliorate the effects predicted by models of climate change.

CONCLUSION

This study provides the first contribution of 16S metabarcoding data to document bacterial endophyte richness associated with Cape *Oxalis* host plants. Even though bacterial endophyte species identities could not be confirmed, the majority of these genera include various well-known plant endophytes. Metabarcoding results confirmed the presence of six out of nine bacterial genera identified with culture-dependent techniques. Importantly, these six genera included various bacterial species with well-known plant growth-promoting and nitrogen-fixing abilities, as do many of the newly identified endophytic genera. Plant endophytes fulfil various vital roles in nutrient cycling and ameliorating the effects of biotic and abiotic stressors, especially in nutrient-depleted habitats such as the Cape. The understanding and preservation of plant endophytes may be crucial to the health and conservation of various species in biodiversity hotspots. Given this wealth of bacterial endophyte richness associated

with Cape *Oxalis*, we propose that plants from the Cape Flora offer unique opportunities to explore highly diverse plant bacterial (and possibly fungal) endophytes with various beneficial traits and potential future applications.

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Declaration by the candidate (for chapter currently under review at BMC Plant Biology)

With regards to Chapter 5 (Nitrogen-fixing bacteria and *Oxalis* – evidence for a vertically inherited bacterial symbiosis), the nature and scope of my contribution were as follows:

Nature of contribution	Extent of contribution (%)
Isolation, culturing and identification of bacterial endophytes associated with *Oxalis* vegetative and reproductive propagules	100
Data analysis, interpretation and manuscript preparation	80

The following co-authors have contributed to Chapter 5:

Name	e-mail address	Nature of contribution	Extent of contribution (%)
Francois Roets	[redacted]	Provided guidance and edited the manuscript	5
Guy F. Midgley	[redacted]	Provided guidance and edited the manuscript	5
Kenneth C. Oberlander	[redacted]	Provided guidance, especially in terms of data analysis, and edited the manuscript	5
Léanne L. Dreyer	[redacted]	Provided guidance, funding and edited the manuscript	5

Signature of candidate: .....……

Declaration by co-authors:

The undersigned hereby confirm that

1. The declaration above accurately reflects the nature and extent of the contributions of the candidate and the co-authors to Chapter 5.
2. No other authors contributed to Chapter 5 than those specified above.
3. There are no conflicts of interest relevant to Chapter 5 of this dissertation.

Signature	Institutional affiliation	Date
Francois Roets	Stellenbosch University	December 2018
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Chapter 5 Nitrogen-fixing bacteria and *Oxalis* – evidence for a vertically inherited bacterial symbiosis

ABSTRACT

Background: Plant-endophyte symbioses often revolve around nitrogen metabolism, and involve varying degrees of intimacy. Although evidence for vertical inheritance of nitrogen-fixing endophytic bacteria is increasing, it is confined mostly to crop plants, and to date no such system has been reported for geophytes.

Methods: Here we have studied bacterial endophytes associated with *Oxalis*, the most species-rich geophytic genus form the Cape Flora, southern Africa. Culturable endophytes were isolated from surface-sterilized vegetative and reproductive plant organs for six host species at three locations. Colonies of microbes on various artificial media were morphotyped, enumerated and identified using sequence data. Filter exclusion experiments were conducted to determine if endophytes were vertically transmitted to seeds, determine if mucilage plays a role to actively attract microbes from the soil and to assess microbial richness isolated from the mucilage of *Oxalis* seedlings. Fluorescent microscopy was implemented in order to visualize endophytic bacteria in cryo-sectioned seeds.

Results: Here we report evidence for a novel, vertically transmitted symbiosis between communities of nitrogen-fixing and plant growth-promoting *Bacillus* endophytes and selected *Oxalis* hosts from nitrogen-deficient environments of the Cape. *Bacillus* endophytes were ubiquitous and diverse across species and plant bodies, and were prominent in seeds. Three common nitrogen-fixing *Bacillus* have known oxalotrophic properties and appear to be housed inside specialised cavities (containing oxalates) within the plant body and seeds.

Conclusions: The discovery of vertical transmission and potential benefits to both host and endophyte suggest a particularly tight mutualism in the *Oxalis*-endophyte system. This discovery suggests unexpected ways in which geophytes might avoid nitrogen deficiency, and suggest that such symbioses are more common than previously expected.

Keywords: *Bacillus*, endophytic bacteria, geophytes, nitrogen fixation, oxalotrophic bacteria, *Oxalis*, vertical transmission

BACKGROUND

The Greater Cape Floristic Region (Cape) biodiversity hotspot of southern Africa is globally renowned for its diverse and extremely species-rich flora [1, 2]. To date, at least some of this remarkable diversity has been attributed to abiotic factors such as palaeoclimatic stability, reliable seasonal water availability, geographical gradients and diverse soil types [3, 4]. The sandstone derivation and low pH of most soils, together with predictable winter rainfall and relatively frequent wildfires all contribute to dystrophic conditions, with severe nitrogen deficiency [4], amongst the lowest nitrogen and phosphorus levels measured globally [5]. Nitrogen is essential to the growth and development of all terrestrial plants [6 - 8]. There is growing evidence that diverse Cape plant lineages have adapted by forming associations with growth promoting and nitrogen-fixing micro-organisms [9]. Recently it has been suggested that plant-microbial interactions play an important role in generating, shaping and maintaining ecosystem diversity within the Cape [10, 11]. Many of the most diverse and ecologically dominant indigenous Cape plant lineages form classical 'textbook' symbioses with beneficial micro-organisms [12 - 17]. Unfortunately, relatively little research attention has been given to these associations; it is evident that limited information is available addressing the mechanisms, diversity and role of microbial associations of Cape plants [18].

The Cape is also renowned for the most diverse geophyte flora in the world, including 2100 species from 20 families [19, 20]. Although the factors driving this remarkable Cape geophyte diversity are still poorly understood, geographical distribution, climatic factors (rainfall quantity and reliability) and plant growth form (storage organ size) have been suggested [21]. The role of plant-microbial interactions has, however, not yet been confirmed [22].

Oxalis is the largest geophyte genus in the Cape (180 spp.), and has undergone an extensive radiation (*ca*. 230 spp.) in southern Africa [1, 2] that likely originated in the Cape [23]. The evolutionary success of this genus in the Cape may partly be attributed to its unique life history (geophytic habit, winter flowering, variable seed strategies), but is still poorly understood. Cape *Oxalis* species are highly unusual in terms of their seed germination strategies – approximately 60% have recalcitrant seeds, with an inverted germination order compared to most angiosperms (foliar leaf development and growth followed by delayed radicle growth, Extended Data Figure 1). This remarkable germination strategy raises the question of how these seedlings are able to photosynthesize and grow without a well-

developed radicle or roots. Here we have uncovered a set of observations that suggest that *Oxalis* has developed a unique association with nitrogen-fixing and/or growth promoting bacterial endophytes (EB). Such associations may help explain the ecological (and evolutionary) success of *Oxalis* under challenging Cape edaphic conditions.

We focused on beneficial plant-microbial interactions by considering: 1) the presence of EB associated with Cape *Oxalis* species, 2) the EB community composition within hosts and between locations, and finally 3) elucidating the nature of relationships between host plants and endosymbionts.

METHODS

Oxalis plant material and sterilization protocol

Six phylogenetically representative *Oxalis* species (three recalcitrant species: *O. glabra* Thunb., *O. hirta* L. and *O. tenuifolia* Jacq.; three dormant species: *O. obtusa* Jacq., *O. pes-caprae* L. and *O. purpurea* L.) were sampled from three locations (Malmesbury [-33.481121, 18.753625], Stellenbosch [-33.932358, 18.874571] and Tulbagh [-33.311688, 19.096747]) in the Western Cape Province, South Africa. All plants were correctly identified and harvested from the wild (research sample collection permit obtained from the Western Cape Nature Conservation Board, South Africa (Permit No. 0028-AAA088-00243)). Five individuals of each species (at least 10 m apart), with no external signs of microbial infections (asymptomatic), were collected at each location during May-June of 2016 and 2017. Plants were dug out with minimal disturbance to the below-ground organs and all excess soil was shaken off, until no soil was visible on roots and bulbs. Before samples were processed for endophyte isolation, plant roots were gently washed in 5 ml sterile water in order to obtain rhizosphere samples. Hereafter roots, bulbs, stems/rhizomes (depending on the above-ground growth forms of species: *O. glabra*, *O. hirta* and *O. tenuifolia* have above ground stems; *O. pes-caprae*, *O. purpurea* and the sampled populations of *O. obtusa* do not), leaves and seeds of all plants were aseptically separated using a scalpel and individually surface sterilized. For surface sterilization, samples were washed in a 33% dilution of household bleach (±5% sodium hypochlorite) for 1 minute and 75% ethanol for 1 minute, interspersed with three one-minute washes in sterile water. As sterilization controls, a few additional samples of each of the plant organs were dabbed onto bacterial plate count agar (Biolab, Merck) and malt extract

agar (Sigma-Aldrich), and incubated for 7 days at 28°C in the dark - no colonies were detected.

Isolation of bacterial colonies

Plant organs were manually cut into small segments using a scalpel and transferred into Eppendorf tubes with five sterile glass beads and sterile water to 1.5 mL, under sterile conditions. Samples were macerated using a TissueLyser (Qiagen TissueLyser, Retsch MM301) at the Central Analytical Facility at Stellenbosch University. 200 µL each of 1:4 sterile-water-diluted macerate was plated onto three agar media: bacterial plate count agar (Biolab, Merck), nutrient broth agar (Sigma-Aldrich) amended with 4g potassium oxalate (BMS Education) per litre of agar and malt extract agar (Sigma-Aldrich). Potassium oxalate was added to nutrient broth agar to re-create the high oxalate content of the host plant (as described by Sahin (2005)). After five days of incubation at 28°C in the dark, all morphologically different colonies (in terms of colour, shape, size and/or texture) were sub-cultured onto fresh plates. This process was repeated after another five days of incubation for each identified morphotype until pure cultures were obtained. All bacterial morphotypes were recorded and photographed. Three individuals of each morphotype per *Oxalis* species from each location were kept to test accuracy of morphotype identification. Each representative morphotype was divided, with 50% used for DNA extraction and sequencing, and the remainder stored in 50% glycerol in cryostorage (-80°C).

DNA extraction, amplification and sequencing

To assess accuracy of endophyte morphotyping, three replicates from 25 different bacterial morphotypes were sequenced, with the expectation that the DNA sequences of each morphotype triplet would be identical. Morphotypes isolated from seeds and bulbs (reproductive propagules) were prioritized for sequencing in this study. Sequencing revealed that 92% of replicates were consistent with morphotyping, with all three replicates showing identical 16S sequences. For the remaining two replicate sets, one sequence differed from the other two. This error margin (2.6% of morphotypes incorrectly assigned), is small enough not to substantially affect our conclusions. We used sequencing to identify bacterial colonies from seeds and referenced our morphotype records to determine the distribution of bacterial species throughout the remainder of plant organs that were not sequenced.

DNA extractions were done following a modified 2X CTAB protocol, as described in Oberlander *et al.* [24]. Amplification and sequencing of the 16S rRNA region was conducted using universal bacterial primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-ACCTTGTTACGACTT-3') [25]. PCR amplification reactions were performed using 25 µL reaction mixtures consisting of 9 µL dH2O, 2.5 µL $MgCl_2$ (25mM), 0.25 µL of each primer (10µM), 12 µL KapaTAQ (KM1000, Kapa Biosystems) and 1 µL DNA (326 to 498 ng/µL). The PCR thermal cycling conditions were: 94°C (5 min), 30 cycles of 94°C (1 min), 49°C (1 min) and 72°C (2 min), with final extension at 72°C (10 min). PCR products were sequenced at the Central Analytical Facility (CAF) at Stellenbosch University. Confirmation of base calling in sequence chromatograms was manually conducted in Chromas v. 2.6.5 (http://www.technelysium.com.au). Sequences were aligned using the embedded ClustalW function [26] in BioEdit v. 7.2.6 [27]. Obtained sequences were compared to GenBank (NCBI) submissions using online BLAST searches.

This study specifically focussed on endophytes that were present in seeds (*Bacillus* species), but it should be noted that seed bacterial diversity was a small subset (on average 25%) of the total bacterial and fungal morphotype diversity isolated per plant (roots, bulbs, stems and leaves). True endophytic diversity of bacteria and fungi colonising *Oxalis* hosts is much larger than reported here, and are discussed in Jooste *et al*., 2018 (Chapters 3 and 4).

Phylogenetic analysis

Phylogenetic trees were reconstructed using our dataset of 16S sequences for endophytic bacteria (excluding duplicate sequences), sequences downloaded from Genbank (three representative BLAST results per *Oxalis* endophyte sequence) as well as four bacterial outgroup species. Tree samples were generated in MrBayes (v. 3.2) using the nst=mixed command for model selection and a gamma rate correction for 2 X 10^7 generations under otherwise default settings. Convergence of parameter values was checked in Tracer (v.1.6) [28] with a 25% burnin. The consensus tree was used to aid species identification (Extended Data Figure 1). Six bacterial endophytes were identified to species level and morphological traits (bacterial cell lengths) were used to distinguish between *B. megaterium* and *B. aryabhattai* that had unresolved relationships based on the consensus tree. There were three instances where we could not distinguish between two closely related species. As these species are morphologically indistinguishable from one another, we have consequently referred to these endophytes as *B. cereus/thuringiensis*, *B. safensis/pumilus* and *B.*

subtilus/siamensis, indicating the two most likely identities of these isolates based on BLAST results. It should be noted that *B. cereus* and *B. subtilus* have previously been isolated from the rhizosphere and roots of two *Oxalis* species from Columbia [29] and were therefore considered as the most likely EB. More conclusive identification for these latter taxa requires additional markers.

Filter exclusion experiments

We aimed to determine if EB were vertically transmitted to seedlings and determine if mucilage plays a role to actively attract microbes from the soil. Filter exclusion experiments were conducted to assess microbial richness isolated from the mucilage of *Oxalis* seedlings. Sterilized gel drying frames (Sigma-Aldrich) were used as a filter barrier between the germinating seed and a selected treatment medium. The pore size (10-15µm) of these filters was large enough to allow movement of microorganisms through the filter. Filters were wet with sterile water in order to prevent an artificial pulling-effect when seeds with mucilage were placed on the filters. Experimental filter treatments were compared to two negative controls (sterile conditions) and one positive environmental control (soil).

Mature seeds were harvested from 12 recalcitrant *Oxalis* species (20 seeds per species) that are known to produce large amounts of hypocotylar mucilage. For each species, a soil sample (10 x 10 cm and 5 cm deep) was collected at each sampling location (10 species sampled from field locations and 2 species sampled from a research collection at the Stellenbosch University Botanical gardens). A research sample collection permit was obtained from the Western Cape Nature Conservation Board, South Africa for all seeds harvested from the wild. Seeds were surface-sterilized according to the above-mentioned protocol. Seeds were randomly divided into four groups of five seeds each and assigned to one of four experimental treatments. Treatment 1 served as a negative control where seeds were placed on sterile agar. Treatment 2 served as an additional negative control where seeds were placed on a sterilized filter and a sample of autoclaved soil within a sterile petri-dish. Treatment 3 consisted of a sample of raw soil within a petri dish with a sterilized filter placed on top. Seeds were then placed on top of the filter to determine if microbes actively move towards the mucilage. Treatment 4 served as a positive environmental control where seeds were placed on a sample of untreated raw soil to determine if microbes passively move towards the mucilage. After three days of exposure to treatments, each seed was individually removed and the mucilage around the base of the hypocotyl was lightly streaked across one

bacteriological and one fungal agar growth medium under sterile conditions. Isolates were sub-cultured to obtain pure culture colonies of each identified morphotype, as described in the protocol above. Morphotypes were visually compared to a reference database created from all sequenced microbes (as described in the section above). We had a 97.4% success rate of accurately identifying morphologically similar colonies, and if morphologically new/unknown colonies were encountered they were given a unique morphotype number. Fungal isolates were documented and identified, but are not discussed in this paper (Jooste *et al*., 2018; Chapter 3).

The total number of unique microbial morphotypes was recorded in order to assess microbial richness associated with the mucilage of all seeds exposed to the four treatments. Microbial morphotype count data were analysed with the best fitting generalized linear mixed effects model using the lme4 package [30] in the R statistical environment, version 3.4.1 (R-Core-Team, 2014). Treatment type and sampling location were entered as fixed effects and *Oxalis* host species as a random effect. Residual plots did not reveal any obvious deviations from normality or homoscedasticity. A *post-hoc* Tukey test was used to compare estimated values between the four treatment groups.

Seed, seedling and plant anatomy

All *Oxalis* bulb, leaf, seed and seedling material (sampled from a research collection and the wild) was fixed in Formalin-Acetic-Acid-Alcohol (FAA), dehydrated in an alcohol series and gradually infiltrated with and embedded in paraffin wax [31]. Samples were sectioned with a rotary microtome (Leitz, Germany). Sections were stained using the Safranin-Alcian-blue or Safranin-Fastgreen differential staining methods [31] and DPX glue was used to preserve these sections as permanent slides. Anatomical traits of embedded plant material were studied using a Nikon ECLIPSE E400 light microscope and photographed using a Leica MC 170 HD camera and LAS CORE software (Leica, Switzerland). Seedling germination and growth were documented using Leica M125 stereo microscope and LAS CORE software. Backgrounds of images and plant debris from preparing slides were removed using the ‘fuzzy select tool’ from Gimp 2.10.2.

Fresh *Oxalis* seeds were surface sterilized following the above-mentioned protocol, embedded and cryo-sectioned using a Leica CM1860 UV cryostat (Leica Biosystems). All seeds were harvested from the wild *Oxalis* populations growing near Stellenbosch. Sections

were mounted on sterilized glass slides. Sectioning and staining were done on the same days as seed harvest. Slides were stained with LIVE/DEAD BacLight Bacterial Viability Kit (Life Technology), using 100 µL per slide. Slides were viewed using a Carl Zeiss Confocal LSM 780 Elyra S1 microscope from the Fluorescence Microscopy Unit in CAF at Stellenbosch University, in order to detect the location of bacteria within seeds. Original red and green light fluorescence images have been changed to a colour-safe combination of magenta and lime-green (Figure 3 di-iii), using the 'colour shift function' (310° to 319°) in Inkscape 0.92.3.

RESULTS

The presence and diversity of EB were assessed amongst vegetative and reproductive organs of six *Oxalis* host species sampled from three different locations (Figure 1a). Pure-culture bacterial colonies isolated from sterilized, macerated plant material were identified using universal 16S bacterial primers. A labile community of cultivable EB (nine of the most abundant and consistent EB species were from the genus *Bacillus* - *B. aryabhattai* Shivaji *et al*. 2009, *B. bataviensis* Heyrman *et al.* 2004, *B. cereus/thuringiensis* Frankland and Frankland 1887/Berliner 1915, *B. licheniformis* Weigmann 1898, *B. megaterium* de Bary 1884, *B. mycoides* Flügge 1886, *B. safensis/pumilus* Satomi et al. 2006/Meyer and Gottheil 1901, *B. simplex* Priest *et al.* 1989 emend. Heyrman *et al.* 2005/Sumpavapol et al. 2010 and *B. subtilus/siamensis* Cohn 1872 (most likely identifications based on 16S sequences and phylogenetic comparison, Supporting Information Figure S1)) were identified from various host plant organs (including roots, bulbs, stems, leaves and seeds), as well as the rhizosphere (soil surrounding plant roots). Most (77.8%) of the EB sampled from plant tissues were also present in the rhizosphere of the specific host plant studied, suggesting a strong link between EB and rhizosphere bacteria. It is possible that the remainder of EB reached plants through colonization events during previous growing seasons, they were vertically transmitted from parent plants or we were not able to successfully isolate or identify specific EB during culturing (either due to isolation protocol or EB was outcompeted by other stains on agar media). The majority of EB (91.1%) occupied all sampled plant organs and all host species contained at least two EB species, across all recalcitrant and dormant species. This indicated that these EB are generalists that are not species-, organ- or germination strategy-specific. Sampling location influenced endophytic community composition, as one or two unique EB were isolated from each site or were shared among two sites (Figure 1b). However, five out

of the nine identified EB were shared among all three locations (Figure 1c), indicating a universal association between *Bacillus* endophytes and *Oxalis* hosts.

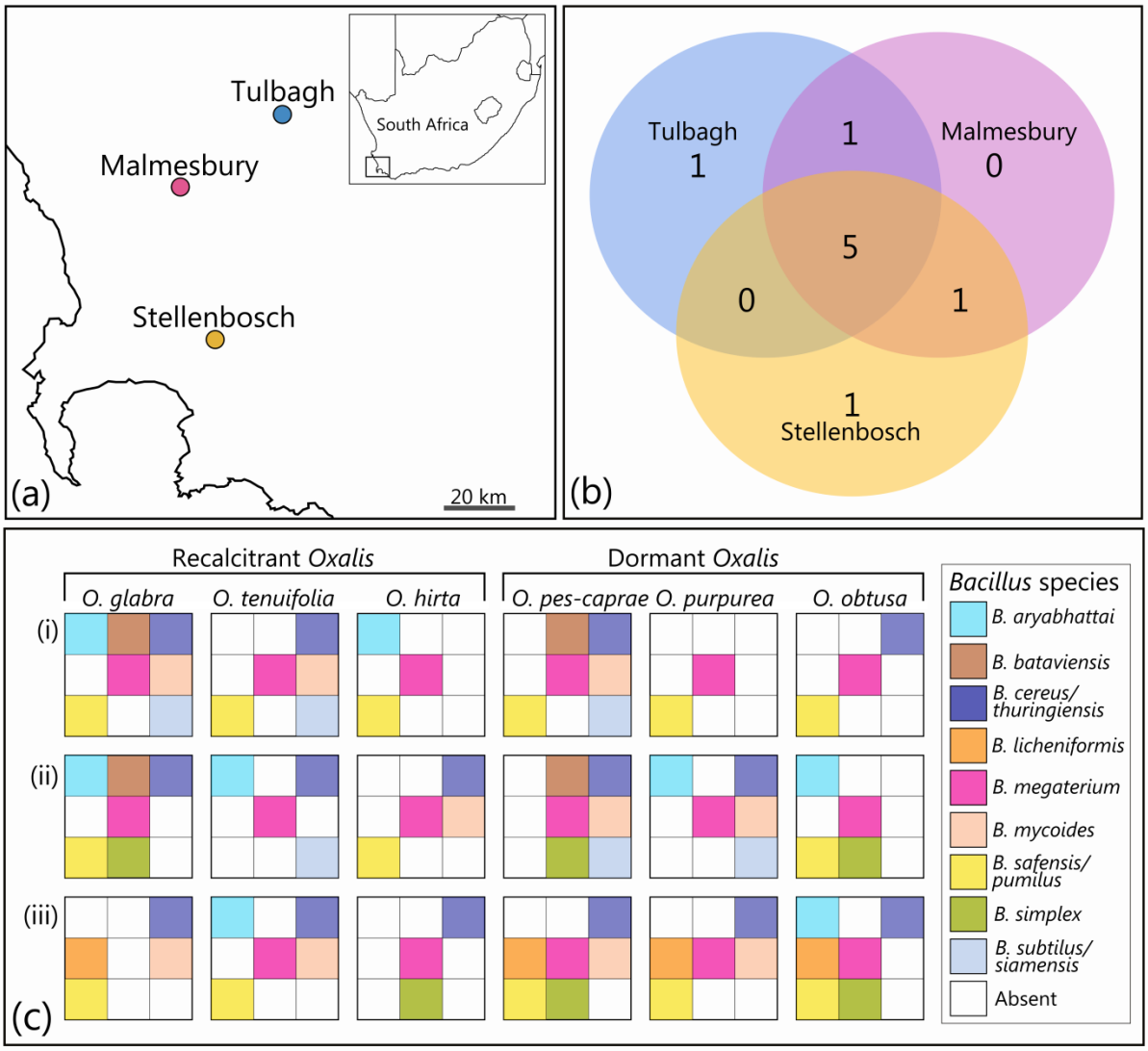

Figure 1: Community composition and diversity of the most abundant and consistent endophytic bacteria (EB) isolated from *Oxalis* hosts. (a) Sampling locations within the Cape of southern Africa, (b) Unique and shared EB diversity across sampling locations, (c) Endophytic *Bacillus* species isolated from reproductive and vegetative plant organs (combined isolates from roots, bulbs, leaves, stems and seeds) and five plant replicates of six *Oxalis* hosts sampled at three locations: (i) Tulbagh, (ii) Malmesbury, (iii) Stellenbosch.

EB were isolated from surface-sterilized, macerated seeds from all host species and locations. Multiple pure-culture colonies isolated from the vegetative (roots, bulbs, leaves, stems) and seed tissues from a single host plant were sequenced using universal bacterial 16S primers. Among 88.9% of studied cases identical sequences of EB isolates were obtained from parent

plant and seed material. Isolation and identification of these EB illustrated vertical transmission from parent plants to their offspring.

Seeds and fruits from 230 angiosperm genera are known to excrete carbohydrate-rich mucilage around the seed coat and base of the hypocotyl [32]. Most recalcitrant Cape *Oxalis* species produce large amounts of mucilage upon germination. Filter exclusion experiments were conducted to assess if EB inhabit the mucilage of germinating seedlings, and to determine if additional microbes from the soil actively move towards the mucilage. Filters had an average pore size of 10-15 µm, while all identified EB had dimensions of 3.19 x 1.33 µm (length SD=1.770 µm, width SD=0.671 µm). Using the sequenced and identified EB from macerated plant material as reference material (97.4% accuracy in morphotype identification), we have shown that the same suite of *Bacillus* species (and no other cultivable bacteria) were present in the mucilage of developing seedlings when germinated on sterile media (Figure 2a-b). The mean number of species obtained from both negative control treatments were significantly greater than zero (sterile agar mean=1.67, ±1.118 SE; sterile soil mean = 1.89, ±1.150 SE, $z=4.592$, $p>0.0001$), confirming that these EB were vertically transmitted from parent plants to their offspring. There was no significant difference between the number of species from the two sterile negative control treatments ($z=0.908$, $p=0.795$). The total diversity of vertically transmitted EB associated with each *Oxalis* host species was higher than the average values reported per seedling, as different combinations of EB species associated with individual seedlings.

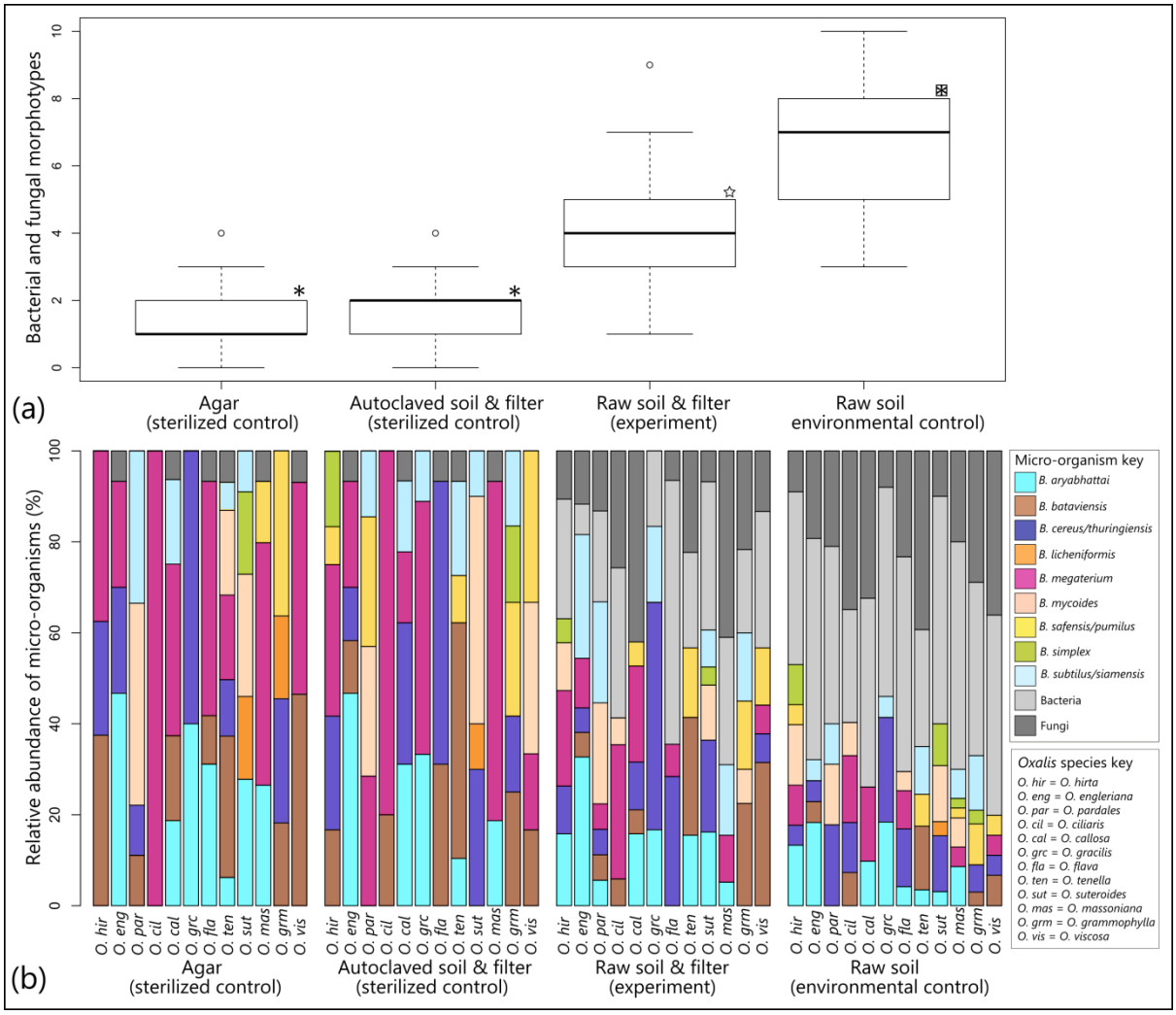

Figure 2: Abundance and community composition of micro-organisms (bacteria and fungi) isolated from mucilage produced by *Oxalis* seedlings. (a) Number of bacterial and fungal morphotypes isolated from the mucilage produced per seedling, averaged across 12 *Oxalis* species per treatment. All symbols are significantly different at $p<0.0001$. (b) Diversity and relative abundance of bacterial and fungal morphotypes associated with *Oxalis* mucilage. Each vertical bar represents percentage of each bacterial/fungal morphotype isolated across five seedling replicates per *Oxalis* species.

Environmental control treatments had the highest number of isolates per seedling (mean=6.80, ±1.121 SE), while treatments with a filter had a significantly lower average number of isolates (mean=4.08, ±1.129 SE) (Figure 2a). Both the filter exclusion treatment and the environmental control had significantly higher microbial diversity ($z=7.399$, $p<0.0001$ and $z=12.343$, $p<0.0001$ respectively) relative to the negative controls. Sampling location may have had an effect on number of isolates as the two *Oxalis* species sampled from the common garden collection had significantly fewer isolates per treatments (mean=1.17, $z=-2.363$, $p=0.018$) than field-species. This may indicate a bottleneck effect in

loss of microbial diversity in the garden soils. This experiment showed that a diverse assemblage of soil bacteria and fungi inhabit the mucilage of *Oxalis* seedlings, some which may be actively recruited from the rhizosphere and others provided (via vertical inheritance) by the seed itself (Figure 2b).

Among bacterial species isolated from macerated *Oxalis* seeds, 90.9% were identified as EB with well-documented growth promoting and/or nitrogen-fixing properties (Extended Data Table 1). Culture-independent 16S metabarcoding techniques revealed the presence of two bacterial genera with various known growth promoting traits (*Acinetobacter* and *Microbacterium*) from *Oxalis* seeds (Jooste *et al*., 2018; Chapter 4). This implies a possible role for selection favouring vertical transmission of beneficial endophytes. Three of the most prevalent endophytic species isolated from all *Oxalis* hosts and locations were the diazotrophic bacterial species *B. cereus/thuringiensis*, *B. megaterium* and *B. safensis/pumilus* (Figure 1c). Symbiosis with diazotrophic and plant growth promoting EB can be considered a highly beneficial association from the host plant's perspective. Preliminary $\delta^{15}N$ data obtained from 83 *Oxalis* species illustrated a wide range of values (16.78 – 2.09$^{o}/_{oo}$), including relatively light values approaching the range reported for plants associated with N-fixing EB. However, due to limited sampling and lack of reference soil samples [33], this requires further testing. To our knowledge this is the first report of a potentially nitrogen-fixing association between EB and geophytes. Future research should explore the extent of N-fixation in both seedlings and mature plants.

Potential benefits to the EB associates have not been tested, but could include a carbon-source for energy and housing inside *Oxalis* plant tissues. To date, nine EB have been isolated from four *Oxalis* species globally [34], and seven of these have known oxalotrophic properties. These include *Azospirillum brasilense* Krieg & Döbereiner, 1978, *B. amyloliquefaciens* Priest *et al.* 1987, *B. cereus, B. subtilis, B. vallismortis* Roberts *et al.* 1996, *Methylobacterium oxalidis* Tani *et al.* 2012 and *Serratia fonticola* Gavini *et al.* 1979 [34, 35]. Oxalotrophic bacteria have the metabolic capacity to utilize oxalates as their only (and often preferred) carbon source [35, 36], and typically form symbioses in order to access these compounds. Oxalotrophic bacteria have been isolated from various habitats [37], but are most commonly found in the rhizosphere and roots of plants that excrete large amounts of oxalate (most often in the form of oxalic acid or calcium oxalate crystals), such as members of the genus *Rumex* and *Oxalis* [37 - 39]. Due to the toxicity and low energy yield of oxalates, most

microbes cannot utilize these as an energy source [35, 40]. Anatomical sections through bulbs, leaves, fruits and seeds have revealed a diversity of crystal containing idioblasts, cavaties and epitelial-cell lined and unlined channels (Figure 3a-e).

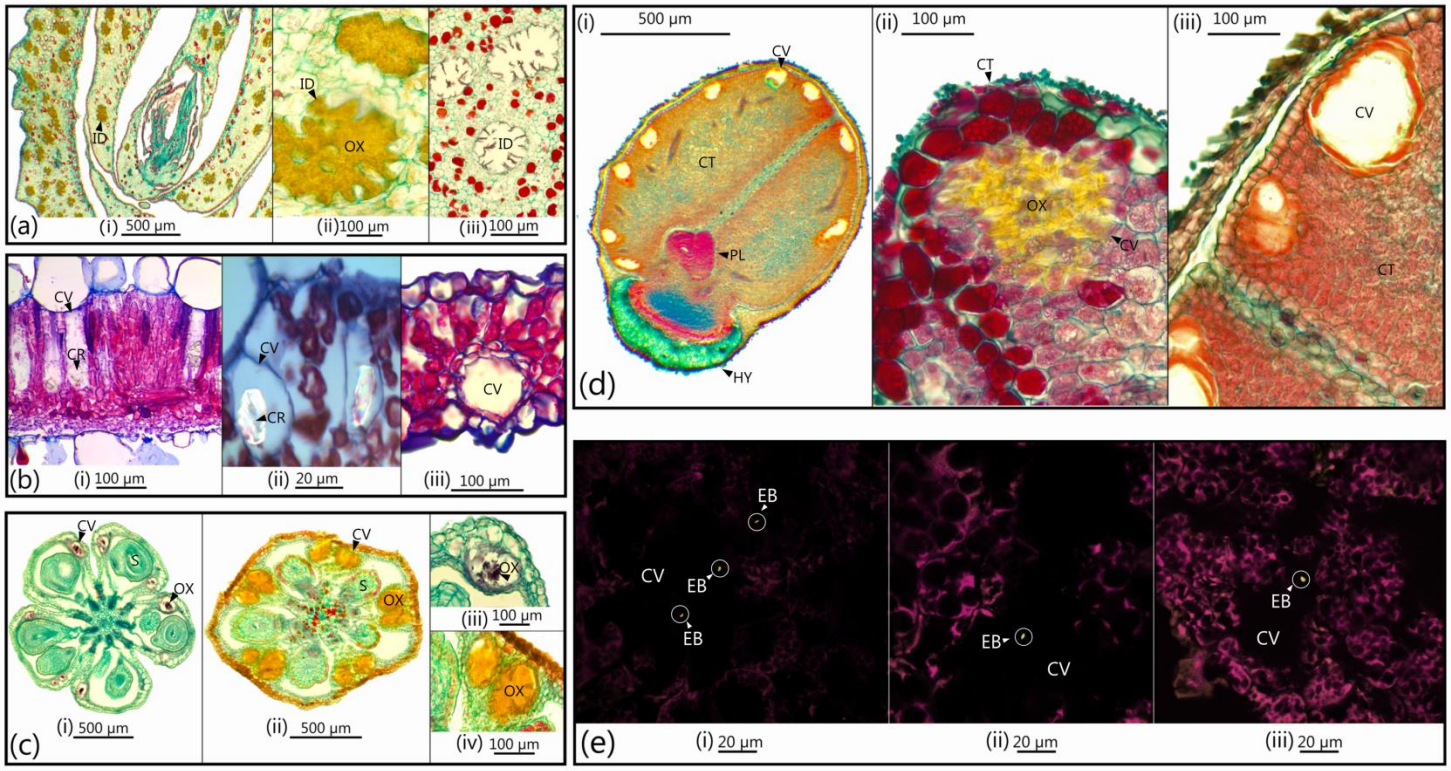

Figure 3: Specialized idioblast cells and cavities containing oxalates, and potential endophytic bacteria within Cape *Oxalis* host plants. (a) Longitudinal sections through bulb fleshy leaves with idioblasts containing oxalates (crystals) (i,ii) and a cross section of empty cavities (iii). (b) Cross sections of photosynthetic leaves with cavities containing oxalates (i,ii) and empty cavities with an epithelial lining (iii). (c) Cross sections of dormant (i) and recalcitrant (ii) fruit capsules with cavities containing oxalates. (d) Longitudinal sections of recalcitrant seeds with multiple cavities in the cotyledons (i), a cavity containing oxalates (ii) and empty cavities with epithelial lining (iii). (e) Confocal staining of cross sections of sterilized seeds indicating bacteria (bright green rods, circled in white) inside cavities. Original red-and-green confocal images are supplied in Extended Data Figure 3. EB=endophytic bacteria, CO=cotyledon, CR=crystals, CV=cavities, HY=hypocotyl, ID=idioblasts or idioblast cavities, OX=oxalates, PL=plumule, S=seeds. A key to all species names is provided in Extended Data Table 2.

DISCUSSION

In our study three EB with known oxalotrophic activity were ubiquitous among Cape *Oxalis*: *B. cereus/thuringiensis*, *B. licheniformis* and *B. subtilis*. Importantly all three of these oxalotrophs have also been reported as nitrogen-fixers, and at least one of each was isolated

from the seeds of every sampled host plant. These findings could indicate a strong association between *Oxalis* plants and diazotrophic oxalotrophic EB. Vertical transmission typically evolves when symbiotic associations are mutualistic, in order to ensure that the beneficial endosymbionts are transferred from one generation to the next [41, 42]. A recent study showed that oxalotrophic properties among EB were required to ensure colonization and transmission within host plants [43].

Oxalates are by-products of photorespiration [44] and high oxalate concentrations could be harmful to plant tissues, especially the photosynthetic system [45]. To avoid such damage, plants often compartmentalize oxalates as deposits within intracellular (ordinary cells or specialized crystal idioblasts) or extracellular structures (cavities) [46, 47]. Crystal idioblasts and cavities harbouring calcium oxalate crystals are well-documented in storage, vegetative and reproductive tissues of *Oxalis* [38, 48], including Cape *Oxalis*. Given that idioblasts and cavities are common among *Oxalis* species, and that oxalotrophic nitrogen-fixing EB were ubiquitous in *Oxalis* hosts, it is possible that endophytes might be housed inside these structures. That *Oxalis*-associated oxalotrophic EB would utilize oxalates as a carbon source, and in turn supply the hosts with biologically fixed nitrogen, is thus an intriguing possibility.

In this study the majority (95.5%) of seeds with large and/or abundant idioblasts or cavities with oxalates were detected among embryo tissue of recalcitrant *Oxalis* species. It is possible that this structural adaptation, together with the mucilage production associated with germination, could help ensure that recalcitrant species host and transfer the beneficial endophytes to their seeds. Even though oxalotrophic diazotrophic EB were detected among all *Oxalis* species, we suggest that recalcitrant seedlings may be most reliant on these associations given their inverted germination sequence. Additional nitrogen could lessen the need for immediate root growth, which could allow a resource allocation shift from root germination to leaf growth instead. It is possible that an association with EB were a pre-adaptation to such an unusual inversed sequence of germination and rapid growth and establishment of seedlings.

If shown to have benefit to the host, such a widespread association with EB could be a key mechanism that allows *Oxalis* to thrive and diversify in such nutrient-depleted environments as those present in the Cape. The discovery of this symbiosis could hold critical implications for our understanding of the distribution of *Oxalis*, its anatomy and physiology, the establishment of seedlings and evolution of this genus within the Cape Flora. Given the lack

of data for endophytic associations in other geophytes, this may be a more widespread phenomenon in the Cape and globally. This knowledge is also directly relevant to all conservation efforts, invasion biology and predictions of the expected response of this genus and other Cape plants under current climate change predictions.

Most importantly, all of these EB strains were isolated from surface-sterilized *Oxalis* seeds, illustrating vertical transmission from parent plants to the next generation. To date, growth promoting and nitrogen-fixing seed endophytes have been recorded among well-studied crop plants [49, 50], but are rarely documented among wild plants [51]. The confirmation that nitrogen-fixing EB are vertically transmitted among geophytes indicates that it may be a far more widespread phenomenon than previously thought. To date the only other known examples of vertical transmission of diazotrophic bacteria occur in the giant cordon cactus (*Pachycereus pringlei* S. Watson) [52] and an invasive grass (*Sorghum halepense* L.) [53], both from North America.

The many beneficial traits associated with diazotrophic and/or oxalotrophic seed EB could be used for applications in natural and agricultural systems, especially for plants grown in harsh and nutrient-depleted conditions [54, 55]. *Bacillus* species are widely known to be safe and highly effective when used to enhance crop growth and yield [56]. The roles and importance of nitrogen-fixing EB capable of vertical transmission, represents a fascinating, yet largely understudied aspect of the Cape Flora.

CONCLUSIONS

The discovery of vertical transmission of *Bacillus* endophytes from parent plants to the next generation and potential benefits to both host and endophyte, suggest a particularly tight mutualism in the *Oxalis*-endophyte system. Given that idioblasts and cavities are common among *Oxalis* species, and that oxalotrophic nitrogen-fixing EB were ubiquitous in *Oxalis* hosts, it is possible that endophytes might be housed inside these structures. Beneficial traits associated with diazotrophic and/or oxalotrophic *Bacillus* species could be implemented in various natural and agricultural systems, especially for plants grown in harsh and nutrient-depleted conditions. This discovery also suggests unexpected ways in which geophytes might avoid nitrogen deficiency, and suggest that such symbioses are more common than previously expected.

Abbreviations

Endophytic bacteria = EB; Nitrogen fixation = N- fixation

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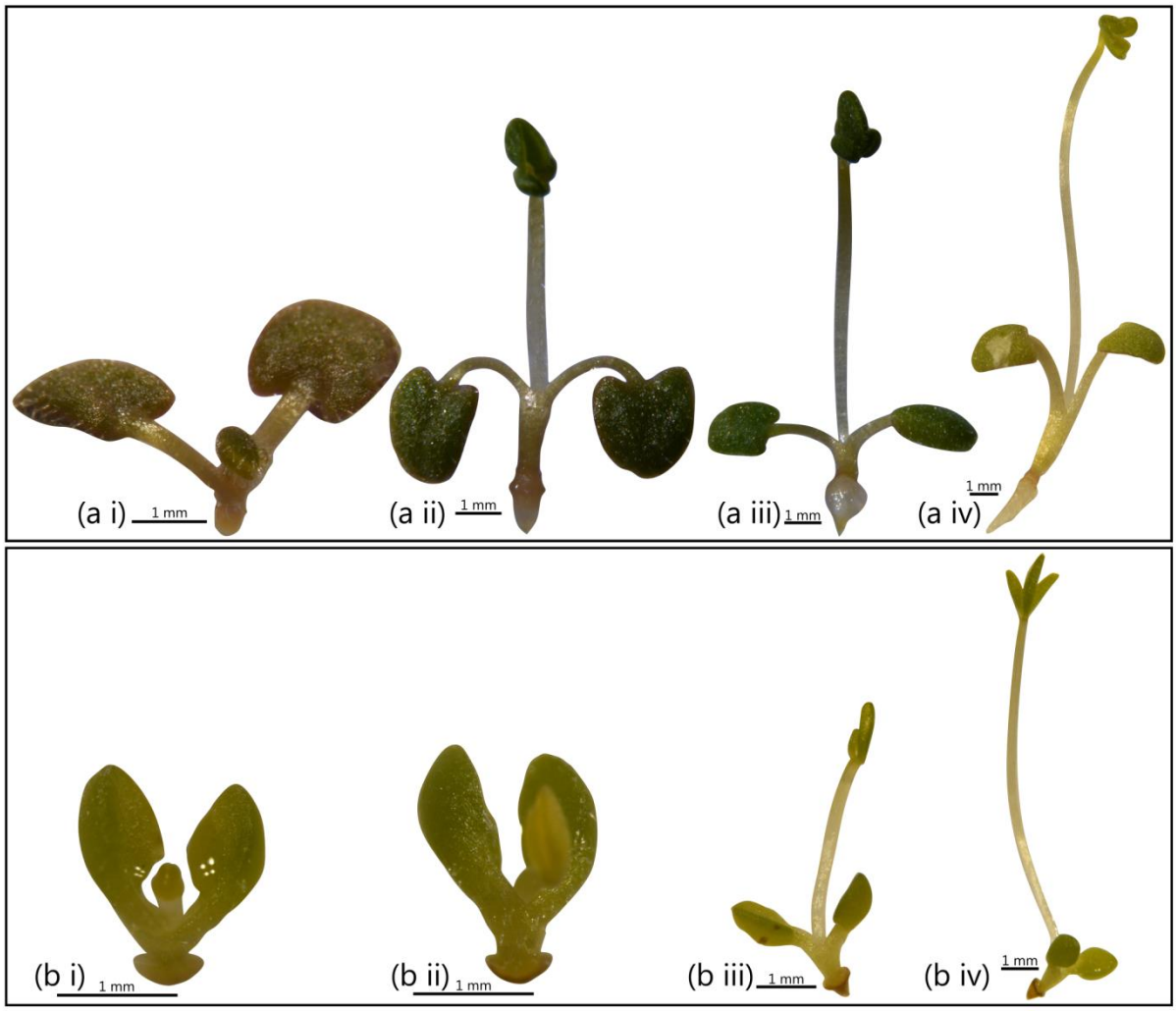
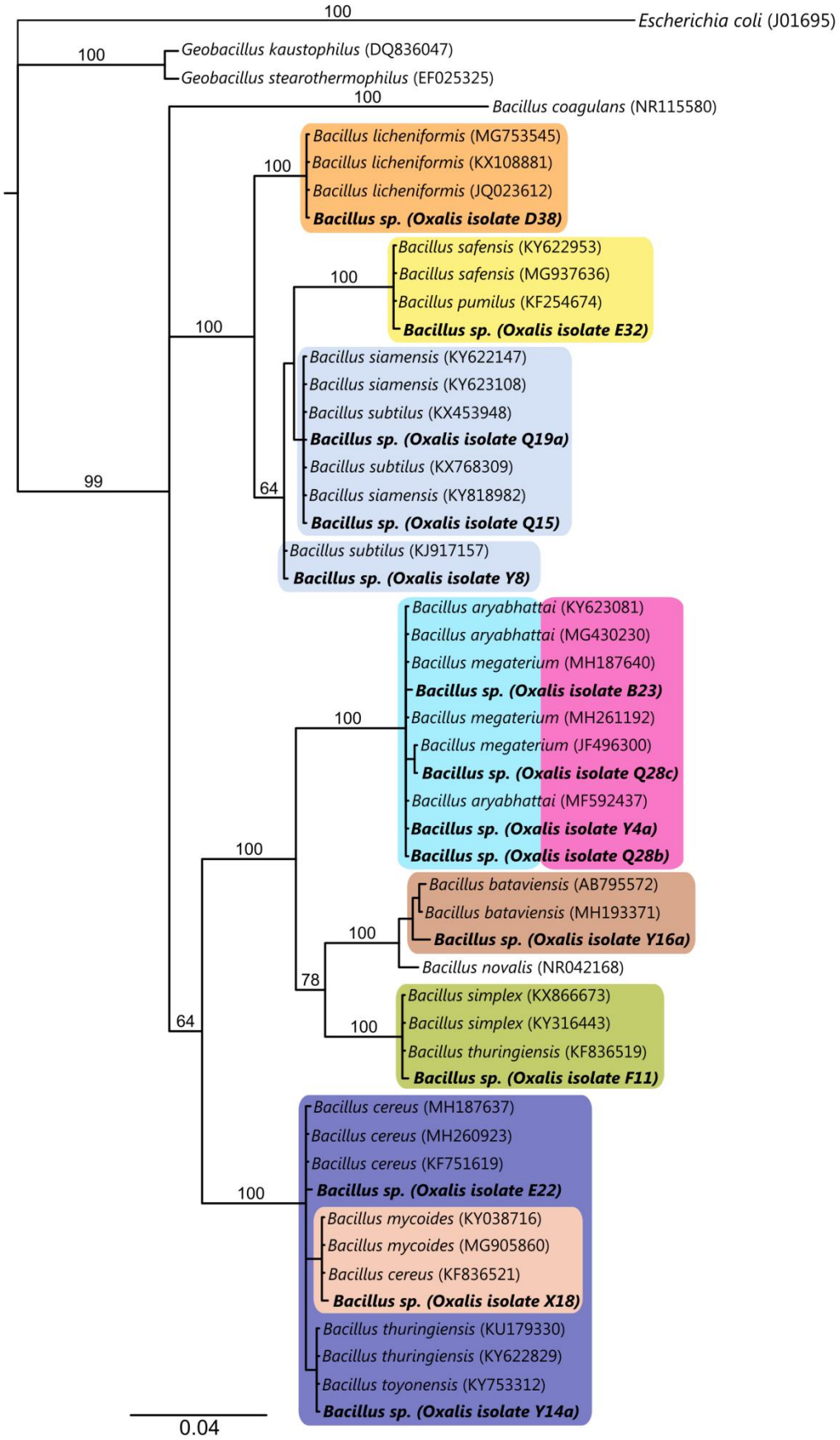
EXTENDED DATA

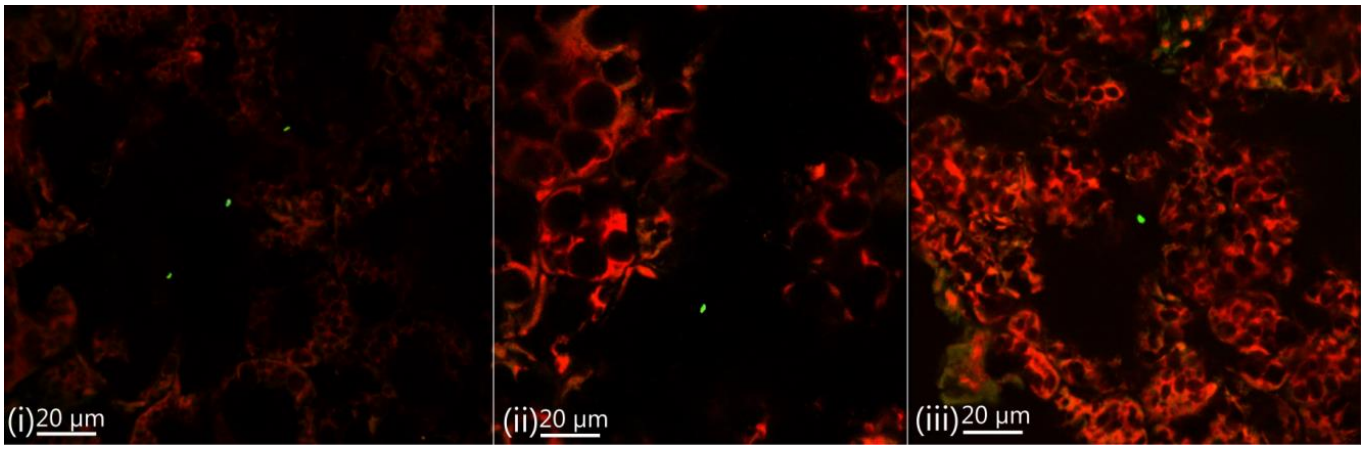


Extended Data Figure 1: Seedling germination and development of recalcitrant Cape *Oxalis*, where foliar leaf development and growth is followed by delayed radicle growth. *O. clavifolia* Sond. (a) and *O. glabra* Thunb. (b) one (i), three (ii), five (iii) and 10 (iv) days after germination. All seedlings oriented with radicle pointing to bottom of figure. CT=cotyledons, FL=foliar leaf, RD=radicle.



Extended Data Figure 2: Phylogenetic consensus tree constructed with universal 16S region sequences for endophytic bacteria isolated from Cape *Oxalis* seeds (boldface font) and

representative GenBank BLAST results. Colour boxes indicate the most likely species identifications of *Oxalis* isolates. *B. megaterium* and *B. aryabhattai* had unresolved relationships based on the consensus tree.



Extended Data Figure 3: Original red-and-green confocal staining images of cross sections of sterilized *Oxalis* seeds indicating bacteria (bright green rods) inside cavities. (i-ii) *O. hirta*, (iii) *O. pes-caprae.*

Extended Data Table 1: Properties of bacterial endophytes isolated from *Oxalis* host plants, as described in literature.

Bacterial endophyte	Known beneficial traits	Oxalotrophic metabolism	References
B. aryabhattai	Mobilization of zinc Various plant growth promoting mechanisms	*No*	(57)
B. bataviensis	Possible nitrogen fixation Improved availability of nitrogen	*No*	(58)
B. cereus	Nitrogen fixation Various plant growth promoting mechanisms	*Yes*	(58), (29)
B. licheniformis	Nitrogen fixation Phosphate solubilization	*Yes*	(60), (61)
B. megaterium	Nitrogen fixation Phosphate solubilization	*No*	(59), (62)
B. safensis	Possible nitrogen fixation Improved availability of nitrogen	*No*	(40)

B. siamensis	Anti-fungal activity	*No*	(63)
B. simplex	Phosphate solubilization Anti-fungal activity	*No*	(64), (65)
B. subtilus	Nitrogen fixation Various plant growth promoting traits Anti-pathogenic fungal activity	*Yes*	(63), (56)
B. thuringiensis	Various plant growth promoting traits Anti-pathogenic fungal activity	*No*	(66), (67)

Extended Data Table 2: A key to all species names relating to Figure 2.

Figure label		*Oxalis* species	Germination strategy
a)	*i-iii*	*O. hirta* L.	Recalcitrant
b)	*i*	*O. pulchella* Jacq.	Dormant
	ii	*O. camelopardalis* Salter	Recalcitrant
	iii	*O. foveolata* Turcz.	Dormant
c)	*i*	*O. cf. purpurea*	Dormant
	ii	*O. cf. hirta*	Recalcitrant
	iii	*O. cf. purpurea*	Dormant
	iv	*O. cf. hirta*	Recalcitrant
d)	*i*	*O. grammophylla* Salter	Recalcitrant
	ii	*O. xantha* Salter	Recalcitrant
	iii	*O. cf. pardales*	Recalcitrant
e)	*i-ii*	*O. hirta*	Recalcitrant
	iii	*O. pes-caprae*	Dormant

Chapter 6 Discussion

General discussion

This dissertation investigated the diversity and nature of symbiotic bacterial and fungal endosymbionts associated with native *Oxalis* species from the Greater Cape Floristic Region (Cape) of southern Africa. By implementing various techniques, we have documented exceptionally high endophytic diversity among *Oxalis* hosts and revealed that various plant growth promoting and nitrogen-fixing bacteria are vertically transmitted from parent plants to their offspring. We have also proposed a re-classification of Cape *Oxalis* germination strategies as a continuum of germination states, where an ancestral dormant strategy evolved towards a maximally recalcitrant peak, with a mosaic of intermediate states reflected in extant taxa. The role of beneficial endophtes in the evolution of such diverse germination strategies, plant biology and behaviour of Cape *Oxalis* were explored, in order to better understand the success of this genus, given the various biotic and abiotic stressors associated with the Cape.

The first data chapter proposes an extensive re-classification of *Oxalis* germination strategies (Chapter 2), into a continuum of dormant, recalcitrant and intermediate states. The high species diversity and intriguing seed biology of the Cape *Oxalis* provided an ideal model system to study the diversity of germination strategies. We explored the potential adaptive significance of observed morphological traits, potential evolutionary trends among germination strategies and environmental factors affecting germination strategies. Recalcitrance and intermediate germination strategies are rare among angiosperms (<11%), therefore insights gained from studying *Oxalis* as model system, promoted our understanding of the evolution of the recalcitrant germination strategy among angiosperms in general.

We documented the remarkable strategy of inverse (foliar-leaf first) germination displayed by the majority of recalcitrant species (*ca.* 60% of Cape *Oxalis*). These seedlings are capable of rapid growth and development without well-established roots to supply seedling with nutrients. Based on the copious mucilage secretion around the hypocotyl of such germinating seeds, a basic need for nutrients for seedling growth, and the pilot study observation of micro-organisms within the mucilage (*pers obs*.), we suspected this unique germination behaviour to be supported by undocumented plant growth promoting microbial associates.

The dissertation thus shifted focus to the diversity and distribution of endophytes associated with six phylogenetically spread *Oxalis* species with different germination strategies. Thereby we provide the first overview of the diversity and nature of endophytic bacteria and fungi associated with *Oxalis* from the Cape. We have found that Cape *Oxalis* harbour a very species rich community of endophytic bacteria and fungi, including 46 culturable bacterial and 39 culturable fungal morphotypes associated with host plants (regardless of seed germination strategy) (Chapter 3). We found that endophytic microbial richness and composition changed according to the surrounding environment. However, the most common and frequently encountered bacterial endophytes included members from the genus *Bacillus* - a group well-known for various plant-growth promoting properties. A surprisingly diverse collection of bacterial and fungal endophytes were also commonly found in the reproductive and vegetative propagules of all hosts.

Bacillus and *Pseudomonas* were the most common bacterial genera cultured from vegetative (roots, bulbs, stems and leaves) and reproductive (seeds) host plant organs, as well as the rhizosphere soil. *Aspergillus, Cladosporium, Fusarium, Talaromyces* and *Trichoderma* were the most common fungal genera cultured from vegetative, reproductive and rhizosphere samples. Many of these genera are known plant endophytes, with various documented plant growth promoting and nitrogen-fixing properties. Some (especially *Bacillus*) appeared to have an intimate association with their hosts.

I attempted to expand on the documentation of the extreme diversity of bacterial endophytes associated with the six *Oxalis* study species by employing metabarcoding techniques to identify non-culturable associates. These techniques proved to be problematic when applied to plant-associated microbes, in terms of a high number of plant chloroplast and/or mitochondrial reads, misidentification of chloroplast and/or mitochondrial sequences as bacteria or cyanobacteria, inconsistent number of bacterial reads among samples and a discrepancy in taxonomic richness based on different variable regions analysed. Chapter 4 explores these issues in some detail. Despite these caveats, metabarcoding did uncover significant insight into the rich collection of bacterial endosymbionts (taxa from 118 genera and various uncultured bacteria from79 families, 39 orders, 19 classes and eight bacterial phyla) associated with Cape *Oxalis*. Metabarcoding results confirmed the presence of six out of nine bacterial genera identified with culture dependent techniques. Importantly, these six

genera included various bacterial species (including *Bacillus*) with well-known plant growth promoting and nitrogen-fixing abilities.

The final data chapter (Chapter 5) constitutes a case study that emanated from the collective insights gained from the preceding chapters. We explore the discovery of a novel, vertically transmitted symbiosis between communities of nitrogen-fixing and plant growth-promoting *Bacillus* endophytes and *Oxalis* hosts in some detail. *Oxalis* plant tissues are known to be oxalate-rich, and we found that three of the ubiquitous nitrogen-fixing endophytic bacteria have known oxalotrophic properties (bacteria capable of using oxalates as their sole carbon source). These beneficial endophytes appeared to be contained inside specialised cavities (containing oxalates) within the plant body and seeds, deeming this a highly specialized symbiosis worthy of further study.

Vertical transmission of these beneficial bacteria may confer a selective advantage on *Oxalis* species within the nitrogen-deficient and seasonally dry environments of the Cape. We suggest that the vast symbiont-pool enable plants to establish in extremely short growing season (± 3-5 months) and allows *Oxalis* to tolerate and even thrive in such a wide range of nutrient-depleted habitats of the Cape. A recent experimental study identified plant nutrient-acquisition strategies as a key trait explaining plant responses to soil microbes, and how these interactions promote diversity in Mediterranean-climate shrublands (Teste *et al*., 2017). Similar interactions and effects could offer another explanation for the high species diversity of *Oxalis* (and other plant lineages) in the unique Cape region.

As the largest geophytic genus in the Cape, *Oxalis* contributes a major component of the Cape flora. More than one third of all Cape *Oxalis* are IUCN red-listed rare or endangered species with very narrow geographic ranges and specific habitat requirements (Raimondo *et al*., 2009). The Cape of southern Africa is globally renowned for its extraordinary biological diversity and the value and importance of biodiversity conservation has increased in recent years. Bacteria and fungi fulfil countless invaluable ecosystem services, which directly influence plants and animals inhabiting almost every niche on earth (Peay *et al*., 2016). Despite the apparent importance of microbial biodiversity and plant endophytic communities, to date these organisms have largely been neglected from conservation strategies (Souza *et al*., 2008). Knowledge and better understanding of southern Africa's rich biodiversity in general, and *Oxalis* in particular, will be vital to conservation planning and management, especially under predicted models of climate change.

Moisture availability plays an important role in in the dynamics of soil microbial communities in arid regions, and notable seasonal fluctuations (during dry and wet seasons) of microbial populations have been documented in the Succulent Karoo (Pieterse, 2017). Current climate change studies predict that rainfall in the Cape will become more irregular, which could have profound effects on the community structure and functionality of soil microbes in the affected areas, which in turn would affect *Oxalis* hosts.

Growth promoting and nitrogen-fixing seed endophytes have been recorded among well-studied crop plants (Lodewyckx *et al*., 2002; Bashan and De-Bashan, 2005), but are rarely documented among wild plants. To date, no such system has been reported for geophytes. The confirmation that nitrogen-fixing endophytes are vertically transmitted is a truly remarkable discovery, as the only other known examples of vertical transmission of diazotrophic bacteria are the giant cordon cactus and an invasive grass from North America. Future research and exploration of possible avenues for sustainable agricultural applications are needed, especially in regions where agriculture is limited by poor soil nutrition.

To date, the majority of research on biological nitrogen fixation among crop plants has been focused on legumes, so the recent discovery of nitrogen-fixing communities associated with maize (non-legume crops) received plenty of media attention (Van Deynze *et al*., 2018). These authors studied maize growing under low-nitrogen nutrient depleted fields, from areas of maize origin in Mexico, and found that these primitive maize plants have evolved strategies to overcome nitrogen deficiencies by forming symbioses with a microbiome capable of biological nitrogen fixation (Van Deynze *et al*., 2018). Maize plants displayed unusual bright red aerial roots that produced carbohydrate rich mucilage (Van Deynze *et al*. 2018). Authors showed that the high levels of recorded atmospheric nitrogen fixation were at least in part supported by the root mucilage, which hosted the nitrogen fixing microbes.

We can draw very strong parallels (mucilage production, red colouration of base of hypocotyl, nutrient poor soils, association with N-fixing bacteria) between this discovery and our own observations and discoveries in a group of plants originating from a very similar nutrient-depleated environment. Van Deynze *et al*. (2018) did, however, not report any possible evidence of vertical transmission and stated that the source of microbial inoculum is still unresolved. *Oxalis* and its endosymbionts therefore provide a truly unique opportunity to study vertically transmitted nitrogen-fixing endophytes, from a group of diverse and highly

successful angiosperms, that seems to thrive despite the low-nitrogen nutrient conditions associated with the Cape.

Future research

Future studies on the intriguing relationships between *Oxalis* hosts and their endosymbionts should start by documenting endophytic associations of the *ca*. 224 other Cape *Oxalis* species. We have literally just scraped the tip of the iceberg of host diversity. More detailed studies should focus on understanding and quantifying the direct effect of plant growth promoting microbes on host plant success. We suggest that the natural abundance of δ^{15} N isotopes in *Oxalis* plants and relevant reference material should be studied, in combination with elemental distribution and visualization of nitrogen and phosphates in seeds, seedlings and mature plants. Assessment of these data at specific intervals throughout the growing season of *Oxalis* (*e.g*. seeds at shedding, 1 week old seedlings vs. 1 month old seedlings vs. 3 month old seedlings vs. 1 year later), under field and experimental conditions would provide important physiological data that may help explain the role of endophytes in *Oxalis* seedling establishment.

Previous studies reported differences in soil and endophytic microbial communities that were significantly affected by seasonality in arid (Pieterse, 2017) and tropical regions (Kivlin and Hawkes, 2016). Seasonal variation of rhizosphere and endophytic microbial communities associated with *Oxalis* should thus be investigated at specific time intervals, for species distributed throughout the winter- and summer-rainfall regions of the Cape.

Interactions between different fungal and bacterial endophytes within a single host plant will also be an interesting topic for future research. Many of the *Oxalis* endophytes identified in our study are known to produce secondary metabolites with known anti-fungal or anti-bacterial properties. Understanding how these microbes interact with one another within a host plant could also help understand the diversity of observed community assemblages. Future studies can also focus on understanding co-evolution among *Oxalis* hosts and their endosymbionts, and how these interactions affected angiosperm species richness in biodiversity hotspots.

Knowledge of vertical transmission of nitrogen-fixing and oxalotrophic bacteria among angiosperms may have far-reaching agricultural and economic applications that definitely warrant future research. Future research into methods of agricultural applications should

establish the composition of seedling secreted mucilage and determine if mucilage and/or oxalic acid (and related idioblasts or cavities) are pre-requisites to host nitrogen fixing endophytes. Experimental inoculations could be done on crop species to determine if endophytes have similar beneficial effects and ultimately determine if the endophytes are successfully vertically transmitted.

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