Do mutualistic organisms associated with wild *Nicotiana benthamiana* plants influence drought tolerance?

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B. Sc. Ag. (Hons.), M. S. (Plant Path.)

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Doctor of Philosophy

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DECLARATION

I declare that this thesis is my own account of my research and contains as its main content work which has not been previously submitted for a degree at any tertiary educational institution.

Khondoker M. G. Dastogeer

22 September 2017

A note on formatting and style

This thesis comprises a number of published papers as well as some submitted or ready to submit manuscripts, each of which represents a chapter. In these cases, the formatting style follows the respective journal guidelines.

This thesis has continuous pagination. For published documents, the original journal page numbers are also provided.

Khondoker M.G. Dastogeer

Statement of Contribution

This thesis includes three original papers published in peer reviewed journals and two unpublished publications. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the school of veterinary and life sciences under the supervision of Prof Michael Jones, Dr Steve Wylie and Dr Hua Li.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

My contribution to the work and those of the co-authors involved the following:

Thesis Chapter	Publication Title	Status	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution
Chapter 2	Differential response of wild-collected Australian *Nicotiana* species to water stress highlights natural variation among the accessions	Under review	65%, Concept, laboratory works, data analysis and writing manuscript	1) Hua Li, Sample collection, concept, input into manuscript, 10% 2) Krishnapillai Sivasithamparam, concept and input into manuscript, 5% 3) Michael Jones, input into manuscript, 5% 4) Stephen Wylie, concept, sample collection and input into manuscript 15%
Chapter 3	Host specificity of endophytic mycobiota of wild *Nicotiana* plants from arid regions of northern Australia.	Published in Microbial Ecology. DOI:10.1007/s00248-00017-01020-00240.	70%, Concept, laboratory works, data analysis and writing manuscript	1) Hua Li, sample collection, input into manuscript 10% 2) Krishnapillai Sivasithamparam input into manuscript 5% 3) Michael Jones, input into manuscript 5%

				4) Stephen Wylie, concept, sample collection and input into manuscript 10%
Chapter 4	A simple and rapid *in vitro* test for large-scale screening of fungal endophytes from drought-adapted Australian wild plants for conferring water deprivation tolerance and growth promotion in *Nicotiana benthamiana* seedlings.	Published in Archives of Microbiology DOI:10.1007/s00203-00017-01411-00200.	65%. Concept, laboratory works, data analysis and writing manuscript	1) Hua Li, concept, input into manuscript 10% 2) Krishnapillai Sivasithamparam, concept and input into manuscript 10% 3) Michael Jones, input into manuscript 5% 4) Stephen Wylie, concept, concept and input into manuscript 10%
Chapter 5	Metabolic responses of endophytic *Nicotiana benthamiana* plants experiencing water stress	Published in Environmental and Experimental Botany 143, 59-71.	65%, Concept, laboratory works, data analysis and writing manuscript	1) Hua Li, concept, input into manuscript 5% 2) Krishnapillai Sivasithamparam, and input into manuscript 5% 3) Michael Jones, input into manuscript 5% 4) Xin Du, laboratory works 5% 5) Yonglin Ren laboratory works 5% 6) Stephen Wylie, concept, concept and input into manuscript 10%
Chapter 6	Fungal endophytes and virus mediated drought tolerance of *Nicotiana benthamiana* plant involves accumulation of	Unpublished	70%, Concept, laboratory works, data analysis and writing manuscript	1) Hua Li, concept, input into manuscript 5% 2) Krishnapillai Sivasithamparam, concept and input into manuscript 5%

	osmolytes and antioxidant enzymes and expression of host drought responsive genes.			3) Michael Jones, input into manuscript 5% 4) Stephen Wylie, concept, and input into manuscript 15%

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The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work.

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	osmolytes and antioxidant enzymes and expression of host drought responsive genes.			3) Michael Jones, input into manuscript 5% 4) Stephen Wylie, concept, and input into manuscript 15%

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Hua Li: Date: 14 Nov 2017

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ABSTRACT

In arid regions, such as those in north-western Australia, plants survive under water deficit, high temperatures, intense solar radiation and nutrient-impoverished soils. They employ various morpho-physiological and biochemical adaptations including interaction with microbial symbionts. Seed from thirty-two accessions of four *Nicotiana* species (*N. benthamiana, N. occidentalis, N. simulans,* and *N. umbratica*) collected from wild plants from northern Australia, were grown and used to assess their responses to water stress. The original wild host plants were selected because they grow in an extremely hot environment where water supply is often unpredictable, and because they share a close genetic relationship to the international model plant *N. benthamiana* research accession 4 (RA-4). Under moderate water stress conditions, shoot fresh weight, shoot dry weight, root fresh weight, root dry weight, root/shoot ratio, and relative water content of leaves was significantly affected. However, the degree to which the accessions were affected varied considerably. Some accessions of *N. simulans*, *N. benthamiana* and *N. occidentalis* were significantly more affected by water stress than others. There was significant inherent variation between accessions in leaf and shoot tip wilting times. Initial symptom expression (leaf wilting) was significantly delayed in two accessions of *N. benthamiana* and in one accession of *N. umbratica*. The least water stress tolerant lines, three accessions each of *N. occidentalis* and *N. simulans* exhibited advanced symptoms of water stress (shoot tip wilting) within 14-17 days of cessation of watering. This stage was significantly delayed in three accessions of *N. benthamiana* and two accessions each of *N. occidentalis* and *N. simulans*, which exhibited tip wilting after only 21-24 days. There were variations among the accessions of *Nicotiana* species on their tolerance to water stress. Plant responses to water stress could not be predicted from their phenotype under well-watered conditions.

We evaluated identity, host and tissue association, and geographical distribution of fungal endophytes isolated from above and below-ground tissues of wild plants of three indigenous Australian *Nicotiana* species. Isolation frequency and α-diversity were significantly higher for root endophyte assemblages than those of stem and leaf tissues. We recorded no differences in endophyte species richness or diversity as a function of sampling location, but did detect differences among different host genotypes and plant tissues. There was a significant pattern of community similarity associated with host genotypes but no consistent pattern of fungal community structuring associated with sampling location and tissue type, regardless of the community similarity measurements used.

We developed and evaluated two rapid screening methods to identify fungal endophytes that enhanced water deprivation stress tolerance in seedlings of *N. benthamiana* RA-4. Sixty-eight endophyte isolates taken from wild *Nicotiana* plants were co-cultivated with *N. benthamiana* RA-4 seedlings on either damp filter paper or on an agar medium before being subjected to water deprivation. The longevity of seedlings was compared under association with different fungal isolates and under the two screening methods. The filter paper method was faster and simpler than the agar-based method. Based on results, 17 isolates were selected for further testing under water deprivation conditions while growing in washed river sand in a glasshouse. Only two fungal isolates, one

resembling *Cladosporium cladosporioides* (E-162) and a fungus not closely related to any described species (E-284), significantly enhanced seedling tolerance to moisture deprivation consistently in both *in vitro* and glasshouse-based tests. Although a strongly significant correlation was observed between any two screening methods, the results of the filter paper test was more strongly reflected ($r = 0.757$, $p < 0.001$) in results of the glasshouse-based test, indicating its relative suitability over the agar-based test. In another experiment, the same 17 isolates were inoculated to *N. benthamiana* plants growing in sand in a glasshouse under nutrient-limiting conditions to test their influence on growth promotion. Isolates resembling *C. cladosporioides*, *Fusarium equiseti*, and *Thozetella* sp. promoted seedling growth, evidenced by increased shoot length and higher biomass than non-inoculated control.

The two promising fungal endophytes identified from wild *Nicotiana* plants, E-162 (*C. cladosporioides*) and E-284 (an unidentified species) were inoculated to plants of *N. benthamiana* RA-4 to examine their metabolic response to endophyte colonisation under adequate water and water deficit conditions. We examined leaf metabolites using gas chromatography-mass spectrometry (GC-MS) to compare levels of sugars, sugar alcohols, amino acids and other metabolites at various stages of plant growth and stress application. Ninety-three metabolites were detected in leaves, including 20 sugars, 13 sugar alcohols, 21 amino acids, 29 organic and fatty acids and ten other compounds. Endophyte colonization caused significant differential accumulation of 17-21 metabolites when the plants were grown under well-watered conditions. The presence of endophytes under water stress conditions caused differential accumulation of cytosine, diethylene glycol, galactinol, glycerol, heptadecanoate, mannose, oleic acid, proline, rhamnose, succinate, and urea. Accumulation of these metabolites suggests that fungal endophytes influence plants to accumulate certain metabolites under water-stress. Further, the two different endophytes tested caused slightly different accumulation patterns of some metabolites.

We evaluated how these two fungal endophytes as well as yellowtail flower mild mottle virus (genus *Tobamovirus*), influenced water stress tolerance in *N. benthamiana* RA-4 plants. The water stress tolerance of fungus-inoculated plants correlated with increased plant biomass, relative water content, soluble sugars, soluble proteins, proline content, increased activity of the antioxidant enzymes catalase, peroxidase and polyphenol oxidase, and decreased production of reactive oxygen species and electrical conductivity in plants under water stress. In addition, we found that there was significant differential upregulation of drought-related genes in the fungus-inoculated plants subjected to water stress. Plants inoculated with the virus exhibited a similar response to those plant inoculated with the fungi in terms of increasing plant osmolytes, antioxidant enzyme activity and gene expression. Although the fungus and virus infection similarly increased plant water stress tolerance by influencing plant physiology and gene expression, their presence together in the same plant did not have an additive effect, nor did they decrease water stress tolerance.

These findings suggest that both fungi and virus influence plant physiology and gene expression under water stress, and it suggests that there is potential to use endophytic fungi, and perhaps virus, to induce greater tolerance to water stress in agricultural production systems.

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LIST OF PUBLICATIONS

1. **Dastogeer, K. M. G.**, Li H., Sivasithamparam K., Jones M. G. K., Wylie S. J. (2017) Host specificity of endophytic mycobiota of wild *Nicotiana* plants from arid regions of northern Australia. *Microbial Ecology*. doi:10.1007/s00248-017-1020-0 (Chapter 3)
2. **Dastogeer, K. M. G.**, Li. H., Sivasithamparam, K., Jones, M. G. K., Wylie, S. J. (2017) A simple and rapid *in vitro* test for large-scale screening of fungal endophytes from drought-adapted Australian wild plants for conferring water deprivation tolerance and growth promotion in *Nicotiana benthamiana* seedlings. *Archives of Microbiology* doi:10.1007/s00203-017-1411-0. (Chapter 4)
3. **Dastogeer, K. M. G.**, Du, B., Li. H., Sivasithamparam, K., Jones, M. G. K., Wylie, S. J. (2017) Metabolic responses of endophytic *Nicotiana benthamiana* plants experiencing water stress. *Environmental and Experimental Botany*. 143:59-71. (Chapter 5)
4. **Dastogeer, K. M. G.**, Li. H., Sivasithamparam, K., Jones, M. G. K., Wylie, S. J. (2017) Differential response of wild-collected Australian *Nicotiana* species to water stress highlights natural variation among the accessions (Chapter 2: *Australian Journal of Botany*-under review).
5. **Dastogeer, K. M. G.**, Li. H., Sivasithamparam, K., Jones, M. G. K., Wylie, S. J. (2017) Fungal endophytes and virus mediated drought tolerance of *Nicotiana benthamiana* plant involves accumulation osmolytes and antioxidant enzymes and expression of host drought responsive genes. (Chapter 6: *Journal of environmental and Experimental Botany*-under review).
6. **Dastogeer, K. M. G**., and Wylie, S. J. (2017). Plant–fungi association: Role of fungal endophytes in improving plant tolerance to water stress. In "Plant-Microbe Interactions in Agro-Ecological Perspectives" (D. P. Singh, H. B. Singh and R. Prabha, eds.), Vol. 1: Fundamental Mechanisms, Methods and Functions, Springer Nature Singapore, pp. 143-159. Doi:10.1007/978-981-10-5813-4 (Appendix I)
7. **Dastogeer, K. M. G.**, Jones, M. G. K., Wylie, S. J. (2017) Fungal endophytes alter plant physiological responses to water stress: a meta-analysis (Appendix II: *Scientific Reports*-will be submitted).

LIST OF PRESENTATIONS

Oral Presentations

- Global microbiome Symposium 2017, Harry Perkins Institute of Medical Research, Perth, WA, Aug 18, 2017.

  -Host species shapes fungal endophyte community structuring in native Australian *Nicotiana* plants. Oral presentation.

- Combined Biological Science Meeting 2017, UniClub, University of Western Australia, Perth, WA, Aug 25, 2017.

  -Mutualistic fungal endophytes enhance host tolerance to water stress through reprogramming host metabolism.

Posters presentations

- Annual Postgraduate Poster Day, School of Veterinary & Life Sciences, Murdoch University, Perth, WA. Nov 09, 2016.

  - Fungal endophytes of Australian *Nicotiana* species: Influence of location, host and tissue types on endophyte community structuring.

- XVII Congress of International Society for Molecular Plant Microbe Interaction (ISMPMI), Portland, Oregon, U.S.A. July 17-21, 2016

  -Harnessing power of fungal endophytes that enhance plants to tolerate abiotic stress. Poster presentation.

- First Annual Symposium of West Coast Microbiome Network, University of Western Australia, Perth, WA, March 11th, 2016

  -Identifying microbes that live inside wild plants: influences on tolerance to abiotic stress.

ABBREVIATIONS

ANOSIM	Analysis of similarity
ANOVA	Analysis of variance
APX	Ascorbate peroxidase
Blast	Basic local alignment search tool
CCI	Chlorophyll concentration index
cDNA	Complementary DNA
df	Degrees of freedom
DMRT	Duncan's new multiple range test
DNA	Deoxyribonucleic acid
dpi	Days post inoculation
dps	Days post-stress
dsRNA	Double-stranded RNA
DW	Dry weight
Fv/Fm	Maximum quantum yield
FW	Fresh weight
GC–MS	Gas chromatography–mass spectrometry
GSH	Glutathione
H_2O_2	Hydrogen peroxide
IPCC	Intergovernmental panel on climate change
ITS	Internal transcribed spacer
MCL	Maximum composite likelihood
MDA	Malondialdehyde
mg	milligram
mg/L	milligrams per Liter
mRNA	Messenger RNA
NCBI	National center for biotechnology information
NMDS	Non-metric multi-dimensional scale
nmol/L	nanomoles per liter
PCA	Principal component analysis
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PLS-DA	Partial least squares-discriminant Analysis
PPO	Polyphenol oxidase
RA4	Research accession 4

RCBD	Randomized complete block design
RDW	Root dry weight
RFW	Root fresh weight
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RSR	Root shoot ratio
RT-qPCR	Quantitative reverse transcription PCR
RWC	Relative water content
SDW	Shoot dry weight
SE	Standard error
SEM	Scanning electron microscopy
SFW	Shoot Fresh Weight
SOD	Superoxide dismutase
ssDNA	single-stranded DNA
TW	Turgid weight
W.A.	Western Australia
WT	Water treatment

Chapter 1

Review of Literature

(Note: A part of this chapter has been published as a book chapter. A copy of the book chapter has been attached in the Appendix-I. I did a systematic review on the effect of fungal endophyte on plants under stress using meta-analysis which will be submitted to '*Scientific Reports'*. A copy of this has been attached in the Appendix-II)

1. Review of Literature

1.1. Impact of water stress on plants

Plants are exposed to environmental (abiotic) stresses, including shortage of water, extreme temperatures, salinity, and chemical toxicity, all of which bring serious consequences to plant production. Abiotic stress collectively causes up to 50% losses in the worldwide agricultural crop (Bray, 2000). In spite of our ability to predict onset of drought and to modify its impact, drought is still the single most crucial abiotic threat disrupting world crop productivity (Lambers et al., 2008; McWilliam, 1986). The most devastating losses of crop production due to droughts occur in developing countries, and the most vulnerable regions to drought are situated in Sub-Saharan Africa and Asia. The African continent has experienced severe episodes of major drought that caused huge losses in crop production (Anonymous, 1984; McWilliam, 1986). A minimum of 23 million hectares in Asia (20% of the total rice area) are prone to droughts of varying degrees (Pandey and Bhandari, 2009). In 2004, many countries in South-east Asia suffered from severe drought that caused loss of millions of dollars (Times, 2005). In 2010–2011, 20% of farmland and 35% of the entire wheat crop were damaged due to drought that affected eight China provinces (Krishnan, 2011). In the USA in 2012, a severe drought affected 80% of cropped land and decreased yields of corn by 27.5% and of soybean by 10%, with massive financial losses (USDA, 2013). The 2010 droughts in Russia caused a reduction in wheat harvest by 32.7% severely diminishing the world's wheat supply (Sternberg, 2011).

Over the last 100 years, Australia has experienced at least eight major extensive droughts and several severe regional droughts (McWilliam, 1986). For example, in 1902, during the period of Australian Federation, Australia suffered a devastating drought when the wheat crop was "all but lost" (BOM, 1999). The erratic behaviour of rainfall of Australia is attributable to the phenomenon called El Niño Southern Oscillation system **(**ENSO**)** which occurs every three to seven years. The drought in the 1914-1915 resulted in the destruction of the 1914 wheat yield (BOM, 2006). The drought in 1982/83 was thought to the most notorious in the 20th Century that caused loss of over US$4000 million in decreased crop production (BAC, 1985; EMA, 1997). Cotton production declined by 66% in comparison with five years earlier which was considered a "normal" year (WAA, 2004). Over next decades the occurrence and severity of drought in Australia are likely to increase due to of global climate change (Quiggin, 2010). Regional predictions advise that Australia will be badly affected by alteration in rainfall patterns, as well as by increasing temperatures, which increase the severity of drought. It is predicted that Australia could confront 40% more drought months by 2070 with worse circumstances in a soaring-emission situation (CSIRO, 2007). It is projected that climate change in the coming decades will alter average temperature and rainfall values and will increase the unpredictability of precipitation events which may lead to even more severe and frequent droughts

with raise from 1% to 30% in extreme drought land area by 2100 (Fischlin et al., 2007; IPCC, 2007; Walter et al., 2011).

Crop growth and development are severely affected by water leading to significant reductions in yield and overall productivity. The main consequences of drought in crop plants are decreased cell division and growth rate, reduced size of leaves, reduced stem enlargement and disrupted stomatal oscillations, and imbalanced water and nutrient ratios (Farooq et al., 2009). If the crop experiences water stress during early stage, germination and seedling establishment are severely arrested mainly owing to low water uptake, low energy supply and hindered enzyme functions (Kaya et al., 2006; Okçu et al., 2005; Taiz and Zeiger, 2010). Water deficit causes reduced dry matter content in all parts of the plant (Asrar and Elhindi, 2011; Liu et al., 2011) and minimize the leaf area index (LAI) in crop plants (Hussain et al., 2009; Kramer and Boyer, 1995). The crop phenology is also affected by limiting water supply that generates a signal to trigger an early switching of plant development from the vegetative stage to reproductive stage and thereby shorten the crop growth cycle (Desclaux and Roumet, 1996) which generally leads to significant yield losses. Moreover, all major attributes of plant water relations viz. relative water contents (RWC), leaf water potential, osmotic potential, pressure potential, and transpiration rate are significantly affected under drought conditions leading to impaired crop productivity (Kirkham, 2005).

1.2. Plant strategies to withstand water stress

The underlying mechanisms of how plants respond to drought stress have been explored to a great extent from molecular to whole-plant levels. Researchers have identified hundreds of genes that are activated in plants in response to stress. A variety of tools including gene expression patterns and the use of transgenic plants has been developed to investigate the particular roles of these genes in plant responses to stress. Transgenic technologies and the advent of genomics and proteomics have offered a comprehensive profiling of the changes in gene and protein expression resulting from exposure to drought.

Plant reactions to water deficit stress are complicated since it is a function of time and space, and it involves multifaceted mechanisms from genomic, molecular and biochemical levels (Blum, 1996; Chaves et al., 2003; Xu et al., 2009). Plants use different mechanisms to cope with the stress, and the way a plant behaves under drought can be explained by the following six broad strategies: i) Escaping from drought by terminating plant life cycle prior to onset of severe stress, e.g. early flowering in annuals before the start of water deficit (Geber and Dawson, 1990), ii) Drought avoidance through increasing water uptake and reducing water loss, e.g. developing root systems and reducing of stomata and canopy area (Jackson et al., 2000; Schulze, 1986), iii) Drought tolerance chiefly through maintaining a better osmotic balance and expanding elasticity of the cell wall to keep the tissue turgid (Morgan, 1984), iv) Drought resistance via changing metabolic routes to thrive under stress condition

(e.g. greater antioxidant metabolism) (Bartoli et al., 1999), v) Drought abandonment by shedding one or more plant organ, e.g. detaching older foliage during drought (Chaves et al., 2003), vi) Drought-adapted physio-biochemical characters developed through plant evolution under long-term drought conditions via mutation and modifications at the genomic level (Hoffmann and Merilä, 1999; Maherali et al., 2010; Sherrard et al., 2009).

1.3. Plant-microbe interactions and drought tolerance in plant

The interaction of microbes with the plant can be traced back to the origin of plants. The early evolution of plants occurred in a diverse microbial world. Archaea, bacteria, fungi, and viruses had been evolving for billions of years (Reid and Greene, 2013). The most well-known plant–microbe interaction is the mutualism between mycorrhizal fungi and plants where both partners generally benefit from each other. However, under natural settings, plants form relationships with endophytic fungi and viruses which can be beneficial or harmful for the partners depending on host types and natural and environmental situations (Bao and Roossinck, 2013). Plants in natural systems and crop lands are simultaneously exposed to both biotic and abiotic stresses. Though stress research is mostly focusing on plant responses to a particular environmental stress, research focusing to both biotic and abiotic stresses together has also been conducted (Garrett et al., 2006; Xu et al., 2008). Unravelling the complex mechanisms of plant–microbe relations and their effects in abiotic stress tolerance in plants could potentially advocate novel tactics to boost the productivity of crops (Schenk et al., 2012).

1.3.1. What are fungal endophytes?

The term ‘endophyte’ refers to fungi that live inside the plant intercellular and intracellular spaces for at least part of their life cycle, causing no concurrent visible symptoms at any specific moment (Purahong and Hyde, 2011; Rodriguez et al., 2009). This definition of endophyte is strictly operational and contextual since it takes into account the result of a specific fungus–host interaction only in a given time under the particular environmental settings, because symptomless endophytes can behave differently (e.g. as pathogens) under altered environmental conditions (Andrew et al., 2012; Márquez et al., 2012). The existence of fungal endophytes from fossil records suggests that endophyte–host associations may have evolved from the time of development of first higher plants on earth (Krings et al., 2012; Rodriguez and Redman, 1997). Based on the survey conducted in the last 20 years on endophytes, it is thought that the majority, if not all plants, have one or more types of these endophytes and numerous endophytic species; in some cases, above a hundred can be found in a certain plant species (Arnold, 2007). Fungal endophytes have been documented from healthy aerial tissues of conifers (Petrini and Fisher, 1986) and grasses (Clay, 1988). Further, fungal endophytes have also been reported from marine algae (Hawksworth, 1988), lichens (Li et al., 2007), mosses and ferns (Fisher, 1996), palms (Fröhlich and Hyde, 1999) and pteridophytes

(Dhargalkar and Bhat, 2009). Fungal endophytes can be grouped into three basic ecological groups: (1) mycorrhizal fungi, (2) balancious or 'grass endophytes' and (3) non-balancious endophytes (Schulz and Boyle, 2005). However, Brundrett (2004) separated mycorrhizal from endophytic interactions in that mycorrhizas have coordinated plant– fungus development and nutrient transfer at specialized interfaces. Later, Rodriguez et al. (2009) classified the endophytes under two major groups, viz. clavicipitaceous and non-clavicipitaceous on the basis of phylogeny and life history traits. Clavicipitaceous fungal endophytes are limited to certain grasses, while non-clavicipitaceous ones have a broad host range including both nonvascular and vascular plant species. In addition, recent reviews propose that members of the non-clavicipitaceous group can be segregated into three subgroups on the basis of host range, type of tissue infected, pattern spread, in planta infection and the establishment, diversity and benefits given to hosts (Purahong and Hyde, 2011; Rodriguez et al., 2009). A diverse kind of relationships exists between the fungal endophytes and plants ranging from mutualistic (Redman et al., 2002), symbiotic and commensal (Deckert et al., 2001) to pathogens (Schulz et al., 1998). However, the state of the interaction between endophyte and host may be transitory, and many factors could make changes in their mode of interaction. In symbiotic associations, balansiaceous endophytes with their hosts are commonly considered as being mutualistic (Schardl and Clay, 1997) even though some of them provide nothing to their hosts and can occasionally be antagonistic (Schardl et al., 2004). Although most of the endophytes are regarded as being mutualistic with their hosts, some fungal endophytes may become pathogenic to plants, depending on the developmental stage of the partners, environmental conditions and plant defence reactions (Schulz and Boyle, 2005). Endophytic fungi have been known to play a vital role in plant growth, especially grasses; however, few reports have elucidated their symbiosis with crops. Recently, the ecological roles of some endophytes have been explained (Arnold and Lutzoni, 2007; Redman et al., 2011; Waller et al., 2005). In addition to providing nutritional benefits, fungal endophytes also confer significant physiological (Malinowski et al., 2004; Malinowski and Belesky, 2000) and ecological (Malinowski et al., 2004) benefits, including protection from environmental stress (Rodriguez et al., 2004) as well as from an attack of pathogens (Zabalgogeazcoa, 2008) and pests (Lewis and Clements, 1986).

1.3.2. Mechanisms of endophyte-mediated plant drought tolerance

Fungal endophytes have been shown to provide fitness benefit to plants when exposed to water-limiting conditions. Perhaps the most widely documented example of endophyte-mediated drought stress tolerance in plants is the enhanced drought tolerance of tall fescue and perennial ryegrass due to infection of the endophyte *Neotyphodium coenophialum*. Kane (2011) studied the leaf-inhabiting endophyte *Neotyphodium lolii* to assess its potential benefits or harm in drought stress tolerance of native perennial ryegrass collections formerly obtained from the Mediterranean regions. Non-grass fungal endophytes have also been described to help plants alleviate drought stress (Khan et al.,

2012; Redman et al., 2011; Waqas et al., 2012). The findings showed that endophyte colonization can help improve abiotic stress tolerance such as drought in that host. It must be noted that endophytic symbiosis in plants does not always benefit the plant under drought or other abiotic stress conditions, endophyte could reduce plant's ability to withstand stresses (Cheplick, 2004; Cheplick, 2007; Cheplick et al., 2000; Eerens et al., 1998). Cheplick (2007) reviewed the role of fungal endophytes on potential drought tolerance and cited some studies where endophytes imparted no improvement in the host's ability to tolerate drought stress. For instance, Zaurov et al. (2001) inoculated fescue plants with *Neotyphodium* isolates collected from dissimilar hosts. They observed that some genotypic combinations affected negatively on plant biomass, some had no effect and others increased plant biomass. Similarly, few combinations improved tolerance to soil aluminium; others have neutral or decreased tolerance compared to endophyte-free clones. This study revealed that genotype-specific interactions may increase or decrease or have no effect on plant adaptation and fitness. Thus, the endophyte-mediated responses to water stress is a complex phenomenon involving various metabolites and metabolic pathways. While the ability of fungal endophytes to provide drought tolerance in host plants has been described in many studies, the underlying mechanism(s) are incompletely characterized. In an effort to illuminate the underlying mechanism by which endophytes cause increased drought tolerance, researchers have reported few observations. Research so far studying the effect of endophyte on plant responses to drought stress have described certain physiological, biological and biochemical modifications such as (a) increased growth and development, (b) enhanced osmotic balance, (c) increased gaseous exchange and water-use efficiency and (d) improved defence against oxidative damage when water-limiting conditions may improve, alleviate and recompense the harmful effects of water stress in endophyte-colonized (EC) plants (Fig. 8.1). The present chapter aimed at outlining the recent advances in the study of improvement of drought tolerance by endophyte colonization in plants subjected to water stress.

Endophyte-mediated plant growth enhancement

Fungal endophytes have been shown to enhance growth and biomass of plants under water-limiting conditions. For example, inoculation of *Fusarium culmorum* and *Curvularia protuberata* resulted in higher biomass of drought-affected rice plants than non-inoculated plants (Redman et al., 2011). Endophytes *Chaetomium globosum* and *Penicillium resedanum* isolated from *Capsicum annuum* plants promoted shoot length and biomass of the host plants subjected to drought stress (Khan et al., 2014; Khan et al., 2012). Drought-challenged tomato plants showed higher root and shoot biomass when inoculated with class 2 fungal endophytes, including *Alternaria* sp. and *Trichoderma harzianum* (Azad and Kaminskyj, 2016). Inoculating a *Trichoderma hamatum* isolate caused increased root fresh weight, dry weight and water content, regardless of water availability in *Theobroma cacao* (cacao) (Bae et al., 2009). The endophyte *Piriformospora indica* colonization in Chinese cabbage promoted root and shoot growth and lateral root development (Sun et al., 2010). Increased growth of plants

under stress is attributed to the production of auxins by endophytes (De Battista et al., 1990). Also, stress-induced endogenous abscisic acid and the genes involved, such as zeaxanthin epoxidase, 9-cis-epoxycarotenoid dioxygenase 3 and ABA aldehyde oxidase 3, have been found to be significantly decreased in endophyte-colonized plants under stress, the effect of which could be comparable to that of exogenous GA3 in terms of promoting plant growth and yield under stressed conditions by manipulating the hosts' physiological processes (Khan et al., 2014). However, in some cases, it was recorded that endophytes do not show positive effects on host growth during drought stress, but they help with rapid recovery of host plant after water became available again (Ren and Clayy, 2009).

Endophyte-mediated improved photosynthesis

Moisture stress causes decreased levels of photosynthesis in plants through decreased synthesis of ATP and enzymes such as rubisco and sucrose–phosphate synthase as water availability decreases (Flexas and Medrano, 2002; Ghannoum et al., 2003; Parry et al., 2002; Vassey and Sharkey, 1989). Plant tolerance to water stress involves the management of extra radiation caused by reduced photosynthesis and CO_2 availability and a greater susceptibility to photo-damage (Chaves et al., 2003; Powles, 1984). The endophyte-colonization results in higher chlorophyll content and leaf area in plants challenged by stress than non-colonized plants. Higher concentration of chlorophyll is associated with higher photosynthetic rate (Davies et al., 1993). The increased rate of photosynthesis was recorded from the drought-stressed *Capsicum annuum* plants colonized by endophytes *Chaetomium globosum* (Khan et al., 2012) and *Penicillium resedanum* (Khan et al., 2014). About twofold increase in chlorophyll content and photosynthetic efficiency in *P. indica*-colonized *Arabidopsis* plants was measured when seedlings were challenged with water-limiting conditions (Sherameti et al., 2008). *P. indica* reduced the drought-induced decline in the photosynthetic rate and the denaturation of chlorophyll and thylakoid proteins (Sun et al., 2010). Although, the Fv/Fm values decreased in the non-EC plants under drought, no significant difference was observed for the *P. indica*-colonized plants indicating that EC plants suffer less from water stress than un-inoculated controls. In the same study, the total chlorophyll level was reported to be reduced by more than 50% in non-EC plant, but colonized plants showed only a slight decrease in total chlorophyll content (Sun et al., 2010). The discrepancy such as the increase in chlorophyll in plants under stress in some cases and decrease in others may be due to the severity of the drought challenged or the position of leaves where the chlorophyll was measured. Additionally, a decrease in the protein levels of representative constituents of the thylakoid membrane and of enzymes situated in the plastid stroma in stressed plants was retarded when colonized with *P. indica* (Sun et al., 2010). Recently, Azad and Kaminskyj (2016) characterized a fungal endophyte that enhanced drought tolerance of the host and increased photosynthesis in the leaf. The mechanism of increased photosynthesis in EC plants under water stress is not fully understood. In one study, it was found that while the photosynthesis rate and stomatal conductance increased in drought-affected EC plants, initial rubisco activity and

carboxylation efficiency did not differ from non-EC plants (Morse et al., 2002). It was suggested that endophyte colonization might result in reduced biochemical damage to the photosynthetic machinery of plants subjected to water stress (Swarthout et al., 2009).

Plant–water relation and osmotic adjustment as mediated by endophytes

In the broad sense, decreasing water loss and maintaining water uptake are the key processes that plants employ to adapt to water-limiting environments. Maintaining water uptake is assisted within plant cells by osmotic adjustment (OA), a biochemical mechanism that helps plants to adapt to drought conditions. OA results in a net accrual of compatible solutes, also known as osmolytes in the cell so as to maintain the favourable gradient for water flow from soil into roots (Sanders and Arndt, 2012). This accumulation of various ions, amino acids and sugars leads to a more negative osmotic potential, which is important for maintaining cell hydration and turgor, cellular development and growth, stomatal opening, photosynthesis and water uptake during drought (Chaves et al., 2003; Sanders and Arndt, 2012). Endophyte-colonized plants consume significantly less water than non-colonized plants. For example, significantly less water use has been reported in endophyte-inoculated panic grass, rice, tomato and dune grass, indicative of their more efficient water usage. Reduced water consumption and improved water-use efficiency may offer a distinctive mechanism for endophyte-mediated drought resistance in plants (Rodriguez et al., 2008). Again, EC plants can maintain significantly greater water content than the non-inoculated under water stress, implying the ability of endophytes to delay desiccation and damage in stress. The endophyte association could help plants access larger volumes of water from sources not reachable to the non-infected plants which suffer from stress (Khan et al., 2013). Endophyte association resulted in a decreased level of electrolytic leakage inside the plant tissues upon exposure to water deficit stress. Altered water potential and improved osmotic balance in drought-affected tall fescue infected with *Neotyphodium coenophialum* endophyte have also been noted in some studies (Elmi and West, 1995). Increased root water content was reported from *Trichoderma hamatum*-inoculated *Theobroma cacao* plants subjected to water deficit stress compared to non-inoculated plants (Bae et al., 2009). A number of fungal endophytes have been reported to produce active biochemicals and metabolites that help the host plant withstand water deficit stress. Under drought conditions, significant upregulation of free glucose, fructose, trehalose, sugar alcohols, proline and glutamic acid was detected in shoots and roots in tall fescue colonized by *N. coenophialum* (Nagabhyru et al., 2013). Variable levels of proline accumulation were observed in EC plants subjected to water stress. While significantly more proline was accumulated in one genotype of tall fescue plant, no differences were observed in another genotype challenged with mimic drought in hydroponic culture (Bayat et al., 2009) when inoculated with *Neotyphodium* grass endophyte. Increased level of proline, soluble sugar and catalase (CAT) was observed in wheat colonized by endophyte *Chaetomium globosum* under water stress (Cong et al., 2015). Concentrations of aspartic acid and

glutamic acid and of alanine and γ-aminobutyric acid were measured in drought-affected *T. cacao* seedlings colonized by an isolate of *T. hamatum* (Bae et al., 2009). The changes in metabolites could be attributed to the strategies of EC plants towards drought tolerance or avoidance. Downregulation in osmolytes has previously been described as a strategy of drought avoidance, whereas the increase of osmoprotectants has been related to drought tolerance (Augé and Moore, 2005; Ruiz-Sánchez et al., 2010).

Endophyte-mediated ROS scavenging

Reactive oxygen species (ROS) act as signalling molecules in plants. ROS is involved in many plant processes, including growth, stress response, cell cycle and programmed cell death by influencing the expression of related genes. Abiotic stresses cause excess synthesis of these highly reactive molecules, causing oxidative stress and damaging proteins, lipids and DNA (Gechev et al., 2006; Gill and Tuteja, 2010). Manufacturing additional ROS, i.e. hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$), singlet oxygen and superoxides ($^1O_2$), is one of the main mechanisms for plant cell damage or death in drought (Smirnoff, 1993). Plants react against excess ROS through an intricate network of direct ROS-quenching activity or indirect hormone-mediated signalling activity. Various enzymatic and non-enzymatic antioxidant molecules are involved in scavenging ROS (revised in (Miller et al., 2010; Scheibe and Beck, 2011). Malfunctioning of these antioxidants' defence system results in oxidative damage in cells (Apel and Hirt, 2004; Kwak et al., 2006). Endophyte colonization simulates a more powerful ROS-scavenging system in host plants under stress and reduces damage of biomolecules at the cellular level. For instance, a lower level ROS production has been documented in endophyte-colonized tomato plants than in control plants following water stress (Azad and Kaminskyj, 2016). When plants were inoculated with *P. indica* and exposed to drought stress, up-regulation of peroxidase (POX), catalase (CAT) and superoxide dismutase (SOD) activities in the leaves was observed (Sun et al., 2010). The level of another biomarker of oxidative stress, namely, malondialdehyde (MDA), was recorded to be lower in *P. indica*-colonized cabbage plants than in control plants. MDA is primarily produced through the ROS-induced degradation of polyunsaturated lipids (Del Rio et al., 2005; Pryor and Stanley, 1975). It is suggested that *P. indica* could prevent or reduce the damage of these lipids by inhibiting excess ROS production under stress conditions. Endophytes that promote drought tolerance have also been found to have high levels of loline alkaloids (Schardl et al., 2004). Further experiments could test if these molecules are associated with the prevention of damage of macromolecules or reduction of ROS effects. Endophyte-induced production of antioxidant enzyme in plants under stress is predominantly observed in leaves (Baltruschat et al., 2008; Vadassery et al., 2009). All these studies demonstrate that endophyte inoculation results in a strong defence response in plants in water stress, in which alleviation of oxidative stress might be a vital part. The study of non-volatile compounds has been the major focus in most plant antioxidant research. However, plant leaves emitting volatile organic

compounds could also play as a further defence role against stresses (Kesselmeier and Staudt, 1999; Peñuelas and Munné-Bosch, 2005). The effect of volatile compounds such as isoprenoids has been described, where these compounds act as protective agent against oxidative stress in plants through direct ROS scavenging and indirect alteration of ROS signalling in arbuscular mycorrhizal plants (Asensio et al., 2012; Baslam and Goicoechea, 2012; López-Ráez et al., 2008; Peñuelas and Munné-Bosch, 2005; Rapparini and Peñuelas, 2014; Vickers et al., 2009; Walter and Strack, 2011). Endophyte-colonized plants could emit similar volatile organic compounds to cope with abiotic stress, but this aspect of the research has not been done till date. Further investigation is necessary to have the information on the fungal side as well as the knowledge of the fungal/plant interaction is paramount to elucidate underlying mechanisms regulating antioxidant defences that are crucial to improve the tolerance of plants to drought stress.

Molecular mechanisms of endophyte-mediated plant drought tolerance

Studies on the beneficial effects of endophyte symbiosis under drought have predominantly focused at the plant morpho-physiological level. Molecular tools have also been included in this type of studies. The responses of EC plants to stress can be regulated by the expression of drought-associated plant genes, e.g. those associated with signalling and regulatory pathways or those producing enzymes that synthesize various metabolic compounds. It was noted that, under drought conditions, EC and non-EC plants differently regulate the expression of several drought genes in the plant tissue, indicating the association of activation of Ca_2P signalling and related proteins (Schäfer et al., 2007) involved in the drought tolerance mechanisms. Among the genes regulated by the endophyte symbiosis during drought, delayed expression of drought-altered ESTs such as TcTPP, TcSOT, TcPR5 and TcNI in the leaves and TcPR5 and TcCESA3 in the roots has been described (Bae et al. 2009). Again, the expression of a diverse array of stress-related genes, including 29A, ANAC072, DEHYDRATION-FINGER1, Ddelta, CBL1, HAT, etc. putatively mediate drought tolerance of *Arabidopsis* plants inoculated with *P. indica* (Sherameti et al., 2008). Similarly, up-regulation of drought-associated genes DREB2A, CBL1, ANAC072 and RD29A was also reported in the drought-challenged leaves of *P. indica*-colonized Chinese cabbage plants. The contribution of endophytes to the enhanced drought tolerance of the host plant can be mediated by CAS protein and the thylakoid membrane CAS mRNA level associated with Ca2+ sensing regulator (Sun et al., 2010). Further research could encompass non-targeted screening of cDNA libraries from both endophyte and host plants. Such an approach could allow the detection of stress-induced genes that offer increased stress tolerance in endophyte-colonized hosts. Employing microarrays and next-generation sequencing technologies to elucidate stress tolerance mechanisms (physiological and molecular) involved in endophyte colonization will be used to compare EC and non-EC plants of the same host genotype.

1.2. Plant viruses and abiotic stress tolerance in plants

Viruses are by far the most abundant biological beings on the planet (Suttle, 2007). Every living being can be infected by at least one and normally several viruses and most prokaryotes and eukaryotes are infected by diverse and largely undescribed groups of viruses. Plants support enormous numbers of viruses that sometimes also replicate in insects. Viruses use the host machinery and resources for their own replication and transmission. Until recently, virus infections were seen as always being harmful. Indeed, this arises from the fact that most plant viruses described to date are studied as pathogens that incite diseases in crop plants (Zaitlin and Palukaitis, 2000). However, in natural non-agricultural environments such as in a tropical forests, viruses are present in both symptomatic or asymptomatic plants (Xu et al., 2008). Depending on the type of virus, host and the environment the interaction between virus and plant could range from mutualistic to pathogenic (Roossinck, 2011).

Beneficial effects of some plant viruses are evident and they display conditional mutualism and confer abiotic stress tolerance to their hosts. *N. benthamiana* plants inoculated with cucumber mosaic virus (CMV), having a very broad host range (Palukaitis et al., 1992; Roossinck, 2001), or tobacco mosaic virus (TMV) and tobacco rattle virus (TRV) both with intermediate host ranges, or brome mosaic virus (BMV), a virus with a very narrow host range (Lane, 1981), they survive longer after water is withdrawn than uninfected plants (Xu et al., 2008). Rice and tobacco plants exhibited better tolerance to drought stress when inoculated with BMV and TMV, respectively. Improved drought tolerance was also recorded in few other cultivated and wild crops like beet, cucumber, *Chenopodium amaranticolor,* pepper, squash, *Solanum habrochaites* (wild relative of tomato), tomato and watermelon as a result of inoculation with CMV (Xu et al., 2008). Furthermore, beets inoculated with CMV were found to tolerate cold treatments but all uninfected plants died (Xu et al., 2008). The underlying mechanism for this noteworthy observation is unknown for the most part. However, the phenomenon of increased drought tolerance in plants could be explained by the effect of the virus on plant morpho-physiological changes.

Virus infection often causes plants to be shorter (Hull, 2013), with lower water requirements, thereby helping plants to survive during severe drought. Viral infection can alter water content and cause the production and movement of metabolic compounds (Hull, 2013), all helping plants to be more tolerant to drought. In their study, Xu et al. (2008) found that CMV infection increased the water content and water retention in infected plants which are indicative of decreased levels of stomatal opening and transpiration in virus affected plants (Keller et al., 1989; Lindsey and Gudauskas, 1975). By metabolite profiling, Xu and associates (Xu et al., 2008) found high levels of salicylic acid and some osmoprotectants and antioxidants in virus-infected plants, causing increased plant tolerance to abiotic stress (Singh and Usha, 2003). Moreover, TMV infection radically increased ABA concentration in tobacco plants (Whenham et al., 1986), which is often regarded as a plant adaptation

strategy to environmental stress, but it is not clear whether this is a universal response of plants to virus infection.

Recent technologies and the development of metagenomics reveal the virus richness in many diverse environments and propose that producing disease is not the usual lifestyle of viruses and that many are probably benevolent and some are clearly beneficial. More research is needed for understanding of the fundamental mechanisms of plant-virus interactions and enhanced plant tolerance to abiotic stress. This area of research will provide potential for agricultural applications and also intuition to the key role of viruses in the adaptation and evolution of their hosts. This is particularly important in the recent era of global climate change when drought is becoming one of the chief limiting factors for agricultural production throughout the world (Wollenweber et al., 2005).

1.5. The *Nicotiana* Species of Australia

Several species of *Nicotiana* have been, and still are, used by Australian Aboriginals either by inhalation of smoke, or by smokeless route such as chewing, snuff, dermal pasting etc. Australasian species of *Nicotiana* belong to *Suaveolentes* and all are originated from allopolyploid (Goodin et al., 2008) which is placed under the subgenus *Petunioides*. These species can be identified by the non-deflexed corolla limb, by the corolla throat not or only slightly dilated, and by the fact that the lowest anther is on the longest filament (not in *N. debneyi*) (Goodspeed, 1947). In terms of distribution, the Australian *Nicotiana* species are widely spread over the continent but no native tobacco has been recorded from New Guinea, New Zealand or Tasmania (Horton, 1981; Symon, 2005). The morphological characteristics of Australian *Nicotiana* species may vary substantially according to different environmental factors making their identification difficult. For example, plants thriving under a dry and harsh habitat may be dwarfed with small, narrow leaves, mostly stems without leaf but others of the same species growing in wetter and favourable conditions may be taller in height with leafy stems and large, lush, broad leaves (Horton, 1981).

The Australian species of *Nicotiana* are real annuals or short-lived perennials. The species have striking habitat preferences and most can be found in specific habitats, such as deep sands or rocky outcrops (Horton, 1981). They are generally associated with disturbed areas, such as recent regrowth on burnt patches or along newly made country roads or places where competition from other plants, especially perennials, is limited (Burbidge, 1960). Most species contain one or more pyridine alkaloids and have occasionally been suspected of poisoning stock if consumed in large quantities (Willaman and Schubert, 1961).

1.5. *Nicotiana benthamiana* - a model plant for plant-microbe interaction research

Nicotiana benthamiana is indigenous to Australia. In its natural state, it is mostly found amongst rocks on hills and cliffs throughout the northern regions of Australia (Goodin et al., 2008). Since it contains nicotine and other alkaloids, *Nicotiana* (and *Duboisia*) species were used by Australian Aboriginals as an intoxicant and drug before the introduction of cultivated tobacco (*N. tabacum* and *N. rustica*). The Aboriginal called it Pituri or Tjuntiwari or Muntju, and other names, depending on the language group and location. It was first collected by Benjamin Bynoe on a voyage of the H.M.S. Beagle from the "N.W. Coast" of New Holland in 1837 (Chase et al., 2003; Clarkson et al., 2004; Goodin et al., 2008; Knapp and Smith, 2001).

N. benthamiana Research accession 4 (RA-4) was adopted as a model plant by plant virologists due to its susceptibility to plant virus infection (Christie and Crawford, 1978; Quacquarelli and Avgelis, 1975). Since then it has been used as a model plant for research on plant-microbe interactions, virus-induced gene silencing (VIGS), and the mechanisms of RNA interference (Angell and Baulcombe, 1997; Baulcombe, 1999; Kumagai et al., 1995). In addition to being highly susceptible to viruses, the plant is also susceptible to attack by many fungi, bacteria, oomycetes, as well as certain insects (Bos et al., 2010; de Jonge et al., 2012; Kamoun et al., 1998; Yoshino et al., 2012). The large leaves of *N. benthamiana* and its susceptibility to microorganisms have been exploited as a way to transiently express proteins, using either engineered viruses or syringe-infiltration of *Agrobacterium tumefaciens* (Ma et al., 2012; Tang et al., 1996; Wagner et al., 2004). Apart from its use in plant-microbe interaction studies this species has also been used for research in many different areas including gene silencing, metabolic engineering, protein-protein interactions and gene function studies (Van der Hoorn et al., 2000). Although this species has been of great interest in the scientific world, the information on the plant itself is scanty. Only single accession is being used by the research community to date. The origins of the *N. benthamiana* accessions used for research are generally not known, although one study using distance based phenetic analysis indicates they are very similar and may have derived from a single source (Goodin et al., 2008; Wylie et al., 2015).

1.6. Research questions

This research project was undertaken to answer the following questions:

i. Are there differences among wild *Nicotiana* accessions with regard to their inherent drought tolerant ability?

ii. Do wild plants of Australian *Nicotiana* species harbour fungal endophytes?

iii. Are there any influences of host genotype, tissue origin and host location on the community structure of fungal endophytes of Australian *Nicotiana* species?

iv. Do endophytic fungi and viruses associated wild *N. benthamiana* plants influence their responses to water stress? If so,

   a. what are the physiological and metabolic changes in fungal endophytic plants undergoing water stress tolerance?

   b. what are the physiological and metabolic changes in virus-infected plants undergoing water stress tolerance?

   c. Are there interactions between responses to water stress by plants infected by both fungal endophytes and viruses?

References

Andrew, D. R., Fitak, R. R., Munguia-Vega, A., Racolta, A., Martinson, V. G., and Dontsova, K. (2012). Abiotic factors shape microbial diversity in Sonoran Desert soils. *Applied and Environmental Microbiology* **78**, 7527–7537.

Angell, S. M., and Baulcombe, D. C. (1997). Consistent gene silencing in transgenic plants expressing a replicating potato virus X RNA. *The EMBO Journal* **16**, 3675-3684.

Anonymous (1984). Southern Africa's drought. *In* "Economist", pp. 70-1.

Apel, K., and Hirt, H. (2004). Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.* **55**, 373-399.

Arnold, A. E. (2007). Understanding the diversity of foliar endophytic fungi: progress, challenges, and frontiers. *Fungal Biology Reviews* **21**, 51–66.

Arnold, A. E., and Lutzoni, F. (2007). Diversity and host range of foliar fungal endophytes: are tropical trees biodiversity hot spots? *Ecology* **88**, 541–549.

Asensio, D., Rapparini, F., and Peñuelas, J. (2012). AM fungi root colonization increases the production of essential isoprenoids vs. nonessential isoprenoids especially under drought stress conditions or after jasmonic acid application. *Phytochemistry* **77**, 149-161.

Asrar, A. W., and Elhindi, K. M. (2011). Alleviation of drought stress of marigold (*Tagetes erecta*) plants by using arbuscular mycorrhizal fungi. *Saudi J Biol Sci* **18**, 93-8.

Augé, R., and Moore, J. (2005). Arbuscular mycorrhizal symbiosis and plant drought resistance. *Mycorrhiza: role and applications. Nagpur: Allied Publishers*, 136-162.

Azad, K., and Kaminskyj, S. (2016). A fungal endophyte strategy for mitigating the effect of salt and drought stress on plant growth. *Symbiosis* **68**, 73-78.

BAC (1985). Bureau of Agricultural Economics (1985) 'Wheat Situation and Outlook 1985'. . Bureau of Agric. Econ.: Canberra.

Bae, H., Sicher, R. C., Kim, M. S., Kim, S. H., Strem, M. D., Melnick, R. L., and Bailey, B. A. (2009). The beneficial endophyte *Trichoderma hamatum* isolate DIS 219b promotes growth and delays the onset of the drought response in Theobroma cacao. *Journal of Experimental Botany* **60**, 3279-3295.

Baltruschat, H., Fodor, J., Harrach, B. D., Niemczyk, E., Barna, B., Gullner, G., Janeczko, A., Kogel, K. H., Schafer, P., Schwarczinger, I., Zuccaro, A., and Skoczowski, A. (2008). Salt tolerance of barley induced by the root endophyte *Piriformospora indica* is associated with a strong increase in antioxidants. *New Phytol* **180**, 501–510.

Bao, X., and Roossinck, M. J. (2013). Multiplexed interactions: viruses of endophytic fungi. *Adv Virus Res* **86**, 37-58.

Bartoli, C. G., Simontacchi, M., Tambussi, E., Beltrano, J., Montaldi, E., and Puntarulo, S. (1999). Drought and watering-dependent oxidative stress: effect on antioxidant content in *Triticum aestivum* L. leaves. *Journal of Experimental Botany* **50**, 375-383.

Baslam, M., and Goicoechea, N. (2012). Water deficit improved the capacity of arbuscular mycorrhizal fungi (AMF) for inducing the accumulation of antioxidant compounds in lettuce leaves. *Mycorrhiza* **22**, 347-359.

Baulcombe, D. C. (1999). Fast forward genetics based on virus-induced gene silencing. *Current opinion in plant biology* **2**, 109-113.

Bayat, F., Mirlohi, A., and Khodambashi, M. (2009). Effects of endophytic fungi on some drought tolerance mechanisms of tall fescue in a hydroponics culture. *Russian Journal of Plant Physiology* **56**, 510-516.

Blum, A. (1996). Crop responses to drought and the interpretation of adaptation. *Plant Growth Regulation* **20**, 135-48.

BOM (1999). Bureau of Meteorology (1999) "The "Federation Drought", 1895–1902". Climate Education.

BOM (2006). Bureau of Meteorology (2006): The 1914–15 drought". Climate Education. . Bureau of Meteorology, Australia.

Bos, J. I., Prince, D., Pitino, M., Maffei, M. E., Win, J., and Hogenhout, S. A. (2010). A functional genomics approach identifies candidate effectors from the aphid species *Myzus persicae* (green peach aphid). *PLoS genetics* **6**, e1001216.

Bray, E. A. (2000). Responses to abiotic stresses. *Biochemistry and molecular biology of plants*, 1158-1203.

Brundrett, M. (2004). Diversity and classification of mycorrhizal associations. *Biological Reviews* **79**, 473-495.

Chase, M. W., Knapp, S., Cox, A. V., Clarkson, J. J., Butsko, Y., Joseph, J., Savolainen, V., and Parokonny, A. S. (2003). Molecular systematics, GISH and the origin of hybrid taxa in *Nicotiana* (Solanaceae). *Annals of Botany* **92**, 107-127.

Chaves, M. M., Maroco, J. P., and Pereira, J. S. (2003). Understanding plant responses to drought-from genes to the whole plant. *Functional plant biology* **30**, 239-264.

Cheplick, G. P. (2004). Recovery from drought stress in *Lolium perenne* (Poaceae): Are fungal endophytes detrimental. *American Journal of Botany* **91**, 1960-1968.

Cheplick, G. P. (2007). Costs of fungal endophyte infection in *Lolium perenne* genotypes from Eurasia and North Africa under extreme resource limitation. *Environmental and Experimental Botany* **60**, 202-210.

Cheplick, G. P., Perera, A., and Koulouris, K. (2000). Effect of drought on the growth of *Lolium perenne* genotypes with and without fungal endophytes. *Functional Ecology* **14**, 657-667.

Christie, S. R., and Crawford, W. E. (1978). Plant virus range of *Nicotiana benthamiana*. *Plant Disease Reporter* **62**, 20-22.

Clarkson, J. J., Knapp, S., Garcia, V. F., Olmstead, R. G., Leitch, A. R., and Chase, M. W. (2004). Phylogenetic relationships in *Nicotiana* (Solanaceae) inferred from multiple plastid DNA regions. *Molecular phylogenetics and evolution* **33**, 75-90.

Clay, K. (1988). Fungal endophytes of grasses: a defensive mutualism between plants and fungi. *Ecology* **69**, 10-16.

Cong, G. Q., Yin, C. L., He, B. I., Li, L., and Gao, K. X. (2015). Effect of the endophytic fungus *Chaetomium globosum* ND35 on the growth and resistance to drought of winter wheat at the seedling stage under water stress. *Shengtai Xuebao/ Acta Ecologica Sinica* **35**, 6120-6128.

CSIRO (2007). Climate change in Australia. Bureau of Meteorology, Department of Environment, Australian Government.

Davies, F., Potter, J., and Linuerman, R. (1993). Drought resistance of mycorrhizal pepper plants independent of leaf P concentration-response in gas exchange and water relations. *Physiologia Plantarum* **87**, 45-53.

De Battista, J. P., Bacon, C. W., Severson, R., Plattner, R. D., and Bouton, J. H. (1990). Indole acetic acid production by the fungal endophyte of tall fescue. *Agronomy Journal* **82**, 878–880.

de Jonge, R., van Esse, H. P., Maruthachalam, K., Bolton, M. D., Santhanam, P., Saber, M. K., Zhang, Z., Usami, T., Lievens, B., and Subbarao, K. V. (2012). Tomato immune receptor Ve1 recognizes effector of multiple fungal pathogens uncovered by genome and RNA sequencing. *Proceedings of the National Academy of Sciences* **109**, 5110-5115.

Deckert, R. J., Melville, L. H., and Peterson, R. L. (2001). Structural features of a Lophodermium endophyte during the cryptic life-cycle phase in the foliage of Pinus strobus. *Mycological Research* **105**, 991-997.

Del Rio, D., Stewart, A. J., and Pellegrini, N. (2005). A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. *Nutrition, Metabolism & Cardiovascular Diseases* **15**, 316–28.

Desclaux, D., and Roumet, P. (1996). Impact of drought stress on the phenology of two soybean (*Glycine max* L. Merr) cultivars. *Field Crops Research* **46**, 61-70.

Dhargalkar, S., and Bhat, D. (2009). *Echinosphaeria pteridis* sp. nov. and its Vermiculariopsiella anamorph. *Mycotaxon* **108**, 115-122.

Eerens, J., Lucas, R., Easton, S., and White, J. (1998). Influence of the endophyte (*Neotyphodium lolii*) on morphology, physiology, and alkaloid synthesis of perennial ryegrass during high temperature and water stress. *New Zealand Journal of Agricultural Research* **41**, 219-226.

Elmi, A. A., and West, C. P. (1995). Endophyte infection effects on stomatal conductance, osmotic adjustment and drought recovery of Tall fescue. *New Phytologist* **131**, 61-67.

EMA (1997). EMA (Emergency Management Australia), Hazards, Disasters and Survival. . pp. 46. EMA, Dickson, ACT.

Farooq, M., Wahid, A., Kobayashi, N., Fujita, D., and Basra, S. (2009). Plant drought stress: effects, mechanisms and management. *Agronomy for sustainable development* **29**, 185-212.

Fischlin, A., Midgley, G. F., Price, J. T., Leemans, R., Gopal, B., and Turley, C. (2007). Ecosystems, their properties, goods, and services. *In* "Climate Change 2007: Impacts, Adaptation and Vulnerability. Contribution of Working Group II to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change" (M. L. Parry, O. F. Canziani, J. P. Palutikof, P. J. van der Linden and C. E. Hanson, eds.), pp. 211–272. Cambridge University Press, Cambridge.

Fisher, P. (1996). Survival and spread of the endophyte Stagonospora pteridiicola in *Pteridium aquilinum*, other ferns and some flowering plants. *New Phytologist* **132**, 119-122.

Flexas, J., and Medrano, H. (2002). Drought-inhibition of photosynthesis in C3 plants: stomatal and non-stomatal limitations revisited. *Annals of botany* **89**, 183-189.

Fröhlich, J., and Hyde, K. D. (1999). Biodiversity of palm fungi in the tropics: are global fungal diversity estimates realistic? *Biodiversity and Conservation* **8**, 977-1004.

Garrett, K. A., Dendy, S. P., Frank, E. E., Rouse, M. N., and Travers, S. E. (2006). Climate change effects on plant disease: genomes to ecosystems. *Annu. Rev. Phytopathol.* **44**, 489-509.

Geber, M. A., and Dawson, T. E. (1990). Genetic variation in and covariation between leaf gas exchange, morphology, and development in *Polygonum arenastrum*, an annual plant. *Oecologia* **85**, 153-158.

Gechev, T. S., Van Breusegem, F., Stone, J. M., Denev, I., and Laloi, C. (2006). Reactive oxygen species as signals that modulate plant stress responses and programmed cell death. *Bioessays* **28**, 1091-1101.

Ghannoum, O., Conroy, J. P., Driscoll, S. P., Paul, M. J., Foyer, C. H., and Lawlor, D. W. (2003). Nonstomatal limitations are responsible for drought-induced photosynthetic inhibition in four C4 grasses. *New Phytologist* **159**, 599-608.

Gill, S. S., and Tuteja, N. (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant physiology and biochemistry* **48**, 909-930.

Goodin, M. M., Zaitlin, D., Naidu, R. A., and Lommel, S. A. (2008). Nicotiana benthamiana: its history and future as a model for plant–pathogen interactions. *Molecular plant-microbe interactions* **21**, 1015-1026.

Goodspeed, T. H. (1947). On the evolution of the genus *Nicotiana*. *Proceedings of the National Academy of Sciences* **33**, 158-171.

Hawksworth, D. (1988). The variety of fungal-algal symbioses, their evolutionary significance, and the nature of lichens. *Botanical journal of the Linnean Society* **96**, 3-20.

Hoffmann, A. A., and Merilä, J. (1999). Heritable variation and evolution under favourable and unfavourable conditions. *Trends in Ecology & Evolution* **14**, 96-101.

Horton, P. (1981). A taxonomic revision of *Nicotiana* (Solanaceae) in Australia. *Journal of the Adelaide Botanic Garden*, 1-56.

Hull, R. (2013). "Plant virology," Academic press.

Hussain, I., Ahsan, M., Saleem, M., and Ahmad, A. (2009). Gene action studies for agronomic traits in maize under normal and water stress conditions. *Pak. J. Agri. Sci* **46**, 107-112.

IPCC (2007). "Climate change 2007: the physical science basis," Cambridge University Press, Cambridge.

Jackson, R. B., Sperry, J. S., and Dawson, T. E. (2000). Root water uptake and transport: using physiological processes in global predictions. *Trends in plant science* **5**, 482-488.

Kamoun, S., van West, P., Vleeshouwers, V. G., de Groot, K. E., and Govers, F. (1998). Resistance of *Nicotiana benthamiana* to Phytophthora infestans is mediated by the recognition of the elicitor protein INF1. *The Plant Cell* **10**, 1413-1425.

Kane, K. H. (2011). Effects of endophyte infection on drought stress tolerance of *Lolium perenne* accessions from the Mediterranean region. *Environmental and Experimental Botany* **71**, 337-344.

Kaya, M. D., Okçu, G., Atak, M., Çıkılı, Y., and Kolsarıcı, Ö. (2006). Seed treatments to overcome salt and drought stress during germination in sunflower (*Helianthus annuus* L.). *European journal of agronomy* **24**, 291-295.

Keller, P., Lüttge, U., Wang, X.-C., and Büttner, G. (1989). Influence of rhizomania disease on gas exchange and water relations of a susceptible and a tolerant sugar beet variety. *Physiological and molecular plant pathology* **34**, 379-392.

Kesselmeier, J., and Staudt, M. (1999). Biogenic volatile organic compounds (VOC): an overview on emission, physiology and ecology. *Journal of atmospheric chemistry* **33**, 23-88.

Khan, A. L., Shin, J. H., Jung, H. Y., and Lee, I. J. (2014). Regulations of capsaicin synthesis in *Capsicum annuum* L. by *Penicillium resedanum* LK6 during drought conditions. *Scientia Horticulturae* **175**, 167-173.

Khan, A. L., Shinwari, Z. K., Kim, Y., Waqas, M., Hamayun, M., Kamran, M., and Lee, I.-J. (2012). Role of endophyte *Chaetomium globosum* LK4 in growth of *Capisum annuum* by production of gibberellins and indole acetic acid. *Pakistan J Bot* **44**, 1601-7.

Khan, A. L., Waqas, M., Hamayun, M., Al-Harrasi, A., Al-Rawahi, A., and Lee, I. J. (2013). Co-synergism of endophyte *Penicillium resedanum* LK6 with salicylic acid helped *Capsicum annuum* in biomass recovery and osmotic stress mitigation. *BMC Microbiology* **13**, 13.

Kirkham, M. B. (2005). "Principles of soil and plant water relations," Elsevier Academic Press, Burlington, MA.

Knapp, A. K., and Smith, M. D. (2001). Variation among biomes in temporal dynamics of aboveground primary production. *Science* **291**, 481-484.

Kramer, P., and Boyer, J. (1995). Water relations of plants and soils. *Academic Press, New York* **1**, 495.

Krings, M., Taylor, T. N., and Dotzler, N. (2012). Fungal endophytes as a driving force in land plant evolution: evidence from the fossil record. *In* "Biocomplexity of plant-fungal interactions" (D. Southworth, ed.), pp. 5-28. Wiley-Blackwell.

Krishnan, A. (2011). In China, record drought brings focus on water security. *In* "The Hindu". The Hindu, India.

Kumagai, M., Donson, J., Della-Cioppa, G., Harvey, D., Hanley, K., and Grill, L. (1995). Cytoplasmic inhibition of carotenoid biosynthesis with virus-derived RNA. *Proceedings of the National Academy of Sciences* **92**, 1679-1683.

Kwak, J. M., Nguyen, V., and Schroeder, J. I. (2006). The role of reactive oxygen species in hormonal responses. *Plant Physiology* **141**, 323-329.

Lambers, H., Chapin III, F. S., and Pons, T. L. (2008). Growth and allocation. *In* "plant physiological ecology", pp. 321-374. Springer.

Lane, L. (1981). Bromoviruses. *Handbook of plant virus infections and comparative diagnosis*, 333-376.

Lewis, G., and Clements, R. (1986). A survey of ryegrass endophyte (*Acremonium loliae*) in the UK and its apparent ineffectually on a seedling pest. *The Journal of Agricultural Science* **107**, 633-638.

Li, W.-C., Zhou, J., Guo, S.-Y., and Guo, L.-D. (2007). Endophytic fungi associated with lichens in *Baihua mountain* of Beijing, China. *Fungal Divers* **25**, 69-80.

Lindsey, D. W., and Gudauskas, R. (1975). Effects of maize dwarf mosaic virus on water relations of corn. *Phytopathology* **65**, 434-440.

Liu, C., Zhao, L., and Yu, G. (2011). The dominant glutamic acid metabolic flux to produce γ-amino butyric acid over proline in *Nicotiana tabacum* leaves under water stress relates to its significant role in antioxidant activity. *Journal of integrative plant biology* **53**, 608-618.

López-Ráez, J. A., Charnikhova, T., Gómez-Roldán, V., Matusova, R., Kohlen, W., De Vos, R., Verstappen, F., Puech-Pages, V., Bécard, G., and Mulder, P. (2008). Tomato strigolactones are derived from carotenoids and their biosynthesis is promoted by phosphate starvation. *New Phytologist* **178**, 863-874.

Ma, L., Lukasik, E., Gawehns, F., and Takken, F. L. (2012). The use of agroinfiltration for transient expression of plant resistance and fungal effector proteins in Nicotiana benthamiana leaves. *Plant Fungal Pathogens: Methods and Protocols*, 61-74.

Maherali, H., Caruso, C. M., Sherrard, M. E., and Latta, R. G. (2010). Adaptive value and costs of physiological plasticity to soil moisture limitation in recombinant inbred lines of *Avena barbata*. *American Naturalist* **175**, 211-224.

Malinowski, D., Zuo, H., Belesky, D., and Alloush, G. (2004). Evidence for copper binding by extracellular root exudates of tall fescue but not perennial ryegrass infected withNeotyphodium spp. endophytes. *Plant and Soil* **267**, 1-12.

Malinowski, D. P., and Belesky, D. P. (2000). Adaptation of endophyte-infected cool-season grasses to environmental stresses: mechanisms of drought and mineral stress tolerance. *Crop Science* **40**.

Márquez, S. S., Bills, G. F., Herrero, N., and Zabalgogeazcoa, Í. (2012). Non-systemic fungal endophytes of grasses. *Fungal Ecology* **5**, 289-297.

McWilliam, J. R. (1986). The national and international importance of drought and salinity effects on agricultural production. *Australian Journal of Plant Physiology* **35**, 1–13.

Miller, G., Suzuki, N., CIFTCI-YILMAZ, S., and Mittler, R. (2010). Reactive oxygen species homeostasis and signalling during drought and salinity stresses. *Plant, cell & environment* **33**, 453-467.

Morgan, J. M. (1984). Osmoregulation and water stress in higher plants. *Annual review of plant physiology* **35**, 299-319.

Morse, L. J., Day, T. A., and Faeth, S. H. (2002). Effect of Neotyphodium endophyte infection on growth and leaf gas exchange of Arizona fescue under contrasting water availability regimes. *Environmental and Experimental Botany* **48**, 257-268.

Nagabhyru, P., Dinkins, R. D., Wood, C. L., Bacon, C. W., and Schardl, C. L. (2013). Tall fescue endophyte effects on tolerance to water-deficit stress. *BMC Plant Biology* **13**, 17.

Okçu, G., Kaya, M. D., and Atak, M. (2005). Effects of salt and drought stresses on germination and seedling growth of pea (*Pisum sativum* L.). *Turkish journal of agriculture and forestry* **29**, 237-242.

Palukaitis, P., Roossinck, M. J., Dietzgen, R. G., and Francki, R. I. (1992). Cucumber mosaic virus. *Advances in virus research* **41**, 281-348.

Pandey, S., and Bhandari, H. (2009). Drought, coping mechanisms and poverty: Insights from rainfed rice farming in Asia. *Occasional Paper* **7**.

Parry, M. A., Andralojc, P. J., Khan, S., Lea, P. J., and Keys, A. J. (2002). Rubisco activity: effects of drought stress. *Annals of botany* **89**, 833-839.

Peñuelas, J., and Munné-Bosch, S. (2005). Isoprenoids: an evolutionary pool for photoprotection. *Trends in plant science* **10**, 166-169.

Petrini, O., and Fisher, P. (1986). Fungal endophytes in *Salicornia perennis*. *Transactions of the British Mycological Society* **87**, 647-651.

Powles, S. B. (1984). Photoinhibition of photosynthesis induced by visible light. *Annual Review of Plant Physiology* **35**, 15-44.

Pryor, W. A., and Stanley, J. P. (1975). A suggested mechanism for the production of malonaldehyde during the autoxidation of polyunsaturated fatty acids. Nonenzymatic production of prostaglandin endoperoxides during autoxidation. *Journal of Organic Chemistry* **40**, 3615–7.

Purahong, W., and Hyde, K. D. (2011). Effects of fungal endophytes on grass and non-grass litter decomposition rates. *Fungal Diversity* **47**, 1-7.

Quacquarelli, A., and Avgelis, A. (1975). *Nicotiana benthamiana* Domin, as host for plant viruses/*Nicotiana benthamiana* Domin, quale ospite di virus vegetali. *Phytopathologia Mediterranea*, 36-39.

Quiggin, J. (2010). Drought, climate change and food prices in Australia. *Melbourne: Australian Conservation Foundation*.

Rapparini, F., and Peñuelas, J. (2014). Mycorrhizal fungi to alleviate drought stress on plant growth. *In* "Use of Microbes for the Alleviation of Soil Stresses, Volume 1", pp. 21-42. Springer.

Redman, R. S., Kim, Y. O., Woodward, C. J. D. A., Greer, C., Espino, L., Doty, S. L., and Rodriguez, R. J. (2011). Increased fitness of rice plants to abiotic stress via habitat adapted symbiosis: a strategy for mitigating impacts of climate change. *PLoS ONE* **6**, e14823.

Redman, R. S., Sheehan, K. B., Stout, R. G., Rodriguez, R. J., and Henson, J. M. (2002). Thermotolerance generated by plant/fungal symbiosis. *Science* **298**, 1581-1581.

Reid, A., and Greene, S. E. (2013). How microbes can help feed the world. *Issues*, 33.

Ren, A. Z., and Clayy, K. (2009). Impact of a horizontally transmitted endophyte, *Balansia henningsiana*, on growth and drought tolerance of *Panicum rigidulum*. *International Journal of Plant Sciences* **170**, 599-608.

Rodriguez, R., and Redman, R. (1997). Fungal life-styles and ecosystem dynamics: biological aspects of plant pathogens, plant endophytes and saprophytes. *Advances in botanical research* **24**, 169-193.

Rodriguez, R., White Jr, J., Arnold, A., and Redman, R. (2009). Fungal endophytes: diversity and functional roles. *New phytologist* **182**, 314-330.

Rodriguez, R. J., Henson, J., Van Volkenburgh, E., Hoy, M., Wright, L., Beckwith, F., Kim, Y. O., and Redman, R. S. (2008). Stress tolerance in plants via habitat-adapted symbiosis. *ISME Journal* **2**, 404-416.

Rodriguez, R. J., Redman, R. S., and Henson, J. M. (2004). The role of fungal symbioses in the adaptation of plants to high stress environments. *Mitigation and adaptation strategies for global change* **9**, 261-272.

Roossinck, M. J. (2001). Cucumber mosaic virus, a model for RNA virus evolution. *Molecular Plant Pathology* **2**, 59-63.

Roossinck, M. J. (2011). The good viruses: viral mutualistic symbioses. *Nature reviews. Microbiology* **9**, 99.

Ruiz-Sánchez, M., Aroca, R., Muñoz, Y., Polón, R., and Ruiz-Lozano, J. M. (2010). The arbuscular mycorrhizal symbiosis enhances the photosynthetic efficiency and the antioxidative response of rice plants subjected to drought stress. *Journal of plant physiology* **167**, 862-869.

Sanders, G. J., and Arndt, S. K. (2012). Osmotic adjustment under drought conditions. *In* "Plant Responses to Drought Stress", pp. 199-229. Springer.

Schäfer, P., Khatabi, B., and Kogel, K.-H. (2007). Root cell death and systemic effects of *Piriformospora indica*: a study on mutualism. *FEMS microbiology letters* **275**, 1-7.

Schardl, C., and Clay, K. (1997). Evolution of mutualistic endophytes from plant pathogens. *In* "Plant Relationships Part B", pp. 221-238. Springer.

Schardl, C. L., Leuchtmann, A., and Spiering, M. J. (2004). Symbioses of grasses with seedborne fungal endophytes. *Annu. Rev. Plant Biol.* **55**, 315-340.

Scheibe, R., and Beck, E. (2011). Drought, desiccation, and oxidative stress. *In* "Plant desiccation tolerance", pp. 209-231. Springer.

Schenk, P. M., Carvalhais, L. C., and Kazan, K. (2012). Unraveling plant–microbe interactions: can multi-species transcriptomics help? *Trends in biotechnology* **30**, 177-184.

Schulz, B., and Boyle, C. (2005). The endophytic continuum. *Mycological research* **109**, 661-686.

Schulz, B., Guske, S., Dammann, U., and Boyle, C. (1998). Endophyte-host interactions. II. Defining symbiosis of the endophyte-host interaction. *Symbiosis, Philadelphia, Pa.(USA)*.

Schulze, E. (1986). Carbon dioxide and water vapor exchange in response to drought in the atmosphere and in the soil. *Annual Review of Plant Physiology* **37**, 247-274.

Sherameti, I., Tripathi, S., Varma, A., and Oelmuller, R. (2008). The root-colonizing endophyte Pirifomospora indica confers drought tolerance in *Arabidopsis* by stimulating the expression of drought stress-related genes in leaves. *Molecular Plant-Microbe Interactions* **21**, 799-807.

Sherrard, M. E., Maherali, H., and Latta, R. G. (2009). Water stress alters the genetic architecture of functional traits associated with drought adaptation in *Avena barbata*. *Evolution* **63**, 702-715.

Singh, B., and Usha, K. (2003). Salicylic acid induced physiological and biochemical changes in wheat seedlings under water stress. *Plant Growth Regulation* **39**, 137-141.

Smirnoff, N. (1993). The role of active oxygen in the response of plants to water deficit and desiccation. *New Phytologist* **125**, 27-58.

Sternberg, T. (2011). Regional drought has a global impact. *Nature* **472**, 169-169.

Sun, C. A., Johnson, J., Cai, D. G., Sherameti, I., Oelmuller, R., and Lou, B. G. (2010). *Piriformospora indica* confers drought tolerance in Chinese cabbage leaves by stimulating antioxidant enzymes, the expression of drought-related genes and the plastid-localized CAS protein. *Journal of Plant Physiology* **167**, 1009-1017.

Suttle, C. A. (2007). Marine viruses-major players in the global ecosystem. *Nature reviews. Microbiology* **5**, 801.

Swarthout, D., Harper, E., Judd, S., Gonthier, D., Shyne, R., Stowe, T., and Bultman, T. (2009). Measures of leaf-level water-use efficiency in drought stressed endophyte infected and non-infected tall fescue grasses. *Environmental and Experimental Botany* **66**, 88-93.

Symon, D. (2005). Native tobaccos (Solanaceae: *Nicotiana* spp.) in Australia and their use by Aboriginal peoples. *Beagle: Records of the Museums and Art Galleries of the Northern Territory, The* **21**, 1.

Taiz, L., and Zeiger, E. (2010). Plant physiology 5th Ed. *Sunderland, MA: Sinauer Associates*.

Tang, X., Frederick, R. D., Zhou, J., and Halterman, D. A. (1996). Initiation of plant disease resistance by physical interaction of AvrPto and Pto kinase. *Science* **274**, 2060.

Times, A. (2005). Grim reaping for Thai farmers: A news report on Southeast Asia [OL].

USDA (2013). U.S. Drought 2012: Farm and Food Impacts. USDA.

Vadassery, J., Ranf, S., Drzewiecki, C., Mitho¨fer, A., Mazars, C., Scheel, D., Lee, J., and Oelmu¨ller, R. (2009). A cell wall extract from the endophytic fungus *Piriformospora indica* promotes growth of Arabidopsis seedlings and induces intracellular calcium elevation in roots. *Plant Journal* **59**, 193–206.

Van der Hoorn, R. A., Laurent, F., Roth, R., and De Wit, P. J. (2000). Agroinfiltration is a versatile tool that facilitates comparative analyses of Avr 9/Cf-9-induced and Avr 4/Cf-4-induced necrosis. *Molecular Plant-Microbe Interactions* **13**, 439-446.

Vassey, T. L., and Sharkey, T. D. (1989). Mild water stress of *Phaseolus vulgaris* plants leads to reduced starch synthesis and extractable sucrose phosphate synthase activity. *Plant Physiology* **89**, 1066-1070.

Vickers, C. E., Gershenzon, J., Lerdau, M. T., and Loreto, F. (2009). A unified mechanism of action for volatile isoprenoids in plant abiotic stress. *Nature Chemical Biology* **5**, 283-291.

WAA (2004). Water Account Australia 2004/05 *In* "Water and Cotton Fact Sheet ". Cotton Australia.

Wagner, B., Fuchs, H., Adhami, F., Ma, Y., Scheiner, O., and Breiteneder, H. (2004). Plant virus expression systems for transient production of recombinant allergens in *Nicotiana benthamiana*. *Methods* **32**, 227-234.

Waller, F., Achatz, B., Baltruschat, H., Fodor, J., Becker, K., Fischer, M., Heier, T., Huckelhoven, R., Neumann, C., Wettstein, D., Franken, P., and Kogel, K. H. (2005). The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 13386–13391.

Walter, J., Nagy, L., Hein, R., Rascher, U., Beierkuhnlein, C., Willner, E., and Jentsch, A. (2011). Do plants remember drought? Hints towards a drought-memory in grasses. *Environmental and Experimental Botany* **71**, 34-40.

Walter, M. H., and Strack, D. (2011). Carotenoids and their cleavage products: biosynthesis and functions. *Natural product reports* **28**, 663-692.

Waqas, M., Khan, A. L., Kamran, M., Hamayun, M., Kang, S.-M., Kim, Y.-H., and Lee, I.-J. (2012). Endophytic fungi produce gibberellins and indoleacetic acid and promotes host-plant growth during stress. *Molecules* **17**, 10754-10773.

Whenham, R., Fraser, R., Brown, L., and Payne, J. (1986). Tobacco-mosaic-virus-induced increase in abscisic-acid concentration in tobacco leaves. *Planta* **168**, 592-598.

Willaman, J. J., and Schubert, B. G. (1961). "Alkaloid-bearing plants and their contained alkaloids," Agricultural Research Service, US Department of Agriculture.

Wylie, S. J., Zhang, C., Long, V., Roossinck, M. J., Koh, S. H., Jones, M. G., Iqbal, S., and Li, H. (2015). Differential responses to virus challenge of laboratory and wild accessions of Australian species of Nicotiana, and comparative analysis of RDR1 gene sequences. *PloS one* **10**, e0121787.

Xu, P., Chen, F., Mannas, J. P., Feldman, T., Sumner, L. W., and Roossinck, M. J. (2008). Virus infection improves drought tolerance. *New Phytologist* **180**, 911-921.

Xu, Z. Z., Zhou, G. S., and Shimizu, H. (2009). Effects of soil drought with nocturnal warming on leaf stomatal traits and mesophyll cell ultrastructure of a perennial grass. *Crop Science* **49**, 1843-1851.

Yoshino, K., Irieda, H., Sugimoto, F., Yoshioka, H., Okuno, T., and Takano, Y. (2012). Cell death of Nicotiana benthamiana is induced by secreted protein NIS1 of Colletotrichum orbiculare and is suppressed by a homologue of CgDN3. *Molecular plant-microbe interactions* **25**, 625-636.

Zabalgogeazcoa, I. (2008). Fungal endophytes and their interaction with plant pathogens: a review. *Spanish Journal of Agricultural Research* **6**, 138-146.

Zaitlin, M., and Palukaitis, P. (2000). Advances in understanding plant viruses and virus diseases. *Annual review of phytopathology* **38**, 117-143.

Zaurov, D., Bonos, S., Murphy, J., Richardson, M., and Belanger, F. (2001). Endophyte infection can contribute to aluminum tolerance in fine fescues. *Crop science* **41**, 1981-1984.

Chapter 2

Differential responses of accessions of native Australian *Nicotiana* species to water stress

2. Differential responses of accessions of native Australian *Nicotiana* species to water stress

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2.1. Abstract

Thirty-two accessions of four *Nicotiana* species (*N. benthamiana, N. occidentalis, N. simulans,* and *N. umbratica*) collected from wild plants in northern Australia were assessed for responses to water stress. Under moderate water stress conditions, shoot fresh weight, shoot dry weight, root fresh weight, root dry weight, root/shoot ratio, and relative water content of leaves were significantly affected. However, the degree to which the accessions were affected varied considerably. Some accessions of *N. simulans*, *N. benthamiana* and *N. occidentalis* were significantly more affected by water stress than others. There was significant variation between accessions in leaf and shoot tip wilting times. Initial symptom expression (leaf wilting) was significantly delayed in three accessions of *N. benthamiana*, and in one accession of each of *N. glutinosa* and *N. umbratica*. The least water stress tolerant lines, two accessions each of *N. benthamiana*, *N. occidentalis* and *N. simulans,* exhibited advanced symptoms of water stress (shoot tip wilting) within 14-17 days of cessation of watering. This stage was significantly delayed in three accessions of *N. benthamiana* and two accessions *N. occidentalis* and one accession of each of *N. simulans* and *N. umbratica*, which showed tip wilting only after 21-24 days. There were variations among the accessions of same *Nicotiana* species on their tolerance to water stress. Plant responses to water stress could not be predicted from

their plant biomass and leaf relative water content under well-watered conditions. Leaf chlorophyll content was variable under water stress, but did not correlate with water stress tolerance.

Key words: *Nicotiana* spp.; biomass, chlorophyll content; leaf wilting; shoot tip wilting; RWC.

2. 2. Introduction

Water stress represents one of the most important limitations on agricultural productivity (McWilliam 1986), leading to significant reductions in crop yield and overall productivity (Kirkham 2005). It is predicted that climate change in the coming decades will increase average temperatures, and rainfall patterns will become less predictable (IPCC 2007). Plant responses to water stress are complicated and involve escape, avoidance, and tolerance (Chaves *et al.* 2003; Morgan 1984). Changes in gene expression in response to water stress lead to changes in metabolism (Chaves *et al.* 2009; Chaves *et al.* 2003). Some responses result in differential influences on carbon assimilation, photosynthesis and growth (Pereira and Chaves 1993). The type and degree of physiological response can differ in different plant species and between genotypes within species. Parameters of biomass, relative water content and chlorophyll content have been used as indices for screening plants for stress tolerance (Chaves *et al.* 2009; Ings *et al.* 2013).

Breeding programs have narrowed the genetic diversity in cultivated plants, and in some cases, inadvertently reduced tolerance to environmental stress (Ings *et al.* 2013). Wild plants represent sources of new genetic diversity. Thus, the identification of wild plant germplasm with genes for better tolerance to stresses may be valuable for introgression to related cultivars (Ings *et al.* 2013).

All indigenous Australian species of *Nicotiana* are fast-growing, short-lived annuals. Most are ephemeral, appearing only when soil moisture conditions are favourable, and remaining dormant for long periods as seed when it is not. Thus, plants attempt to reduce their risk of experiencing water stress by escaping from it by controlling their time of germination. When water stress occurs during growth, the plant may die before reproducing. Annual plants have developed a suite of physical (structural) and physiological (genetic, metabolic) strategies to prolong survival under stress until the

progenitor of the next generation, the seed, is mature (Ledbetter *et al.* 1996). The present study used parameters of leaf relative water content, shoot weight, root weight, and chlorophyll content to screen accessions of wild *Nicotiana* species for tolerance to water stress. Plant wilting is commonly used as a visual assessment of relative levels of tolerance to water stress (Bohnert and Sheveleva 1998). The initial stage of wilting is characterized by folding, rolling or changes in leaf surface structure followed by a change of leaf angle or protrusion of veins on the leaf surface. After leaf wilting, the plant shoot tip starts to wilt, and later the wilted shoot tip becomes necrotic, and application of water will no longer recover the plant (Engelbrecht *et al.* 2007).

N. benthamiana, and to a lesser extent *N. occidentalis*, are regarded as model plants in plant pathology and for gene expression studies. However, unlike another model plant species, *Arabidopsis thaliana*, only one accession of *N. benthamiana* (research accession 4, RA4) is widely available to the scientific community (Goodin *et al.* 2008; Wylie *et al.* 2015). Very little is known about the phenotypic range of *N. benthamiana* in its natural range in northern Australia. The objective of this study was to assess new wild-collected accessions of *Nicotiana* species, including *N. benthamiana*, for tolerance to moisture deprivation.

2.3. Materials and Methods

2.3.1. *Collection of Nicotiana accessions*

Plants of 32 accessions that belonged to six *Nicotiana* species were used in this study. Of them, plants of 29 accessions belonging to four species viz. *N. benthamiana*, *N. occidentalis*, *N. simulans* and *N. umbratica* that were collected from six natural populations in arid regions of northern Western Australia (Table 1). The laboratory accession of *N. benthamiana* (RA4) and plants of *N. glutinosa* and *N. tabacum* (unknown accession names/cultivars) were also used. The latter are species originating in the Andes region of South America.

Table 1: List of *Nicotiana* accessions used for drought tolerance study with description of the collection location

SL No.	Accession Code	Plant Species	Collection Location	Altitude (meter above sea level)	Coordinate
1. 2. 3.	Ham-1 KL-1 Kx-1	*N. benthamiana*	Karijini	649	22° 15' 3.5136" S 117° 58' 24.0132" E
4.	Weanoo-1	*N. umbratica*			
5.	Ft-1	*N. occidentalis*			
6.	HCK-1	*N. simulans*	Karratha	18	20° 44' 11.8140" S 116° 50' 48.3540" E
7.	VL552B.01	*N. occidentalis ssp obliqua*			
8. 9.	VL552B.2.1-1 VL552B.2.2	*N. benthamiana*			
10. 11. 12. 13.	PPM1 Mill-1(17.23) Mill-5(17.26) Mill-7(17.28)	*N. benthamiana*	Millstream	10	20° 46' 8.9976" S 117° 8' 47.6664" E
14.	Mill-2 (17.25)	*N. simulans*			
15. 16. 17.	MtA-4 MtA-8 MtA-11	*N. occidentalis*	Mount Augustus	514	26° 35' 28.3488" S 118° 29' 48.2964" E
18. 19. 20.	MtA-1 MtA-5 MtA-7	*N. benthamiana*			
21. 22. 23. 24.	MtA-2 MtA-9 MtA-10 MtA-12	*N. simulans*			
25.	MtG-11	*N. occidentalis ssp occidentalis*	Mount Gould	396	25° 47' 48.0012" S 117° 20' 39.9984" E
26.	MtG-10	*N. simulans*			
27. 28. 29.	Nt-1 Nt-4 Nt-5	*Nicotiana occidentalis ssp hesperis*	Nanutarra	98	22° 35' 27.4020" S 115° 55' 41.5020" E
30.	RA-4	*N. benthamiana*	Laboratory collection		
31.	Nglu	*N. glutinosa*			
32.	Ntab	*N. tabacum*			

2.3.2. *Water stress and plant physiology: Experiment-1*

Moderate water deficit experiment

Wild collected seeds of 32 *Nicotiana* accessions were sown on soil in trays, and after 4 weeks, the seedlings were transferred to square-shaped pots (8 cm^2; 18 cm depth) containing 800g air-dried washed river sand. Sand was selected as the growth medium because it allows even, uniform and rapid drying, and simplifies harvesting of roots. All plants were grown in an air-conditioned greenhouse under natural lighting conditions, with temperatures set to 25°C/22°C (day/night). There was one seedling per pot. All plants were watered daily and supplied nutrients for 21 d after transplanting before imposing stress. The pots were arranged in a randomized complete block design (RCBD). There were 24 pots per accession; half of them underwent water stress and the rest received adequate watering (control) to drip point. Location of blocks of pots and/or individual pots was changed weekly to control for effects micro-environmental variation. At 21 days post-transplantation, all plants from both groups were watered to saturation, and then the treatment groups were challenged by withholding water supply from that day for five days. Control plants were watered as before until the end of the experiment.

Sample collection for RWC and biomass

At five days post-stress (dps) imposition, most of the plants started exhibiting symptoms of water stress by visibly wilting in the first 2/3 leaves from the base of the plant. At this time, the youngest two fully developed leaves were harvested from six plants per accession for the purpose of determining fresh, turgid and dry weights. After measuring the fresh weight of leaves, they were kept in a bowl containing water for 48 h at 4°C and a second weight was taken at saturation for the turgid weight. For taking dry weight, the leaves were dried in an oven at 80°C until a constant weight was attained. Measuring water content was based on the maximum amount of water a tissue can hold, referred to as relative water content. Therefore, the leaf relative water content (RWC) (Engelbrecht *et al.* 2007) was calculated: $RWC\ (\%) = [(FW - DW)/(TW - DW)] \times 100$

FW, DW, and TW are the fresh, dry and turgid weights, respectively, of the leaves.

The remaining plants (six plants per accession per treatment group) were harvested with their roots for shoot and root biomass measurements on the same day. The sand attached to the roots was removed gently, and the roots and shoots were separated by cutting. Since the growth substrate was removed manually, small errors are expected in root weights. Fresh weight of shoots and roots were taken, and the material was loosely wrapped in aluminium foil and dried at 80° C, and then weighed again. The root to shoot ratio was calculated from following formula: RSR=DWr/DWs, where DWr and DWs are the dry weights of the roots and shoots, respectively.

Chlorophyll measurement

Chlorophyll content was measured on the last three fully developed leaves by a hand-held chlorophyll meter (CCM-200 plus, Opti-Sciences, Inc., USA). Three readings per plant were averaged, and the resulting figure was taken as the chlorophyll content of that plant. Twelve plants per accession were investigated, six of non-stressed and six of stressed plants. This estimation of the chlorophyll content involves a non-destructive method by using absorbance of light of the intact leaf. It gives a chlorophyll content index (CCI, dimensionless) value obtained from the absorbance of two wavelengths (660nm - red, 940nm – near infrared), which is proportional to the amount of chlorophyll in the sample. Chlorophyll data measurement was carried out at 5 days after stress was imposed, just before the plants were harvested.

2.3.3. *Severe water stress and plant wilting response: Experiment-2*

Essentially the same procedures and growth conditions were adopted as in the moderate drought treatment experiment described above. The difference with the first experiment was that the plants were not harvested during the moderate stress period. Rather, water stress was continued until the plants exhibited shoot tip wilting. The following stress responses were recorded: (i) days to first lower leaf wilting (initial visible stress response), (ii) days to upper leaves wilting but shoot tip still upright, and (iii) days to shoot tip wilting. The wilting of the shoot tip is considered as the point when irreversible damage to the plant occurs (Barrs 1968). We measured the chlorophyll content of stressed

and control plants at 0, 5 and 9 dps imposition by following the same procedure described above in experiment 1. Experiment 2 was repeated once using the same procedure except that chlorophyll measurements were not taken. The terms ‘trial-1’ and ‘trial-2’ refer the repeats of experiment-2.

2.3.4. *Statistical Analysis*

A principal component analysis was run with all biomass parameters, RWC and chlorophyll data of both moderately stressed and control plants from experiment 1 on the software “R version 3.2.3” (R Core Team 2015) using the co-relation matrices since the data were with different scales. Plant accessions were clustered separately based on data from watered and stressed conditions. Dendrograms were created by agglomerative hierarchical clustering algorithms (Ward’s linkage) using PAST v 3.15 Statistical Software (Hammer *et al.* 2001). This software package originally designed for paleontological data analysis has been variously used in many other fields (Garcia-Mazcorro *et al.* 2017; Hirsch *et al.* 2017; McKay *et al.* 2016; Vialet-Chabrand *et al.* 2017; Zaura *et al.* 2009; Zhang *et al.* 2004). PAST, a user friendly and powerful statistical tool, produces Ward’s dendrograms identical to those made by Stata (Stata Corp 2017). A two-factor ANOVA was performed to measure the effect of water stress and the interactions of accessions and stress. The significance of changes in biomass due to stress in each accession was determined with a non-parametric test (Mann–Whitney U test). For comparing the plant wilting response under severe stress, a two-factor ANOVA (accession and trial) was run for the three parameters days to the 2nd leaf wilting, days to final leaf wilting and days to shoot tip wilting. The ‘trial’ was considered as a categorical variable (independent) which had two levels, *viz*. trial-1 and trial-2 to determine if the results of the two trials were significantly different. Since no significant difference was observed between the trials for any of the parameters we combined the data to make a single dataset and treated them as replications (n=12) for subsequent analyses. Plant responses to severe stress with leaf and shoot tip wilting were compared using ANOVA and pairwise comparison with a post-hoc Duncan's new multiple range test (DMRT) test. All ANOVA tests, bar charts and boxplots were done using IBM SPSS Statistics 24 (IBM 2012) software and Microsoft excel 2016.

2.4. Results

2.4.1. *Physiological traits affected by moderate water stress reveal variable responses within and between Nicotiana species*

In experiment 1, water stress was moderate and was applied for a relatively short period (5 days). There was a significant effect on all physiological parameters measured (except root dry weight; Table 2). Physiological responses varied between accessions and a significant accession × treatment interaction was observed for RWC and shoot and root fresh weight (Table 2). To group the accessions based on their response to moderate water stress, a principal component analysis (PCA) was performed on both the control and stressed plants. Fig. 1a illustrates the first factorial plane delimited by the Dim1, which accounted for 57% of the observed variation. *N. occidentalis* accessions Ft-1, *N. simulans* accessions MtG-10, MtA-10, MtA-12, HCK-4 and the *N. umbratica* accession Weanoo-1 are located far from the origin of the axis in Fig. 1a, showing that these plants had different responses to water stress to the average of the studied accessions. The variable factor map (Fig. 1b) produced by Dim1 and Dim2 shows that characters under watered or stressed conditions are located closely except RWC and RSR, meaning that the quantitatively important variation of 69% (57% for Dim1 and 12% for Dim2) is on the same relative value for accessions in watered and stressed conditions: for example, accessions showing more shoot fresh weight in watered conditions still had more shoot biomass under stressed conditions. However, the relative water content, and to a lesser extent the root/shoot ratio, responded differently (Fig.1b) indicating a negative correlation between plants in watered and stressed conditions. On the second plane, explaining 12% of the variation, outliers are one accession of each of *N. glutinosa (Nglu)*, *N. occidentalis* (MtG-11), two accessions of *N. benthamiana* (KL-1 and Mill-5) and two accessions of *N. simulans* (Mill-2 and MtA-2). The variables factor map showed that except for relative water content (RWC) and root shoot ratio (RSR), there is no distinct separation of parameters measured in watered and stressed conditions.

Each parameter considered in this study was examined carefully after a general assessment of accessions on their response to water stress. As predicted, a systematic lower level of relative water

content was documented for all accessions following drought. This decrease in RWC during drought compared to control conditions was distinct and was significant for most accessions but not very conspicuous for six accessions of *N. benthamiana* (VL552B.2.1-1, MtA-5, Mill-7, RA4, Mill-1 and PPM1); one accession of each of *N. glutinosa* and *N. tabacum*; two accessions of *N. occidentalis* (VL552B.01, and Nt-4) and two accessions of *N. simulans* (MtA-2 and MtA-10) (Table 3).

Shoot fresh weight (SFW) was also affected among accessions under water deficit conditions (Table 3). Although most of the accessions had a substantial reduction in SFW due to moderate water deficit, three accessions of *N. benthamiana* (VL552B.2.1-1, RA4 and MtA-7) two accessions of *N. occidentalis* (VL552B.01 and Nt-1), and two of *N simulans* (MtA-2 and MtA-10) were not significantly affected.

Shoot dry biomass of the accessions was also affected by water stress, but this trait was not significantly affected across many accessions, the exceptions being the three accessions of *N. benthamiana* (VL552B.2.1-1, Mill-5 and Mill-5), and three of *N. simulans* (HCK-4, MtG-10 and MtA-2), which did show a significant reduction in shoot dry weight. Most accessions produced fewer roots as evident from their decreased root fresh weight. On the other hand, RDW increased in response to water deficit in all accessions despite the fact that this was statistically significant in only a few accessions, i.e. VL552B.2.1-1, MtA-1, PPM1 (*N. benthamiana*), HCK-4 (*N. simulans*) and Ntab (*N. tabacum*). As consequence of decreased shoot dry weight (SDW) and increased RDW in the stress affected plants, the root shoot ratio was increased in most accessions and was significant for certain accessions (Table 3). Variation occurred within species. For example, *N. benthamiana* accessions Kx-1 and MtA-1were affected significantly under stress, whereas VL552B.2.1-1 and MtA-7 were more tolerant (Table 3).

2.4.2. *Plant reaction to water stress cannot be predicted from their phenotype in well-watered conditions*

Accessions were grouped by cluster algorithms using data obtained from the plants in watered and water-stressed conditions (Fig. 2).

Table 2: Two-factor ANOVA table* showing the effect of moderate water stress on *Nicotiana* accessions on biomass traits (error df= 320)

Source of variation	**Relative water content**		**Shoot fresh weight**		**Root fresh weight**		**Shoot dry weight**		**Root dry weight**		**Root shoot ratio**		**Chlorophyll content**	
	F	P	F	P	F	P	F	P	F	P	F	P	F	P
WT (df=1)	307.56	**0.000**	349.53	**0.000**	45.53	**0.000**	16.63	**0.000**	0.01	0.91	51.31	**0.000**	7.93	**.005**
A (df= 31)	5.81	**0.000**	24.56	**0.000**	15.48	**0.000**	16.57	**0.000**	14.58	**0.000**	1.52	**0.042**	5.85	**0.000**
WT× A (df= 31)	4.474	**0.000**	4.923	**0.000**	1.870	**0.004**	0.362	0.999	0.722	0.863	1.471	0.055	1.458	0.059

*ANOVA has been run on the whole set of accessions, showing the effect of ‘‘Water Treatment (WT)’’ and ‘‘Accession (A)’’ factors on the measured parameters: Leaf Relative Water content, Shoot fresh weight, Root fresh weight, Shoot dry weight, Root dry weight, Root shoot ratio and Chlorophyll Content. WT= Water treatment, A= Accession, Significant *P*- values (≤0.05) are indicated by bold

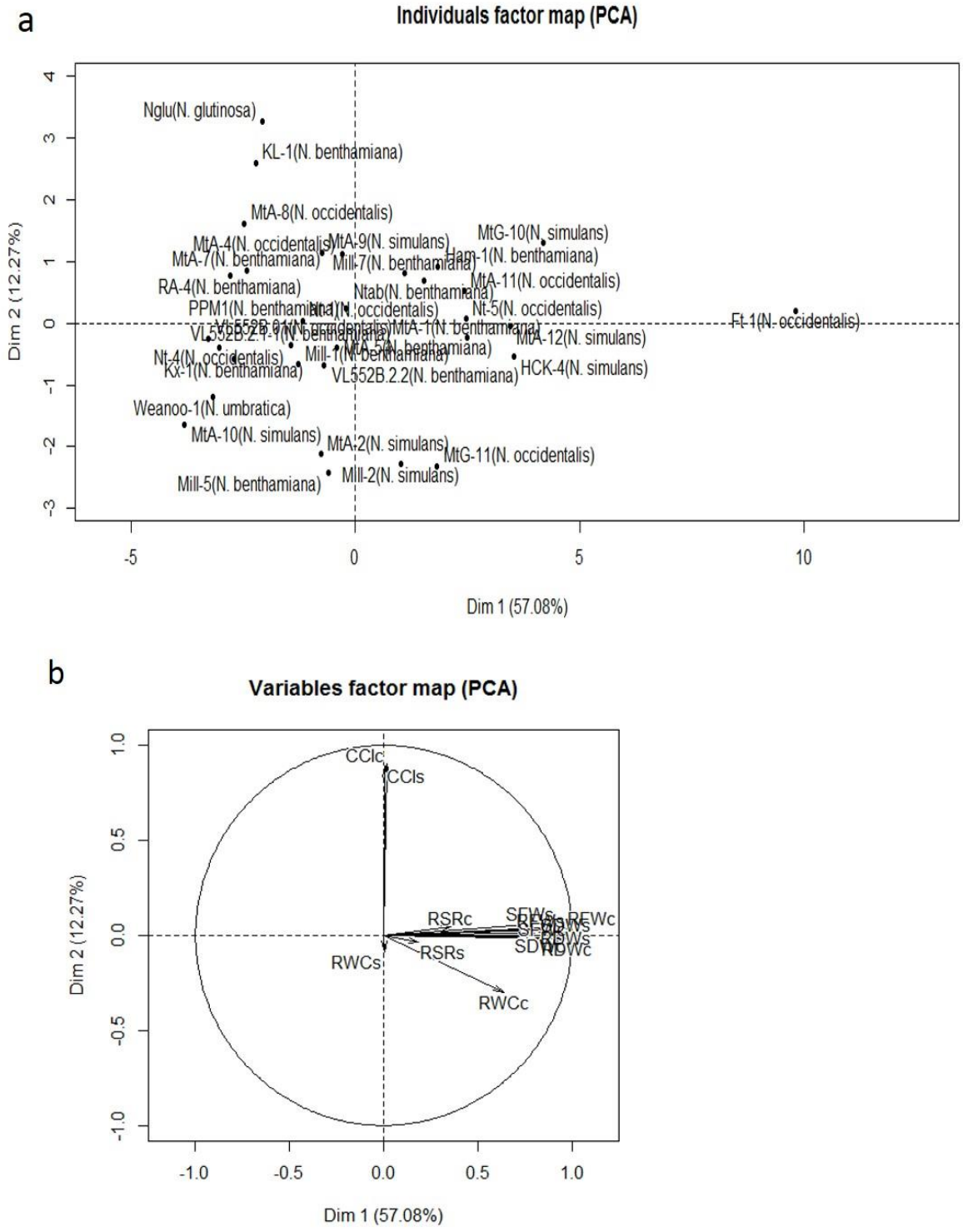

Fig 1. A Principal Component Analysis of control and stressed plants. (1a) Distribution of accessions on Dim1 (Dimension1) and Dim2 (Dimension2) axis. (1b) Variable factor map plots for PCA planes on correlation circles. The measured traits are specified respectively for watered (c) and stress (s) plants: RWC = Relative Water Content, SFW = Fresh weight of shoot, SDW=Shoot Dry Weight, RFW = Root Fresh Weight, RDW= Root Dry Weight, RSR=Root Shoot Ratio and CCI=Chlorophyll content index.

Table 3. Per cent reduction or increase of biomass parameter in moderately stressed (5 days) plants over the control

Accession		% Changes in biomass trait in moderate drought*					
	RWC	SFW	SDW	RFW	RDW	RSR	CCI
Ft-1	**-8.63**	**-55.69**	-9.21	**-45.8**	-6.82	5.86	-4.4
Ham-1	0.1	**-31.83**	-9.77	-28.5	14.17	39.2	22.07
HCK-4	**-12.01**	**-25.09**	**-19.57**	-14.79	**29.86**	**58.53**	**30.4**
KL-1	**-6.83**	**-26.69**	-5.7	-23.62	-23.44	**-17.3**	17.22
Kx-1	**-5.87**	**-63.58**	**-48.06**	5.02	-5.18	**83.6**	17.9
Mill-1	-4.07	**-31.55**	-18.62	-31.47	**68.93**	**183.74**	19.55
Mill-2	-0.89	-48.24	-33	**-38.18**	-10.54	27.91	37.39
Mill-5	**-13.37**	**-60.1**	-14.55	**-29.05**	19.25	**60.69**	7.36
Mill-7	**-6.59**	**-55.93**	**-15.6**	**-37.93**	**50.47**	**78.12**	-15.47
MtA-1	**-5.26**	**-43.24**	-16.2	-27.37	-5.74	23.86	41.55
MtA-10	**-10.91**	**-63.55**	**-31.27**	**-48.09**	26.44	**79.16**	2.93
MtA-11	-1.52	-42.56	-6.92	9.67	66.61	**81.79**	-14.25
MtA-12	**-14.29**	**-64.52**	-24.42	-48.65	-19.93	15.31	**-29.38**
MtA-2	**-9.2**	**-62.35**	**-31.37**	-43.8	-3.97	37.9	6.85
MtA-4	-6.52	-59.22	-18.19	**-74.46**	-62.69	46.26	1.04
MtA-5	**-5.57**	**-79.09**	-34.45	**-50.5**	-25.29	66.21	-13.31
MtA-7	**-9.95**	**-67.66**	-32.83	**-42.6**	-29.41	46.14	**72.29**
MtA-8	**-5.42**	**-59**	-14.8	-31.59	24.91	61.78	19.23
MtA-9	**-9.12**	**-52.27**	-15.08	-34.61	17.58	43.2	15.01
MtG-10	-3.07	**-39.01**	-5.39	**-42.19**	**-20.99**	**-16.82**	41.94
MtG-11	**-12.96**	**-63.37**	-17.91	**-46.48**	2.92	32.81	**-26.27**
Nglu	**-4.81**	**-58.85**	**-24.98**	-8.65	10.28	**47.55**	6.16
Nt-1	-7.44	**-70.69**	-4.28	-13.24	-36.4	42.22	16.27
Nt-4	**-6.99**	**-67.07**	-18.63	-16.96	10.52	**39.21**	25.61
Nt-5	**-6.62**	-29.38	-8.99	10.86	-5.46	6.23	-2.05
Ntab	**-11.41**	**-64.31**	-15.52	**-55.08**	16.03	45.45	**49.34**
PPM1	1.47	**-51.76**	-2.85	**-32.25**	-25.1	-24.38	-28.27
RA-4	-2.77	**-63.96**	-8.91	**-37.31**	1.94	11.51	-15.53
VL552B.01	-2.68	-55.92	-11.48	-27.27	-23.07	**7.74**	-2.78
VL552B.2.1-1	-0.23	-58.89	-23.2	29.1	**2.35**	62.29	12.71
VL552B.2.2	-1.53	-41.14	-5.99	-21.04	13.1	14.18	-10.82
Weanoo-1	**-6.16**	**-39.16**	-8.77	-25.78	-13.67	-4.94	19.46

*Percent biomass changes were calculated by following formula: per cent biomass change = (Bs-Bw)/Bw × 100, where Bs = biomass weight of stressed plants and Bw=biomass weight of well-watered

plant. RWC= Leaf relative water content, SFW= Shoot fresh weight, SDW= Shoot dry weight, RFW= Root fresh weight, RDW=Root dry weight, RSR= root shoot ratio and CCI=Chlorophyll content index of 32 accessions. The bold indicates that changes in biomass due to moderate drought is statistically significant at $P \leq 0.05$ level, determined with a non-parametric test (Mann-Whitney U test). The minus (-) sign before the mean value indicates the decrease in biomass trait under mild drought than in control.

The dendrogram formed by the accessions grown under well-watered conditions, grouped accessions KL-1, RA4 (N. *benthamiana*) and Nglu (*N. glutinosa*) together on one side of the tree. Under water stress, clusters changed in the dendrogram; KL-1 (*N. simulans*) and Nglu (*N. glutinosa*) remained close together but RA4 separated from them. Accessions, Weanoo-1 (*N. umbratica*), Kx-1 (*N. benthamiana*), Nt-1, Nt-4, MtA-4 and MtA-8 (*N. occidentalis*), formed another group in well-watered conditions, but they did not group together under water stress (Fig. 2). The accessions PPM1 and MtA-5 (*N. benthamiana*) were close to each other in tree 1, but they were far from each other in tree 2 (Fig. 2a and 2b). Similarly, the overall response of accessions MtA-2 and Mill-2 (*N. simulans*) was different under well-watered conditions, but was similar under water deficit conditions (Fig. 2a and 2b).

2.4.3. *Nicotiana accessions vary in response to severe stress*

When the plants were exposed to severe water deficit conditions, significant variations in response were observed among the accessions ($P \leq 0.001$) in the three parameters measured. Accessions were grouped based on their initial response to stress by wilting of second leaves after they were challenged with water stress (Fig 3a)**.** Stress symptoms began significantly later in accessions RA4, MtA-5 (*N. benthamiana*), Nglu (*N. glutinosa*), Ntab (*N. tabacum*) and Weanoo-1 (*N. umbratica*) which took 7-9 days to first exhibit visible stress responses, whereas the majority of accessions started showing symptoms from 4-6 days after stress was imposed (Fig 3a).

Accessions where leaf wilting occurred early showed wilting of all their leaves earlier in general. For example, Weanoo-1 (*N. umbratica*), MtA-5, Ham-1 (*N. benthamiana*); MtA-2 (*N. simulans*) and Nglu (*N. glutinosa*) took 15-17 days to complete leaf wilting whereas for some accessions wilting finished

in 10-13 days (44% of the total number of accessions) or earlier (7-9 days, 40% of the total number of accessions) (Fig. 3b).

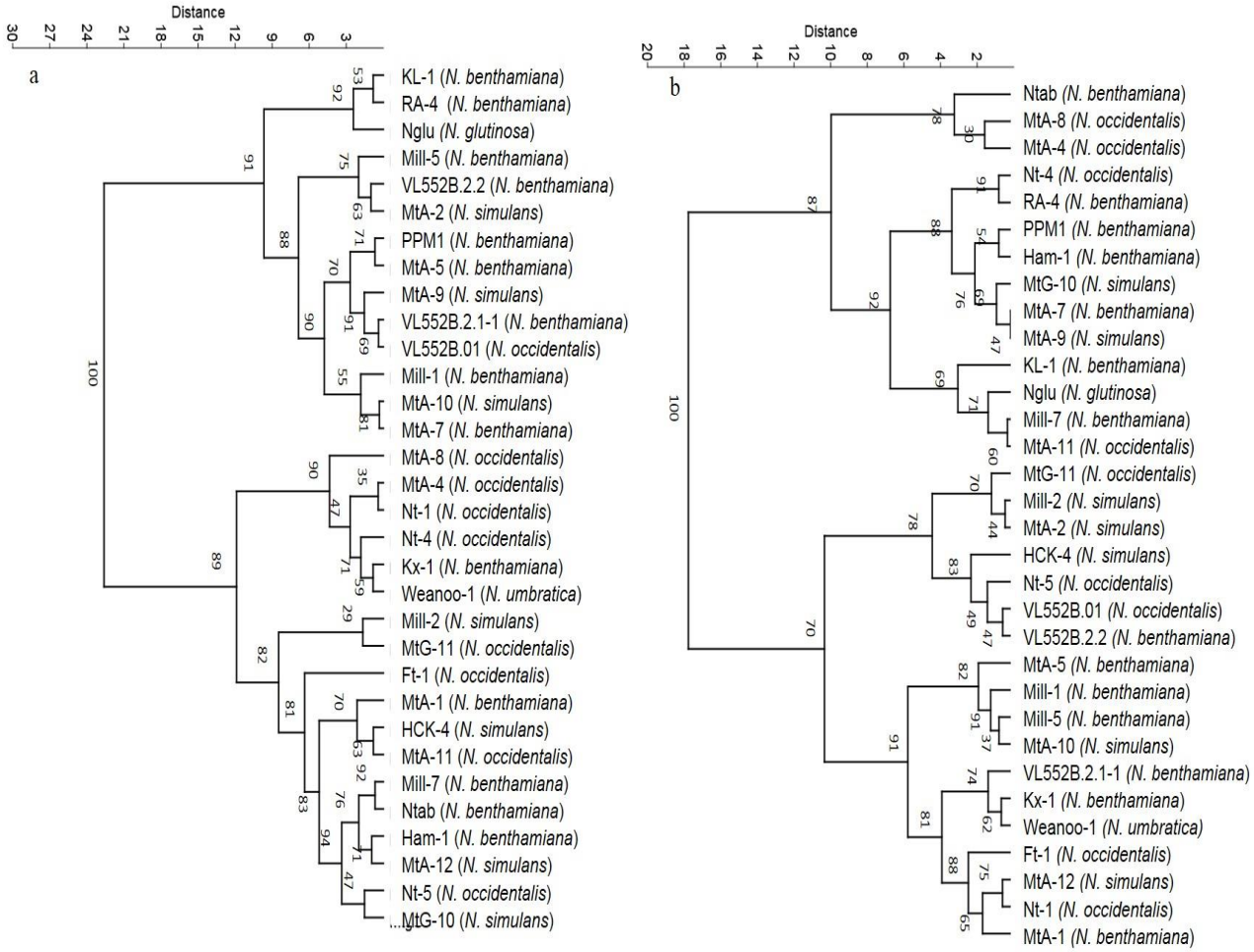

Fig.2. Dendrogram of hierarchical classification of the whole set of accessions using the data of shoot and root fresh and dry weight, root/shoot ratio and chlorophyll content of leaf under (a) well-watered and (b) moderate water stress at five days-post stress. The species names of the accessions are indicated in parenthesis. Numbers corresponding to nodes represent percent bootstrap values (N=1000 replicates).

For the final responses to stress, three *N. benthamiana* accessions, MtA-5, VL552B.2.1-1 and RA4; two *N. occidentalis* accessions, Nt-1, VL552B.01 and two *N. simulans* accessions HCK-4 and MtA-2 and the accession of *N. umbratica* Weanoo-1 were the most stress tolerant because they took 21-24 days for their shoot tips to wilt. In contrast, among the least tolerant ones were five *N. benthamiana* accessions (Ham-1, MtA-7, Mill-1, PPM1and VL552B.2.2), six *N. occidentalis* accessions (Nt-5, MtA-8, Ft-1, MtA-4, MtA-11 and MtG-11), and four *N. simulans* accessions (MtG-10, MtA-9, MtA-12, Mill-2), which showed shoot tip wilting within 14-17 days (Fig. 3c).

All the accessions grouped in six clusters based on similarity of stress responses with respect to the three wilting parameters measured. For example, accessions Weanoo-1 (*N. umbratica*), Nglu (*N. glutinosa*), MtA-2 (*N. simulans*) and MtA-5 (*N. benthamiana*) formed cluster 1, being the most stress tolerant accessions (Fig 4). Clusters 2 and 3 were the moderately tolerant accessions, whereas cluster 4 accessions were moderately susceptible. The most stress susceptible accessions were PPM1 (*N. benthamiana*), MtA-8 (*N. occidentalis*), MtA-9, and MtA-12 (*N. simulans*) (Fig. 4). The dendrogram clearly indicates that there were significant differences between accessions of the same species (Fig. 4). For example, while an accession of *N. simulans* (MtA-2) showed the highest stress tolerance, another two accessions of the same species (MtA-9 and MtA-12) were among the most susceptible accessions (Fig. 4). Similarly, *N. benthamiana* accessions MtA-5 and Mill-5 were grouped under cluster 1 (most tolerant) and Cluster 5 (susceptible), respectively (Fig. 4).

2.4.4. Effect of species and locations on water stress among *Nicotiana* populations

Significant variation in stress response was observed as a function of location and of the two-way interaction of species and location with respect to final leaf and shoot tip wilting (Table 4). Plants of *N. occidentalis* in Karratha and *N. benthamiana* in Mt Augustus took longer days to start leaf wilting (Fig 5a). In the case of final leaf wilting, *N. benthamiana* plants of Karijini, Millstream and Mt Augustus; *N. simulans* of Karratha and Mt Gould took longer days (Fig 5b). No difference in shoot tip wilting response as a function of plant species in any location except in Mt Augustus where *N.*

benthamiana plants showed higher stress tolerance ($P \leq 0.05$) than did plants of the other two *Nicotiana* species tested (Fig. 5c).

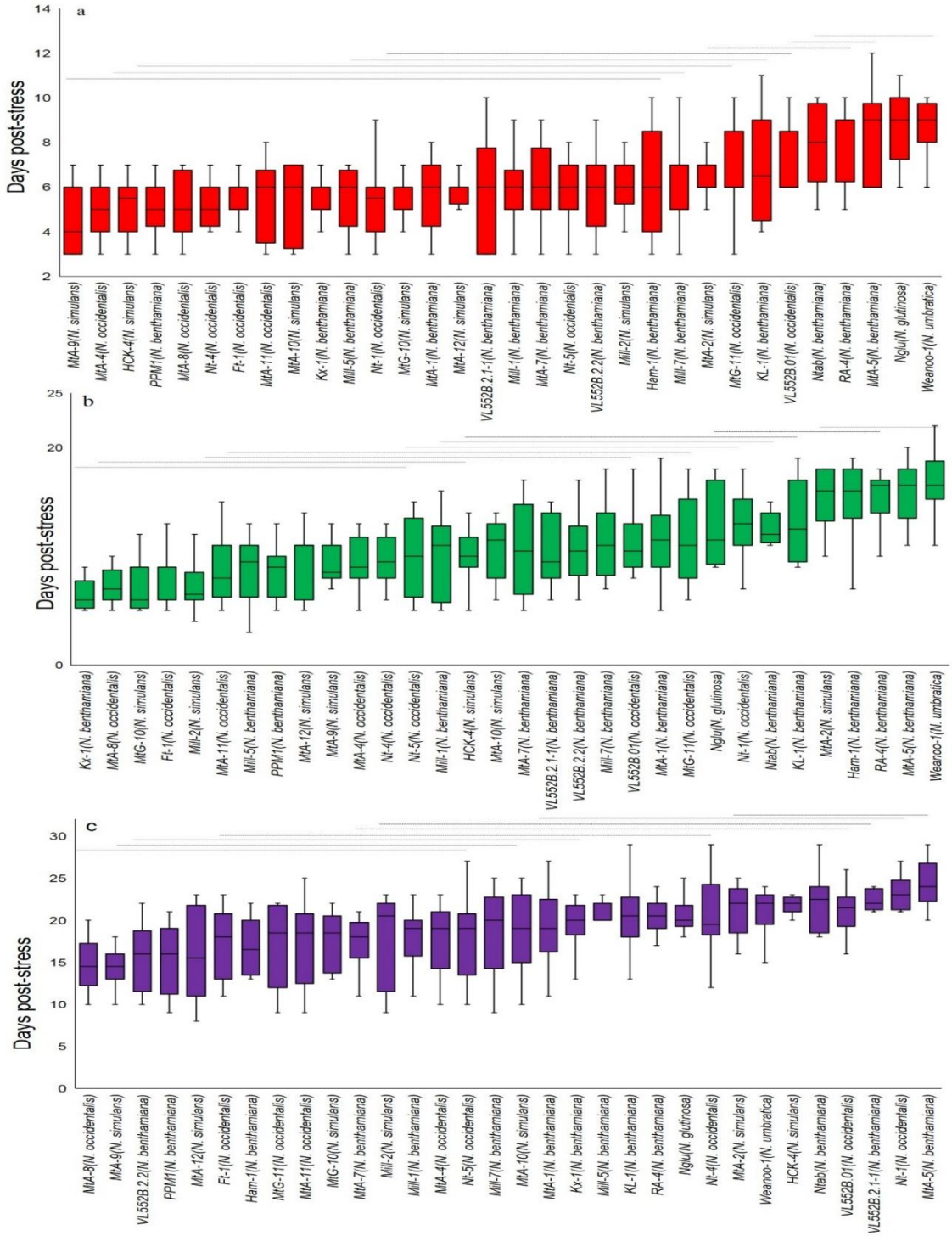

Fig 3. Box plots showing the effect of water stress on wilting response of *Nicotiana* accessions as exhibited by days to (a) First leaf wilting (b) Final leaf wilting and (c) Shoot tip wilting of plants. The accessions that are grouped together and are not significantly different ($P \leq 0.05$, n=12) as obtained by post-hoc test by Duncan new multiple range test (DMRT) are indicated by a line above the bars.

Table 4: ANOVA table showing the effect of water stress on *Nicotiana* accessions on wilting response

Source of variation	**Second leaf wilting**		**Final leaf wilting**		**Shoot tip wilting**	
	F	P	F	P	F	P
Trial (df=1, 382)	0.119	0.730	2.039	0.154	0.848	0.358
Accessions (df=31, 352)	5.536	**0.000**	9.454	**0.000**	4.505	**0.000**
Species (df= 2, 322)	3.028	**0.049**	6.673	**0.001**	0.837	0.434
Location (df= 5, 322)	0.517	0.763	6.268	**0.000**	4.384	**0.000**
Species × Location (df=6, 322)	3.057	**0.002**	6.004	**0.000**	3.094	**0.006**

2.4.5 *Chlorophyll content fluctuates under stress but does not correlate with stress tolerance*

Chlorophyll was measured from watered and stressed plants at 0, 5 and 9 dps. In watered plants, chlorophyll increased steadily. In stressed plants, the chlorophyll content increased initially but then decreased. There was significant variation in chlorophyll among the accessions at day 0, and it ranged from 4.0 to 11.0 among the accessions of the stressed group and Mill-5, MtA-1 and HCK-4 had higher value and MtA-9 and MtA-5 had lower (Fig. 6a). During moderate stress, chlorophyll increased above that of the corresponding control set (Fig. 6b). As stress progressed, chlorophyll decreased (Fig. 6c). At severe stress (9dps) the tolerant accessions MtaS1 and MtA-5 had the lowest chlorophyll, whereas Weanoo-1 and Nglu had higher values (Fig. 6c). On the contrary, MtA-8 and MtA-9-had higher chlorophyll levels even though they were among the most susceptible accessions, indicating chlorophyll level is not correlated to water stress tolerance.

2.4.6. *Plant responses to moderate stress correlated with their tolerance to severe water stress*

Stress reduced the relative water content in MtA-12 by 9%, whereas it increased (although not significant) in MtA-5 and Nglu plants (Table 3). These changes were correlated with their tolerance to

severe stress. The MtA-12 was the most susceptible accession, whereas MtA-5 and Nglu were among the most tolerant accessions. Accessions most affected by moderate water stress were MtA-8, MtA-12, MtA-9 (Table 3). The tolerant accessions were less affected by moderate stress such as in the case of Weanoo-1and Nglu. Tolerant accessions were less affected in the root to shoot ratio under moderate stress (Table 3).

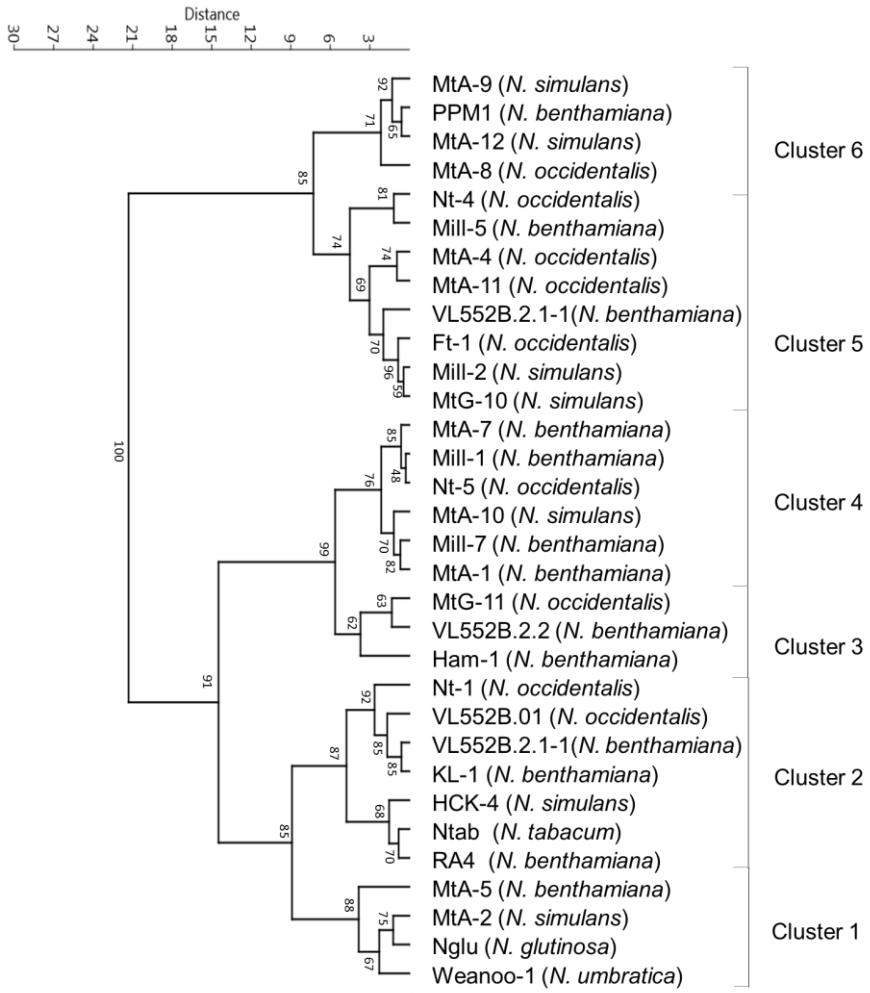

Fig 4. Dendrogram using average linkage (Ward's method) of the hierarchical cluster analysis of *Nicotiana* accessions under severe water deficit conditions based on the data of days to leaf wilting, final leaf wilting and shoot tip wilting after stress was challenged. Numbers corresponding to nodes represent percent bootstrap values (N=1000 replicates).

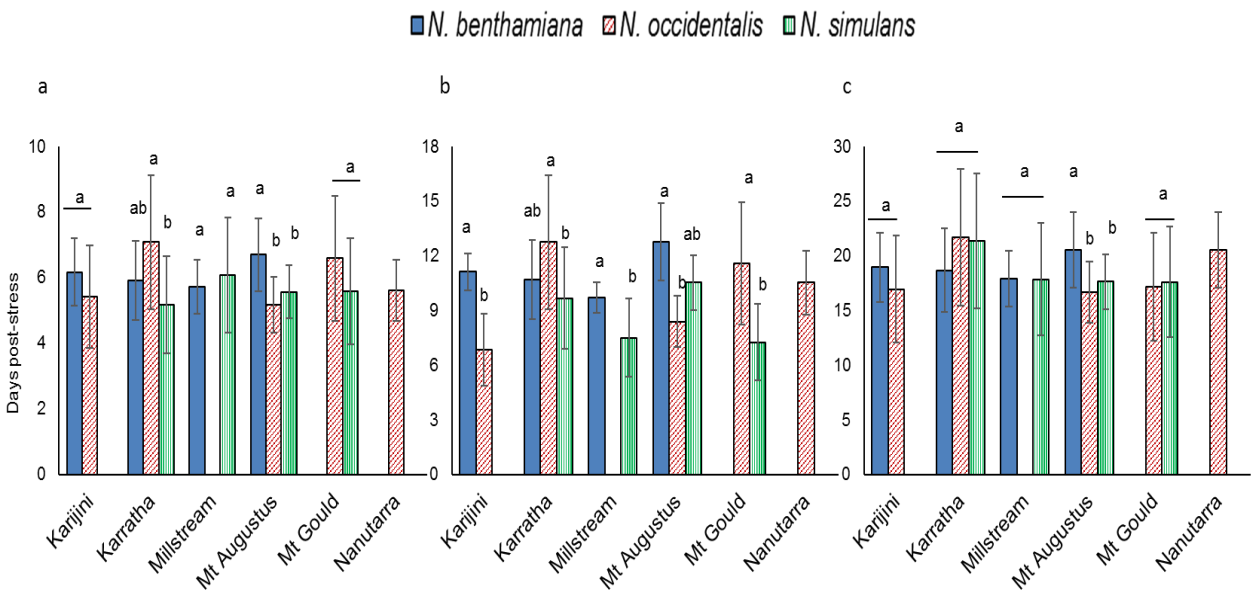

Fig. 5: Variations among the accessions in response to water stress across species and across locations based on days to wilting of a) Second leaf wilting, B) Final leaf wilting and C) Shoot tip wilting of plants. Significant difference among species within a location is indicated by letter where same letters indicate that they are not significantly different ($P \leq 0.05$) as obtained by a post-hoc test by Duncan new multiple range test (DMRT).

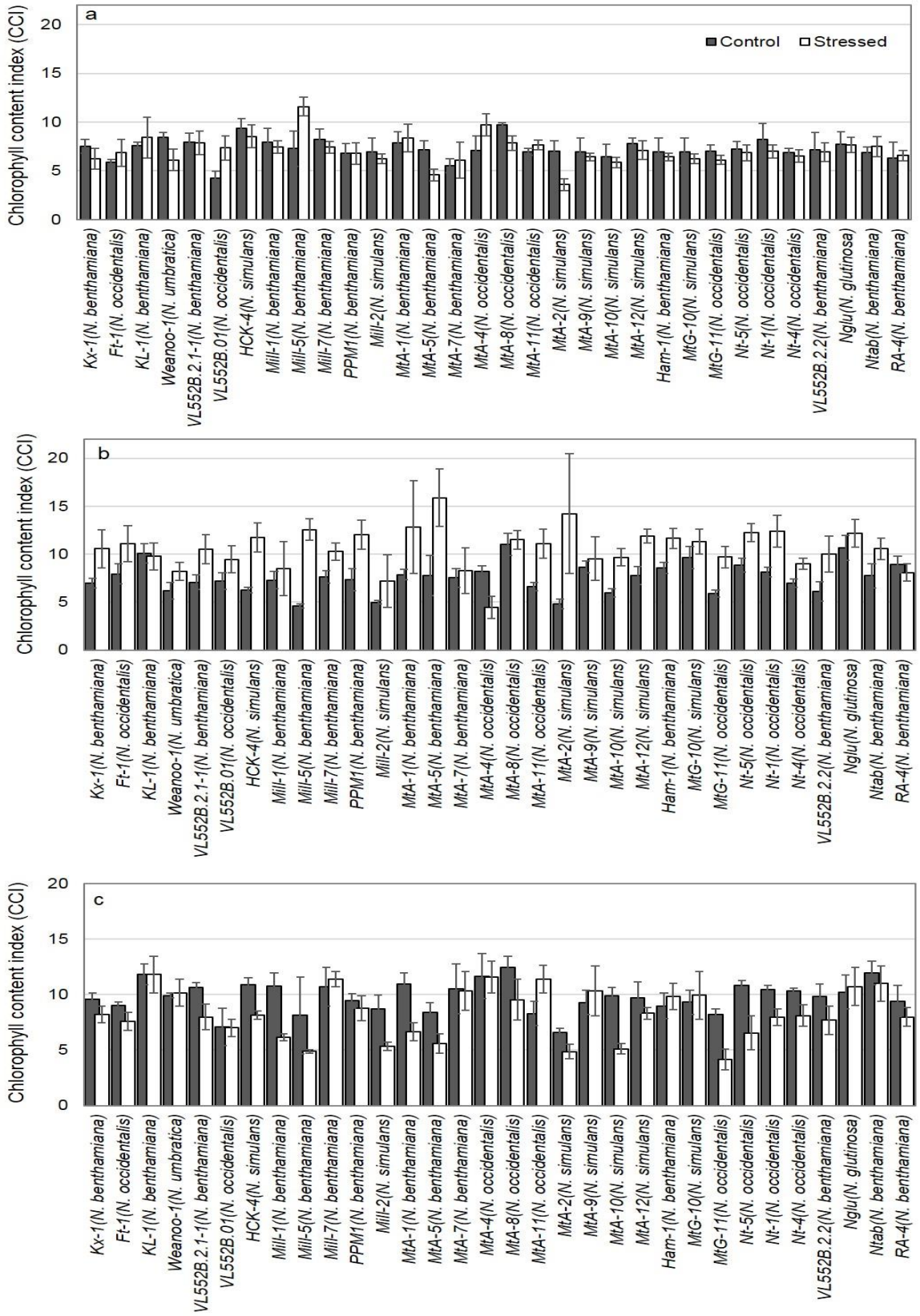

Fig.6. Effect of water stress on changes in chlorophyll content (CCI) at (a) 0-days post stress, (b) 5-days post stress and (c) 9-days post stress in control and drought stressed plants.

An attempt was also made to see if plant water stress tolerance related to their above-ground biomass weight. No significant positive or negative correlation of days to shoot tip wilting under stress was found with either shoot fresh or dry biomass (Fig. 7).

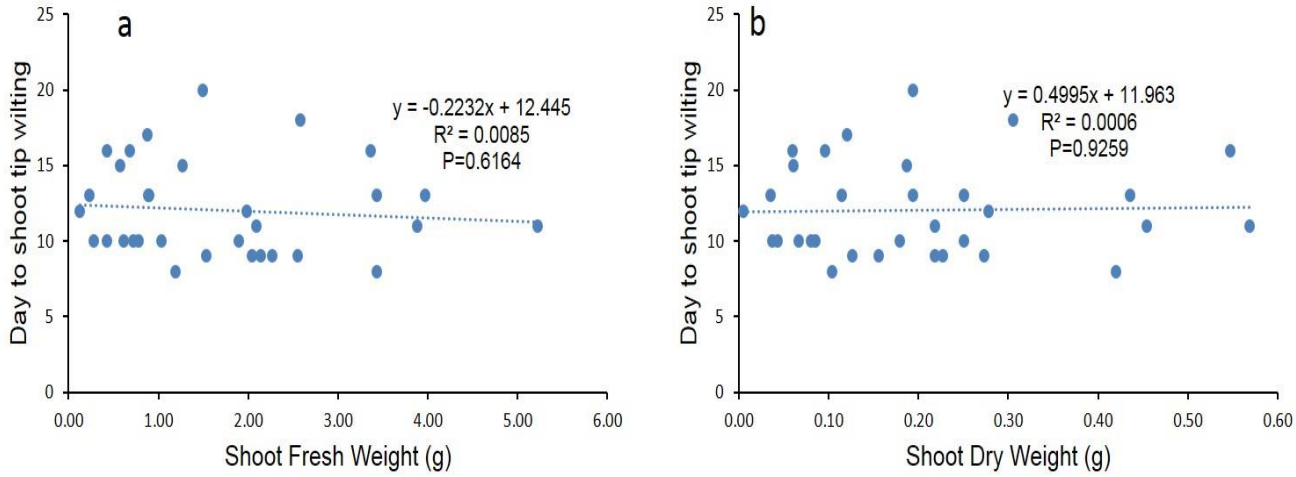

Fig. 7: Correlation between (a) Days to shoot tip wilting and Shoot fresh weight and (b) Days to shoot tip wilting and shoot dry weight of *Nicotiana* accessions subjected to water stress. The shoot biomass weight was taken from the well-watered control plants after 5-days post stress to the stressed group.

2.5. Discussion

Nicotiana plants were collected from wild populations in northern Western Australia (Table 1). Considering the differences in species and their geographical, climatic and edaphic factors across the locations, accessions are potentially subjected to different constraints and selective pressures. Under these circumstances, notable variations in responses to water stress would be expected. Indeed, significant variation was noted for most of the parameters measured. Therefore, these accessions might have developed adaptive responses to stress that could be exploited to further discern the inherent variability responsible for these physiological adaptations.

After principal component analysis combining all the traits measured in both control and stress conditions, accessions MtA-1 (*N. benthamiana*), Nt-1, Ft-1, MtA-4, MtA-8 (*N. occidentalis*), MtA-12

(*N. simulans*) and Ntab *(N. tabacum*) were outliers with regards to the effects of water stress on the biomass and relative water content and could be good candidates for further studies, which might reveal unknown genetic factors or other adaptive traits responsible for variable responses to stress.

Plant growth seems to be the most sensitive physiological process to water deficit in plants. Water availability strongly influences growth and biomass accumulation in plants predominantly through its effect on leaf, shoot and root mass accumulation (Beadle *et al.* 1993). A significant reduction in shoot and root fresh biomass was observed in most of the accessions. The dry weight of roots increased in stressed plants by relative degrees among the accessions. This increase in root dry biomass seems to an important trait to be considered for differentiating accessions on their stress tolerance. The root to shoot ratio was observed to be generally higher in stressed than in non-stressed plants, and this may be because the growth priority shifts in favour of the roots when plants are water stressed (Kalloo 1991). Under stress, some plants show rapid growth of roots into deeper soil layers that lead to improved water uptake (Larcher 2003). Water stress tolerant plants may have a greater root-shoot ratio (*viz*. MtA-2 and Nglu), although certain water stress susceptible accessions (*viz*. PPM1 and MtA-9) had high root-shoot ratios. Such difference could be an outcome of different adaptation mechanisms between the accessions. Increased root-shoot ratio occurrence in water deficit-stressed plants was also reported by Chaves *et al.* (2003), who found that an increased root/shoot ratio is a long-term response to stress. Tomato, also a solanaceous species, produced extra roots under soil water deficit conditions (Larcher 2003).

In our study, lower leaf relative water content was observed in most accessions following stress. This finding is in accordance with Ramos *et al.* (2003) and Allestrofa (2014) who reported that RWC was significantly lower in water stressed bean plants. Schonfeld *et al.* (1988) showed that wheat cultivars having high RWC are more resistant to water stress, although another study with tomato found the RWC decreased (Lazacano-Ferrat and Lovat 1999).

2.5.1 *Chlorophyll*

Chlorophyll is one of the major components of chloroplasts, and relative chlorophyll content of leaves is an indicator of the photosynthetic efficiency of plant tissues. Although there exist arguments about the contribution of chlorophyll content to growth and yield of plants under stress conditions (Allestrofa, 2014), many studies indicated that plants that stay green are associated with better yield and transpiration efficiency under water deficit environments. Previous studies showed there is a strong correlation between chlorophyll content index (CCI) and the amount of chlorophyll in annual and perennial plants. Chlorophyll content increased with time for all accessions in both stressed and well-watered plants, but at varying rates (Fig. 6). It was observed that until 5 dps, chlorophyll increased in non-watered, although as stress progressed past 5 dps, the plant's chlorophyll decreased. Nikolaeva *et al.* (2010) found similar responses in wheat where differential stress treatment showed variable influence on chlorophyll content of leaves. They found that in the first stages of stress (3 dps), the chlorophyll content increased slightly until 5 dps when the chlorophyll content decreased slightly until 7 dps where it suddenly decreased by 13–15%. Rong-hua *et al.* (2006), however, reported that water stress caused decreased levels of chlorophyll in all genotypes of barley, although the rate of decrease was less in stress tolerant genotypes than in stress susceptible ones. Similar results were reported from wheat, maize and sunflower. The decrease in chlorophyll under severe stress is mainly the result of damage to chloroplasts caused by the exposure to active oxygen species.

2.5.2. *Variations in plant stress tolerance*

Accessions showed variations in their responses to severe water deficit stress. Leaf and shoot tip wilting are the most prominent physiological responses of plants to water deficit conditions (Blum 1998) and can be considered to be an indicator of plant stress tolerance (Xu *et al.* 2008).
There is an hypothesis that domestication presents trade-offs between stress tolerance and certain plant growth traits, and thus a less stress tolerant genotype may be a result of the evolution of plants during domestication. In our experiment, the cultivated tobacco (*N. tabacum*, code Ntab) showed higher stress tolerance than most Australian species of *Nicotiana*. This discrepancy could be

explained by the geographic origin of *N. tabacum;* tobacco is thought to be originated from the tropical regions of South America.

Differences exist among the accessions within species and significant variation was observed as a function of location and as a function of a two-way interaction between species and location on water stress tolerance of native Australian *Nicotiana* plants. In general, an adaptation is a phenotypic trait which is functionally designed by past natural selection, and which explains Darwinian fitness relative to alternative features (Williams 1966). There are various studies that describe the within-species plant adaptations to their environment (Malyshev 2015). Within-species variation happens practically in every trait and in response to every environmental gradient or stress factor, just as in different species (Malyshev 2015). Creation of within-species variation via adaptation marks the early stage of speciation events, and is based on the ground that populations become more adapted to their local environment through natural selection (Hereford 2009).

In summary, it was observed that the *Nicotiana* accessions tested showed variable responses to moderate and severe water deficit conditions. Accessions Weanoo-1 (*N. umbratica*), Nglu (*N. glutinosa*), MtA-2 (*N. simulans*), MtA-5 (*N. benthamiana*) were most stress tolerant, whereas accessions PPM-1 (*N. benthamiana*), MtA-8, MtA-2 and MtA-12 (*N. simulans*) were the least. Moderate water stress tolerance is an important trait for crops because plants in most regions of the world receive at least short periods of water stress, which affects yield. Screening plants for responses to severe water deficit stress was more definitive than screening for moderate stress. Screening based on leaf wilting behaviour is sometimes uninformative because some plants show leaf wilting quickly but survive for longer periods under severe stress. In our experiments, we counted days of survival as indicated by wilting shoot tip under stress. Observation of plant survival or recovery after re-watering plants is another approach used by plant stress physiologists. This approach has some implications in agriculture where plants are subjected to water stress for a period and then recover when water is available. No single parameter was indicative of plant water stress tolerance, so it is necessary to use multiple parameters.

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Conflict of Interest Statement: The authors declare no conflicts of interest.

References

Allestrofa V (2014) Evaluation of drought adaptation of different tomato genotypes. dissertation].[Vienna]: University of Natural Resources and Life Sciences,

Barrs HD (1968) Determination of water deficits in plant tissue. In 'Water deficits and plant growth. Vol. 1. (Ed. TT Kozlowski) pp. 235-368. (Academic Press: New York).

Beadle CL, Ludlow MM, Honeysett L (1993) Water relations. In 'Photosynthesis and Production in a Changing Environment. (Eds DO Hall, JMO Scurlock, HR Bolhàr-Nordenkampf, RC Leegood and SP Long) pp. 113-127. (Chapman & Hall,: London, England).

Blum A (1998) Improving wheat grain filling under stress by stem reserve mobilization. *Euphytica* **100**, 77-83.

Bohnert HJ, Sheveleva E (1998) Plant stress adaptations-making metabolism move. *Current Opinion in Plant Biology* **1**, 267–274.

Chaves MM, Flexas J, Pinheiro C (2009) Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. *Annals of Botany* **103**, 551–560.

Chaves MM, Maroco JP, Pereira JS (2003) Understanding plant responses to drought – from genes to the whole plant. *Functional Plant Biology* **30**, 239-264.

Engelbrecht BMJ, Comita L, Condit R, Kursar TA, Tyree MT, Turner BL, Hubbell SP (2007) Drought sensitivity shapes species distribution patterns in tropical forests. *Nature* **447**, 80–83.

Garcia-Mazcorro JF, Castillo-Carranza SA, Guard B, Gomez-Vazquez JP, Dowd SE, Brigthsmith DJ (2017) Comprehensive molecular characterization of bacterial communities in feces of pet birds using 16S marker sequencing. *Microbial ecology* **73**, 224-235.

Goodin MM, Zaitlin D, Naidu RA, Lommel SA (2008) Nicotiana benthamiana: its history and future as a model for plant–pathogen interactions. *Molecular plant-microbe interactions* **21**, 1015-1026.

Hammer Ø, Harper DAT, Ryan PD (2001) PAST: paleontological statistics software package for education and data analysis. *Palaeontologia Electronica* **4**.

Hereford J (2009) A quantitative survey of local adaptation and fitness trade-offs. *The American Naturalist* **173**, 579-588.

Hirsch PR, Jhurreea D, Williams JK, Murray PJ, Scott T, Misselbrook TH, Goulding KW, Clark IM (2017) Soil resilience and recovery: rapid community responses to management changes. *Plant and Soil* **412**, 283-297.

IBM C (2012) IBM SPSS Statistics for Windows. In. ' 24 edn. (IBM Corp: Armonk, NY)

Ings J, Mur LAJ, Robson PRH, Bosch M (2013) Physiological and growth responses to water deficit in the bioenergy crop Miscanthus x giganteus. *Frontiers in Plant Science* **4**, 468.

IPCC (2007) 'Climate change 2007: the physical science basis.' (Cambridge University Press: Cambridge).

Kalloo G (1991) Breeding for environmental stress resistance in tomato. In 'Genetic Improvement of Tomato. (Ed. G Kalloo) pp. 153-165. (Springer: Berlin).

Kirkham MB (2005) 'Principles of soil and plant water relations.' (Elsevier Academic Press: Burlington, MA).

Larcher W (2003) 'Physiological Plant Ecology.' 4 edn. (SpringerVerlag: Berlin, Heidelberg, New York).

Lazacano-Ferrat I, Lovat CJ (1999) Relationship between relative water content, nitrogen pools, and growth of Phaseolus vulgaris L. and P. acutifoolius A. Gray during water deficit. *Crop Science* **39**, 467-475.

Ledbetter CA, Peterson S, Palmquist D (1996) *In vitro* osmotic tolerance of six clonally propagated *Prunus* accessions. *Journal of Genetics and Breeding* **50**, 1-6.

Malyshev AV (2015) Plant growth responses to winter climate change: from among-and within-species variation to plant-soil interactions. Universität Bayreuth,

McKay C, Filipsson H, Romero O, Stuut JB, Björck S (2016) The interplay between the surface and bottom water environment within the Benguela Upwelling System over the last 70 ka. *Paleoceanography* **31**, 266-285.

McWilliam JR (1986) The national and international importance of drought and salinity effects on agricultural production. *Australian Journal of Plant Physiology* **35**, 1–13.

Morgan JM (1984) Osmoregulation and water stress in higher plants. *Annual Review of Plant Physiology* **35**, 299-319.

Nikolaeva MK, Maevskaya SN, Shugaev AG, Bukhov NG (2010) Effect of drought on chlorophyll content and antioxidant enzyme activities in leaves of three wheat cultivars varying in productivity. *Russian Journal of Plant Physiology* **57**, 87–95.

Pereira JS, Chaves MM (1993) 'Plant water deficits in Mediterranean ecosystems.' (Bios Scientific Publications: Oxford).

R Core Team (2015) R: A language and environment for statistical computing. In. ' 3.2.3 edn. (R foundation for statistical computing: Vienna, Austria)

Ramos MLG, Parsons R, Sprent JI, Games EK (2003) Effect of water stress on nitrogen fixation and nodule structure of common bean. *Pesquisa Agropecuária Brasileira* **38**, 339-347.

Rong-hua L, Pei-po G, Baum M, Grando S, Ceccarelli S (2006) Evaluation of chlorophyll content and fluorescence parameters as indicators of drought tolerance in barley. *Agricultural Sciences in China* **5**, 751-757.

Schonfeld MA, Johnson RC, Carwer BF, Mornhinweg DW (1988) Water relations in winter wheat as drought resistance indicators. *Crop Science* **28**, 526-531.

Stata Corp L (2017) 'Stata Statistical Software Release 15.' (Stata Press Publication).

Vialet-Chabrand SR, Matthews JS, Simkin A, Raines CA, Lawson T (2017) Importance of fluctuations in light on pant photosynthetic acclimation. *Plant Physiology*, pp. 01767.2016.

Williams GC (1966) Adaptation and natrual selection: a critique of some current evolutionary thought.

Wylie SJ, Zhang C, Long V, Roossinck MJ, Koh SH, Jones MG, Iqbal S, Li H (2015) Differential responses to virus challenge of laboratory and wild accessions of Australian

species of Nicotiana, and comparative analysis of RDR1 gene sequences. *PloS one* **10**, e0121787.

Xu P, Chen F, Mannas JP, Feldman T, Sumner LW, Roossinck MJ (2008) Virus infection improves drought tolerance. *New Phytologist* **180**, 911–921.

Zaura E, Keijser BJ, Huse SM, Crielaard W (2009) Defining the healthy" core microbiome" of oral microbial communities. *BMC microbiology* **9**, 259.

Zhang C, Li H, Shober A, McGrath J Greenhouse Gas Emissions of Corn Fielded with Broilers Litter Treated by Ammonia Control Technology. In 'NABEC Papers', 2004, p. 1

Chapter 3

Host Specificity of Endophytic Mycobiota of Wild *Nicotiana* Plants from Arid Regions of Northern Australia

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FUNGAL MICROBIOLOGY

Host Specificity of Endophytic Mycobiota of Wild *Nicotiana* Plants from Arid Regions of Northern Australia

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Abstract In arid regions of northern Australia, plants survive under water deficit, high temperatures, intense solar radiation and nutrient-impoverished soils. They employ various morpho-physiological and biochemical adaptations including interaction with microbial symbionts. We evaluated identity, host and tissue association with geographical distribution of fungal endophytes isolated from above- and below-ground tissues of plants of three indigenous Australian *Nicotiana* species. Isolation frequency and α-diversity were significantly higher for root endophyte assemblages than those of stem and leaf tissues. We recorded no differences in endophyte species richness or diversity as a function of sampling location, but did detect differences among different host genotypes and plant tissues. There was a significant pattern of community similarity associated with host genotypes but no consistent pattern of fungal community structuring associated with sampling location and tissue type, regardless of the community similarity measurements used.

Keywords Arid land · Community structure · Endophyte diversity · Fungi

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Introduction

In arid lands such as those of the North-Western Australia, plants cope with drought, high temperatures, strong solar radiation, nutrient-impoverished soils and other challenges with various morpho-physiological and biochemical adaptations [1, 2]. Less well studied are the interactions of such plants with microorganisms, which may convey thermo-tolerance, drought resistance, and other benefits that increase growth, primary productivity and plant community structure in such challenging environments [3, 4]. Potential roles of fungal symbionts of plants such as mycorrhizal and non-mycorrhizal endophytes have received growing attention from researchers in the growth and survival of plants in inhospitable landscapes [5–8]. Studies have focussed on fungi associated with grasses and sedges [5, 9], gypsophilous plants [10], dicots, [11] and some cacti [12].

Endophytic fungi are a polyphyletic group of predominantly ascomycetous fungi that live within inter- and intracellular spaces for at least part of their life cycle and cause no visible symptoms of disease on the host [13]. Endophytes are often of diverse species composition [14], and their composition usually differs as a function of host species [15], among geographically separated individuals of the same host species [16], and also within the tissues or organs of a host plant [17]. Several studies reported that fungal endophytes may confer physiological [18] and ecological [19] advantages to the host, including protection from environmental stress [20] as well as from attack by pathogens [21] and pests [22]. Although long overlooked due to their cryptic nature, endophytes are increasingly being recognized for their ecological roles, especially in extreme environments [23]. Therefore, knowledge on their nature and distributions is essential both for revealing features of plant ecology and evolution in natural and managed

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ecosystems, and for developing effective survey schemes for tapping their biochemical capabilities [24].

Symbiotic relationships, between plants and fungi are thought to manifest their utmost significance under stressful conditions [23, 25] where close interaction of fungi with plants could alleviate or eliminate the effects of particular abiotic or biotic challenges. Studies of endophytic fungal symbionts associated with plants in arid and semi-arid regions increased our knowledge on the host, tissue and geographic association of endophytes of important plants [5, 9–12, 26–29]. Lower fungal diversity and higher colonization frequency was reported from some plants [12, 29], which were heavily colonized by dark septate fungi [5, 8–10, 12, 30–33]. Investigations on endophytes in arid lands that have diverse plant communities as well as experience relatively extreme conditions would provide an opportunity to assess the potential of previously unknown endophyte communities would broaden insights in endophyte-plant associations.

Little is known about the endophyte communities associated with Australian native plants, and no study has yet been done on the fungal endophytic communities of indigenous *Nicotiana* species native to the hot and arid regions of northwest Australia. *Nicotiana* is a genus in the *Solanaceae* and is divided into 13 sections comprising 86 species of which about 27 species and subspecies are indigenous to Australia [34] Some species from the region, notably *N. benthamiana* and *N. occidentalis*, are model species used in plant science laboratories internationally.

This study aimed to investigate the occurrence and species diversity of fungal endophytes in *Nicotiana* plants growing in their natural environments in arid parts of northern Australia. We also wanted to examine if endophyte varies across sampling location, host and tissue types and if endophyte community structure is influenced by location, host and tissue types.

Collection of *Nicotiana* Species

Plants of three species of indigenous *Nicotiana* species (*N. benthamiana*, *N. occidentalis* and *N. simulans*) were collected under licence from four different locations (Carnarvon, Karratha, Mount Augustus and Mount Gould) in north-western Australia. Three plants of each species were used for endophyte isolation except for the Carnarvon site where no *N. benthamiana* were available. The collection sites were chosen based on the availability and accessibility of *Nicotiana* plants. The sampling locations were characterized by arid and hot environments. Details of the collection sites are presented in Table 1.

Fungal Endophyte Isolation

Asymptomatic plant tissues (leaves, stems and roots) of *Nicotiana* plants were collected and used for endophyte isolation. Three or four fully expanded leaves were collected from shoot tip of the main stem and top two branches at about 60–70 cm above the ground, and stems were cut from the lower part of the main stem from above 10 cm off the ground. All the samples were collected over the course of 2–3 weeks. Tissues were rinsed under running tap water to remove surface debris before being subjected to surface sterilization. Samples were cut into short segments and surface sterilized by immersion in 0.5% sodium hypochlorite for 2 min for root or leaf and 3 min for stem, before immersing in 70% ethanol for 3 min. After three washes in sterile distilled water and blotting with sterile filter paper, samples were cut into 2 mm^2 segments using a sterile scalpel. There were 55 segments from each plant per tissue/host/location. Accordingly, a total of 5445 segments were incubated. To evaluate the efficacy of surface sterilization procedure, the imprint method, i.e. pressing sterilized tissue segments gently onto potato dextrose agar (PDA) was used [35] in order to confirm that the isolates only originated from the internal tissues of the segments. Also, the final water that used to wash the surface-sterilized tissues was incubated in PDA to observe any fungal growth. For the fungal isolation, segments of leaf, stem or root were plated in a petridish containing media composed of 0.1× PDA (PDB 2.4 g (Catalogue # P6685, Sigma-Aldrich, USA) plus 17 g agar in 1 L water) amended with 100 μg/mL streptomycin sulphate (Sigma, St Louis, MO, USA). Each plate contained five tissue fragments with a total of 55 fragments assayed per tissue type. Plates were incubated at 25 °C in the dark and checked periodically for fungal growth. The growing margins (hyphal tips) of colonies from tissue segments were transferred to plates of 0.25× PDA supplemented with streptomycin (100 μg/mL) [36]. The fungal isolates were numbered and stored temporarily at 4 °C and the mother culture permanently at −80 °C in 80% sterile glycerol.

Endophyte Identification

A combination of morphological and molecular techniques was employed for fungal identification due to the large number of isolates obtained [37]. Fungal endophytes from different tissues of all plant species of a particular location were grouped based on cultural characteristics such as isolate age, colony appearances and mycelial textures. We obtained 41, 43, 43 and 33 distinct morphotypes from 477, 523, 622 and 304 fungal isolates from Karratha, Mt. Augustus, Mt. Gould and Carnarvon, respectively. We obtained ITS sequence for 60 representative isolates from Carnarvon and 80 representative isolates from each of the other three locations with a total of 300 sequences out of the 1926 isolates (15.6% of the isolates recovered).

For molecular identification, fungal mycelium taken from an agar plate was inoculated into a 250-mL flask containing 100 mL PDB and incubated on a shaker for 7–21 days in the dark. At the harvest, the mycelium was rinsed under tap water

Table 1 Description of collection sites of the *Nicotiana* plants used in the present study

Sampling location	Coordinates	Altitude (m)	Average annual temperature (°C)		Average annual rainfall (mm)
			Minimum	Maximum	
Carnarvon	24° 53′ 02.40″ S 113° 39′ 39.60″ E	4	17.2	27.3	226.0
Karratha	20° 44′ 07.26″ S 116° 50′ 44.89″ E	6	20.8	32.4	298.6
Mount Augustus	24° 19′ 30.00″ S 116° 50′ 30.00″ E	500	17.2	31.8	233.8
Mt. Gould	25° 48′ 12.03″ S 117° 22′ 48.35″ E	240	14.6	30.5	247.9

and squeezed dry on a sterile paper towel and genomic DNA was extracted following a phenol/chloroform/isoamyl alcohol (24:24:1) protocol. Briefly, fungal mycelia (100 mg) were ground in liquid nitrogen and the cells were lysed in 450 μL of extraction buffer (200 mM Tris-HCl pH 7.5, 25 mM EDTA, 200 mM NaCl, 1% SDS) followed by mixing thoroughly with continuous shaking for 5 min. The lysate was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (24:24:1) and centrifuged at 20,000×*g* for 5 min, repeating this process twice. The aqueous phase was transferred to a sterile 1.5-μL microfuge tube. The genomic DNA was precipitated in an equal volume of iso-propanol at −20 °C for 15–30 min followed by centrifugation at 20,000×*g* for 15 min at 4 °C. The resulting pellet was washed twice with 70% ethanol, air dried and dissolved in 50 μL of sterile RNase-free Water or 50 μL EB buffer (10 mM Tris-Cl, pH 8.5).

The ITS regions of the fungi were amplified with the universal primers ITS4 and ITS1 or ITS1F [38, 39] using PCR. The PCR conditions used were 95 °C for 3 min followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and a final extension at 72 °C for 7 min. The 20 μL reaction mixture contained 10 μL of 2× Promega Go Taq™ Master Mix, 10 μM of each primer, 1 μL of template DNA and 7 μL water. Amplicons were purified by AxyPrep Mag PCR Clean-up kit (Axygen Biosciences, USA). Each sequencing reaction contained 1 μL of BigDye® Terminator sequence mix (Applied Biosystems, USA), 1.5 μL of 5× Sequencing Buffer, 1 μL of the forward primer (3.2 μM), 4 μL cleaned PCR amplicons and 2.5 μL water. Cycling conditions were 35 cycles of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min.

The fungal sequences were aligned as query sequences with the databases GenBank (NCBI) and UNITE [40]. The last two are specially compiled and used for fungal identification. In all cases, the isolates were identified to the species level if their sequences ≥97% similar to any identified accession from the databases analysed. When the similarity percentage was between 95 and 96%, only the genus name was accepted; and for sequence identities <95%, the isolates were classified according to family/order or as "fungal sp." [41]. The strains that ended up with same taxa were aligned using MAFFT (multiple alignment using fast Fourier transform) algorithm [42] and percent similarity was obtained using EMBL-EBI (http://www.ebi.ac.uk/Tools/msa/mafft/) platform. We used ≥97% similarity threshold to distinguish taxa that yielded same blast hit and noted them as "A" or "B" at the end of taxa name.

Analysis of culture and sequence results indicated that certain genera were isolated frequently in all types of samples such as *Aspergillus*, *Alternaria*, *Chaetomium*, *Cladosporium, Lecythophora*, *Penicillium*, *Setophom* and *Trichoderma*. We inferred the phylogenetic relationships of these endophytes obtained here in the context of currently recognized species isolated as endophytes in other studies to examine the whether endophytes of these genera reflect a single evolutionary origin. The sequences were aligned using MAFFT v7.123b [43] and trimmed to consistent start and end points. The resulting cured alignment was analysed by Bayesian inference [44] using MrBayes 3.2.2 [45] in Geneious 8.1.8 [46] for 5 million generations, with sampling every 1000th generation. A majority-rule consensus tree was inferred from 500 trees from the posterior, with support defined by Bayesian posterior probabilities.

Observation of Endophyte Colonization of *Nicotiana* Seedlings

Seeds of *N. benthamiana* (laboratory accession RA4 [47]) were surface sterilized using 0.5% sodium hypochloride for 2 min. The seeds were washed three times in sterile distilled water, blot dried onto sterilized filter paper and pre-germinated on moist filter paper for 2 days at 25 °C. Selected fungal strains were grown on petridish containing 0.25× PDA medium for 3 days at 25 °C in the dark. Pre-germinated (2 days) seeds were placed on the growing margin of fungi and incubated in growth chamber with photoperiod of 16 h:8 h (light/dark) at 23 °C. Control seeds were placed on the same medium without fungi. Seven to 10 days after inoculation, seedlings, roots and stems were separated and surface sterilized in 0.5% sodium

hypochloride for 1 min and washed in sterile water. Microscopic observation of colonization was done using trypan blue staining method as described by [48] with some modifications. The tissues were cut into 1 × 1 cm^2 segments, cleared with acetic acid/ethanol (1:3, *v*/*v*) solution overnight for the first time. Then a second clearing was done using in an acetic acid/ethanol/glycerol (1:5:1, *v*/*v*/*v*) solution for at least 2 h. The samples were subsequently incubated for at least 5 h to overnight in a staining solution of 0.01% (*w*/*v*) trypan blue in lactophenol. Then the stained samples were rinsed with 60% sterile and stored in the same until examination. Specimens were transferred onto microscopic slides and examined under a compound microscope. Five to six segments were assessed per fungal inoculation. Specimens were examined under an Olympus BX 51 optical microscope (Olympus, Japan).

Isolation Frequency

Isolation frequency (IF) was defined as the percentage of tissue fragments yielding an endophyte in culture. We used generalized linear models to observe variation in isolation frequency from as a function of sampling location, host genotype and tissue type. The final model used the Pearson chi-square as scale parameter method and incorporated all main effects and relevant interaction terms (Table 2). Analyses were performed in SPSS 22 (IBM Corp. Armonk, NY).

Diversity and Community Composition

The species accumulation curves of fungi in root, stems and leaves were estimated by individual based rarefaction curve with using Coleman rarefaction and Chao1 estimator [49, 50]. Diversity measures used were the Shannon-Wiener diversity index (*H′*), Fisher's alpha diversity, evenness (H/S), Menhinick index and Simpson dominance. These were used to measure endophyte species diversity within samples (α-diversity). Since the results were consistent in all cases, only the Shannon-Wiener biodiversity, the most commonly used index, are shown in the index (*H′*). An ANOVA test was performed to compare *H′* values across locations, host species and tissue (Table 3). We used a Shapiro-Wilk test before analysis to ensure that the distribution of *H′* values did not vary significantly from normal.

We compared differences in community composition and structure among hosts, tissues and locations (β-diversity) using analysis of similarity (ANOSIM). Use of three different pairwise similarity measures was attempted because they take in account different types of information that can provide different insights for comparing communities [51]. We calculated (i) the Jaccard's index, which considers only the presence or absence of fungal taxa among samples; (ii) the Bray-Curtis coefficient, which considers the abundance of taxa along with the presence or absence of particular taxa and (iii) the Morisita index, another abundance-based measure, is also a highly suggested measure because of its relative independence from size and diversity of sample [52].

Matrices obtained from pairwise similarity measures of endophyte communities were visualized using non-metric multidimensional scale (NMDS). Multidimensional scaling is intended to graphically present interactions between objects in multidimensional space. NMDS is a robust visual analysis method applicable to various data types, and it is amenable to a number of user-defined standardizations and transformations of the data and flexible with respect to which dissimilarity or similarity measure is used [53]. NMDS plots use rank-order information in a dissimilarity matrix [54]. A test statistic *R* was calculated as the difference of mean ranks between vs. within groups. Significance was calculated by 10,000 permutations of group membership. The diversity measures (α-diversity and β-diversity including NMDS plots) analyses and species accumulation curves were estimated using PAST ver. 3.15 [55].

Table 2 Results of generalized linear models for analyses of isolation frequency of indigenous Australian *Nicotiana* plants. Whole-model test: chi-square = 792.4, DF = 32, $p < 0.0001$, AICc = 3473.86

Source of variation	Chi-square	DF	*p*
Location	32.357	3	0.000***
Host	10.615	2	0.005**
Tissue	660.851	2	0.000***
Location × host	21.958	5	0.001**
Location × tissue	19.534	6	0.003**
Host × tissue	6.074	4	0.194
Location × host × tissue	8.772	10	0.554

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

Results

Isolation and Identification of Endophytes

The absence of fungal or bacterial growth in the tissue imprints onto the PDA media indicated that surface sterilization protocol was effective enough to eliminate epiphytic fungi. A total of 1926 isolates of fungal endophytes were recovered from 5445 tissue segments representing root, stem and tissues of *N. occidentalis*, *N. benthamiana* and *N. simulans* plants from four sampling locations (isolation frequency = 35.4%). Here, an isolates means a visible fungal growth from a 2- m m ^{2} segment/organ/plant/location. Seedlings inoculated with certain fungal strains were examined under a light microscopic for evidence of endophytic colonization. Stained tissues highlighted the presence of fungal hyphae, most of which

Table 3 Results of ANOVA of Shannon-Wiener diversity of indigenous Australian *Nicotiana* plants

Source of variation	Mean square	DF	Mean square	*F*	*p*
Host	0.113	2	0.113	3.002	0.057
Tissue	4.875	2	4.875	129.390	0.000***
Location	0.047	3	0.047	1.240	0.302
Host × tissue	0.075	4	0.075	1.979	0.108
Host × location	0.119	5	0.119	3.171	0.013*
Tissue × location	0.021	6	0.021	0.560	0.761
Host × tissue × location	0.034	10	0.034	0.914	0.526

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

penetrated the intercellular spaces of the root and stem tissues (Figs. 1 and 2).

The generalized linear model analysis (SPSS 21.0, IBM North America, NY, USA) of isolation frequencies indicated that the number of isolates recovered varied significantly as a function of sampling location, host species and tissue types (Table 2). In particular, endophytes were isolated in culture about 3.3 and 2.3 times more frequently from root tissue (IF = 60.8%) than from stem and leaf tissue overall (from all host and location), respectively (chi-square = 660.85, DF = 2, $p < 0.001$). In addition, isolation frequency also differed as function of two-way interaction between location × host (chi-square = 21.95, DF = 5, $p < 0.01$) and location × tissue (chi-square = 19.53, DF = 6, $p < 0.01$) but not as a function of host × tissue types or as a function of three-way interaction (location × host × tissue types) (Table 2, Fig. 3).

In aggregate across all regions, 300 representative fungal isolates were selected for molecular identification based on morphological characteristics in cultures. Molecular identification using ITS rDNA sequences resulted in a total of 68 different endophytic fungal taxa that belonged to 40 different genera with few taxa not identified to genus level. All endophytes recovered from *Nicotiana* spp. were members of the Dikarya. Ninety-eight percent of endophyte isolates recovered here represented the

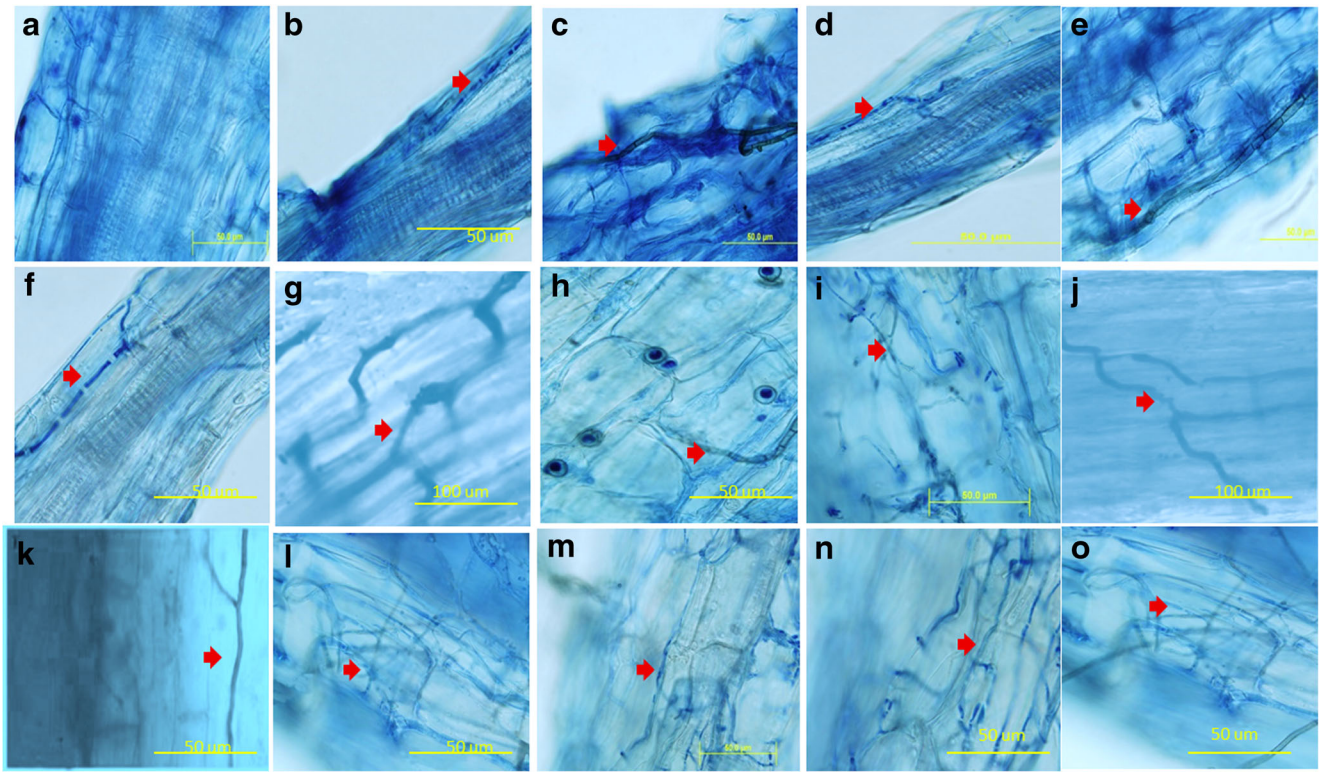


Fig. 1 Detection of endophytic fungal structures between and within root cells in *Nicotiana benthamiana* observed in longitudinal section under light microscopy of **a** non-colonized and colonized by fungal strain, **b** E-210.1 (*Acremonium* sp.), **c** E-141 (Ascomycota sp.-A), **d** E-529 (*A. fumigatiaffinis*), **e** *Chaetomium* sp.-B, **f** E-284 (Ascomycota sp.-B), **g** E-470 (*P. cucumerina*), **h** E-162 (*C. cladosporioides*), **i** E-145 (*A. ochraceus*), **j** E-14 (*Lecythophora* sp.-A), **k** E-152 (*Thozetella* sp.), **l** E-172.1 (*P. simplicissimum*), **m** E-503 (*A. quadrilineatus*), **n** E-163 (*Setophoma* sp.), **o** E-133 (*Zopfiella latipes*)

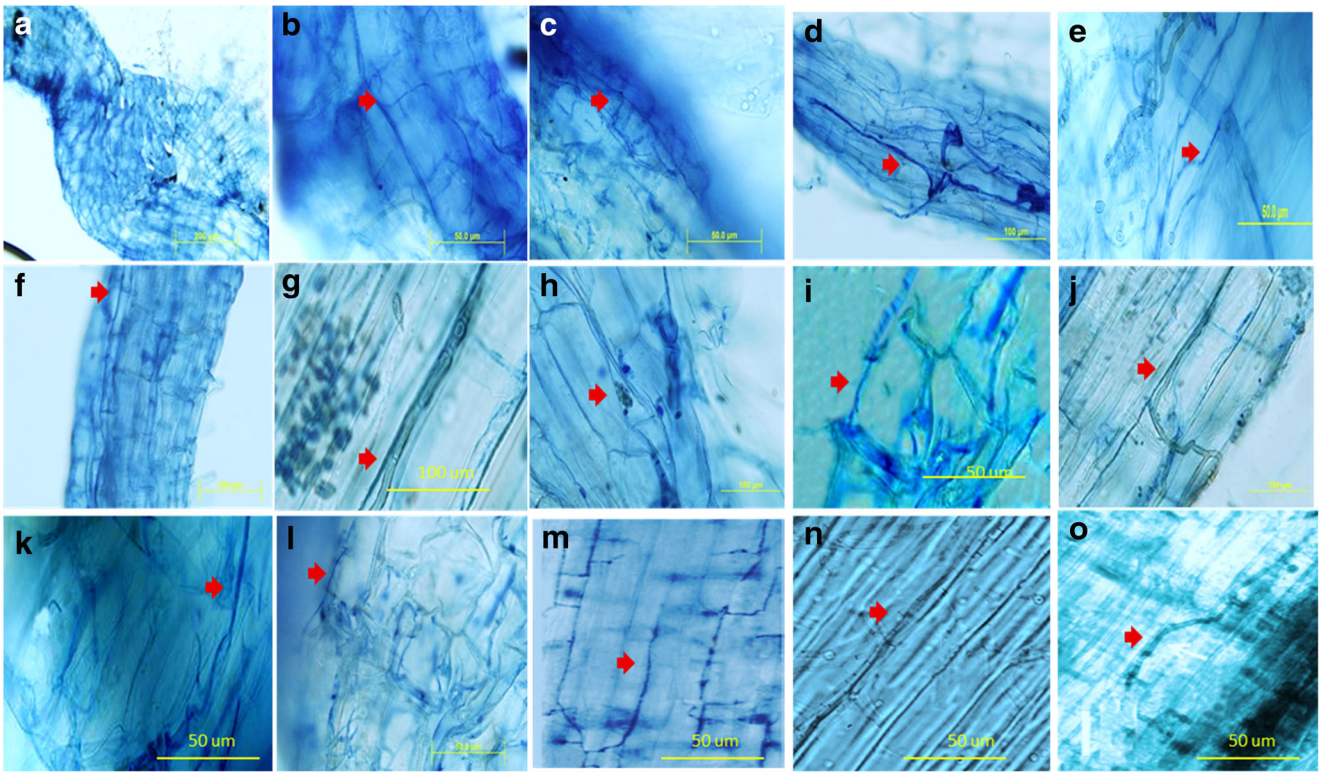


Fig. 2 Detection of endophytic fungal structures between and within stem cells in *Nicotiana benthamiana* observed in longitudinal section under light microscopy of **a** non-colonized and colonized by fungal strain, **b** E-210.1 (*Acremonium* sp.), **c** E-141 (Ascomycota sp.-A), **d** E-529 (*A. fumigatiaffinis*), **e** *Chaetomium* sp.-B, **f** E-284 (Ascomycota sp.-B, **g** E-470 (*P. cucumerina*), **h** E-162 (*C. cladosporioides*), **i** E-145 (*A. ochraceus*), **j** E-14 (*Lecythophora* sp.-A), **k** E-152 (*Thozetella* sp.), **l** E-172.1 (*P. simplicissimum*), **m** E-503 (*A. quadrilineatus*), **n** E-163 (*Setophoma* sp.), **o** E-133 (*Zopfiella latipes*)

Ascomycota and were distributed in four classes of the Pezizomycotina. The majority represented the Pleosporales (26%), Eurotiales (19%) and Hypocreales (18%) (Fig. 4). Overall, the most dominant fungal genus was *Penicillium* (8.68%), whereas the most dominant genera isolated from *N. benthamiana*, *N. occidentalis* and *N. simulans* were species

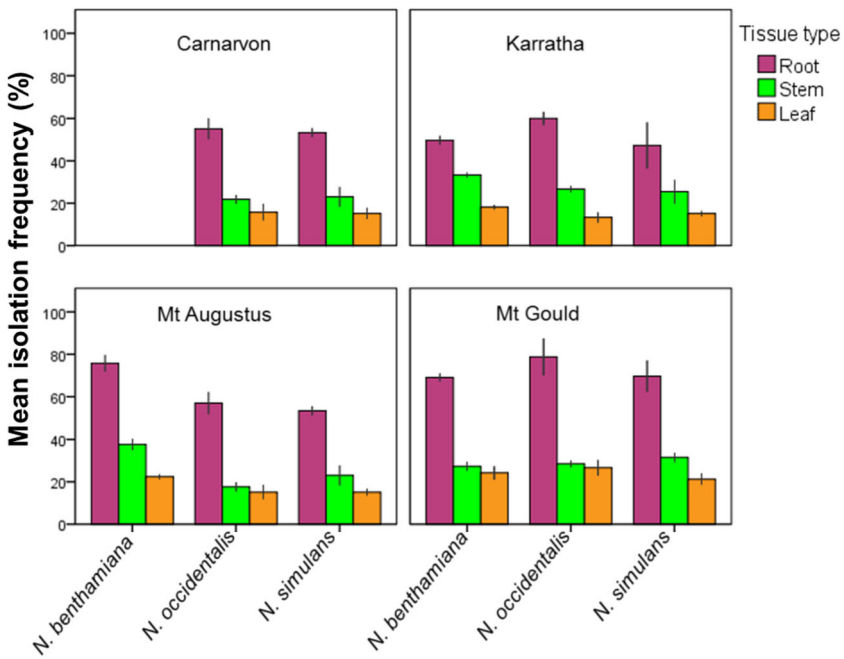


Fig. 3 Isolation frequency of fungal endophytes expressed as the mean percentage (%). Data indicate the percentage of different tissue fragments of three indigenous Australian *Nicotiana* species that yielded endophytes in culture, as a function of sampling location (Carnarvon, Karratha, Mt. Augustus and Mt. Gould), and host species (*N. benthamiana*, *N. occidentalis*, *N. simulans*)

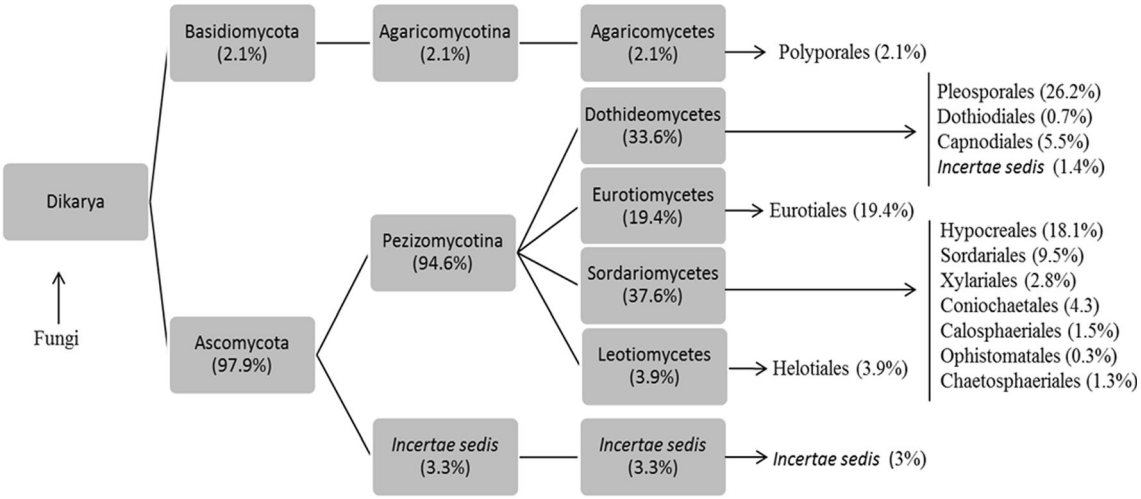


Fig. 4 Schematic representation of phylogenetic placement of 68 fungal taxa identified from ITS sequences of endophytes isolated from *Nicotiana* plants from different locations of Australia. Classification follows Hibbett et al. (2007). The genotypes represent the crown fungal group Dikarya (Ascomycota plus Basidiomycota). Isolates were distributed in only one subphylum of each of the Ascomycota (Pezizomycotina) and Basidiomycota. Percentages indicate the total number of isolates obtained out of a total of 1926 isolates for indicated group

of *Cladosporium*, *Chaetomium* and *Penicillium*, respectively. Members of four genera viz. *Alternaria*, *Aspergillus*, *Lecythophora* and *Penicillium* were recorded from plants of all four *Nicotiana* species tested (Fig. 5).

Diversity and Species Richness of Endophyte Communities (α-Diversity)

The diversity of each endophytic fungal community was assessed in relation to sampling location, host species and tissue type. Regarding the intensity of sampling, individual based rarefaction curve of fungal taxa accumulation curves became asymptotic in most tissue types regardless of the host species and sampling location except in some cases signifying that our sampling was adequate enough to obtain rare fungal taxa (Supplementary Figs. 1, 2 and 3). Thus, it provided a basis for the analyses as presented below.

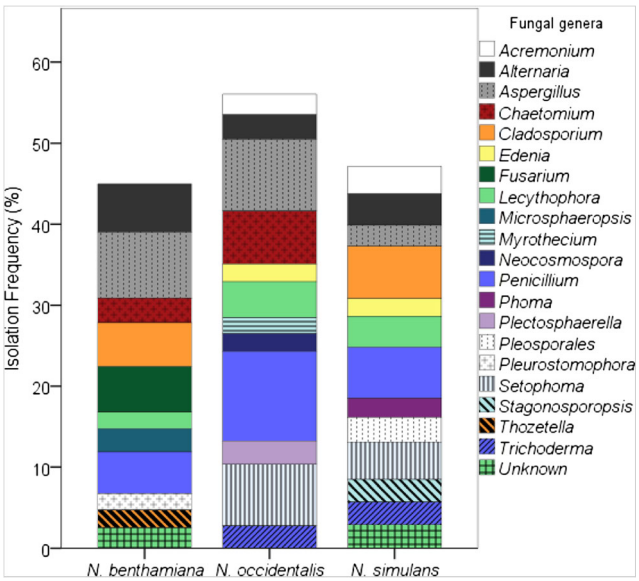


Fig. 5 Isolation frequency of dominant fungal genera (>2%) recovered from different *Nicotiana* species. Isolation frequency (%) indicates the total number of isolates obtained out of a total of 1926 isolates for indicated genera

The Shannon-Wiener diversity index (*H'*) value was used to compare diversity across sampling location, host species and tissue types. Diversity of the endophyte communities did not differ significantly as a function of sampling location and host species type but their interaction did. There were clear differences in fungal diversity among the tissue types where the *H'* value had a range from 2.61 ± 0.17 (root) to 1.84 ± 0.22 (Fig. 6, Table 3). Difference in diversity was evident neither for a host × tissue and location × tissue interactions nor for the location × host × tissue type interaction (Table 3).

Variation in Endophyte Communities Across Location, Host and Tissue (β-Diversity)

Ordination analysis was performed to investigate patterns of endophyte assemblages on various host species, host organs and locations of the plants. Two-dimensional non-metric multidimensional scale (NMDS) and ANOSIM-based clustering of fungal communities revealed that host species had a significant effect on the structuring of endophyte communities at the sampling sites (Fig. 7). The ANOSIM statistic *R* ranged from 0.35 to 0.60, indicating a dissimilarity of the endophyte communities among the host species and that the endophyte communities at particular locations appeared to be specific to a particular host. On the other hand, endophyte communities did not vary among different tissue types at various sampling sites (Fig. 8). However, in the Mount Gould community,

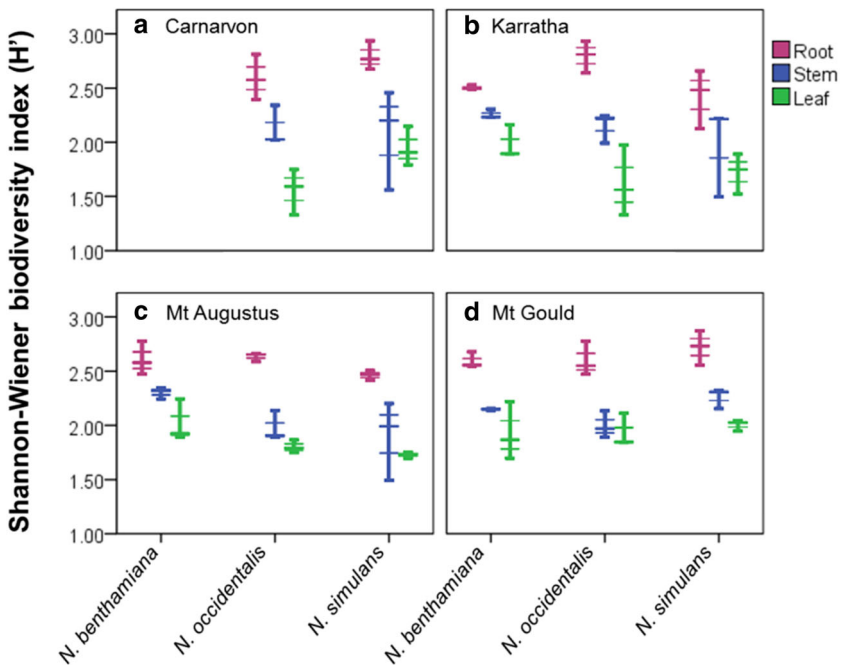


Fig. 6 Fungal endophyte biodiversity analysis. The effects of host species and tissue types on fungal endophyte biodiversity as measured by the Shannon-Wiener index (*H'*). Endophytes were isolated from root, stem and leaf tissues of different *Nicotiana* species sampled at four different sampling locations: **a** Carnarvon, **b** Karratha, **c** Mt. Augustus and **d** Mt. Gould

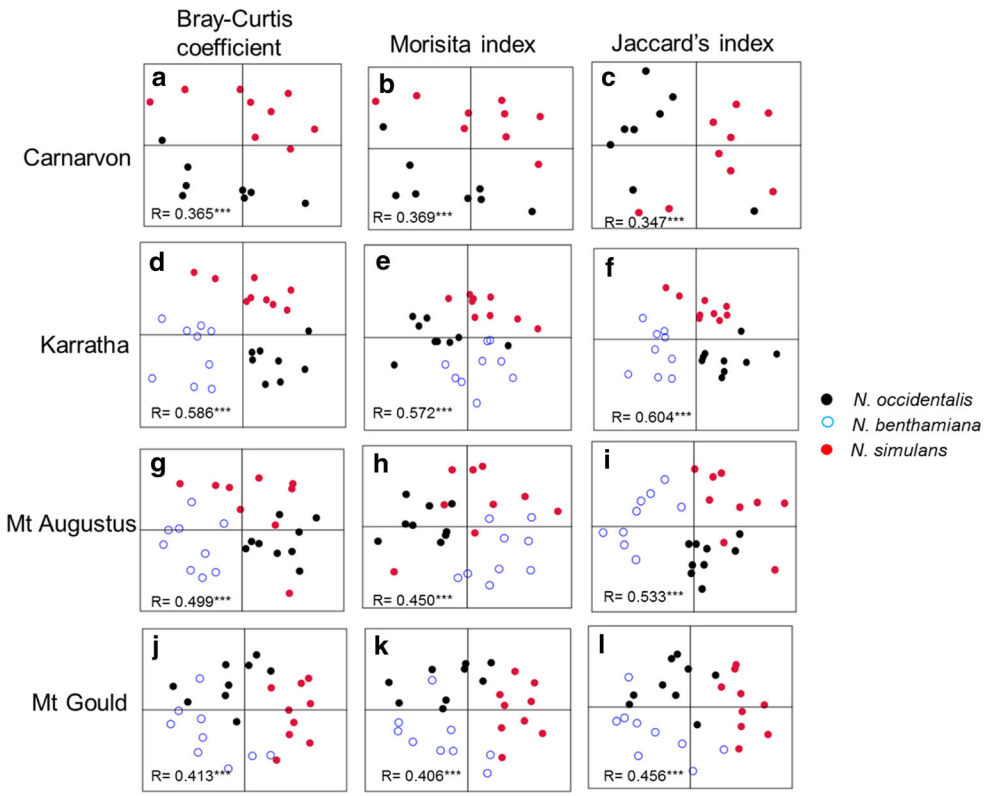


Fig. 7 Fungal endophyte communities as influenced by host species. Non-metric multidimensional scaling (NMDS) and ANOSIM analysis of endophyte communities recovered three *Nicotiana* species as indicated by colours at four locations. For clustering analysis, three different community similarity indices were computed: **a**, **d**, **g**, **j** the Bray-Curtis coefficient and **b**, **e**, **h**, **k** Morisita index both of which compares fungal taxa presence or absence along with the abundance among groups and **c**, **f**, **i**, **l** the Jaccard's index uses only presence and absence data for comparing fungal community similarity among groups. The ANOSIM statistic *R* values; large positive *R* (up to 1) signifies dissimilarity between groups. The *triple asterisk* represents the significance of dissimilarity (*R*) at $p < 0.001$ obtained by permutation of group membership, with 9999 replicates

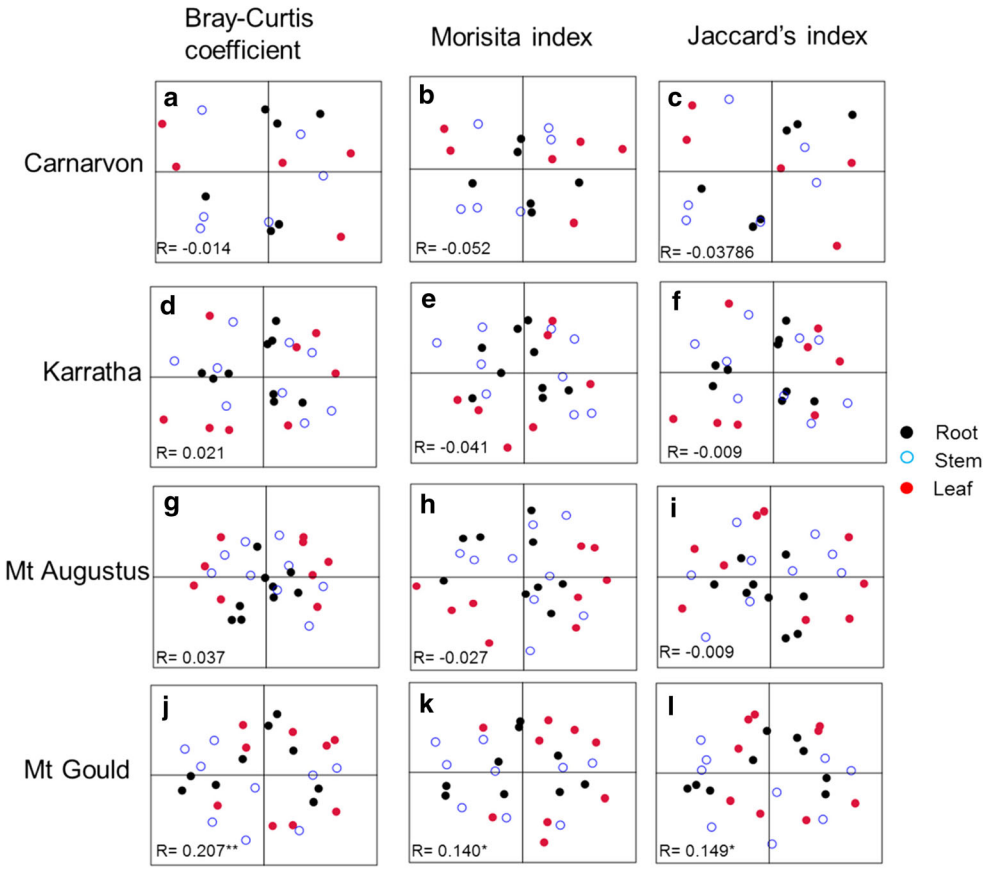


Fig. 8 Fungal endophyte communities as influenced by tissue types. Non-metric multidimensional scaling (NMDS) and ANOSIM analysis of endophyte communities recovered from root, stem and leaf tissues as indicated by different colours from three *Nicotiana* species at four sampling location. For clustering analysis, three different community similarity indices were computed: **a**, **d**, **g**, **j** the Bray-Curtis coefficient and **b**, **e**, **h**, **k** the Morisita index both of which compares fungal taxa presence or absence along with the abundance among groups and **c**, **f**, **i**, **l** the Jaccard's index uses only presence and absence data for comparing fungal community similarity among groups. The ANOSIM statistic *R* values; large positive *R* (up to 1) signifies dissimilarity between groups. The *single asterisk* and *double asterisk* represent the significance of dissimilarity (*R*) at $p < 0.05$ and $p < 0.01$ respectively, obtained by permutation of group membership, with 9999 replicates

structuring by tissue type was apparent, although the *R* value was very low (~0.2) (Fig. 8j–l). Various ecological similarity indices did show noticeable differences in luster formation in all cases.

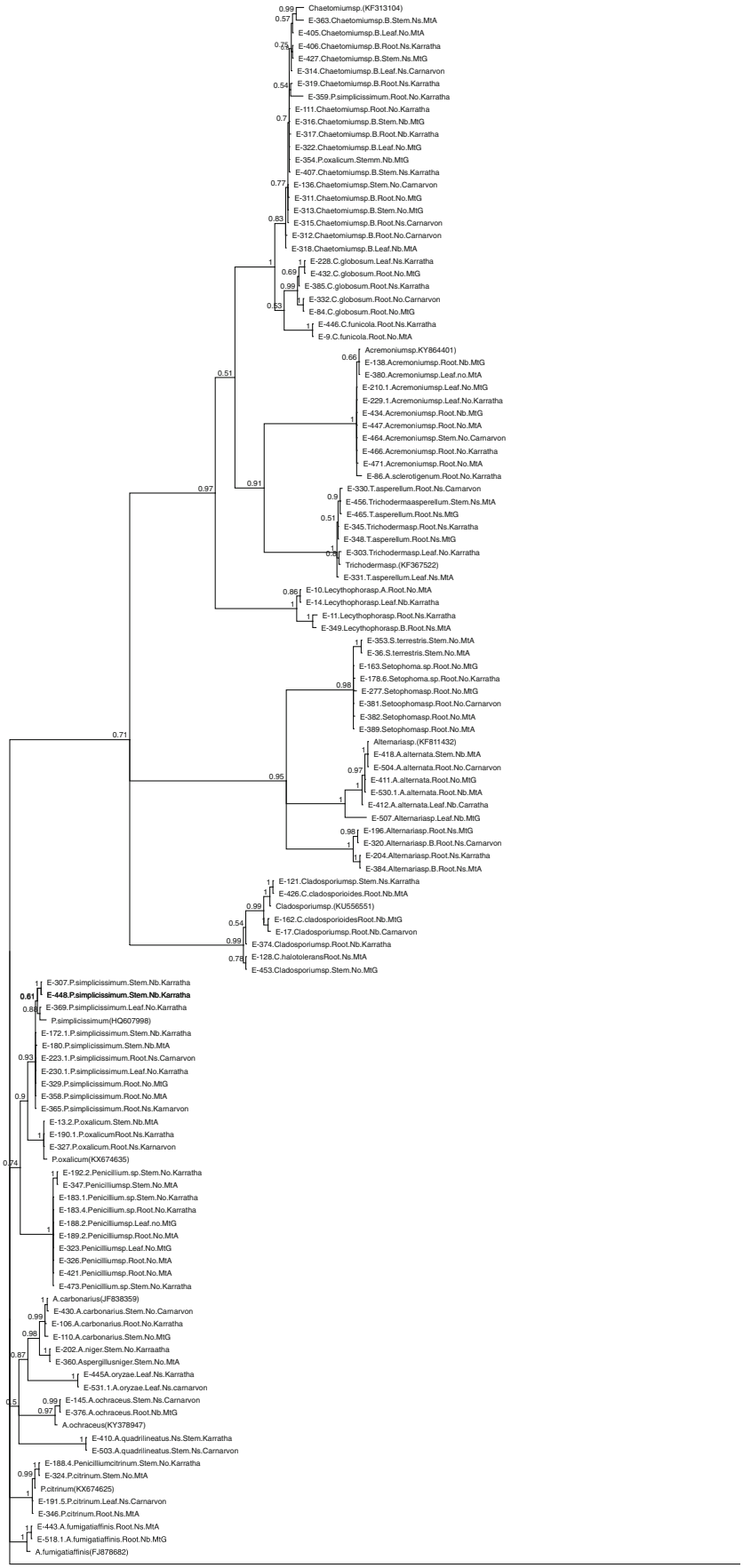
An attempt was made to determine the influence of a particular factor on structuring fungal communities by plotting an overall NMDS plot for each location and host and tissue types obtained by clustering fungal communities of particular location, host and tissue types, respectively. As predicted, host had a significant effect but the sampling location did not (Suppl. Fig. 3 A, B). However, the tissue types did show a degree of specificity in overall clustering. The specificity (although very low) of tissue-communities in Mt. Gould could affect this overall prediction (Suppl. Fig. 3C). However, tissue specificity of fungal communities was not consistent as depicted in (Fig. 8j–l).

Phylogenetic Analyses

Phylogenetic analyses of the most abundant genera revealed low phylogenetic richness endophytes of the Australian *Nicotiana* species within these genera (Fig. 9). Further, most endophytes of particular genera associated with the plants surveyed here mostly form a single monophyletic group along with other known endophyte taxa from other plants. Consistent with the community-level analysis, no phylogenetic pattern was evident with regard to the tissue, or sampling site from which the isolates was recovered (Fig. 9). Although, community analyses revealed a strong effect of host species, endophytes of the most frequent isolated genera did not show a pattern in terms of host affiliations (Fig. 9).

Discussion

Microbial symbionts of plants including endophytes are increasingly recognized for their potential in agriculture and biotechnology [20, 36, 56]. Fungal endophyte communities have been recorded from a broad range of wild and agricultural plants. The object of our current study was to examine for the first time the composition, biodiversity, spatial variation


Chaetomiumsp.(KF313104)
E-363.Chaetomiumsp.B.Stem.Ns.MtA
E-405.Chaetomiumsp.B.Leaf.No.MtA
E-406.Chaetomiumsp.B.Root.Ns.Karratha
E-427.Chaetomiumsp.B.Stem.Ns.MtG
E-314.Chaetomiumsp.B.Leaf.Ns.Carnarvon
E-319.Chaetomiumsp.B.Root.Ns.Karratha
E-359.P.simplicissimum.Root.No.Karratha
E-111.Chaetomiumsp.Root.No.Karratha
E-316.Chaetomiumsp.B.Stem.Nb.MtG
E-317.Chaetomiumsp.B.Root.Nb.Karratha
E-322.Chaetomiumsp.B.Leaf.No.MtG
E-354.P.oxalicum.Stemm.Nb.MtG
E-407.Chaetomiumsp.B.Stem.Ns.Karratha
E-136.Chaetomiumsp.Stem.No.Carnarvon
E-311.Chaetomiumsp.B.Root.No.MtG
E-313.Chaetomiumsp.B.Stem.No.MtG
E-315.Chaetomiumsp.B.Root.Ns.Carnarvon
E-312.Chaetomiumsp.B.Root.No.Carnarvon
E-318.Chaetomiumsp.B.Leaf.Nb.MtA
E-228.C.globosum.Leaf.Ns.Karratha
E-432.C.globosum.Root.No.MtG
E-385.C.globosum.Root.Ns.Karratha
E-332.C.globosum.Root.No.Carnarvon
E-84.C.globosum.Root.No.MtG
E-446.C.funicola.Root.Ns.Karratha
E-9.C.funicola.Root.No.MtA
Acremoniumsp.KY864401)
E-138.Acremoniumsp.Root.Nb.MtG
E-380.Acremoniumsp.Leaf.no.MtA
E-210.1.Acremoniumsp.Leaf.No.MtG
E-229.1.Acremoniumsp.Leaf.No.Karratha
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E-466.Acremoniumsp.Root.No.Karratha
E-471.Acremoniumsp.Root.No.MtA
E-86.A.sclerotigenum.Root.No.Karratha
E-330.T.asperellum.Root.Ns.Carnarvon
E-456.Trichodermaasperellum.Stem.Ns.MtA
E-465.T.asperellum.Root.Ns.MtG
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E-303.Trichodermasp.Leaf.No.Karratha
Trichodermasp.(KF367522)
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E-14.Lecythophorasp.Leaf.Nb.Karratha
E-11.Lecythophorasp.Root.Ns.Karratha
E-349.Lecythophorasp.B.Root.Ns.MtA
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E-36.S.terrestris.Stem.No.MtA
E-163.Setophoma.sp.Root.No.MtG
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E-382.Setophomasp.Root.No.MtA
E-389.Setophomasp.Root.No.MtA
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E-418.A.alternata.Stem.Nb.MtA
E-504.A.alternata.Root.No.Carnarvon
E-411.A.alternata.Root.No.MtG
E-530.1.A.alternata.Root.Nb.MtA
E-412.A.alternata.Leaf.Nb.Carratha
E-507.Alternariasp.Leaf.Nb.MtG
E-196.Alternariasp.Root.Ns.MtG
E-320.Alternariasp.B.Root.Ns.Carnarvon
E-204.Alternariasp.Root.Ns.Karratha
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E-426.C.cladosporioides.Root.Nb.MtA
Cladosporiumsp.(KU556551)
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E-374.Cladosporiumsp.Root.Nb.Karratha
E-128.C.halotoleransRoot.Ns.MtA
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E-448.P.simplicissimum.Stem.Nb.Karratha
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P.simplicissimum(HQ607998)
E-172.1.P.simplicissimum.Stem.Nb.Karratha
E-180.P.simplicissimum.Stem.Nb.MtA
E-223.1.P.simplicissimum.Root.Ns.Carnarvon
E-230.1.P.simplicissimum.Leaf.No.Karratha
E-329.P.simplicissimum.Root.No.MtG
E-358.P.simplicissimum.Root.No.MtA
E-365.P.simplicissimum.Root.Ns.Karnarvon
E-13.2.P.oxalicum.Stem.Nb.MtA
E-190.1.P.oxalicumRoot.Ns.Karratha
E-327.P.oxalicum.Root.Ns.Karnarvon
P.oxalicum(KX674635)
E-192.2.Penicillium.sp.Stem.No.Karratha
E-347.Penicilliumsp.Stem.No.MtA
E-183.1.Penicillium.sp.Stem.No.Karratha
E-183.4.Penicillium.sp.Root.No.Karratha
E-188.2.Penicilliumsp.Leaf.no.MtG
E-189.2.Penicilliumsp.Root.No.MtA
E-323.Penicilliumsp.Leaf.No.MtG
E-326.Penicilliumsp.Root.No.MtA
E-421.Penicilliumsp.Root.No.MtA
E-473.Penicillium.sp.Stem.No.Karratha
A.carbonarius(JF838359)
E-430.A.carbonarius.Stem.No.Carnarvon
E-106.A.carbonarius.Root.No.Karratha
E-110.A.carbonarius.Stem.No.MtG
E-202.A.niger.Stem.No.Karraatha
E-360.Aspergillusniger.Stem.No.MtA
E-445A.oryzae.Leaf.Ns.Karratha
E-531.1.A.oryzae.Leaf.Ns.carnarvon
E-145.A.ochraceus.Stem.Ns.Carnarvon
E-376.A.ochraceus.Root.Nb.MtG
A.ochraceus(KY378947)
E-410.A.quadrilineatus.Ns.Stem.Karratha
E-503.A.quadrilineatus.Stem.Ns.Carnarvon
E-188.4.Penicilliumcitrinum.Stem.No.Karratha
E-324.P.citrinum.Stem.No.MtA
P.citrinum(KX674625)
E-191.5.P.citrinum.Leaf.Ns.Carnarvon
E-346.P.citrinum.Root.Ns.MtA
E-443.A.fumigatiaffinis.Root.Ns.MtA
E-518.1.A.fumigatiaffinis.Root.Nb.MtG
A.fumigatiaffinis(FJ878682)

◀ **Fig. 9** Phylogenetic analysis of the nine most frequently isolated endophyte genera obtained from wild Australian *Nicotiana* plants. Taxa are annotated to indicate isolate number, taxon name, tissue of origin, host site, and sampling location. Taxa with accession number indicate these sequences were obtained from GenBank. The tree depicts the results of MrBayes analysis of ITSrDNA data; support values are Bayesian posterior probabilities

and host affiliation of the *Nicotiana* plants, studied as representative of indigenous Australian plans collected from hot and arid regions. The present study compared root, leaf and stem fungal endophytes obtained from plants in their native habitat. The resulting data set will augment the available knowledge on root and shoot associated fungal endophytes of plants in deserts and grasslands [6, 8, 9, 29, 57].

The value of studies on endophytes is dependent on the methods used [58]. The identity and isolation efficiency can be affected by various experimental factors that, in turn, may affect the comparability datasets obtained. Our surface sterilization protocol was sufficient to eliminate fungal epiphytes and bacteria, as shown by the absence of microbial growth on control media following tissue imprints of sterilized tissue fragments [59]. Moreover, our use of PDA might also have affected the isolation if it was unsuitable for the growth of certain taxa. Given that the same sterilization protocol, media and growth conditions were applied to all samples in this study, our comparisons of communities across location, host and tissue type are expected to be unaffected by any bias related to the specific fungal isolation methods. Problems, however, remain in the identification of culturable endophytes using ITS region-based approaches [60]. There is no assurance that the sequences listed on GenBank have always been correctly identified [61]. Given these limitations, ITS sequence analysis is still the most widely used method of fungus identification, which is why the current study adopted this approach of identification. The names of taxa were proposed on the basis of maximum similarity with curated database, UNITE [40] (≥97% similarity threshold applied), as was done in other studies [62, 63].

Composition of Endophytes

On average, endophytes were isolated in culture from 35% of the tissue segments plated. We found that endophytes associated with stems and leaves were isolated roughly two- to threefold less frequently than those with root tissues. Various factors could influence isolation frequency from particular tissues. Low endophyte isolation frequency of above-ground tissues has been reported to be correlated with UV radiation and atmospheric aridity [59, 64].

The DNA sequence data revealed 68 distinct fungal taxa of which the majority belonged to the Ascomycota (98%). The predominance of Ascomycota seems to be characteristic of endophytic mycota identified from other plant species [65, 66]. Basidiomycota have also been reported as endophytes in many plant species, although, as we found, in lower numbers and frequencies [67]. The dominant endophytic fungi identified here contained several isolates of the genera *Acremonium*, *Aspergillus*, *Penicillium*, *Chaetomium*, *Cladosporium*, *Fusarium*, *Phoma* and *Trichoderma*; most of them represent common saprophytes or plant pathogens. However, they were also reported to be the plant symbionts and some of them are playing significant roles in plant growth, nutrient uptake, tolerance to harsh environments, defence against pathogens and herbivores [33, 56, 65, 68–80]. Our results are in accordance with a previous study by Spurr and Welty [81, 82] who reported the frequent occurrence of species of *Alternaria* sp., *Penicillium* sp., *Aspergillus* sp. and *Cladosporium* sp. from *N. rustica*, *N. glutinosa* and *N. sylvestris*. In one study, it was reported that Australian native *Gossypium* (cotton) species harbour similar genera as those identified in our study [63]. Zhou et al. [83] isolated 38 endophytic fungi from the healthy roots and stems of tobacco (*N. tabacum*) in China, of which dominant genera were *Acremonium*, *Fusarium*, *Plectosphaerella*, *Penicillium* and *Cladosporium*. They also identified the genera such as *Clonostachys*, *Ilyonectria*, *Mortierella*, *Myriodontium*, *Petriella*, *Podospora*, *Purpureocillium*, *Rhizopycnis*, *Stephanonectria* and *Thielavia*; all genera were absent from the plants studied here. This indicates that *Nicotiana* species worldwide may be colonized by a large range of endophytic fungi.

Diversity and Structuring of the Endophyte Community

Endophytic composition and diversity can be influenced by several factors such as host genotype, tissue origin, local environmental conditions, nutrient availability and interactions with soil fungi and bacteria [49, 84]. Therefore, we wanted to examine if endophyte diversity would exhibit any variation across sampling location, host and tissue types. The Shannon-Wiener diversity index (*H'*) values, which considers both richness (the number of species) and evenness (relative abundance) of the individuals present in a sample [49] were not significantly different among the locations sampled and also among the host genotypes, but we did observe a difference for the location × host interaction, implying that both host genotype and geography together have impacts on diversity of fungal communities (Table 3).

As with isolation frequency, the diversity of culturable, horizontally transmitted endophytes in above-ground plant parts is often negatively correlated with factors including UV radiation and aridity [59, 64]. We tested to see if endophytes occupying stem and leaf tissues of these plants show lower diversity than root tissue. Our findings indeed suggest that fungal community diversity of stem and leaf was much lower than the root tissue (Table 3). Our results agree with

previous studies showing there were differences in endophyte diversity among various host tissues [17, 33].

Within a particular sampling location, we sought to examine for an effect of host and tissue types on endophyte community structuring by comparing three different indices of community similarity (β-diversity), each of which conveys different information and explanation. The Bray-Curtis coefficient uses quantitative species abundance data while Jaccard's similarity measure takes into account only binary presence-absence data [51, 85]. Another abundance-based measure, the Morisita index, though not as widely used as the Bray-Curtis, was used because of its relative independence from size and diversity of sample [52].

Irrespective of the community similarity index employed, we observed an obvious clustering of endophyte communities associated with different *Nicotiana* species at any of the sampled localities (Fig. 5). It implied that endophyte community structure can be highly influenced by host genotypes.

Regarding tissue types, we did not observe any clustering at any sampling sites except in Mt. Gould (Fig. 6) indicating a neutral effects of tissue types on endophyte community structuring at any particular location.

We also wanted to test for regional variation in endophyte community assemblage across all locations by using pooled taxa across host within each location to make a single community for comparisons across sampling locations. The species accumulation curves obtained to assess sampling intensity (Supplementary Figs. 1 and 2) showed that our sampling was adequate enough to recover rare fungal taxa irrespective of the host and tissue being assessed. Since we could not sample *N. benthamiana* from Carnarvon, we did not include this host species in our regional analysis. Assessing the whole endophyte community assemblage across all location did not demonstrate any strong pattern of similarity indicating that spatial position did not have strong role in fungal endophyte community structuring. These results are at par with one of the few studies that specifically investigate the spatial turnover in local community composition [86]. In that study, endophyte communities in tropical ferns were not strongly related to spatial position. In contrast, Higgins et al. [87] found that endophyte community similarity reduced significantly over distances as short as 1 km in tropical forest grasses.

It should be noted that our inferences have limitations because we focussed only on cultivable fungi, and specifically on those that could be cultured on PDA, we are likely to have underestimated the numbers of fungi present in our samples. Previous research indicates that plants harbour many species of endophytes including other microbiomes that remain undetected in culture-based approaches, but can be detected in culture-independent approaches [88]. Such tools may be particularly important in harsh environments where obligate symbionts may be favoured and are less likely to be recovered easily by culturing [5, 9, 10]. Therefore, culture-independent identification approaches should ideally be combined with culture-dependant methods.

To sum up, we assessed identity, host, tissue association and geographical association tolerance of fungal endophytes isolated from root, stem and leaf tissues Australian *Nicotiana* plants. Endophyte isolation frequency and α-diversity were significantly higher for below-ground than above-ground tissues. We found no variation in endophyte species diversity as a function of sampling location, but as a function of host genotype and tissue origin. A significant pattern of community structuring was observed due to host genotypes but no consistent pattern of fungal community structuring was recorded to be associated with sampling location and tissue type.

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References

1. Hopper SD, Brown AP, Marchant NG (1997) Plants of Western Australian granite outcrops. J. R. Soc. West. Aust. 80:141–158
2. Pepper M, Keogh JS (2014) Biogeography of the Kimberley Western Australia: a review of landscape evolution and biotic responses in an ancient refugium. J. Biogeogr. 41:1443–1445
3. Marasco R, Rolli E, Ettoumi B, Vigani G, Mapelli F, Borin S, Abou-Hadid AF, El-Behairy UA, Sorlini C, Cherif A, Zocchi G, Daffonchio D (2012) A drought resistance-promoting microbiome is selected by root system under desert farming. PLoS One 7: e48479
4. Yang J, Kloepper JW, Ryu CM (2009) Rhizosphere bacteria help plants tolerate abiotic stress. Trends Plant Sci. 14:1–4. doi:10.1016/j.tplants.2008.10.004
5. Khidir HK, Eudy DM, Porras-Alfaro A, Herrera J, Natvig DO, Sinsabaugh RL (2010) A general suite of fungal endophytes dominate the roots of two dominant grasses in a semiarid grassland. J. Arid Environ. 74:35–42. doi:10.1016/j.jaridenv.2009.07.014
6. Lucero ME, Barrow JR, Osuna P, Reyes I (2006) Plant-fungal interactions in arid and semi-arid ecosystems: large-scale impacts from microscale processes. J. Arid Environ. 65:276–284
7. Lugo MA, Reinhart KO, Menoyo E, Crespo EM, Urcelay C (2015) Plant functional traits and phylogenetic relatedness explain variation in associations with root fungal endophytes in an extreme arid environment. Mycorrhiza 25:85–95
8. Porras-Alfaro A, Herrera J, Sinsabaugh RL, Odenbach K, Lowrey T, Natvig DO (2008) Novel root fungal consortium associated with a dominant desert grass. Appl. Environ. Microbiol. 74:2805–2813
9. Herrera J, Khidir HH, Eudy DM, Porras-Alfaro A, Natvig DO, Sinsabaugh RL (2010) Shifting fungal endophyte communities colonize *Bouteloua gracilis*: effect of host tissue and geographical distribution. Mycologia 102:1012–1026
10. Porras-Alfaro A, Raghavan S, Garcia M, Sinsabaugh RL, Natvig DO, Lowrey TK (2014) Endophytic fungal symbionts associated with gypsophilous plants. Bot 92:295–301
11. Lucero ME, Unc A, Cooke P, Dowd S, Sun S (2011) Endophyte microbiome diversity in micropropagated *Atriplex canescens* and *Atriplex torreyi* var *griffithsii*. PLoS One 6:e17693

12. Suryanarayanan TS, Wittlinger SK, Faeth SH (2005) Endophytic fungi associated with cacti in Arizona. Mycol. Res. 109:635–639
13. Bacon CW, White JF (2000) Microbial endophytes. CRC Press. Marcel Dekker Inc., New York
14. U'Ren JM, Dalling JW, Gallery RE, Maddison DR, Davis EC, Gibson CM, Arnold AE (2009) Diversity and evolutionary origins of fungi associated with seeds of a neotropical pioneer tree: a case study for analyzing fungal environmental samples. Mycol. Res. 113:432–449
15. Saikkonen K (2007) Forest structure and fungal endophytes. Fungal Biology Reviews 21:67–74
16. Collado J, Platas G, González I, Peláez F (1999) Geographical and seasonal influences on the distribution of fungal endophytes in *Quercus ilex*. New Phytol. 144:525–532
17. Kumar DSS, Hyde KD (2004) Biodiversity and tissue-recurrence of endophytic fungi in *Tripterygium wilfordii*. Fungal Divers. 17:69–90
18. Malinowski DP, Belesky DP (2000) Adaptations of endophtye-infected cool-season grasses to environmental stresses: mechanisms of drought and mineral stress tolerance. Crop Sci. 40:923–940
19. Malinowski DP, Belesky DP (2006) Ecological importance of Neotyphodium spp. grass endophytes in agroecosystems. Grassl. Sci. 52:23–28
20. Rodriguez RJ, Redman RS, Henson JM (2004) The role of fungal symbioses in the adaptation of plants to high stress environments. Mitig. Adapt. Strateg. Glob. Chang. 9:261–272
21. Zabalgogeazcoa I (2008) Fungal endophytes and their interactions with plant pathogens. Span. J. Agric. Res. 6:138–146
22. Lewis GC, Clement RO (1986) A survey of ryegrass endophyte in the U.K. and its apparent ineffectuality on a seedling pest. J. Agric. Sci. 107:633–638
23. Rodriguez EAE, Jonkers W, Kistler HC, May G (2012) Interactions between *Fusarium verticillioides*, *Ustilago maydis*, and *Zea mays*: an endophyte, a pathogen, and their shared plant host. Fungal Genet. Biol. 49:578–587
24. Higginbotham SJ, Arnold AE, Ibañez A, Spadafora C, Coley PD, Kursar TA (2013) Bioactivity of fungal endophytes as a function of endophyte taxonomy and the taxonomy and distribution of their host plants. PLoS One 8:e73192
25. Rodriguez RJ, White J, Arnold AE, Redman RS (2009) Fungal endophytes: diversity and ecological roles. New Phytol. 182:314–330
26. Barrow JR, Osuna P (2002) Phosphorus solubilization and uptake by dark septate fungi in fourwing saltbush, *Atriplex canescens* (Pursh) Nutt. J. Arid Environ. 51:449–459
27. Bezerra JDP, Santos MGS, Svedese VM, Lima DMM, Fernandes MJS, Paiva LM, Souza-Motta CM (2012) Richness of endophytic fungi isolated from *Opuntia ficus-indica* Mill. (Cactaceae) and preliminary screening for enzyme production. World J. Microbiol. Biotechnol. 28: 1989–1995.
28. Lopez BR, Bashan Y, Bacilio M (2011) Endophytic bacteria of *Mammillaria fraileana*, an endemic rock-colonizing cactus of the southern Sonoran Desert. Arch. Microbiol. 193:527–541
29. Massimo NC, Nandi Devan MM, Arendt KR, Wilch MH, Riddle JM, Furr SH, Steen C, U'Ren JM, Sandberg DC, Arnold AE (2015) Fungal endophytes in aboveground tissues of desert plants: infrequent in culture, but highly diverse and distinctive symbionts. Microb. Ecol. 70:61–76. doi:10.1007/s00248-014-0563-6
30. Jumpponen A, Trappe JM (1998) Dark septate endophytes: a review of facultative biotrophic root-colonizing fungi. New Phytol. 140:295–310. doi:10.1046/j.1469-8137.1998.00265.x
31. Porras-Alfaro A, Herrera J, Natvig DO, Lipinski K, Sinsabaugh RL (2011) Diversity and distribution of soil fungal communities in a semiarid grassland. Mycologia 103:10–21
32. Porras-Alfaro A, Herrera J, Natvig DO, Sinsabaugh RL (2007) Effect of long-term nitrogen fertilization on mycorrhizal fungi associated with a dominant grass in a semiarid grassland. Plant Soil 296:65–75. doi:10.1007/ s11104-007-9290-9
33. Sun Y, Wang Q, Lu X, Okane I, Kakishima M (2012) Endophytic fungal community in stems and leaves of plants from desert areas in China. Mycol. Prog. 11:781–790
34. Marks CE, Newbigin E, Ladiges PY (2011) Comparative morphology and phylogeny of *Nicotiana* section Suaveolentes (Solanaceae) in Australia and the South Pacific. Aust. Syst. Bot. 24:61–86
35. Schulz B, Wanke U, Draeger S, Aust HJ (1993) Endophytes from herbaceous plants and shrubs: effectiveness of surfuse sterilization methods. Mycol. Res. 97:1447–1450
36. Strobel GA (2003) Endophytes as sources of bioactive products. Microbes Infect. 5:535–544
37. Arnold AE, Maynard Z, Gilbert GS, Coley PD, Kursar TA (2000) Are tropical fungal endophytes hyperdiverse? Ecol. Lett. 3:267–274
38. Gardes M, Bruns TD (1993) ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. Mol. Ecol. 2:113–118
39. White TJ, Bruns TD, Lee SB, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Academic Press, New York
40. Abarenkov K, Nilsson RH, Larsson KH, Alexander IJ, Eberhardt U (2010) The UNITE database for molecular identification of fungi-recent updates and future perspectives. New Phytol. 186:281–285
41. Rosa LH, Almeida Vieira Mde L, Santiago IF, Rosa CA (2010) Endophytic fungi community associated with the dicotyledonous plant Colobanthus quitensis (Kunth) Bartl. (Caryophyllaceae) in Antarctica. FEMS Microbiol. Ecol. 73:178–189
42. Katoh K, Asimenos G, Toh H (2009) Multiple alignment of DNA sequences with MAFFT. Humana Press, New Jersey
43. Katoh K, Standley DM (2014) MAFFT: iterative refinement and additional methods. Methods Mol. Biol. 1079:131–146
44. Kia SH, Glynou K, Nau T, Thines M, Piepenbring M, Maciá-Vicente JG (2017) Influence of phylogenetic conservatism and trait convergence on the interactions between fungal root endophytes and plants. The ISME journal 11:777–790. doi:10.1038/ismej.2016.140
45. Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17:754–755
46. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Mentjies P, Drummond A (2012) Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28:1647–1649
47. Wylie SJ, Zhang C, Long V, Roossinck MJ, Koh SH, Jones MGK, Iqbal S, Li H (2015) Differential responses to virus challenge of laboratory and wild accessions of Australian species of *Nicotiana*, and comparative analysis of RDR1 gene sequences. PLoS One 10: e0121787. doi:10.1371/journal.pone.0121787
48. Chung CL, Longfellow JM, Walsh EK, Kerdieh Z, Van Esbroeck G (2010) Resistance loci affecting distinct stages of fungal pathogenesis: use of introgression lines for QTL mapping and characterization in the maize–Setosphaeria turcica pathosystem. BMC Plant Biol. 10:103
49. Colwell RK, Chao A, Gotelli NJ, Lin S-L, Mao CX, Chazdon RL, Longino JT (2012) Models and estimators linking individual-based and sample-based rarefaction, extrapolation and comparison of assemblages. J. Plant Ecol. 5:3–21
50. Krebs CJ (1989) Ecological methodology. Harper & Row, New York
51. Quinn GP, Keough MJ (2002) Multidimensional scaling and cluster analysis. Cambridge University Press, Cambridge
52. Wolda H (1981) Similarity indices, sample size and diversity. Oecologia 50:296–302

53. Fisher PJ, Petrini O, Petrini LE, Sutton BC (1994) Fungal endophytes from the leaves and twigs of *Quercus ilex* L. from England, Majorca and Switzerland. The New phytologist 127:133–147
54. Gauch HG (1982) Multivariate analysis in community ecology. Cambridge University Press, Cambridge
55. Hammer Ø, Harper DAT, Ryan PD (2001) PAST: paleontological statistics software package for education and data analysis. Palaeontol. Electron. 4:9
56. Bae H, Roberts DP, Lim HS, Strem MD, Park SC, Ryu CM, Melnick RL, Bailey BA (2011) Endophytic Trichoderma isolates from tropical environments delay disease onset and induce resistance against Phytophthora capsici in hot pepper using multiple mechanisms. Molecular plant-microbe interactions : MPMI 24: 336–351
57. Andrew DR, Fitak RR, Munguia-Vega A, Racolta A, Martinson VG, Dontsova K (2012) Abiotic factors shape microbial diversity in Sonoran Desert soils. Appl. Environ. Microbiol. 78:7527–7537
58. Hyde KD, Soytong K (2008) The fungal endophyte dilemma. Fungal Divers. 33:163–173
59. Arnold AE, Lutzoni F (2007) Diversity and host range of foliar fungal endophytes: are tropical trees biodiversity hot spots? Ecology 88:541–549
60. Pinruan U, Rungjindamai N, Choeyklin R, Lumyong S, Hyde K, Jones E (2010) Occurrence and diversity of basidiomycetous endophytes from the oil palm, *Elaeis guineensis* in Thailand. Fungal Divers. 41:71–88
61. Cai L, Hyde KD, Taylor PWJ, Weir BS, Waller J, Abang MM, Zhang JZ, Yang YL, Phoulivong S, Liu ZY, Prihastuti H, Shivas RG, McKenzie EHC, Johnston PR (2009) A polyphasic approach for studying *Colletotrichum*. Fungal Divers. 39:183–204
62. Blaalid R, Kumar S, Nilsson RH, Abarenkov K, Kirk PM (2013) ITS1 versus ITS2 as DNA metabarcodes for fungi. Mol. Ecol. Resour. 13:218–224
63. Op De Beeck M, Lievens B, Busschaert P, Declerck S, Vangronsveld J, Colpaert JV (2014) Comparison and validation of some ITS primer pairs useful for fungal metabarcoding studies. PLoS One 9:e97629
64. U'Ren JM, Lutzoni F, Miadlikowska J, Laetsch AD, Arnold AE (2012) Host- and geographic structure of endophytic and endolichenic fungi at a continental scale. Am. J. Bot. 99:898–9143
65. Sakayaroj J, Preedanon S, Supaphon O, Jones EBG, Phongpaichit S (2010) Phylogenetic diversity of endophyte assemblages associated with the tropical seagrass *Enhalus acoroides* in Thailand. Fungal Divers. 42:27–45
66. Stone JK, Polishook JD, White JRJ (2004) Endophytic fungi. Elsevier, Burlington
67. Thomas SE, Crozier J, Aime MC, Evans HC, Holmes KA (2008) Molecular characterization of fungal endophytic morphospecies associated with the indigenous forest tree, *Theobroma gileri*, in Ecuador. Mycol. Res. 112:852–860
68. Albrectsen BR, Björkén L, Varad A, Hagner A, Wedin M, Karlsson J, Jansso S (2010) Endophytic fungi in European aspen (*Populus tremula*) leaves-diversity, detection, and a suggested correlation with herbivory resistance. Fungal Divers. 41. doi:10.1007/s13225-009-0011-y
69. Zhang ZB, Zeng QG, Yan RM, Wang Y, Zou ZR, Zhu D (2011) Endophytic fungus *Cladosporium cladosporioides* LF70 from *Huperzia serrata* produces Huperzine A. World J. Microbiol. Biotechnol. 27:479–486
70. Botella L, Diez JJ (2011) Phylogenic diversity of fungal endophytes in Spanish stands of *Pinus halepensis*. Fungal Divers. 47:9–18. doi: 10.1007/s13225-010-0061-1
71. Cannon CD, Simmons CM (2002) Diversity and host preference of leaf endophytic fungi in the Iwokrama forest reserve, Guyana. Mycologia 94:210–220
72. Clement SL, Graves W, Cunningham P, Nebling V, Bounejmate W, Saidi S, Baya B, Chakroun M, Mezni A, Porqueddu C (1997) Acremonium endophytes in Mediterranean tall fescue. In: Bacon CW, Hill NS (eds) Neotyphodium/grass interactions. Springer, New York, pp 49–51
73. Ghimire SR, Charlton ND, Bell JD, Krishnamurthy YL, Craven KD (2011) Biodiversity of fungal endophyte communities inhabiting switchgrass (*Panicum virgatum* L.) growing in the native tallgrass prairie of northern Oklahoma. Fungal Divers. 47:19–27. doi:10.1007/s13225-010-0085-6
74. Gond SK, Verma VC, Kumar A, Kharwar RN (2007) Study of endophytic fungal community from different parts of *Aegle marmelos* Correae (Rutaceae) from Varanasi (India). World J Microb Biot 23:1371–1375
75. Harman GE (2011) Multifunctional fungal plant symbionts: new tools to enhance plant growth and productivity. New Phytol. 189: 647–649
76. Kusari P, Kusari S, Spiteller M, Kayser O (2013) Endophytic fungi harbored in *Cannabis sativa* L.: diversity and potential as biocontrol agents against host plant-specific phytopathogens. Fungal Divers. 60:137–151. doi:10.1007/s13225-012-0216-3
77. Macia'-Vicente JG, Jansson HB, Mendgen K, Lopez-Llorca LV (2008) Colonization of barley roots by endophytic fungi and their reduction of take-all caused by *Gaeumannomyces graminis* var. *tritici*. Can. J. Microbiol. 54:600–609. doi:10.1139/w08-047
78. Nalini MS, Sunayana N, Prakash HS (2014) Endophytic fungal diversity in medicinal plants of Western Ghats, India. International Journal of Biodiversity 9:494213
79. Paparu P, Dubois T, Coyne D, Viljoen A (2009) Dual inoculation of Fusarium oxysporum endophytes in banana: effect on plant colonisation, growth and control of the root burrowing nematode and the banana weevil. Biocontrol Sci Techn 19:639–655
80. Rosmana A, Samuels GJ, Ismaiel A, Ibrahim ES, Chaverri P, Herawati Y, Asman A (2015) *Trichoderma asperellum*: a dominant endophyte species in cacao grown in sulawesi with potential for controlling vascular streak dieback disease. Tropical Plant Pathology 40:19–25
81. Spurr HWJ, Welty RE (1975) Characterization of endophytic fungi in healthy leaves of *Nicotiana* spp. Phytopathology 65:417–422
82. Wang B, Priest MJ, Davidson A, Brubaker CL, Woods MJ, Burdon JJ (2007) Fungal endophytes of native *Gossypium* species in Australia. Mycol. Res. 111:347–354
83. Zhou K, Wang W, Peng Y, Yu R, Yue Y, Lai D, Zhou L (2015) Endophytic fungi from *Nicotiana tabacum* L.and their antibacterial activity. Natural Product Research and Development 27:1847–1852
84. Wilson D (2000) Ecology of woody plant endophytes. Mercel Dekker Inc., New York
85. McKenna JEJ (2003) An enhanced cluster analysis program with bootstrap significance testing for ecological community analysis. Environ. Model Softw. 18:205–220
86. Del Olmo RM, Arnold AE (2014) Inter annual variation and host affiliations of endophytic fungi associated with ferns at la Selva, Costa Rica. Mycologia 106:8–21
87. Higgins KL, Arnold AE, Coley PD, Kursar TK (2014) Communities of fungal endophytes in tropical forest grasses: highly diverse host- and habitat generalists characterized by strong spatial structure. Fungal Ecol. 8:1–11
88. Chobba BI, Elleuch A, Ayadi I, Khannous L, Namsi A, Cerqueira F, Drira N, Gharsallah N, Vallaeys T (2013) Fungal diversity in adult date palm (*Phoenix dactylifera* L.) revealed by culture-dependent and culture-independent approaches. J Zhejiang Univ Sci B 14: 1084–1099

Supplementary Information

Supplementary Table 1: Identity of endophytic fungal isolates of wild Australian Nicotiana species along with GenBank Accession numbers.

Sl No.	Isolate Code	*OTU name assigned*	GenBank Accession	Host Species	Host Tissue
1	E-86	*Acremonium sclerotigenum*	KU059866	*N. occidentalis*	Root
2	E-9.1	*Acremonium sp.*	KU059954	*N. occidentalis*	Root
3	E-98	*Acremonium sp.*	KU059854	*N. occidentalis*	Stem
4	E-114	*Acremonium sp.*	KU059861	*N. occidentalis*	Leaf
5	E-138	*Acremonium sp.*	KU059812	*N. rosulata*	Root
6	E-143	*Acremonium sp.*	KU059970	*N. occidentalis*	Root
7	E-144	*Acremonium sp.*	KU059885	*N. occidentalis*	Leaf
8	E-150	*Acremonium sp.*	KU059971	*N. occidentalis*	Stem
9	E-178.5	*Acremonium sp.*	KU059978	*N. occidentalis*	Root
10	E-210.1	*Acremonium sp.*	KU059865	*N. occidentalis*	Leaf
11	E-229.1	*Acremonium sp.*	KU059872	*N. occidentalis*	Leaf
12	E-723	*Acremonium sp.*	KU059973	*N. rosulata*	Root
13	E-504	*Alternaria alternata*	KU059918	*N. occidentalis*	Root
14	E-505	*Alternaria alternata*	KU059919	*N. benthamiana*	Leaf
15	E-515.1	*Alternaria alternata*	KU059925	*N. benthamiana*	Stem
16	E-520.1	*Alternaria alternata*	KU059929	*N. occidentalis*	Root
17	E-530.1	*Alternaria alternata*	KU059951	*N. benthamiana*	Root
18	E-507	*Alternaria sp.-A*	KU059920	*N. benthamiana*	Leaf
19	E-196	*Alternaria sp.-B*	KU059824	*N. simulans*	Root
20	E-204	*Alternaria sp.-B*	KU059889	*N. simulans*	Root
21	E-288	*Alternaria sp.-B*	KU059938	*N. simulans*	Root
22	E-219	*Apodus oryza*	KU059868	*N. rosulata*	Stem
23	E-106	*Aspergillus carbonarius*	KU059964	*N. occidentalis*	Root
24	E-110	*Aspergillus carbonarius*	KU059937	*N. occidentalis*	Stem
25	E-529	*Aspergillus fumigatiaffinis*	KU059950	*N. rosulata*	Root
26	E-518.1	*Aspergillus fumigatiaffinis*	KU059926	*N. benthamiana*	Root
27	E-202	*Aspergillus niger*	KU059864	*N. occidentalis*	Stem
28	E-134	*Aspergillus ochraceus*	KU059881	*N. benthamiana*	Root
29	E-145	*Aspergillus ochraceus*	KU059883	*N. simulans*	Stem
30	E-531.1	*Aspergillus oryzae*	KU059952	*N. simulans*	Leaf
31	E-503	*Aspergillus quadrilineatus*	KU059917	*N. simulans*	Stem
32	E-135	*Aurantiporus alborubescens*	KU059953	*N. simulans*	Root
33	E-506	*Aureobasidium pullulans*	KU059941	*N. benthamiana*	Root
34	E-526	*Bartalinia pondoensis*	KU059948	*N. benthamiana*	Stem
35	E-247	*Botrytis cinerea*	KU059891	*N. benthamiana*	Root
36	E-9	*Chaetomium funicola*	KU059876	*N. occidentalis*	Root
37	E-84	*Chaetomium globosum*	KU059836	*N. occidentalis*	Root
38	E-228.1	*Chaetomium globosum*	KU059890	*N. simulans*	Root

39	E-824	*Chaetomium globosum*	KU059939	*N. occidentalis*	Root
40	E-521.1	*Chaetomium sp.-A*	KU059945	*N. occidentalis*	Root
41	E-5	*Chaetomium sp.-B*	KU059912	*N. benthamiana*	Leaf
42	E-101	*Chaetomium sp.-B*	KU059913	*N. occidentalis*	Root
43	E-111	*Chaetomium sp.-B*	KU059860	*N. occidentalis*	Root
44	E-120	*Chaetomium sp.-B*	KU059816	*N. occidentalis*	Root
45	E-136	*Chaetomium sp.-B*	KU059817	*N. occidentalis*	Stem
46	E-137	*Chaetomium sp.-B*	KU059818	*N. occidentalis*	Root
47	E-139	*Chaetomium sp.-B*	KU059819	*N. occidentalis*	Root
48	E-146	*Chaetomium sp.-B*	KU059820	*N. occidentalis*	Stem
49	E-151	*Chaetomium sp.-B*	KU059821	*N. occidentalis*	Root
50	E-156	*Chaetomium sp.-B*	KU059822	*N. benthamiana*	Leaf
51	E-186.1	*Chaetomium sp.-B*	KU059825	*N. occidentalis*	Root
52	E-187.1	*Chaetomium sp.-B*	KU059826	*N. benthamiana*	Leaf
53	E-187.10	*Chaetomium sp.-B*	KU059914	*N. occidentalis*	Stem
54	E-195	*Chaetomium sp.-B*	KU059823	*N. occidentalis*	Root
55	E-216.1	*Chaetomium sp.-B*	KU059867	*N. occidentalis*	Stem
56	E-226	*Chaetomium sp.-B*	KU059934	*N. occidentalis*	Stem
57	E-600	*Chaetomium sp.-B*	KU059815	*N. occidentalis*	Root
58	E-162	*Cladosporium cladosporioides*	KU059933	*N. benthamiana*	Root
59	E-128	*Cladosporium halotolerans*	KU059905	*N. simulans*	Root
60	E-283	*Cladosporium halotolerans*	KU059910	*N. simulans*	Root
61	E-17	*Cladosporium sp.*	KU059878	*N. benthamiana*	Root
62	E-121	*Cladosporium sp.*	KU059960	*N. simulans*	Stem
63	E-516.1	*Curvularia tsudae*	KU059944	*N. benthamiana*	Leaf
64	E-527	*Curvularia tsudae*	KU059949	*N. simulans*	Root
65	E-280	*Cylindrocarpon pauciseptatum*	KU059935	*N. benthamiana*	Stem
66	E-509	*Dothideomycetes sp.*	KU059942	*N. occidentalis*	Root
67	E-511	*Edenia gomezpompae*	KU059943	*N. benthamiana*	Root
68	E-279	*Exserohilum sp.*	KU059895	*N. benthamiana*	Leaf
69	E-519.1	*Exserohilum sp.*	KU059927	*N. occidentalis*	Stem
70	E-523	*Exserohilum sp.*	KU059946	*N. occidentalis*	Leaf
71	E-522	*Fusarium equiseti*	KU059931	*N. rosulata*	Root
72	E-177.1	*Fusarium oxysporum*	KU059856	*N. simulans*	Root
73	E-225.1	*Fusarium oxysporum*	KU059870	*N. benthamiana*	Root
74	E-253	*Fusarium oxysporum*	KU059892	*N. benthamiana*	Root
75	E-712	*Fusarium oxysporum*	KU059848	*N. benthamiana*	Stem
76	E-179.5	*Fusarium sp.*	KU059845	*N. benthamiana*	Root
77	E-180.3	*Fusarium sp.*	KU059956	*N. benthamiana*	Root
78	E-501	*Gelasinospora saitoi*	KU059940	*N. simulans*	Root
79	E-502	*Gelasinospora saitoi*	KU059916	*N. benthamiana*	Root
80	E-131	*Hongkongmyces sp.*	KU059969	*N. benthamiana*	Stem
81	E-08	*Setophoma sp.*	KU059911	*N. occidentalis*	Root
82	E-720	*Hyaloscyphaceae sp.*	KU059859	*N. simulans*	Root

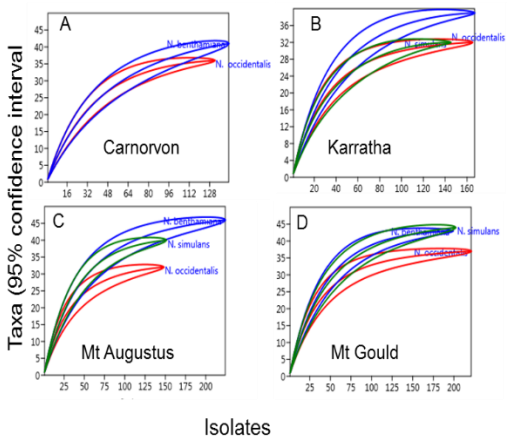
83	E-812	*Hyaloscyphaceae sp.*	KU059884	*N. simulans*	Root
84	E-92	*Hypomyces sp.*	KU059902	*N. occidentalis*	Leaf
85	E-93	*Hypomyces sp.*	KU059879	*N. simulans*	Stem
86	E-14	*Lecythophora sp.-A*	KU059832	*N. benthamiana*	Leaf
87	E-11	*Lecythophora sp.-B*	KU059853	*N. simulans*	Root
88	E-513.1	*Microsphaeropsis arundinis*	KU059924	*N. benthamiana*	Root
89	E-508	*Myrothecium verrucaria*	KU059921	*N. simulans*	Leaf
90	E-512	*Myrothecium verrucaria*	KU059923	*N. benthamiana*	Leaf
91	E-135.2	*Neocosmospora rubicola*	KU059882	*N. simulans*	Stem
92	E-178.3	*Paramyrothecium roridum*	KU059976	*N. occidentalis*	Leaf
93	E-182.2	*Paramyrothecium roridum*	KU059979	*N. benthamiana*	Stem
94	E-188.4	*Penicillium citrinum*	KU059828	*N. occidentalis*	Stem
95	E-191.5	*Penicillium citrinum*	KU059850	*N. simulans*	Leaf
96	E-13.2	*Penicillium oxalicum*	KU059858	*N. benthamiana*	Stem
97	E-190.1	*Penicillium oxalicum*	KU059831	*N. simulans*	Root
98	E-172.1	*Penicillium simplicissimum*	KU059811	*N. benthamiana*	Stem
99	E-180	*Penicillium simplicissimum*	KU059955	*N. benthamiana*	Stem
100	E-190.2	*Penicillium simplicissimum*	KU059833	*N. occidentalis*	Root
101	E-193.1	*Penicillium simplicissimum*	KU059862	*N. occidentalis*	Root
102	E-193.2	*Penicillium simplicissimum*	KU059863	*N. occidentalis*	Root
103	E-223.1	*Penicillium simplicissimum*	KU059869	*N. simulans*	Root
104	E-230.1	*Penicillium simplicissimum*	KU059873	*N. occidentalis*	Leaf
105	E-87	*Penicillium sp.*	KU059928	*N. occidentalis*	Root
106	E-183.1	*Penicillium sp.*	KU059980	*N. occidentalis*	Stem
107	E-183.4	*Penicillium sp.*	KU059803	*N. occidentalis*	Root
108	E-188.2	*Penicillium sp.*	KU059827	*N. occidentalis*	Leaf
109	E-189.2	*Penicillium sp.*	KU059830	*N. occidentalis*	Root
110	E-192.2	*Penicillium sp.*	KU059851	*N. occidentalis*	Stem
111	E-16	*Phanerochaete sp.*	KU059877	*N. occidentalis*	Root
112	E-161	*Phanerochaete sp.*	KU059932	*N. occidentalis*	Root
113	E-206	*Phlebia acerina*	KU059900	*N. occidentalis*	Root
114	E-521.1	*Phoma sp.-A*	KU059930	*N. rosulata*	Root
115	E-127.1	*Plectosphaerella cucumerina*	KU059904	*N. occidentalis*	Root
116	E-178.4	*Plectosphaerella cucumerina*	KU059977	*N. occidentalis*	Stem
117	E-183.2	*Plectosphaerella cucumerina*	KU059981	*N. benthamiana*	Leaf
118	E-185.5	*Plectosphaerella cucumerina*	KU059809	*N. occidentalis*	Root
119	E-232.1	*Plectosphaerella cucumerina*	KU059874	*N. occidentalis*	Stem
120	E-233.1	*Plectosphaerella cucumerina*	KU059875	*N. occidentalis*	Leaf
121	E-835	*Plectosphaerella cucumerina*	KU059936	*N. occidentalis*	Root
122	E-903	*Plectosphaerella cucumerina*	KU059968	*N. occidentalis*	Leaf
123	E-510	*Pleosporales*	KU059922	*N. occidentalis*	Root
124	E-218	*Pleurostomophora repens*	KU059915	*N. occidentalis*	Root
125	E-172	*Rasamsonia piperina*	KU059908	*N. benthamiana*	Leaf
126	E-124	*Sarocladium sp.*	KU059839	*N. benthamiana*	Stem
127	E-163	*Setophoma sp.*	KU059886	*N. occidentalis*	Root

128	E-178.6	*Setophoma sp.*	KU059887	*N. occidentalis*	Root
129	E-277	*Setophoma sp.*	KU059894	*N. occidentalis*	Root
130	E-36	*Setophoma terrestris*	KU059857	*N. occidentalis*	Stem
131	E-119	*Sporothrix schenckii*	KU059903	*N. simulans*	Stem
132	E-270	*Stagonosporopsis cucurbitacearum*	KU059893	*N. simulans*	Root
133	E-271	*Stagonosporopsis cucurbitacearum*	KU059901	*N. simulans*	Root
134	E-123	*Thozetella sp.*	KU059967	*N. benthamiana*	Root
135	E-152	*Thozetella sp.*	KU059907	*N. simulans*	Leaf
136	E-167	*Thozetella sp.*	KU059840	*N. benthamiana*	Root
137	E-170	*Thozetella sp.*	KU059810	*N. benthamiana*	Root
138	E-185.4	*Thozetella sp.*	KU059808	*N. benthamiana*	Root
139	E-80	*Trichoderma asperellum*	KU059834	*N. simulans*	Root
140	E-83	*Trichoderma asperellum*	KU059835	*N. simulans*	Leaf
141	E-100	*Trichoderma asperellum*	KU059963	*N. simulans*	Root
142	E-173	*Trichoderma asperellum*	KU059972	*N. rosulata*	Root
143	E-198	*Trichoderma asperellum*	KU059852	*N. rosulata*	Root
144	E-228	*Trichoderma hamatum*	KU059871	*N. simulans*	Leaf
145	E-185.1	*Trichoderma sp.*	KU059806	*N. simulans*	Root
146	E-116	*Trichoderma sp.*	KU059965	*N. simulans*	Root
147	E-117	*Trichoderma sp.*	KU059966	*N. simulans*	Root
148	E-191.3	*Trichoderma sp.*	KU059849	*N. simulans*	Root
149	E-96	*Uncultured ascomycota*	KU059855	*N. benthamiana*	Leaf
150	E-141	*Uncultured ascomycota*	KU059813	*N. occidentalis*	Root
151	E-157	*Uncultured ascomycota*	KU059814	*N. occidentalis*	Root
152	E-177.2	*Uncultured ascomycota*	KU059841	*N. benthamiana*	Leaf
153	E-177.4	*Uncultured ascomycota*	KU059843	*N. simulans*	Root
154	E-177.5	*Uncultured ascomycota*	KU059844	*N. occidentalis*	Root
155	E-178.1	*Uncultured ascomycota*	KU059974	*N. benthamiana*	Leaf
156	E-178.2	*Uncultured ascomycota*	KU059975	*N. benthamiana*	Root
157	E-181.3	*Uncultured ascomycota*	KU059957	*N. simulans*	Root
158	E-181.5	*Uncultured ascomycota*	KU059959	*N. benthamiana*	Root
159	E-182.4	*Uncultured ascomycota*	KU059846	*N. simulans*	Root
160	E-184.4	*Uncultured ascomycota*	KU059804	*N. benthamiana*	Stem
161	E-184.5	*Uncultured ascomycota*	KU059805	*N. occidentalis*	Root
162	E-185.3	*Uncultured ascomycota*	KU059888	*N. benthamiana*	Root
163	E-189.1	*Uncultured ascomycota*	KU059829	*N. benthamiana*	Leaf
164	E-189.10	*Uncultured ascomycota*	KU059899	*N. benthamiana*	Root
165	E-282	*Uncultured ascomycota*	KU059896	*N. occidentalis*	Root
166	E-285.4	*Uncultured ascomycota*	KU059898	*N. benthamiana*	Leaf
167	E-524	*Uncultured ascomycota*	KU059947	*N. benthamiana*	Stem
168	E-702	*Uncultured ascomycota*	KU059847	*N. benthamiana*	Leaf
169	E-750	*Uncultured ascomycota*	KU059958	*N. benthamiana*	Root
170	E-896	*Uncultured ascomycota*	KU059961	*N. benthamiana*	Leaf

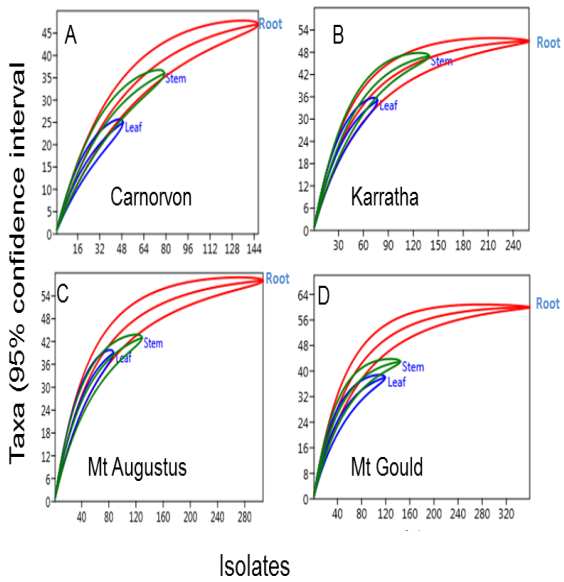
171	E-901	*Uncultured ascomycota*	KU059962	*N. simulans*	Root
172	E-126	*Unknown fungus-A*	KU059837	*N. occidentalis*	Root
173	E-284	*Unknown fungus-B*	KU059897	*N. benthamiana*	Root
174	E-133	*Zopfiella latipes*	KU059838	*N. simulans*	Root
175	E-177.3	*Zopfiella latipes*	KU059842	*N. occidentalis*	Leaf
176	E-10	*Lecythophora sp.-A*	KY582065	*N. benthamiana*	Stem
177	E-13	*Pleurostomophora richardsiae*	KY582066	*N. simulans*	Stem
178	E-175.3	*Fusarium oxysporum*	KY582067	*N. simulans*	Stem
179	E-239	*Phoma sp.-A*	KY582068	*N. occidentalis*	Root
180	E-263	*Talaromyces islandicus*	KY582069	*N. simulans*	Root
181	E-300	*Penicillium sp.*	KY582070	*N. occidentalis*	Leaf
182	E-303	*Trichoderma sp.*	KY582071	*N. simulans*	Leaf
183	E-304	*Thozetella sp.*	KY582072	*N. benthamiana*	Root
184	E-305	*Plectosphaerella cucumerina*	KY582073	*N. occidentalis*	Root
185	E-306	*Thozetella sp.*	KY582074	*N. benthamiana*	Root
186	E-307	*Penicillium simplicissimum*	KY582075	*N. benthamiana*	Stem
187	E-311	*Chaetomium sp.-B*	KY582076	*N. occidentalis*	Root
188	E-312	*Chaetomium sp.-B*	KY582077	*N. occidentalis*	Root
189	E-313	*Chaetomium sp.-B*	KY582078	*N. occidentalis*	Stem
190	E-314	*Chaetomium sp.-B*	KY582079	*N. simulans*	Root
191	E-315	*Chaetomium sp.-B*	KY582080	*N. occidentalis*	Root
192	E-316	*Chaetomium sp.-B*	KY582081	*N. occidentalis*	Stem
193	E-317	*Chaetomium sp.-B*	KY582082	*N. occidentalis*	Root
194	E-318	*Chaetomium sp.-B*	KY582083	*N. benthamiana*	Leaf
195	E-319	*Chaetomium sp.-B*	KY582084	*N. occidentalis*	Root
196	E-320	*Alternaria sp.-B*	KY582085	*N. simulans*	Root
197	E-321	*Chaetomium sp.-B*	KY582086	*N. occidentalis*	Root
198	E-322	*Chaetomium sp.-B*	KY582087	*N. benthamiana*	Leaf
199	E-323	*Penicillium sp.*	KY582088	*N. occidentalis*	Leaf
200	E-324	*Penicillium citrinum*	KY582089	*N. occidentalis*	Stem
201	E-326	*Penicillium sp.*	KY582090	*N. occidentalis*	Root
202	E-327	*Penicillium oxalicum*	KY582091	*N. simulans*	Root
203	E-329	*Penicillium simplicissimum*	KY582092	*N. occidentalis*	Root
204	E-330	*Trichoderma asperellum*	KY582093	*N. simulans*	Root
205	E-331	*Trichoderma asperellum*	KY582094	*N. simulans*	Leaf
206	E-332	*Chaetomium globosum*	KY582095	*N. occidentalis*	Root
207	E-336	*Thozetella sp.*	KY582096	*N. benthamiana*	Root
208	E-341	*Fusarium sp.*	KY582097	*N. benthamiana*	Root
209	E-344	*Fusarium oxysporum*	KY582098	*N. benthamiana*	Stem
210	E-345	*Trichoderma sp.*	KY582099	*N. simulans*	Root
211	E-346	*Penicillium citrinum*	KY582100	*N. simulans*	Leaf
212	E-347	*Penicillium sp.*	KY582101	*N. occidentalis*	Stem
213	E-348	*Trichoderma asperellum*	KY582102	*N. rosulata*	Root
214	E-349	*Lecythophora sp.-B*	KY582103	*N. simulans*	Root
215	E-352	*Fusarium oxysporum*	KY582104	*N. simulans*	Root

216	E-353	*Setophoma terrestris*	KY582105	*N. occidentalis*	Stem
217	E-354	*Penicillium oxalicum*	KY582106	*N. benthamiana*	Stem
218	E-355	*Hyaloscyphaceae sp.*	KY582107	*N. simulans*	Root
219	E-356	*Chaetomium sp.-B*	KY582108	*N. occidentalis*	Root
220	E-358	*Penicillium simplicissimum*	KY582109	*N. occidentalis*	Root
221	E-359	*Penicillium simplicissimum*	KY582110	*N. occidentalis*	Root
222	E-360	*Aspergillus niger*	KY582111	*N. occidentalis*	Stem
223	E-363	*Chaetomium sp.-B*	KY582112	*N. occidentalis*	Stem
224	E-365	*Penicillium simplicissimum*	KY582113	*N. simulans*	Root
225	E-366	*Fusarium oxysporum*	KY582114	*N. benthamiana*	Root
226	E-369	*Penicillium simplicissimum*	KY582115	*N. occidentalis*	Leaf
227	E-370	*Plectosphaerella cucumerina*	KY582116	*N. occidentalis*	Stem
228	E-371	*Plectosphaerella cucumerina*	KY582117	*N. occidentalis*	Leaf
229	E-373	*Zopfiella latipes*	KY582118	*N. occidentalis*	Root
230	E-374	*Cladosporium sp.*	KY582119	*N. benthamiana*	Root
231	E-375	*Hypomyces sp.*	KY582120	*N. simulans*	Stem
232	E-376	*Aspergillus ochraceus*	KY582121	*N. benthamiana*	Root
233	E-377	*Neocosmospora rubicola*	KY582122	*N. simulans*	Stem
234	E-379	*Hyaloscyphaceae sp.*	KY582123	*N. simulans*	Root
235	E-380	*Acremonium sp.*	KY582124	*N. occidentalis*	Leaf
236	E-381	*Setophoma sp.*	KY582125	*N. occidentalis*	Root
237	E-382	*Setophoma sp.*	KY582126	*N. occidentalis*	Root
238	E-384	*Alternaria sp.-B*	KY582127	*N. simulans*	Root
239	E-385	*Chaetomium globosum*	KY582128	*N. simulans*	Root
240	E-386	*Botrytis cinerea*	KY582129	*N. benthamiana*	Root
241	E-387	*Fusarium oxysporum*	KY582130	*N. benthamiana*	Root
242	E-389	*Setophoma sp.*	KY582131	*N. occidentalis*	Root
243	E-390	*Exserohilum sp.*	KY582132	*N. benthamiana*	Leaf
244	E-395	*Neocosmospora rubicola*	KY582133	*N. occidentalis*	Root
245	E-397	*Hypomyces sp.*	KY582134	*N. occidentalis*	Leaf
246	E-399	*Plectosphaerella cucumerina*	KY582135	*N. occidentalis*	Root
247	E-401	*Thozetella sp.*	KY582136	*N. simulans*	Leaf
248	E-404	*Hyaloscyphaceae sp.*	KY582137	*N. occidentalis*	Root
249	E-405	*Chaetomium sp.-B*	KY582138	*N. benthamiana*	Leaf
250	E-406	*Chaetomium sp.-B*	KY582139	*N. occidentalis*	Root
251	E-407	*Chaetomium sp.-B*	KY582140	*N. occidentalis*	Stem
252	E-409	*Gelasinospora saitoi*	KY582141	*N. benthamiana*	Root
253	E-410	*Aspergillus quadrilineatus*	KY582142	*N. simulans*	Stem
254	E-411	*Alternaria alternata*	KY582143	*N. occidentalis*	Root
255	E-412	*Alternaria alternata*	KY582144	*N. benthamiana*	Leaf
256	E-414	*Myrothecium verrucaria*	KY582145	*N. simulans*	Leaf
257	E-416	*Myrothecium verrucaria*	KY582146	*N. benthamiana*	Leaf
258	E-417	*Microsphaeropsis arundinis*	KY582147	*N. benthamiana*	Root
259	E-418	*Alternaria alternata*	KY582148	*N. benthamiana*	Stem
260	E-420	*Exserohilum sp.*	KY582149	*N. occidentalis*	Stem

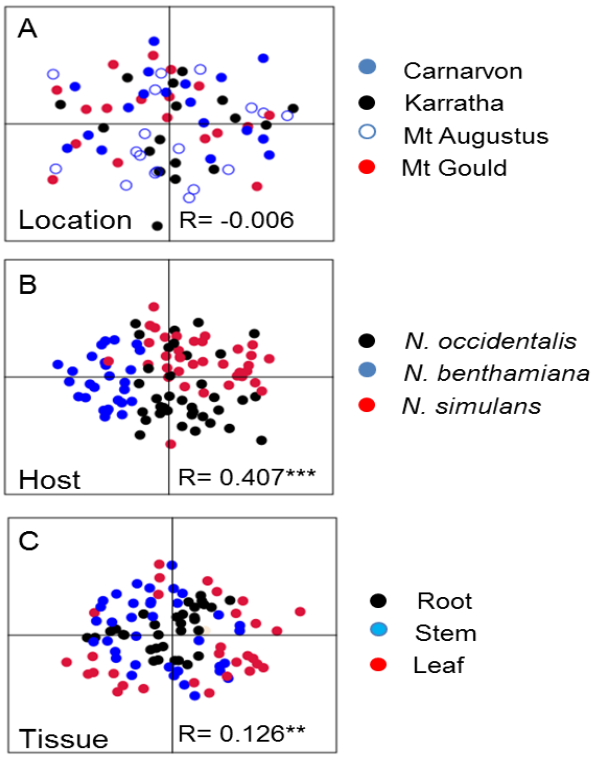
261	E-421	*Penicillium sp.*	KY582150	*N. occidentalis*	Root
262	E-424	*Fusarium equiseti*	KY582151	*N. rosulata*	Root
263	E-425	*Zopfiella latipes*	KY582152	*N. occidentalis*	Root
264	E-426	*Cladosporium cladosporioides*	KY582153	*N. benthamiana*	Root
265	E-427	*Chaetomium sp.-B*	KY582154	*N. occidentalis*	Stem
266	E-428	*Cylindrocarpon pauciseptatum*	KY582155	*N. benthamiana*	Stem
267	E-429	*Plectosphaerella cucumerina*	KY582156	*N. occidentalis*	Root
268	E-430	*Aspergillus carbonarius*	KY582157	*N. occidentalis*	Stem
269	E-432	*Chaetomium globosum*	KY582158	*N. occidentalis*	Root
270	E-433	*Gelasinospora saitoi*	KY582159	*N. simulans*	Root
271	E-434	*Acremonium sp.*	KY582160	*N. benthamiana*	Root
272	E-437	*Curvularia tsudae*	KY582161	*N. benthamiana*	Leaf
273	E-439	*Exserohilum sp.*	KY582162	*N. occidentalis*	Leaf
274	E-441	*Bartalinia pondoensis*	KY582163	*N. benthamiana*	Stem
275	E-442	*Curvularia tsudae*	KY582164	*N. simulans*	Root
276	E-443	*Aspergillus fumigatiaffinis*	KY582165	*N. rosulata*	Root
277	E-445	*Aspergillus oryzae*	KY582166	*N. simulans*	Leaf
278	E-446	*Chaetomium funicola*	KY582167	*N. simulans*	Root
279	E-447	*Acremonium sp.*	KY582168	*N. occidentalis*	Root
280	E-448	*Penicillium simplicissimum*	KY582169	*N. benthamiana*	Stem
281	E-449	*Fusarium sp.*	KY582170	*N. benthamiana*	Root
282	E-453	*Cladosporium sp.*	KY582171	*N. simulans*	Stem
283	E-456	*Trichoderma asperellum*	KY582172	*N. simulans*	Root
284	E-457	*Aspergillus carbonarius*	KY582173	*N. occidentalis*	Root
285	E-458	*Trichoderma sp.*	KY582174	*N. simulans*	Root
286	E-459	*Trichoderma sp.*	KY582175	*N. simulans*	Root
287	E-460	*Thozetella sp.*	KY582176	*N. benthamiana*	Root
288	E-461	*Plectosphaerella cucumerina*	KY582177	*N. occidentalis*	Leaf
289	E-462	*Neocosmospora rubicola*	KY582178	*N. benthamiana*	Stem
290	E-463	*Acremonium sp.*	KY582179	*N. occidentalis*	Root
291	E-464	*Acremonium sp.*	KY582180	*N. occidentalis*	Stem
292	E-465	*Trichoderma asperellum*	KY582181	*N. rosulata*	Root
293	E-466	*Acremonium sp.*	KY582182	*N. rosulata*	Root
294	E-469	*Paramyrothecium roridum*	KY582183	*N. occidentalis*	Leaf
295	E-470	*Plectosphaerella cucumerina*	KY582184	*N. occidentalis*	Stem
296	E-471	*Acremonium sp.*	KY582185	*N. occidentalis*	Root
297	E-472	*Paramyrothecium roridum*	KY582186	*N. benthamiana*	Stem
298	E-473	*Penicillium sp.*	KY582187	*N. occidentalis*	Stem
299	E-474	*Plectosphaerella cucumerina*	KY582188	*N. benthamiana*	Leaf
300	E-500	*Phoma sp.-B*	KY582189	*N. benthamiana*	Root



Supplementary figure 1. Species accumulation curves of fungal taxa recovered from three species of *Nicotiana* plants. The graphs depict the relationship between the number of fungal taxa obtained and the number of isolates recovered in each of the species at (A) Carnorvon, (B) Karratha (C) Mt Augustus and (D) Mt Gould. Whole endophyte communities were considered per host species at particular location.



Supplementary figure 2. Species accumulation curves of fungal taxa recovered from root, stem and leaf tissues of *Nicotiana*. The graphs depict the relationship between the number of fungal taxa obtained and the number of isolates recovered from different tissues from all host at (A) Carnorvon, (B) Karratha (C) Mt Augustus and (D) Mt Gould. Whole endophyte communities were considered per tissue for all species at each particular location.



Supplementary figure 3. Structuring of fungal endophyte communities as influenced by location, host and tissue types. Non-metric multidimensional scaling (NMDS) obtained by and ANOSIM analysis of endophyte communities recovered from root, stem and leaf tissues as indicated by different colours from three *Nicotiana* species at four sampling location. For clustering analysis, Bray Curtis similarity as computed: (A) Fungal communities of all host and tissue type were pooled to obtain the overall fungal community of a particular location as indicated by colours; (B) Fungal communities of all tissue type from all locations were pooled to obtain the overall fungal community of a particular host species as indicated by colours (C) Fungal communities of all host from all sampling location were pooled to obtain the overall fungal community of a tissue type as indicated by colours. The ANOSIM statistic R values; Positive R (up to 1) signifies dissimilarity between groups. ** and *** represents the significance of dissimilarity (R) at $p < 0.01$ and $p< 0.001$ respectively, obtained by permutation of group membership, with 9,999 replicates.

Chapter 4

A simple and rapid *in vitro* test for large scale screening of fungal endophytes from drought-adapted Australian wild plants for conferring water deprivation tolerance and growth promotion in *Nicotiana benthamiana* seedlings

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ORIGINAL PAPER

A simple and rapid in vitro test for large-scale screening of fungal endophytes from drought-adapted Australian wild plants for conferring water deprivation tolerance and growth promotion in *Nicotiana benthamiana* seedlings

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Abstract Some fungal endophytes confer novel phenotypes and enhance existing ones in plants, including tolerance to water deprivation stress. A range of fungal endophytes was isolated from wild *Nicotiana* plants growing in arid parts of northern Australia. These were screened for ability to enhance water deprivation stress tolerance by inoculating seedlings of the model plant *N. benthamiana* in two in vitro tests. Sixty-eight endophyte isolates were co-cultivated with *N. benthamiana* seedlings on either damp filter paper or on agar medium before being subjected to water deprivation. Seventeen isolates were selected for further testing under water deprivation conditions in a sand-based test in a glasshouse. Only two fungal isolates, *Cladosporium cladosporioides* (E-162) and an unknown fungus (E-284), significantly enhanced seedling tolerance to moisture deprivation consistently in both in vitro and sand-based tests. Although a strongly significant correlation was observed between any two screening methods, the result of filter paper test was more strongly reflected ($r = 0.757$, $p < 0.001$) in results of the glasshouse test, indicating its relative suitability over the agar-based test. In another experiment, the same 17 isolates carried forward to the sand-based test used in the glasshouse screening test were inoculated to *N. benthamiana* plants in pots in a nutrient-limiting environment to test their influence on growth promotion. Isolates related to *C. cladosporioides*, *Fusarium equiseti*, and *Thozetella* sp. promoted seedling growth by increasing shoot length and biomass. The fungal isolate E-162 (*C. cladosporioides*) significantly enhanced moisture deprivation tolerance as well as promoted seedling growth.

Keywords Agar media · Filter paper · Glasshouse · Growth promotion · Water deficit · Wilting

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Introduction

All macroscopic organisms living in nature share close associations with microorganisms (Moran 2006). This association includes the bacterial microflora of animal digestive systems to the mycorrhizal fungi of plants. Plants, owing to their sessile lifestyle, are exposed to continually fluctuating environmental conditions. It may be this that has led to symbiotic interactions with various microorganisms to adapt to these conditions. These microbes are believed to have vital functions in the structure, function, and fitness of plant communities (Clay and Holah 1999). Despite the fact that almost 300,000 plant species are recorded to be associated with one or more endophytes (endo = within, phyte = plant), few associations have been studied in detail, and the main exceptions being the legume–rhizobia symbiosis (Long et al. 2008) and mycorrhizae (Herrera et al. 2010; Lugo et al. 2015). Endophytes are increasingly reported for their ecological importance, especially in extreme environments (Rodriguez et al. 2004). Unlike mycorrhizal

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fungi that inhabit plant roots and grow into the rhizosphere, endophytes live predominantly inside plant roots, stems, and/or leaves (Sherwood and Carroll 1974; Carroll 1988; Stone et al. 2004). Of the two major groups, clavicipitaceous endophytes are isolated from some grasses, whereas the non-clavicipitaceous endophytes can be isolated from tissues of many plant types (Rodriguez et al. 2009). Non-clavicipitaceous endophytes represent a highly diverse group of fungi that may have a role in improving abiotic stress tolerance of plants (Redman et al. 2002; Schulz and Boyle 2005; Rodriguez et al. 2008), and in promoting growth of host (Bacon and White 2000). The mechanism by which endophytes confer drought tolerance generally entails one or more of the following mechanisms: (1) aiding the plants to evade or alleviate the impacts of the stress by stimulating early host stress response systems (Redman et al. 1999); (2) producing anti-stress biochemicals (Strobel et al. 2001; Miller et al. 2002), and (3) activation of host stress response systems by acting as biological triggers (Rodriguez et al. 2004).

Plants of the deserts of northwest Australia cope with aridity, thermal fluctuations, and nutrient impoverished soils using various morphological, physiological, and biochemical strategies (Hopper et al. 1997; Wende 1997; Pepper and Keogh 2014). Less well documented are the biological associations of these plants with microorganisms, which may provide additional levels of adaptation to stress (Yang et al. 2009; Marasco et al. 2012). There is potential that this knowledge could be applied to agricultural systems to improve tolerance to abiotic stressors.

We isolated fungal endophytes from wild Australian *Nicotiana* plants growing in arid parts of northwest Australia. *Nicotiana benthamiana* is a model plant used internationally in plant virology and gene expression studies (Goodin et al. 2008, Wylie et al. 2015). *N. benthamiana* is an indigenous plant of parts of subtropical and tropical zones of northern Australia where it inhabits a range of habitats, from offshore islands to arid inland regions. Although it has been used as a model plant in laboratories for 70 years, very little is known about its ecology. We hypothesized that fungal endophytes could influence tolerance of *N. benthamiana* plants to moisture stress conditions. One of the major setbacks for studying endophyte-conferred stress tolerance is lack of suitable methods for screening endophytes involved in stress tolerance from those that may not be. Our study focused on the facility of developing a large-scale procedure to rapidly screen for the ability of non-clavicipitaceous fungal endophytes to confer water deprivation tolerance to seedlings in vitro, before testing more fully in sand-based tests.

Materials and methods

Endophyte isolates

The fungal endophytes were isolated from root, stem, and leaf tissue of plants belonging to three indigenous Australian *Nicotiana* species collected in natural habitats in northern Western Australia. Endophytes were isolated from surface sterilized plant tissue on 0.1X potato dextrose agar (PDA) medium from symptomless plant tissue (Schulz et al. 1993). A combined morphological (colony appearance, mycelial texture, hyphae, and conidial structures) and molecular approach was used for fungal identification. Molecular identification of isolates was done by Sanger sequencing of PCR products of ITS regions using the universal primers ITS1 and ITS4 or ITS1F and ITS4 (White et al. 1990) (Table 1). Fungal sequences were aligned as query sequences with GenBank (NCBI) and UNITE (Abarenkov et al. 2010) databases. Fungal species names were given if their sequences were >98% similar to an accession identified from the databases. If the similarity was between 95% and 97%, only the genus name was accepted, and for sequence identities <95%, the isolates were classified according to family, or order, or as 'unknown fungus' (Rosa et al. 2010). While there are limitations to using ITS for species identification, it is the most widely used region for fungal DNA barcoding (Schoch et al. 2012; Yahr et al. 2016), which is why we adopted this approach. Fungal mother cultures were stored at −80 °C in potato dextrose broth (PDB) containing 15% (v/v) glycerol. Fungi were sub-cultured from the frozen stock to potato dextrose agar (PDA) and incubated at 25°C in the dark prior to use in experiments.

Filter paper-based test

Three round filter papers (Whatman 10312209, Grade 598) were saturated in 2 ml sterile water and placed in a sterile disposable petri dish. *Nicotiana benthamiana* seeds (Research accession RA-4) (Goodin et al. 2008; Wylie et al. 2015) were surface-sterilized in 1% NaOCl for 1 min, rinsed in 70% ethanol 1 min, and serially rinsed in sterile water six times. Five seeds were placed on the soaked filter paper in the petri dish, which was sealed with parafilm (Parafilm® M, P7793, Sigma). Petri dishes were incubated at 25 °C and exposed to a 16 h light and 8 h dark cycle in an inclined position to facilitate downward root movement. At day 9 post-germination, a 5 mm^3 agar plug made from the growing edge of fungal colony was placed along the roots of each seedling and 100 µl of sterile water was added to each seedling. Dishes were sealed again with parafilm and incubated as before. At 10 dpi (days post-inoculation), the parafilm was removed

Table 1 Endophytes used in this study, showing original host species and the organ where it was isolated

Isolate Code	Predicted identity	GenBank Accession code of ITS region	Host species	Tissue origin
E-9	*Chaetomium funicola*	KU059876	*N. occidentalis*	Root
E-11	*Lecythophora sp.-B*	KU059853	*N. simulans*	Root
E-13	*Pleurostomophora richardsiae*	KY582066	*N. simulans*	Leaf
E-13.2	*Penicillium oxalicum*	KU059858	*N. benthamiana*	Stem
E-14	*Lecythophora sp.-A*	KU059832	*N. benthamiana*	Leaf
E-16	*Phanerochaete sp.*	KU059877	*N. occidentalis*	Root
E-17	*Cladosporium sp.*	KU059878	*N. benthamiana*	Root
E-84	*Chaetomium globosum*	KU059836	*N. occidentalis*	Root
E-86	*Acremonium sclerotigenum*	KU059866	*N. occidentalis*	Root
E-93	*Hypomyces sp.*	KU059879	*N. simulans*	Stem
E-100	*Trichoderma asperellum*	KU059963	*N. simulans*	Root
E-106	*Aspergillus carbonarius*	KU059964	*N. occidentalis*	Root
E-119	*Sporothrix schenckii*	KU059903	*N. simulans*	Stem
E-124	*Sarocladium sp.*	KU059839	*N. benthamiana*	Stem
E-126	*Fungal sp.*	KU059837	*N. occidentalis*	Root
E-128	*Cladosporium halotolerans*	KU059905	*N. simulans*	Root
E-131	*Hongkongmyces sp.*	KU059969	*N. benthamiana*	Stem
E-134	*Aspergillus ochraceus*	KU059881	*N. benthamiana*	Leaf
E-135	*Aurantiporus sp.*	KU059953	*N. simulans*	Root
E-139	*Chaetomium sp.-A*	KU059819	*N. occidentalis*	Leaf
E-143	*Acremonium sp.*	KU059970	*N. occidentalis*	Root
E-162	*Cladosporium cladosporioides*	KU059880	*N. benthamiana*	Root
E-172	*Rasamsonia piperina*	KU059908	*N. benthamiana*	Leaf
E-172.1	*Penicillium simplicissimum*	KU059811	*N. benthamiana*	Stem
E-177.1	*Fusarium oxysporum*	KU059856	*N. simulans*	Leaf
E-184.4	*Ascomycota sp.-A*	KU059804	*N. benthamiana*	Stem
E-185.1	*Trichoderma sp.*	KU059806	*N. simulans*	Stem
E-189.2	*Penicillium sp.*	KU059830	*N. occidentalis*	Root
E-202	*Aspergillus niger*	KU059864	*N. occidentalis*	Stem
E-204	*Alternaria sp.-B*	KU059889	*N. simulans*	Root
E-206	*Phlebia acerina*	KU059900	*N. occidentalis*	Root
E-218	*Pleurostoma repens*	KU059915	*N. occidentalis*	Root
E-219	*Apodus oryzae*	KU059868	*N. rosulata*	Stem
E-228	*Trichoderma hamatum*	KU059871	*N. simulans*	Leaf
E-233.1	*Plectosphaerella cucumerina*	KU059875	*N. occidentalis*	Leaf
E-239	*Phoma sp.-A*	KY582068	*N. occidentalis*	Root
E-247	*Botrytis cinerea*	KU059891	*N. benthamiana*	Root
E-263	*Talaromyces islandicus*	KY582069	*N. simulans*	Root
E-271	*Stagonosporopsis cucurbitacearum*	KU059901	*N. simulans*	Leaf
E-277	*Setophoma sp.*	KU059894	*N. occidentalis*	Stem
E-284	*Ascomycota sp.-B*	KU059897	*N. benthamiana*	Root
E-306	*Thozetella sp.*	KY582074	*N. benthamiana*	Root
E-322	*Chaetomium sp.-B*	KY582087	*N. occidentalis*	Leaf
E-341	*Fusarium sp.*	KY582097	*N. benthamiana*	Stem
E-346	*Penicillium citrinum*	KY582100	*N. simulans*	Leaf
E-353	*Setophoma terrestris*	KY582105	*N. occidentalis*	Root
E-373	*Zopfiella latipes*	KY582118	*N. occidentalis*	Root
E-379	*Hyaloscyphaceae sp.*	KY582123	*N. simulans*	Stem

Table 1 (continued)

Isolate Code	Predicted identity	GenBank Accession code of ITS region	Host species	Tissue origin
E-390	*Exserohilum sp.*	KY582132	*N. benthamiana*	Root
E-395	*Neocosmospora rubicola*	KY582133	*N. occidentalis*	Root
E-409	*Gelasinospora saitoi*	KY582141	*N. benthamiana*	Stem
E-414	*Albifimbria verrucaria*	KY582145	*N. simulans*	Root
E-424	*Fusarium equiseti*	KY582151	*N. rosulata*	Leaf
E-428	*Dactylonectria pauciseptata*	KY582155	*N. benthamiana*	Leaf
E-437	*Curvularia tsudae*	KY582161	*N. benthamiana*	Root
E-469	*Paramyrothecium roridum*	KY582183	*N. occidentalis*	Root
E-500	*Phoma sp.-B*	KY582189	*N. benthamiana*	Root
E-503	*Aspergillus quadrilineatus*	KU059917	*N. simulans*	Stem
E-505	*Alternaria alternata*	KU059919	*N. benthamiana*	Leaf
E-506	*Aureobasidium pullulans*	KU059941	*N. benthamiana*	Root
E-507	*Alternaria sp.-A*	KU059920	*N. benthamiana*	Leaf
E-509	*Dothideomycetes sp.*	KU059942	*N. occidentalis*	Root
E-510	*Pleosporales sp.*	KU059922	*N. occidentalis*	Root
E-511	*Edenia gomezpompae*	KU059943	*N. benthamiana*	Root
E-513.1	*Microsphaeropsis arundinis*	KU059924	*N. benthamiana*	Root
E-518.1	*Aspergillus fumigatiaffinis*	KU059926	*N. benthamiana*	Root
E-526	*Bartalinia pondoensis*	KU059948	*N. benthamiana*	Stem
E-531.1	*Aspergillus oryzae*	KU059952	*N. simulans*	Leaf

from the petri dishes to induce slow drying of the filter paper. Seedling responses were checked daily until seedling death. The control treatment was mock inoculation with an agar plug containing no fungus. Each fungal inoculation or control treatment plate was replicated at least four times.

Agar-based test

Screening of fungal cultures was done on potato dextrose agar plates. Agar plugs (5 mm^3) containing mycelia of each fungal isolate were placed in the middle of each petri dish containing 1/4th strength PDA media (each petri dish was filled 2/3rd with media and assumed variation was minimized by replication). *Nicotiana benthamiana* seeds (RA-4) were surface-sterilized as above. Five seeds were plated on each agar plate surrounding the fungal inoculum at similar distance and the petri dishes were sealed with parafilm. The petri dishes were then incubated at room temperature (25 °C) and exposed to a 16 h light and 8 h dark cycle. At day 14, the parafilm was removed to initiate slow drying-up of the medium to impose moisture stress conditions. The seedlings were observed daily and days to seedling death recorded. Controls contained no fungus. Each fungal inoculation or control was replicated at least four times.

Sand-based test

Spore or mycelial suspensions were prepared from 10-day-old fungal cultures growing in 1/4th strength PDB and incubated on a shaker. The mycelial pellicle was washed in sterile water to remove residual broth, then macerated in a blender, and filtered through sterile cotton wool. The number of spores or mycelial fragments was counted using a haemocytometer and diluted to 5×10^4 spores/mycelial fragments mL^{-1}.

Seventeen fungal isolates were screened in a greenhouse. Isolates screened were E-11, E-84, E-86, E-100, E-128, E-162, E-177.1, E-284, E-306, E-373, E-379, E-409, E-424, E-500, E-505, E-507, and E-509 (Table 1). Three-week-old seedlings of *N. benthamiana* grown in steam treated soil were washed to remove soil adhering to roots. Seedlings were inoculated by placing roots in the spore suspension for 4–5 h. Four seedlings were planted per pot, and there were four pots per treatment. Inoculated seedlings were transplanted in steamed washed yellow sand and watered daily. The position of pots was changed at 7 dpi to minimize the influence of microenvironments. At 14 dpi, all seedlings were watered to field capacity and no more water was applied from that day onwards. Plants were scored daily until the shoot tip wilted, which was the point from which they could not recover if water was applied (Engelbrecht et al. 2007; Xu et al. 2008). Days until wilting

of 1st leaf from the bottom, days till wilting of all leaves, and the days to shoot tip wilting were recorded.

Root colonisation

Trypan blue staining was used to identify fungal mycelium within root tissues (Chung et al. 2010). Inoculated seedlings on filter paper were collected 7 day post-inoculation (dpi). Roots were cut into 0.3–0.5 cm segments and cleared with acetic acid:ethanol (1:3, v/v) solution overnight. A second clearing was done by soaking tissues in acetic acid:ethanol:glycerol (1:5:1, v/v/v) solution for 2 h. The samples were subsequently incubated 5 h to overnight in a staining solution of 0.01% (w/v) trypan blue in lactophenol. Stained tissues were rinsed with 60% sterile glycerol and stored in the same until examination. Specimens were examined under an Olympus BX 51 optical microscope (Olympus, Japan). Five segments were assessed per fungal inoculation treatment.

Root segments from *N. benthamiana* seedlings inoculated (in filter paper screening) with the fungal isolates E-162 and E-284 were fixed in 3% glutaraldehyde in 0.025 M phosphate buffer (pH 7.0) overnight at 4 °C. After rinsing three times with the same buffer, they were fixed with osmium tetraoxide in 0.025 M phosphate buffer (pH 7.0) for 1 h at room temperature. After washing in three changes in the same buffer, the specimens were dehydrated in an ascending series of ethanol through 30, 50, 70, 90, and 100% with two changes of each solution, each 10 min. The specimens were incubated in two changes of amyl acetate. After critical point drying with CO_2 (Critical point dryer 11 120, Balzers Union), samples were mounted on stubs and coated with gold (sputting device 07 120, Balzers Union). The specimens were visualized under a scanning electron microscope (JEOL JCM-6000 NeoScope Benchtop SEM).

Plant growth promotion study

Fungal isolates that were used for the water deprivation tolerance study were also used for a growth promotion study. The same methods for inoculation were used, and the same steam-treated washed yellow sand medium was used. This experiment was done twice, with differences in data collection time. In the first experiment, above ground seedling height was measured at 7, 11, and 15 dpi. Since no significant differences were noted at days 7 and 11 in the first experiment, in the second experiment, seedling height measurement began at day 14 and continued until day 26. At day 26, seedlings were harvested and roots were washed to remove adhering sands. The fresh and dry weights of roots and shoots were recorded.

Statistical analysis

Statistical analysis was done using IBM SPSS statistics 21 (SPSS Inc., Chicago, IL, USA). All data sets were tested for normal distribution based on the Shapiro–Wilk test and the significance of treatment effects and the biomass were analysed using one-way ANOVA. If the main effects were significant, treatment means were compared using a post-hoc test "Tukey's HSD (Honestly Significant Difference)". The regression equation, correlation co-efficient, and corresponding *p* value were calculated, and the bar charts, line graphs, and the box plots were created using Microsoft Excel 2016 and its Data Analysis ToolPak.

Results

Effect of endophytes on seedling water deprivation tolerance in vitro

Filter paper screening

Plants co-cultivated with three fungal isolates, viz., unknown fungus-B (E-284), *Cladosporium*

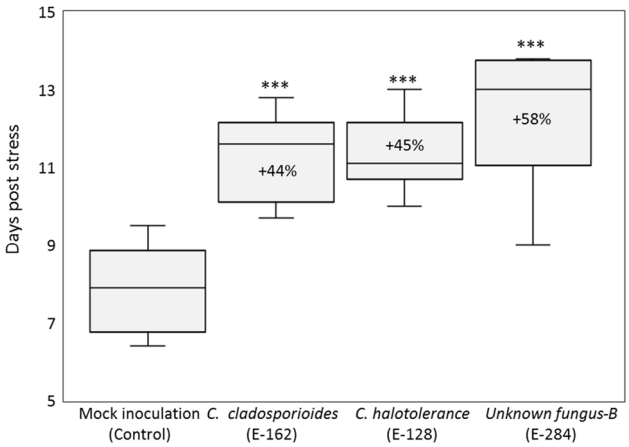

Fig. 1 Boxplots showing the number of days of survival after challenging *N. benthamiana* seedlings inoculated with three fungal isolates with moisture stress in a filter paper test. Significant differences to the control are indicated by an asterisk above the box. The *** denotes that the mean is significantly different from control at $p < 0.001$ obtained from *post-hoc* test using Tukey's HSD (Honestly significant difference) test. The value with a "+" symbol inside the box indicates longer seedling survivability under water stress when colonised with the respective fungal isolate as a percentage over control. Effects of the isolates that significantly increased seedling stress tolerance are shown and the results of other isolates are presented in Supplementary Table 1

cladosporioides (E-162), and *Cladosporium halotolerans* (E-128) were more tolerant to water deprivation than controls, as manifested by their significantly longer survival in water stress. These isolates caused seedlings to survive 58, 45, and 44% longer (E-284, E-128, and E-162, respectively) over mock-inoculated seedlings in water deficit conditions, which were statistically significant at $p < 0.001$ (Fig. 1). Of the 68 isolates tested, more than 60% either killed the seedling upon inoculation or decreased seedling tolerance by causing death of seedlings significantly earlier than controls under water deficit (Fig. 2a). About 34% of the isolates exhibited no measurable influence on moisture stress tolerance (Fig. 2a and Supplementary Table 1). In relation to the tissue origin of the endophytes, all three isolates that significantly enhanced seedling moisture stress tolerances were isolated from roots of either *N. benthamiana* (E-162 and E-284) *or N. simulans* (E-128*)* plants. Most of the isolates obtained from roots or leaves had neutral effects (36 and 50%, respectively), whereas those from the stem often acted as pathogens to the seedlings (69%) (Fig. 2a).

Agar media screening

Four isolates E-84 (*Chaetomium globosum*), E-500 (*Phoma* sp.-B), E-284 (unknown fungus-B), and E-162 (*C. cladosporioides*) enhanced tolerance of *N. benthamiana* seedlings to water deprivation as manifested by their significantly longer survivability (22, 22, 23, and 33%, respectively) over non-inoculated seedlings (Fig. 3) on agar medium. Of the 68 fungal isolates inoculated to *N. benthamiana* seedlings on agar, more than 60% of isolates either became pathogenic (32%) or decreased seedling longevity under water limitation (32%) (Fig. 2b). No isolates from leaf or stem tissues were found to increase seedling moisture tolerance. About 50% of the leaf isolates had neutral effect on seedling moisture stress tolerance. However, isolates from stem tissues were either pathogenic (44%), or showed negative influence on seedling stress tolerance (44%) (Fig. 2b and Supplementary Table 1). All isolates enhancing seedling moisture stress tolerances had been isolated from roots of either *N. benthamiana* (E-162, E-284, and E-500) or *N. occidentalis* (E-84) plants.

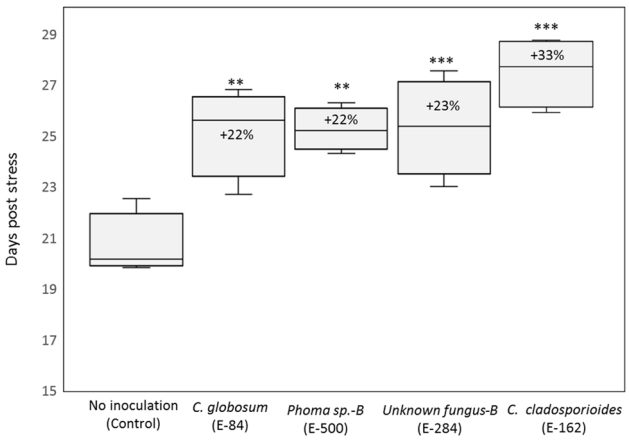

Fig. 3 Boxplots showing the number of days which the seedlings survived after challenging with moisture stress in the agar media screening. Significant differences to the control are indicated by an asterisk above the box. The ** and *** denote that the mean is significantly different from control at $p < 0.01$, and $p < 0.001$, respectively, obtained from post-hoc test using Tukey's HSD (honestly significant difference) test. The value with "+" symbol inside the box indicates longer seedling survivability in stress when colonised with respective isolates as a percentage over control. Effects of the isolates that significantly increased seedlings stress tolerances are shown and the results of other isolates are presented in Supplementary Table 1

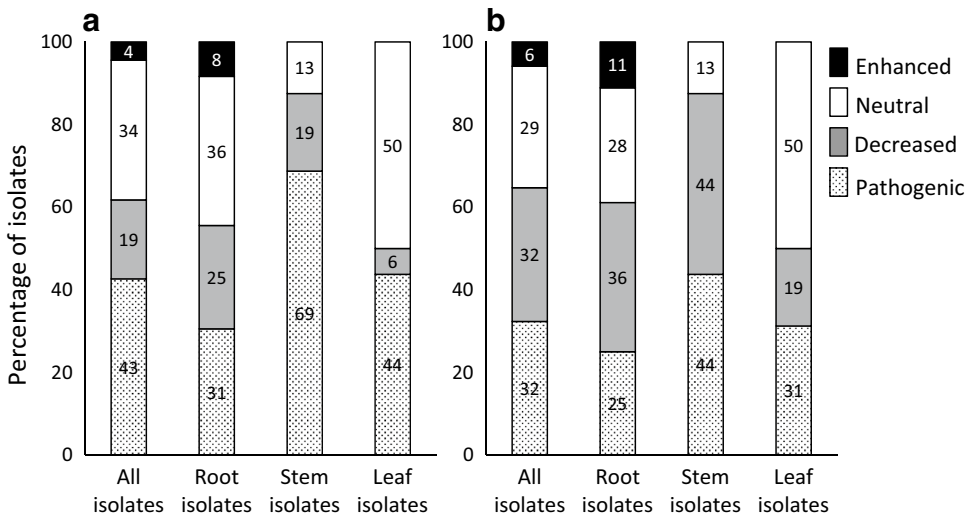

Fig. 2 Percentage of endophytic isolates recovered from different tissues showing differential reactions when co-cultivated with *N. benthamiana* seedlings in **a** filter paper screening and **b** agar media screening to study the moisture deprivation tolerance. The number inside each stack bar indicates the relative percentage of endophyte isolates corresponding to particular tissue with their reaction

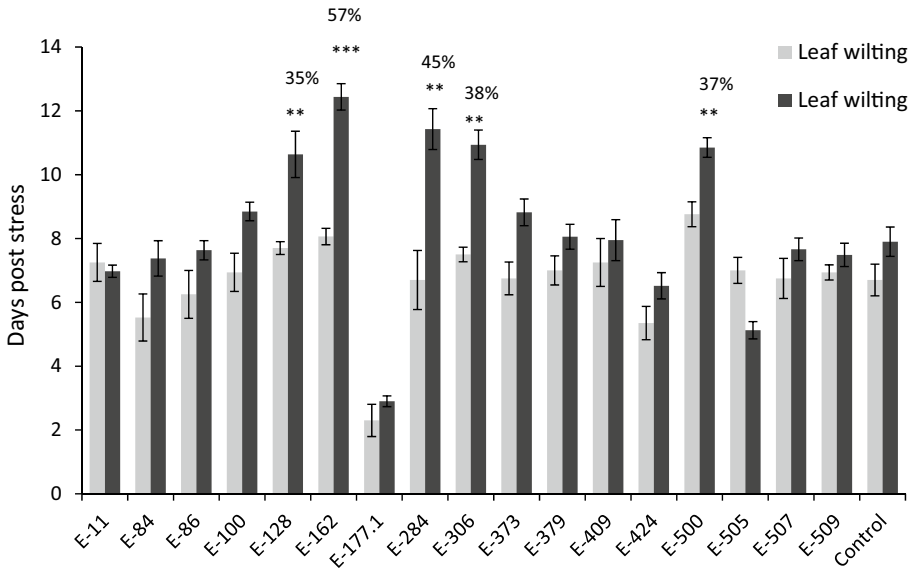

Fig. 4 Multiple *bar plots* indicating number of days to leaf and shoot tip wilting of seedlings inoculated with different isolates and control when challenging moisture stress in glasshouse trial. Significant differences to the control are indicated by an *asterisk* above the *box*. The ** and *** denote that the mean is significantly different from control at $p < 0.01$, and $p < 0.001$, respectively, obtained from post-hoc test using Tukey's HSD (honestly significant difference) test. The above the bar indicates longer seedling survivability in stress when colonised with respective isolates as a percentage over control

Effect of endophytes on seedling water deprivation tolerance in sands in a glasshouse

The longest shoot tip survivability was recorded from the seedlings inoculated with the isolate E-162 (*C. cladosporioides*), which delayed wilting time by 57% ($p < 0.001$) (Fig. 4). This was followed by the seedlings colonised by isolates, viz., E-284 (unknown fungus-B), E-306 (*Thozetella* sp.), and E-500 (*Phoma* sp.-B) and E-128 (*C. halotolerans*), which also enhanced tolerance to water deprivation as manifested by their effect on significantly delaying shoot tip wilting by 35, 37, 38, 45, and 57%, respectively, over non-inoculated control seedlings under water deficit stress (Fig. 4). Of the two parameters considered, time to leaf wilting was not significantly different between treatments and the control seedlings (Fig. 4).

Comparison and correlation between screening methods

There were positive and significant regression lines and correlation co-efficients among the three methods used to screen isolates. The highest correlation ($r = 0.757$) was found between filter paper screening and glasshouse screening, followed by that between the filter paper screen and the agar plate screen ($r = 0.697$), and both were statistically significant at $p < 0.001$ (Fig. 5). The significant positive correlations between the trials indicated that the results of in vitro trials are comparable with the glasshouse trial. Despite significant correlations between the trials, some fungi behaved differently under different screening methods. For example, the fungal isolates E-177.1 (*F. oxysporum*) and E-100 (*T. asperellum*) either killed seeds or seedlings or decreased tolerance in both in vitro trials but did not kill seedlings in the glasshouse trial and even the isolate E-100 did not differ significantly with controls regarding its effect on days to leaf and shoot tip wilting in the glasshouse trial. The fungal strain E-500 (*Phoma* sp.-B) did not confer tolerance in the filter paper test as it did in other tests. The isolates E-84 (*C. globosum*) and E-306 (*Thozetella* sp.) did not significantly enhance water deprivation tolerance to seedlings in any other tests except in the agar media and the glasshouse test. An attempt was made to group isolates based on their effects on water deprivation tolerance using average linkage cluster analysis. The dendrogram grouped isolates into three major clusters in which E-84 (*C. globosum*), E-128 (*C. halotolerans*), E-162 (*C. cladosporioides*), E-284 (unknown fungus-B), and E-500 (*Phoma* sp.-B) formed one cluster, indicating these fungi had a significant influence on increasing tolerance to water deprivation (Fig. 6). The remaining isolates formed another cluster along with the control, indicating they had a neutral influence on the seedling tolerance to moisture deprivation under nearly all conditions. The other isolates showed poor performance in that they either killed the seedlings or increased sensitivity of seedlings to moisture stress (E-86 and E-177.1) or showed variable effects in different trials (E-100).

Endophyte colonisation of roots

Roots of plants inoculated with isolates E-84, E-86, E-128, E-162, E-284, E-306, E-373, E-379, E-424, and E-507 were examined under a light microscope for evidence of endophytic colonisation. Stained roots highlighted the presence of a network of hyphae, most of which penetrated the intercellular spaces of the root cortex (Fig. 7a, arrows). Colonisation of two fungal isolates, namely E-162

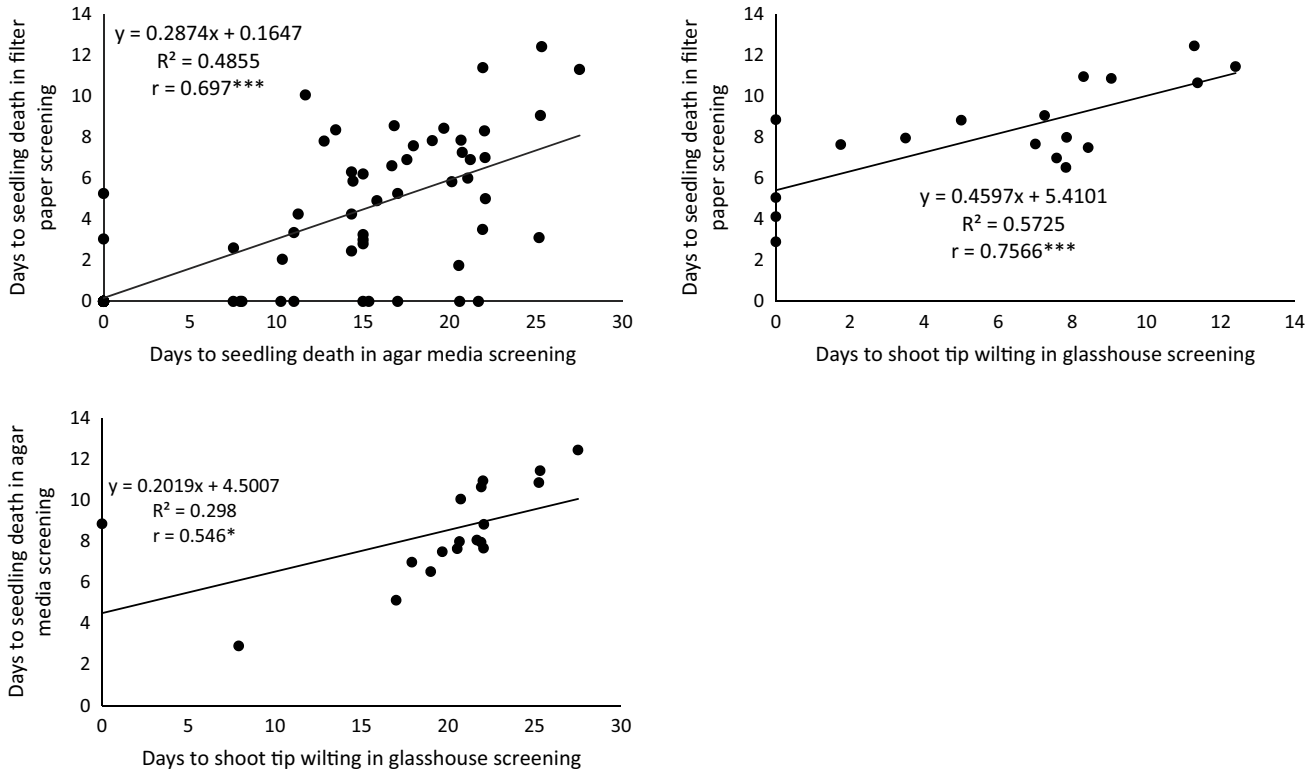

Fig. 5 Relationship between agar media screening (top left) and filter paper screening, glasshouse screening and filter paper screening (top right), and glasshouse trial and agar media screening (lower left). To measure the co-correlation between the in vitro tests, a bivariate Pearson co-relation co-efficient (*r*) was obtained using values of days to death of seedlings inoculated with all 68 fungal isolates and that of the controls after exposed to moisture stress. To obtain the correlation of the in vitro tests with the glasshouse screening, the same correlation co-efficients were obtained using the value of days to seedling death and days to shoot tip wilting, respectively, inoculated with the same 17 isolates for all tests

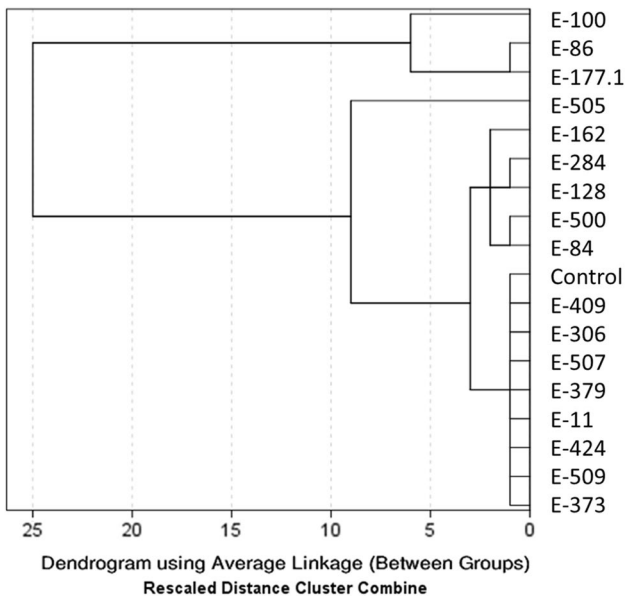

Fig. 6 Clustering of 17 endophyte isolates based on their interaction with seedlings in both in vitro and glasshouse experiments for drought tolerance screening. The average linkage clustering was done using squared Euclidean distance

(*C. cladosporioides*) and E-284 (unknown fungus-B), was confirmed by sections from resin-embedded roots used for scanning electron microscopy (Fig. 7b).

Plant growth promotion by endophytes

Two experiments tested the influence of fungal inoculation on the promotion of growth of *N. benthamiana* seedlings. In experiment 1, no significant variation was observed between inoculated and un-inoculated plants in terms of shoot length at 7 and 11 dpi. At 15 dpi seedlings inoculated with E-162 (*C. cladosporioides*), E-306 (*Thozetella* sp.), and E-424 (*F. equiseti*) showed significantly ($p < 0.05$) increased shoot length (39%) over non-inoculated controls (Fig. 8). Inoculation with other fungal isolates did not significantly increase growth. In experiment 2, consistently higher shoot length was measured at 14–26 dpi from the seedlings inoculated with the above three isolates. At 14 dpi along with these three isolates, E-100 also promoted shoot length growth (Fig. 9). Isolates E-162, E-306, and E-424 resulted in >47% longer

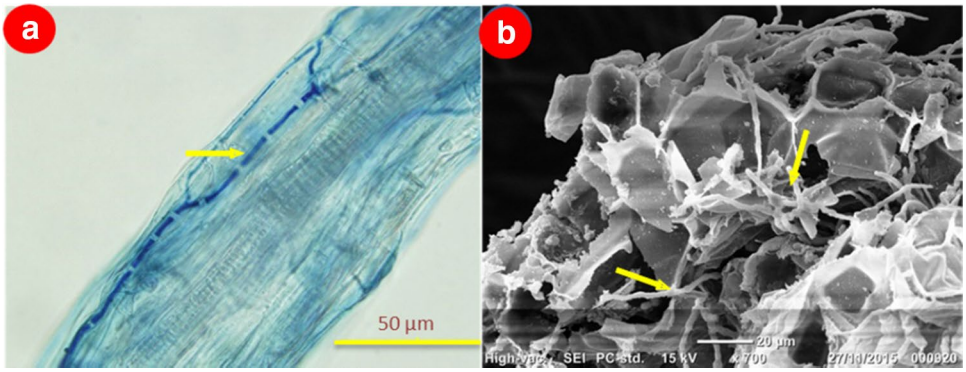

Fig. 7 Colonization by the fungal isolate E-162 (*C. cladosporioides*-B) inside the root tissue of *Nicotiana benthamiana* inoculated in the filter paper screening test. The yellow-coloured arrow head indicates the presence of fungal mycelia as observed under **a** compound microscope stained with trypan blue and **b** scanning electron microscope

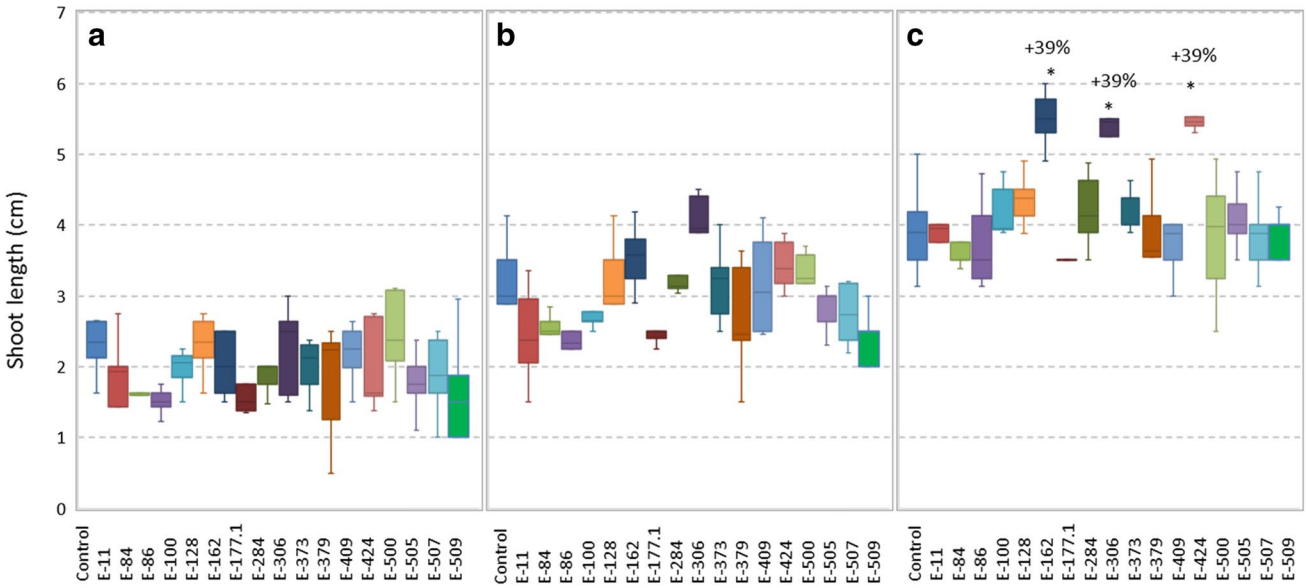

Fig. 8 Box plot shows the effect of inoculation of seventeen selected fungal endophytes on growth of shoots of *N. benthamiana* seedlings growing in pots under glasshouse conditions in experiment 1 at **a** 7 days, **b** 11 days, and **c** 15 days post-inoculation. Significant differences to the control are indicated by an asterisk above the box. The asterisk denotes the mean is significantly different from control at $p < 0.05$ obtained from post-hoc test using Tukey's HSD test. Percentage increase of shoot length over control is presented above the bar for the isolates that showed significant tolerance

shoot length at 20 dpi and >57% longer shoot length at 26 dpi compared to the non-inoculated control ($p < 0.05$) (Fig. 9).

Differences in biomass of seedlings subjected to different treatments also occurred. Inoculation with the isolate E-424 (*F. equiseti*) stimulated the highest shoot biomass, which was 88% higher ($p < 0.05$) than the control (Table 2). In addition, E-162 (*C. cladosporioides*-B) and E-100 (*T. asperellum*) also significantly enhanced (>74%) the shoot biomass (Table 2). Other isolates did not enhance shoot growth of seedlings. With regard to root growth, isolates E-100 (*T. asperellum*), E-162 (*C. cladosporioides*), and E-424 (*F. equiseti*) increased root biomass by 118, 121, and 146%, respectively ($p < 0.05$) (Table 2).

Discussion

Endophytes conferred plant water deprivation tolerance

In the current study, we described two simple in vitro methods and compared them with a glasshouse test as rapid screening methods of endophytic fungal isolates to determine their influence on conferring plant tolerance to water deprivation. Sixty-eight fungal endophytes isolated from wild *Nicotiana* species were screened using these methods. Of these, two isolates, viz., E-162 (*C. cladosporioides*) and E-284 (unknown fungus-B), consistently conferred tolerance to water deprivation in *N. benthamiana* seedlings. Additional isolates also improved plant water deprivation

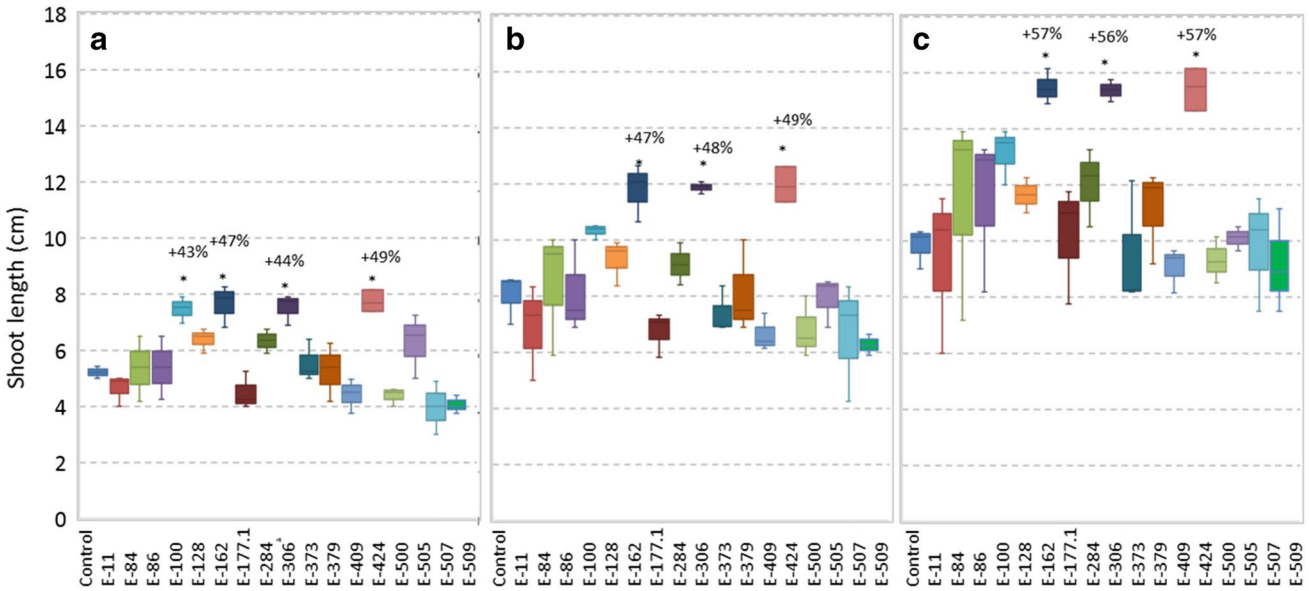

Fig. 9 *Box plot* shows the effect of inoculation of seventeen selected fungal endophytes on growth of shoot of *N. benthamiana* seedlings growing in pots under glasshouse conditions in experiment 2 at **a** 14 day, **b** 20 day, and **c** 26 day post-inoculation. Significant differences to the control are indicated by *asterisk* above the *box*. The *asterisk* denotes that the mean is significantly different from control at $p < 0.05$ and $p < 0.01$, respectively, obtained from a post-hoc test using Tukey's HSD (honestly significant difference). Percentage increase of shoot length over control is presented above the bar for the isolates that showed significant tolerance

Table 2 Effects of inoculation with endophytic fungi on shoot and root dry weights of plants and root/shoot ratios of glasshouse grown *Nicotiana benthamiana* plants

Isolate code	Shoot dry weight (SDW) (g)	% Increase/decrease of SDW over control	Root dry weight (RDW)	% Increase/decrease of RDW over control	Root/shoot ratio (RSR)	% Increase/decrease of RSR over control
E-11	0.184 ± 0.037c	−20.39	0.034 ± 0.0103d	−18.03	0.183 ± 0.026a	−0.4
E-84	0.251 ± 0.048bc	8.64	0.048 ± 0.0116bcd	14.1	0.187 ± 0.009a	2.02
E-86	0.251 ± 0.048bc	8.65	0.048 ± 0.012bcd	14.11	0.188 ± 0.009a	2.02
E-100	0.404 ± 0.014ab	74.9	0.091 ± 0.008ab	118.17	0.225 ± 0.012a	22.88
E-128	0.258 ± 0.009bc	11.63	0.046 ± 0.003bcd	9.52	0.178 ± 0.0083a	−3.1
E-162	0.408 ± 0.008ab	76.62	0.103 ± 0.010a	146.44	0.253 ± 0.027a	38.03
E-177.1	0.144 ± 0.073c	−37.6	0.040 ± 0.008d	−4.7	0.182 ± 0.019a	−0.48
E-284	0.247 ± 0.035bc	6.8	0.048 ± 0.013bcd	15.58	0.189 ± 0.024a	3.27
E-306	0.278 ± 0.047abc	20.61	0.056 ± 0.012abcd	34.69	0.203 ± 0.022a	10.7
E-373	0.192 ± 0.019c	−16.95	0.039 ± 0.008d	−7.85	0.196 ± 0.023a	7.18
E-379	0.250 ± 0.047bc	8.64	0.047 ± 0.011bcd	14.1	0.187 ± 0.0094a	2.01
E-409	0.193 ± 0.021c	−27.7	0.042 ± 0.003cd	−39.48	0.220 ± 0.011a	−15.01
E-424	0.433 ± 0.035a	87.72	0.092 ± 0.018a	120.89	0.230 ± 0.024a	14.59
E-500	0.167 ± 0.029c	−4.35	0.026 ± 0.003d	7.84	0.156 ± 0.012a	10.61
E-505	0.220 ± 0.011c	−15.55	0.045 ± 0.007cd	−6.16	0.203 ± 0.024a	7.82
E-507	0.195 ± 0.016c	−27.7	0.039 ± 0.008d	−39.48	0.198 ± 0.022a	−15.01
E-509	0.167 ± 0.029c	−16.29	0.025 ± 0.002d	0.88	0.156 ± 0.012a	20.27
Control	0.231 ± 0.018c	–	0.042 ± 0.006cd	–	0.183 ± 0.028a	–

In a column, treatment means having a common letter(s) are not significantly different at the 5% level by Duncan's Multiple Range Test. Values in the table refer to mean ± SD, the minus sign (—) indicates that the growth was inhibited. Values are the means of five replications

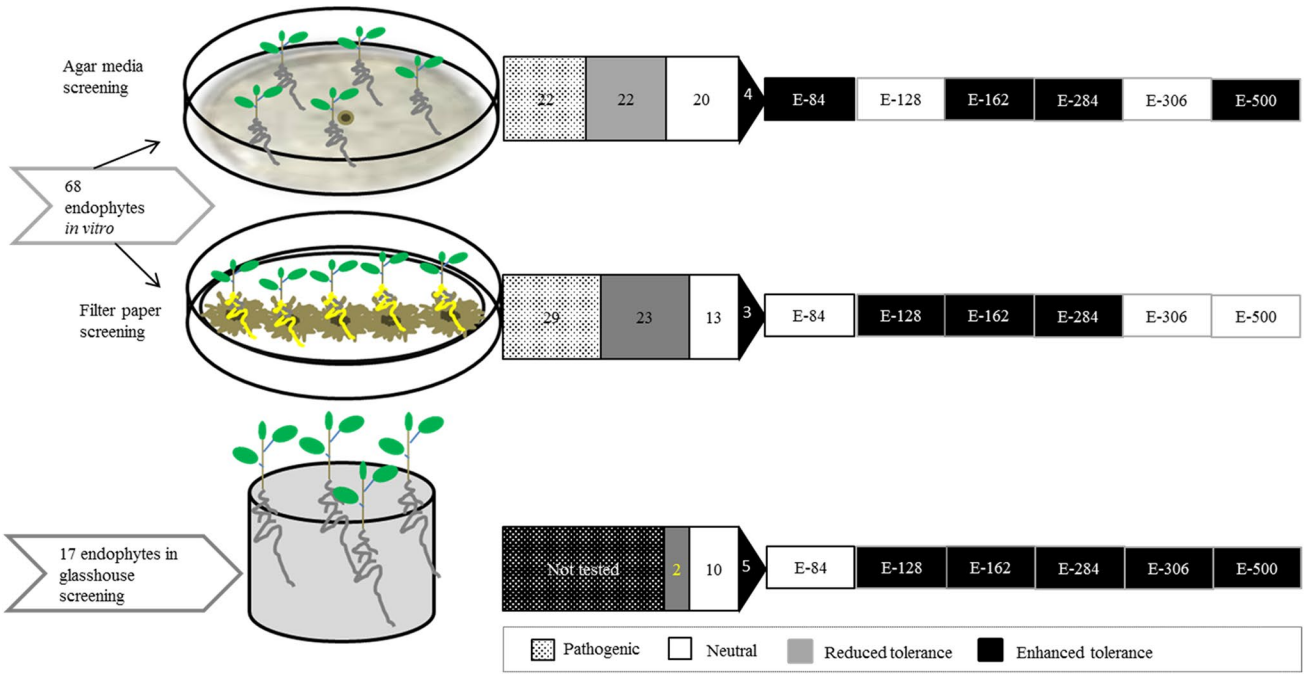

Fig. 10 Cartoon summarizing the results of in vitro and glasshouse screening approaches for identifying fungal endophytes of *Nicotiana* spp. that conferred water deprivation tolerance to seedlings. The number indicates the count of isolates showing respective effects as indicated by *colors* when inoculated onto the *N. benthamiana* seedlings. Isolate codes are presented for those that enhanced water deprivation tolerance in seedling in any of the tests

tolerance, but the results were inconsistent between tests (Fig. 10).

Although a strong and significant correlation was observed between any two screening methods in our study, the result of the filter paper screening method was more strongly reflected in the result of the glasshouse test ($r = 723$, $p < 0.01$), indicating its relative suitability over the agar-based screening method (Fig. 5). The filter paper screening method has additional benefits over the agar-based method. The set-up of the filter paper test is very simple and inexpensive, requiring only petri dishes and filter paper and sterile water. The time required for drying the paper and thus entering the stress phase is much quicker than for the agar test. A measured amount of water is applied in the paper, and therefore, the experimental error between treatments is negligible. Since the paper contains no nutrients, it does not provide any significant nutritional benefit to either fungi or seedling. A possible limitation with this technique is the difficulty in standardizing the amount of inoculum present in each plug. The amount of inoculum present may be a factor in pathogenicity (Dangl and Jones 2001).

On the other hand, the relatively longer time to dry agar makes it slower and less precise than the filter paper method. Although the glasshouse screening technique may be more closely resemble plant growth and plant–microbe interactions in nature, the longer time required for assessment, the possibility of contamination with other microbes, relative heterogeneity of moisture stress application in sand/soil based media, the greater space requirement, and the cost involved makes it less attractive as an initial and rapid screening method of choice in large-scale experiments. The in vitro screening methods developed identified fungi that enhanced water stress tolerance in plants growing in soil. However, in some cases, fungi responded to hosts differently in sand than they did in in vitro tests, indicating that in large-scale screens, some potentially beneficial fungal lines could be discarded at the in vitro test stage because they did not perform well, but they may perform differently in a more complex biological situation in the field. In the present screening methods, only one fungus was inoculated to each plant, which does not closely mimic the natural situation where multiple organisms are interacting internally and externally on the plant. Experiments with *Arabidopsis thaliana* infected with *Piriformospora indica* showed enhanced root and shoot growth in natural soil containing other microbial species, but not when interacting in sterile growth medium (Sirrenberg et al. 2007).

Endophyte-conferred benefits to hosts are dependent upon the host's genetic background and ecological habitats (Rodriguez et al. 2009). *N. benthamiana* is an indigenous Australian species, as presumable are the endophytes used

here. The region where the original hosts, the experimental host and the fungal endophytes were collected experiences periodic extreme heat and drought (Wheeler 2016). We hypothesized that annual plants growing under such conditions would host endophytes if they provided a measure of assistance in enabling survival long enough to set seed. The phenomenon of endophyte-mediated moisture stress tolerance occurs in some grasses (Arechavaleta et al. 1989; Malinowski and Belesky 2000; Hubbard et al. 2014). The effect of endophyte-mediated moisture stress tolerance on non-grasses species is less well studied. One notable example is the study of Redman et al. (2001) who described that the non-grass endophytes *Colletotrichum magna* and *Curvularia protuberata* significantly increased drought tolerance of wheat, tomato, and watermelon plants. The present study identified certain fungal isolates such as E-128 (*C. halotolerans*), E-84 (*Chaetomium* sp.-B), E-162 (*C. cladosporioides*), E-306 (*Thozetella* sp.), E-284 (unknown fungus-B), and E-500 (*Phoma sp.*-B) which positively influenced moisture deprivation tolerance in *N. benthamiana* seedlings. None of these fungal species has previously been reported to increase plant moisture stress tolerance except in a report where a higher incidence (33%) of *C. cladosporioides* was found to be associated with drought affected rice grains than in water grown rice (Morillo et al. 2011). An isolate of *Chaetomium globosum* conferred water stress tolerance to wheat and *Chrysanthemum* plants (Song et al. 2011, Cong et al. 2015).

Endophyte–host interaction and tissue origin

Although fungal taxa of the isolates used in this study had been isolated as endophytes, inoculation of some of these isolates showed pathogenic effects. About 43% of isolates in the filter paper screening and 32% in the agar media screening became pathogenic to seeds or seedlings (Fig. 2a, b). This could be explained by the fact that the phenomenon of endophytism is influenced by various factors and a host-endophyte interaction is subject to change over time (Saikkonen et al. 1998; Schulz and Boyle 2005). This interaction can change from mutualistic to a pathogenic interaction, or vice versa, depending on various factors including host identity, host physiological status, environmental conditions, etc (Millar 1980; Fisher and Petrini 1992). It is also interesting to note that certain endophyte isolates decreased the seedling tolerance to stress in both in vitro trials, although they did not show pathogenicity initially. Previous authors postulated that some fungal endophytes remain latent in plant tissues, but when environmental factors change or host defence mechanisms are compromised, they can become pathogenic (Bayman 2007). Another observation was that most isolates recovered from the leaf tissue showed no influence on seedling stress tolerance.

Growth promotion and water deprivation tolerance as conferred by endophytes

In the current study, few endophyte isolates promoted growth of *N. benthamiana* seedlings under glasshouse conditions. Plant growth promotion mediated by *C. cladosporioides*, *F. equiseti,* and *Trichoderma* spp. was previously reported (Baker et al. 1984; Chang et al. 1986; Hyakumachi and Kubota 2003; Saldajeno and Hyakumachi 2011; Paul and Park 2013). Several mechanisms were proposed to explain the effect of microbes on plant growth promotion (Harman et al. 2004; Ting et al. 2008; Tucci et al. 2011). This study detected isolates that conferred water deprivation tolerance to the host without promoting growth, e.g., E-128 (*C. halotolerans*), E-284 (unknown fungus-B), and E-500 (*Phoma* sp.-B), while E-424 (*F. equiseti*) and E-100 (*T. asperellum*) promoted growth promotion but did not confer water deprivation tolerance. However, the only isolate that promoted the growth of *N. benthamiana* seedlings as well as enhanced water deprivation tolerance consistently in all screening experiments was E-162 (*C. cladosporioides*). It is unclear if growth promotion is related to the stress tolerance response. Providing a fitness benefit to the plant through the promotion of growth could be a way to mediate tolerance to abiotic stress. A larger plant would probably have a greater root surface, and, therefore, can explore the soil to a greater depth and absorb more water. On the other hand, a larger plant would have a greater leaf area from which to lose water.

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References

Abarenkov K, Nilsson RH, Larsson K-H, Alexander IJ, Eberhardt U, Erland S, Høiland K, Kjøller R, Larsson E, Pennanen T, Sen R, Taylor AFS, Tedersoo L, Ursing BM, Vrålstad T, Liimatainen K, Peintner U, Kõljalg U (2010) The UNITE database for molecular identification of fungi-recent updates and future perspectives. New Phytol 186:281–285

Arechavaleta M, Bacon CW, Hoveland CS, Radcliffe DE (1989) Effect of the tall fescue endophyte on plant response to environmental stress. Agron 81:83–90

Bacon CW, White JF (2000) Microbial endophytes. Marcel Dekker, Inc., New York

Baker R, Elad Y, Chet I (1984) The controlled experiment in the scientific method with special emphasis on biological control. Phytopathology 74:1019–1021

Bayman P (2007) Fungal endophytes. In: Kubicek CP, Druzhinina IS (eds) The mycota IV: environmental and microbial relationships, 2nd edn. Springer-Verlag, New York

Carroll G (1988) Fungal endophytes in stems and leaves- from latent pathogen to mutualistic symbiont. Ecology 69:2–9

Chang YC, Baker R, Kleifeld O, Chet I (1986) Increased growth of plants in the presence of the biological control agent *Trichoderma harzianum*. Plant Dis 70:145–148

Chung CL, Longfellow JM, Walsh EK, Kerdieh Z, Van Esbroeck G (2010) Resistance loci affecting distinct stages of fungal pathogenesis: use of introgression lines for QTL mapping and characterization in the maize–*Setosphaeria turcica* pathosystem. BMC Plant Biol 10:103

Clay K, Holah J (1999) Fungal endophyte symbiosis and plant diversity in successional fields. Science 285:1742–1744

Cong GQ, Yin CL, He BL, Li L, Gao KX (2015) Effect of the endophytic fungus *Chaetomium globosum* ND35 on the growth and resistance to drought of winter wheat at the seedling stage under water stress. Acta Ecol Sin 35:6120–6128

Dangl JL, Jones JD (2001) Plant pathogens and integrated defence responses to infection. Nature 411:826–833

Engelbrecht BMJ, Comita L, Condit R, Kursar TA, Tyree MT, Turner BL, Hubbell SP (2007) Drought sensitivity shapes species distribution patterns in tropical forests. Nat 447:80–83

Fisher PJ, Petrini O (1992) Fungal saprobes and pathogens as endophytes of rice (*Oryza sativa* L.). New Phytol 120:137–143

Goodin MM, Zaitlin D, Naidu RA, Lommel SA (2008) *Nicotiana benthamiana*: its history and future as a model for plant-pathogen interactions. Mol Plant Microbe Interact 21:1015–1026

Harman GE, Howell CR, Viterbo A, Chet I, Lorito M (2004) *Trichoderma* species: opportunistic avirulent plant symbionts. Nat Rev Microbiol 2:43–56

Herrera J, Khidir HH, Eudy DM, Porras-Alfaro A, Natvig DO, Sinsabaugh RL (2010) Shifting fungal endophyte communities colonize *Bouteloua gracilis*: effect of host tissue and geographical distribution. Mycologia 102:1012–1026

Hopper SD, Brown AP, Marchant NG (1997) Plants of Western Australian granite outcrops. J R Soc West Austral 80:141–158

Hubbard M, Germida JJ, Vujanovic V (2014) Fungal endophytes enhance wheat heat and drought tolerance in terms of grain yield and second-generation seed viability. J Appl Microbiol 116:109–122

Hyakumachi M, Kubota M (2003) Fungi as plant growth promoter and disease suppressor. In: Arora DK (ed) Fungal Biotechnology in Agricultural, Food and Environmental Application. Marcel Dekker, CRC Press, pp 101–110

Long HH, Schmidt DD, Baldwin IT (2008) Native bacterial endophytes promote host growth in a species-specific manner; phytohormone manipulations do not result in common growth responses. PLoS One 3:e2702

Lugo MA, Reinhart KO, Menoyo E, Crespo EM, Urcelay C (2015) Plant functional traits and phylogenetic relatedness explain variation in associations with root fungal endophytes in an extreme arid environment. Mycorrhiza 25:85–95

Malinowski DP, Belesky DP (2000) Adaptations of endophtye-infected cool-season grasses to environmental stresses: mechanisms of drought and mineral stress tolerance. Crop Sci 40:923–940

Marasco R, Rolli E, Ettoumi B, Vigani G, Mapelli F, Borin S, Abou-Hadid AF, El-Behairy UA, Sorlini C, Cherif A, Zocchi G, Daffonchio D (2012) A drought resistance-promoting microbiome is selected by root system under desert farming. PLoS One 7:e48479

Millar CS (1980) Infection processes on conifer needles. In: Blakeman JP (ed) Microbial ecology of the phylloplane. Academic Press, London, pp 185–209

Miller JD, MacKenzie S, Foto M, Adams GW, Findlay JA (2002) Needles of white spruce inoculated with rugulosin-producing endophytes contain rugulosin reducing spruce budworm growth rate. Mycol Res 106:471–479

Moran NA (2006) Symbiosis. Curr Biol 16:R866–R871

Morillo K, Rodríguez I, Mazzani C, Trujillo de Leal A (2011) Mycobiota associated with grains of rice harvested in cycles of drought and rainfall in Guárico State Venezuela. Fitopatol Venez 24:42–45

Paul D, Park KS (2013) Identification of volatiles produced by *Cladosporium cladosporioides* CL-1 a fungal biocontrol agent that promotes plant growth. Sensors 13:13969–13977

Pepper M, Keogh JS (2014) Biogeography of the Kimberley Western Australia: a review of landscape evolution and biotic responses in an ancient refugium. J Biogeog 41:1443–1455

Redman RS, Freeman S, Clifton DR, Morrel J, Brown G, Rodriguez RJ (1999) Biochemical analysis of plant protection afforded by a nonpathogenic endophytic mutant of *Colletotrichum magna*. Plant Physiol 119:795–804

Redman RS, Dunigan DD, Rodriguez RJ (2001) Fungal symbiosis: from mutualism to parasitism who controls the outcome host or invader? New Phytol 151:705–716

Redman RS, Sheehan KB, Stout RG, Rodriguez RJ, Henson JM (2002) Thermotolerance conferred to plant host and fungal endophyte during mutualistic symbiosis. Science 298:1581

Rodriguez RJ, Redman RS, Henson JM (2004) The role of fungal symbioses in the adaptation of plants to high stress environments. Mitig Adapt Strateg Glob Chang 9:261–272

Rodriguez RJ, Henson J, Van Volkenburgh E, Hoy M, Wright L, Beckwith F (2008) Stress tolerance in plants via habitat-adapted symbiosis. ISME J 2:404–416

Rodriguez RJ, White JF, Arnold AE, Redman RS (2009) Fungal endophytes: diversity and functional roles. New Phytol 182:314–330

Rosa LH, Almeida Vieira MDL, Santiago IF, Rosa CA (2010) Endophytic fungi community associated with the dicotyledonous plant *Colobanthus quitensis* (Kunth) Bartl. (*Caryophyllaceae*) in Antarctica. FEMS Microbiol Ecol 73(1):178–189

Saikkonen K, Faeth SH, Helander M, Sullivan TJ (1998) Fungal endophytes: a continuum of interactions with host plants. Annu Rev Ecol Syst 29:319–343

Saldajeno MGB, Hyakumachi M (2011) The plant growth-promoting fungus *Fusarium equiseti* and the arbuscular mycorrhizal fungus *Glomus mosseae* stimulate plant growth and reduce severity of anthracnose and damping-off diseases in cucumber (*Cucumis sativus*) seedlings. Ann Appl Biol 159:28–40

Schoch CL, Seifert KA, Huhndorf C, Robert V, Spouge JL, Levesque CA, Chen W, Fungal Barcoding Consortium (2012) Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proc Natl Acad Sci USA 109:6241–6246. doi:10.1073/pnas.1117018109

Schulz B, Boyle C (2005) The endophytic continuum. Mycol Res 109:661–686

Schulz B, Wanke U, Draeger S, Aust HJ (1993) Endophytes from herbaceous plants and shrubs: effectiveness of surface sterilization methods. Mycol Res 97:1447–1450

Sherwood M, Carroll G (1974) Fungal succession on needles and young twigs of old-growth Douglas fir. Mycologia 66:499–506

Sirrenberg A, Göbel C, Grond S, Czempinski N, Ratzinger A, Karlovsky P, Santos P, Feussner I, Pawlowski K (2007) *Piriformospora indica* affects plant growth by auxin production. Physiol Planta 131:581–589

Song W, Liu X, Cai X, Sun D, Dai C (2011) Effect of PEG stress on plantlets of *Chrysanthemum morifolium* induced by endophytic botrytis sp. (c1) and *Chaetomium globosum* (c4). Zhongguo Zhong Yao Za Zhi 36:302–306

Stone JK, Polishook JD, White JRJ (2004) Endophytic fungi. In: Mueller G, Bills GF, Foster MS (eds) Biodiversity of fungi: inventory and monitoring methods. Elsevier, Burlington, pp 241–270

Strobel GA, Dirksie E, Sears J, Markworth C (2001) Volatile antimicrobials from a novel endophytic fungus. Microbiol 147:2943–2950

Ting ASY, Meon S, Kadir J, Radu S, Singh G (2008) Endophytic microorganisms as potential growth promoters of banana. Bio Control 53:541–553

Tucci M, Ruocco M, De Masi L, De Palma M, Lorito M (2011) The beneficial effect of Trichoderma spp on tomato is modulated by the plant genotype. Mol Plant Pathol 12:341–354

Wende R (1997) Aspects of the fluvial geomorphology of the Eastern Kimberley Plateau Western Australia. Ph.D. Dissertation, University of Wollongong

Wheeler HM (2016) *Nicotiana occidentalis.* FloraBase. Western Australian Government Department of Parks and Wildlife https://florabase.dpaw.wa.gov.au/browse/profile/6979#. Accessed 24 Oct 2016

White TJ, Bruns TD, Lee SB, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic Press, Cambridge, pp 315–322

Wylie SJ, Zhang C, Long V, Roossinck MJ, Koh SH, Jones MGK, Iqbal S, Li H (2015) Differential responses to virus challenge of laboratory and wild accessions of Australian species of *Nicotiana*, and comparative analysis of RDR1 Gene sequences. PLoS One 30(10):e0121787. doi:10.1371/journal.pone.0121787

Xu P, Chen F, Mannas JP, Feldman T, Sumner LW, Roossinck MJ (2008) Virus infection improves drought tolerance. New Phytol 180:911–921

Yahr R, Schoch CL, Dentinger BTM (2016) Scaling up discovery of hidden diversity in fungi: impacts of barcoding approaches. Philos Trans R Soc B Biol Sci 371:20150336

Yang J, Kloepper JW, Ryu CM (2009) Rhizosphere bacteria help plants tolerate abiotic stress. Trends Plant Sci 141–4:92

Supplementary Table 1: Mean days to death of seedling after applying moisture stress to seedling colonised by different fungal isolates and the control along with their corresponding rank in *in vitro* trial

	Filter paper screening			**Agar media screening**		
Sl No	Isolates code	Mean days to death of seedling	Rank*	Isolates code	Mean days to death of seedling	Rank*
1	E-284	12.4	a	E-162	27.52	a
2	E-128	11.38	ab	E-284	25.33	ab
3	E-162	11.29	ab	E-500	25.25	ab
4	E-93	10.05	a-c	E-84	25.18	ab
5	E-500	9.05	b-d	E-373	22.07	bc
6	E-409	8.55	b-e	E-507	22.05	bc
7	E-509	8.43	b-e	E-306	22.02	bc
8	E-131	8.35	b-f	E-128	21.93	b-d
9	E-306	8.3	b-f	E-409	21.91	b-d
10	Mock inoculation (control)	7.84	c-g	E-379	21.67	b-d
11	E-424	7.83	c-g	E-93	21.2	b-e
12	E-395	7.8	c-g	E-14	21.05	b-f
13	E-11	7.58	c-g	E-322	20.73	c-f
14	E-84	7.25	c-h	Control	20.66	c-f
15	E-507	7	c-h	E-143	20.58	c-f
16	E-13.1	6.9	d-h	E-86	20.53	c-f
17	E-379	6.9	d-h	E-206	20.13	c-g
18	E-16	6.6	d-i	E-509	19.67	c-h
19	E-271	6.3	d-j	E-424	19	c-i
20	E-106	6.2	d-k	E-11	17.91	c-j
21	E-14	6	d-l	E-390	17.53	d-k
22	E-506	5.85	e-m	E-505	17	e-l
23	E-206	5.83	e-m	E-518.1	17	e-l
24	E-172.1	5.25	f-n	E-139	16.8	e-l
25	**E-228**	5.25	f-n	E-172.1	16.67	f-l
26	E-189.2	5	g-o	E-126	15.8	g-m
27	E-263	4.9	g-o	E-119	15.33	h-n
28	E-247	4.25	h-p	E-414	15	i-n
29	E-518.1	4.25	h-p	E-503	15	i-n
30	E-139	3.5	i-p	E-511	15	i-n
31	E-204	3.35	j-p	E-526	15	i-n
32	E-526	3.25	j-p	E-16	14.99	i-n
33	E-322	3.1	k-p	E-506	14.42	j-o
34	E-135	3.04	l-p	E-134	14.33	j-o
35	E-503	3	l-p	**E-228**	14.33	j-o
36	E-511	2.8	m-p	E-437	14.33	j-o
37	E-346	2.6	n-p	E-277	13.42	k-o
38	E-437	2.45	n-p	E-395	12.75	l-o

39	E-177.1	2.05	Op	**E-428**	11.67	m-p
40	E-86	1.75	p	E-510	11.25	n-p
41	E-9	-	-	E-184.4	11	n-p
42	E-13.2	-	-	E-204	11	n-p
43	E-17	-	-	E-172	10.33	op
44	**E-100**	-	-	E-189.2	10.25	op
45	E-119	-	-	E-346	8	p
46	E-124	-	-	E-271	7.9	p
47	E-126	-	-	E-185.1	7.5	p
48	E-134	-	-	E-9	-	-
49	E-143	-	-	E-13	-	-
50	E-172	-	-	E-13.2	-	-
51	E-184.4	-	-	E-17	-	-
52	E-185.1	-	-	**E-100**	-	-
53	E-202	-	-	E-106	-	-
54	E-218	-	-	E-124	-	-
55	E-219	-	-	E-131	-	-
56	E-233.1	-	-	E-135	-	-
57	E-239	-	-	E-177.1	-	-
58	E-277	-	-	E-202	-	-
59	E-341	-	-	E-218	-	-
60	E-353	-	-	E-219	-	-
61	E-373	-	-	E-233.1	-	-
62	E-390	-	-	E-239	-	-
63	E-414	-	-	E-247	-	-
64	**E-428**	-	-	E-263	-	-
65	E-469	-	-	E-341	-	-
66	E-505	-	-	E-353	-	-
67	E-510	-	-	E-469	-	-
68	E-513.1	-	-	E-513.1	-	-
69	E-531.1	-	-	E-531.1	-	-

*the ranking was done on the basis of a multiple comparison test obtained using Tukey's HSD test. The mean sharing similar letter in the rank column are did not differ statistically from each other and those with different letters did.

**n/a indicates that the colonisation of these isolates was lethal to seeds upon or prior germination (agar media screening) or seedling upon inoculation (filter paper screening) and therefore application of moisture stress was not possible.

Chapter 5

Metabolic responses of endophytic *Nicotiana benthamiana* plants experiencing water stress

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Research Paper

Metabolic responses of endophytic *Nicotiana benthamiana* plants experiencing water stress

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ABSTRACT

Endophytic fungal colonization may influence how plants respond to environmental stress. Two promising fungal isolates, one resembling *Cladosporium cladosporioides* and another unidentified ascomycetous fungus, isolated from wild *N. benthamiana* plants in northern Australia were inoculated to plants of the research accession of *N. benthamiana* (RA-4). Inoculated seedlings were grown under adequate or water deficit conditions. We examined leaf metabolites using gas chromatography-mass spectrometry (GC–MS) to compare levels of sugars, sugar alcohols, amino acids and other metabolites at various stages of plant growth and stress application. Ninety-three metabolites were detected in leaves, including 20 sugars, 13 sugar alcohols, 21 amino acids, 29 organic and fatty acids and ten other compounds. Endophyte colonization caused significantly differential accumulation of 17–21 metabolites when the plants were grown under well-watered condition. The presence of endophytes under water stress conditions caused differential accumulation of cytosine, diethylene glycol, galactinol, glycerol, heptadecanoate, mannose, oleic acid, proline, rhamnose, succinate, and urea. Accumulation of these metabolites suggests that fungal endophytes influence plants to accumulate certain metabolites under water-stress. Further, plants colonised by the two different endophytes tested, showed some differences in the metabolites they accumulated. Colonization with endophytic fungi significantly increased root dry mass and relative water content in plants under severe water stress condition, suggestive of a symbiotic relationship between these fungi and *N. benthamiana* plants, a species adapted to the hot and unpredictable soil moisture conditions of northern Australia. We reveal that endophyte colonization triggers reprogramming of host metabolism and indices changes in host development. This study sheds lights on the mechanisms underlying increased tolerance to water stress in plants conferred by fungal endophytes. Fungal endophytes have the potentials for application to increase the inherent water stress tolerance of crops.

1. Introduction

In nature, plants are exposed to various environmental stresses that may have significant impacts on size, lifespan and fecundity. Water deficit (commonly referred to as drought) is one of the most widespread abiotic stresses limiting plant growth in many parts of the world (Chaves et al., 2003; Lawlor, 2012). Current climate change models predict that soil water availability in some regions will be significantly reduced (Stocker, 2014). Plant strategies to cope with water stress can broadly be divided into tolerance and avoidance (Claeys and Inzé, 2013). Plant drought tolerance involves detoxification of reactive oxygen species (ROS) and the accumulation of solutes called osmolytes such as sugars, the amino acid proline, and other compounds that maintain the cellular turgor pressure required for cell expansion under stress conditions (Chen and Jiang, 2010; Claeys and Inzé, 2013; Hoekstra et al., 2001; Morgan, 1984; Rodriguez and Redman, 2005). Also, microbial symbionts may play a role in plant adaptation to stress (Coleman-Derr and Tringe, 2014; Rodriguez et al., 2009).

Fungal endophytes live in association with plants while inducing no visible symptoms of pathogenicity. In some cases, fungal endophytes confer benefits to plants exposed to water scarcity (Upson et al., 2009). Possibly the most well-known example endophyte-mediated plant water stress tolerance is the mutualism of tall fescue and perennial ryegrass with the grass endophyte (Class 1, *sensu*: Rodriguez et al. 2009)

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Epichloë coenophiala (Kane, 2011). Non-grass fungal endophytes (Class 2, *sensu*: Rodriguez et al. 2009) have also been reported to improve plant tolerance under water deficit stress (Khan et al., 2013, 2015; Redman et al., 2011; Waqas et al., 2012). Although the role of fungal endophytes to mediate plant water stress tolerance has been described in several studies, the key mechanism(s) are incompletely understood. Endophyte colonization causes (a) increased growth and development (Khan et al., 2013; Redman et al., 2011) (b) enhanced osmotic adjustment (Grover et al., 2001), (c) increased gas exchange and water use efficiency (Bae et al., 2009; Elmi and West, 1995) and (d) improved defence against oxidative stress in host plants (Azad and Kaminskyj, 2016). Metabolomic studies in *Festuca arundinacea* (tall fescue) colonised with *E. coenophiala* have reported a significant impact of the endophyte on primary and secondary metabolism under water deficit conditions (Nagabhyru et al., 2013; Rasmussen et al., 2008). Stress-induced accumulation of sugars, sugar alcohols, amino acids, and mineral ions has been documented in plants (Chen and Jiang, 2010; Hanson and Smeekens, 2009; Loescher, 1987). Class 1 endophytes have a significant effect on the accumulation of simple sugars in the plant under water stress. Effects of the endophyte on accumulations of amino acids, organic acids other metabolites in plants under water stress have not been well characterised.

To the best of our best knowledge, no study has yet been undertaken to investigate the role of Class 2 endophytes on water stress tolerance of hosts at the metabolic level. Our preliminary (Dastogeer et al., 2017b) study identified two ascomycetous fungal endophytes isolated from indigenous Australian *Nicotiana* plants growing in arid conditions that provided significantly improved water stress tolerance in *Nicotiana benthamiana* plants tested in both *in vitro* and greenhouse trials. Analysing these endophyte effects on host plants at the metabolite level using high-throughput metabolite profiling is an approach to elucidate the mechanisms of endophyte-enhanced plant growth and survival under water deficit conditions. The objectives of this study were to investigate and compare the metabolic responses of endophytic and non-endophytic *N. benthamiana* plants grown under well-watered, moderately water stressed and severely water stressed conditions.

2. Materials and methods

2.1. Fungal isolates

Two fungal endophytes, named E-162 (GenBank ID: KU059880) and E-284, (GenBank ID: KU059897) were selected from our previous experiments (Dastogeer et al., 2017b) which indicated that they increased water stress tolerance of *N. benthamiana* plants both *in vitro* and in greenhouse conditions. These endophytes were originally isolated from the roots of wild *Nicotiana benthamiana* plants species collected in northern Western Australia. The strains were isolated from surface-sterilized plant tissue on 0.1 × potato dextrose agar (PDA) medium from symptomless root tissue (Dastogeer et al., 2017a). Combined morphological (colony appearance, mycelial texture, hyphae and conidial structure, etc.) and molecular techniques were used to help in identification of the fungi studied. Molecular identification was done by Sanger sequencing of PCR amplified products of the ITS regions rDNA of the fungi using the universal primers ITS1 or ITS1F with ITS4 (Gardes and Bruns, 1993; White et al., 1990). Based on the closest match (> 98% similarity) from a Blastn search of the NCBI nucleotide UNITE (Abarenkov et al., 2010) database, isolate E-162 was identified as closely resembling isolates of *Cladosporium cladosporioides,* whereas E-284 was not identified below the subkingdom level of Ascomycota. The closest matching sequences (≥95%) to isolate E-284 were all labeled as unidentified Ascomycota (Supplementary Fig. 1). Since the isolate did not sporulate on PDA media, we went no further in describing E-284 in greater detail.

Fungal cultures were stored at −80 °C in potato dextrose broth (PDB) containing 15% (v/v) glycerol. Fungi were sub-cultured from the frozen stock to potato dextrose agar (PDA) and incubated at 25 °C in the dark, prior to use in experiments.

2.2. Inoculum preparation

For inoculum preparation, the fungi were seeded in 250 ml Erlenmeyer flasks containing 0.1 × potato dextrose broth (PDB), with continuous shaking at 100 rpm at 25 °C. Seven-day-old mycelial pellets were harvested and macerated in a liquid homogenizer-mixer into uniform fragments. The suspension was filtered through sterile absorbent cotton wool plugs to remove large hyphal fragments. The inoculum concentration was determined by using a haemocytometer with a compound microscope and was adjusted to 5×10^4 fragments mL^{-1} through sterile dilution. This inoculum concentration was chosen based on our previous findings (Dastogeer et al., 2017b). To assess the viability of the fragments, a germination test was carried out on PDA after incubation for 48 h at 25 °C.

2.3. Stress treatment

Seeds of the research accession of *N. benthamiana* (research accession 4 (RA-4)) (Goodin et al., 2008; Wylie et al., 2015) were surface-sterilised by submerging them in 3% sodium hypochlorite for 3 min and then in 75% ethanol for 2 min, then rinsed with sterile water three times. The seeds were sown in steam-treated sand for germination. Steam sterilization of soil was done at 99 °C for 4 h twice with a gap of 48 h in between steaming sessions to eliminate other fungi. Three-week-old seedlings were removed from the soil, and the roots were washed to remove adhering soil. For inoculation, the seedling roots were submerged in the inoculum suspension for 5 h before planting. The control seedlings were immersed in sterile distilled water for 5 h. Treated seedlings were transplanted in steam-treated sand in pots (10 cm in diameter and 12 cm deep). We used sterilised media to see the effect only these strains in absence of other microbes. In non-sterilised soil the effect of these fungal stains could have been modified by the interaction with indigenous microbial communities and we would not be able to measure the sole effect of these strains. To improve the efficacy of inoculation, the inoculum suspension was also applied to the root-zone of the seedling at the time of planting. The root-zone application was done by spraying of the inoculum suspension into depressions in the sand where the seedlings were to be planted. The plants were maintained in a greenhouse at 22–24 °C, 60 ± 5% RH, and with natural photoperiod. Seedlings were watered to drip point for three weeks before stress treatment was imposed to allow acclimation of plants after transplanting and to allow sufficient growth of plants. The pots were arranged randomly; the position of pots was rearranged every week to minimise environmental variation within the greenhouse, until the imposition of stress. Plant infection status was checked at the end of the experiments by culturing surface-sterilized roots and identifying isolated fungi by microscopy as well as by sequencing of the ITS region as described above.

2.4. Sampling

The first sampling was done at 21 days post-inoculation (dpi), referred to as harvest one (H1, 0 days post-stress). In the following days, half of the pots from each inoculated and non-inoculated group received stress by withholding watering and other half continued to receive watering as before. There were four plants per pot, five pots per replication and four replications in each endophyte or non-endophyte treatment under well-watered condition. The same number of plants was treated with water stress. When the non-inoculated groups started showing stress symptoms as manifested by wilting of one or two leaves from the bottom at 4 days post-stress (at 25 dpi), we considered it as moderate water stress and harvested the second sample (H2). As the stress continued, the non-inoculated group showed shoot tip wilting.

We considered this stage as severe water stress and collected the final sample (H3) at 8 d post-stress imposition (29 dpi). The 3rd and 4th mature leaves from the top of a single plant per pot were harvested during each sampling (a total of five seedlings from each replication from each endophytic and non-endophytic plants under both well-watered and stressed groups). Leaves sampled for metabolite extraction were wrapped in aluminium foil and frozen in liquid nitrogen immediately after harvest and then stored at −80 °C.

2.5. Relative water content and biomass

One seedling in each pot was harvested at the final sampling to measure the relative water content (RWC) of the leaves and shoot and root biomass. Seedlings were divided into roots and shoots, and soil was washed from roots by hand. Samples were desiccated for 48 h at 80 °C, and dry weight (mg) was recorded. Because of limitations of number of plants, we measured RWC at the end only. We considered other physiological parameter e.g. wilting behaviour to determine the stress level as described under "Sampling", which we thought could be an alternative or supplementary to RWC. RWC of the leaf samples was evaluated based on the method of Gonzalez (2003). One leaf from each plant was weighed just after sampling to obtain fresh weight (W_f). Leaves were then soaked in 100 ml of distilled water for 24 h at 4 °C in the dark, and turgid weight (W_t) was noted. Subsequently, samples were dried in the oven at 70 °C for 24 h, and dry weight (W_d) was measured. RWC was determined as: $RWC = (W_f - W_d)/(W_t - W_d) \times 100$.

2.6. Metabolite extraction and derivation

Leaf metabolite extraction was done following the protocol of Du et al. (2012) with modifications. Leaf tissue from five seedlings from five pots in one replication were mixed for each endophyte treatment and water treatment. Leaf tissues (100 mg) from the mix which contained similar amount (20 mg) from each five seedlings was pulverised in liquid nitrogen and extracted in a 1.4 mL solution of methanol:water:ribitol (20:2:1). The ribitol was added in the extraction solution as an internal standard and was prepared in water (0.4 mg mL^{-1}). The extract was incubated in a thermo-mixer (Eppendorf ThermoMixer® C) with shaking (500 g) at ambient temperature for an hour and then at 80 °C for 20 min. The extraction solution was centrifuged for 3 min at 3000*g*, the supernatant was transferred to new tubes, and 1.4 ml of water and 0.75 ml of chloroform was added. The mixture was vortexed and centrifuged for 2 min at 3000 × *g*. The polar phase (methanol/water) was decanted into 1.5 ml high-performance liquid chromatography vials and dried in a centrifugal concentrator (Labconco Corporation, Kansas City, MI). Before methoximation, the polar phase was dried under nitrogen gas. The dried polar phase was methoximated for 90 min at 30 °C with 50 µl of 20 mg mL^{-1} methoxyamine hydrochloride in pyridine (made fresh) and was trimethylsilylated (TMS) with 30 µl MSTFA (*N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane) and 40 ml pyridine for 30 min at 70 °C whilst shaking at 200 × g. The reaction was allowed to sit for 30 min at room temperature before being subjected to Gas chromatography–mass spectrometry (GC–MS) analysis.

2.7. Gas chromatography–mass spectrometry analysis

Extracted metabolites were analysed on an Agilent HP5890II GC fitted with an Agilent 5972 MSD system. A derivatized aliquot (1 µl) of the extracts was injected into a Varian Factor 4 capillary column (VF-5ms, 30 m × 0.25 mm, 0.25 µm plus 10 m EZ-Guard) (Agilent J & W Scientific, Folsom, CA). The inlet temperature was set at 300 °C. After a 5-min solvent delay, initial GC oven temperature was set at 70 °C for 1 min, ramped at 1 °C/min until 76 °C and then the GC oven temperature was raised to 325 °C at a rate of 6 °C/min with a final hold for 8 min. The MSD transfer line heater was set to 300 °C, MS quadruple at 150 °C and the ion source temperature was adjusted to 230 °C. Hydrogen was used as the carrier gas with a constant flow rate set at 1 ml min^{-1}.

2.8. Data analysis

The spectral data files were processed using AMDIS (Automated Mass Spectral Deconvolution and Identification System) software for metabolite identification. Metabolites were identified by retention index and spectral comparison using the commercial mass spectral library NIST 14 (National Institute Standard and Technology). Metabolites that had a confirmed identity and were present in the majority of samples were used for further data analysis; other compounds present at a low abundance across the dataset were not used for statistical analysis. Data were normalised by dividing the response values for each compound in each sample by the mean value of the reference sample for the metabolite in the sample batch run. The reference sample was made by combining leaf tissues from a selection of the entire sample range (covering all time points and treatments) that was extracted in bulk, and four of these reference samples were run for each batch. Relative response ratios were calculated based on internal standard extracted per gram fresh weight for each analysed metabolite as described by Roessner et al. (2000). Data were tested for normality, and a multivariate ANOVA was performed on log transformed values using SPSS version 24 (IBM SPSS, USA). In the ANOVA for each of the metabolites the response ratio was the dependent variable. There were three factors (dependent variables) such as the water treatment (two levels; well-watered, stressed) endophyte status (three levels; inoculation with NE, E-162, E-284) and the days to sampling (three levels; H1, H2 and H3). Both the main effects and possible interaction effects were determined. Since the sampling was done in a destructive way, repeated measures ANOVA was not possible. The response ratios were autoscaled to improve the biological interpretability (van den Berg et al., 2006) and a global principal component analysis (PCA) was initially applied to the whole dataset to verify statistically the differences between the metabolic profiles of the watered and stressed tissues, and to identify the main metabolites responsible for the differences. In a subsequent step, partial least squares discriminant analyses (PLS-DA) using regressions were done separately for the watered, moderately and severely stressed samples with both endophytic and non-endophytic samples using Metaboanalyst 3.0 (Xia and Wishart, 2016). The x-fold changes of metabolite were presented for endophytic samples relative to non-endophytic samples at H1 and for the stressed samples at each time point relative to respective watered control at each time point as well as for the stress-treated endophytic samples relative to stress-treated non-endophytic samples at each time point. Statistical differences in metabolites between treatment means were measured by Mann-Whitney test algorithms at $p < 0.05$ probability. Plant metabolic pathway map was constructed in VANTED (Junker et al., 2006) using normalised log-transformed response ratio of the metabolites identified.

3. Results

3.1. Overview of metabolite analysis results

Fungal re-isolation data at the end of the experiment showed that there was successful recovery of both fungal strains in the inoculated plant roots. Our investigation confirmed that infection was successful for most of the sample for both strains which was about 90% and 82% for E-162 and E-284, respectively. We did not find any major differences in infection rate due to stress treatment. Non-infection of some seedlings was present, and this may influence the results to a small extent. We did not check the fungal growth in shoots, but our previous study *in vitro* (Dastogeer et al., 2017b) did show that any of the fungal

Table 1
Three-factor ANOVA [F_{df} (4, 54)] values of all metabolites detected from endophytic and non-endophytic *N. benthamiana* plant grown under well watered, moderately and severely stressed conditions.

Sl No.	Metabolites	WT*	E	D	WT × D	WT × E	D × E	WT × D × E
		F	F	F	F	F	F	F
	Sugars							
1	Arabinose	70.08	232.26	9.73	9.63	26.24	2.97	3.91
2	Cellobiose	27.91	2.09	1.71	5.58	1.94	0.28	0.58
3	Fructose	351.90	15.43	18.74	36.33	5.66	1.61	2.88
4	Fucose	75.18	1.54	3.03	1.96	1.00	0.50	0.23
5	Galactopyranose	27.88	9.60	1.94	0.90	5.78	0.29	0.26
6	Galactose	8.70	0.06	0.02	0.01	0.08	0.05	0.20
7	Gentibiose	1.63	5.91	13.61	13.56	2.54	3.13	2.15
8	Glucose	115.06	11.51	9.95	21.09	9.61	1.20	2.23
9	Maltose	21.63	19.71	10.71	0.27	0.88	1.18	0.62
10	Mannose	0.19	44.79	2.95	0.00	1.18	0.36	0.56
11	Melibiose	7.29	2.36	3.18	2.13	1.40	0.18	0.76
12	Psicose	5.20	11.61	2.18	2.82	0.18	0.59	0.06
13	Raffinose	16.00	12.48	8.40	6.07	7.13	2.41	8.27
14	Rhamnose	4.22	8.20	3.76	1.87	8.66	0.19	1.19
15	Ribose	0.08	2.72	2.03	0.29	4.05	0.77	2.56
16	Sucrose	71.21	0.15	18.55	22.69	2.29	1.81	1.80
17	Talose	6.18	4.49	1.24	0.08	2.56	1.13	1.48
18	Threose	1.58	0.77	0.56	0.80	0.12	0.18	0.16
19	Trehalose	272.97	2.28	20.29	47.39	1.01	1.72	1.56
20	Xylose	5.75	10.41	0.90	0.95	7.14	1.95	3.06
	Organic acid							
1	Aconitate	2.00	2.68	7.55	2.27	0.45	4.10	0.58
2	Arachidonic acid	1.63	0.30	1.93	0.00	0.42	0.21	0.20
3	Ascorbate	6.11	1.03	9.06	0.50	0.16	2.72	0.19
4	Benzoate	11.12	0.10	0.59	1.75	0.93	0.35	0.36
5	Citrate	91.78	0.04	7.72	8.74	2.06	1.60	0.30
6	Docosanoate	69.11	0.74	10.28	18.08	1.28	1.07	3.07
7	Dodecanoate	61.37	0.67	8.33	15.56	0.27	0.23	0.50
8	Fumarate	110.55	12.20	7.99	1.55	0.95	2.88	2.52
9	Gluconate	33.82	2.77	10.78	15.76	2.22	0.59	0.88
10	Glycerate	78.66	4.27	11.70	2.36	0.66	2.72	0.49
11	Glycolate	19.45	40.21	2.46	11.77	0.18	0.22	0.52
12	Gulonate	7.14	3.28	2.32	2.77	1.43	1.10	0.64
13	Hepta-decanoate	1.88	55.32	0.11	0.03	0.23	2.01	0.00
14	Hexa-decanoate	2.07	5.27	3.71	64.30	9.45	2.17	5.74
15	Isocitrate	0.36	8.93	5.99	9.15	7.91	2.12	6.61
16	Lactate	40.68	0.44	0.75	0.60	0.28	0.13	0.28

17	Linoleic acid	5.49	0.69	0.00	0.41	0.14	0.17	0.06
18	Malate	67.38	18.53	2.93	3.32	1.82	0.50	0.75
19	Maleate	2.06	0.30	0.11	0.68	0.31	0.33	0.40
20	Malonate	147.95	5.31	5.19	21.35	3.12	1.51	3.90
21	Nonanoate	54.89	0.15	2.51	4.69	0.15	0.23	0.59
22	Octa-decanoate	3.83	1.98	10.79	5.16	1.24	2.53	1.80
23	Oleic acid	27.27	67.28	5.26	12.66	2.41	3.07	6.35
24	Oxalate	13.91	1.24	1.25	18.18	1.83	0.36	0.49
25	Oxaloacetate	48.18	0.21	1.49	1.05	0.06	1.52	0.01
26	Pipecolate	16.08	28.16	0.41	2.45	0.31	0.14	0.30
27	Succinate	0.33	92.59	1.33	0.10	10.15	4.29	1.72
28	Tartarate	6.11	0.03	1.09	0.41	0.07	0.50	0.26
29	Threonate	35.51	3.04	2.28	3.18	1.98	0.16	0.01
	Sugar Alcohol							
1	Digalactosyl glycerol	0.00	6.49	1.81	1.30	5.41	1.03	0.14
2	Fucitol	15.28	0.12	4.17	0.91	0.13	0.27	0.16
3	Galactinol	8.89	19.20	2.57	0.53	0.06	0.17	0.00
4	Galactitol	5.94	0.80	1.06	0.30	0.10	0.48	0.05
5	Galactosyl glycerol	8.52	0.54	0.02	0.36	0.01	0.12	0.01
6	Glycerol	18.91	17.81	6.06	0.32	0.01	0.57	0.00
7	Glycerol-3-P	11.82	19.88	1.54	3.02	0.01	0.52	0.06
8	Mannitol	39.27	165.32	1.81	3.98	3.06	0.97	2.35
9	Myoinositol	599.62	0.28	43.77	57.05	0.08	0.24	0.49
10	Ribonate	5.14	0.06	0.32	0.12	0.10	0.21	0.05
11	Sorbitol	298.28	0.27	39.98	40.37	0.13	0.43	0.73
12	Threitol	28.37	0.99	3.28	0.45	0.24	0.28	0.05
13	Xylitol	34.34	54.90	3.38	1.31	0.33	0.10	0.00
	Amino acid							
1	Alanine	0.04	0.05	3.19	0.00	0.00	0.73	0.05
2	Asparagine	299.11	3.18	4.93	9.97	4.45	0.43	1.18
3	Aspartate	0.17	0.46	0.86	0.11	0.05	0.51	0.18
4	Cystathionine	85.06	1.92	3.53	9.15	2.87	0.52	1.97
5	GABA	0.58	74.53	0.15	0.38	0.28	0.80	0.28
6	Glutamate	47.22	1.96	6.88	4.64	1.43	0.11	0.03
7	Glutamine	50.02	0.18	2.10	1.82	0.20	0.47	1.12
8	Glycine	21.84	2.92	5.89	4.24	2.61	0.21	0.26
9	Homoserine	32.57	2.57	4.72	7.53	1.85	0.10	0.00
10	Isoleucine	58.28	0.21	3.08	3.12	0.30	0.39	0.56
11	Leucine	0.01	43.49	0.24	1.97	2.69	1.48	5.41
12	Lysine	239.36	8.84	5.72	10.67	4.84	0.17	0.03
13	Methionine	104.80	0.56	3.92	10.49	0.32	0.51	0.91
14	Phenylalanine	546.01	1.12	5.00	11.16	0.11	0.27	0.09
15	Proline	459.71	5.76	23.32	33.89	4.22	0.10	0.05
16	Pyroglutamate	0.15	0.22	3.92	9.13	0.11	0.36	0.07
17	Serine	20.23	0.46	2.80	2.97	0.77	0.60	0.43
18	Threonine	72.33	3.77	3.44	9.88	4.06	1.79	1.56
19	Tryptophan	141.11	0.02	6.07	8.22	0.04	0.33	0.36
20	Tyrosine	633.03	2.07	23.47	44.64	1.90	0.05	0.13
21	Valine	14.92	6.91	0.05	3.77	1.76	0.25	0.28
	Other compounds							
1	Adenosine	25.87	2.17	2.33	10.55	0.65	0.16	0.32
2	Allantoin	5.96	8.49	12.00	1.09	0.15	8.06	0.07
3	Cytosine	0.57	17.61	5.35	1.26	4.38	1.12	0.53
4	Diethylene glycol	7.60	27.60	0.78	1.29	0.15	0.42	0.02
5	Ethanolamine	123.80	0.06	2.49	4.57	0.09	0.10	0.11
6	Guanine	6.88	0.08	7.04	15.05	0.06	1.19	0.16
7	Octa-decanol	0.84	0.67	3.72	0.04	1.73	1.67	0.47
8	Thiourea	1.57	0.56	7.92	13.78	0.49	1.34	1.85
9	Uracil	0.29	0.01	0.85	1.24	0.29	0.52	0.43
10	Urea	3.69	41.02	0.46	0.92	3.71	0.54	1.46

*WT = Water Treatment, E = Endophyte, D = Day (sample harvesting time points H1, H2 and H3). Significant F-values are indicated by shading the box where the dark, medium dark or the light shades indicate that the F-value is significant at $p \leq 0.001$, at $p \leq 0.01$ to > 0.001 or at $p \leq 0.05$ to > 0.01, respectively.

strains could not grow in shoots. In the metabolite analysis, a total of 93 distinct metabolites were detected in leaves of *N. benthamiana,* including 20 sugars, 13 sugar alcohols, 21 amino acids, 29 organic and fatty acids and 10 other compounds. Of these, 43–74% exhibited differential accumulation as a function of single factors such as endophyte infection, stress treatment and days to sampling (40, 47 and 74 metabolites, respectively) (Table 1). Some metabolites showed a differential response ($p < 0.05$) to two factors, i.e. as a function of the interaction between water treatment × endophyte infection and endophyte infection × days to sampling, or as a function of three-way interaction of endophyte infection × water treatment × days to sampling (17, 10 and 7 metabolites, respectively) (Table 1).

Under well-watered conditions, higher total amounts of all metabolites were measured in both E-162- and E-284 endophytic plants as compared to non-endophytic plants at sampling points H1, H2 and H3. Water stress induced significant changes in the levels of total amounts of all compounds categories regardless of inoculation status. The magnitudes of change, however, varied between endophytic and non-endophytic samples. For example, under moderate stress, the total content of sugars increased by 105% and 71% in E-162 and E-284 endophytic plants, respectively, whereas it increased by only 62% in non-endophytic plants compared with the well-watered and non-endophytic control plants sampled at H1 (Fig. 1). Under severe stress, the differences between stressed versus watered and endophytic versus non-endophytic became more pronounced (Fig. 1).

Principal component analysis (PCA) showed a good separation of watered, moderately-stressed and severely stressed samples (Fig. 2a). This separation seems to be due to the water treatment (watered vs. moderate water stress vs. severe water stress) with further separation between endophytic and non-endophytic samples. The separation between samples of watered and stressed plants appeared to be mostly due to differential levels of threonine, malonate, oxaloacetate, docosanoate, cystathionine, tyrosine, myoinositol, lysine, phenylalanine, citrate, sorbitol and tryptophan (Fig. 2b). The heatmap revealed the patterns of fluctuation of metabolite levels among treatments, showing an overview of the effect of the various treatments on the metabolism of the plant (Fig. 2c).

PLS-DA analysis revealed the strong influence of endophyte inoculation regardless of water treatment on metabolite composition. There was a good separation of endophytic samples from non-endophytic samples under well-watered conditions (Fig. 3a). The separation was more pronounced under moderate and severe water stress (Fig. 3c and d). The corresponding metabolite-biomarkers of the plant response to endophyte treatments displayed in the corresponding loading plots revealed the patterns of variation of metabolite levels, showing the effect of the endophyte treatment on the metabolism of the plant under both wet and dry conditions (Fig. 3b, d and f).

3.2. Effects of endophyte colonisation under well-watered condition

In well-watered plants at H1, 17–21 metabolites expressed differentially ($p < 0.05$) in endophytic plants of which 11 metabolites were

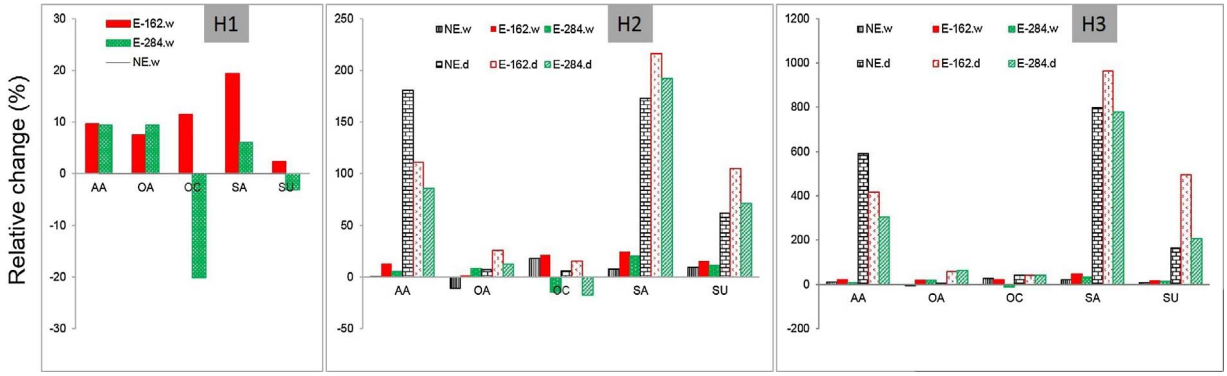

Fig. 1. Percent relative changes of the total amount of different metabolite groups for both endophytic and non-endophytic *Nicotiana benthamiana* leaf samples collected at different sampling points over the amount in non-endophytic well-watered samples at H1. NE = no endophyte, E-162; inoculation with fungal isolate E-162; E-284; inoculation with fungal isolate E-284. W; well-watered, d = drought stressed. H1, H2, and H3 indicate sample harvesting time points. AA = Amino Acid, OA = Organic Acid, OC = Other Compounds, SA = Sugar Alcohol, SU = Sugar.

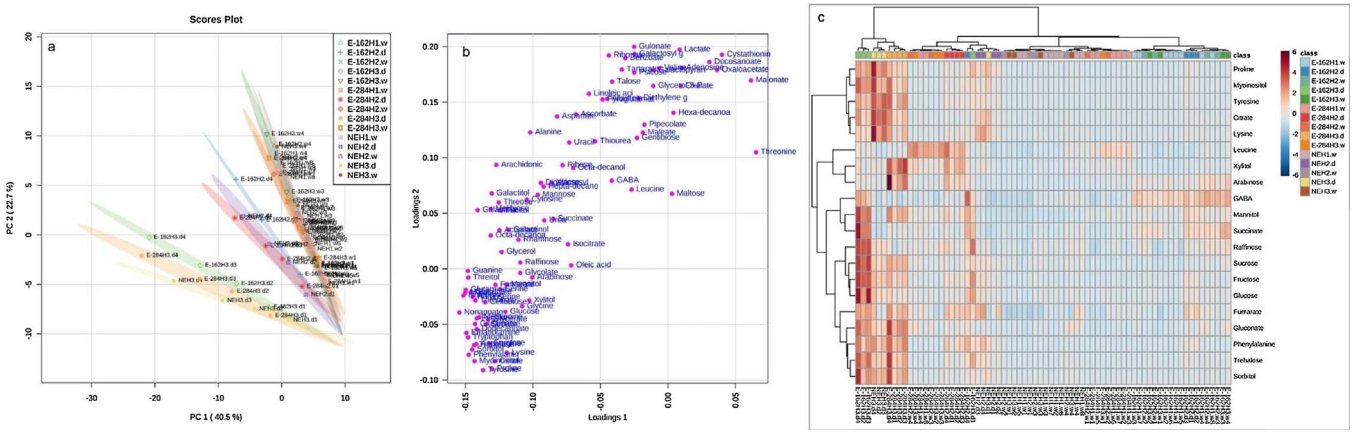

Fig. 2. Principal component analysis (PCA) and hierarchical clustering analysis of 93 metabolites in the leaves from endophytic and non-endophytic *N. benthamiana* plants grown under well-watered, moderate and severe water stressed conditions. Score (a) and loading plot (b) of samples (c) heat-map constructed using average linkage hierarchical clustering. NE = no endophyte, E-162; inoculation with E-162; E-284; inoculation with E-284. W = well-watered, d = drought stressed. H1, H2, and H3 indicate sample harvesting time points.

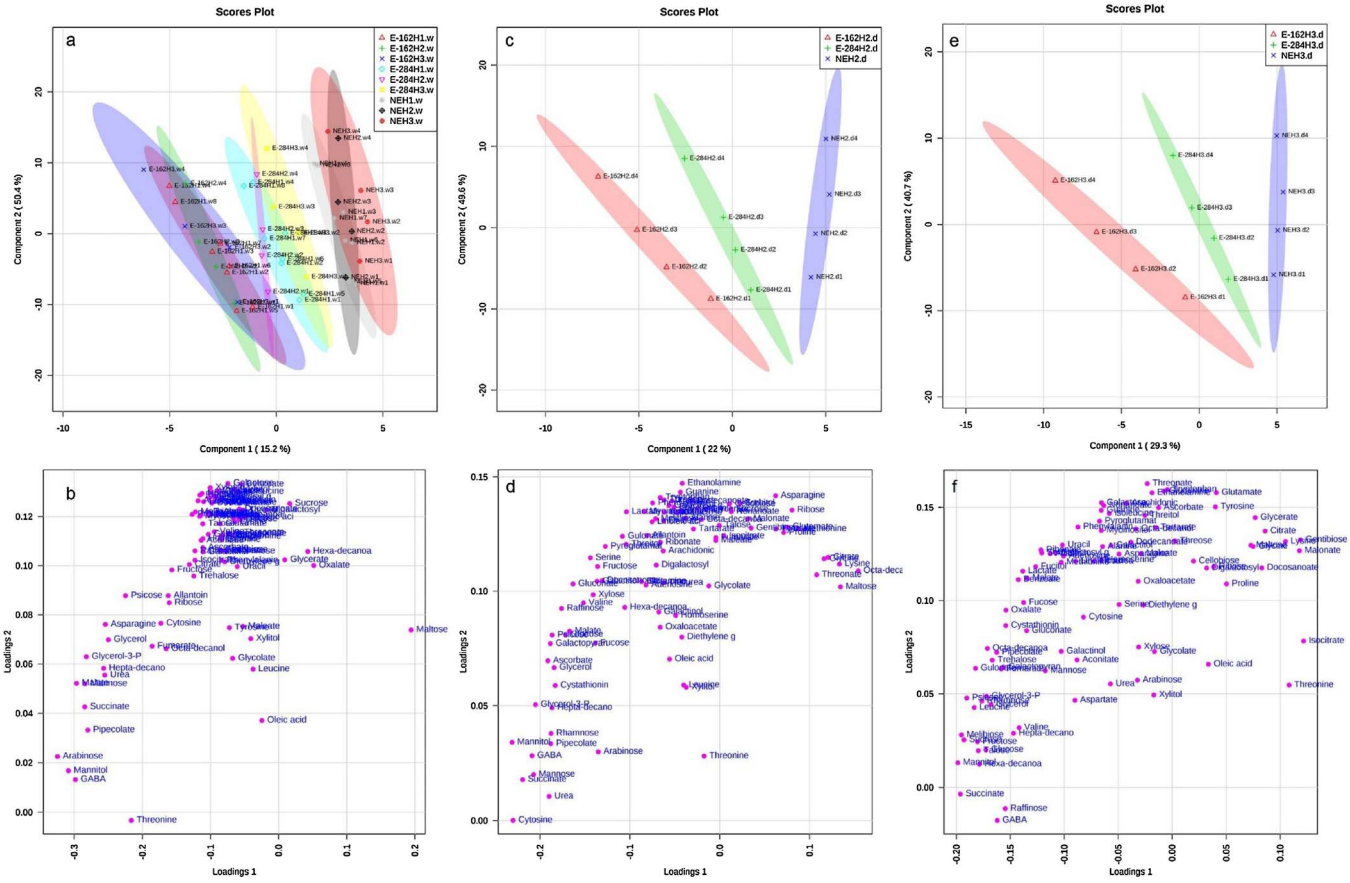

Fig. 3. Partial least squares-discriminant analyses (PLS-DA); component 1/2 score plots (a, c, e); and loading plots (b, d, f) obtained from hierarchical average linkage clustering for well-watered samples (a and b), moderately stressed samples (c and d) and severely stressed samples (e and f). NE = no endophyte, E-162; inoculation with E-162; E-284; inoculation with E-284. W; well-watered, d = drought stressed.

common to both fungal inoculation and the other ten and six metabolites were exclusive to the effect of strains E-162 and E-284, respectively (Supplementary Table 1). For example, E-162-endophytic plants had one to three-fold (log_2fc) increases in allantoin, citrate, GABA, glycerol, glycerol-3-P, hexadecanoate, mannitol, mannose, pipecolate, and threonine and inoculation with E-284 caused one to three-fold (log_2fc) increases in allantoin, arabinose, citrate, glycolate, heptadecanoate, leucine, mannitol, oleic acid, and xylitol (Fig. 4; Supplementary Table 1).

Among the metabolites, only arabinose and mannitol showed a differential response due to inoculation of either of the fungi throughout all the sampling points under well-watered conditions, and plant age did not affect their response. The majority of metabolites in non-endophytic plants did not show any significant changes with the age of the plants, with the exceptions of allantoin, ascorbate, maltose, alanine and aspartate. Similarly, none of the metabolites except glycerate, succinate, and octadecanol in E-162 endophytic plants and glutamate, mannitol, malonate, aconitate maltose, rhamnose and mannose in E-284 endophytic plants exhibited a substantial change ($P < 0.05$) as the plants grew older over time under well-watered condition (Supplementary Table 2).

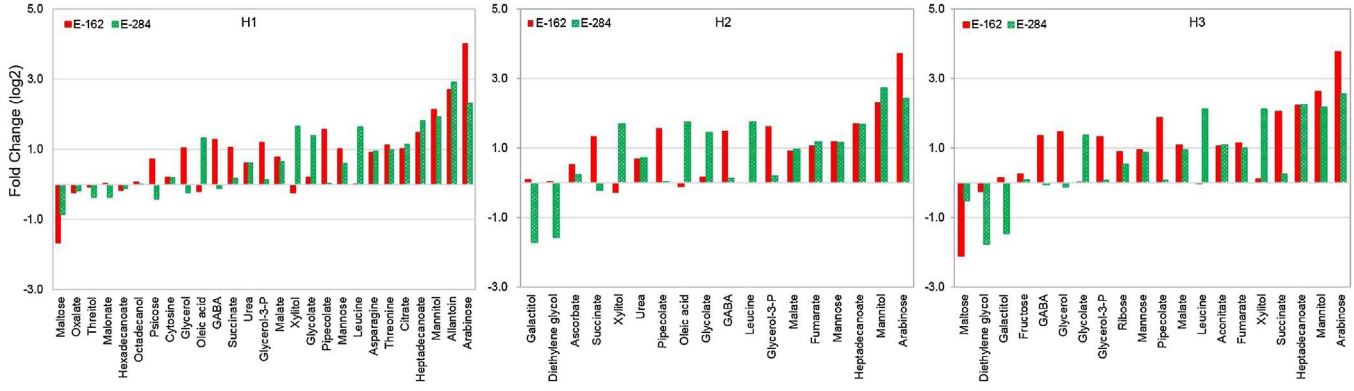

Fig. 4. Fold changes (log_2) of metabolites in endophytic samples over non-endophytic samples under well-watered conditions throughout the course of sampling, NE = no endophyte, E-162; inoculation with E-162; E-284; inoculation with E-284; H1, H2, and H3 indicates the samples harvesting time points. Only the metabolites that showed significant differences ($p < 0.05$) between non-endophytic and either of the endophytic samples at the respective sampling point are shown.

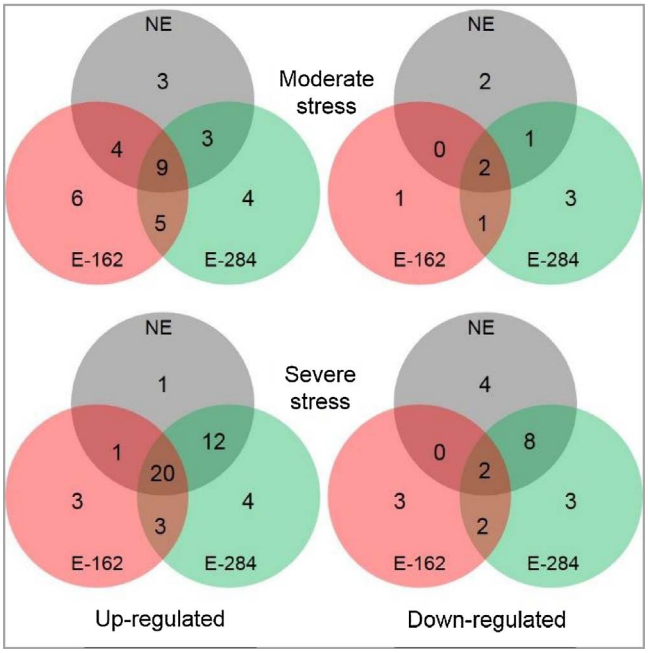

Fig. 5. Venn diagrams showing number of metabolites up-and down regulated in endophytic and non-endophytic samples and their overlaps under both moderate (H2) and severe water stress (H3). NE = no endophyte, E-162; inoculation with E-162; E-284; inoculation with E-284.

3.3. Effects of endophyte colonization under water stress condition

Under moderate water stress, 11 and nine metabolites were up-regulated in E-162 and E-284 plants respectively of which five compounds were common. Under severe water stress, six and seven metabolites were significantly up-regulated in E-162 and E-284 endophytic plants respectively of which five compounds were common. Under severe stress, only two compounds were downregulated in both endophytic and non-endophytic plants (Fig. 5).

3.3.1. Amino acids

The majority of amino acids increased in both endophytic and non-endophytic plants under moderate and severe water stress (Table 2, Fig. 6). The most pronounced increase (2–5-fold) was observed for asparagine, phenylalanine, lysine, methionine, proline, tryptophan and tyrosine in all samples regardless of endophytic colonization. On the other hand, threonine, cystathionine, pyroglutamate, valine decreased significantly in most samples under stress (Table 2). Endophyte inoculation caused differences in accumulation of certain amino acids under stress. For example, proline accumulated at a significantly ($P < 0.05$) lower level, valine was higher in E-284 plants, whereas GABA and leucine accumulated higher amount in E-162 plants as compared to non-endophytic plants under severe stress (Fig. 6)

3.3.2. Organic acids

Approximately 20–24% of organic acids in E-162 and E-284 endophytic plants, and 34% of organic acids in the non-endophytic plants, increased under moderate stress (Table 2). Under severe stress, 27–70% of the organic acids showed differential accumulation ($p < 0.05$). The most pronounced increase over watered samples were citrate (2–5-fold), dodecanoate (1- to 4-fold) and fumarate for all samples regardless of fungal treatments. In addition, docosanoate, malonate and oxaloacetate decreased following stress in all samples (Table 2).

Endophyte inoculation caused differences in accumulation of certain organic acids under stress. For example, heptadecanoate, succinate and pipecolate in E-162 and heptadecanoate, oleic acid and succinate in E-284 endophytic plants exhibited significantly higher amounts (1–2-

Table 2
Fold change (log_2) of metabolites under moderate and severe stress in endophytic and non-endophytic plants over corresponding well-watered plants. Fold change was calculated by dividing the response value of a particular compound under stress condition with that under well-watered condition and the log_2 value was obtained. Metabolites that were differently expressed ($P \leq 0.05$) are represented with their fold-change bolded.

Metabolites	Moderate stress			Severe Stress		
	NE	E-162	E-284	NE	E-162	E-284
Sugar						
Arabinose	0.24	−0.08	**2.27**	**2.38**	−0.36	**3.23**
Cellobiose	1.00	0.44	1.25	**2.00**	0.95	**3.07**
Fructose	**0.92**	**1.32**	**1.34**	**1.99**	**2.95**	**2.00**
Fucose	**1.04**	**1.95**	**2.64**	**2.36**	2.86	**2.84**
Galactopyranose	**−2.19**	−0.43	−0.60	**−2.96**	−0.37	**−1.33**
Galactose	0.65	0.44	1.03	0.81	0.97	0.80
Gentibiose	0.43	0.28	0.03	−0.09	**−1.13**	−0.35
Glucose	0.61	**1.25**	0.48	**1.43**	**3.06**	**1.24**
Maltose	−1.12	−0.81	**−1.89**	**−1.71**	**−0.64**	−1.07
Mannose	−0.07	0.10	0.13	−0.21	0.07	0.54
Melibiose	0.29	0.25	0.03	0.17	1.24	0.53
Psicose	−0.36	−0.02	0.03	−1.54	−0.89	−0.85
Raffinose	−0.10	0.16	0.31	**0.42**	**1.22**	−0.15
Rhamnose	−0.17	**2.40**	−0.95	−0.09	**2.41**	0.80
Ribose	0.15	−0.19	−0.29	0.62	**−1.08**	0.69
Sucrose	0.47	0.53	0.40	**1.11**	**2.36**	**1.62**
Talose	−0.05	−0.22	**−0.90**	**−0.98**	0.17	−1.06
Threose	0.01	0.18	0.13	0.71	0.13	1.07
Trehalose	**1.31**	1.31	**1.20**	**2.31**	**3.05**	**3.23**
Xylose	−0.92	−0.28	−0.03	−1.94	−1.05	1.61
Organic acids						
Aconitate	0.04	0.03	−0.08	0.41	0.26	**1.13**
Arachidonic acid	0.06	0.05	0.54	0.27	0.19	0.40
Ascorbate	−0.35	−0.24	−0.27	−0.47	−0.27	−0.58
Benzoate	−0.57	−0.29	−0.63	**−1.89**	−0.57	−0.84
Citrate	**3.25**	**1.74**	1.62	**5.01**	**3.81**	**4.13**
Docosanoate	**−1.50**	−0.94	−0.97	**−2.13**	−3.84	**−4.37**
Dodecanoate	**1.23**	0.87	1.35	**3.35**	**3.14**	**2.39**
Fumarate	**2.11**	**0.99**	**0.92**	**1.75**	**1.68**	**1.79**
Gluconate	−0.07	0.53	**0.16**	0.82	1.50	**1.85**
Glycerate	**1.42**	**1.63**	**1.23**	**2.54**	**1.94**	**1.66**
Glycolate	**0.57**	0.03	−0.02	**1.22**	**1.17**	**1.40**
Gulonate	−0.51	−0.15	−0.19	−2.33	−0.32	−0.97
Heptadecanoate	0.15	0.18	0.51	−0.12	0.27	0.50
Hexadecanoate	**0.21**	0.33	**0.30**	**−0.69**	0.06	**−0.62**
Isocitrate	−0.15	−0.26	−0.19	**1.17**	−0.16	−0.14
Lactate	−1.23	−0.90	**−1.22**	−1.91	−1.15	**−1.52**
Linoleic acid	−0.50	−0.22	−0.43	−0.74	−0.37	−0.20
Malate	**1.37**	0.99	0.37	**1.64**	**1.36**	**1.53**
Maleate	−0.24	−0.34	−0.30	−0.25	−0.27	**−0.17**
Malonate	**−0.63**	−0.65	**−0.95**	**−1.05**	−2.37	**−1.58**
Nonanoate	**1.42**	0.90	1.53	2.07	**2.30**	**2.38**
Octa-decanoate	0.02	−0.05	−0.07	−0.06	0.92	**0.71**
Oleic acid	0.29	0.43	0.04	**0.84**	0.40	**2.05**
Oxalate	−0.01	0.12	0.05	**−0.69**	−0.25	**−0.69**
Oxaloacetate	**−0.60**	**−0.75**	**−0.59**	−0.84	**−0.94**	**−0.80**
Pipecolate	−0.49	−0.45	**−0.59**	−0.75	−1.13	−0.82
Succinate	−0.52	0.40	0.58	**−1.43**	0.91	0.65
Tartarate	−0.50	−0.51	**−0.47**	−0.68	−0.81	**−0.93**
Threonate	**1.94**	0.64	**1.25**	**2.82**	2.26	**2.55**
Sugar Alcohol						
Digalactosyl glycerol	−0.04	0.31	−0.92	0.75	0.68	−0.70
Fucitol	0.80	0.76	0.40	0.22	0.94	0.70
Galactinol	0.70	0.78	**0.83**	1.00	0.56	0.51
Galactitol	0.21	**0.88**	0.56	0.98	**1.34**	1.01
Galactosyl-glycerol	−0.79	−0.73	−0.47	−1.08	−0.69	−0.79
Glycerol	0.84	1.03	0.93	1.30	**1.33**	**1.21**
Glycerol-3-P	−0.69	−0.37	−0.33	−1.25	**−1.33**	**−1.45**
Mannitol	0.67	**1.31**	0.36	0.59	**1.86**	**1.88**
Myoinositol	**1.88**	**1.92**	**2.03**	**3.82**	**3.83**	**3.51**
Ribonate	−0.60	−0.46	−0.45	−0.99	−0.26	−0.59
Sorbitol	**2.09**	**1.80**	**1.49**	**3.67**	**4.13**	**4.05**
Threitol	0.56	**0.86**	0.59	0.95	**0.92**	0.76
Xylitol	0.91	1.02	**1.40**	**1.57**	1.50	**1.83**

(continued on next page)

Table 2 *(continued)*

Metabolites	Moderate stress			Severe Stress		
	NE	E-162	E-284	NE	E-162	E-284
Amino acid						
Alanine	0.06	0.01	0.00	−0.03	0.04	0.06
Asparagine	**4.43**	**2.56**	**2.46**	**5.28**	**4.47**	**4.26**
Aspartate	0.16	0.20	0.00	−0.12	0.03	−0.01
Cystathionine	**−0.78**	−0.43	**−0.83**	**−1.83**	**−1.15**	**−0.98**
GABA	−0.06	0.20	0.40	−0.01	0.14	0.02
Glutamate	1.28	**0.55**	**1.20**	**2.34**	**1.45**	**2.17**
Glutamine	0.56	**1.07**	0.80	**1.26**	0.93	**1.39**
Glycine	1.40	0.38	0.31	**2.97**	1.31	**1.24**
Homoserine	0.38	0.31	1.41	**1.64**	**1.77**	**2.59**
Isoleucine	0.52	**0.99**	0.74	**1.19**	1.12	**1.30**
Leucine	0.17	0.08	0.38	−0.08	0.83	**−1.66**
Lysine	**3.13**	**1.65**	**2.08**	**4.24**	**2.76**	**3.32**
Methionine	**2.24**	**1.56**	1.34	**3.22**	**3.61**	**3.21**
Phenylalanine	**3.23**	**3.35**	**3.39**	**4.06**	**4.24**	**4.48**
Proline	**2.17**	**1.60**	**1.49**	**3.52**	2.99	**2.66**
Pyroglutamate	0.50	1.00	0.84	−1.30	−1.01	−0.74
Serine	0.44	0.99	0.87	1.25	1.86	**3.14**
Threonine	−0.78	**−1.42**	**−1.25**	**−1.33**	**−3.78**	**−2.50**
Tryptophan	2.91	**3.03**	**3.11**	**4.36**	**3.81**	**4.29**
Tyrosine	**2.70**	**2.01**	**2.71**	**4.64**	**3.92**	**4.37**
Valine	−1.75	−0.32	−0.27	**−2.80**	−1.07	**−0.83**
Other Compounds						
Adenosine	−0.11	−0.10	−0.18	**−0.64**	−0.39	**−0.64**
Allantoin	−0.34	−0.25	−0.27	−1.09	**−0.62**	−0.59
Cytosine	−0.18	0.02	0.09	−0.12	0.03	0.36
Diethylene glycol	−0.34	−0.24	−0.48	−0.86	−0.77	−0.90
Ethanolamine	1.78	**1.67**	**1.89**	**2.69**	**2.40**	**2.39**
Guanine	−0.22	−0.16	−0.21	**1.16**	1.04	**1.32**
Octadecanol	**0.06**	**−0.01**	0.00	0.04	0.02	−0.03
Thiourea	**1.09**	**1.15**	0.51	−0.87	−0.35	0.04
Uracil	−0.04	0.00	0.00	0.04	0.06	−0.01
Urea	−0.01	0.05	**0.23**	−0.11	−0.10	0.90

fold, $p < 0.05$) as compared to non-endophytic plants under moderate stress (Fig. 6). Under severe stress, nine to ten organic acids accumulated at significantly ($p < 0.05$) different levels in endophytic plants than in non-endophytic plants (Fig. 6; Supplementary Table 3).

3.3.3. Sugars

In addition to fructose and fucose, which were significantly upregulated in all the samples, glucose and rhamnose were upregulated only in E-162 plants, arabinose and trehalose only in E-284 plants, and trehalose in non-endophytic plants under moderate stress (Table 2). Total sugars increased by 55, 30 and 40% under severe stress. The most pronounced changes were in accumulation of glucose, fructose, fucose, sucrose and trehalose regardless of endophyte status (Table 2).

Arabinose, galactopyranose, fucose, rhamnose accumulated at significantly higher amounts (2–5-fold) in both E-162 and E-284 plants than in non-endophytic plants under moderate stress (Fig. 6). It is interesting to note that 13 sugars out of the 20 tested showed a variable response in E-162 plants, but only four sugars had a variable response in E-284 plants (Fig. 6, Supplementary Table 4). Arabinose, mannose and sucrose the three sugars accumulated substantially higher in endophytic plants than in non-endophytic plants under stress (Fig. 6; Supplementary Table 3).

3.3.4. Sugar alcohols

Some sugar alcohols showed varied response under stress in non-endophytic plants whereas 35–55% of the total number of sugar alcohols analysed showed differential accumulation in endophytic plants (Table 2). Myinositol and sorbitol increased following stress in all samples irrespective of colonisation status. Mannitol and glycerol and glycerol-3-P were accumulated differentially under stress in endophytic plants but not in non-endophytic plants (Table 2).

In endophytic plants under moderate and severe water stress, we found that several sugar alcohols accumulated to higher levels than in non-endophytic plants. For example, mannitol, xylitol and glycerol in E-162 and mannitol and xylitol in E-284 plants showed a significantly higher value over non-endophytic plants under moderate stress (Fig. 6; Supplementary Table 3).

3.3.5. Other compounds

Most of the other compounds identified did not show any significant change under stress. Exceptions were ethanolamine, which rose under stress (1–2-fold, $p < 0.05$) in all samples. Levels of cytosine and urea were detected significantly higher ($p < 0.05$) in endophytic plants than in non-endophytic plants at both moderate and severe water stress. Compounds that decreased were diethylene glycol in both E-162 and E-284 plants, and octadecanol and thiourea in E-162 plants (Fig. 6; Supplementary Table 3).

3.4. Relative water content (RWC) and biomass traits

Under well-watered conditions, no significant differences were observed in seedling biomass, or leaf RWC by endophyte colonization except that E-162 plants had higher dry mass in shoots (Fig. 7). Water stress significantly affected the shoot and root dry weights as well as RWC and increased the root to shoot ratio in both endophytic and non-endophytic plants. When the plants were subjected to severe stress, both endophyte plants produced significantly higher root biomass but not shoot biomass and accumulated higher RWC in leaves compared to non-endophytic plants (Fig. 7).

4. Discussion

To characterise the metabolic responses of *N. benthamiana* to water stress and the potential effect of fungal endophytes on this response, we evaluated the differences in metabolite accumulation in leaves of endophytic or non-endophytic *Nicotiana benthamiana* plants under well-watered, moderately and severely stressed conditions. Analyses identified a separation between endophytic and non-endophytic plants, between the watering levels (watered and stressed) and among the harvest times.

Arabinose and mannitol were significantly and consistently upregulated in both E-162- and E-284 plants irrespective of plant age under well-watered, moderate and severe stressed conditions, indicating that accumulation of these metabolites is directly associated with fungal inoculation. Similarly, accumulation of GABA was found to be directly associated only with E-162 plants and xylitol only with E-284 plants, irrespective of age and water treatment status.

There were significantly higher levels of several amino acids in water-stressed plants. Amino acids synthesis is influenced because of the role as the precursor proteins as well as for diverse kinds of compounds with various functions in plant growth and adaptation to stress (Less and Galili, 2008). Abiotic stress causes protein degradation leading to an internal ammonium build-up in plants (Krasensky and Jonak, 2012; Vierstra, 1993). Under stress, production of amides, particularly asparagine, inhibits ammonium toxicity (Bowne et al., 2012; Díaz et al., 2005; Rare, 1990). Accumulation of various amino acids is associated with plant tolerance to water stress (Díaz et al., 2005; Mayer et al., 1990; Yoshiba et al., 1997).

Differential accumulation of several amino acids occurred in endophytic plants. For example, threonine and GABA in E-162 plants and leucine in E-284 plants increased significantly under well-watered conditions. A previous study also reported a higher level of some amino acids in tall fescue inoculated with endophytes, and they postulated that endophytes could have directly synthesised these amino acids (Lyons et al., 1990). Contrasting results were reported by Redman et al. (2011) where endophyte infection decreased the level of certain amino acids in grasses, and they described that endophyte effects on plant

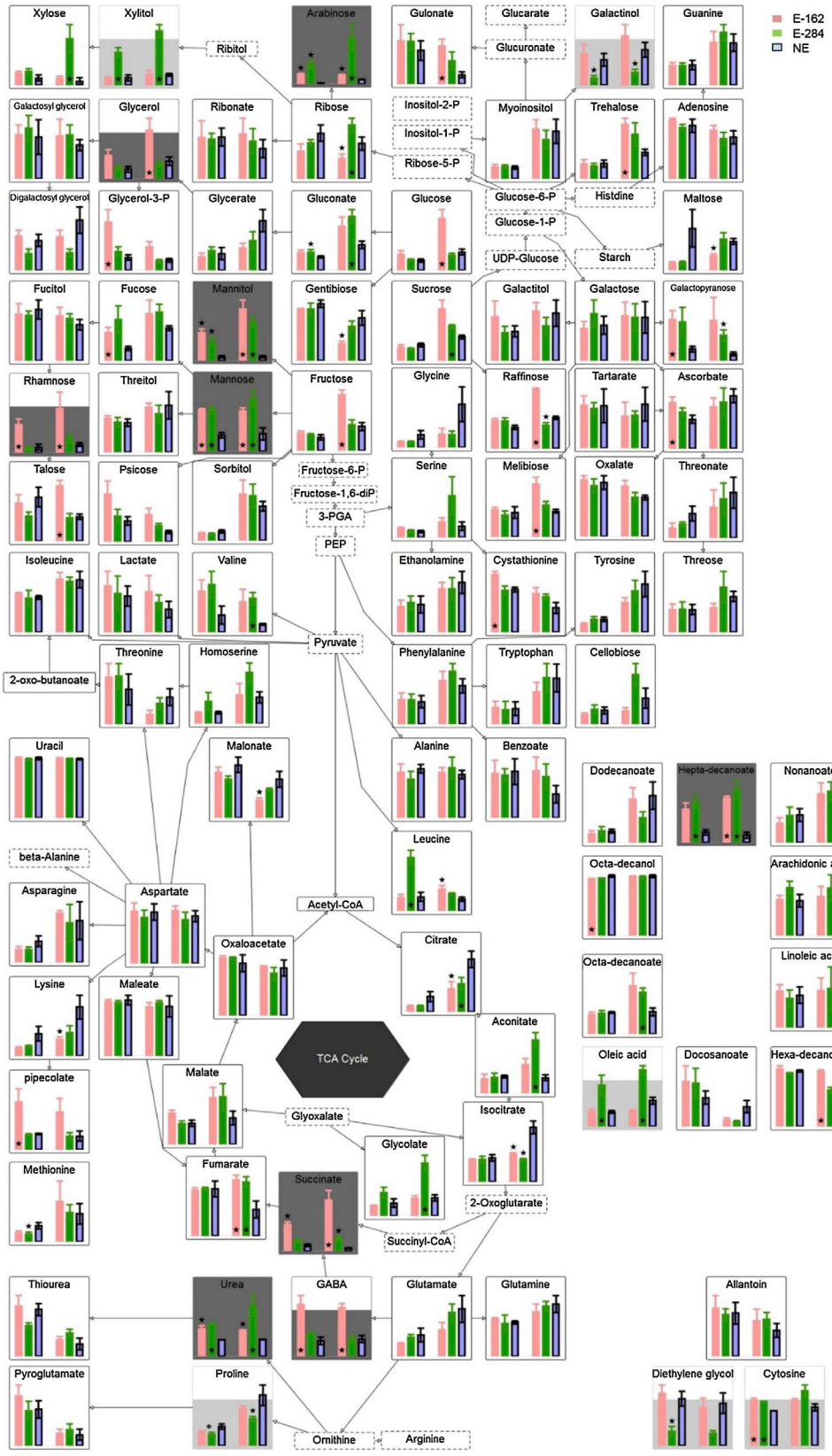

Fig. 6. Metabolic pathway map for endophytic and non-endophytic samples under mild and severe water-stressed conditions. Bars with * indicate significant difference from corresponding non-endophytic samples. Plots filled with a dim grey color indicate that metabolite accumulated differentially in both fungal inoculated samples, and those partly filled with dim gray and light grey color indicate metabolite accumulated differentially only in E-162 or E-284 inoculated plants, respectively, throughout all the time points of sampling. The significant differences were obtained by ANOVA test at the 95% confidence interval after normalizing data using log transformation.

metabolite were dependant of host characteristics and nutrient supply. Nagabhyru et al. (2013) found that several amino acids were differentially expressed in endophytic plants under water stress.

In accordance with earlier investigations (Verslues and Sharma, 2010), higher proline levels occurred in plants in response to moisture stress. However, proline levels were higher in non-endophytic plants than in endophytic plants under stress, indicating that proline accumulation is somewhat inhibited by colonization by endophytic fungi. Proline is a stress-related amino acid, which may function as an osmoregulator (Yoshiba et al., 1997) as well as an ROS scavenger (Chen

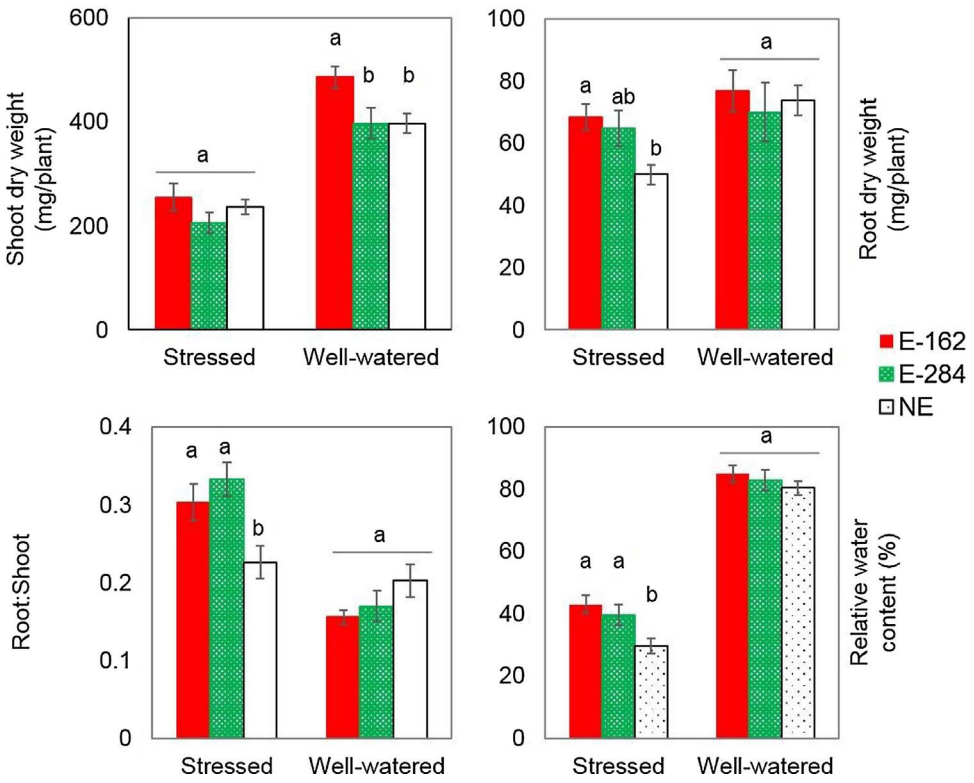

Fig. 7. Effect of endophyte colonization and water stress on biomass and RWC of *N. benthamiana* seedlings. Bars with same letter are not statistically different according to Tukey's multiple comparisons test ($P < 0.05$ n = 20). Plant biomass and leaf RWC were from seedling harvested at H3 (8 days post-stress) from well-watered and stressed samples. NE = no endophyte, E-162; inoculation with E-162; E-284; inoculation with E-284.

and Dickman, 2005). Its accumulation correlates with both osmotic stress tolerance and responses to stress conditions involving dehydration (Aspinall and Paleg, 1981; Gzik, 1996; Verbruggen and Hermans, 2008). However, it is still debatable if its presence is an adaptive trait that confers higher stress tolerance or if its accumulation is a symptom of stress damage (Ashraf and Foolad, 2007). The relatively lower accumulation of proline in endophytic plants could, therefore, be an indication that there is less damage in endophytic drought-stressed plants. Relatively lower levels of proline in endophytic plants than non-endophytic plants under stress has been reported by others (Elbersen and West, 1997; Hahn et al., 2008; Pandey et al., 2016; Shukla et al., 2012; Vazquez-de-Aldana et al., 2013; Yang et al., 2014), as has increased proline levels in endophytic plants under stress (Bae et al., 2009; Cong et al., 2015; Molina-Montenegro et al., 2016; Nagabhyru et al., 2013 Zhang and Nan, 2007) and no change in proline (Bayat et al., 2009; Nagabhyru et al., 2013). The proline response may depend on the host-endophyte interaction and the level of stress.

Methionine, besides its role in protein synthesis, acts as the precursor for S-adenosylmethionine (SAM), the principal methyl donor in transmethylation reactions (Chiang et al., 1996). SAM also serves as a precursor for compounds including 3-dimethylsulphoniopropionate which is an osmoprotectant (Amir et al., 2002).

Tyrosine and phenylalanine are produced through the shikimate pathway and act as precursors for a varied range of secondary metabolites, including ROS scavengers (Gill and Tuteja, 2010; Less and Galili, 2008). Water stress increases the production of ROS and the regulation of the activity of enzymes involved in detoxifying ROS, to escape cellular damage, is considered to be vital in plant dehydration tolerance (Chaves et al., 2003).

Accumulation of some of the amino acids such as asparagine, cytosine, lysine, methionine, phenylalanine, pyroglutamate, mentioned above did not appear to be associated with endophyte inoculation since we observed no consistent endophyte effects on these amino acids levels under stress. A more or less comparable trend was described for water stressed tall fescue infected with the fungal endophyte *E. coenophiala* (Nagabhyru et al., 2013).

Soluble sugars such as glucose, fructose and sucrose play critical roles in plant structure and metabolism at the cellular and whole-organism levels (Couée et al., 2006). Sugars commonly accumulate in plants under stress, and the level of accumulation is associated with plant water stress tolerance (Bowne et al., 2012; Hoekstra et al., 2001). These sugars influence osmotic adjustment and thus help regulate plant water uptake and maintain cell turgor under water deficit conditions (Krasensky and Jonak, 2012; Morgan, 1984) and can act as regulatory molecules in various signalling pathways (Hanson and Smeekens, 2009) involved in maintaining redox balance and ROS scavengers (Couée et al., 2006; Deryabin et al., 2007). Our findings showed that endophytic plants accumulated a higher level of different osmotically active sugars. For example, endophytic plants generally accumulated more total sugars in leaves compared to non-endophytic plants (Fig. 1C). Endophyte presence increased arabinose, mannose and sucrose with few others indicating such increases could be a part of endophyte-mediated water stress tolerance of *N. benthamiana*. It implies that endophytes could have contributed to increased synthesis of sugars to allow better osmotic adjustment and thus alleviate the impact of stress on the host plant. Comparable results were obtained in other plant-endophyte systems where endophytes improved water stress tolerance of tall fescue, maize and grapevine plants with higher and faster accumulation of stress-related metabolites (Fernandez et al., 2012; Nagabhyru et al., 2013; Vardharajula et al., 2011).

Due to hydroxyl groups, sugar alcohols can imitate the structure of water and can make an artificial sphere of hydration surrounding the macromolecules (Schobert, 1977). Sugar alcohol can also act as scavengers of ROS for impeding lipid peroxidation and the consequent cellular destruction (Smirnoff and Cumbes, 1989). Two sugar alcohols, myoinositol and sorbitol increased due under water stress in all plants regardless of colonisation status. Endophyte colonisation resulted in a higher accumulation of mannitol, xylitol and glycerol-3-P. Higher accumulation of mannitol in endophyte-endophytic plants was also reported by Richardson et al. (1992). These authors did not observe any effect on the mannitol levels when the plants were challenged with polyethylene glycol, which mimics water stress. Mannitol accumulation in endophytic

plants in our study could be because this metabolite is common filamentous fungi (Solomon et al., 2007). Mannitol can act as an osmoprotectant in plants and fungi under abiotic stress, as well as serve as an antioxidant (Patel and Williamson, 2016), so mannitol in the endophytic plants may have contributed to their tolerance to water stress (Chan et al., 2011; Hu et al., 2005; Sickler et al., 2007; Tarczynski et al., 1993)

Increased levels of some tricarboxylic acid cycle (TCA) intermediates under stress such as aconitate, citrate, fumarate, and malate could be reflective of the plant's mechanisms to withstand water stress. Moreover, higher levels of aconitate, fumarate, and succinate in endophytic plants over non-endophytic plant could be an indicative of better mitochondrial activity leading to increased formation of reducing agents and ATP (Vasquez-Robinet et al., 2008).

Endophytic plants had significantly higher root weight and RWC under stress. A similar result was reported by Hassan et al. (2014) who demonstrated that presence of *Trichoderma* spp. significantly increased root length and root dry mass in millet plants. Higher root biomass, which is frequently noted in drought-tolerant plants, could also contribute to avoidance in endophyte-colonized plants (Harman, 2000; Mastouri et al., 2012). Endophyte induced higher RWC in water stressed plants is in line with the earlier investigations (Bae et al., 2009; Zhang and Nan, 2007). These results suggested that endophyte colonization may help maintain the host cell protoplasm water to support metabolization (Malinowski and Belesky, 2000).

Production of bioactive plant secondary metabolite by fungal endophytes is well-known (see review by Aly et al., 2013) but the endophyte induced changes in plant metabolism particularly under water stress and their molecular mechanisms have not been studied well. In a study on the effects of *E. festucae* infection on ryegrass revealed that endophyte has significant effects on host responses to stress through reprogramming of host metabolism and altering stress responsive gene expression and substantially alters host development (Dupont et al., 2015). Rasmussen et al. (2008) reported of roles *E. coenophiala* on the primary and secondary metabolism of *Lolium perenne* (perennial ryegrass). The necessity of more works to identify robust metabolic traits and pathways associated with stress tolerance in plants have been emphasized in reviews (Rasmussen et al., 2012). As we observed in the endophytic plants, the fungi in the infected plants may have induced, or rapidly activated, the plant biochemical reactions to accumulate the metabolites during stress conditions, and this may be one of the ways that the presence of the endophyte helps reduce the effects of water deficit stress. It supports the view that beneficial effects of fungal endophytes on plant is context dependent (Rodriguez et al., 2009; Yang et al., 2014). It should be mentioned that the generalization of our findings to natural environments entail further investigation, since the experimental design, e.g., glasshouse or controlled conditions and interaction with other biotic and abiotic factors may significantly impact the outcome of this type of experiment.

Fungal endophytes may play a role in persistence of annual Australian *N. benthamiana* plants, an ephemeral species that lives in the hot, dry and unpredictable conditions of northern Australia. If endophytes enable these plants to survive somewhat longer than non-endophytic plants when water stress occurs, their opportunity for seed set is increased.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.envexpbot.2017.08.008.

References

Abarenkov, K., Henrik Nilsson, R., Larsson, K.H., Alexander, I.J., Eberhardt, U., Erland, S., Høiland, K., Kjøller, R., Larsson, E., Pennanen, T., 2010. The UNITE database for molecular identification of fungi—recent updates and future perspectives. New Phytol. 186, 281–285.

Aly, A., Debbab, A., Proksch, P., 2013. Fungal endophytes-secret producers of bioactive plant metabolites. Die Pharm. Int. J. Pharm. Sci. 68, 499–505.

Amir, R., Hacham, Y., Galili, G., 2002. Cystathionine γ-synthase and threonine synthase operate in concert to regulate carbon flow towards methionine in plants. Trends Plant Sci. 7, 153–156.

Ashraf, M., Foolad, M., 2007. Improving plant abiotic-stress resistance by exogenous application of osmoprotectants glycine betaine and proline. Environ. Exp. Bot. 59, 206–216.

Aspinall, D., Paleg, L., 1981. Proline accumulation: physiological aspects. Physiology and Biochemistry of Drought Resistance in Plants.

Azad, K., Kaminskyj, S., 2016. A fungal endophyte strategy for mitigating the effect of salt and drought stress on plant growth. Symbiosis 1, 73–78.

Bae, H., Sicher, R.C., Kim, M.S., Kim, S.-H., Strem, M.D., Melnick, R.L., Bailey, B.A., 2009. The beneficial endophyte *Trichoderma hamatum* isolate DIS 219b promotes growth and delays the onset of the drought response in *Theobroma cacao*. J. Exp. Bot. 60, 3279–3295.

Bayat, F., Mirlohi, A., Khodambashi, M., 2009. Effects of endophytic fungi on some drought tolerance mechanisms of tall fescue in a hydroponics culture. Russ. J. Plant Physiol. 56, 510–516.

Bowne, J.B., Erwin, T.A., Juttner, J., Schnurbusch, T., Langridge, P., Bacic, A., Roessner, U., 2012. Drought responses of leaf tissues from wheat cultivars of differing drought tolerance at the metabolite level. Mol. Plant 5, 418–429.

Chan, Z., Grumet, R., Loescher, W., 2011. Global gene expression analysis of transgenic, mannitol-producing, and salt-tolerant *Arabidopsis thaliana* indicates widespread changes in abiotic and biotic stress-related genes. J. Exp. Bot. 62, 4787–4803.

Chaves, M.M., Maroco, J.P., Pereira, J.S., 2003. Understanding plant responses to drought—from genes to the whole plant. Funct. Plant Biol. 30, 239–264.

Chen, C., Dickman, M.B., 2005. Proline suppresses apoptosis in the fungal pathogen Colletotrichum trifolii. Proc. Natl. Acad. Sci. U. S. A. 102, 3459–3464.

Chen, H., Jiang, J.-G., 2010. Osmotic adjustment and plant adaptation to environmental changes related to drought and salinity. Environ. Rev. 18, 309–319.

Chiang, P.K., Gordon, R.K., Tal, J., Zeng, G., Doctor, B., Pardhasaradhi, K., McCann, P.P., 1996. S-Adenosylmethionine and methylation. FASEB J. 10, 471–480.

Claeys, H., Inzé, D., 2013. The agony of choice: how plants balance growth and survival under water-limiting conditions. Plant Physiol. 162, 1768–1779.

Coleman-Derr, D., Tringe, S.G., 2014. Building the crops of tomorrow: advantages of symbiont-based approaches to improving abiotic stress tolerance. Front. Microbiol. 5.

Cong, G.Q., Yin, C.L., He, B.I., Li, L., Gao, K.X., 2015. Effect of the endophytic fungus *Chaetomium globosum* ND35 on the growth and resistance to drought of winter wheat at the seedling stage under water stress. Shengtai Xuebao Acta Ecol. Sin. 35, 6120–6128.

Couée, I., Sulmon, C., Gouesbet, G., El Amrani, A., 2006. Involvement of soluble sugars in reactive oxygen species balance and responses to oxidative stress in plants. J. Exp. Bot. 57, 449–459.

Díaz, P., Monza, J., Márquez, A., 2005. Drought and saline stress. Lotus Japonicus Handbook. pp. 39–50.

Dastogeer, K.M.G., Li, H., Sivasithamparam, K., Jones, M.G.K., Wylie, S.J., 2017a. Host specificity of endophytic mycobiota of wild *Nicotiana* plants from arid regions of Northern Australia. Microb. Ecol. http://dx.doi.org/10.1007/s00248-017-1020-0.

Dastogeer, K.M.G., Li, H., Sivasithamparam, K., Jones, M.G.K., Wylie, S.J., 2017b. A simple and rapid in vitro test for large-scale screening of fungal endophytes from drought-adapted Australian wild plants for conferring water deprivation tolerance and growth promotion in *Nicotiana benthamiana* seedlings. Arch. Microbiol. http://dx.doi.org/10.1007/s00203-017-1411-0.

Deryabin, A., Sin'kevich, M., Dubinina, I., Burakhanova, E., Trunova, T., 2007. Effect of sugars on the development of oxidative stress induced by hypothermia in potato plants expressing yeast invertase gene. Russ. J. Plant Physiol. 54, 32–38.

Du, H., Wang, Z., Yu, W., Huang, B., 2012. Metabolic responses of hybrid bermudagrass to short-term and long-term drought stress. J. Am. Soc. Hortic. Sci. 137, 411–420.

Dupont, P.Y., Eaton, C.J., Wargent, J.J., Fechtner, S., Solomon, P., Schmid, J., Day, R.C., Scott, B., Cox, M.P., 2015. Fungal endophyte infection of ryegrass reprograms host metabolism and alters development. New Phytol. 208, 1227–1240.

Elbersen, H.W., West, C.P., 1997. Endophyte Effects on Growth and Water Relations of Tall Fescue. Plenum Press Div Plenum Publishing Corp, New York.

Elmi, A., West, C., 1995. Endophyte effects on tall fescue stomatal response, osmotic adjustment, and tiller survival. New Phytol. 131, 61–67.

Fernandez, O., Theocharis, A., Bordiec, S., Feil, R., Jacquens, L., Clément, C., Fontaine, F., Barka, E.A., 2012. Burkholderia phytofirmans PsJN acclimates grapevine to cold by modulating carbohydrate metabolism. Mol. Plant Microbe Interact. 25, 496–504.

Gardes, M., Bruns, T.D., 1993. ITS primers with enhanced specificity for basidiomycetes-application to the identification of mycorrhizae and rusts. Mol. Ecol. 2, 113–118.

Gill, S.S., Tuteja, N., 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol. Biochem. 48, 909–930.

Goodin, M.M., Zaitlin, D., Naidu, R.A., Lommel, S.A., 2008. Nicotiana benthamiana: its history and future as a model for plant-pathogen interactions. Mol. Plant Microbe Interact. 21, 1015–1026.

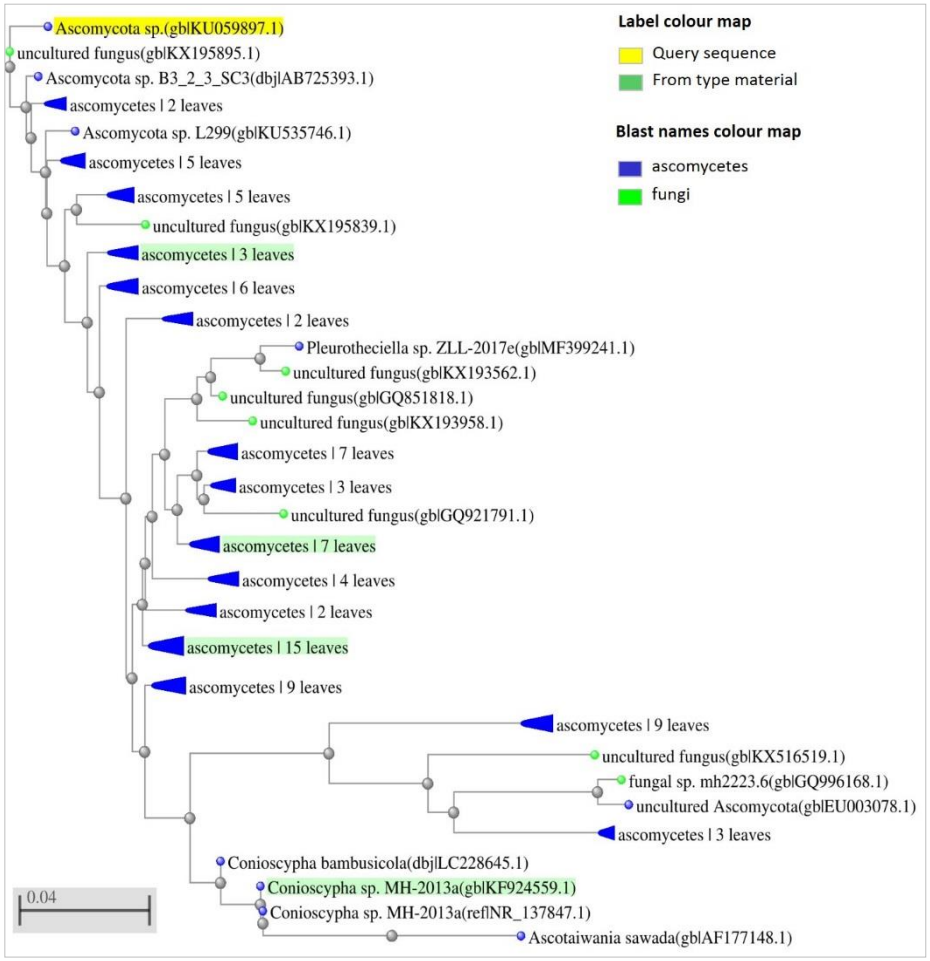
Grover, A., Kapoor, A., Lakshmi, O.S., Agarwal, S., Sahi, C., Katiyar-Agarwal, S., Agarwal, M., Dubey, H., 2001. Understanding molecular alphabets of the plant abiotic stress responses. Curr. Sci. India 206–216.

Gzik, A., 1996. Accumulation of proline and pattern of α-amino acids in sugar beet plants in response to osmotic, water and salt stress. Environ. Exp. Bot. 36, 29–38.

Hahn, H., McManus, M.T., Warnstorff, K., Monahan, B.J., Young, C.A., Davies, E., Tapper, B.A., Scott, B., 2008. Neotyphodium fungal endophytes confer physiological protection to perennial ryegrass (*Lolium perenne* L.) subjected to a water deficit. Environ.

Exp. Bot. 63, 183–199.
Hanson, J., Smeekens, S., 2009. Sugar perception and signaling—an update. Curr. Opin. Plant Biol. 12, 562–567.
Harman, G.E., 2000. Myths and dogmas of biocontrol changes in perceptions derived from research on *Trichoderma harzinum* T-22. Plant Dis. 84, 377–393.
Hassan, M.M., Daffalla, H.M., Modwi, H.I., Osman, M.G., Ahmed, I.I., Gani, M.E.A., Abdel El Gabar, E.B., 2014. Effects of fungal strains on seeds germination of millet and *Striga hermonthica*. Univers. J. Agric. Res. 2, 83–88.
Hoekstra, F.A., Golovina, E.A., Buitink, J., 2001. Mechanisms of plant desiccation tolerance. Trends Plant Sci. 6, 431–438.
Hu, L., Lu, H., Liu, Q., Chen, X., Jiang, X., 2005. Overexpression of mtl D gene in transgenic *Populus tomentosa* improves salt tolerance through accumulation of mannitol. Tree Physiol. 25, 1273–1281.
Junker, B.H., Klukas, C., Schreiber, F., 2006. VANTED: a system for advanced data analysis and visualization in the context of biological networks. BMC Bioinform. 7, 109.
Kane, K.H., 2011. Effects of endophyte infection on drought stress tolerance of *Lolium perenne* accessions from the Mediterranean region. Environ. Exp. Bot. 71, 337–344.
Khan, A.L., Waqas, M., Khan, A.R., Hussain, J., Kang, S.-M., Gilani, S.A., Hamayun, M., Shin, J.-H., Kamran, M., Al-Harrasi, A., 2013. Fungal endophyte *Penicillium janthinellum* LK5 improves growth of ABA-deficient tomato under salinity. World J. Microbiol. Biotechnol. 29, 2133.
Khan, A.L., Waqas, M., Lee, I.-J., 2015. Resilience of *Penicillium resedanum* LK6 and exogenous gibberellin in improving *Capsicum annuum* growth under abiotic stresses. J. Plant Res. 128, 259.
Krasensky, J., Jonak, C., 2012. Drought, salt, and temperature stress-induced metabolic rearrangements and regulatory networks. J. Exp. Bot. 63, 1593–1608.
Lawlor, D.W., 2012. Genetic engineering to improve plant performance under drought: physiological evaluation of achievements, limitations, and possibilities. J. Exp. Bot. 64, 83–108.
Less, H., Galili, G., 2008. Principal transcriptional programs regulating plant amino acid metabolism in response to abiotic stresses. Plant Physiol. 147, 316–330.
Loescher, W.H., 1987. Physiology and metabolism of sugar alcohols in higher plants. Physiol. Plant. 70, 553–557.
Lyons, P.C., Evans, J.J., Bacon, C.W., 1990. Effects of the fungal endophyte *Acremonium coenophialum* on nitrogen accumulation and metabolism in tall fescue. Plant Physiol. 92, 726–732.
Malinowski, D.P., Belesky, D.P., 2000. Adaptations of endophyte-infected cool-season grasses to environmental stresses: mechanisms of drought and mineral stress tolerance. Crop Sci. 40, 923–940.
Mastouri, F., Björkman, T., Harman, G.E., 2012. *Trichoderma harzianum* Trichoderma harzianum enhances antioxidant defense of tomato seedlings and resistance to water deficit. Mol. Plant Microbe Interact. 25, 1264–1271.
Mayer, R.R., Cherry, J.H., Rhodes, D., 1990. Effects of heat shock on amino acid metabolism of cowpea cells. Plant Physiol. 94, 796–810.
Molina-Montenegro, M.A., Oses, R., Torres-Diaz, C., Atala, C., Zurita-Silva, A., Ruiz-Lara, S., 2016. Root-endophytes improve the ecophysiological performance and production of an agricultural species under drought condition. Aob Plants 8, 11.
Morgan, J.M., 1984. Osmoregulation and water stress in higher plants. Annu. Rev. Plant Physiol. 35, 299–319.
Nagabhyru, P., Dinkins, R.D., Wood, C.L., Bacon, C.W., Schardl, C.L., 2013. Tall fescue endophyte effects on tolerance to water-deficit stress. BMC Plant Biol. 13, 127.
Pandey, V., Ansari, M.W., Tula, S., Yadav, S., Sahoo, R.K., Shukla, N., Bains, G., Badal, S., Chandra, S., Gaur, A.K., Kumar, A., Shukla, A., Kumar, J., Tuteja, N., 2016. Dose-dependent response of *Trichoderma harzianum* in improving drought tolerance in rice genotypes. Planta 243, 1251–1264.
Patel, T.K., Williamson, J.D., 2016. Mannitol in plants, fungi, and plant-fungal interactions. Trends Plant Sci. 21, 486–497.
Rare, E., 1990. Stress physiology: the functional significance of the accumulation of nitrogen-containing compounds. J. Hortic. Sci. 65, 231–243.
Rasmussen, S., Parsons, A.J., Fraser, K., Xue, H., Newman, J.A., 2008. Metabolic profiles of Lolium perenne are differentially affected by nitrogen supply, carbohydrate content, and fungal endophyte infection. Plant Physiol. 146, 1440–1453.
Rasmussen, S., Parsons, A.J., Jones, C.S., 2012. Metabolomics of forage plants: a review. Ann. Bot. 110, 1281–1290.
Redman, R.S., Kim, Y.O., Woodward, C.J., Greer, C., Espino, L., Doty, S.L., Rodriguez, R.J., 2011. Increased fitness of rice plants to abiotic stress via habitat adapted symbiosis: a strategy for mitigating impacts of climate change. PLoS One 6, e14823.
Richardson, M., Chapman, G., Hoveland, C., Bacon, C., 1992. Sugar alcohols in endophyte-infected tall fescue under drought. Crop Sci. 32, 1060–1061.
Rodriguez, R., Redman, R., 2005. Balancing the generation and elimination of reactive oxygen species. Proc. Natl. Acad. Sci. U. S. A. 102, 3175–3176.
Rodriguez, R., White Jr., J., Arnold, A., Redman, R., 2009. Fungal endophytes: diversity and functional roles. New Phytol. 182, 314–330.
Roessner, U., Wagner, C., Kopka, J., Trethewey, R.N., Willmitzer, L., 2000. Simultaneous analysis of metabolites in potato tuber by gas chromatography–mass spectrometry. Plant J. 23, 131–142.
Schobert, B., 1977. Is there an osmotic regulatory mechanism in algae and higher plants? J. Theor. Biol. 68, 17–26.
Shukla, N., Awasthi, R.P., Rawat, L., Kumar, J., 2012. Biochemical and physiological responses of rice (Oryza sativa L.) as influenced by Trichoderma harzianum under drought stress. Plant Physiol. Biochem. 54, 78–88.
Sickler, C.M., Edwards, G.E., Kiirats, O., Gao, Z., Loescher, W., 2007. Response of mannitol-producing *Arabidopsis thaliana* to abiotic stress. Funct. Plant Biol. 34, 382–391.
Smirnoff, N., Cumbes, Q.J., 1989. Hydroxyl radical scavenging activity of compatible solutes. Phytochemistry 28, 1057–1060.
Solomon, P.S., Waters, O.D., Oliver, R.P., 2007. Decoding the mannitol enigma in filamentous fungi. Trends Microbiol. 15, 257–262.
Stocker, T., 2014. Climate Change 2013: The Physical Science Basis: Working Group I Contribution to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press.
Tarczynski, M.C., Jensen, R.G., Bohnert, H.J., 1993. Stress protection of transgenic tobacco by production of the osmolyte mannitol. Science-New York, Washington 259 508–508.
Upson, R., Read, D.J., Newsham, K.K., 2009. Nitrogen form influences the response of *Deschampsia antarctica* to dark septate root endophytes. Mycorrhiza 20, 1–11.
van den Berg, R.A., Hoefsloot, H.C., Westerhuis, J.A., Smilde, A.K., van der Werf, M.J., 2006. Centering, scaling, and transformations: improving the biological information content of metabolomics data. BMC Genomics 7, 142.
Vardharajula, S., Zulfikar Ali, S., Grover, M., Reddy, G., Bandi, V., 2011. Drought-tolerant plant growth promoting *Bacillus* spp.: effect on growth osmolytes, and antioxidant status of maize under drought stress. J. Plant Interact. 6, 1–14.
Vasquez-Robinet, C., Mane, S.P., Ulanov, A.V., Watkinson, J.I., Stromberg, V.K., De Koeyer, D., Schafleitner, R., Willmot, D.B., Bonierbale, M., Bohnert, H.J., 2008. Physiological and molecular adaptations to drought in Andean potato genotypes. J. Exp. Bot. 59, 2109–2123.
Vazquez-de-Aldana, B.R., Garcia-Ciudad, A., Garcia-Criado, B., Vicente-Tavera, S., Zabalgogeazcoa, I., 2013. Fungal endophyte (Epichloe festucae) alters the nutrient content of Festuca rubra regardless of water availability. PLoS One 8, 14.
Verbruggen, N., Hermans, C., 2008. Proline accumulation in plants: a review. Amino Acids 35, 753–759.
Verslues, P.E., Sharma, S., 2010. Proline metabolism and its implications for plant-environment interaction. Arabidopsis Book e0140.
Vierstra, R.D., 1993. Protein degradation in plants. Annu. Rev. Plant Biol. 44, 385–410.
Waqas, M., Khan, A.L., Kamran, M., Hamayun, M., Kang, S.-M., Kim, Y.-H., Lee, I.-J., 2012. Endophytic fungi produce gibberellins and indoleacetic acid and promotes host-plant growth during stress. Molecules 17, 10754–10773.
White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR Protocols: A Guide to Methods and Applications, vol. 18. pp. 315–322.
Wylie, S.J., Zhang, C., Long, V., Roossinck, M.J., Koh, S.H., Jones, M.G., Iqbal, S., Li, H., 2015. Differential responses to virus challenge of laboratory and wild accessions of Australian species of Nicotiana, and comparative analysis of RDR1 gene sequences. PLoS One 10, e0121787.
Xia, J., Wishart, D.S., 2016. Using metaboanalyst 3.0 for comprehensive metabolomics data analysis. Curr. Protoc. Bioinformatics 14.10.11–14.10.91.
Yang, T., Ma, S., Dai, C.C., 2014. Drought degree constrains the beneficial effects of a fungal endophyte on Atractylodes lancea. J. Appl. Microbiol. 117, 1435–1449.
Yoshiba, Y., Kiyosue, T., Nakashima, K., Yamaguchi-Shinozaki, K., Shinozaki, K., 1997. Regulation of levels of proline as an osmolyte in plants under water stress. Plant Cell Physiol. 38, 1095–1102.
Zhang, Y.P., Nan, Z., 2007. Growth and anti-oxidative systems changes in *Elymus dahuricus* is affected by *Neotyphodium* endophyte under contrasting water availability. J. Agron. Crop Sci. 193, 377–386.

Supplementary Information:



Supplementary Fig.1: Distance tree of results obtained from NCBI BLAST search of the ITS-based query sequence of the fungal strain E-284. Tree was created using Fast Minimum Evolution method (Desper and Gascuel, 2004). The maximum allowed fraction of mismatched bases in the aligned region between any pair of sequences was 0.75. Number of nodes were reduced in the tree by collapsing subtrees composed of nodes that belong to the same Blast name.

Desper, R., Gascuel, O., 2004. Theoretical foundation of the balanced minimum evolution method of phylogenetic inference and its relationship to weighted least-squares tree fitting. Molecular Biology and Evolution 21, 587-598.

Supplementary Table 1: Fold change (Log 2) of metabolites in the endophytic plants over the non-endophytic plants at various time points under well-watered conditions.

	Metabolites	NEH1.w	E-162H1.w	E-284H1.w	NEH2.w	E-162H2.w	E-284H2.w	NEH3.w	E-162H3.w	E-284H3.w
	Sugar									
1	Melibiose	1.00	0.10	0.04	1.00	0.19	0.10	1.00	0.00	-0.03
2	Arabinose	1.00	4.00	2.32	1.00	3.73	2.44	1.00	3.78	2.55
3	Fructose	1.00	0.13	-0.09	1.00	0.00	-0.23	1.00	0.25	0.09
4	Fucose	1.00	-0.13	-0.06	1.00	0.31	0.16	1.00	0.04	0.11
5	Galactopyranose	1.00	0.09	0.34	1.00	0.09	0.16	1.00	0.04	0.34
6	Galactose	1.00	-0.15	-0.17	1.00	0.08	0.02	1.00	-0.09	0.01
7	Glucose	1.00	-0.06	-0.02	1.00	0.14	0.25	1.00	-0.11	-0.11
8	Gentibiose	1.00	0.18	0.14	1.00	0.02	0.26	1.00	-0.32	-0.04
9	Mannose	1.00	1.03	0.60	1.00	1.20	1.17	1.00	0.94	0.87
10	Psicose	1.00	0.71	-0.41	1.00	0.86	-0.09	1.00	0.89	0.09
11	Raffinose	1.00	-0.03	-0.15	1.00	0.16	-0.03	1.00	0.14	0.17
12	Rhamnose	1.00	0.04	-0.50	1.00	-0.22	0.45	1.00	-0.04	-0.09
13	Maltose	1.00	-1.67	-0.85	1.00	-2.95	-1.78	1.00	-2.10	-0.51
14	Ribose	1.00	0.42	0.21	1.00	-0.40	0.11	1.00	0.89	0.54
15	Sucrose	1.00	-0.41	-0.16	1.00	-0.31	-0.35	1.00	-0.12	0.03
16	Talose	1.00	0.16	-0.34	1.00	-0.04	0.02	1.00	0.03	0.02
17	Threose	1.00	-0.25	-0.05	1.00	-0.17	-0.09	1.00	0.03	-0.02
18	Trehalose	1.00	0.01	-0.05	1.00	-0.03	-0.17	1.00	0.29	-0.18
19	Cellobiose	1.00	-0.11	-0.10	1.00	-0.06	-0.11	1.00	0.06	-0.11
20	Xylose	1.00	0.02	-0.02	1.00	0.09	0.09	1.00	-0.10	0.04
	Organic acids									
21	Arachidonicacid	1.00	0.17	0.06	1.00	0.06	-0.02	1.00	-0.10	-0.03
22	Ascorbate	1.00	0.59	0.47	1.00	0.55	0.23	1.00	-0.53	-0.07
23	Benzoate	1.00	-0.01	0.01	1.00	-0.35	-0.05	1.00	-0.31	-0.23
24	Citrate	1.00	1.01	1.15	1.00	0.01	0.02	1.00	0.00	-0.02
25	Aconitate	1.00	-0.25	-0.32	1.00	-0.25	0.05	1.00	1.05	1.08
26	Docosanoate	1.00	-0.47	-0.21	1.00	0.10	0.07	1.00	0.11	-0.02
27	Dodecanoate	1.00	-0.02	0.09	1.00	-0.04	-0.09	1.00	0.08	0.05
28	Fumarate	1.00	0.06	0.03	1.00	1.07	1.18	1.00	1.15	0.99
29	Glycerate	1.00	-0.28	-0.45	1.00	-0.51	0.44	1.00	-0.55	0.15
30	Glycolate	1.00	0.20	1.38	1.00	0.19	1.44	1.00	0.01	1.37
31	Gulonate	1.00	0.00	0.00	1.00	0.01	0.02	1.00	0.06	0.01
32	Heptadecanoate	1.00	1.48	1.82	1.00	1.71	1.68	1.00	2.23	2.26
33	Hexadecanoate	1.00	-0.17	-0.11	1.00	-0.06	-0.15	1.00	-0.08	0.00
34	Isocitrate	1.00	-0.04	-0.18	1.00	0.02	-0.06	1.00	0.37	-0.01
35	Gluconate	1.00	0.05	-0.17	1.00	-0.20	0.23	1.00	0.10	0.05
36	Lactate	1.00	0.03	0.12	1.00	0.03	0.09	1.00	0.08	0.00
37	Linoleic acid	1.00	-0.25	-0.35	1.00	-0.11	-0.22	1.00	0.17	0.08

38	Maleate	1.00	0.01	0.05	1.00	0.05	0.00	1.00	0.01	0.03
39	Malate	1.00	0.77	0.64	1.00	0.93	0.97	1.00	1.09	0.96
40	Malonate	1.00	0.03	-0.35	1.00	-0.21	-0.13	1.00	0.16	0.06
41	Succinate	1.00	1.06	0.16	1.00	1.34	-0.20	1.00	2.05	0.25
42	Nonanoate	1.00	0.26	0.14	1.00	0.01	-0.13	1.00	-0.13	-0.12
43	Octa-decanoate	1.00	-0.06	-0.12	1.00	-0.10	0.02	1.00	0.14	0.15
44	Oleic acid	1.00	-0.20	1.31	1.00	-0.13	1.76	1.00	-0.30	-0.07
45	Oxalate	1.00	-0.24	-0.19	1.00	-0.06	-0.14	1.00	-0.08	0.00
46	Oxaloacetate	1.00	-0.35	-0.15	1.00	0.28	0.14	1.00	0.14	-0.21
47	Pipecolate	1.00	1.56	0.02	1.00	1.57	0.04	1.00	1.87	0.07
48	Threonate	1.00	-0.31	-0.28	1.00	-0.06	-0.15	1.00	-0.01	0.06
49	Tartarate	1.00	0.32	0.12	1.00	0.03	-0.12	1.00	-0.34	-0.18
	Sugar Alcohols									
50	Fucitol	1.00	0.22	0.02	1.00	0.03	-0.26	1.00	0.11	-0.17
51	Galactinol	1.00	0.23	-1.00	1.00	-0.12	-0.20	1.00	-0.02	-0.04
52	Galactitol	1.00	0.23	-0.02	1.00	0.11	-1.71	1.00	0.14	-1.46
53	Galactosyl-glycerol	1.00	-0.03	-0.01	1.00	0.01	-0.04	1.00	-0.04	0.09
54	Digalactosyl glycerol	1.00	-0.18	-0.31	1.00	-0.16	0.01	1.00	-0.53	-0.08
55	Glycerol	1.00	1.04	-0.23	1.00	1.15	0.10	1.00	1.46	-0.13
56	Glycerol-3-P	1.00	1.19	0.14	1.00	1.62	0.20	1.00	1.32	0.07
57	Mannitol	1.00	2.13	1.93	1.00	2.32	2.73	1.00	2.63	2.19
58	Myoinositol	1.00	0.08	0.07	1.00	0.03	-0.05	1.00	0.06	0.02
59	Ribonate	1.00	0.32	0.26	1.00	-0.15	-0.22	1.00	-0.16	-0.08
60	Sorbitol	1.00	-0.06	-0.21	1.00	-0.06	0.01	1.00	-0.03	0.01
61	Threitol	1.00	-0.08	-0.36	1.00	-0.11	0.01	1.00	-0.05	-0.10
62	Xylitol	1.00	-0.24	1.66	1.00	-0.29	1.69	1.00	0.12	2.13
	Amino acids									
63	Alanine	1.00	0.11	0.23	1.00	-0.07	-0.29	1.00	0.00	0.11
64	Asparagine	1.00	0.91	0.96	1.00	1.15	1.22	1.00	1.03	0.94
65	Aspartate	1.00	0.01	0.13	1.00	-0.01	0.00	1.00	0.00	-0.23
66	Cystathionine	1.00	-0.19	0.00	1.00	0.09	0.02	1.00	0.00	-0.29
67	GABA	1.00	1.29	-0.10	1.00	1.50	0.13	1.00	1.35	-0.05
68	Glutamate	1.00	0.03	-0.03	1.00	-0.09	-0.08	1.00	0.03	0.05
69	Glutamine	1.00	-0.01	0.17	1.00	-0.37	-0.27	1.00	0.08	-0.18
70	Glycine	1.00	0.18	0.00	1.00	-0.27	-0.19	1.00	-0.07	-0.03
71	Homoserine	1.00	0.17	0.04	1.00	-0.02	0.03	1.00	0.00	0.01
72	Isoleucine	1.00	-0.10	0.08	1.00	-0.34	-0.25	1.00	0.07	-0.17
73	Leucine	1.00	0.01	1.64	1.00	0.03	1.75	1.00	0.00	2.12
74	Lysine	1.00	-0.09	-0.07	1.00	-0.14	-0.15	1.00	-0.08	-0.17
75	Methionine	1.00	0.10	0.03	1.00	-0.21	-0.21	1.00	0.13	0.08
76	Phenylalanine	1.00	0.13	0.15	1.00	-0.01	-0.05	1.00	0.00	0.03
77	Proline	1.00	0.11	0.05	1.00	0.03	-0.08	1.00	0.09	-0.06
78	Pyroglutamate	1.00	0.21	-0.06	1.00	-0.03	-0.38	1.00	-0.09	0.02
79	Serine	1.00	0.17	-0.33	1.00	-0.03	-0.37	1.00	-0.17	0.01

80	Threonine	1.00	1.13	0.98	1.00	1.08	0.95	1.00	0.93	0.77
81	Tryptophan	1.00	0.18	-0.09	1.00	-0.02	-0.27	1.00	0.02	0.08
82	Tyrosine	1.00	0.02	0.01	1.00	0.00	-0.01	1.00	0.03	0.05
83	Valine	1.00	0.17	-0.03	1.00	-0.16	0.03	1.00	0.22	0.15
	Other compounds									
84	Adenosine	1.00	-0.03	-0.06	1.00	0.15	0.03	1.00	-0.03	-0.05
85	Allantoin	1.00	2.70	2.92	1.00	0.06	-0.11	1.00	-0.02	0.01
86	Cytosine	1.00	0.21	0.19	1.00	0.19	0.06	1.00	0.06	0.04
87	Diethylene glycol	1.00	0.21	-0.87	1.00	0.05	-1.57	1.00	-0.25	-1.76
88	Ethanolamine	1.00	0.03	-0.02	1.00	0.00	-0.02	1.00	0.10	0.10
89	Guanine	1.00	0.03	-0.32	1.00	-0.08	-0.05	1.00	0.14	0.16
90	Octadecanol	1.00	0.07	0.01	1.00	0.00	0.00	1.00	0.02	0.04
91	Thiourea	1.00	-0.07	-0.02	1.00	0.04	-0.03	1.00	-0.12	0.02
92	Uracil	1.00	0.01	0.01	1.00	-0.03	-0.06	1.00	0.00	0.06
93	Urea	1.00	0.62	0.61	1.00	0.70	0.72	1.00	0.59	0.60

Supplementary table 2: Fold change (Log 2) of metabolites at various time points over that of H1 in the endophytic and non-endophytic plants grown under well-watered conditions.

	Metabolites	**NEH1.w**	**NEH2.w**	**NEH3.w**	**E-162H1.w**	**E-162H2.w**	**E-162H3.w**	**E-284H1.w**	**E-284H2.w**	**E-284H3.w**
	Sugar									
1	Melibiose	1.00	0.01	0.19	1.00	0.10	0.09	1.00	0.06	0.12
2	Arabinose	1.00	0.18	0.35	1.00	-0.10	0.13	1.00	0.30	0.59
3	Fructose	1.00	0.13	-0.06	1.00	0.00	0.07	1.00	0.00	0.13
4	Fucose	1.00	0.09	0.19	1.00	0.53	0.36	1.00	0.31	0.36
5	Galactopyranose	1.00	0.05	-0.01	1.00	0.04	-0.06	1.00	-0.14	-0.01
6	Galactose	1.00	-0.14	-0.01	1.00	0.10	0.05	1.00	0.06	0.17
7	Glucose	1.00	0.11	0.23	1.00	0.31	0.18	1.00	0.37	0.14
8	Gentibiose	1.00	0.23	0.33	1.00	0.06	-0.17	1.00	0.34	0.15
9	Mannose	1.00	0.06	0.31	1.00	0.24	0.23	1.00	0.63	0.59
10	Psicose	1.00	-0.10	-0.09	1.00	0.05	0.09	1.00	0.21	0.41
11	Raffinose	1.00	0.01	0.04	1.00	0.20	0.22	1.00	0.13	0.36
12	Rhamnose	1.00	-0.15	0.32	1.00	-0.41	0.25	1.00	0.80	0.74
13	Maltose	1.00	1.04	1.08	1.00	-0.24	0.65	1.00	0.11	1.42
14	Ribose	1.00	0.64	-0.19	1.00	-0.18	0.28	1.00	0.54	0.14
15	Sucrose	1.00	0.19	0.14	1.00	0.29	0.43	1.00	-0.01	0.33
16	Talose	1.00	0.15	0.23	1.00	-0.06	0.10	1.00	0.51	0.59
17	Threose	1.00	0.10	0.06	1.00	0.17	0.34	1.00	0.05	0.09
18	Trehalose	1.00	0.21	0.02	1.00	0.16	0.30	1.00	0.08	-0.11
19	Cellobiose	1.00	0.10	0.04	1.00	0.15	0.22	1.00	0.10	0.04
20	Xylose	1.00	0.06	0.21	1.00	0.12	0.09	1.00	0.17	0.28

	Organiuc acids									
21	Arachidonic acid	1.00	0.26	0.39	1.00	0.14	0.12	1.00	0.18	0.30
22	Ascorbate	1.00	0.28	1.25	1.00	0.24	0.13	1.00	0.04	0.70
23	Benzoate	1.00	0.27	0.60	1.00	-0.07	0.30	1.00	0.21	0.36
24	Citrate	1.00	0.87	0.91	1.00	-0.14	-0.10	1.00	-0.26	-0.26
25	Aconitate	1.00	0.04	-0.47	1.00	0.05	0.84	1.00	0.41	0.94
26	Docosanoate	1.00	-0.21	-0.13	1.00	0.36	0.46	1.00	0.07	0.07
27	Dodecanoate	1.00	0.09	0.07	1.00	0.08	0.18	1.00	-0.09	0.02
28	Fumarate	1.00	-0.96	-1.43	1.00	0.05	-0.35	1.00	0.20	-0.47
29	Glycerate	1.00	-0.95	-0.47	1.00	-1.19	-0.75	1.00	-0.06	0.13
30	Glycolate	1.00	0.10	-0.05	1.00	0.09	-0.25	1.00	0.16	-0.06
31	Gulonate	1.00	0.00	-0.07	1.00	0.01	-0.02	1.00	0.02	-0.07
32	Heptadecanoate	1.00	-0.07	-0.26	1.00	0.17	0.49	1.00	-0.21	0.18
33	Hexadecanoate	1.00	-0.24	-0.02	1.00	-0.13	0.06	1.00	-0.27	0.09
34	Isocitrate	1.00	-0.01	-0.12	1.00	0.06	0.29	1.00	0.11	0.06
35	Gluconate	1.00	0.11	0.17	1.00	-0.13	0.22	1.00	0.51	0.39
36	Lactate	1.00	0.18	0.18	1.00	0.18	0.23	1.00	0.16	0.07
37	Linoleic_acid	1.00	-0.08	-0.19	1.00	0.06	0.23	1.00	0.04	0.24
38	Maleate	1.00	0.17	0.00	1.00	0.22	0.01	1.00	0.12	-0.02
39	Malate	1.00	-0.19	-0.13	1.00	-0.02	0.19	1.00	0.14	0.18
40	Malonate	1.00	-0.07	-0.10	1.00	-0.30	0.02	1.00	0.15	0.32
41	Succinate	1.00	0.21	-0.03	1.00	0.49	0.95	1.00	-0.16	0.05
42	Nonanoate	1.00	0.26	0.34	1.00	0.01	-0.05	1.00	0.00	0.08
43	Octa-decanoate	1.00	-0.01	0.12	1.00	-0.05	0.32	1.00	0.13	0.39
44	Oleic acid	1.00	-0.39	-0.11	1.00	-0.31	-0.21	1.00	0.05	-1.50
45	Oxalate	1.00	-0.31	-0.10	1.00	-0.13	0.06	1.00	-0.27	0.09
46	Oxaloacetate	1.00	-0.31	-0.21	1.00	0.32	0.28	1.00	-0.01	-0.27
47	Pipecolate	1.00	-0.07	-0.04	1.00	-0.06	0.27	1.00	-0.06	0.01
48	Threonate	1.00	-0.37	-0.36	1.00	-0.13	-0.06	1.00	-0.25	-0.03
49	Tartarate	1.00	0.32	0.56	1.00	0.03	-0.10	1.00	0.08	0.25
	Sugar Alcohol									
50	Fucitol	1.00	0.56	0.62	1.00	0.22	0.38	1.00	0.34	0.56
51	Galactinol	1.00	0.27	0.45	1.00	0.15	0.36	1.00	-0.44	-0.02
52	Galactitol	1.00	0.34	0.27	1.00	0.14	0.14	1.00	0.11	0.12
53	Galactosyl-glycerol	1.00	-0.03	-0.06	1.00	0.01	-0.06	1.00	-0.07	0.04
54	Digalactosyl glycerol	1.00	0.38	0.35	1.00	0.40	0.00	1.00	0.70	0.57
55	Glycerol	1.00	0.04	0.48	1.00	0.15	0.90	1.00	0.37	0.57
56	Glycerol-3-P	1.00	-0.04	0.16	1.00	0.39	0.28	1.00	0.02	0.08
57	Mannitol	1.00	-0.18	-0.13	1.00	0.01	0.37	1.00	0.62	0.12
58	Myoinositol	1.00	-0.02	0.13	1.00	-0.08	0.11	1.00	-0.14	0.08

59	Ribonate	1.00	0.34	0.25	1.00	-0.14	-0.23	1.00	-0.14	-0.09
60	Sorbitol	1.00	0.08	0.46	1.00	0.08	0.50	1.00	0.30	0.68
61	Threitol	1.00	-0.03	0.25	1.00	-0.05	0.28	1.00	0.33	0.51
62	Xylitol	1.00	0.33	0.19	1.00	0.29	0.55	1.00	0.36	0.66
	Amino acids									
63	Alanine	1.00	0.54	0.45	1.00	0.36	0.34	1.00	0.02	0.33
64	Asparagine	1.00	-0.21	-0.06	1.00	0.03	0.06	1.00	0.06	-0.07
65	Aspartate	1.00	0.20	0.36	1.00	0.17	0.35	1.00	0.06	0.00
66	Cystathionine	1.00	-0.05	0.18	1.00	0.23	0.37	1.00	-0.02	-0.11
67	GABA	1.00	-0.16	-0.05	1.00	0.05	0.02	1.00	0.07	0.00
68	Glutamate	1.00	0.14	0.25	1.00	0.01	0.25	1.00	0.08	0.33
69	Glutamine	1.00	0.16	0.09	1.00	-0.21	0.18	1.00	-0.29	-0.26
70	Glycine	1.00	0.25	0.44	1.00	-0.20	0.19	1.00	0.05	0.40
71	Homoserine	1.00	0.00	0.04	1.00	-0.18	-0.13	1.00	-0.01	0.01
72	Isoleucine	1.00	0.09	0.03	1.00	-0.15	0.20	1.00	-0.24	-0.22
73	Leucine	1.00	0.09	0.08	1.00	0.11	0.07	1.00	0.20	0.57
74	Lysine	1.00	-0.04	0.01	1.00	-0.09	0.01	1.00	-0.12	-0.10
75	Methionine	1.00	0.16	0.00	1.00	-0.15	0.04	1.00	-0.08	0.05
76	Phenylalanine	1.00	0.19	0.15	1.00	0.06	0.03	1.00	0.00	0.04
77	Proline	1.00	0.02	0.09	1.00	-0.06	0.07	1.00	-0.10	-0.01
78	Pyroglutamate	1.00	0.30	0.35	1.00	0.06	0.04	1.00	-0.01	0.43
79	Serine	1.00	0.31	0.45	1.00	0.11	0.11	1.00	0.26	0.78
80	Threonine	1.00	0.05	0.24	1.00	0.00	0.04	1.00	0.02	0.03
81	Tryptophan	1.00	0.26	0.42	1.00	0.06	0.27	1.00	0.08	0.59
82	Tyrosine	1.00	0.00	-0.01	1.00	-0.02	0.00	1.00	-0.02	0.03
83	Valine	1.00	0.10	0.03	1.00	-0.23	0.08	1.00	0.16	0.21
	Other Compounds									
84	Adenosine	1.00	-0.06	0.12	1.00	0.13	0.12	1.00	0.03	0.13
85	Cytosine	1.00	0.18	0.26	1.00	0.24	0.17	1.00	-0.16	-0.02
86	Allantoin	1.00	2.88	2.89	1.00	0.16	0.11	1.00	0.05	0.11
87	Diethylene glycol	1.00	0.30	0.67	1.00	0.14	0.21	1.00	-0.40	-0.22
88	Ethanolamine	1.00	-0.05	-0.12	1.00	-0.09	-0.05	1.00	-0.05	0.01
89	Guanine	1.00	0.25	-0.04	1.00	0.14	0.06	1.00	0.52	0.43
90	Octadecanol	1.00	-0.02	0.00	1.00	-0.08	-0.05	1.00	-0.02	0.03
91	Thiourea	1.00	-0.03	0.00	1.00	0.08	-0.04	1.00	-0.04	0.04
92	Uracil	1.00	0.07	-0.02	1.00	0.03	-0.03	1.00	0.01	0.03
93	Urea	1.00	0.00	0.12	1.00	0.08	0.09	1.00	0.11	0.11

Supplementary Table 3: Fold change (Log 2) of metabolites in the endophytic plants over the non-endophytic plants at various time points under moderate and severe stress conditions

	Metabolites	**NEH2.d**	**E-162H2.d**	**E-284H2.d**		**NEH3.d**	**E-162H3.d**	**E-284H3.d**
	Sugar							
1	Melibiose	1.00	0.16	-0.16		1.00	1.07	0.33
2	Arabinose	1.00	3.41	4.47		1.00	1.04	3.40
3	Fructose	1.00	0.40	0.19		1.00	1.22	0.10
4	Fucose	1.00	1.22	1.77		1.00	0.54	0.58
5	Galactopyranose	1.00	1.85	1.74		1.00	2.63	1.96
6	Galactose	1.00	-0.13	0.41		1.00	0.06	-0.01
7	Glucose	1.00	0.78	0.12		1.00	1.52	-0.30
8	Gentibiose	1.00	-0.14	-0.14		1.00	-1.36	-0.31
9	Mannose	1.00	1.37	1.37		1.00	1.22	1.62
10	Psicose	1.00	1.20	0.29		1.00	1.54	0.78
11	Raffinose	1.00	0.42	0.38		1.00	0.94	-0.40
12	Rhamnose	1.00	2.35	-0.33		1.00	2.46	0.80
13	Maltose	1.00	-2.64	-2.55		1.00	-1.03	0.13
14	Ribose	1.00	-0.74	-0.33		1.00	-0.80	0.61
15	Sucrose	1.00	-0.24	-0.42		1.00	1.14	0.54
16	Talose	1.00	-0.21	-0.83		1.00	1.18	-0.06
17	Threose	1.00	0.00	0.03		1.00	-0.56	0.33
18	Trehalose	1.00	-0.03	-0.29		1.00	1.04	0.75
19	Cellobiose	1.00	-0.62	0.14		1.00	-0.99	0.95
20	Xylose	1.00	0.73	0.98		1.00	0.79	3.59
	Organic acids							
21	Arachidonicacid	1.00	0.05	0.46		1.00	-0.18	0.11
22	Ascorbate	1.00	0.66	0.32		1.00	-0.33	-0.18
23	Benzoate	1.00	-0.07	-0.11		1.00	1.01	0.82
24	Citrate	1.00	-1.51	-1.61		1.00	-1.20	-0.90
25	Aconitate	1.00	-0.26	-0.07		1.00	0.90	1.80
26	Docosanoate	1.00	0.66	0.61		1.00	-1.60	-2.26
27	Dodecanoate	1.00	-0.40	0.03		1.00	-0.12	-0.91
28	Fumarate	1.00	-0.05	0.00		1.00	1.08	1.03
29	Glycerate	1.00	-0.31	0.24		1.00	-1.15	-0.73
30	Glycolate	1.00	-0.36	0.85		1.00	-0.04	1.56
31	Gulonate	1.00	0.36	0.34		1.00	2.07	1.36
32	Heptadecanoate	1.00	1.74	2.03		1.00	2.62	2.87
33	Hexadecanoate	1.00	0.06	-0.06		1.00	0.67	0.07
34	Isocitrate	1.00	-0.09	-0.11		1.00	-0.96	-1.31
35	Gluconate	1.00	0.41	0.47		1.00	0.78	1.08
36	Lactate	1.00	0.36	0.11		1.00	0.85	0.39
37	Linoleic acid	1.00	0.16	-0.16		1.00	0.53	0.62
38	Maleate	1.00	-0.04	-0.07		1.00	-0.01	0.10

39	Malate	1.00	0.55	-0.03		1.00	0.81	0.85
40	Malonate	1.00	-0.22	-0.45		1.00	-1.16	-0.46
41	Succinate	1.00	2.26	0.89		1.00	4.38	2.33
42	Nonanoate	1.00	-0.52	-0.02		1.00	0.11	0.19
43	Octa-decanoate	1.00	-0.16	-0.06		1.00	1.12	0.92
44	Oleic acid	1.00	0.02	1.51		1.00	-0.74	1.14
45	Oxalate	1.00	0.07	-0.09		1.00	0.36	0.00
46	Oxaloacetate	1.00	0.14	0.15		1.00	0.04	-0.17
47	Pipecolate	1.00	1.61	-0.06		1.00	1.49	0.00
48	Threonate	1.00	-1.36	-0.84		1.00	-0.56	-0.21
49	Tartarate	1.00	0.01	-0.09		1.00	-0.47	-0.44
	Sugar Alcohol							
50	Galactitol	1.00	0.58	-0.07		1.00	0.48	-1.46
51	Fucitol	1.00	-0.13	-0.17		1.00	0.32	0.24
52	Galactinol	1.00	0.29	-1.85		1.00	0.07	-0.46
53	Galactosyl-glycerol	1.00	0.07	0.29		1.00	0.35	0.38
54	Digalactosyl glycerol	1.00	0.20	-0.87		1.00	-0.59	-1.53
55	Glycerol	1.00	1.34	0.19		1.00	1.50	-0.22
56	Glycerol-3-P	1.00	1.94	0.57		1.00	1.24	-0.14
57	Mannitol	1.00	2.96	2.42		1.00	3.89	3.47
58	Myoinositol	1.00	0.07	0.10		1.00	0.07	-0.29
59	Ribonate	1.00	-0.01	-0.07		1.00	0.58	0.33
60	Sorbitol	1.00	-0.35	-0.60		1.00	0.43	0.39
61	Threitol	1.00	0.20	0.03		1.00	-0.08	-0.28
62	Xylitol	1.00	-0.17	2.19		1.00	0.04	2.39
	Amino acid							
63	Alanine	1.00	-0.12	-0.35		1.00	0.07	0.20
64	Asparagine	1.00	-0.72	-0.75		1.00	0.23	-0.07
65	Aspartate	1.00	0.03	-0.16		1.00	0.15	-0.12
66	Cystathionine	1.00	0.44	-0.02		1.00	0.68	0.56
67	GABA	1.00	1.76	0.59		1.00	1.51	-0.02
68	Glutamate	1.00	-0.82	-0.16		1.00	-0.85	-0.12
69	Glutamine	1.00	0.13	-0.03		1.00	-0.24	-0.06
70	Glycine	1.00	-1.28	-1.28		1.00	-1.72	-1.76
71	Homoserine	1.00	-0.10	1.06		1.00	0.13	0.96
72	Isoleucine	1.00	0.13	-0.03		1.00	0.01	-0.05
73	Leucine	1.00	-0.07	1.95		1.00	0.91	0.55
74	Lysine	1.00	-1.62	-1.21		1.00	-1.57	-1.09
75	Methionine	1.00	-0.89	-1.11		1.00	0.52	0.06
76	Phenylalanine	1.00	0.11	0.11		1.00	0.18	0.45
77	Proline	1.00	-0.54	-0.76		1.00	-0.44	-0.92
78	Pyroglutamate	1.00	0.46	-0.04		1.00	0.20	0.58
79	Serine	1.00	0.52	0.06		1.00	0.44	1.90
80	Threonine	1.00	0.44	0.47		1.00	-1.52	-0.40

81	Tryptophan	1.00	0.09	-0.07		1.00	-0.52	0.01
82	Tyrosine	1.00	-0.69	0.00		1.00	-0.69	-0.22
83	Valine	1.00	1.27	1.51		1.00	1.95	2.12
	Other Compounds							
84	Adenosine	1.00	0.15	-0.05		1.00	0.22	-0.06
85	Allantoin	1.00	0.15	-0.04		1.00	0.45	0.51
86	Cytosine	1.00	0.39	0.33		1.00	0.22	0.52
87	Diethylene glycol	1.00	0.15	-1.71		1.00	-0.16	-1.80
88	Ethanolamine	1.00	-0.11	0.10		1.00	-0.20	-0.20
89	Guanine	1.00	-0.02	-0.05		1.00	0.02	0.31
90	Octadecanol	1.00	-0.07	-0.06		1.00	0.00	-0.03
91	Thiourea	1.00	0.10	-0.61		1.00	0.41	0.94
92	Uracil	1.00	0.00	-0.02		1.00	0.02	0.00
93	Urea	1.00	0.76	0.97		1.00	0.59	1.61

	The fold change value is significantly different at P≤0.001 and positive
	The fold change value is significantly different at P≤0.01 and positive
	The fold change value is significantly different at P≤0.05 and positive
	The fold change value is significantly different at P≤0.001 and negative
	The fold change value is significantly different at P≤0.01 and negative
	The fold change value is significantly different at P≤0.05 and negative

Chapter 6

Fungal endophytes and a virus confer drought tolerance to *Nicotiana benthamiana* plants through modulating osmolytes, antioxidant enzymes and expression of drought responsive genes

6. Fungal endophytes and a virus confer drought tolerance to *Nicotiana benthamiana* plants through modulating osmolytes, antioxidant enzymes and expression of host drought responsive genes

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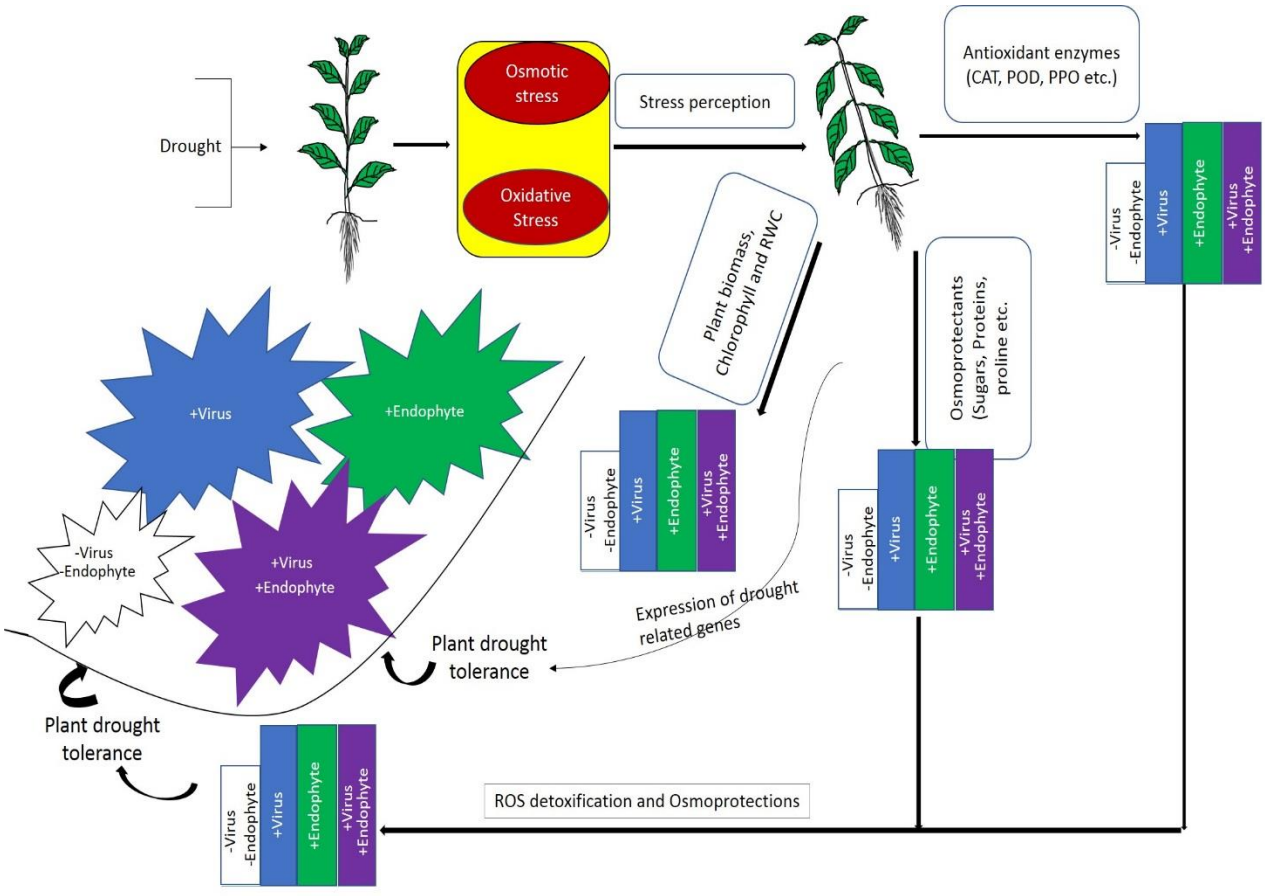
6.1. Graphical Abstract



Highlights:

- Water stress tolerance of endophyte-colonized plants was correlated with increases in plant biomass, RWC, osmolytes, and antioxidant enzymes.
- There was significant upregulation of drought-related genes in endophyte colonized plants.
- Both fungi and the virus reprogram plant responses to water stress in a similar way.
- Co-infection of fungi and virus neither had an additive nor a regressive effect on plant drought responses.

6.2. Abstract

Microbial symbionts increase plant growth and eco-physiological performance under abiotic stress. In this study, we evaluated how the colonization of two fungal endophytes isolated from wild *Nicotiana* species from areas of drought-prone northern Australia, and a plant virus, yellowtail flower mild mottle virus (genus *Tobamovirus*), improved water stress tolerance in *N. benthamiana* plants. Inoculation with both of the two fungal strains used and the virus significantly increased plants tolerance to water stress as manifested by their significant delay in wilting of shoot tips. The water stress tolerance of fungus-inoculated plants was correlated with increases in plant biomass, relative water content, soluble sugar, soluble protein, proline content, increased activities of the antioxidant enzymes catalase, peroxidase and polyphenol oxidase, decreased production of reactive oxygen species, and decreased electrical conductivity. In addition, there was significant upregulation of several genes previously identified as drought induced. The influence of the virus was similar to the fungi in terms of increasing the plant osmolytes, antioxidant enzyme activity and gene expression. Although separate infection of fungi and virus increased plant water stress tolerance responses, their co-infection in plants did not have an additive effect on water stress responses. These findings show that both fungi and viruses reprogram plant responses to water stress in a similar way.

Keywords: Antioxidants; chlorophyll; drought tolerance; gene expression; fungi; osmolytes; plant biomass; ROS; wilting.

6.3. Introduction

Plants, being sessile, are continuously challenged with environmental stimuli and stresses, which significantly deter their growth and survival. Of these, water deficit or drought stress is one of the most important limiting factors for the growth of plants in both natural and agricultural settings (Passioura, 2006). Current climate change models are predicting a substantial decrease in soil water availability for plants in the coming years (IPCC, 2015).

The way plants respond to water stress is rather complex and involves various physiological and morphological adaptations (Chaves et al., 2009; Flexas et al., 2006; Nobel, 1999). Water deprivation causes an imbalance in the osmotic potential of the plant tissues, and induces the synthesis of reactive oxygen species (ROS) (Jakab et al., 2005). Plants accumulate compatible solutes or osmolytes such as sugars, proteins, proline, etc. to maintain their cellular redox potential and osmoregulation (Dar et al., 2016; Joshi et al., 2016; Lisar et al., 2012; Munns, 1988; Serraj and Sinclair, 2002a; Slama et al., 2015). The plant also produces reduced glutathione (GSH), polyphenols, and several antioxidant enzymes like catalase (CAT), peroxidase (POD) and polyphenol oxidase (PPO), etc. to counter the detrimental effects of increased ROS (Claeys and Inzé, 2013; Hoekstra et al., 2001). Many drought-related genes have been identified (Seki et al., 2002) that can be grouped into two major classes: i) proteins that function directly in abiotic stress tolerance, and ii) regulatory proteins, which are involved in signal transduction or expression of stress-responsive genes (Shinozaki et al., 2003).

Several microorganisms such as bacteria and fungi have been found to increase plant tolerance to water stress. Fungal induced plant water stress tolerance has been reported for both mycorrhizae (reviewed by Rapparini and Peñuelas (2014)) and endophytes (reviewed by (Dastogeer and Wylie, 2017); Rodriguez et al. (2009). In particular, fungal endophytes have been shown to increase drought-tolerance in many crops through morphological and biochemical mechanisms such as increased photosynthetic rate and water use efficiency (Rozpądek et al., 2015; Swarthout et al., 2009), higher accumulation of osmoprotectants or compatible solutes (Grover et al., 2011), improving the plant nutrient availability and root growth (Malinowski and Belesky, 2000b) and regulation of genes implicated in homeostasis (Estrada et al., 2013).

The study of plant viruses has been biased towards investigating pathogens. The study on the positive role of virus induced plant stress tolerance is, however, limited. Several viruses provide benefits to their hosts undergoing stress. For instance, a three-way mutualism between a virus, a fungus and a plant conferred thermal tolerance (Márquez et al., 2007). Infection with some RNA plant viruses such as brome mosaic virus (BMV), cucumber mosaic virus (CMV), tobacco mosaic virus and tobacco rattle virus increase drought tolerance through the accumulation of osmoprotectants and antioxidants (Xu et al., 2008). Plant tissue contains various RNA viruses, but their roles in plant ecophysiology to a large extent are still unstudied. Because plants simultaneously interact with biotic and abiotic agents, biotic and abiotic signalling pathways share common nodes and their output has substantial overlaps. This may be why infection with some microbes primes plants to survive under complex environmental conditions (Chini et al., 2004; Timmusk and Wagner, 1999; Xu et al., 2008).

We aimed to study the effects of infection by two ascomycete fungal endophytes and yellow tail flower mild mottle virus that were identified previously (Dastogeer et al., 2017a). *N. benthamiana* is an important model plant in plant virology and other branches of plant science (Goodin et al., 2008).

Here we described the changes in plant biomass, relative water content, chlorophyll content, electrolyte leakage, accumulation of osmolytes and some antioxidant enzymes as well as expression of drought related genes in *N. benthamiana* plants caused by fungal and virus infection and drought stress and by their interactions.

6.4. Materials and methods

6.4.1. Endophyte strain, virus and plant materials

Two ascomycete endophytic fungal strains; E-162 (*Cladosporium cladosporioides*, GenBank accession: KU059880) and E-284, (unidentified member of the Ascomycota, GenBank accession: KU059897) were previously isolated from sterilised root tissues of native *Nicotiana benthamiana* plants (Dastogeer et al., 2017c). These isolates were chosen based on their positive influence on water stress tolerance of *N. benthamiana* plants (Dastogeer et al., 2017a). We used yellow tailflower mild mottle virus isolate Cervantes (YTMMV, genus *Tobamovirus*, GenBank accession KF495565) which was originally collected from a wild plant of Yellow Tailflower (*Anthocercis littoria*, family Solanaceae) at Cervantes, Western Australia (Wylie et al., 2014). For the current study, we chose an accession of wild *N. benthamiana* plant, Mta5, originally isolated from an Arid region near Mt Augustus, Western Australia. This accession was identified to have the highest inherent tolerance to water stress from a number of accessions of Australian *Nicotiana* species tested (unpublished). This virus and the plant accession were chosen because this virus was not lethal to the wild accessions of *N. benthamiana* plants we used. Instead, it results in systemic spread within plants as was confirmed by RT-PCR, and produces moderate symptoms of chlorosis, leaf mosaic and deformation. It causes moderate stunting of growth and reduces flower production (Wylie et al., 2015).

6.4.2. Plant growth conditions and inoculation

Surface-sterilised seeds were prepared by treatment with 3% sodium hypochlorite for 3 min and then 75% ethanol for 2 min, then rinsed with sterile water three times. Three weeks after sowing, seedlings were uprooted and roots were washed in running water. Before transplanting each seedling in an individual pot filled with sterilised soil, the roots were inoculated with fungal mycelium. For inoculum preparation, fungal strains were grown on potato dextrose broth (0.1X) at 25°C for 7 d. The mycelia were collected, and the mycelial suspension was prepared in a liquid homogenizer-mixer by maceration. The large hyphal bits were removed by filtration through sterile absorbent cotton wool plugs. With the help of a haemocytometer and a compound microscope, the inoculum concentration was counted and adjusted to the desired level (5×10^4 fragments mL^{-1}) through sterile dilution (Dastogeer et al., 2017a). The viability of mycelial fragments was assessed by a germination test on PDA after incubation for 48 h at 25°C.

Inoculation of *N. benthamiana* seedling was done by dipping the roots in the inoculum suspension for 5 h before planting. The control seedlings were mock inoculated with sterile distilled water. The seedlings then were transplanted in steam-treated soil in pots with one seedling per pot (10 cm in diameter and 12 cm deep, 800 g soil per pot). Steam treatment of soil was done at 99°C for 4 h twice with a gap of 48 h in between steaming sessions to eliminate other microbial contaminants. Sterilised media was used to observe the effects only of these strains in the absence of other fungi. Inoculation efficiency was achieved by applying the inoculum suspension by spraying at the root-zone of the seedling at the time of planting. The plants were grown in an insect proof greenhouse at 22°C days and 17°C night, 60±5% RH, with a natural photoperiod. They were watered regularly and received weekly nutrient feeds.

After two weeks of fungal inoculation, half of each group was subjected to mock inoculation with 0.1M phosphate buffer (pH 7) and diatomaceous earth (Sigma) and another half as above with the addition of macerated leaf material from YTMMV infected plants (Wylie et al., 2015). After two weeks when the YTMMV inoculated plants showed mild mosaic symptoms on the leaves, water stress was applied to half of each group by withholding watering. Plants were grown under the condition described above except that the water stress group did not receive watering. The fungal infection status was checked by culturing surface-sterilized roots and identifying the isolated fungi by sequencing of the ITS region of the rDNA (Dastogeer et al., 2017c).

6.4.3. Biomass and relative water content (RWC) measurement

Biomass is an overall measure of plant fitness. For the measurement of biomass, six plants from each treatment combination (watered vs. stressed; YTMMV vs. mock inoculated; E-162 vs. E-284 vs. non-inoculated, NE) were removed from the potting medium at 11 d after water stress application. The roots were washed clean, and the plants were cut at the crown to separate root and shoot. Biomass dry weight was measured after oven drying the samples at 75°C for 48 h.

To measure RWC, fully expanded leaves from twelve plants from each treatment combinations were sampled at d11 after water stress treatments. The leaves were weighed just after sampling to obtain fresh weight (Wf) and were submerged in tubes containing distilled water. The tubes were then stored at 4°C in darkness for 24 h to achieve full turgidity of the leaves. Then, the leaves were removed from the tubes, and the turgid weight (Wt) was determined. Finally, the leaves were oven-dried (48 h at 75°C) to obtain dry weight (Wd). The leaf relative water content was determined as $RWC = [(Wd - Wd)/(Wt - Wd)]$ (Barrs and Weatherley, 1962)

6.4.4. Determination of electrolyte conductivity (EC) and chlorophyll content (ChlC)

We followed the techniques of Sullivan (1971) with modifications to determine the membrane stability of leaves. Fully expanded leaves were collected from plants at 0, 4, 8 and 11 days after stress treatment. Leaves were washed with deionized water to remove leaf surface-adhered electrolytes. To allow electrolyte diffusion from the leaf tissue, leaf discs (5 mm diameter) were placed in a test tube containing 10 ml of deionized water and incubated at 25°C for 4 h. After incubation, tubes were brought to 25°C, shaken, and the initial conductance (EC1) was determined with a digital electric conductivity meter. Tubes were then placed in an autoclave at 121°C for 20 min to completely kill leaf tissues, releasing all electrolytes. After cooling to 25°C, shaken, and final conductance was remeasured (EC2). There were twelve plants from each treatment combinations (watered plus stressed; YTMMV plus mock inoculated; E-162 plus E-284 plus NE inoculated). Leaf electrolyte leakage was calculated as a percentage as follows: $\mathrm{LEL} = \mathrm{EC1}/\mathrm{EC2} \times 100$.

Chlorophyll content of leaves was measured by using a hand-held chlorophyll meter (CCM-200plus, Opti-Science) at 0, 4, 8 and 11 d after stress treatment from six plants of each treatment combination. Chlorophyll measurement was taken during mid-day from three fully expanded leaves from the top, and the readings were averaged.

6.4.5. Determination of sugar, protein and proline

Leaf samples for sugar, protein and proline determination were collected at 0, 4, 8 and 11 d after water stress treatments from six plants per treatment combination. Immediately after harvesting leaves were snap frozen in liquid nitrogen and preserved at -80°C until use.

Proline content (µmols/gram).

Proline was estimated according to the method described by Bates et al. (1973) with minor modifications. Approximately 0.05 g of frozen leaf tissue was homogenised in 5 ml of 3% aqueous sulfosalicylic acid. The homogenate was centrifuged at 4000 xg for 10 min at 4°C. One millilitre of the supernatant was mixed with 1 ml of acid ninhydrin and 1 ml of glacial acetic acid in a test tube. The mixture was placed in a boiling water bath for 1 h. The reaction mixture was extracted with 3 ml of toluene, and the absorbance was measured at 520 nm with a spectrometer (Lambda 25, PerkinElmer). Appropriate proline standards were used for calculation of proline levels in the samples.

Soluble sugar content (mg/g)

Total soluble sugar content was estimated using the method of Dubois et al. (1951). Frozen leaves (0.05 g) of each sample were homogenised in 5 ml of 80% ethanol and incubated for 1 h at 80°C in water bath. In 0.5 ml extracts, 0.5 ml distilled water and 1 ml of 5% phenol were added and incubated for 1 hr at 25°C. After adding 2.5 ml sulphuric acid in the solution, the absorbance was read at 490 nm with a spectrometer (Lambda 25, PerkinElmer). Glucose was used as a standard.

Soluble protein content (mg/g):
The total soluble protein was estimated by a dye-binding assay as described in Bradford and Williams (1976) with some modification. Leaf materials (0.05 g) were homogenised in 1.5 ml of phosphate buffer solution and centrifuged at 4000 xg for 5 min. Then, 0.1 ml of extract was placed in a test tube containing 5 ml protein reagent. One litre of protein reagent was prepared with 100 mg Coomassie Brilliant Blue-G250 in 50 ml of 95% ethanol and 100 ml of 85% phosphoric acid in water. The absorbance of samples was read at 595 nm against a blank with a spectrometer (Lambda 25, PerkinElmer). Bovine Serum Albumin (BSA) was used to prepare the standard curve.

6.4.6. Determination of reactive oxygen species (ROS) sensitivity.

For ROS determination leaves were harvested at 11 d after stress treatment. Using a cork borer, discs (2 mm) from fresh leaves were obtained from each of the treatment combinations and placed in a solution of 100 uM of the herbicide paraquat (N,N'-Dimethyl–4,49-bipyridinium dichloride, Sigma, CAS No.# 856177-) and incubated at 22 °C under fluorescent lights. After 48 hr exposure to paraquat, leaf discs were observed to record chlorophyll oxidation visualised by tissue bleaching (Redman et al., 2011).

6.4.7. Measurement of the antioxidant enzyme activity

The activity of catalase (CAT), peroxidase (POD) and polyphenol oxidase (PPO) were chosen as representative antioxidants to estimate the plant's responses to water stress, endophytes and virus infection. Leaf samples were collected at 0, 4, 8 and 11 d after water stress treatments from twelve plants per treatment combination. Immediately after harvesting, leaves were snap frozen in liquid nitrogen and preserved at -80 °C until use. Plant leaves (100 mg) were homogenised in 5 ml phosphate buffer **(**0.1M, pH 7.0) and then centrifuged (4000xg rpm for 15 min at 4°C). The supernatant was used for all enzymatic analyses. All parameters were expressed as activity per mg protein. Total proteins were determined according to Bradford method as described above (Bradford and Williams, 1976).

The CAT activity was estimated as described by Aebi (1984). In the 0.5 ml crude enzyme supernatant 0. 5 ml phosphate butter (10 mM, 7.0 pH and 0.5 ml H_2O_2 (0.2M) were added. After 1 min incubation, the reaction was stopped by adding 4 ml H_2SO_4 (2%, v/v). The absorbance of the reaction solution was measured at 240 nm. An absorbance change of 1 min was defined as µg of H_2O_2 released per mg protein per min.

The POD and PPO activities were measured as described by Kar and Mishra (1976) with a little modification. To the 0.1 ml crude enzyme supernatant, 0.4 ml phosphate buffer (0.1 M), 50 µl pyrogallol (1 M, Sigma, CAS No.# 254002) and 50 µl H_2O_2 (30%) were added. After incubation (1 min at 25°C), the reaction was stopped by adding 5% (v/v) H_2SO_4 (4 ml). The amount of purpurogallin synthesised during the reaction was measured by the absorbance at 420 nm. A similar

assay mixture (without H_2O_2) was prepared for determination of polyphenol oxidase and the absorbance of purpurogallin formed was read at 420 nm after 5 min incubation at 25°C.

6.4.8. Gene expression analysis using RT-qPCR

The effect of fungi and virus infection on relative expression of drought related genes was studied. Leaf samples were collected at 8 d after initiation of water stress and kept under -80°C until use. Total RNA was extracted using QIAGEN RNeasy plant mini kit per the manufacturer's instructions. The quantitative reverse transcription-polymerase chain reaction (RT-qPCR) analysis was performed using QIAGEN OneStep RT-PCR kit in Rotor-Gene Q instrument (QIAGEN) and the gene-specific primers described in Table 1. Thermal cycling conditions utilized a reverse transcription step at 50°C for 30 min; 95°C for 15 min (RT inactivation and initial denaturation step), followed by 40 PCR cycles at 94°C for 40 sec, 60°C for 30 sec and 72 for 1 min and final extension at 72°C for 10 min.
The full coding sequence of genes of interest was obtained from the NCBI (National Center for Biotechnology Information) database, and primers were designed using primer3 in accordance with the criteria required for quantitative PCR primer design (Udvardi et al., 2008). *Actin* was chosen as the reference gene due to its constitutive and stable expression (Staiger, 2000; Tian et al., 2015a; Vergne et al., 2007). The expression levels of each gene were normalised using *actin* as a reference gene, and relative expression of genes were derived using $2^{\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

6.4.9. Plant wilting behaviour

Three different parameters were considered: days to showing first leaf wilting symptoms; days to final leaf wilting and days to shoot tip wilting (irrecoverable damage) after initiating the water stress. Six plants from each of the treatment combinations (YTMMV vs. Mock; E-162 vs. E-284 vs. NE and all possible combinations) were chosen, and water stress applied. to the plant. This experiment was repeated once again under same experiment condition and experimental design.

6.4.10. Statistical analysis

The three-way ANOVA was performed separately on data under well-watered and stressed conditions. The variables were sugar content, protein content, proline, CAT, POD, PPO, EC, ChlC, while the independent variables were days of water stress, fungal inoculation, and virus inoculation status. For parameters, such as RWC, biomass traits, ROS sensitivity and gene expression analysis the data were obtained only at a single time point, so the independent variable was not present in the three-way ANOVA analysis. Instead, water treatment, fungal inoculation, and virus inoculation were the three independent variables considered. The multiple comparisons among the treatments were computed using Tukey's HSD tests. For analysing the wilting behaviour of the plants in response to water stress, the data from the two separate experiments were pooled because we did not find any

Table 1: Primers used for quantitative reverse transcriptase-polymerase chain reaction analysis of *Nicotiana benthamiana* drought associated genes

Gene Code	Description	GenBank Accession	Forward Primer (5'-3')	Reverse Primer (3'-5')
NbPAL1	*N. benthamiana* cDNA similar to Phenylalanine ammonialyase 1	JK739025.1	CCCATGTTTGCCTCTCCGTG	GCCCGTTGAAACACCTTGC
NbMYB	*N. benthamiana* cDNA similar to Myb-related transcription factor	JK739024.1	TGGCCTGGAGACTGATAACG	GATGTAGGTGGTGGTGGTGG
NbMYC	*N. benthamiana* cDNA similar to MYC related transcription factor	JK739021.1	GATCAATGCCAAACTGGAAGC	GACTTAGCGGTGGTTAAGAGTC
NbGBP16	*N. benthamiana* cDNA similar to DNA-binding protein GBP16	JK739018.1	TTGAGGCTTGTGAGACCAGG	GGCTTTCCTTCACCAGTGCT
NbSOS1	*N. benthamiana* cDNA similar to SOS1 mRNA for Na+/H+ antiporter	JK739016.1	GACAAGGGCAAGGGTGATTA	CGTCTTCGCTATTATCCCACTC
NbPDH1	*N. benthamiana* cDNA similar to Proline oxidase/dehydrogenase 1 (PROX1)	JK739015.1	GCCGTTTAAACTCAGCTCCG	GAACGCCGTAGAACAGCTCC
NbCAT3	*N. benthamiana* cDNA similar to Catalase (CAT3)	JK739008.1	GGAGCAAATCATAGTCACGCC	GCAAGATGTCCTCAGGCCAG
NbHSP101	*N. benthamiana* cDNA similar to Heat shock protein 101 kDa (HSP101)	JK739013.1	CTGAGGCAGCTCTAGATTTCATAC	ATTAGGAGATGGTTGGAGAGGA
NbZfP	*N. benthamiana* zinc finger protein mRNA	AY899938.1	AGGCACAAAACGGAAAACAC	CTGGCATTTGATTGAGCAGA
NbDreb2a	*N. benthamiana* mRNA for transcription factor DREB2a	FN649467.1	TGAAAGCGCTTGAACCTTTT	CTACACGGCCCATAGGGTTA
NbWrky	*N. benthamiana* cDNA 5- similar to WRKY transcription factor 44 (WRKY DNA-binding protein 44)	EH367381.1	GTCTGAGGCATCCAAGACAA	CCTCATTCCGGCACAATAAATG
NbActin	*N. benthamiana* actin (act) mRNA, act-b allele	JQ256516.1	GAGCGGGAAATTGTCAGGGA	GAAACGCTCAGCACCAATGG

significant difference between the experiments. A two-way ANOVA was performed to determine the effect of fungi and virus, and their interaction on plants stress tolerance. The ANOVA and multiple comparison tests were performed in statistical software R using package agricolae (De Mendiburu, 2016). The line graphs, bar charts and box plots were created in Microsoft Excel-2016, and the mean and standard error values were calculated in PAST (Hammer et al., 2008).

Results

6.5.1. Plant Biomass, relative water content and chlorophyll content

Water stress significantly affected plant biomass, and it caused a decreased shoot and root dry mass as well as root/shoot ratio (Table 2). The presence of the fungal endophyte did not show any effect on biomass traits under well-watered conditions, but both the fungal strains produced greater root biomass and root/shoot ratio under stress only in the absence of YTMMV (Table 2). It was noted that the effect of the YTMMV infection was significant and it caused lower accumulation of shoot and root biomass under the well-watered condition and a lower accumulation of shoot biomass under stressed conditions irrespective of fungal inoculation (Table 3). Infection of the virus had no apparent effect on root/shoot ratio in any case (Table 2). We did not find any interaction between fungi and virus or three-way interaction of virus, fungus and water stress on plant biomass accumulation (Table 2).

The RWC was dependent on water stress treatment and endophyte status, water stress × endophyte status and water stress × virus inoculation on plants (Table 2). There was no interaction between endophyte infection status and virus inoculation status and three-way interactions of water stress, endophyte infection and virus inoculation (Table 2). A significantly lower level of water content was measured from all samples subjected to stress (Fig. 1). Non-endophytic samples, however, showed substantially decreased RWC compared to the endophytic plants with either of the strains (E-162 or E-284) regardless of virus inoculation. Virus infection did significantly influence RWC of plants under stress. The YTMMV significantly negatively affected leaf chlorophyll pigment under all conditions (Fig. 2a). Water stress caused a reduction in leaf chlorophyll only at d11 in the absence of virus in NE and E-284 plants but did not affect in E-162 plants (Fig. 2a).

6.5.2. Reactive Oxygen Species

Excised leaf discs from *N. benthamiana* seedlings grown under well-watered and under stress conditions with or without fungus and/or virus were analysed for ROS sensitivity. The photobleaching of leaf disc by herbicide paraquat is mimicking to ROS sensitivity.

Leaves that were not subjected to water stress remained green indicating that ROS was not produced by exposure to paraquat and the effect of fungi and virus was neutral. When exposed to water stress, plants colonized with either of the fungal strains showed no significantly different in photobleaching than those uncolonized (Table 3) in the absence of virus. Interestingly, under water stress, virus infected plants showed significantly lower photobleaching than non-infected plants, and there was no interaction effect of the virus and fungal colonization in any condition (Table 3).

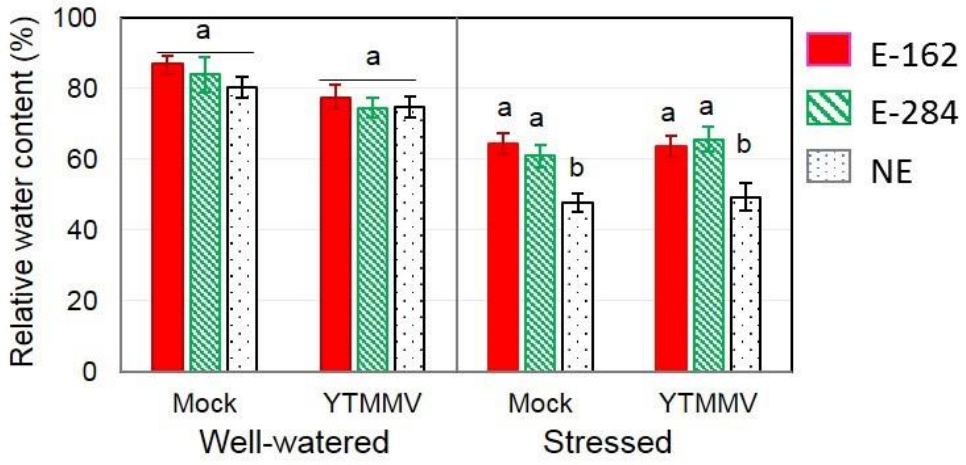


Fig 1. Relative water content of *N. benthamiana* leaf at 8 d after stress application. Bar indicates mean with standard error (SE), N=12. Bar with different letters a significantly different at $p \leq 0.05$ under a condition (well-watered or stressed) in presence or absence of virus (YTMMV or Mock) as obtained by Tukey's honestly significant difference (HSD) test. The E-162 indicates a plant inoculated with the fungal strain E-162, E-284 indicates a plant inoculated with fungal strain E-284, NE = No fungal inoculation, Mock = in absence of virus and YTMMV = plant inoculated with YTMMV (yellowtail flower mild mottle virus).

6.5.3. Electrical conductivity

Under well-watered conditions, there were no differences in electrolyte conductivity (EC) of leaves among the treatments across sampling points (Fig. 2b). Water stress significantly influenced EC of leaves, and it increased as the stress progressed in all plants. However, the rate of the increase in non-endophytic plants was significantly higher as compared to the endophytic plants at 8 d and 11d. This increasing trend continued in all groups as the stress progressed in absence of virus. It was noted that the YTMMV virus inoculation increased the EC under water stress at d4 in the non-endophytic plants but not significantly so in endophytic plants. Under well-watered condition, YTMMV increased EC in NE and E-284 plants but not in E-162 plants at d11 (Fig. 2b).

Table 2: Results of the three-way ANOVA of relative water content and biomass data of *N. benthamiana* plants subjected to water stress and inoculated with fungal endophytes and virus[1].

	RWC (n=12, error df=132)	**Biomass traits (n=6, error df=60)**		
	F value	Shoot	Root	Root: Shoot
		F value	F value	F value
WT	121.51***	35.68***	9.19**	27.09***
VI	2.66	32.36***	25.77***	0.00
EI	10.42***	0.15	0.59	1.28
WT×VI	7.01**	4.92*	8.45**	6.06*
WT×EI	4.46*	0.22	0.93	3.55*
VI×EI	0.25	0.02	0.07	0.64
WT×VI×EI	0.32	0.03	0.28	0.53

[1]WT= Water treatment, EI= Endophyte inoculation, VI= Virus inoculation, RWC= Relative water content. The "*, ** and ***" symbols indicate the F-value is significant at $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$, respectively.

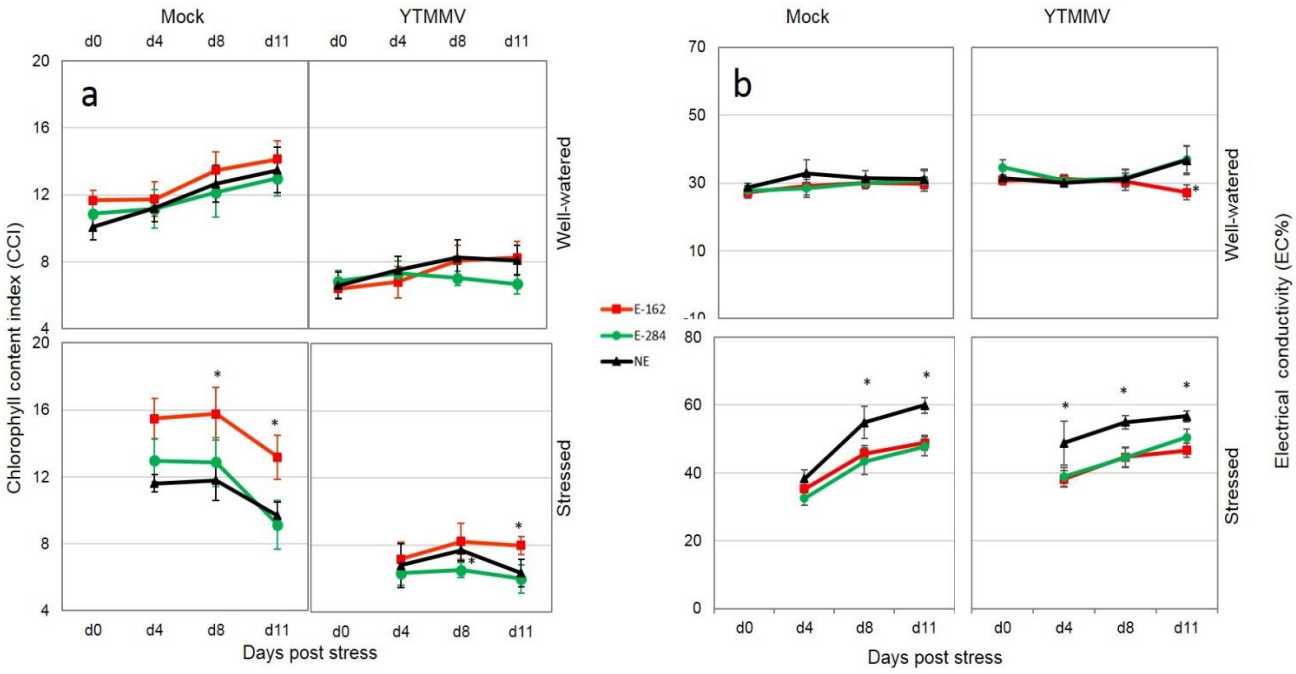


Fig. 2. (a) Chlorophyll Content Index and (b) Electrical conductivity values of *N. benthamiana* leaf at 0, 4, 8 and 11 d after water stress application. The bars indicate standard error (SE), N=6. The "*" symbol above any line indicates the value is significantly different (p≤0.05) from the other treatments at the corresponding time point under a condition (well-watered or stressed) in presence or absence of virus (YTMMV or Mock) as obtained by Tukey's HSD test. The E-162 indicates plants inoculated with the fungal strain E-162, E-284 indicates plants inoculated with fungal strain E-284, NE = No fungal inoculation, Mock = in absence of virus, and YTMMV = plant inoculated with YTMMV (yellowtail flower mild mottle virus).

6.5.4. Accumulation of osmolytes

Soluble Sugars:
None of the endophyte strains had any noticeable influence on the plant soluble sugar levels across sampling points under well-watered conditions. Inoculation with YTMMV seemed to increase the sugar level in endophytic plants to a certain extent under well-watered conditions (Fig. 3a, Table 4).

When drought was applied, sugar levels showed a steady increase in all the groups irrespective of endophyte or virus inoculation status even though there were significant differences in the level of sugar between endophytic and non-endophytic plants (Fig. 3a, Table 4). In contrast, virus inoculation did not influence sugar accumulation in the plant under water stress (Fig. 3a, Table 4).

Soluble protein:
The amount of soluble protein in leaves showed a sharp increase under water stress conditions regardless of fungal or viral inoculation (Fig. 3b). When there was no virus inoculation, endophyte treatment alone resulted in significantly higher protein accumulation than non-endophytic plants. YTMMV infection resulted in a steeper increase (> four times the level under control condition) in the level of protein in both endophytic and non-endophytic plants (Fig. 3b). The YTMMV inoculation increased soluble proteins in the plants also under the well-watered condition, and no influence of any of the endophyte isolates was observed (Fig. 3b and Table 4).

Proline content:
Proline levels measured low and were constant in well-watered (Fig. 3c). None of the endophyte strains had any influence on proline in well-watered plants, but inoculation with YTMMV increased proline levels to some extent as the plants grew older (Fig. 3c). The levels of proline increased dramatically in plants challenged with increasing water stress in all treatments (Fig. 3c). There were significant differences in proline levels between endophytic and non-endophytic plants in the absence of virus inoculation. But this difference was not observed when the plants were inoculated with the YTMMV (Fig. 3c). Therefore, it was noted that virus inoculation had a substantial impact on leaf proline levels in both stressed and well-watered conditions (Fig. 3c).

6.5.5. Changes in antioxidant enzymes

Catalase
Catalase activity increased under water stress treatment. The rate of increase was highest in E-162 inoculated plants (Fig. 4a). Infection of YTMMV caused a steady increase in CAT activity, which became more prominent as water stress progressed (Fig. 4a). No additive effect of virus and fungal inoculation was noted regardless of water status in the plants except at d8 under stress (Table 4 and Fig. 4a).

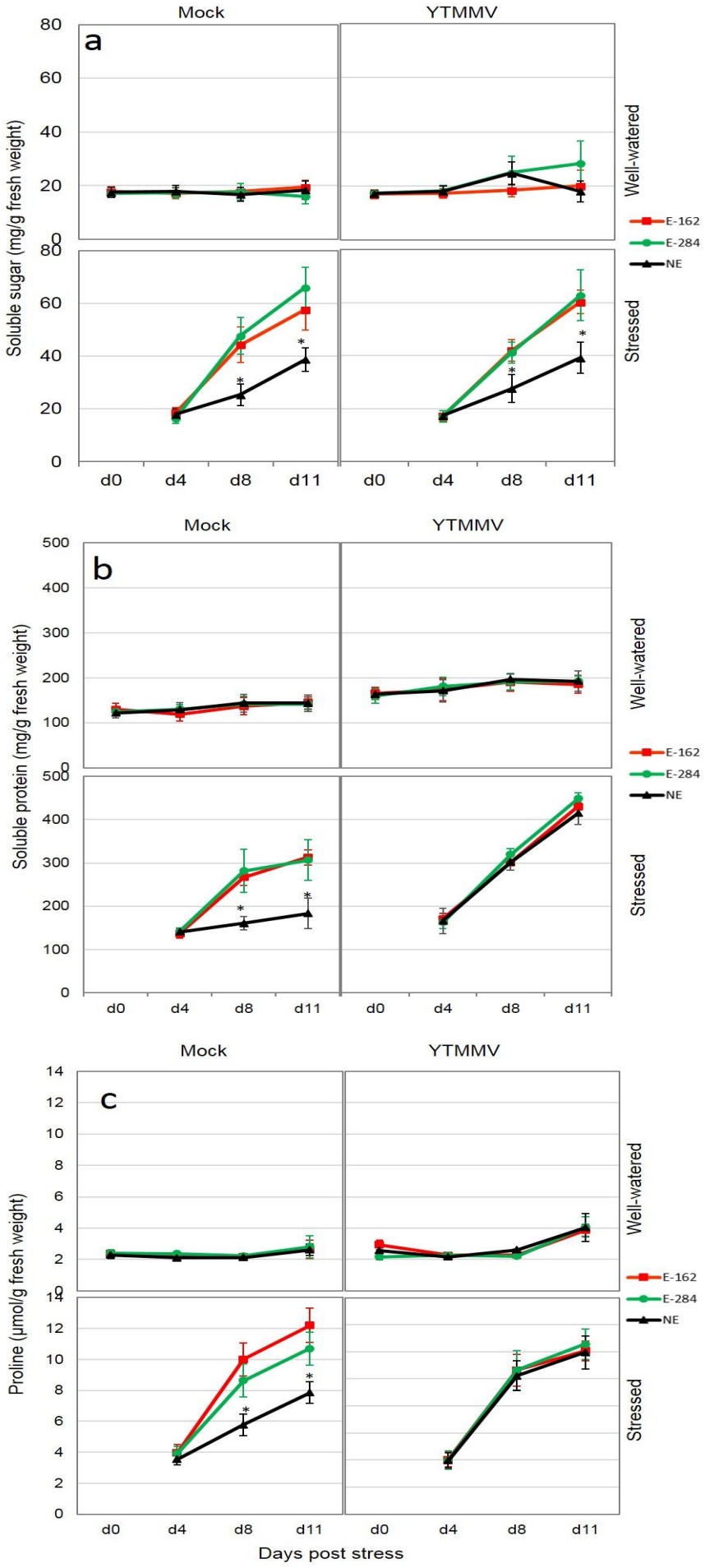

Mock
YTMMV
a
Soluble sugar (mg/g fresh weight)
Well-watered
Stressed
E-162
E-284
NE
d0
d4
d8
d11
b
Soluble protein (mg/g fresh weight)
c
Proline (µmol/g fresh weight)
Days post stress

Fig. 3. (a) Soluble sugar (b) Soluble protein and (c) Proline content of *N. benthamiana* leaves at 0, 4, 8 and 11 d after water stress application. The bars indicate standard error (SE), N=12. The "*" symbol above any line indicates the value is significantly different ($p \leq 0.05$) from the other treatments at the corresponding time point under a condition (well-watered or stressed) in presence or absence of virus (YTMMV or Mock) as obtained by Tukey's HSD test. E-162 indicates plants inoculated with fungal strain E-162, E-284 indicates plants inoculated with fungal strain E-284, NE = No fungal inoculation, Mock = in absence of virus, and YTMMV = plant inoculated with YTMMV (yellowtail flower mild mottle virus).

Peroxidase

Water stress caused increased peroxidase activity in plants in all treatments. At severe water stress, the peroxidase activity was significantly higher in endophytic plants with either of the fungal strains (Fig. 4b). The virus inoculation resulted in a higher peroxidase activity regardless of water availability although the increase was much more pronounced under water stress conditions (Fig. 4b). We did not observe any interaction effect of the virus and fungal inoculation on peroxidase activity under any condition except at d11 (Table 4 and Fig. 4b).

Polyphenol oxidase

Water stress increased polyphenol oxidase activity in all plants. Plants inoculated with E-284 had a significantly higher polyphenol oxidase activity under both conditions in presence of virus (Fig. 4c). Virus inoculated plants had a higher PPO activity under normal watering or stress conditions even though the rate of increase was much more prominent under water stress (Fig. 4c). We did not observe any interaction effect of the virus and fungal inoculation on peroxidase activity under well-watered conditions but did observe the interaction of E-284 and the virus under stress condition (Table 4 and Fig. 4c).

Table 3: Effect of fungal endophyte and virus infection on biomass and ROS sensitivity in water-stressed *N. benthamiana* plants at d8*

Water treatment	**Biomass trait**	**Shoot (mg)**		**Root (mg)**		**Root: Shoot**		**ROS**	
	EI	Mock	YTMMV	Mock	YTMMV	Mock	YTMMV	Mock	YTMMV
WS	E-162	273.82±53.9aA	160.03±31.6aA	51.42±6.1aA	38.17±8.1aA	5.20±0.7bA	4.18±0.51aA	7.6±0.87aA	5±0.84aB
	E-284	241.87±34.2aA	131.23±25.8aB	49.78±7.8aA	31.23±5.8aA	4.94±0.2bA	4.65±0.67aA	8.4±0.51aA	3.8±0.58aB
	NE	243.03±23.3aA	136.53±22.2aB	31.58±1.7bA	27.60±6.1aA	7.80±0.9aA	5.66±0.89aA	10.4±0.51bA	4.8±0.58aB
WW	E-162	502.20±58.9aA	269.50±32.7aB	74.72±15.1aA	29.48±3.9aB	7.32±0.8aA	9.33±0.8aA	1.4±0.51aA	0.6±0.24aA
	E-284	536.83±135.7aA	273.60±59.5aA	77.75±21.0aA	37.33±8.3aA	7.29±0.5aA	7.94±0.9aA	2.2±0.86aA	1±0.32aA
	NE	501.03±53.2aA	242.77±17.4aB	78.27±9.5aA	32.23±2.9aB	7.12±1.4aA	7.79±.07aA	0.8±0.37aA	0.8±0.37aA

*The values with different letters are significantly different ($p \leq 0.05$) as obtained by Tukey's honestly significant different test; small letters compare across column s separately under well-watered (WW) and water stress (WS) condition and the capital letters compare across rows between Mock and YTMMV. E-162=plants inoculated with fungal strain E-162, E-284-plants inoculated with fungal strain E-284, NE=No fungal inoculation, Mock = in absence of virus and YTMMV = plants inoculated with YTMMV; ROS=reactive oxygen species.

Table 4: F-values obtained from the three-way ANOVA of data of various parameters under well-watered and stressed conditions.[1]

		df	EC	Proline	Protein	Sugar	Catalase	Peroxidase	Polyphenol oxidase
Well-watered (n=12, error df=336)	VI	1	9.07*	9.65*	46.19***	4.18*	145.55***	49.82***	111.83***
	EI	2	1.3	0.29	0.05	0.59	0.02	1.34	0.62
	Day	3	1.77	12.69***	3.35*	1.66	7.60***	16.94***	6.33***
	VI×EI	2	0.89	0.04	0.001	1.55	1.51	0.55	0.51
	VI×Day	3	0.61	4.29*	0.3	1.51	1.78	2.4	1.59
	EI×Day	6	0.96	0.36	0.11	0.37	0.66	0.74	0.46
	VI × EI × Day	6	0.63	0.46	0.05	0.76	0.46	1.41	0.4
Stressed (n=12, error df=198	VI	1	1.02	11.87**	60.52***	0.13	50.14***	124.45***	94.03***
	EI	2	14.31***	3.32*	7.32**	13.42***	5.91*	6.54**	4.50*
	Day	2	37.29***	101.75***	103.01***	76.25***	107.59***	75.30***	55.91***
	VI×EI	2	0.86	5.01*	4.08*	0.21	0.33	1.86	2.68
	VI×Day	2	2.57	2.33	12.85***	0.08	2.5	11.71***	16.15***
	EI×Day	4	0.36	0.56	1.98	3.86**	1.69	2.43*	0.48
	VI × EI × Day	4	0.79	0.99	1.18	0.17	0.099	1.27	0.54

[1]EI= Endophyte inoculation, VI= Virus inoculation, EC= Electrical conductivity. The "*, ** and ***" symbols indicate the F-value is significant at $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$, respectively.

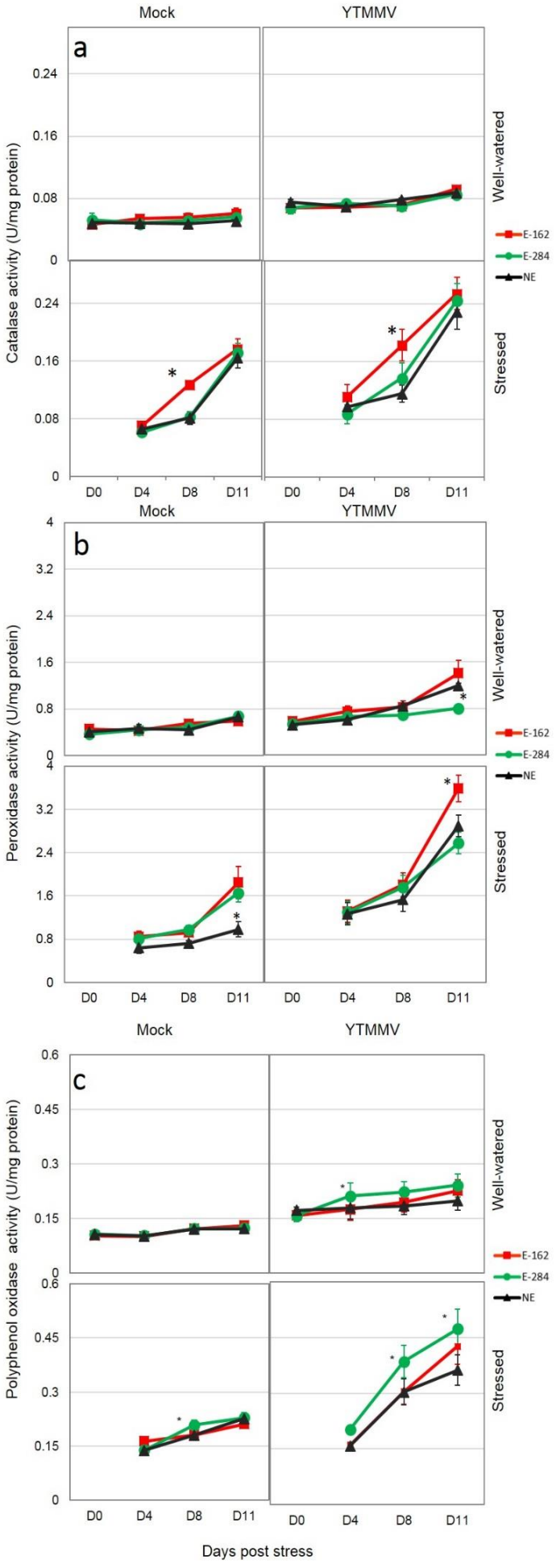

Mock
YTMMV
a
Catalase activity (U/mg protein)
0.24
0.16
0.08
0
Well-watered
Stressed
E-162
E-284
NE
*
D0
D4
D8
D11
b
Peroxidase activity (U/mg protein)
4
3.2
2.4
1.6
0.8
0
c
Polyphenol oxidase activity (U/mg protein)
0.6
0.45
0.3
0.15
Days post stress

Fig. 4. Activity of (a) catalase, (b) and peroxidase (c) polyphenol oxidase enzymes in *N. benthamiana* leaf at 0, 4, 8 and 11 d after water stress application. The bars indicate standard error (SE), N=12. The "*" symbol above any line indicates the value is significantly different ($p \leq 0.05$) from the other treatment at the corresponding time point under a condition (well-watered or stressed) in presence or absence of virus (YTMMV or Mock) as obtained by Tukey's HSD test. E-162 indicates plants inoculated with fungal strain E-162, E-284 indicates plants inoculated with fungal strain E-284, NE = No fungal inoculation, Mock = in absence of virus and YTMMV = plants inoculated with YTMMV (yellowtail flower mild mottle virus).

6.6.6. Expression of drought stress related genes

Water stress and virus infection separately caused differential expression of most of the genes under investigation. There was a significant interaction between water stress and YTMMV inoculation on NbCAT3, NbDreb2a, NbGBP16, NbMYB, NbPAL1, and NbSOS1 (Table 5). The NbCAT3 was significantly upregulated (8-11 fold) in YTMMV infected plant under well-watered conditions, which increased even more (17-20 fold) under stress (Fig. 5). The fungus-infected plants did not show any difference in expression compared to non-inoculated plants under well-watered conditions in absence of virus. Under stress however. However, under stress, NbCAT3 expression differed as a function of fungal inoculation (Fig. 5) in absence of the virus. NbDreb2a expression was significantly downregulated under stress and due to YTMMV inoculation (Fig. 5), and there was a significant interaction of stress and virus infection (Table 5). The expression of NbGBP16 was upregulated under water stress, and E-162 inoculation caused a further increase in its expression in the absence of virus. Interacting with YTMMV, water stress resulted in higher expression (20-24-fold compared to non-inoculated control) regardless of endophyte inoculation. While water stress caused in general a higher expression of NbHSP101 gene its expression was much higher in non-endophytic plants than corresponding endophytic plants (Fig. 5).

There were significant three-way interaction effects of water treatment, endophyte inoculation and virus inoculation as well as all possible three-way interaction effects on the expression of this gene (Table 5). E-162 caused significant upregulation of NbMYB gene under stress but only in the absence of virus. In presence of virus, stress induced NbMYB expression did not differ among endophytic or non-endophytic plants. (Fig. 5). E-162 had a marked influence on NbMYC expression in the absence of YTMMV (Fig. 5). Expression of NbPAL1 was upregulated by virus infection in well-watered condition only (Fig. 5). In absence of virus fungal inoculation increased NbPAL1 expression under stressed and watered conditions. NbSOS1 expression was increased by YTMMV infection in well-watered plants regardless of fungal presence. The gene NbPDH1 and NbWRKY were significantly upregulated in endophytic plants under water stress irrespective of YTMMV infection (Fig. 5).

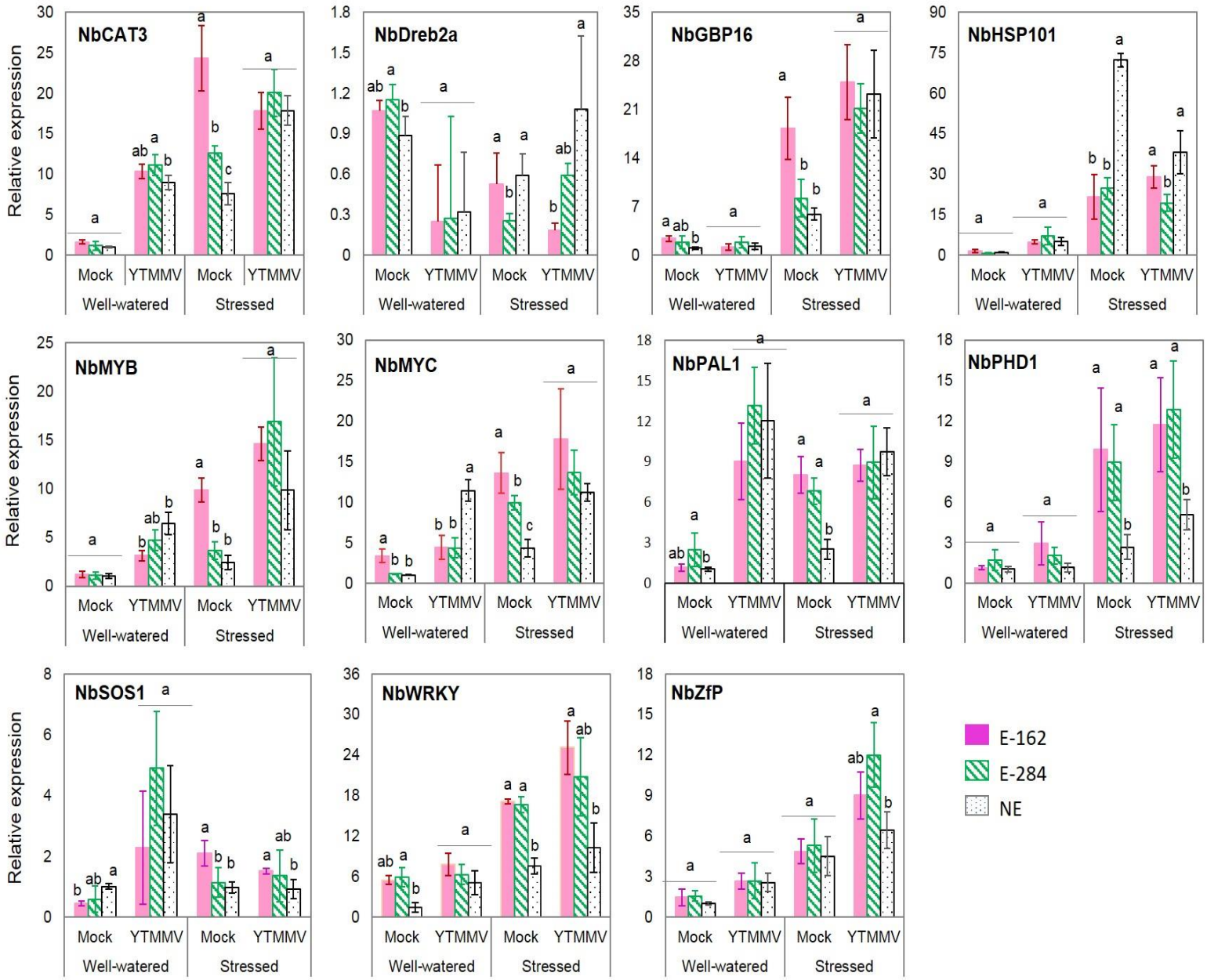

NbCAT3
NbDreb2a
NbGBP16
NbHSP101
NbMYB
NbMYC
NbPAL1
NbPHD1
NbSOS1
NbWRKY
NbZfP
Relative expression
Mock
YTMMV
Well-watered
Stressed
E-162
E-284
NE

Fig 5. Relative changes in expression of drought related genes in *N. benthamiana* leaves under water stress after 8 d. The bars indicate standard error (SE), N=3. Bars with different letters are significantly different ($p \leq 0.05$) under a condition (well-watered or stressed) in presence or absence of virus (YTMMV or Mock) as obtained by Tukey's HSD test. E-162 indicates plant inoculated with fungal strain E-162, E-284 indicates plant inoculated with fungal strain E-284, NE = No fungal inoculation, Mock = in absence of virus, and YTMMV = plants inoculated with YTMMV (yellowtail flower mild mottle virus).

The expression of NbZfP was increased by water treatment and virus infection, but their interaction was not significant (Fig. 5, Table 5) and the role of fungal inoculation was not apparent on its expression (Table 5).

Table 5. F values obtained from three-way ANOVA of the data of relative expression of the *N. benthamiana* drought associated genes. *

Gene Code	WT	VI	EI	WT×VI	WT×EI	VI×EI	WT×VI×EI
Degrees of freedom	1	1	2	1	2	2	2
NbCAT3	111.25	36.75	6.78	6.16	4.34	6.24	6.34
NbDreb2a	0.9	5.45	1.00	13.29	1.95	1.55	0.79
NbGBP16	75.87	11.55	1.92	12.74	1.63	1.01	0.57
NbHSP101	173.96	1.73	19.57	10.82	21.33	6.98	6.9
NbMYB	22.9	19.25	0.96	3.04	2.63	1.15	0.75
NbMYC	32.4	14.05	1.85	0	5.42	2.07	0.54
NbPAL1	0.68	30.86	0.58	7.51	0.96	1.39	0.36
NbPDH1	28.45	1.82	4.16	0.59	2.53	0.04	0.17
NbSOS1	1.93	5.98	0.25	7.25	1.21	0.76	0.25
NbWrky	55.94	5.81	10.86	0.92	3.44	0.34	0.45
NbZfP	43.61	13.74	1.8	3.64	1.21	0.68	1

*The Ct value for each gene was normalised with the Ct value of NbActin and relative expression value was obtained using $2^{\Delta\Delta CT}$ method. A three-way ANOVA was performed to detect whether the effect of WT= water treatment, VI=virus inoculation and EI= Endophyte inoculation and all possible interactions between them were significant. The values provided are F-ratio. The cells marked with dark are significant at $p \leq 0.001$ and those with grey and light grey are significant at $p \leq 0.01$ and $p \leq 0.05$.

6.5.7. Plant wilting behaviour under water stress:

Plants respond to water stress by wilting of leaves followed by wilting of the shoot tip and ultimately death at the severe stage. In our experiment, we found that inoculation with YTMMV delayed plant initial responses to stress. Virus-inoculated plants took a significantly longer time to begin leaf wilting irrespective of fungal inoculation. None of the fungal strains had an apparent effect on the plants early

response to water stress (Fig. 6). As wilting progressed, virus inoculated plants took significantly more days to fully wilt. Regarding the shoot tip, both fungal strains significantly delayed plant shoot tip wilting in the absence of the virus. For example, plants inoculated with E-162 or E-284 took an average of 19 days for the shoot tip to wilt, whereas non-inoculated plants wilted within 14 dps (days post stress) (Fig. 6). However, in the presence of YTMMV infection, all the plant significantly delayed shoot tip wilting (average: 23-25 dps) compared to virus free plants (14-19 dps) and the effect of fungal infection was not apparent (Fig. 6).

6.5. Discussion

6.6.1. Plant responses to water stress

None of the fungal strains increased plant shoot biomass either under well-watered or stressed conditions. This result is in line with several other studies showing a neutral effect of fungal endophytes on shoot biomass under stress (Briggs et al., 2013; He L, 2017; Hesse et al., 2003, 2005; Hill et al., 1996; Jia et al., 2015; Kane, 2011; Khan et al., 2013; Oberhofer et al., 2014; Rudgers and Swafford, 2009). In contrast, certain endophytic strains were also reported to increase (Ghabooli et al., 2013; Gibert et al., 2012; Hill et al., 1996; Malinowski et al., 2005; Vazquez-de-Aldana et al., 2013; Zhang and Nan, 2007) or decrease (Assuero et al., 2006; He L, 2017; Rahman et al., 2015; Yang et al., 2014; Zhang, 2017) plant shoot biomass under water stress. Previously, we reported increased shoot biomass increased of *N. benthamiana* plants under stress when inoculated with the same fungal strains that we used in the present study (Dastogeer et al., 2017a). This could be because of the differences in plant genotype and their inherent drought tolerance level suggesting that the endophyte-plant interaction is context dependent. Increased root biomass of endophytic plants under stress could be a mechanism to uptake water from a greater area and increase tolerance. A similar mechanism could be associated with virus-infected plants in the current study, which was supported by the findings that virus infection did not change plant root biomass under stress, although it reduced the shoot biomass substantially under stress.

As can be expected, the RWC of *Nicotiana* seedlings significantly diminished under stress, while colonization with either of the fungal strains enhanced the leaf water levels to a small extent. This is in accordance with previous studies. For example, inoculation with a strain of *Chaetomium globosum* resulted in an increased water content of wheat leaves subjected to water stress as compared to non-endophytic counterparts (Cong et al., 2015). Also, *Trichoderma atrovride* colonization in maize (Guler et al., 2016) and *T. hamatum* colonization in cacao (Bae et al. 2009) showed a relative increase in water status under stress conditions. In similar research, it was shown that endophytic plants use significantly less water than non-endophytic plants (Chepsergon et al. 2014). Martinez-Medina et al. (2014) also showed that endophytic *T. hamatum* drew water from deeper soil to enhance plant water potential. Endophytes could also help plants reduce the losses of water as the stress progresses to

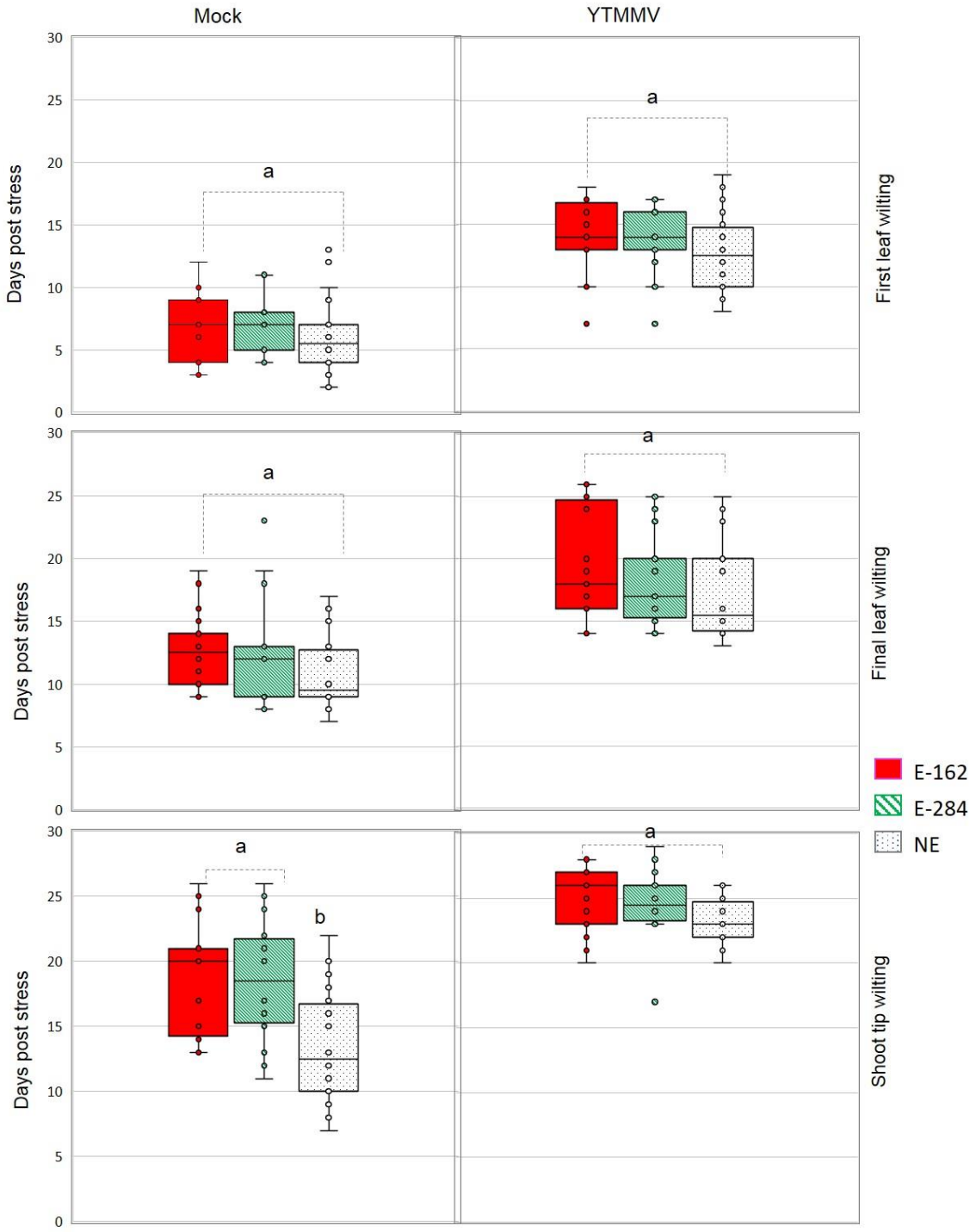


Fig. 6. Wilting behaviour of *N. benthamiana* under water stress. The bars indicate standard error (SE), N=12. Bars with different letters are significantly different ($p \leq 0.05$) as obtained by Tukey's HSD test. E-162 indicates plants inoculated with fungal strain E-162, E-284 indicates plants inoculated with fungal strain E-284, NE = No fungal inoculation, Mock = in absence of virus, and YTMMV = plant inoculated with YTMMV (yellowtail flower mild mottle virus).

better the plant performance by reducing transpiration and through osmotic and metabolite adjustment (Dastogeer et al., 2017b; Morsy et al., 2010; Rodriguez et al., 2010; Waller et al., 2005).

Reduction in chlorophyll content under water stress regimes is the main cause of inactivation of photosynthesis (Shukla et al., 2012). Root colonization with the fungal endophyte E-162 alleviated the water stress effect by improving chlorophyll pigment contents in *N. benthamiana* seedlings. A similar result has been reported by Bae et al. (2009) who reported an increase in chlorophyll content in seedlings colonized by *T. hamatum*. Harman et al. (2004) reported that a strain of *T. harzianum* increased leaf greenness in maize, which enhanced the vigour when an adequate carbon source was available for plant development. In our study, the isolate E-284 did now show any effect on chlorophyll content, while a positive effect of fungal endophytes on plant chlorophyll is common (Bayat et al., 2009; Guler et al., 2016; Khan et al., 2013; Khan et al., 2015; Mastouri et al., 2012; Shukla et al., 2012; Sun et al., 2010b; Waqas et al., 2012; Zhang and Nan, 2007), neutral or negative (Pandey et al., 2016; Ren et al., 2011; Tian et al., 2015b). It is understandable that virus infection severely reduces plant chlorophyll content (Dai et al., 2009; Guo et al., 2005; Plattt et al., 1979). However, it is interesting that in our study, water stress did not influence chlorophyll content in virus infected plants. One possible explanation is that initial plant drought response includes wilting of lower leaves and plant diverts most available water and energy to the upper leaves from where we measure chlorophyll to increase photosynthesis to support growth. The virus accumulation may not keep pace with the activity of these leaves as they can do in the well-watered plant leaves resulting in slower symptom expression (such as yellowing) and hence the disruption of chlorophyll. However, we did not come across any literature explaining this phenomenon and this may be an interesting area of future studies.

A higher level of EC in the plant under water stress is an indication of damage to the cell membrane. Higher leakage of solutes could be associated with increased H_2O_2 production and lipid peroxidation under stress causing membrane destruction and metabolic toxicity leading to higher solutes leakage (Deshmukh et al., 1991; Dionisio-Sese and Tobita, 1998; Premchandra et al.). An increased EC in non-endophytic plants suggests more tissue damage, while this is reduced by the presence of endophytic fungi under stress conditions. Endophyte-mediated reduced EC in plants under stress has been reported in other studies (Bayat et al., 2009; Khan et al., 2013; Shukla et al., 2012; Tian et al., 2015b).

6.6.2. Osmolytes under water stress

Increased sugar accumulation in plants in response to water stress has been documented (Assuero et al., 2006; Ren et al., 2006). Accumulation of soluble sugars in plants is an adaptive response to stress, which functions as an osmotic adjustment balance (Ren et al., 2006). Research on the influence of

fungal endophytes on plant sugar accumulation under water deficit stress reported variable results. For example, endophytes may increase (Assuero et al., 2006; Ren et al., 2006; Richardson et al., 1992; Yang et al., 2014) or decrease (Hill et al., 1990) or exert no apparent influence (Cong et al., 2015; Hill et al., 1990) sugar levels in stressed plants, depending on the host species, breeds and genotypes. Higher sugar accumulation could contribute to the osmotic potential of endophytic plants under stress, which might be initiated by altered carbohydrate balance or by carbohydrate re-metabolization from sugars of senescing leaves (Assuero et al., 2006).

Stressed increased plant soluble proteins. This could be related to their roles in permeation and antioxidation, for example, antioxidant enzymes, phytohormone receptors, etc (Yang et al., 2014). Endophyte-mediated increased soluble proteins and various amino acids in response to water stress has also been reported (Bae et al., 2009; Yang et al., 2014).

Stress induced proline accumulation is common in plants, although its role as an osmoprotectant is still debated (Delauney and Verma, 1993; Serraj and Sinclair, 2002b). Proline accumulation may or may not have any relation with plant drought tolerance (Cha-Um et al., 2010; Hien et al., 2003; Roy et al., 2009). Similar to our findings, endophyte-induced increase in proline was described from drought stressed grass species (Abernethy and McManus, 1998; Bandurska and Jóźwiak, 2010; Elbersen and West, 1996; Malinowski and Belesky, 2000a; Nagabhyru et al., 2013). The higher proline accumulation in the endophytic plant could be due to the role of endophytes under stress conditions to shield plants from the severity of damage through increased accumulation of osmoprotectants (such as proline). It was noted that virus infection increased proline in both watered and stressed plants. This might be because virus infection put the plant under stress, stimulating proline accumulation which was intensified even more under water stress.

6.6.3. ROS and antioxidant enzymes under water stress

When photosynthetic tissue is exposed to paraquat, it generates superoxide ions and causes photobleaching through the reduction of the electron transfer from the plants photosystem I and oxidation by molecular oxygen (Vaughn and Duke, 1983). ROS production is associated with early events in the plant stress response mechanism. The current study indicated that water stress tolerance in endophytic or virus inoculated plants correlated with reduced ROS activity. Higher ROS synthesis is common to all stresses because of stress-induced metabolic imbalances (Apel and Hirt, 2004; Vaughn and Duke, 1983). Decreased ROS activity in endophytic or YTMMV-infected plants could be achieved by improved ROS scavenging of antioxidation systems, which correlate strongly with stress tolerance and may play a critical role in the process.

Plants tend to produce additional ROS in response to various environmental stimuli. Various pathways are involved in detoxifying ROS in plants (Mittler, 2002). Plant cellular antioxidative

enzymes are produced to scavenge more H_2O_2 (Tran et al., 2012) . While the interaction between endophytes and the elements of defence mechanisms is not well-understood, activities of CAT, POD, and PPO were more significantly increased in endophytic plants than in non-endophytic plants under water stress. These enzymes counteract ROS induced oxidative damage. Peroxidase and polyphenol oxidase protect cells against the toxic effects of H_2O_2 by catalysing its decomposition through the oxidation of phenolic osmolytes (Elmi and West, 1995). Our results correlate with those of others that suggest these enzyme activities are increased in endophytic plants challenged with abiotic and biotic stress (Harman, 2011; Khan et al., 2013; Waller et al., 2005). Endophyte-inoculated plants recruited higher amount of oxidative stress enzymes that successively protect plants against ROS formation and membrane damage under stress (Baltruschat et al., 2008; Guler et al., 2016; Harman, 2006; Hashem et al., 2014; Khan et al., 2014; Khan et al., 2013; Mastouri et al., 2012; Shukla et al., 2012; Waller et al., 2005). Virus-induced changes CAT, POD and PPO activity in plants have been reported (Amoako et al., 2015; Buonaurio and Montalbini, 1993; Chatterjee and Ghosh, 2008; Madhusudhan et al., 2009; Riedle-Bauer, 1998; Srivastava and Singh, 2010). In the current study, the activity of all these antioxidant enzymes was increased in the presence of the virus. This could be one of the mechanism the plant uses for virus resistance. The plant we used is a wild accession of native Australian *N. benthamiana* which responded with moderate symptoms to YTMMV infection. The virus produces mosaic or mottling symptoms on leaves of *N. benthamiana* plants, but is not lethal to them. Under water stress, the virus resulted in a more pronounced increase in these enzymes. We hypothesise that when the plant was challenged with water stress and YTMMV simultaneously, the plant produces more antioxidant enzymes to protect it from increased ROS production. We assumed that the effect of fungal infection was masked by virus infection and therefore its influence could not be distinguished from the effect of the virus.

6.6.4. Drought related gene expressions

It is known that catalase is one of the key enzymes participating in the regulation of H_2O_2 in cells. The upregulation of NbCAT3 is correlated with increased CAT activity during water stress as well as virus infection. This could be a plant strategy to shield the toxic influence of elevated ROS under stress. At the same time, activation of CAT may be beneficial for the virus, by reducing the efficiency of the plant defence system. It is still unclear if activation of NbCAT3 is a consequence of viral infection, or if it is a common response to ROS upregulation under any stress stimulus. Some plant viruses are known to influence the expression of antioxidant defence-related genes (Kogovšek et al., 2010; Pompe-Novak et al., 2006; Yergaliyev et al., 2016).

Several transcription factors, MYB, MYC, DREB, and ZFP, have roles in plant development and stress tolerance (Kizis et al., 2001; Mengiste et al., 2003; Meshi and Iwabuchi, 1995; Narusaka et al., 2003; Xiang et al., 1997). The MYB family of proteins is large, functionally diverse and represented

across all eukaryotes. Different MYB transcription factors have been characterised in many plant species, and their involvement in drought responses have been described (Ambawat et al., 2013; Baldoni et al., 2015). NbPHAN, a MYB transcriptional factor, was found to regulate leaf development and affects drought tolerance in *N. benthamiana* plants (Huang et al., 2013).

The WRKY transcription factors are unique to plants (Eulgem et al., 2000; Kalde et al., 2003). The WRKY proteins have been implicated in cellular defence against a variety of biotic and abiotic stressors, including drought and salt (Jiang and Deyholos, 2006; Tripathi et al., 2014). In the present study, we found that NbWRKy, an NTEIG-D homolog (WERKY-like gene), is upregulated under stress and was upregulated under fungus infection. The earlier study suggests that NbWRKY overexpressed in *N. benthamiana* plants under water stress (Archana et al., 2009).

Most genes investigated in this study were upregulated in response to water stress in fungus- and virus-colonized seedlings. Endophyte-induced upregulation of drought related genes was reported in *Piriformospora indica*-infected *Arabidopsis* seedlings (Sherameti et al., 2008a; Sherameti et al., 2008b; Sun et al., 2010a; Xu et al., 2017). It is likely that a more comprehensive analysis will uncover more fungus- and virus-responsive genes and proteins involved in water stress tolerance.

Taking these results together, both fungal endophytes and the virus may confer water stress tolerance to *N. benthamiana* seedlings by increased accumulation of sugar, protein and proline as osmolytes, increased antioxidative enzyme activity, reduced membrane damage, and enhanced expression of drought-related genes. Plant drought tolerance is a complex trait. Roles of compatible solutes in plant drought stress tolerance have been discussed elaborately. At physiological level, osmotic adjustment is an adaptive mechanism involved in drought tolerance and permits the maintenance of turgor pressure under stress conditions. Also, these osmoprotectants (sugars, prolines, proteins etc) and antioxidant enzymes detoxify adverse effect of reactive oxygen species and alleviate drought stresses in plants (reviewed by Singh et al. (2015). Our results suggest, the fungal endophytes and the virus helps plant increase plant biomass, chlorophyll, RWC and osmoprotectants as well as antioxidant enzymes and thus enhance the adaptive drought tolerance mechanisms in plant.

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6.7. References

Abernethy, G.A., McManus, M.T., 1998. Biochemical responses to an imposed water deficit in mature leaf tissue of *Festuca arundinacea*. Journal of Experimental and Environmental Botany 40, 17–28.

Aebi, H., 1984. [13] Catalase in vitro. Methods in enzymology 105, 121-126.

Ambawat, S., Sharma, P., Yadav, N.R., Yadav, R.C., 2013. MYB transcription factor genes as regulators for plant responses: an overview. Physiol Mol Biol Pla 19, 307-321.

Amoako, S., Yahaya, A., Sarfo, J.K., 2015. Catalase activity of cassava (Manihot esculenta) plant under African cassava mosaic virus infection in Cape coast, Ghana. African Journal of Biotechnology 14, 1201-1206.

Apel, K., Hirt, H., 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu. Rev. Plant Biol. 55, 373-399.

Archana, K., Rama, N., Mamrutha, H., Nataraja, K.N., 2009. Down-regulation of an abiotic stress related Nicotiana benthamiana WRKY transcription factor induces physiological abnormalities.

Assuero, S.G., Tognetti, J.A., Colabelli, M.R., Agnusdei, M.G., Petroni, E.C., Posse, M.A., 2006. Endophyte infection accelerates morpho-physiological responses to water deficit in tall fescue. New Zealand Journal of Agricultural Research 49, 359-370.

Bae, H., Sicher, R.C., Kim, M.S., Kim, S.H., Strem, M.D., Melnick, R.L., Bailey, B.A., 2009. The beneficial endophyte Trichoderma hamatum isolate DIS 219b promotes growth and delays the onset of the drought response in Theobroma cacao. Journal of experimental botany 60, 3279-3295.

Baldoni, E., Genga, A., Cominelli, E., 2015. Plant MYB transcription factors: their role in drought response mechanisms. International journal of molecular sciences 16, 15811-15851.

Baltruschat, H., Fodor, J., Harrach, B.D., Niemczyk, E., Barna, B., Gullner, G., Janeczko, A., Kogel, K.H., Schäfer, P., Schwarczinger, I., 2008. Salt tolerance of barley induced by the root endophyte Piriformospora indica is associated with a strong increase in antioxidants. New Phytol. 180, 501-510.

Bandurska, H., Jóźwiak, W., 2010. A comparison of the effects of drought on proline accumulation and peroxidases activity in leaves of *Festuca rubra* L. and *Lolium perenne*. Acta Soc Bot Pol 79, 111–116.

Barrs, H., Weatherley, P., 1962. A re-examination of the relative turgidity technique for estimating water deficits in leaves. Aust J Biol Sci 15, 413-428.

Bates, L., Waldren, R., Teare, I., 1973. Rapid determination of free proline for water-stress studies. Plant Soil 39, 205-207.

Bayat, F., Mirlohi, A., Khodambashi, M., 2009. Effects of endophytic fungi on some drought tolerance mechanisms of tall fescue in a hydroponics culture. Russian Journal of Plant Physiology 56, 510-516.

Bradford, M., Williams, W., 1976. New, rapid, sensitive method for protein determination, Federation Proceedings. Federation of American Societies for Experimental Biology 9650 Rockville Pike, Bethesda, MD 20814, USA, pp. 274-274.
Briggs, L., Crush, J., Ouyang, L., Sprosen, J., 2013. Neotyphodium endophyte strain and superoxide dismutase activity in perennial ryegrass plants under water deficit. Acta Physiol Plant 35, 1513-1520.
Buonaurio, R., Montalbini, P., 1993. Peroxidase, superoxide dismutase and catalase activities in tobacco plants protected against Erysiphe cichoracearum by a necrotic strain of potato virus Y. Rivista di Patologia vegetale, 23-31.
Cha-Um, S., Yooyongwech, S., Supaibulwatana, K., 2010. Water deficit stress in the reproductive stage of four indica rice (*Oryza sativa* L.) genotypes. Pak. J. Bot. 42, 3387–3398
Chatterjee, A., Ghosh, S.K., 2008. Alterations in biochemical components in mesta plants infected with yellow vein mosaic disease. Brazilian Journal of Plant Physiology 20, 267-275.
Chaves, M.M., Flexas, J., Pinheiro, C., 2009. Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. Annals of botany 103, 551–560.
Chini, A., Grant, J.J., Seki, M., Shinozaki, K., Loake, G.J., 2004. Drought tolerance established by enhanced expression of the CC–NBS–LRR gene, ADR1, requires salicylic acid, EDS1 and ABI1. The Plant Journal 38, 810-822.
Claeys, H., Inzé, D., 2013. The agony of choice: how plants balance growth and survival under water-limiting conditions. Plant physiology 162, 1768-1779.
Cong, G.Q., Yin, C.L., He, B.I., Li, L., Gao, K.X., 2015. Effect of the endophytic fungus chaetomium globosum ND35 on the growth and resistance to drought of winter wheat at the seedling stage under water stress. Shengtai Xuebao Acta Ecol. Sin. 35, 6120-6128.
Dai, Y., Shen, Z., Liu, Y., Wang, L., Hannaway, D., Lu, H., 2009. Effects of shade treatments on the photosynthetic capacity, chlorophyll fluorescence, and chlorophyll content of Tetrastigma hemsleyanum Diels et Gilg. Environmental and experimental botany 65, 177-182.
Dar, M.I., Naikoo, M.I., Rehman, F., Naushin, F., Khan, F.A., 2016. Proline accumulation in plants: roles in stress tolerance and plant development, Osmolytes and Plants Acclimation to Changing Environment: Emerging Omics Technologies. Springer, pp 155-166.
Dastogeer, K.M., Li, H., Sivasithamparam, K., Jones, M.G., Wylie, S.J., 2017a. A simple and rapid in vitro test for large-scale screening of fungal endophytes from drought-adapted Australian wild plants for conferring water deprivation tolerance and growth promotion in Nicotiana benthamiana seedlings. Archives of Microbiology, 1-14.
Dastogeer, K.M., Wylie, S.J., 2017. Plant–Fungi Association: Role of Fungal Endophytes in Improving Plant Tolerance to Water Stress, Plant-Microbe Interactions in Agro-Ecological Perspectives. Springer, pp 143-159.

Dastogeer, K.M.G., Li, H., Sivasithamparam, K., Jones, M.G.K., Du, X., Ren, Y., Wylie, S.J., 2017b. Metabolic responses of endophytic *Nicotiana benthamiana* plants experiencing water stress. Environmental and Experimental Botany 143, 59-71.
Dastogeer, K.M.G., Li, H., Sivasithamparam, K., Jones, M.G.K., Wylie, S.J., 2017c. Host specificity of endophytic mycobiota of wild *Nicotiana* plants from arid regions of northern Australia. Microb. Ecol., DOI:10.1007/s00248-00017-01020-00240.
Delauney, A.J., Verma, D.P.S., 1993. Proline biosynthesis and osmoregulation in plants. Plant J 4, 215–223.
Deshmukh, P.S., Sairam, R.K., Shukla, D.S., 1991. Measurement of ion leakage as a screening technique for drought resistance in wheat genotypes. Indian journal of Plant Physiology 35, 89-91.
Dionisio-Sese, M.L., Tobita, S., 1998. Antioxidant responses of rice seedlings to salinity stress. Plant Sci. 135, 1-9.
Dubois, M., Gilles, K., Hammiltron, J., Robers, P., Smith, F., 1951. Estimation of soluble sugar contents through phenol-silphuric acid method. Anal. Chemistry 26, 351-356.
Elbersen, H.W., West, C.P., 1996. Growth and water relations of field-grown tall fescue as influenced by drought and endophyte. Grass Forage Science 51, 333–342.
Elmi, A., West, C., 1995. Endophyte infection effects on stomatal conductance, osmotic adjustment and drought recovery of tall fescue. New Phytol. 131, 61-67.
Estrada, B., Aroca, R., Maathuis, F.J., Barea, J.M., Ruiz-Lozano, J., 2013. Arbuscular mycorrhizal fungi native from a Mediterranean saline area enhance maize tolerance to salinity through improved ion homeostasis. Plant, cell & environment 36, 1771-1782.
Eulgem, T., Rushton, P.J., Robatzek, S., Somssich, I.E., 2000. The WRKY superfamily of plant transcription factors. Trends in plant science 5, 199-206.
Flexas, J., Bota, J., Galmes, J., Medrano, H., Ribas-Carbó, M., 2006. Keeping a positive carbon balance under adverse conditions: responses of photosynthesis and respiration to water stress. Physiologia plantarum 127, 343-352.
Ghabooli, M., Khatabi, B., Ahmadi, F.S., Sepehri, M., Mirzaei, M., Amirkhani, A., Jorrin-Novo, J.V., Salekdeh, G.H., 2013. Proteomics study reveals the molecular mechanisms underlying water stress tolerance induced by Piriformospora indica in barley. J. Proteomics 94, 289-301.
Gibert, A., Volaire, F., Barre, P., Hazard, L., 2012. A fungal endophyte reinforces population adaptive differentiation in its host grass species. New Phytol. 194, 561-571.
Goodin, M.M., Zaitlin, D., Naidu, R.A., Lommel, S.A., 2008. Nicotiana benthamiana: its history and future as a model for plant–pathogen interactions. Mol Plant Microbe In 21, 1015-1026.
Grover, M., Ali, S.Z., Sandhya, V., Rasul, A., Venkateswarlu, B., 2011. Role of microorganisms in adaptation of agriculture crops to abiotic stresses. World Journal of Microbiology and Biotechnology 27, 1231-1240.

Guler, N.S., Pehlivan, N., Karaoglu, S.A., Guzel, S., Bozdeveci, A., 2016. Trichoderma atroviride ID20G inoculation ameliorates drought stress-induced damages by improving antioxidant defence in maize seedlings. Acta Physiol Plant 38.

Guo, D.-P., Guo, Y.-P., Zhao, J.-P., Liu, H., Peng, Y., Wang, Q.-M., Chen, J.-S., Rao, G.-Z., 2005. Photosynthetic rate and chlorophyll fluorescence in leaves of stem mustard (Brassica juncea var. tsatsai) after turnip mosaic virus infection. Plant Sci. 168, 57-63.

Harman, G.E., 2006. Overview of Mechanisms and Uses of Trichoderma spp. Phytopathology 96, 190-194.

Harman, G.E., 2011. Multifunctional fungal plant symbionts: new tools to enhance plant growth and productivity. New Phytol. 189, 647-649.

Harman, G.E., Howell, C.R., Viterbo, A., Chet, I., Lorito, M., 2004. Trichoderma species--opportunistic, avirulent plant symbionts. Nature reviews. Microbiology 2, 43.

Hashem, A., Abd_Allah, E., Alqarawi, A., Al Huqail, A.A., Egamberdieva, D., 2014. Alleviation of abiotic salt stress in Ochradenus baccatus (Del.) by Trichoderma hamatum (Bonord.) Bainier. J Plant Interact 9, 857-868.

He L, M.C., Jones S S, and Hatier J H B, 2017. Productivity in simulated drought and post-drought recovery of eight ryegrass cultivars and a tall fescue cultivar with and without Epichloë endophyte. Crop Pasture Sci.

Hesse, U., Schoberlein, W., Wittenmayer, L., Forster, K., Warnstorff, K., Diepenbrock, W., Merbach, W., 2003. Effects of Neotyphodium endophytes on growth, reproduction and drought-stress tolerance of three *Lolium perenne* L. genotypes. Grass Forage Sci. 58, 407-415.

Hesse, U., Schoberlein, W., Wittenmayer, L., Forster, K., Warnstorff, K., Diepenbrock, W., Merbach, W., 2005. Influence of water supply and endophyte infection (*Neotyphodium* spp.) on vegetative and reproductive growth of two Lolium perenne L. genotypes. European Journal of Agronomy 22, 45-54.

Hien, D.T., Jacobs, M., Angenon, G., Hermans, C., Thu, T.T., Son, L.V., Roosens, N.H., 2003. Proline accumulation and D1-pyrroline-5-carboxylate synthetase gene properties in three rice cultivars differing in salinity and drought tolerance. Plant Sci. 165, 1059–1068

Hill, N.S., Pachon, J.G., Bacon, C.W., 1996. *Acremonium coenophialum*-mediated short- and long-term drought acclimation in tall fescue. Crop Sci. 36, 665-672.

Hill , N.S., Stringer, W.C., Rottinghaus, G.E., Belesky, D.P., Parrott, W.A., Pope, D.D., 1990. Growth, Morphological, and Chemical Component Responses of Tall Fesue to Acremonium coenophialum. Crop Sci. 30, 156-161.

Hoekstra, F.A., Golovina, E.A., Buitink, J., 2001. Mechanisms of plant desiccation tolerance. Trends in plant science 6, 431-438.

Huang, C., Hu, G., Li, F., Li, Y., Wu, J., Zhou, X., 2013. NbPHAN, a MYB transcriptional factor, regulates leaf development and affects drought tolerance in Nicotiana benthamiana. Physiologia plantarum 149, 297-309.

IPCC, I.P.o.C.C., 2015. Climate change 2014: mitigation of climate change. Cambridge University Press.
Jakab, G., Ton, J., Flors, V., Zimmerli, L., Métraux, J.-P., Mauch-Mani, B., 2005. Enhancing Arabidopsis salt and drought stress tolerance by chemical priming for its abscisic acid responses. Plant physiology 139, 267-274.
Jia, T., Shymanovich, T., Gao, Y.B., Faeth, S.H., 2015. Plant population and genotype effects override the effects of Epichloe endophyte species on growth and drought stress response of *Achnatherum robustum* plants in two natural grass populations. J. Plant Ecol. 8, 633-641.
Jiang, Y., Deyholos, M.K., 2006. Comprehensive transcriptional profiling of NaCl-stressed Arabidopsis roots reveals novel classes of responsive genes. Bmc Plant Biol 6, 25.
Joshi, R., Wani, S.H., Singh, B., Bohra, A., Dar, Z.A., Lone, A.A., Pareek, A., Singla-Pareek, S.L., 2016. Transcription factors and plants response to drought stress: current understanding and future directions. Frontiers in plant science 7.
Kalde, M., Barth, M., Somssich, I.E., Lippok, B., 2003. Members of the Arabidopsis WRKY group III transcription factors are part of different plant defense signaling pathways. Mol Plant Microbe In 16, 295-305.
Kane, K.H., 2011. Effects of endophyte infection on drought stress tolerance of Lolium perenne accessions from the Mediterranean region. Environmental and Experimental Botany 71, 337-344.
Kar, M., Mishra, D., 1976. Catalase, peroxidase, and polyphenoloxidase activities during rice leaf senescence. Plant physiology 57, 315-319.
Khan, A.L., Shin, J.H., Jung, H.Y., Lee, I.J., 2014. Regulations of capsaicin synthesis in Capsicum annuum L. by Penicillium resedanum LK6 during drought conditions. Scientia Horticulturae 175, 167-173.
Khan, A.L., Waqas, M., Hamayun, M., Al-Harrasi, A., Al-Rawahi, A., Lee, I.J., 2013. Co-synergism of endophyte *Penicillium resedanum* LK6 with salicylic acid helped *Capsicum annuum* in biomass recovery and osmotic stress mitigation. BMC microbiology 13, 13.
Khan, A.L., Waqas, M., Lee, I.J., 2015. Resilience of Penicillium resedanum LK6 and exogenous gibberellin in improving Capsicum annuum growth under abiotic stresses. J Plant Res 128, 259-268.
Kizis, D., Lumbreras, V., Pages, M., 2001. Role of AP2/EREBP transcription factors in gene regulation during abiotic stress. FEBS Lett 498, 187-189.
Kogovšek, P., Pompe-Novak, M., Baebler, Š., Rotter, A., Gow, L., Gruden, K., Foster, G., Boonham, N., Ravnikar, M., 2010. Aggressive and mild Potato virus Y isolates trigger different specific responses in susceptible potato plants. Plant Pathol 59, 1121-1132.
Lisar, S.Y., Motafakkerazad, R., Hossain, M.M., Rahman, I.M., 2012. Water stress in plants: causes, effects and responses, Water stress. InTech.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. methods 25, 402-408.

Madhusudhan, K., Srikanta, B., Shylaja, M., Prakash, H., Shetty, H.S., 2009. Changes in antioxidant enzymes, hydrogen peroxide, salicylic acid and oxidative stress in compatible and incompatible host-tobamovirus interaction. J Plant Interact 4, 157-166.
Malinowski, D.P., Belesky, D.P., 2000a. Adaptations of endophyte-infected cool-season grasses to environmental stresses: mechanisms of drought and mineral stress tolerance. Crop Sci. 40, 923–940.
Malinowski, D.P., Belesky, D.P., 2000b. Adaptations of endophyte-infected cool-season grasses to environmental stresses: mechanisms of drought and mineral stress tolerance. Crop Sci. 40, 923-940.
Malinowski, D.P., Zuo, H., Kramp, B.A., Muir, J.P., Pinchak, W.E., 2005. Obligatory summer-dormant cool-season perennial grasses for semiarid environments of the southern Great Plains. Agron. J. 97, 147-154.
Márquez, L.M., Redman, R.S., Rodriguez, R.J., Roossinck, M.J., 2007. A virus in a fungus in a plant: three-way symbiosis required for thermal tolerance. Science 315, 513-515.
Mastouri, F., Bjorkman, T., Harman, G.E., 2012. *Trichoderma harzianum* Enhances Antioxidant Defense of Tomato Seedlings and Resistance to Water Deficit. Mol Plant Microbe In 25, 1264-1271.
Mengiste, T., Chen, X., Salmeron, J., Dietrich, R., 2003. The BOTRYTIS SUSCEPTIBLE1 gene encodes an R2R3MYB transcription factor protein that is required for biotic and abiotic stress responses in Arabidopsis. The Plant cell 15, 2551-2565.
Meshi, T., Iwabuchi, M., 1995. Plant Transcription Factors. Plant Cell Physiol 36, 1405-1420.
Mittler, R., 2002. Oxidative stress, antioxidants and stress tolerance. Trends in plant science 7, 405-410.
Morsy, M.R., Oswald, J., He, J., Tang, Y., Roossinck, M.J., 2010. Teasing apart a three-way symbiosis: transcriptome analyses of Curvularia protuberata in response to viral infection and heat stress. Biochemical and biophysical research communications 401, 225-230.
Munns, R., 1988. Why measure osmotic adjustment? Funct Plant Biol 15, 717-726.
Nagabhyru, P., Dinkins, R.D., Wood, C.L., Bacon, C.W., Schardl, C.L., 2013. Tall fescue endophyte effects on tolerance to water-deficit stress. Bmc Plant Biol 13, 17.
Narusaka, Y., Nakashima, K., Shinwari, Z.K., Sakuma, Y., Furihata, T., Abe, H., Narusaka, M., Shinozaki, K., Yamaguchi-Shinozaki, K., 2003. Interaction between two cis-acting elements, ABRE and DRE, in ABA-dependent expression of Arabidopsis rd29A gene in response to dehydration and high-salinity stresses. Plant J 34, 137-148.
Nobel, P.S., 1999. Physicochemical & environmental plant physiology. Academic press.
Oberhofer, M., Gusewell, S., Leuchtmann, A., 2014. Effects of natural hybrid and non-hybrid Epichloe endophytes on the response of Hordelymus europaeus to drought stress. New Phytol. 201, 242-253.
Pandey, V., Ansari, M.W., Tula, S., Yadav, S., Sahoo, R.K., Shukla, N., Bains, G., Badal, S., Chandra, S., Gaur, A.K., Kumar, A., Shukla, A., Kumar, J., Tuteja, N., 2016. Dose-dependent

response of Trichoderma harzianum in improving drought tolerance in rice genotypes. Planta 243, 1251-1264.
Passioura, J., 2006. The drought environment: physical, biological and agricultural perspectives. Journal of experimental botany 58, 113-117.
Plattt, S.G., Henriques, F., Rand, L., 1979. Effects of virus infection on the chlorophyll content, photosynthetic rate and carbon metabolism of Tolmiea menziesii. Physiol Plant Pathol 15, 351-365.
Pompe-Novak, M., Gruden, K., Baebler, Š., Krečič-Stres, H., Kovač, M., Jongsma, M., Ravnikar, M., 2006. Potato virus Y induced changes in the gene expression of potato (Solanum tuberosum L.). Physiol Mol Plant P 67, 237-247.
Premchandra, G.S., Saneoka, h., Ogata, S., 1990. Cell membrane stability an indicator of drought tolerance as affected by applied nitrogen in soybean. J. Agric. Sci. 115, 1332-1343.
Rahman, M., Simpson, W., Matthew, C., Sabreen, S., Okubo, A., Islam, K., 2015. Response of diploid perennial ryegrass to fungal endophyte AR29 infections under water stress. Commun. Soil Sci. Plant Anal. 46, 845–860.
Rapparini, F., Peñuelas, J., 2014. Mycorrhizal fungi to alleviate drought stress on plant growth, Use of Microbes for the Alleviation of Soil Stresses, Volume 1. Springer, pp 21-42.
Redman, R.S., Kim, Y.O., Woodward, C.J., Greer, C., Espino, L., Doty, S.L., Rodriguez, R.J., 2011. Increased fitness of rice plants to abiotic stress via habitat adapted symbiosis: a strategy for mitigating impacts of climate change. PloS one 6, e14823.
Ren, A., Gao, Y., Wang, W., Wang, J., 2006. Photosynthetic pigments and photosynthetic products of endophyte-infected and endophyte-free Lolium perenne L. under drought stress conditions. Front. Biol. China 1, 168-173.
Ren, A.Z., Li, X., Han, R., Yin, L.J., Wei, M.Y., Gao, Y.B., 2011. Benefits of a symbiotic association with endophytic fungi are subject to water and nutrient availability in Achnatherum sibiricum. Plant Soil 346, 363-373.
Richardson, M.D., Chapman, G.W., Hoveland, C.S., Bacon, C.W., 1992. Sugar alcohols in endophyte-infected tall fescue under drought. Crop Sci. 32, 1060-1061.
Riedle-Bauer, M., 1998. Activities of antioxidant enzymes in cucumber plants infected with cucumber mosaic virus. Phyton.
Rodriguez, R., White Jr, J., Arnold, A., Redman, R., 2009. Fungal endophytes: diversity and functional roles. New Phytol. 182, 314-330.
Rodriguez, R.J., Woodward, C., Redman, R.S., 2010. Adaptation and survival of plants in high stress habitats via fungal endophyte conferred stress tolerance, Symbioses and Stress. Springer, pp 461-476.
Roy, R., Mazumder, P.B., Sharma, G.D., 2009. Proline, catalase and root traits as indices of drought resistance in bold grained rice (*Oryza sativa*) genotypes. African Journal of Biotechnology 8, 6521–6528.

Rozpądek, P., Wężowicz, K., Nosek, M., Ważny, R., Tokarz, K., Lembicz, M., Miszalski, Z., Turnau, K., 2015. The fungal endophyte Epichloë typhina improves photosynthesis efficiency of its host orchard grass (Dactylis glomerata). Planta 242, 1025-1035.
Rudgers, J.A., Swafford, A.L., 2009. Benefits of a fungal endophyte in Elymus virginicus decline under drought stress. Basic and Applied Ecology 10, 43-51.
Seki, M., Narusaka, M., Ishida, J., Nanjo, T., Fujita, M., Oono, Y., Kamiya, A., Nakajima, M., Enju, A., Sakurai, T., 2002. Monitoring the expression profiles of 7000 Arabidopsis genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. The Plant Journal 31, 279-292.
Serraj, R., Sinclair, T., 2002a. Osmolyte accumulation: can it really help increase crop yield under drought conditions? Plant, cell & environment 25, 333-341.
Serraj, R., Sinclair, T.R., 2002b. Osmolyte accumulation: can it really help increase crop yield under drought conditions? . Plant Cell Environ 25, 333–341.
Sherameti, I., Tripathi, S., Varma, A., Oelmuller, R., 2008a. The root-colonizing endophyte Pirifomospora indica confers drought tolerance in Arabidopsis by stimulating the expression of drought stress-related genes in leaves. Mol Plant Microbe In 21, 799-807.
Sherameti, I., Venus, Y., Drzewiecki, C., Tripathi, S., Dan, V.M., Nitz, I., Varma, A., Grundler, F.M., Oelmuller, R., 2008b. PYK10, a beta-glucosidase located in the endoplasmatic reticulum, is crucial for the beneficial interaction between Arabidopsis thaliana and the endophytic fungus Piriformospora indica. Plant J 54, 428-439.
Shinozaki, K., Yamaguchi-Shinozaki, K., Seki, M., 2003. Regulatory network of gene expression in the drought and cold stress responses. Current opinion in plant biology 6, 410-417.
Shukla, N., Awasthi, R.P., Rawat, L., Kumar, J., 2012. Biochemical and physiological responses of rice (Oryza sativa L.) as influenced by Trichoderma harzianum under drought stress. Plant Physiol Bioch 54, 78-88.
Singh, M., Kumar, J., Singh, S., Singh, V.P., Prasad, S.M., 2015. Roles of osmoprotectants in improving salinity and drought tolerance in plants: a review. Reviews in Environmental Science and Bio/Technology 14, 407-426.
Slama, I., Abdelly, C., Bouchereau, A., Flowers, T., Savouré, A., 2015. Diversity, distribution and roles of osmoprotective compounds accumulated in halophytes under abiotic stress. Annals of botany 115, 433-447.
Srivastava, S., Singh, A.K., 2010. Changes in catalase activity and total protein content in Urdbean [Vigna mungo (L.) Hepper] plants as a result of UL CV Infection. Indian Journal of Scientific Research 1, 67-69.
Staiger, C.J., 2000. Signaling to the actin cytoskeleton in plants. Annual review of plant biology 51, 257-288.
Sullivan, C.Y., 1971. Techniques for measuring plant drought stress. Crop Science Society of America.

Sun, C., Johnson, J.M., Cai, D., Sherameti, I., Oelmuller, R., Lou, B., 2010a. Piriformospora indica confers drought tolerance in Chinese cabbage leaves by stimulating antioxidant enzymes, the expression of drought-related genes and the plastid-localized CAS protein. Journal of plant physiology 167, 1009-1017.
Sun, C.A., Johnson, J., Cai, D.G., Sherameti, I., Oelmuller, R., Lou, B.G., 2010b. Piriformospora indica confers drought tolerance in Chinese cabbage leaves by stimulating antioxidant enzymes, the expression of drought-related genes and the plastid-localized CAS protein. Journal of plant physiology 167, 1009-1017.
Swarthout, D., Harper, E., Judd, S., Gonthier, D., Shyne, R., Stowe, T., Bultman, T., 2009. Measures of leaf-level water-use efficiency in drought stressed endophyte infected and non-infected tall fescue grasses. Environmental and Experimental Botany 66, 88-93.
Tian, C., Jiang, Q., Wang, F., Wang, G.-L., Xu, Z.-S., Xiong, A.-S., 2015a. Selection of suitable reference genes for qPCR normalization under abiotic stresses and hormone stimuli in carrot leaves. PloS one 10, e0117569.
Tian, Z., Huang, B., Belanger, F.C., 2015b. Effects of Epichloë festucae fungal endophyte infection on drought and heat stress responses of strong creeping red fescue. J. Am. Soc. Hortic. Sci. 140, 257-264.
Timmusk, S., Wagner, E.G.H., 1999. The plant-growth-promoting rhizobacterium Paenibacillus polymyxa induces changes in Arabidopsis thaliana gene expression: a possible connection between biotic and abiotic stress responses. Mol Plant Microbe In 12, 951-959.
Tran, L.T., Taylor, J.S., Constabel, C.P., 2012. The polyphenol oxidase gene family in land plants: Lineage-specific duplication and expansion. BMC genomics 13, 395.
Tripathi, P., Rabara, R.C., Rushton, P.J., 2014. A systems biology perspective on the role of WRKY transcription factors in drought responses in plants. Planta 239, 255-266.
Udvardi, M.K., Czechowski, T., Scheible, W.-R., 2008. Eleven golden rules of quantitative RT-PCR. The Plant cell 20, 1736-1737.
Vaughn, K., Duke, S., 1983. In situ localization of the sites of paraquat action [herbicide]. Plant Cell Environ.
Vazquez-de-Aldana, B.R., Garcia-Ciudad, A., Garcia-Criado, B., Vicente-Tavera, S., Zabalgogeazcoa, I., 2013. Fungal Endophyte (Epichloe festucae) Alters the Nutrient Content of Festuca rubra Regardless of Water Availability. PloS one 8, 14.
Vergne, E., Ballini, E., Marques, S., Sidi Mammar, B., Droc, G., Gaillard, S., Bourot, S., DeRose, R., Tharreau, D., Nottéghem, J.L., 2007. Early and specific gene expression triggered by rice resistance gene Pi33 in response to infection by ACE1 avirulent blast fungus. New Phytol. 174, 159-171.
Waller, F., Achatz, B., Baltruschat, H., Fodor, J., Becker, K., Fischer, M., Heier, T., Hückelhoven, R., Neumann, C., von Wettstein, D., 2005. The endophytic fungus Piriformospora indica reprograms

barley to salt-stress tolerance, disease resistance, and higher yield. Proceedings of the National Academy of Sciences of the United States of America 102, 13386-13391.
Waqas, M., Khan, A.L., Kamran, M., Hamayun, M., Kang, S.M., Kim, Y.H., Lee, I.J., 2012. Endophytic Fungi Produce Gibberellins and Indoleacetic Acid and Promotes Host-Plant Growth during Stress. Molecules 17, 10754-10773.
Wylie, S.J., Li, H., Jones, M.G., 2014. Yellow tailflower mild mottle virus: a new tobamovirus described from Anthocercis littorea (Solanaceae) in Western Australia. Archives of virology 159, 791-795.
Wylie, S.J., Zhang, C., Long, V., Roossinck, M.J., Koh, S.H., Jones, M.G., Iqbal, S., Li, H., 2015. Differential responses to virus challenge of laboratory and wild accessions of Australian species of Nicotiana, and comparative analysis of RDR1 gene sequences. PloS one 10, e0121787.
Xiang, C., Miao, Z., Lam, E., 1997. DNA-binding properties, genomic organization and expression pattern of TGA6, a new member of the TGA family of bZIP transcription factors in Arabidopsis thaliana. Plant Mol Biol 34, 403-415.
Xu, L., Wang, A., Wang, J., Wei, Q., Zhang, W., 2017. Piriformospora indica confers drought tolerance on Zea mays L. through enhancement of antioxidant activity and expression of drought-related genes. The Crop Journal 5, 251-258.
Xu, P., Chen, F., Mannas, J.P., Feldman, T., Sumner, L.W., Roossinck, M.J., 2008. Virus infection improves drought tolerance. New Phytol. 180, 911-921.
Yang, T., Ma, S., Dai, C.C., 2014. Drought degree constrains the beneficial effects of a fungal endophyte on Atractylodes lancea. Journal of applied microbiology 117, 1435-1449.
Yergaliyev, T.M., Nurbekova, Z., Mukiyanova, G., Akbassova, A., Sutula, M., Zhangazin, S., Bari, A., Tleukulova, Z., Shamekova, M., Masalimov, Z.K., 2016. The involvement of ROS producing aldehyde oxidase in plant response to Tombusvirus infection. Plant Physiol Bioch 109, 36-44.
Zhang, Q., Gong, M., Yuan, J., Hou, Y., Zhang, H., Wang, Y., and Hou, X., 2017. Dark septate endophyte improves drought tolerance in sorghum. International Journal of Agriculture & Biology 19, 53–60.
Zhang, Y.P., Nan, Z.B., 2007. Growth and anti-oxidative systems changes in Elymus dahuricus is affected by Neotyphodium endophyte under contrasting water availability. Journal of Agronomy and Crop Science 193, 377-386.

Chapter 7

General Discussion

7. General Discussion

Plants interact with diverse microorganisms including fungal endophytes. The roles of these endophytes in the broader context of evolution and ecology remains largely unknown, as well as their specific effects on the host. In our study, we assessed identity, host, tissue association and geographical association of fungal endophytes isolated from root, stem and leaf tissues of native Australian *Nicotiana* plants. The diversity of fungal endophytes did not vary as a function of sampling location but it varied as a function of host genotype and tissue origin. A significant pattern of community structuring was observed due to host genotypes but no consistent pattern of fungal community structure was recorded to be associated with sampling location and tissue type. Although endophytes were recovered from symptomless tissues, inoculation of the isolated endophytes showed that the majority of these fungi acted as commensals or pathogens. Among the endophytes, only two isolates significantly enhanced drought tolerance in *Nicotiana* seedlings. The presence of these endophytes under water stress resulted in differential accumulation of certain plant metabolites. Drought tolerance of fungus-inoculated plants was correlated with increased plant biomass, relative water content, soluble sugar, soluble protein, proline content, and increased activity of antioxidant enzymes. Additionally, fungus-mediated plant drought tolerance was associated with decreased production of reactive oxygen species and electrical conductivity and differential regulation of drought-related genes. The influence of infection by yellow tailflower mild mottle virus (YTMMV) was similar, but virus infection and drought together did not have a positive additive effect on plants, nor did they decrease the plant's drought tolerance. The summary and major findings described in different chapters are depicted in a conceptual diagram (Fig. 7.1).

7.1. Drivers of fungal endophyte community structuring: consistent or variable

Our study revealed that fungal endophyte community structering was shaped primarily by *Nicotiana* host species. The results of previous studies have been inconsistent with respect to endophyte host specificity (Cannon and Simmons, 2002; Davis and Shaw, 2008; De Errasti et al., 2010; Higgins et al., 2007; Ragazzi et al., 2003; Suryanarayan and Kumaresan, 2000; Suryanarayanan et al., 2005; Walker et al., 2011). The reliability of studies on endophytes depends on the methods used (Hyde and Soytong, 2008). The identity and isolation efficiency can be affected by various experimental factors that, in turn, may affect the comparability of results. Our inferences on host specificity may have limitations because we focussed only on cultivable fungi, and specifically, those that could be cultured on PDA, we are likely to have underestimated the numbers and diversity of fungi present in our samples. Previous research indicates that plants harbour many species of endophytes, including microbiomes other than fungi, that remain undetected in culture-based approaches but can be detected in a culture-independent approach (Chobba et al., 2013). Therefore, culture-independent identification approaches should be combined with culture-dependent approaches. Such tools may be particularly

important in harsh environments where obligate symbionts may be favoured and are less likely to be recovered easily by culturing (Herrera et al., 2010; Khidir et al., 2010; Porras-Alfaro et al., 2014).

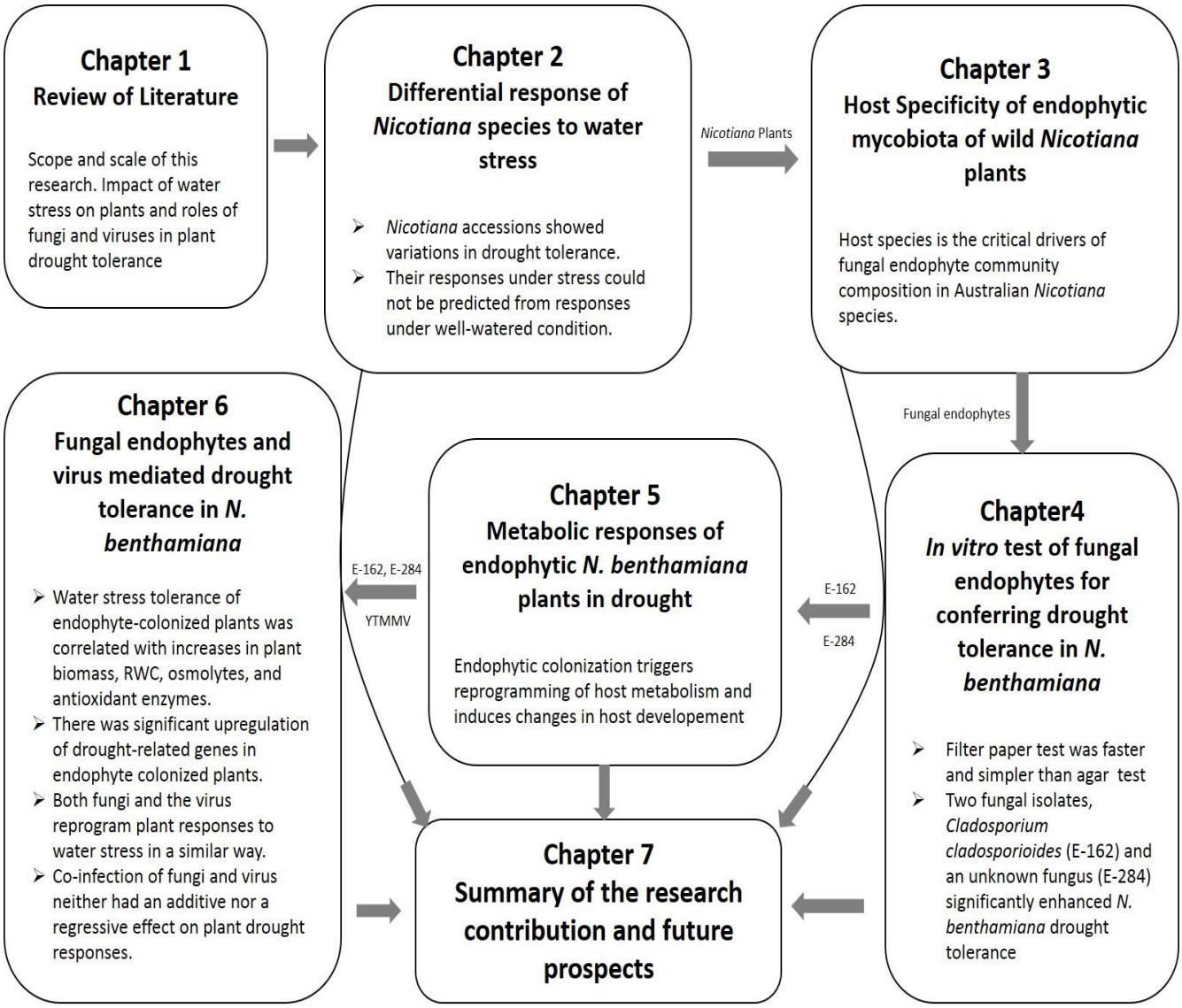

Fig. 7.1: Summary of thesis structure with key findings

Host specificity has also been reported to be the major determinant of bacterial endophytes (Ding et al., 2013; Wagner et al., 2016). The host effect on microbial endophyte communities can be explained by the fact that the majority of endophytes enter into plants *via* roots. Different plant species are characterized by different root exudation patterns, which are likely to attract different microorganisms to the rhizoplane, that can subsequently gain entry into the plant. Furthermore, plant physiology and chemical or physical characteristics are likely to play a major role (Hardoim et al., 2015).

In our study, we did not consider other factors such as soil types, soil water content, time of the year, surrounding vegetation, anthropogenic intervention etc. We controlled these factors by collecting the same number of plants from all locations studied and assumed that there were little variations within a location. It would be interesting to know if associated soil or rhizosphere fungi and other microbes show similar or different pattern of community structuring. There are numerous gaps in our knowledge about plant-endosymbiont relationships. For example, is host specificity a general phenomenon among endophytic microorganisms? Is the host specificity of endophytes of a plant species stable over ranges of environments? Is it stable across host generations? Is host specificity a factor with other microbes (e.g. bacteria and archaea), and viruses that associate with plants? Do agricultural practices affect the microbiome of cropped plant species? Are there any temporal variations? What host and microbial factors are associated with endophyte-host associations? What kind of relationship exists between microbial endosymbionts the co-occur in a host? With high throughput technologies, such as next-generation sequencing and metagenomics, we can begin to study endophyte microbiomes across hosts, environmental conditions, and at different time points and focus on mechanisms of the plant-endophyte association.

Measuring host specificity using physiological parameters combined with sequence data may provide a solid platform for delineating morphologically cryptic host-specific microorganisms. The degree of plant genetic control over endophytic community structuring is of interest to plant breeders and evolutionary biologists, since heritability of the microbiome determines whether it can evolve in response to selection on host plants (Bordenstein and Theis, 2015; Moran and Sloan, 2015; Whitham et al., 2006; Whitham et al., 2003). For the researcher, host specificity provides a unique opportunity to investigate co-evolution of host and endophyte. Manipulative experiments in the laboratory and under field conditions will be needed to unravel the molecular interactions and learn how each affects plant microbiome composition and function (Anderson et al., 2014). Use of genome-wide association studies, RNAseq, metagenomics and experimental re-inoculations will be necessary. An improved understanding of how host genetic variation affects associated microbial compositions will be rewarding for future efforts to integrate microbiome biology into evolutionary ecology and agricultural biotechnology.

7.2. Endophyte-mediated plant stress amelioration: implications for sustainable agriculture

Global agriculture is under pressure to provide increased production to feed the growing population, which is projected to reach up to 12 billion by the end of this century (DESA, 2015). Both abiotic and biotic stresses place limitations on production. Moreover, climate change is predicted to intensify the frequency, extent and severity of limiting factors. Emphasis must be given towards sustainable intensification of agriculture under variable and unpredictable conditions. Plants and their microbial symbionts co-evolved around 400 million years ago (Rodriguez and Redman, 2008), and so it is likely

that mutual benefits arise. Recently, a paradigm shift has taken place by considering plants as a holobiont, an ecological and evolutionary unit encompassing both the host and its associated microbiome (Vandenkoornhuyse et al., 2015). Mutualistic interactions between endophytic fungi and plants are ecologically important and globally prevalent. Certain endophytic microbes residing within plant tissues have been shown to promote plant growth and endow protection against biotic and abiotic stresses (Baltruschat et al., 2008; Hubbard et al., 2014; Khan et al., 2017; Šišić et al., 2017). These interactions are not currently actively exploited in agricultural systems. One reason is that the underlying mechanisms of beneficial plant-endophyte interactions are not well understood. Our study revealed that a subset of ascomycete fungal endophytes protects plant growth and promotes water stress tolerance through manipulating host metabolic pathways, and the production of osmolytes, antioxidant enzymes, and through altered gene expression. This proved our hypothesis that beneficial fungal endophytes interact with Australian *Nicotiana* plants from regions where aridity prevails and where selection favours interactions that increase survival under drought. Endophyte-mediated drought tolerance in these *Nicotiana* plants may be explained by plant-fungus mutualism under adverse climatic conditions.

Most published studies, including ours, were performed under controlled laboratory conditions. The results of these controlled experiments may not always reflect field conditions (Serfling et al., 2007). Fungal endophytes have been tested singly with a few pure isolates, but our isolations revealed large co-associations with their hosts. The outcome could be variable under microbe-microbe competition for space and nutrients, mycoparasitism, activation of host defences or antagonism involving the production of antifungal or antibacterial compounds (Saunders et al., 2010). To assess the likelihood of such an inadvertent outcome, co-inoculations with multiple mutualistic microorganisms are merited. Another key consideration would be to study the behaviours of endophytes under a range of conditions and stages of the life cycle. It is important to understand these factors in order to manage the risk of endophytes becoming pathogenic, either through a change in environmental conditions or adaptation to an alternative host (Redman et al., 2001). An innovative biotechnology strategy might be to modify root exudation chemistry to attract beneficial microbial communities, once these are identified. Studying function and structure of the whole microbiome will enable comparisons between different microbiomes on isogenic plant lines (Busby et al., 2017). This may be more meaningful than assessing endophytes individually.

Achieving agricultural sustainability will involve a greater understanding of the plant microbiome, and more research to determine if findings in wild systems can be applied to managed systems. A number of questions need to be addressed. How much correlation is there between the results in controlled environments and the natural field situation? Will effects of individual fungal isolates on host fitness, such as those examined here, be meaningful in the presence of other interacting microbes

and under field conditions? What drives the outcomes of the plant-microbe association? What is the molecular basis of these outcomes? Are these outcomes specific to the particular host-microbe system or could that be utilized in a new system after modification? Are these benefits sufficient to include endophytes in existing farming systems? Are there any unexpected harmful effects of these microorganisms in human and animal health? What are the public and scientist perceptions on using inocula in food production?

7.3. Virus and plant stress tolerance

Plant-microbe mutualistic symbiosis is a not new concept (Bronstein, 1994; Long, 1989; Redman et al., 2001; Schardl et al., 2004; Soto et al., 2009). Although the concept of viruses as mutualists is relatively new, the positive interactions between viruses and other organisms is common (Roossinck, 2011). Plant viruses are commonly studied as pathogens. Recent studies indicate that there is more to viruses than simply causing diseases (Xu et al., 2008). In our present study, we found that infection with a plant virus improved the tolerance of the host under water limiting conditions, indicating the potential of a mutualistic association between virus and plant. Earlier studies reported that several acute plant viruses improved plant responses to drought, salinity and cold in host plants under greenhouse conditions. The mechanism is unknown, but we found that some osmoprotectants, and oxidative enzymatic enzymes were stimulated in virus infected plants under stress. Also, there was differential expression of certain drought related genes in virus infected plants. In addition, plant viruses impact biotic stress factors. For example, zucchini yellow mosaic virus infects wild gourds reducing infestation of beetles and transmission of wilt bacteria. Viral beneficial mutualists have also been reported in human and insect viruses, and microbes (Bhattarai and Stapleton, 2012; Mai-Prochnow et al., 2015; Márquez and Roossinck, 2012; Xu et al., 2014). Looking at viruses as pathogens is an incomplete view on their roles. The virus-host interaction could be on a continuum between mutualism and antagonism, depending on the circumstances (Bao and Roossinck, 2013a; Roossinck, 2015).

Conditional mutualistic interactions have been described in other symbiotic systems (Bronstein, 1994; Clay and Schardl, 2002; Redman et al., 2001; Schardl et al., 2004). For instance, certain pathogenic fungi such as *Colletotrichum* spp. can express a mutualistic lifestyle depending on the host genotype (Redman et al., 2001). Endophyte-colonised tall fescue increased plant recovery following drought stress (Schardl et al., 2004). The DpAV4 ascovirus is beneficial in some *Diadromus* wasps but is pathogenic to other species of this genus (Stasiak et al., 2005). Even mycorrhizal associations can be antagonistic depending on environmental conditions and plant physiology (Johnson et al., 1997). Therefore, symbioses can be variable particularly under complex natural conditions. Symbiosis generally involves sharing of either benefits or costs among the partners depending on host species and environmental conditions. From an evolutionary point of view, a true mutualism is a reciprocated

increase in fitness for both partners. We did not test reproductive fitness of the plant host *pe se*. The death of virus-free plants versus survival of virus-infected plants under extreme drought stress represents a conditional difference in fitness if the surviving plants can subsequently produce offspring.

YTMMV used in our study is a plant virus. The possible interaction effect of a mycovirus and a fungal endophyte on plant stress tolerance may not be predicted. Certain mycoviruses have been reported to affect their fungal or plant hosts, causing hypovirulence, disease or being beneficial. For example, a virus-infected endophyte was reported to increase heat tolerance to tomato plants (Márquez et al., 2007). A review of fungal endophytic viruses by Bao and Roossinck (2013b) outlined an excellent and exhaustive account on viruses with their putative roles. They argued that viruses can be detected from all different kinds of fungal endophytes and their species richness is probably high in endophytes. However, our attempt to detect mycoviruses in 80 representative strains of all fungal taxa in the present study using high- throughput sequencing was not successful (data not provided). We concluded that viruses in fungal endophytes may not be present as frequent as previously claimed or viruses were lost during pure culturing of fungi (Bao and Roossinck, 2013b). In the future, it would be interesting to study the effect of fungal endophytes with or without mycoviruses on plant fitness under stress conditions.

Plants are simultaneously exposed to numerous environmental stresses. Stress research often focuses on plant responses to a single stress such as resistance to biotic attack, whereas cross tolerance to multiple abiotic stresses have been less described. Plant responses to simultaneous abiotic and biotic stresses are complex (Garrett et al., 2006) and it can sometimes be synergistic (Diourte et al., 1995; Mayek-PÉrez et al., 2002). The mechanisms of interaction and underlying factors warrant critical examination in a case by case basis. Studying viruses in the context of ecology and evolutionary biology (Dennehy, 2014) provides a foundation for a deeper understanding of the intricate relationships of all life. It is expected that more examples of mutualistic viruses will be discovered as we continue this exciting phase of virus discovery. More in-depth studies through metabolite profiling and RNAseq analysis would provide a detailed picture of the virus-host interaction under stress. This will open new opportunities for agricultural applications which is especially important in the future as we face changes to the earth's climate.

References

Anderson, J., Wagner, M., Rushworth, C., Prasad, K., and Mitchell-Olds, T. (2014). The evolution of quantitative traits in complex environments. *Heredity* **112**, 4.

Baltruschat, H., Fodor, J., Harrach, B. D., Niemczyk, E., Barna, B., Gullner, G., Janeczko, A., Kogel, K. H., Schäfer, P., and Schwarczinger, I. (2008). Salt tolerance of barley induced by the root endophyte Piriformospora indica is associated with a strong increase in antioxidants. *New Phytologist* **180**, 501-510.

Bao, X., and Roossinck, M. J. (2013a). A life history view of mutualistic viral symbioses: quantity or quality for cooperation? *Current opinion in microbiology* **16**, 514-518.

Bao, X., and Roossinck, M. J. (2013b). Multiplexed interactions: viruses of endophytic fungi. *Adv Virus Res* **86**, 37-58.

Bhattarai, N., and Stapleton, J. T. (2012). GB virus C: the good boy virus? *Trends in microbiology* **20**, 124-130.

Bordenstein, S. R., and Theis, K. R. (2015). Host biology in light of the microbiome: ten principles of holobionts and hologenomes. *PLoS Biology* **13**, e1002226.

Bronstein, J. L. (1994). Conditional outcomes in mutualistic interactions. *Trends in Ecology & Evolution* **9**, 214-217.

Busby, P. E., Soman, C., Wagner, M. R., Friesen, M. L., Kremer, J., Bennett, A., Morsy, M., Eisen, J. A., Leach, J. E., and Dangl, J. L. (2017). Research priorities for harnessing plant microbiomes in sustainable agriculture. *Plos Biology* **15**.

Cannon, P. F., and Simmons, C. M. (2002). Diversity and host preference of leaf endophytic fungi in the Iwokrama Forest Reserve, Guyana. *Mycologia* **94**, 210-220.

Chobba, B. I., Elleuch, A., Ayadi, I., Khannous, L., Namsi, A., Cerqueira, F., Drira, N., Gharsallah, N., and Vallaeys, T. (2013). Fungal diversity in adult date palm (*Phoenix dactylifera* L.) revealed by culture-dependent and culture-independent approaches. *Journal of Zhejiang University Science B* **14**, 1084–1099.

Clay, K., and Schardl, C. (2002). Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. *the american naturalist* **160**, S99-S127.

Davis, E. C., and Shaw, A. J. (2008). Biogeographic and phylogenetic patterns in diversity of liverwort-associated endophytes. *American Journal of Botany* **95**, 914-924.

De Errasti, A., Carmarán, C., and Novas, M. V. (2010). Diversity and significance of fungal endophytes from living stems of naturalized trees from Argentina. *Fungal Diversity* **41**, 29-40.

Dennehy, J. J. (2014). What ecologists can tell virologists. *Annual review of microbiology* **68**, 117-135.

DESA (2015). "World population prospects: The 2015 revision, key findings and advance tables. Working paper no ESA/P/WP. 241." Department of Economic and Social Affairs (DESA), Population Division., New York: United Nations.

Ding, T., Palmer, M. W., and Melcher, U. (2013). Community terminal restriction fragment length polymorphisms reveal insights into the diversity and dynamics of leaf endophytic bacteria. *BMC microbiology* **13**, 1.

Diourte, M., Starr, J., Jeger, M., Stack, J., and Rosenow, D. (1995). Charcoal rot (Macrophomina phaseolina) resistance and the effects of water stress on disease development in sorghum. *Plant Pathology* **44**, 196-202.

Garrett, K. A., Dendy, S. P., Frank, E. E., Rouse, M. N., and Travers, S. E. (2006). Climate change effects on plant disease: genomes to ecosystems. *Annu. Rev. Phytopathol.* **44**, 489-509.

Hardoim, P. R., Van Overbeek, L. S., Berg, G., Pirttilä, A. M., Compant, S., Campisano, A., Döring, M., and Sessitsch, A. (2015). The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. *Microbiology and Molecular Biology Reviews* **79**, 293-320.

Herrera, J., Khidir, H. H., Eudy, D. M., Porras-Alfaro, A., Natvig, D. O., and Sinsabaugh, R. L. (2010). Shifting fungal endophyte communities colonize Bouteloua gracilis: effect of host tissue and geographical distribution. *Mycologia* **102**, 1012-1026.

Higgins, K. L., Arnold, A. E., Miadlikowska, J., Sarvate, S. D., and Lutzoni, F. (2007). Phylogenetic relationships, host affinity, and geographic structure of boreal and arctic endophytes from three major plant lineages. *Molecular phylogenetics and evolution* **42**, 543-555.

Hubbard, M., Germida, J., and Vujanovic, V. (2014). Fungal endophytes enhance wheat heat and drought tolerance in terms of grain yield and second-generation seed viability. *Journal of applied microbiology* **116**, 109-122.

Hyde, K. D., and Soytong, K. (2008). The fungal endophyte dilemma. *Fungal Diversity* **33**, 163–173.

Johnson, N., Graham, J., and Smith, F. (1997). Functioning of mycorrhizal associations along the mutualism–parasitism continuum. *The New Phytologist* **135**, 575-585.

Khan, A. L., Waqas, M., Asaf, S., Kamran, M., Shahzad, R., Bilal, S., Khan, M. A., Kang, S.-M., Kim, Y.-H., and Yun, B.-W. (2017). Plant growth-promoting endophyte Sphingomonas sp. LK11 alleviates salinity stress in Solanum pimpinellifolium. *Environmental and Experimental Botany* **133**, 58-69.

Khidir, H. K., Eudy, D. M., Porras-Alfaro, A., Herrera, J., Natvig, D. O., and Sinsabaugh, R. L. (2010). A general suite of fungal endophytes dominate the roots of two dominant grasses in a semiarid grassland. *Journal of Arid Environments* **74**, 35-42.

Long, S. R. (1989). Rhizobium-legume nodulation: life together in the underground. *Cell* **56**, 203-214.

Mai-Prochnow, A., Hui, J. G. K., Kjelleberg, S., Rakonjac, J., McDougald, D., and Rice, S. A. (2015). Big things in small packages: the genetics of filamentous phage and effects on fitness of their host. *FEMS microbiology reviews* **39**, 465-487.

Márquez, L. M., Redman, R. S., Rodriguez, R. J., and Roossinck, M. J. (2007). A virus in a fungus in a plant: three-way symbiosis required for thermal tolerance. *science* **315**, 513-515.

Márquez, L. M., and Roossinck, M. J. (2012). Do persistent RNA viruses fit the trade-off hypothesis of virulence evolution? *Current opinion in virology* **2**, 556-560.

Mayek-PÉrez, N., GarcÍa-Espinosa, R., LÓpez-CastaÑeda, C., Acosta-Gallegos, J. A., and Simpson, J. (2002). Water relations, histopathology and growth of common bean (Phaseolus vulgaris L.) during pathogenesis of Macrophomina phaseolina under drought stress. *Physiological and Molecular Plant Pathology* **60**, 185-195.

Moran, N. A., and Sloan, D. B. (2015). The hologenome concept: helpful or hollow? *PLoS biology* **13**, e1002311.

Porras-Alfaro, A., Raghavan, S., Garcia, M., Sinsabaugh, R. L., Natvig, D. O., and Lowrey, T. K. (2014). Endophytic fungal symbionts associated with gypsophilous plants. *Botany* **92**, 295–301.

Ragazzi, A., Moricca, S., Capretti, P., Dellavalle, I., and Turco, E. (2003). Differences in composition of endophytic mycobiota in twigs and leaves of healthy and declining Quercus species in Italy. *Forest Pathology* **33**, 31-38.

Redman, R. S., Dunigan, D. D., and Rodriguez, R. J. (2001). Fungal symbiosis from mutualism to parasitism: who controls the outcome, host or invader? *New Phytologist* **151**, 705-716.

Rodriguez, R., and Redman, R. (2008). More than 400 million years of evolution and some plants still can't make it on their own: plant stress tolerance via fungal symbiosis. *Journal of Experimental Botany* **59**, 1109-1114.

Roossinck, M. J. (2011). The good viruses: viral mutualistic symbioses. *Nature reviews. Microbiology* **9**, 99.

Roossinck, M. J. (2015). Plants, viruses and the environment: ecology and mutualism. *Virology* **479**, 271-277.

Saunders, M., Glenn, A. E., and Kohn, L. M. (2010). Exploring the evolutionary ecology of fungal endophytes in agricultural systems: using functional traits to reveal mechanisms in community processes. *Evolutionary Applications* **3**, 525-537.

Schardl, C. L., Leuchtmann, A., and Spiering, M. J. (2004). Symbioses of grasses with seedborne fungal endophytes. *Annu. Rev. Plant Biol.* **55**, 315-340.

Serfling, A., Wirsel, S. G., Lind, V., and Deising, H. B. (2007). Performance of the Biocontrol Fungus Piriformospora indica on Wheat Under Greenhouse and Field Conditions. *Phytopathology* **97**, 523-31.

Šišić, A., Baćanović, J., and Finckh, M. R. (2017). Endophytic Fusarium equiseti stimulates plant growth and reduces root rot disease of pea (Pisum sativum L.) caused by Fusarium avenaceum and Peyronellaea pinodella. *European Journal of Plant Pathology* **148**, 271-282.

Soto, M. J., Domínguez-Ferreras, A., Pérez-Mendoza, D., Sanjuán, J., and Olivares, J. (2009). Mutualism versus pathogenesis: the give-and-take in plant–bacteria interactions. *Cellular microbiology* **11**, 381-388.

Stasiak, K., Renault, S., Federici, B., and Bigot, Y. (2005). Characteristics of pathogenic and mutualistic relationships of ascoviruses in field populations of parasitoid wasps. *Journal of insect physiology* **51**, 103-115.

Suryanarayan, T., and Kumaresan, V. (2000). Endophytic fungi of some halophytes from an estuarine mangrove forest. *Mycological Research* **104**, 1465-1467.

Suryanarayanan, T. S., Wittlinger, S. K., and Faeth, S. H. (2005). Endophytic fungi associated with cacti in Arizona11Dedicated to John Webster on the occasion of his 80th birthday. *Mycological Research* **109**, 635-639.

Vandenkoornhuyse, P., Quaiser, A., Duhamel, M., Le Van, A., and Dufresne, A. (2015). The importance of the microbiome of the plant holobiont. *New Phytologist* **206**, 1196-1206.

Wagner, M. R., Lundberg, D. S., Tijana, G., Tringe, S. G., Dangl, J. L., and Mitchell-Olds, T. (2016). Host genotype and age shape the leaf and root microbiomes of a wild perennial plant. *Nature communications* **7**.

Walker, J. F., Aldrich-Wolfe, L., Riffel, A., Barbare, H., Simpson, N. B., Trowbridge, J., and Jumpponen, A. (2011). Diverse Helotiales associated with the roots of three species of Arctic Ericaceae provide no evidence for host specificity. *New Phytologist* **191**, 515-527.

Whitham, T. G., Bailey, J. K., Schweitzer, J. A., Shuster, S. M., Bangert, R. K., LeRoy, C. J., Lonsdorf, E. V., Allan, G. J., DiFazio, S. P., and Potts, B. M. (2006). A framework for community and ecosystem genetics: from genes to ecosystems. *Nature reviews. Genetics* **7**, 510.

Whitham, T. G., Young, W. P., Martinsen, G. D., Gehring, C. A., Schweitzer, J. A., Shuster, S. M., Wimp, G. M., Fischer, D. G., Bailey, J. K., and Lindroth, R. L. (2003). Community and ecosystem genetics: a consequence of the extended phenotype. *Ecology* **84**, 559-573.

Xu, P., Chen, F., Mannas, J. P., Feldman, T., Sumner, L. W., and Roossinck, M. J. (2008). Virus infection improves drought tolerance. *New Phytologist* **180**, 911-921.

Xu, P., Liu, Y., Graham, R. I., Wilson, K., and Wu, K. (2014). Densovirus is a mutualistic symbiont of a global crop pest (Helicoverpa armigera) and protects against a baculovirus and Bt biopesticide. *PLoS pathogens* **10**, e1004490.

Appendix I

(Note: Content of this book chapter has some overlaps with part of the literature review section. A copy is attached below)

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Plant–Fungi Association: Role of Fungal Endophytes in Improving Plant Tolerance to Water Stress

8

Khondoker M.G. Dastogeer and Stephen J. Wylie

Abstract

Plants are constantly being challenged with various biotic and abiotic stresses throughout their life cycle that exert profound deleterious effects on growth, development and health. Plants employ various physiological, biochemical and molecular mechanisms to combat these stress factors. Microorganism-mediated plant stress tolerance, particularly plant drought tolerance, is important in the study of plant–microbe interactions. Although relatively less well-known, fungal endophyte-mediated plant drought tolerance has been described for several cases. Unlike mycorrhizal fungi, non-mycorrhizal fungi may mediate the effects of water stress by adjusting, regulating or modifying plant physiological, biochemical and metabolic activities. We review the evidence for fungal endophyte-mediated plant drought tolerance and mechanisms.

Keywords

Abiotic stress • Water deficit • Endophyte • Growth • Photosynthesis • ROS • Osmotic adjustment

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8.1 Introduction

Abiotic stress tolerance plays a vital role in determining crop productivity and distribution of plant species across the environment (Boyer 1982; Chaves et al. 2003). Environmental stresses such as drought, extreme temperatures, salinity or chemical toxicity bring serious consequences to crop production, causing collectively more than 50% yield losses worldwide (Bray et al. 2000; Wang and Frei 2011). Due to global climate change, abiotic stresses are expected to become more widespread in the coming decades and will pose serious threats to global food security (Ashmore et al. 2006; Battisti and Naylor 2009). Among the environmental stresses, water stress commonly, known as 'drought', is considered as one of the major challenges to crop production worldwide (Yue et al. 2006; IPCC 2007). If the crop is subjected to stress, particularly drought during its early stage of growth, germination and seedling establishment are severely arrested mainly owing to low water uptake, low energy supply and hindered enzyme functions (Okcu et al. 2005; Taiz and Zeiger 2010). The crop phenology is also affected by triggering a premature shifting of plant development from the vegetative stage to reproductive stage. This shortens the crop growth cycle (Desclaux and Roumet 1996). Moreover, all major attributes of plant–water relations, viz. leaf relative water contents (RWC), water potential, osmotic potential, pressure potential and transpiration rate, are significantly affected by drought, leading to impaired crop productivity (Kirkham 2005). Improving plant resistance to water stress and maintaining crop productivity are great challenges for achieving sustainable agriculture. Given the importance of drought stress to agriculture, plant reactions to stresses have been studied extensively. Such studies have added considerably to our understanding of plant response to stress at the whole-plant, morphological, physiological, cellular and molecular levels (Grover et al. 2001). Considerable research has been done to understand the mechanisms of abiotic stress responses in a wide variety of model and crop plant species. Now scientists are recognising that microbial partnerships are a ubiquitous part of plant biology. The presence and roles of microbes in plants are becoming clearer with high-throughput technologies such as genomics, functional genomics, proteomics and metabolomics. Plants form various associations with diverse kinds of microorganisms such as fungi, bacteria, viruses, archaea, protozoa, etc, and the form relationships ranging from mutualism to pathogenicity. One such interaction is the association of plants with fungal endophytes, which have been recorded from most plants studied in natural ecosystems. Fungal endophytes remain inside plant tissues without showing any disease symptoms (Rodriguez et al. 2009; Purahong and Hyde 2011). Besides mycorrhizal endophytes, non-mycorrhizal endophytes have been recovered from most plants. Non-mycorrhizal fungal endophytes (hereafter referred to as endophytes) form an intimate relationship with the host and provide various benefits including protection from drought stress (Lewis and Clements 1986; Rodriguez et al. 2004; Malinowski et al. 2004; Malinowski and Belesky 2006;

Zabalgogeazcoa 2008). Endophyte-mediated drought tolerance is associated with improving growth and productivity of the host. Endophytes also improve osmolyte production; influence plant–water relations and photosynthesis; adjust plant water potential, electrolyte balance, antioxidant synthesis and other structural and functional parameters; and thus enhance the plant's ability to tolerate stresses. This chapter presents an outline of the main studies in the area of water deficit stress responses in plants mediated by non-mycorrhizal fungal endophytes.

8.2 Plant Strategies to Withstand Water Stress

The underlying mechanisms of how plants respond to drought stress have been explored to a great extent from molecular to whole-plant levels. Researchers have identified hundreds of genes that are activated in plants in response to stress. A variety of tools including gene expression patterns and the use of transgenic plants has been developed to investigate the particular roles of these genes in plant responses to stress. Transgenic technologies and the advent of genomics and proteomics have offered a comprehensive profiling of the changes in gene and protein expression resulting from exposure to drought.

Plant reactions to water deficit stress are complicated since it is a function of time and space, and it involves multifaceted mechanisms from genomic, molecular and biochemical levels (Blum 1996; Chaves et al. 2003; Xu et al. 2009). Plants use different mechanisms to cope with the stress, and the way a plant behaves under drought can be explained by the following six broad stategies:

1. Escaping from drought by terminating plant life cycle prior to onset of severe stress, e.g. early flowering in annuals before the start of water deficit (Geber and Dawson 1990)
2. Drought avoidance through increasing water uptake and reducing water loss, e.g. developing root systems and reducing of stomata and canopy area (Schulze 1986; Jackson et al. 2000)
3. Drought tolerance chiefly through maintaining better osmotic balance and expanding elasticity of the cell wall to keep the tissue turgid (Morgan 1984)
4. Drought resistance via changing metabolic routes to thrive under stress condition (e.g. greater antioxidant metabolism) (Bartoli et al. 1999)
5. Drought abandonment by shedding one or more plant organ, e.g. detaching older foliage during drought (Chaves et al. 2003)
6. Drought-adapted physio-biochemical characters developed through plant evolution under long-term drought conditions via mutation and modifications at the genomic level (Hoffmann and Merilä 1999; Sherrard et al. 2009; Maherali et al. 2010)

8.3 Plant–Microbe Interactions and Drought Tolerance in Plant

The interaction of microbes with the plant can be traced back to the origin of plants. The early evolution of plants occurred in a diverse microbial world. Archaea, bacteria, fungi, and viruses had been evolving for billions of years (Reid and Greene 2012). The most well-known plant–microbe interaction is the mutualism between mycorrhizal fungi and plant where both partners generally benefit from each other. However, under natural settings, plants form relationships with endophytic fungi and viruses which can be beneficial or harmful for the partners depending on host types and natural and environmental situations (Bao and Roossinck 2013). Plants in natural systems and crop lands are simultaneously exposed to both biotic and abiotic stresses. Though stress research is mostly focusing on plant response to a particular environmental stress, research focusing to both biotic and abiotic stresses together has also been conducted (Xu et al. 2008; Garrett et al. 2006). Unravelling the complex mechanisms of plant–microbe relations and their effects in abiotic stress tolerance in plants could potentially advocate novel tactics to boost the productivity of crops (Schenk et al. 2012).

8.3.1 What Are Fungal Endophytes?

The term ‘endophyte’ refers to the fungi that live inside the plant intercellular and intracellular spaces for at least part of life cycle, causing no concurrent visible symptoms at any specific moment (Rodriguez et al. 2009; Purahong and Hyde 2011). This definition of endophyte is strictly operational and contextual since it takes into account the result of a specific fungus–host interaction only in a given time under the particular environmental settings, because symptomless endophytes can behave differently (e.g. as pathogens) under altered environmental conditions (Andrew et al. 2012; Sanchez-Marquez et al. 2012). The existence of fungal endophytes from fossil records suggests that endophyte–host associations may have evolved from the time of development of first higher plants on earth (Rodriguez and Redman 1997; Krings et al. 2012). Based on the survey conducted in the last 20 years on endophytes, it is thought that the majority, if not all plants, have one or more types of these endophytes and numerous endophytic species; in some cases, above a hundred can be found in a certain plant species (Arnold 2007). Fungal endophytes have been documented from healthy aerial tissues of conifers (Petrini and Fisher 1986) and grasses (Clay 1988). Further, fungal endophytes have also been reported from marine algae (Hawksworth 1988), lichens (Li et al. 2007), mosses and ferns (Fisher 1996), palms (Frohlich and Hyde 1999) and pteridophytes (Dhargalkar and Bhat 2009). Fungal endophytes can be grouped into three basic ecological groups: (1) mycorrhizal fungi, (2) balancious or ‘grass endophytes’ and (3) non-balancious endophytes (Schulz and Boyle 2005). However, Brundrett (2004) separated mycorrhizal from endophytic interactions in that mycorrhizas

have coordinated plant–fungus development and nutrient transfer at specialized interfaces. Later, Rodriguez et al. (2009) classified the endophytes under two major groups, viz. clavicipitaceous and non-clavicipitaceous on the basis of phylogeny and life history traits. Clavicipitaceous fungal endophytes are limited to certain grasses, while non-clavicipitaceous ones have a broad host range including both nonvascular and vascular plant species. In addition, recent reviews propose that members of the non-clavicipitaceous group can be segregated into three subgroups on the basis of host range, type of tissue infected, pattern spread, in planta infection and the establishment, diversity and benefits given to hosts (Rodriguez et al. 2009; Purahong and Hyde 2011). A diverse kind of relationships exists between the fungal endophytes and plant ranging from mutualistic (Redman et al. 2002), symbiotic and commensal (Deckert et al. 2001) to pathogens (Schulz et al. 1998). However, the state of the interaction between endophyte and host may be transitory, and many factors could make changes in their mode of interaction. In symbiotic associations, balansiaceous endophytes with their hosts are commonly considered as being mutualistic (Schardl and Clay 1997) even though some of them provide nothing to their hosts and can occasionally be antagonistic (Schardl et al. 2004a). Although most of the endophytes are regarded as being mutualistic with their hosts, some fungal endophytes may become pathogenic to plants, depending on the developmental stage of the partners, environmental conditions and plant defence reactions (Schulz and Boyle 2005). Endophytic fungi have been known to play a vital role in plant growth, especially grasses; however, few reports have elucidated their symbiosis with crops. Recently, the ecological roles of some endophytes have been explained (Redman et al. 2001; Waller et al. 2005; Arnold et al. 2007). In addition to providing nutritional benefits, fungal endophytes also confer significant physiological (Malinowski and Belesky 2000; Malinowski et al. 2004) and ecological (Malinowski and Belesky 2006) benefits, including protection from environmental stress (Rodriguez et al. 2004) as well as from an attack of pathogens (Zabalgogeazcoa 2008) and pests (Lewis and Clements 1986).

8.3.2 Mechanisms of Endophyte-Mediated Plant Drought Tolerance

Fungal endophytes have been shown to provide fitness benefit to plant when exposed to water-limiting conditions. Perhaps the most widely documented example of endophyte-mediated drought stress tolerance in plants is the enhanced drought tolerance of tall fescue and perennial ryegrass due to infection of the endophyte *Neotyphodium coenophialum*. Kane (2011) studied with the leaf-inhabiting endophyte *Neotyphodium lolii* to assess its potential benefits or harm in drought stress tolerance of native perennial ryegrass collections formerly obtained from the Mediterranean regions. Non-grass fungal endophytes have also been described to help plants alleviate drought stress (Redman et al. 2011; Khan et al. 2012; Waqas et al. 2012). The findings showed that endophyte colonization can help improve

abiotic stress tolerance such as drought in that host. It must be noted that endophytic symbiosis in plants does not always benefit the plant under drought or other abiotic stress conditions, and their interactions could cost for plants in terms of their ability to stand in stresses (Eerens et al. 1998; Cheplick et al. 2000; Cheplick 2004, 2006). Cheplick (2006) reviewed the role of fungal endophytes on potential drought tolerance and cited some studies where endophytes imparted no improvement in the host's ability to tolerate drought stress. For instance, Zaurov et al. (2001) inoculated fescue plants with *Neotyphodium* isolates collected from dissimilar hosts. They observed that some genotypic combinations affected negatively on plant mass, some had no effect and others increased plant biomass. Similarly, few combinations improved tolerance to soil aluminium; others have neutral or decreased tolerance compared to endophyte-free clones. This study revealed that genotype-specific interactions may increase or decrease or have no effect on plant adaptation and fitness. Thus, endophyte-mediated response to water stress is a complex phenomenon involving various metabolites and metabolic pathways. While the ability of fungal endophytes to provide drought tolerance in host plants has been described in many studies, the underlying mechanism(s) are incompletely characterized. In an effort to illuminate the underlying mechanism by which endophyte causes increased drought tolerance, researchers have reported few observations. Research so far studying the effect of endophyte on plant responses to drought stress have described certain physiological, biological and biochemical modifications such as (a) increased growth and development, (b) enhanced osmotic balance, (c) increased gaseous exchange and water-use efficiency and (d) improved defence against oxidative damage when water-limiting conditions may improve, alleviate and recompense the harmful effects of water stress in endophyte-colonized (EC) plants (Fig. 8.1). The present chapter aimed at outlining the recent advances in the study of improvement of drought tolerance by endophyte colonization in plant subjected to water stress.

8.3.2.1 Endophyte-Mediated Plant Growth Enhancement

Fungal endophytes have been shown to enhance growth and biomass of plants under water-limiting conditions. For example, inoculation of *Fusarium culmorum* and *Curvularia protuberata* resulted in higher biomass of drought-affected rice plants than non-inoculated plants (Redman et al. 2011). Endophytes *Chaetomium globosum* and *Penicillium resedanum* isolated from *Capsicum annuum* plants promoted shoot length and biomass of the host plants subjected to drought stress (Khan et al. 2012; Khan et al. 2014). Drought-challenged tomato plants showed higher root and shoot biomass when inoculated with class 2 fungal endophytes, including *Alternaria* sp. and *Trichoderma harzianum* (Azad and Kaminskyj 2016). Inoculating a *Trichoderma hamatum* isolate caused increased higher root fresh weight, dry weight and water content, regardless of water availability in *Theobroma cacao* (cacao) (Bae et al. 2009). The endophyte *Piriformospora indica* colonization in Chinese cabbage promoted root and shoot growth and lateral root development (Sun et al. 2010). Production of auxins by fungal endophytes is attributed to the increased growth of plants under stress (De Battista et al. 1990). Also, stress-induced endogenous abscisic acid and the genes involved, such as zeaxanthin epoxidase,

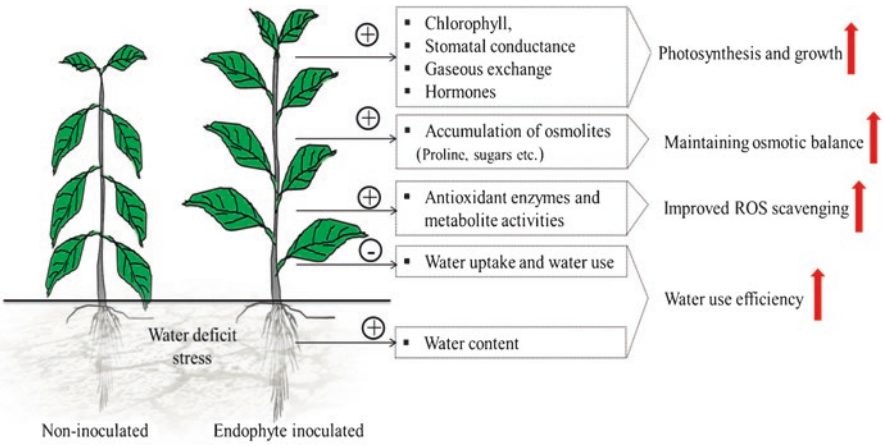

Fig. 8.1 Endophyte colonization can help plants better withstand in water deficit stress by exerting their effects, directly or indirectly, on plant functions at both above- and belowground. The plant on the left side which represents a drought-stressed non-colonized plant shows reduced growth and biomass due to lower photosynthetic rate, higher amount of oxidative damage, reduced uptake water and unbalanced osmoregulation. On the *right* side, a fungal endophyte-colonized plant under water deprivation stress is shown. Endophyte colonization shows increased growth and biomass due to enhanced photosynthetic rate, increased water-use efficiency and better osmotic balance. There is higher accumulation of osmolytes and lower degree of oxidative damage in the EC plants

9-cis-epoxycarotenoid dioxygenase 3 and ABA aldehyde oxidase 3, have been found to be significantly decreased in endophyte-colonized plants under stress, the effect of which could be comparable to that of the exogenous GA3 in terms of promoting plant growth and yield under stressed conditions by manipulating hosts' physiological processes (Khan et al. 2014). However, in some cases, it was recorded that endophytes do not show positive effects on host growth during drought stress, but they help with rapid recovery of host plant after water became available again (Ren and Clay 2008).

8.3.2.2 Endophyte-Mediated Improved Photosynthesis

Moisture stress causes decreased levels of photosynthesis in plants through decreased synthesis of ATP and other enzymes such as rubisco and sucrose–phosphate synthase as water availability decreases (Vassey and Sharkey 1989; Flexas and Medrano 2002; Parry et al. 2002; Ghannoum et al. 2003). Plant tolerance to water stress involves the management of extra radiation caused by reduced photosynthesis and CO_2 availability and a greater susceptibility to photo-damage (Powles 1984; Chaves et al. 2003). The endophyte-colonization results in higher chlorophyll content and leaf area in plants challenged by stress than non-colonized plant. Higher concentration of chlorophyll is associated with higher photosynthetic rate (Davies et al. 1993). The increased rate of photosynthesis was recorded from the drought-stressed *Capsicum annuum* plants colonized by endophytes *Chaetomium globosum* (Khan et al. 2012) and *Penicillium resedanum* (Khan et al. 2014). About twofold

increase in chlorophyll content and photosynthetic efficiency in *P. indica*-colonized *Arabidopsis* plants was measured when seedlings were challenged with water-limiting conditions (Sherameti et al. 2008). *P. indica* reduced the drought-induced decline in the photosynthetic rate and the denaturation of chlorophyll and thylakoid proteins (Sun et al. 2010). Although, the Fv/Fm values decreased in the non-EC plants under drought, no significant difference was observed for the *P. indica*-colonized plants indicating that EC plants suffer less from water stress than un-inoculated controls. In the same study, the total chlorophyll level was reported to be reduced by more than 50% in non-EC plant, but colonized plants showed only a slight decrease in total chlorophyll content (Sun et al. 2010). Additionally, a decrease in the protein levels of representative constituents of the thylakoid membrane and of enzymes situated in the plastid stroma in stressed plants was retarded when colonized with *P. indica* (Sun et al. 2010). Recently, Azad and Kaminskyj (2016) characterized a fungal endophyte that enhanced drought tolerance of the host and increased photosynthesis in the leaf. The mechanism of increased photosynthesis in EC plant under water stress is not fully understood. In one study, it was found that while the photosynthesis rate and stomatal conductance increased in drought-affected EC plants, initial rubisco activity and carboxylation efficiency did not differ from non-EC plants (Morse et al. 2002). It was suggested that endophyte colonization might result in reduced biochemical damage to the photosynthetic machinery plants subjected to water stress (Swarthout et al. 2009).

8.3.2.3 Plant–Water Relation and Osmotic Adjustment as Mediated by Endophyte

In the broad sense, decreasing water loss and maintaining water uptake are the key processes that plants employ to adapt to water-limiting environments. Maintaining water uptake is assisted within plant cells by osmotic adjustment (OA), a biochemical mechanism that helps plants to adapt to drought conditions. OA results in a net accrual of compatible solutes, also known as osmolytes in the cell so as to maintain the favourable gradient for water flow from soil into roots (Sanders and Arndt 2012). This accumulation of various ions, amino acids and sugars leads to a more negative osmotic potential, which is important for maintaining cell hydration and turgor, cellular development and growth, stomatal opening, photosynthesis and water uptake during drought (Chaves et al. 2003; Sanders and Arndt 2012). Endophyte-colonized plants consume significantly less water than non-colonized plants. For example, significantly less water use has been reported in endophyte-inoculated panic grass, rice, tomato and dune grass, indicative of their more efficient water usage. Reduced water consumption and improved water-use efficiency may offer a distinctive mechanism for endophyte-mediated drought resistance in plants (Rodriguez et al. 2008). Again, EC plants can maintain significantly greater water content than the non-inoculated under water stress, implying the ability of endophytes to delay desiccation and damage in stress. The endophyte association could help plant access larger volumes of water from sources not reachable to the non-infected plants which suffered from stress (Khan et al. 2013). Endophyte association resulted in a decreased level of electrolytic leakage inside the plant tissues upon exposure to water deficit

stress. Altered water potential and improved osmotic balance in drought-affected tall fescue infected with *N. coenophialum* endophyte have also been noted in some studies (Elmi and West 1995). Increased root water content was reported from *T. hamatum*-inoculated *T. cacao* plant subjected to water deficit stress compared to non-inoculated plants (Bae et al. 2009). A number of fungal endophytes have been reported to produce active biochemicals and metabolites that help the host plant withstand water deficit stress. Under drought conditions, significantly upregulation of free glucose, fructose, trehalose, sugar alcohols, proline and glutamic acid was detected in shoots and roots in tall fescue colonized by *Neotyphodium coenophialum* (Nagabhyru et al. 2013). Variable levels of proline accumulation were observed in EC plants subjected to water stress. While significantly more proline was accumulated in one genotype of tall fescue plant, no differences were observed in another genotype challenged with mimic drought in hydroponic culture (Bayat et al. 2009) when inoculated with *Neotyphodium* grass endophyte. Increased level of proline, soluble sugar and catalase (CAT) was observed in wheat colonized by endophyte *Chaetomium globosum* under water stress (Cong et al. 2015). Concentrations of aspartic acid and glutamic acid and of alanine and γ-aminobutyric acid were measured in drought-affected *Theobroma cacao* seedlings colonized by an isolate of *Trichoderma hamatum* (Bae et al. 2009). The changes in metabolites could be attributed to the strategies of EC plants towards drought tolerance or avoidance. Downregulation in osmolytes has previously been described as a strategy of drought avoidance, whereas the increase of osmoprotectants has been related to drought tolerance (Augé and Moore 2005; Ruiz-Sánchez et al. 2010).

8.3.2.4 Endophyte-Mediated ROS Scavenging

Reactive oxygen species (ROS) act as signalling molecules in plants. ROS is involved in many plant processes, including growth, stress response, cell cycle and programmed cell death by influencing the expression of related genes. Abiotic stresses cause excess synthesis of these highly reactive molecules, these ROS causing oxidative stress and damaging proteins, lipids and DNA (Gechev et al. 2006; Gill and Tuteja 2010). Manufacturing additional ROS, i.e. hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$), singlet oxygen and superoxides ($^1O_2$), is one of the main mechanisms for plant cell damage or death in drought (Smirnoff 1993). Plants react against excess ROS through an intricate network of direct ROS-quenching activity or indirect hormone-mediated signalling activity. Various enzymatic and non-enzymatic antioxidant molecules are involved in scavenging ROS (revised in Miller et al. 2010; Scheibe and Beck 2011). Malfunctioning of these antioxidants' defence system results in oxidative damage in cells (Apel and Hirt 2004; Kwak et al. 2006). Endophyte colonization simulates a more powerful ROS-scavenging system in host plants under stress and reduces damage of biomolecules at the cellular level. For instance, a lower level ROS production has been documented in endophyte-colonized tomato plants than in control plants following water stress (Azad and Kaminskyj 2016). When plants were inoculated with *P. indica* and exposed to drought stress, up-regulation of peroxidase (POX), catalase (CAT) and superoxide dismutase (SOD) activities in the leaves was observed (Sun et al. 2010).

The level of another biomarker of oxidative stress, namely, malondialdehyde (MDA), was recorded to be lower in *P. indica*-colonized cabbage plants than in control plants. MDA is primarily produced through the ROS-induced degradation of polyunsaturated lipids (Pryor and Stanley 1975; Del Rio et al. 2005). It is suggested that *P. indica* could prevent or reduce the damage of these lipids by inhibiting excess ROS production under stress conditions. Endophytes that promote drought tolerance have also been found to have high levels of loline alkaloids (Schardl et al. 2004b). Further experiments could test if these molecules are associated with the prevention of damage of macromolecules or reduction of ROS effects. Endophyte-induced production of antioxidant enzyme in plants under stress is predominantly observed in leaves (Baltruschat et al. 2008; Vadassery et al. 2009). All these studies demonstrate that endophyte inoculation results in a strong defence response in plant in water stress, in which alleviation of oxidative stress might be a vital part. The study of nonvolatile compounds has been the major focus in most plant antioxidant research. However, plant leaves emitting volatile organic compounds could also play as a further defence system against stresses (Kesselmeier and Staudt 1999; Peñuelas and Munné-Bosch 2005). The effect of volatile compounds such as isoprenoids has been described, where these compounds act as protective agent against oxidative stress in plant through direct ROS scavenging and indirect alteration of ROS signalling in arbuscular mycorrhizal plants (Peñuelas and Munné-Bosch 2005; Rapparini et al. 2008; Lopez-Ráez et al. 2008; Vickers et al. 2009; Walter and Strack 2011; Asensio et al. 2012; Baslam and Goicoechea 2012). Endophyte-colonized plants could emit similar volatile organic compounds to cope with abiotic stress, but this aspect of the research has not been done till date. Further investigation is necessary to have the information on the fungal side as well as the knowledge of the fungal/plant interaction is paramount to elucidate underlying mechanisms regulating antioxidant defences that are crucial to improve the tolerance of plants to drought stress.

8.3.2.5 Molecular Mechanisms of Endophyte-Mediated Plant Drought Tolerance

Studies on the beneficial effects of endophyte symbiosis under drought have predominantly focused at the plant morpho-physiological level. Molecular tools have also been included in this type of studies. The responses of EC plants to stress can be regulated by the expression of drought-associated plant genes, e.g. those associated with signalling and regulatory pathways or those producing enzymes that synthesize various metabolic compounds. It was noted that, under drought conditions, EC and non-EC plants differently regulate the expression of several drought genes in the plant tissue, indicating the association of activation of Ca_2P signalling and related proteins (Singh et al., 2011) involved in the drought tolerance mechanisms. Among the genes regulated by the endophyte symbiosis during drought, delayed expression of drought-altered ESTs such as TcTPP, TcSOT, TcPR5 and TcNI in the leaves and TcPR5 and TcCESA3 in the roots has been described (Bae et al. 2009). Again, the expression a diverse array of stress-related genes, including 29A, ANAC072, DEHYDRATION-FINGER1, Ddelta, CBL1, HAT, etc. putatively

mediate drought tolerance of *Arabidopsis* plants inoculated with *P. indica* (Sherameti et al. 2008). Similarly, up-regulation of drought-associated genes DREB2A, CBL1, ANAC072 and RD29A was also reported in the drought-challenged leaves of *P. indica*-colonized Chinese cabbage plants. The contribution of endophyte to the enhanced drought tolerance of the host plant can be mediated by CAS protein and the thylakoid membrane CAS mRNA level associated with Ca2+ sensing regulator (Sun et al. 2010). Further research could encompass non-targeted screening of cDNA libraries from both endophyte and host plants. Such an approach could allow the detection of stress-induced genes that offer increased stress tolerance in endophyte-colonized hosts. Employing microarrays and next-generation sequencing technologies to elucidate stress tolerance mechanisms (physiological and molecular) involved in endophyte colonization will be used to compare EC and non-EC plants of the same host genotype.

8.4 Future Directions

Studies indicate that fungal endophytes occur in most plant species studied so far. Endophytes that exhibit non-mutualistic lifestyles in particular hosts may form mutualistic symbioses with genetically dissimilar plant species and confer stress tolerance. If this is true for all the endophytes, it may be promising to isolate endophytes from the plant living in harsh environments and exploit their role in genetically different stress-sensitive plant species. To achieve this, identification of novel endophytes from plant of diverse habitat and genotypes is paramount since it is assumed that many endophytes have not yet been identified, and the ecological functions have not been thoroughly studied. The effects of endophytes in improving of drought stress on plants have typically been investigated using pot cultures under greenhouse or growth chamber conditions where interactions between the partners were studied in a controlled manner. However, under natural conditions, endophyte colonization is affected by factors that are absent in controlled greenhouse or laboratory conditions. With a view to fully comprehend the endophyte effects on plant stress tolerance, future research must include field trials. These investigations could include varying levels of stress treatment and nutrition supplement as well as at various geographical locations so as to reveal the effects of endophyte, stress, soil nutrition and their interaction effects. Promising endophyte isolates could also be tested with various crop species under various cropping practices that resembles those used by growers. The proportion of fungal endophytes capable of forming an effective symbiosis with the host under drought stress and enhance tolerance is generally unknown. A thorough investigation of endophyte colonization of various plants and extensive screening of endophyte isolates to select the most promising ones is the first step towards utilizing their full potential. Morphologically, similar strains of the same fungal species can have differential roles on host growth and development as influenced by temperature, pH, water, nutrient availability and other factors (Picone 2003). Such conditional phenomena demand that beneficial endophyte isolates may need to be tested with various host genotypes and local

agroecosystem settings. Again, most studies have been taken place on the plant side, but efforts should be made to study the effect on the endophyte side and how they function at the different circumstances. Therefore, mutants of both partners will be valuable tools to elucidate the fundamental processes involved. Combined efforts where various disciplines as plant physiology, ecology, mycology, biochemistry, molecular biology and biotechnology could meet together are still needed. These investigations should also be united with a thorough analysis of the transmission of this knowledge to natural environments, considering the fact that knowledge of the roles endophytic fungi play in ecosystems is important as parts of the earth are warm and dry.

8.5 Concluding Notes

In nature, plants do not live as independent entities, but form a complex community with diverse organisms including microbes. These organisms, in particular, fungal endophytes, provide significant advantage to the plants that grow in inhospitable environments. From the studies reviewed in this chapter, it is evident that endophyte colonization can significantly improve plant drought stress tolerance. We focused our review on plant growth, photosynthesis, osmotic balance, water relation, metabolic changes and antioxidant production. All these parameters are interrelated and will influence each other, especially at the plant physiological level. How endophytic fungi affect these parameters under drought is still unclear. Molecular approaches will help elucidate the whole response of plant–endophyte interactions at different levels. Further, in-depth investigation involving a combination of approaches, including physiological, biochemical and molecular data and 'omics' techniques, will clarify the interrelated molecular mechanisms and novel metabolic pathways of endophyte-mediated plant drought tolerance.

References

Andrew M, Barua R, Short SM, Kohn LM et al (2012) Evidence for a common toolbox based on necrotrophy in a fungal lineage spanning necrotrophs, biotrophs, endophytes, host generalists and specialists. PLoS One 7:e29943

Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol 55:373–399

Arnold AE (2007) Understanding the diversity of foliar endophytic fungi: progress, challenges, and frontiers. Fung Biol Rev 21:51–66

Asensio D, Rapparini F, Peñuelas J et al (2012) AM fungi root colonization increases the production of essential isoprenoids vs nonessential isoprenoids especially under drought stress conditions or after jasmonic acid application. Phytochemistry 77:149–161

Ashmore M, Toet S, Emberson L et al (2006) Ozone-a significant threat to future world food production? New Phytol 170:201–204

Augé RM, Moore JL (2005) Arbuscular mycorrhizal symbiosis and plant drought resistance. In: Mehrotra VS (ed) Mycorrhiza: role and applications. Allied Publishers Limited, New Delhi, pp 136–157

Azad K, Kaminskyj S (2016) A fungal endophyte strategy for mitigating the effect of salt and drought stress on plant growth. Symbiosis 68(1):73–78. doi:10.1007/s13199-015-0370-y

Bae H, Sicher RC, Kim MS, Kim SH, Strem MD, Melnick RL et al (2009) The beneficial endophyte *Trichoderma hamatum* isolate DIS 219b promotes growth and delays the onset of the drought response in *Theobroma cacao*. J Exp Bot 60:3279–3295. http://dx.doi.org/10.1093/jxb/erp165

Baltruschat H, Fodor J, Harrach BD, Niemczyk E, Barna B, Gullner G, Janeczko A, Kogel KH, Schäfer P, Schwarzinger I, Zuccaro A, Skoczowski et al (2008) Salt tolerance of barley induced by the root endophyte *Piriformospora indica* is associated with a strong increase in antioxidants. New Phytol 180(2):501–510. doi:10.1111/j.1469-8137.2008.02583.x

Bao X, Roossinck MJ (2013) Multiplexed interactions: viruses of endophytic fungi. Adv Virus Res 86:37–58. doi:10.1016/B978-0-12-394315-6.00002-7

Bartoli CG, Simontacchi M, Tambussi E, Beltrano J, Montaldi E, Puntarulo S (1999) Drought and watering-dependent oxidative stress: effect on antioxidant content in *Triticum aestivum* L. leaves. J Exp Bot 50:375–385

Baslam M, Goicoechea N (2012) Water deficit improved the capacity of arbuscular mycorrhizal fungi (AMF) for inducing the accumulation of antioxidant compounds in lettuce leaves. Mycorrhiza 22:347–359

Battisti DS, Naylor RL (2009) Historical warnings of future food insecurity with unprecedented seasonal heat. Science 323:240–244

Bayat F, Mirlohib A, Khodambashic M et al (2009) Effects of endophytic fungi on some drought tolerance mechanisms of tall fescue in a hydroponics. Russ J Plant Physiol 56(4):510–516

Blum A (1996) Crop responses to drought and the interpretation of adaptation. Plant Growth Regul 20:135–148

Boyer JS (1982) Plant productivity and environment. Science 218:443–448

Bray EA, Bailey-Serres J, Weretilnyk E et al (2000) Responses to abiotic stresses. In: Gruissem W, Buchannan B, Jones R (eds) Biochemistry and molecular biology of plants. Am Soc Plant Physiol, Rockville, pp 1158–1249

Brundrett MC (2004) Diversity and classification of mycorrhizal associations. Biol Rev 79:473–495

Chaves MM, Maroco JP, Pereira JS et al (2003) Understanding plant responses to drought- from genes to the whole plant. Funct Plant Biol 30:239–264

Cheplick GP (2004) Recovery from drought stress in *Lolium perenne* (Poaceae): are fungal endophytes detrimental? Am J Bot 91:1960–1968

Cheplick GP (2006) Costs of fungal endophyte infection in *Lolium perenne* genotypes from Eurasia and North Africa under extreme resource limitation. Environ Exp Bot 60:202–210

Cheplick GP, Perera A, Koulouris K et al (2000) Effect of drought on the growth of *Lolium perenne* genotypes with and without fungal endophytes. Funct Ecol 14:657–667

Clay K (1988) Fungal endophytes of grasses-a defensive mutualism between plants and fungi. Ecology 69:10–16

Cong GQ, Yin CL, He BL, Li L, Gao KX et al (2015) Effect of the endophytic fungus *Chaetomium globosum* ND35 on the growth and resistance to drought of winter wheat at the seedling stage under water stress. Acta Ecol Sin 35:6120–6128

Davies FT, Potter JR, Linderman RG et al (1993) Drought resistance of mycorrhizal pepper plants independent of leaf P concentration response in gas exchange and water relations. Physiol Plant 87:45–53

De Battista JP, Bacon CW, Severson RF, Plattner RD, Bouton JH et al (1990) Indole acetic acid production by the fungal endophyte of tall fescue. Agron J 82:878e880

Deckert RJ, Melville LH, Peterson L et al (2001) Structural features of a *Lophodermium* endophyte during the cryptic life-cycle phase in the foliage of *Pinuss trobus*. Mycol Res 105:991–997

Del Rio D, Steward AJ, Pellegrini N et al (2005) A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. Nutr Metab Cardiovasc Dis 15:316–328

Desclaux D, Roumet P (1996) Impact of drought stress on the phenology of two soybean (*Glycine max* L. Merril) cultivars. Field Crops Res 46:61–70

Dhargalkar S, Bhat DJ (2009) *Echinosphaeria pteridis* sp. and its *Vermiculariopsiella* anamorph. Mycotaxon 108:115–122

Eerens JPJ, Lucas RJ, Easton S, White JGH et al (1998) Influence of the endophyte (*Neotyphodium lolii*) on morphology, physiology, and alkaloid synthesis of perennial ryegrass during high temperature and water stress. N Z J Agric Res 41(2):219–226. doi:10.1080/00288233.1998.9513305

Elmi AA, West CP (1995) Endophyte effects on tall fescue stomatal response, osmotic adjustment, and tiller survival. New Phytol 131:61–67

Fisher PJ (1996) Survival and spread of the endophyte *Stagonospora pteridiicola* in *Pteridium aquilinum*, other ferns and some flowering plants. New Phytol 132:119–122

Flexas J, Medrano H (2002) Drought-inhibition of photosynthesis in C3 plants: stomatal and non-stomatal limitations revisited. Ann Bot 89:183–189

Frohlich J, Hyde KD (1999) Biodiversity of palm fungi in the tropics: are global fungal diversity estimates realistic? Biodivers Conserv 8:97Z–1004

Garrett KA, Dendy SP, Frank EE, Rouse MN, Travers SE et al (2006) Climate change effects of plant disease: genomes to ecosystems. Annu Rev Phytopathol 44:489–509

Geber MA, Dawson TE (1990) Genetic variation in and covariation between leaf gas exchange, morphology and development in *Polygonum arenastrum*, an annual plant. Oecologia 85:153–158

Gechev TS, Van Breusegem F, Stone JM, Denev I, Laloi C et al (2006) Reactive oxygen species as signals that modulate plant stress responses and programmed cell death. BioEssays 28:1091–1101

Ghannoum O, Conroy JP, Driscoll SP, Paul MJ, Foyer CH, Lawlor DW et al (2003) Non-stomatal limitations are responsible for drought-induced photosynthetic inhibition in four C_4 grasses. New Phytol 159:835–844

Gill S, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol Biochim 48:909–930

Grover A, Kapoor A, Laksmi OS, Agarwal S, Sahi C, Katiyar-Agarwal S, Agarwal M, Dubey H et al (2001) Understanding molecular alphabets of the plant abiotic stress responses. Curr Sci 80(2):206–216

Hawksworth DL (1988) The variety of fungal-algal symbioses, their evolutionary significance, and the nature of lichens. Bot J Linn Soc 96:3–20

Hoffmann AA, Merilä J (1999) Heritable variation and evolution under favourable and unfavourable conditions. Trends Ecol Evol 14:96–101

IPCC (2007) Climate change 2007: synthesis report. In: Pachauri RK, Reisinger A (eds) Contribution of working groups I, II and III to the fourth assessment report of the intergovernmental panel on climate change. IPCC, Geneva

Jackson RB, Sperry JS, Dawson TE et al (2000) Root water uptake and transport: using physiological processes in global predictions. Trends Plant Sci 5:482–488

Kane KH (2011) Effects of endophyte infection on drought stress tolerance of *Lolium perenne* accessions from the Mediterranean region. Environ Exp Bot 71(3):337–344

Kesselmeier J, Staudt M (1999) Biogenic volatile organic compounds (VOC): an overview on emission, physiology and ecology. J Atmos Chem 33:23–88

Khan AL, Shinwari ZK, Kim Y, Waqas M, Hamayun M, Kamran M, Lee IJ et al (2012) Role of endophyte *Chaetomium globosum* lk4 in growth of *Capsicum annuum* by production of gibberellins and indole acetic acid. Pak J Bot 44:1601–1607

Khan AL, Waqas M, Khan AR, Hussain J, Kang SM, Gilani SA, Hamayun M, Shin JH, Kamran M, Al-Harrasi A, Yun BW, Adnan M, Lee IJ et al (2013) Fungal endophyte *Penicillium janthinellum* LK5 improves growth of ABA-deficient tomato under salinity. World J Microbiol Biotechnol 29(11):2133–2144. doi:10.1007/s11274-013-1378-1

Khan AL, Waqas M, Lee IJ et al (2014) Resilience of *Penicillium resedanum* LK6 and exogenous gibberellin in improving *Capsicum annuum* growth under abiotic stresses. J Plant Res 128(2):259–268. doi:10.1007/s10265-014-0688-1

Kirkham MB (2005) Principles of soil and plant water relations. Elsevier Academic Press, Burlington
Krings M, Taylor TN, Dotzler N et al (2012) Fungal endophytes as a driving force in land plant evolution: evidence from the fossil record. In: Southworth D (ed) Biocomplexity of plant-fungal interactions. Wiley, New York, pp 5–28
Kwak JM, Nguyen V, Shroeder JI et al (2006) The role of active oxygen species in hormonal responses. Plant Physiol 141:323–329
Lewis GC, Clements RO (1986) A survey of ryegrass endophyte (*Acremonium loliae*) in the U.K. and its apparent ineffectuality on a seedling pest. J Agric Sci 107:633–638
Li WC, Zhou J, Guo SY, Guo LD et al (2007) Endophytic fungi associated with lichens in Baihua mountain of Beijing, China. Fungal Divers 25:69–80
Lopez-Ráez JA, Charnikhova T, Gomez-Roldan V, Matusova R, Kohlen W, De Vos R et al (2008) Tomato strigolactones are derived from carotenoids and their biosynthesis is promoted by phosphate starvation. New Phytol 178:863–874
Maherali H, Caruso CM, Sherrard ME, Latta RG et al (2010) Adaptive value and costs of physiological plasticity to soil moisture limitation in recombinant inbred lines of A*vena barbata*. Am Nat 175:211–224
Malinowski DP, Belesky DP (2000) Adaptations of endophyte-infected cool-season grasses to environmental stresses: mechanisms of drought and mineral stress tolerance. Crop Sci 40:923–940
Malinowski DP, Belesky DP (2006) Ecological importance of *Neotyphodium* spp. grass endophytes in agroecosystems. Grassl Sci 52(1):1–14. doi:10.1111/j.1744-697X.2006. 00041.x
Malinowski DP, Zuo H, Belesky DP et al (2004) Evidence for copper binding by extracellular root exudates of tall fescue but not perennial ryegrass infected with *Neotyphodium* spp. endophytes. Plant Soil 267(1):1–12. doi:10.1007/s11104-005-2575-y
Miller G, Suzuki N, Ciftci-Yilmaz S, Mittler R et al (2010) Reactive oxygen species homeostasis and signalling during drought and salinity stresses. Plant Cell Environ 33(4):453–467
Morgan JM (1984) Osmoregulation and water stress in higher plants. Ann Rev Plant Physiol 35:299–319
Morse LJ, Day TA, Faeth SH et al (2002) Effect of *Neotyphodium* endophyte infection on growth and leaf gas exchange of Arizona fescue under contrasting water availability regimes. Environ Exp Bot 48:257–268
Nagabhyru P, Dinkins RD, Wood CL, Bacon CW, Schardl CL et al (2013) Tall fescue endophyte effects on tolerance to water-deficit stress. BMC Plant Biol 13:127
Okcu G, Kaya DM, Atak M et al (2005) Effects of salt and drought stresses on germination and seedling growth of pea (*Pisum sativum* L.) Turk J Agric For 29:237–242
Parry MA, Andralojc PJ, Khan S, Lea PJ, Keys AJ et al (2002) Rubisco activity: effects of drought stress. Ann Bot 89:833–839
Peñuelas J, Munné-Bosch S (2005) Isoprenoids: an evolutionary pool for photoprotection. Trends Plant Sci 10:166–169
Petrini O, Fisher PJ (1986) Fungal endophytes in *Salicornia perennis*. Trans Br Mycol Soc 87(4):647–651
Picone C (2003) Managing mycorrhizae for sustainable agriculture in the tropics. In: Vandermeer JH (ed) Tropical agroecosystems. CRC, Boca Raton, pp 95–132
Powles SB (1984) Photoinhibition of photosynthesis induced by visible light. Annu Rev Plant Physiol 35:15
Pryor WA, Stanley JP (1975) Letter: a suggested mechanism for the production of malonaldehyde during the autoxidation of polyunsaturated fatty acids. Non-enzymatic production of prostaglandin endoperoxides during autoxidation. J Org Chem 40:3615–3617
Purahong W, Hyde KD (2011) Effects of fungal endophytes on grass and non-grass litter decomposition rates. Fungal Divers 47:1–7
Rapparini F, Llusià J, Peñuelas J et al (2008) Effect of arbuscular mycorrhizal (AM) colonization on terpene emission and content of *Artemisia annua*. Plant Biol 10:108–122
Redman RS, Dunigan DD, Rodriguez RJ (2001) Fungal symbiosis: from mutualism to parasitism, who controls the outcome, host or invader? New Phytol 151:705–716

Redman RS, Kim YO, Woodward CJDA, Greer C, Espino L, Doty S, Rodriguez RJ et al (2011) Increased fitness and adaptation of rice plants to cold, drought and salt stress via habitat adapted symbiosis: a strategy for mitigating impacts of climate change. PLoS One 6:E14823
Redman RS, Sheehan KB, Stout RG, Rodriguez RJ, Henson JM et al (2002) Thermotolerance generated by plant/fungal symbiosis. Science 298:1581
Reid A, Greene SE (2012) How microbes can help feed the world. Report on an American Academy of Microbiology Colloquium. Washington, DC
Ren A, Clay K (2008) Impact of a horizontally transmitted endophyte, *Balansia henningsiana*, on growth and drought tolerance of *Panicum rigidulum*. Int J Plant Sci 170:599e608
Rodriguez RJ, Redman RS (1997) Fungal life-styles and ecosystem dynamics: biological aspects of plant pathogens, plant endophytes and saprophytes. In: Andrews JH, Tommerup L (eds) Advances in botanical research. Academic, London
Rodriguez RJ, Redman RS, Henson JM et al (2004) The role of fungal symbioses in the adaptation of plants to high stress environments. Mitig Adapt Strateg Glob Chang 9(3):261–272
Rodriguez RJ, Henson J, Van Volkenburgh E, Hoy M, Wright L, Beckwith F et al (2008) Stress tolerance in plants via habitat-adapted symbiosis. ISME J 2:404–416
Rodriguez RJ, White JF, Arnold AE, Redman RS et al (2009) Fungal endophytes: diversity and functional roles. New Phytol 182:314–330
Ruiz-Sánchez M, Aroca R, Muñoz Y, Armada E, Polón R, Ruiz-Lozano JM et al (2010) The arbuscular mycorrhizal symbiosis enhances the photosynthetic efficiency and the antioxidative response of rice plants subjected to drought stress. J Plant Physiol 167:862–869
Sanchez-Marquez S, Bills GF, Herrero N, Zabalgogeazcoa I et al (2012) Nonsystemic fungal endophytes of grasses. Fungal Ecol 5:289–297
Sanders GJ, Arndt SK (2012) Osmotic adjustment under drought conditions. In: Aroca R (ed) Plant responses to drought stress. Springer, New York, pp 199–229
Schardl CL, Clay K (1997) Evolution of mutualistic endophytes from plant pathogens. In: Carroll, Tudzynski (eds) The mycota V: Part B. Springer, Berlin, pp 1–17
Schardl CL, Leuchtmann A, Spiering MJ et al (2004a) Annu Rev Plant Biol 55:315–340
Schardl CL, Leuchtmann A, Spiering MJ et al (2004b) Symbioses of grasses with seed borne fungal endophytes. Ann Rev Plant Biol 55:315–340
Scheibe R, Beck E (2011) Drought, desiccation, and oxidative stress. In: Lüttge U, Beck E, Bartels D (eds) Plant desiccation tolerance, Ecological studies, vol 215. Springer, Heidelberg, pp 209–232
Schenk PM, Carvalhais LC, Kazan K et al (2012) Unraveling plant–microbe interactions: can multi-species transcriptomics help? Trends Biotechnol 30(3):177–184
Schulz B, Boyle C (2005) The endophytic continuum. Mycol Res 109:661–686
Schulz B, Guske S, Dammann U, Boyle C et al (1998) Endophyte-host interactions. II Defining symbiosis of the endophyte-host interaction. Symbiosis 25:213–227
Schulze ED (1986) Carbon dioxide and water vapor exchange in response to drought in the atmosphere and the soil. Ann Rev Plant Physiol 37:247–274
Sherameti I, Tripathi S, Varma A, Oelmuller R et al (2008) The root-colonizing endophyte *Piriformospora indica* confers drought tolerance in *Arabidopsis* by stimulating the expression of drought stress-related genes in leaves. Mol Plant-Microbe Interact 21:799–807. doi:10.1094/MPMI-21-6-0799
Sherrard ME, Maherali H, Latta RG et al (2009) Water stress alters the genetic architecture of functional traits associated with drought adaptation in *Avena barbata*. Evolution 63:702–715
Singh LP, Gill SS, Tuteja N (2011) Unraveling the role of fungal symbionts in plant abiotic stress tolerance. Plant Signal Behav 6:175–191
Smirnoff N (1993) The role of active oxygen in the response of plants to water deficit and desiccation. New Phytol 125:27–58
Sun CA, Johnson J, Cai DG, Sherameti I, Oelmuller R, Lou BG et al (2010) *Piriformosapora indica* confers drought tolerance in Chinese cabbage leaves by stimulating antioxidant enzymes, the expression of drought-related genes and the plastid-localized CAS protein. J Plant Physiol 167:1009–1017. doi:10.1016/j.jplph.2010.02.013

Swarthout D, Harper E, Judd S, Gonthier D, Shyne R, Stowe T, Bultman T et al (2009) Measures of leaf-level water-use efficiency in drought stressed endophyte infected and non-infected tall fescue grasses. Environ Exp Bot 66(1):88–93

Taiz L, Zeiger E (2010) Plant physiology, 5th edn. Sinauer Associates, Sunderland

Vadassery J, Ranf S, Drzewiecki C, Mithöfer A, Mazars C, Scheel D, Lee J, Oelmüller R (2009) A cell wall extract from the endophytic fungus Piriformospora indica promotes growth of Arabidopsis seedlings and induces intracellular calcium elevation in roots. Plant J 59:193–206

Vassey TL, Sharkey TD (1989) Mild water stress leads to reduced extractable sucrose-phosphate synthase activity in leaves of *Phaseolus vulgaris* L. Plant Physiol 89:1066–1070

Vickers CE, Gershenzon J, Lerdau MT, Loreto F et al (2009) A unified mechanism of action for volatile isoprenoids in plant abiotic stress. Nat Chem Ecol 5:283–291

Waller F, Achatz B, Baltruschat H, Fodor J, Becker K, Fischer M, Heier T, Huckelhoven R, Neumann C, Wettstein D, Franken P, Kogel KH et al (2005) The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. Proc Natl Acad Sci U S A 102:13386–13391. doi:10.1073/pnas.0504423102

Walter MH, Strack D (2011) Carotenoids and their cleavage products: biosynthesis and functions. Nat Prod Rep 28:663–692

Wang Y, Frei M (2011) Stressed food—the impact of abiotic environmental stresses on crop quality. Agric Ecosyst Environ 141:271–286

Waqas M, Khan AL, Kamran M, Hamayun M, Kang SM, Kim YH, Lee IJ et al (2012) Endophytic fungi produce gibberellins and indole acetic acid and promotes host-plant growth during stress. Molecules 17:10754–10773. doi:10.3390/molecules170910754

Xu P, Chen F, Mannas JP, Feldman T, Sumner LW et al (2008) Virus infection improves drought tolerance. New Phytol 180:911–921

Xu ZZ, Zhou GS, Shimizu H (2009) Effects of soil drought with nocturnal warming on leaf stomatal traits and mesophyll cell ultrastructure of a perennial grass. Crop Sci 49:1843–1851

Yue B, Xue W, Xiong L, Yu X, Luo L, Cui K, Jin D et al (2006) Genetic basis of drought resistance at reproductive stage in rice: separation of drought tolerance from drought avoidance. Genetics 172(2):1213–1228

Zabalgogeazcoa I (2008) Fungal endophytes and their interaction with plant pathogens. Span J Agric Res 6:138–146

Zaurov DE, Bonos S, Murphy JA, Richardson M, Belanger FC et al (2001) Endophyte infection can contribute to aluminium tolerance in fine fescues. Crop Sci 41:1981–1984

Appendix II

(Note: This is a systematic review on endophyte mediated plant drought tolerance. Due to limitation in word counts in the main body of the thesis this is provided as an appendix and may serve as an extension to the literature review section)

Fungal endophytes alter plant physiological responses to water stress: a meta-analysis

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Abstract

Water limitation often hinders plant productivity in both natural and agricultural settings. Endophytic fungal symbionts can mediate plant water stress responses by enhancing drought tolerance and avoidance, but these effects have not been quantified across plant-endophyte studies. We performed a meta-analysis of published studies to determine how endophytic fungal symbionts influence plant response under non-stressed vs. water-stressed conditions. A significantly positive or neutral overall effect of fungal endophyte was noted under water stressed condition. In contrast, under non-stressed conditions, the overall effect of fungi on plant was mostly neutral. In general, the presence of fungal endophytes increased plant total biomass, chlorophyll content and stomatal conductance irrespective of water availability. In addition, plant shoot biomass, tiller density, plant height, maximum quantum yield (Fv/Fm), net photosynthesis, relative water content (RWC), amounts of ascorbate peroxidase (APX), glutathione (GSH), polyphenol oxidase (PPO), superoxide dismutase (SOD), and phenolics were significantly increased. The malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) reduced in endophytic plants under stress as compared to non-endophytic counterparts. Categorical analysis revealed that accumulation in plant biomass is influenced by factors such as host and fungi identity, the magnitude of which are greater under stress than under non-stress conditions. Therefore, plant and their fungal symbionts appear to interact in a context-dependent manner, varying with biotic and abiotic conditions. Plant-endophyte symbioses considerably alter plant response to stress, and they could play a role in responses to climate change.

Introduction

Water limitation is one of the major constraints to plant productivity in many natural ecosystems and agroecosystems[1]. As predicted by climate change models, the intensity and frequency of drought may increase in some places[2,3]. Water deficit stress exerts detrimental effects on general plant physiology, i.e., growth, plant water status, gas exchange; photosynthesis as well as metabolism depending on the speed, severity, and duration of the stress event[4-7].

Plants employ a series of interconnected morpho-physiological, cellular, and molecular mechanisms to perceive and respond to water stress[8]. Plant responses are modulated by the severity, duration, and speed of development of the imposed stress[9]. Strategies to cope with stress can be grouped into three broad categories: (i) changes in plant phenology, (ii) avoidance of stress through reduced evapotranspiration and increased root-to-shoot ratios, and (iii) tolerance to water stress through increasing cellular osmolyte concentrations to improve water uptake and retention[10].

Whereas these direct plant responses to stress have been well studied, plant responses indirectly through plant-microbe interactions have been given less attention. In particular, plant species commonly associated with fungal symbionts such as mycorrhizal fungi and endophytes (within plants) which may influence their responses to environmental stimuli including water stress. Both above- and belowground fungal endophytes can change how plants respond to stress and thus have the potential to ameliorate the effects of water stress. These fungal endophytes interact with hosts and increase tolerance to water stress through increasing plant growth and productivity[11-15], increasing osmolyte production and plant-water relations[16-20], improving photosynthetic activity[11,13,15,21,22], reducing oxidative damage [11,15,23,24] and other structural and functional modifications.

Endophyte effects on these physiological processes are not always predictable. For instance, the plant-fungus symbioses have resulted in increased plant growth[13,14,22,25-27], decreased plant growth [28] or have no effect on plant growth [29,30]. The same is true for relative water content, photosynthesis efficiency, and metabolite accumulation [31,32]. These discrepancies were primarily as a result of intricate relationships between plant and fungal symbionts which are modulated by the nature of the interaction and the nature and severity of the stress, among other factors. Thus, it is delicate to infer the findings in general context from individual studies. Therefore, to determine the central tendency and identify different patterns of endophyte effects on plants under stress and compare with them under control, it may be useful integrate results across studies in order to determine if general factors can be identified. To this end, we carried out a meta-analysis to estimate the overall strength and direction of summary effects size of endophyte symbiosis on important plant characteristics associated with stress tolerance mechanisms.

A meta-analysis is a synthetic approach which analyses data from different experimental studies using weighted statistical methods to determine a mean effect size for treatment across a range of studies[33]. It helps understand the findings of any study in the context of all other comparable studies to determine if the effect of a particular treatment is consistent across studies or if it varies considerably among studies and which factor might cause this variation[34]. Categorical variables or "moderators" are frequently examined in meta-analyses to know how experimental conditions modify the treatment effect of interest. This form of analysis has been, for example, used to determine the effect of arbuscular mycorrhizae (AM) and endophytic fungi on plant response to water stress[32] and the effect of dark septate root endophytes on plant response under adequate water[35]. In their analysis, Worcel et al. [32] reported that although AM had significant overall effects on plant biomass, the effect of endophytes was neutral under water stress and that the plant photosynthetic pathway (C3 and C4), as well as fungal phylogeny, were important moderators on that effect. However, in their study, they included only clavicipitaceous endophytes and measured only few growth parameters.

In the present study, we accumulated data from all studies to date and measured effects of endophytes on 32 plant response parameters that encompass plant growth, photosynthesis, water relations, metabolites, and enzymatic activities that are subjected to change under water stress conditions. Our purpose was to answer following questions:

1) What is the overall impact of endophyte colonization on various physiological parameters of plants exposed to water stress?
2) Is the plant-fungal relationship different under stressed conditions compared to unstressed conditions?
3) Are influences mediated by particular combinations of host and symbionts?

Results

We examined endophyte influence on 32 effect sizes in plants exposed to water stress. Summary effect sizes for non-stressed plants from the studies were also considered for comparison. Plant hosts were represented by 26 species in 22 genera and five families across the 67 articles. Ryegrass and Tall fescue were the most commonly studied hosts. The most studied fungal genus was *Epichloë* (45 articles) followed by *Penicillium* and *Trichoderma* (seven articles each) out of the 13 fungal genera recorded from all the studies. We use the genus name *Epichloë* when the fungal genus was reported as *Neotyphodium*[36].

Fungal endophytes on plant growth parameter

Endophyte colonization had a significant effect on five of the nine plant growth characteristics measured during water stress conditions. The symbiosis significantly stimulated plant height, shoot

biomass, tiller density, total biomass, and it diminished the specific leaf mass parameter (Fig. 1). The largest impact was on plant height with an effect size of 0.9307. Under non-stressed conditions, endophytes had neutral effects on all the plant growth parameters except the root/shoot ratio, seed germination, and total biomass which was increased by endophyte colonization (Fig. 1).

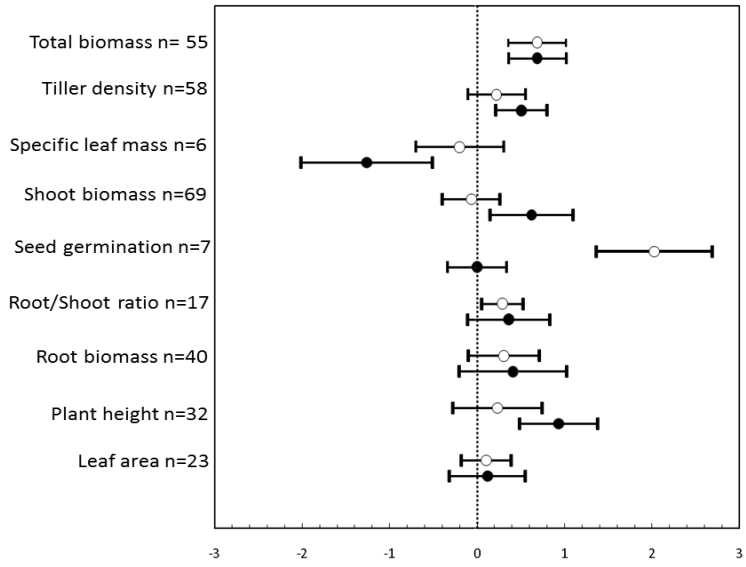

Fig. 1 Growth and biomass responses of endophyte-inoculated plants under water stress (filled circle) and under non-stressed (open circle) condition. Error bars are effect size means ±95 % bootstrap CIs. Where the CIs do not overlap the vertical dashed lines, the effect size for a parameter is significant. n=number of studies included in the meta-analysis

Total biomass

Five categorical variables considered for analysis indicated that endophyte infection differentially influenced plant total biomass. For example, class-2 endophytes increased total plant biomass under stressed and non-stressed conditions, whereas for class-1 endophytes relatively lower effect size values were recorded under both conditions and the effect was only significant under stressed condition (Fig. 2a). Taxonomically, the effect of *Trichoderma* followed by *Epichloë* outweighed the effect of other endophytes with a positive effect on plant total biomass under water stress (Fig. 2b). It was noted that categorically none of the fungal taxa expressed any significant impact on plant biomass under non-stressed conditions (Fig. 2b).

Endophyte colonization positively influenced both dicotyledonous and monocotyledonous plants under water stress. The effect size (ES=2.64) was greater for dicotyledonous plants than monocots (Fig. 2c). Under sufficient water, effects on both groups were neutral even though dicots had higher 95% CIs values than the monocots (Fig. 2c). The effect of endophyte colonization on annual plants tended to be greater under both stressed and non-stressed conditions than on perennial plants (Fig.

2d). Plant total biomass was increased significantly in the annual plants when infected with endophytes irrespective of water stress, whereas in the perennial plants the effect of fungal colonization was only striking under stress but not when plants were grown under non-stressed conditions (Fig. 2d)

It is interesting to note that plant identity was crucial in determining the effect of endophytes on stress tolerances. As is evident in figure 2e, t endophyte effects on the non-grass plant species were higher than on grass species, and it was significantly different from the ryegrass when the plants were subjected to water stress. Under non-stressed conditions, plant total biomass was decreased by endophyte infection in ryegrass whereas it was significantly increased in the other grass species and neutrally influenced in the non-grass species (Fig. 2e)

Shoot biomass

Categorical variables considered for analysis indicated that endophyte infection differentially affected plant shoot biomass. For example, whereas class-2 endophytes increased plant shoot biomass under both stress conditions, they had neutral effect under non-stressed conditions. No significant effect of class-1 endophytes was recorded under any conditions (Fig. 2f). Taxonomically, the effect of *Penicillium* was higher and it significantly positively influenced shoot biomass under stress but not under non-stressed conditions (Fig. 2g). It was noted that categorically none of the other fungal taxa expressed any significant impact on plant shoot biomass under either condition (Fig. 2g)

Monocotyledonous plants were significantly positively influenced by endophyte colonization under water stress although the effect the shoot biomass tended to decrease due to endophyte colonization under non-stressed conditions (Fig. 2h). Endophyte infection did not significantly influence plant shoot biomass under any circumstances in the dicotyledonous plants (Fig. 2h). The effect of endophyte colonization on annual plants tended to be higher under both stressed condition than on the perennial plants (Fig. 2i). Plant shoot biomass increased significantly in the annual plants when infected with endophytes under stress, but endophytes had neutral effect on the perennial plants under both non-stressed and stress conditions (Fig. 2i)

The effect of endophytes on plant shoot biomass varied greatly among plant species. Endophyte effects on grass species other than ryegrass and tall fescue were positively significant under conditions of water deficit stress (Fig. 2j, 3e). Interestingly, shoot biomass increased significantly in tall fescue and decreased in ryegrass even if the effect under stress was not significant in both species. Non-grass plant species were neutrally influenced by fungal endophytes in either condition (Fig. 2j).

Root biomass

Most categorical variables considered for analysis indicated that endophyte infection differentially affected plant root biomass. For example, whereas class-2 endophytes increased root biomass under stress conditions, no significant effect of class-2 endophytes was recorded under non-stressed conditions, but class-1 endophyte significantly increased root biomass (Fig. 2k). The effect of fungal taxa other than *Epichloe* was higher and it significantly positively influenced root biomass under stress and not under non-stressed conditions (Fig. 2l). *Epichloe* increased root biomass significantly under well-watered conditions, but it had neutral influence under stress (Fig. 2l)

Endophyte presence increased root biomass in dicotyledonous plants, and the effect of endophytes on dicots was significantly higher than on monocots, although the influence of endophytes on root biomass tended to decrease in dicots under non-stressed conditions (Fig. 2m). Endophyte colonization did not significantly influence monocot root biomass under any conditions (Fig. 2m). The effect of endophyte colonization on annual plants tended to be higher under stress condition (Fig. 2n). Plant root biomass increased significantly under stress in annual plants but not under non-stressed conditions (Fig. 2n).

Effect on plant root biomass did not vary significantly with plant identity. As depicted in figure 2o, endophyte infection did not yield significant impacts on root biomass on any grass species irrespective of water treatment. In contrast, root biomass increased significantly in endophyte-infected non-grass plant species under water limiting environment even though the effect was neutral under sufficient water conditions (Fig. 2o).

Fungal endophytes on plant photosynthetic activity

Endophyte colonization significantly increased stomatal conductance and total chlorophyll content in plants under both stressed and non-stressed conditions (Fig. 3). Endophyte symbiosis significantly increased Parameter, Fv/Fm, and net photosynthesis only under water stress conditions. All photosynthetic parameters considered, however, tended to be influenced more under stress than under non-stress as evident from their larger effect sizes (Fig. 3).

Fungal endophytes on plant water relations

Most of the plant-water relation parameters seemed to be influenced significantly by endophyte symbiosis both in stressed or non-stressed conditions (Fig. 4). Leaf relative water content (RWC) was increased significantly by

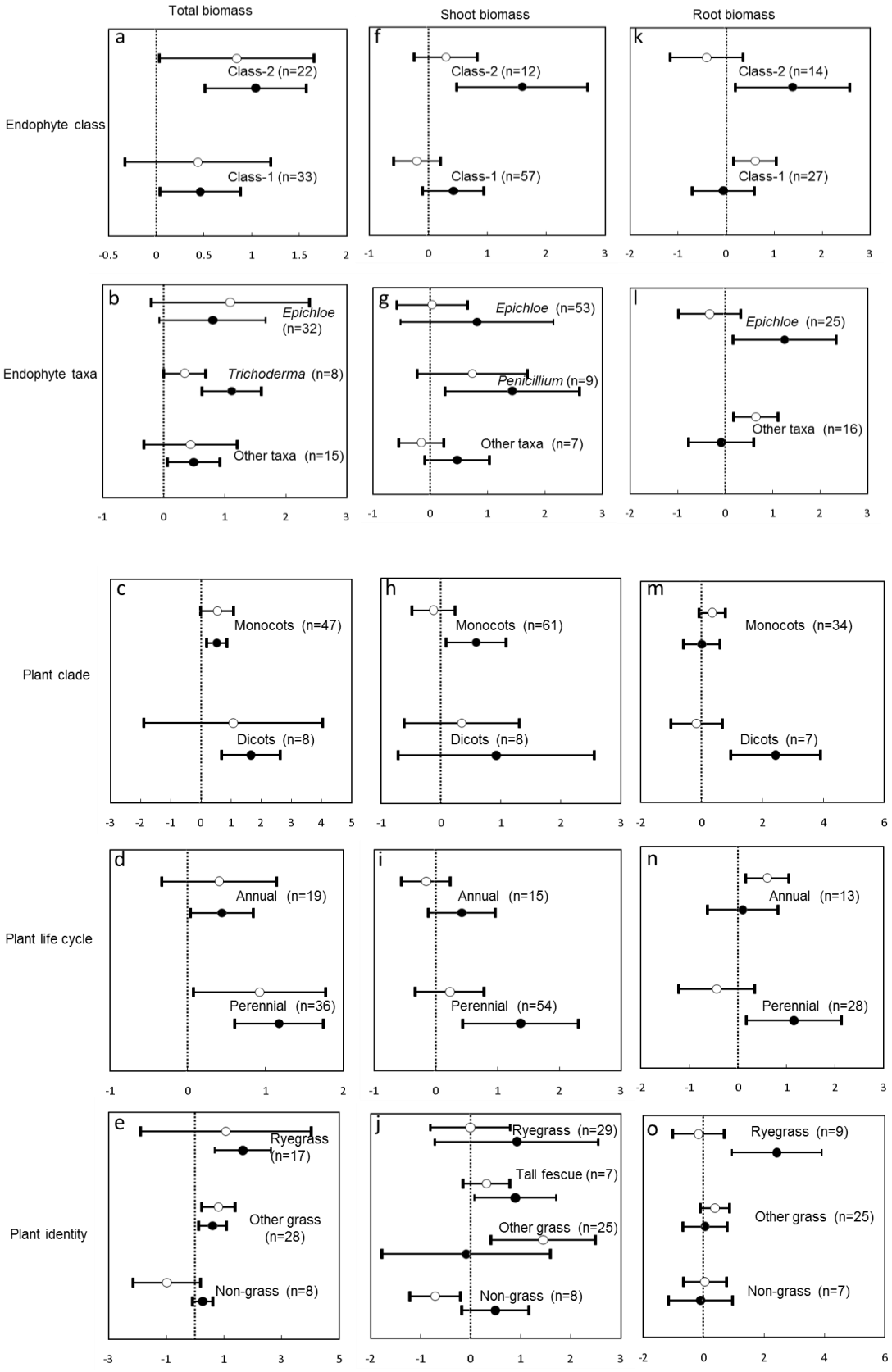

Fig. 2 Effect of endophytes on plants photosynthetic ability under water stress (filled circle) and under non-stressed (open circle) condition. Error bars are effect size means±95 % bootstrap CIs. Where the

CIs do not overlap the vertical dashed lines, the effect size for a parameter is significant. n=number of studies included in the meta-analysis

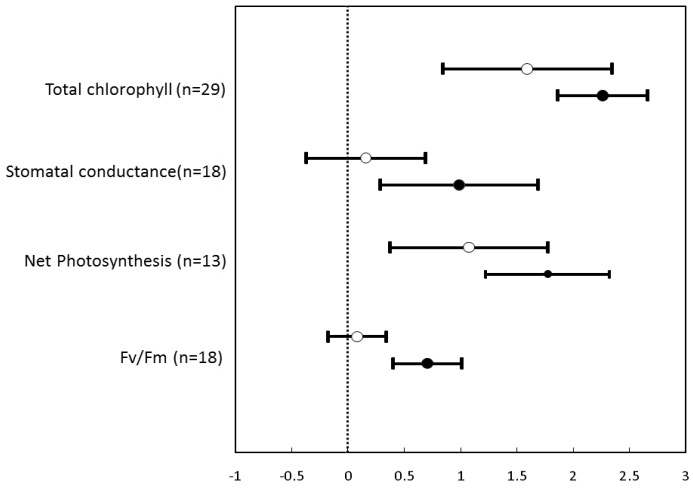

Fig. 3 Effect of endophytes on photosynthetic ability under water stress (filled circle) and non-stressed (open circle) conditions. Error bars are means±95 % bootstrap CIs. Where the CIs do not overlap the vertical dashed lines, the effect size for a parameter is significant. n=number of studies included in the meta-analysis.

fungal colonization only under watered conditions (Fig. 4). Endophyte-colonized plant showed a higher rate of transpiration and hence significantly lower water use efficiency (WUE) when grown under adequate water, but no such effect was evident under stress. It is interesting to note that the effects of endophytes on most of the plant-water relation characteristics were much more variable under stress than those under non-stressed conditions as is evident from their larger confidence interval values (Fig. 4).

Fungal endophytes on plant metabolites

Plant metabolite profiles were not, in general, influenced significantly by endophyte symbioses either in water stressed or non-stressed conditions. The exception was the soluble sugar content, which significantly increased under non-stressed condition (Fig. 5).

Proline

Categorical variables considered for analysis indicated that endophyte infection differentially influenced plant proline content. Both class-1 and-2 endophytes had neutral effects on leaf proline content under both non-stressed and stress conditions. However, interactions with class-2 endophytes tended to decrease proline content under water stress (Fig. 6a). The effect of most fungal taxa on proline content was neutral irrespective of water stress, although the effect under stress was variable

as evident from their wide 95% CIs values (Fig. 8b). It was noted that *Trichoderma* reduced leaf proline significantly under water-limiting environments (Fig. 6b).

No significant categorical variation was observed in proline content in monocotyledonous and dicotyledonous plants under either stress or non-stressed conditions (Fig. 6c). Endophyte colonization tended to lower the proline content in both annual and perennial plants under stress conditions even if the difference was not significant under any conditions (Fig. 6d).

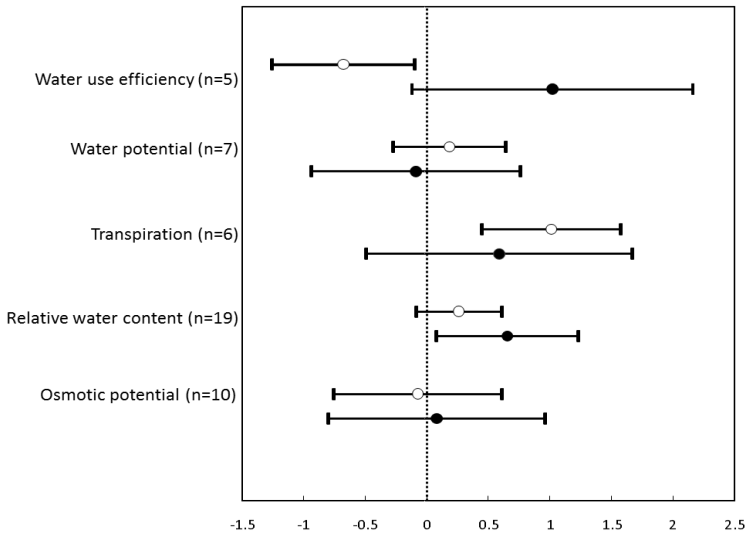

Fig. 4 Effect of endophytes on plants water relation under water stress (filled circle) and under non-stressed (open circle) condition. Error bars are means±95 % bootstrap CIs. Where the CIs do not overlap the vertical dashed lines, the effect size for a parameter is significant. n=number of studies included in the meta-analysis.

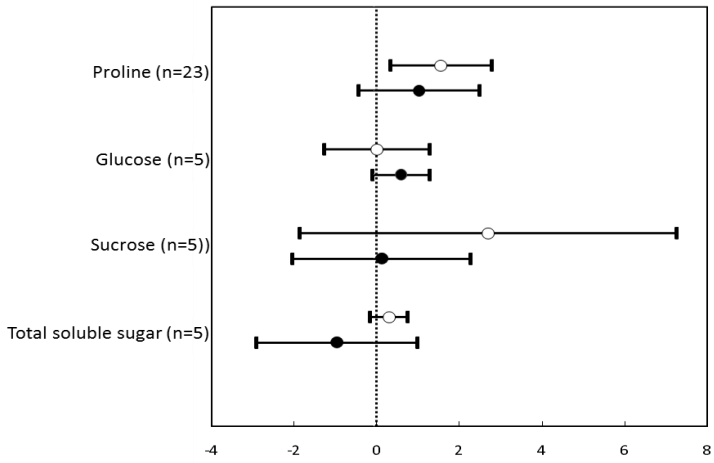

Fig. 5 Effect of endophytes on plant metabolites under water stress (filled circle) and under non-stressed (open circle) condition. Error bars are means±95 % bootstrap CIs. Where the CIs do not

overlap the vertical dashed lines, the effect size for a parameter is significant. n=number of studies included in the meta-analysis.

Effect endophytes on plant root biomass did not vary greatly by plant identity. As depicted in figure 5e, endophyte infection did not significantly impact on proline on any plant species irrespective of water treatment. However, endophytes tended to decrease proline in grasses other than tall fescue and the non-grass plant species (Fig. 6e).

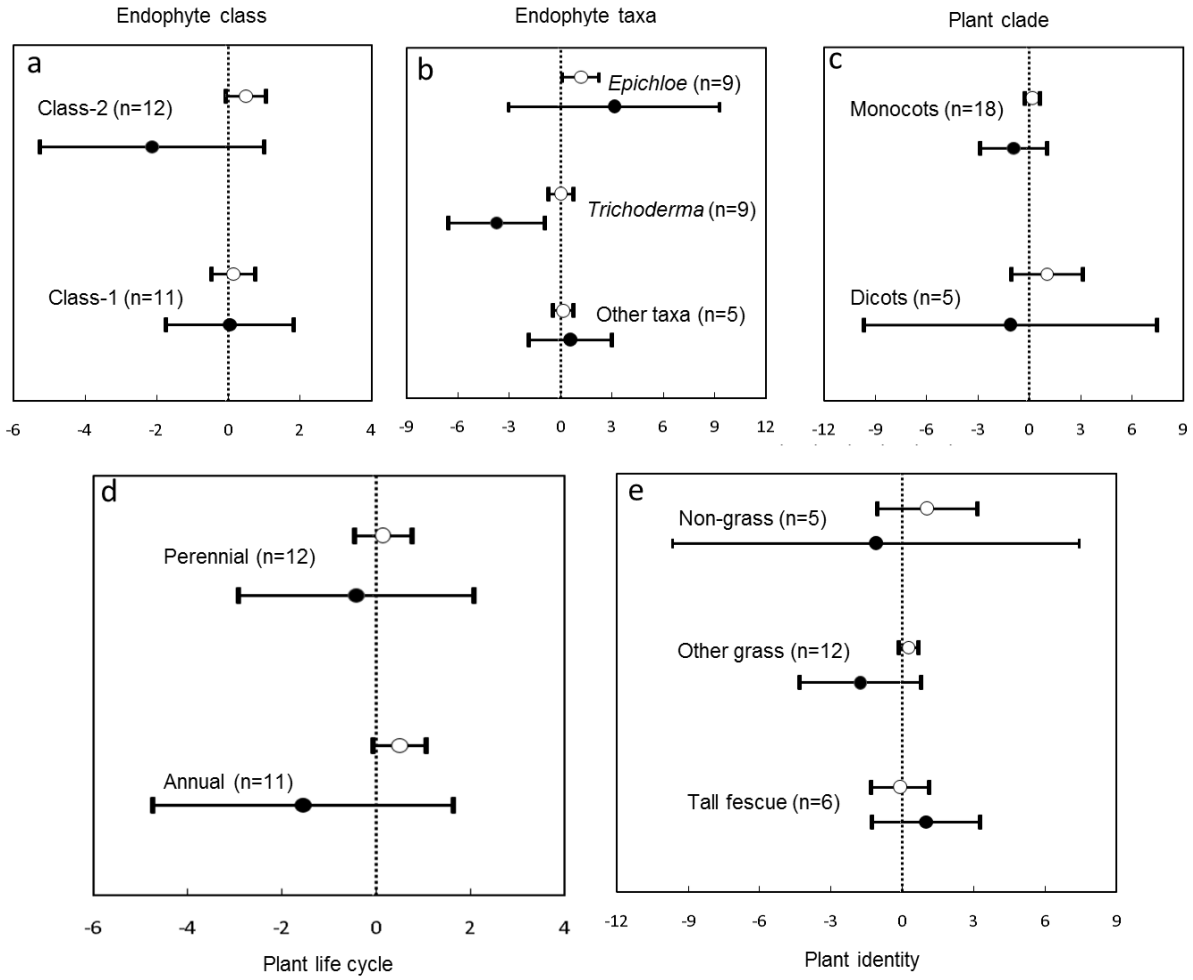

Fig. 6 Effect of endophytes on plant metabolites under water stress (filled circle) and under non-stressed (open circle) condition. Error bars are means±95 % bootstrap CIs. Where the CIs do not overlap the vertical dashed lines, the effect size for a parameter is significant. n=number of studies included in the meta-analysis.

Fungal endophytes on plant enzyme activities and other parameters

Symbioses significantly stimulated APX (ascorbate peroxidase), GSH (*glutathione)*, phenolics, PPO (polyphenol oxidase) and SOD (superoxide dismutase) activity and decreased EC (Electrical Conductivity), H_2O_2 (hydrogen peroxide) and MDA (malondialdehyde) activity, but had neutral effect

on CAT (Catalase) and POD (peroxidase) under water limiting conditions (Fig. 7). The largest positive impact was on APX activity with an effect size of 5.0105 (Fig. 7). Interestingly, MDA activity was found to be significantly increased under non-stressed condition although its activity was decreased under stress in the endophyte colonized plants compared to non-endophytic plants. No other enzymatic activities were influenced by endophyte colonization when the plants were growing under adequate watering environments except POD activity (Fig. 7).

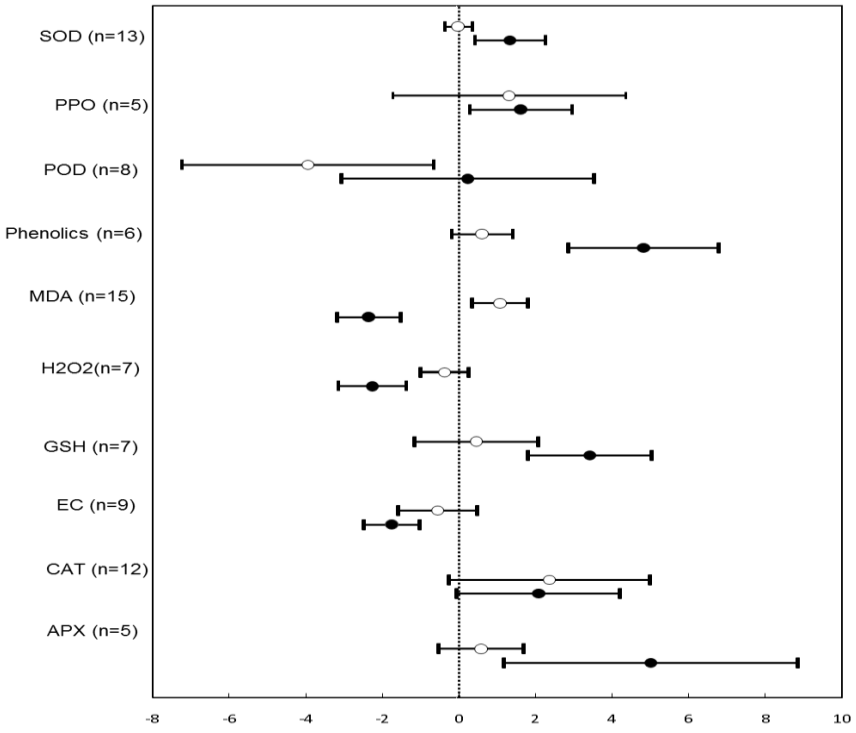

Fig. 7 Effect of endophytes on plant enzymatic activity under water stress (filled circle) and under non-stressed (open circle) conditions. Error bars are means ±95 % bootstrap CIs. Where the CIs do not overlap the vertical dashed lines, the effect size for a parameter is significant. n=number of studies included in the meta-analysis. APX= Ascorbate peroxidase, CAT= Catalase, EC= Electrical conductivity, GSH= Glutathione, H_2O_2= Hydrogen peroxide, MDA=Malondialdehyde, POD= Peroxidase, PPO= Polyphenol oxidase, SOD=Superoxide Dismutase.

Discussion

Meta-analysis combined data from independent studies to estimate the degree of effects across similar studies and locate common factors contributing variations among them (Gurevitch and Hedges 1999). This study indicated fungal endophyte inoculation has a significant impact on various plant physiological variables relating to plant growth, plant water relations, metabolite accumulation, and enzyme activities under water stress.

Endophyte inoculation significantly increased plant height, shoots biomass, tiller density and total biomass under water stress. However, influences under stress were context dependent with several factors playing important roles. For example, class-2 endophytes increased plant shoot, biomass, root biomass and total biomass more so than class-1 endophytes under stress. The effects of *Trichoderma* on total plant biomass and that of *Penicillium* on shoot biomass were more pronounced under stress than any other fungal taxa studied. Endophyte colonization, in general, had a substantially positive impact on the shoot and root biomass in monocotyledonous and dicotyledonous plants, respectively whereas their effect on total biomass was significant in both plant clades under stress. The stage of the plant life cycle did not seem to play a major role in plant-fungal associations under stressed or non-stressed conditions. Plant identity was crucial in determining the plant-endophytes interactions under both well-watered and stresses conditions. For instance, in non-grass plant species, total biomass increased much more than in the grass species in the endophyte colonized stressed plants. Conversely, regarding shoot biomass grass species were more influenced by endophyte symbiosis under stress and no significant difference on root biomass among plant species was observed.

In most cases effect of endophytes on plant growth parameters were more visible under stress than under well-watered conditions. It indicated that plant growth response to endophyte colonization is an outcome of moisture availability. The beneficial effect of fungal symbiosis is increased under extreme environments (Redman et al. 2002; Bunn et al. 2009). A meta-analysis with AM fungi and leaf endophytes and plant growth parameters reported similar results where effects of fungi increased as moisture stress increased [32]. Therefore, the mode of the plant-microbe interaction with regard to mutualism, commensalism, amensalism and parasitism may be dependent on moisture availability along with various factors such as nutritional status, plant/fungal partners, temperature, etc.[32,37]. Increased nutrient availability and uptake efficiency are the proposed mechanisms involved in endophyte-induced plant growth promotion. Fungus mediated plant growth enhancements under stress could also be attributable to the production of auxins by fungal endophytes[38]. Endophyte colonization under stress involves the expression of endogenous abscisic acid and the related genes zeaxanthin epoxidase, 9-cis-epoxycarotenoid dioxygenase-3, and ABA aldehyde oxidase-3. Expression of these compounds promotes plant growth and yield through the production of exogenous GA_3[13].

Plant photosynthetic parameters were also influenced by fungal colonization, which was greater under conditions of stress. Improvement in the photosynthetic ability of endophytic plants is reflected directly by the higher plant biomass of inoculated plants. Water stress exerts an adverse impact on photosynthetic machinery and gaseous exchange of plants leading to altered physiological and biochemical processes. Early response to water stress could result in accelerated stomatal closure and reduced water loss[39] in plants colonized by endophytic fungi. Also, increased chlorophyll pigments

and stomatal conductance in endophytic plants under stress reflects their better photosynthesis which co-relate with their stress tolerance[12,40,41].

Endophyte symbioses stimulated APX, GSH, phenolics, PPO, and SOD activity and decreased EC, H_2O_2, and MDA under water stress. Plants produce additional ROS when exposed to stresses. Water stress causes enhanced H_2O_2 accumulation and generation of ROS (reactive oxygen species) leading to disruption of cell membranes and metabolic toxicity which resulted in leakage of solutes[42-44]. Several pathways are involved in plants transforming toxic ROS to a less toxic form[45]. Alleviating ROS has been described as an important mechanism in fungus mediated stress tolerance[46]. Lower H_2O_2 in endophytic plants under stress could be a protection mechanism from the effect of stress by lowering ROS production. Moreover, a link may exist between the ability of osmotic balance and the degree of membrane protection from the influence of dehydration[47]. The lower H_2O_2 content was also found to be associated with over-expression of glutathione S-transferase, glutathione-dependent formaldehyde dehydrogenase genes in the stress associated pathways[48]. MDA accumulation is indicative of the rate of lipid peroxidation and oxidative stress in plants due to water stress[49]. The overall level of MDA was lower in fungus-colonized plants, and thus the fungus influences this stress response. MDA formation is associated with ROS-induced degradation of polyunsaturated lipids[50,51]. Endophytes could inhibit or impede the deterioration of these lipids by preventing excess ROS formation under stress conditions.

Antioxidant enzymes (POD, SOD, PPO) increased in endophytic plants under stress. Triggering the antioxidant system is an adaptive mechanism to reduce ROS generation and minimize oxidative damage under stress. Our analysis revealed a significantly higher level of ROS scavenging enzymes such as SOD, CAT, APX and GR in endophytic plants under stress. These enzymes are the elements of the antioxidation system that helps plants manage the redox balance. Higher levels of enzymatic activities could be related to better antioxidant responses that protect plants from oxidative injury in endophytic plants[26,52-54]. POD belongs to a large group of enzymes that detoxify H_2O_2, organic hydroperoxide, and lipid peroxides to produce alcohols. These enzymes have a

heme cofactor in their active sites that is manufactured in the plastid. Heme is also attached to the iron homeostasis, which may be involved in plant-microbe interactions[55]. Moreover, the presence of redox-active cysteine residues in POD help measure the redox potential of the cell or organelle. Th plastid is an essential organelle in a leaf that modulates the redox potential[56,57]. It is, however, not known if fungi interfere with the iron homeostasis and redox potential of the plant cell and increase plant stress tolerance *via* this mechanism.

Higher proline levels existed in both stressed and non-stressed endophytic plants. Proline is considered to be a stress-related amino acid which may act as an osmoregulator[58] as well as an ROS

scavenger[59], and its accumulation is correlated with both osmotic stress tolerance and responses to stress conditions involving dehydration[60-62]. However, it is still controversial if its presence is an adaptive response that provides greater stress tolerance or if its increase is a symptom of stress injury[63]. Relatively higher levels of proline in the presence of endophytes could, therefore, be an indication that there is less damage in a drought-stressed plant in the presence of endophytes.

Water deficit inhibits plant growth by affecting photosynthesis, osmotic balance, enzymatic activities and metabolic activities. Fungal endophytes consistently help plants reduce the impact of water stress by manipulating physiological processes. Endophyte-induced plant water stress tolerance has broad ecological and agricultural implications. In dryer regions, increased water tolerance can result in higher crop yield under low moisture availability. Continued interest in fungal endophyte research to uncover the underlying mechanisms of plant-fungal interaction appears well-justified.

Materials and methods

Database search

We followed the general guidelines of Field and Gillett[64] for obtaining meta-data. We did a literature search in ISI Web of Science (Thompson Reuters) through March 2017. The search combinations entered were endophyte* inoculation and water stress/drought, fungi* inoculation and water stress/drought. The Boolean truncation ('*') character used to ensure that the variations of the word such as endophyte, endophytic or fungi, fungus and fungal were also included. We did subsequent searches of relevant papers listed in reference sections. We also collected relevant publications from a review by Worcel et al..[32].

Study selection

The data search procedure yielded 314 online references, of which 160 were considered likely to contain significant relevant information. To minimize bias in the selection of publications, we screened the papers based on predefined criteria:

(i) The experiment had to manipulate at least one fungal endophyte irrespective of inoculation method or colonization rate,
(ii) The fungal symbionts were from any class of endophytes other than those considered as mycorrhizal fungi,
(iii) Both endophyte-inoculated and non-inoculated plants were grown under water stressed and non-stressed conditions. Where more than one level of stress was applied, we considered the results of only the most severe level of stress in our analysis,
(iv) Any of the physiological parameters e. g biomass, RWC, metabolites, etc. were measured

(v) The findings reported sample size, means, standard deviations/errors and other relevant statistical information such that the outcome could be converted to a standardized measure of effect size.

Those studies that did not present information on any of our targeted response variables and those that provided unrelated data were excluded. If there was more than one measurement of any trait over time, we only recorded measurement from the final date. We permitted studies to differ in the levels of fertilizer applied, growth situations (greenhouse, growth chamber or field), duration of time before stress was applied, and growth media into our meta-analysis. Among the 160 references, 93 papers were rejected, and we identified only 67 articles that met these selection criteria (Supplementary Material 1). Papers spanned 25 years (1992-2017) and were in English and Chinese (1 article).

Data extraction

Treatment means, sample sizes (replications), standard deviation were collected for each study. When standard errors (SE) were reported, we obtained standard deviation following the equation: $SE=SD(n^{-1/2})$. The 95% CIs (confidence intervals) reported were converted to SD where necessary[65]. When results were presented in a graph, the image was digitized, and data was extracted using WebPlotDigitizer[66]. Multiple treatments or host/endophyte combinations from the same article were considered as independent studies and represented as a separate data unit in the analysis. We were aware that extracting multiple studies from one experiment increases the dependence on that study by assuming, perhaps incorrectly, that studies are independent[67]. However, we included them because they increased the statistical power of the analysis[68]. This approach has been used in various biological meta-analyses[69-73].

Meta-analysis

To quantify the magnitude and direction of plant-fungal interactions under stress and non-stressed conditions, we used all comparisons in our dataset to calculate the mean effect sizes. We used MetaEasy software v1.0.2[74] to compute effect sizes and standard error (SE) from each study. From sample size information and various combinations of summary statistics, for instance, the mean and the related p-values, we calculated the standardized cumulative effect size [74]. We estimated effect sizes with 95% CIs using the Dersimonian-Laird (DL) random effects model because it takes into account heterogeneity (study variation)[75]. A cumulative effect size was considered significant when 95% CIs did not bracket zero. The level of heterogeneity was assessed using Cochran's Q statistic, where Q follows a χ^2_{k-1} distribution with k = number of studies[76]. Q-statistic is a measure of weighted squared deviations which display only presence versus absence of heterogeneity. Therefore, we quantified heterogeneity with an I^2 statistic which estimates the ratio of true heterogeneity to total

heterogeneity across the observed effect sizes[77,78] (Table 1). By convention, an I^2 value >75 % indicates significant between-study heterogeneity[79].

Categorical analyses were also performed on the data to determine the influence of the factors such as plant or endophyte identity, type, etc. for some parameters where sufficient data was available. For a factor to be included in the analysis as a moderator or categorical variable, it had to be reported from at least five studies.

Table 1: Heterogeneity statistics for the 32 summary effect sizes under non-stressed and water stress condition *Q* , total heterogeneity; *p*, significance of Q heterogeneity ; I^2: percentage of heterogeneity due to true variation among effect sizes.

Parameters	**P**arameters	Under stress			Under non-stressed		
		Q	p	I^2	Q	p	I^2 (%)
Plant growth	Leaf Area	75.12	0.00	70.71	29.79	0.10	29.51
	Plant Height	188.90	0.00	83.59	216.76	0.00	86.62
	Root Biomas	414.12	0.00	90.58	105.28	0.00	71.50
	Root Shoot Ratio	45.79	0.00	65.06	44.81	0.03	35.28
	Seed Germiantion	22.76	0.00	73.64	0.00	1.00	0.00
	Shoot Biomas	765.49	0.00	91.12	265.47	0.00	78.15
	Spelicif Leaf Weight	11.78	0.04	57.56	3.93	0.41	0.00
	Tiller Density	195.07	0.00	70.78	215.51	0.00	76.34
	Total Biomas	256.37	0.00	78.94	336.68	0.00	89.31
Photosynthetic activity	Fv/Fm	22.08	0.18	22.99	3.18	1.00	0.00
	Net Photosyntheis	25.60	0.01	53.13	74.68	0.00	77.24
	Stomatal conductance	75.11	0.00	77.37	16.01	0.10	37.52
	Total Chlorophyll	65.48	0.00	57.24	102.61	0.00	82.46
Plant water relation	Osmotic Potential	43.62	0.00	79.37	14.31	0.05	51.10
	Relative Water Content	62.07	0.00	72.61	24.68	0.13	27.06
	Transpiration	19.62	0.00	74.52	5.46	0.36	8.47
	Water Potential	21.82	0.00	72.50	3.80	0.70	0.00
	Water Use Efficiency	16.54	0.00	75.82	1.00	0.80	0.00
Metabilites	Proline	975.17	0.00	97.74	31.88	0.02	46.68
	Glucose	38.57	0.00	89.63	97.43	0.00	96.92
	Sucrose	2.44	0.65	0.00	7.60	0.06	60.53
	Sugar	24.18	0.00	83.46	16.81	0.00	76.21
Enzymatic activity	ascorbate peroxidase (APX)	176.18	0.00	97.73	14.58	0.01	72.57
	Catalase (CAT)	308.05	0.00	96.75	547.05	0.00	97.99
	Electrical conductivity (EC)	15.03	0.06	46.76	30.21	0.00	73.52
	glutathione (GSH	61.96	0.00	90.32	61.21	0.00	90.20
	Hydrogen peroxide (H_2O_2)	16.27	0.01	63.11	8.28	0.22	27.50
	Malondialdehyde (MDA)	63.53	0.00	77.96	34.23	0.00	64.94

	Phenolics	49.36	0.00	89.87	8.03	0.15	37.70
	Peroxidase (POD)	420.52	0.00	98.34	418.46	0.00	98.33
	Polyphenol Oxidase (PPO)	14.16	0.00	78.81	74.05	0.00	95.95
	Superoxide Dismutase (SOD)	90.99	0.00	86.81	7.59	0.58	0.00

References

1 Knapp, A. K. & Smith, M. D. Variation among biomes in temporal dynamics of aboveground primary production. *Sci* **291**, 481-484, doi:10.1126/science.291.5503.481 (2001).

2 Easterling, D. R. *et al.* Climate extremes: observations, modeling, and impacts. *Sci* **289**, 2068–2074 (2000).

3 Sheffield, J. & Wood, E. F. Characteristics of global and regional drought, 1950–2000: Analysis of soil moisture data from off-line simulation of the terrestrial hydrologic cycle. *J Geophys Res* **112**, 1984–2012 (2007).

4 Hura, T., Hura, K., Grzesiak, M. & Rzepka, A. Effect of long-term drought stress on leaf gas exchange and fluorescence parameters in C3 and C4 plants. *Acta Physiol Plant* **29**, 103-113 (2007).

5 Jones, H. G. Stomatal control of photosynthesis and transpiration. *J Exp Bot* **49**, 387–398 (1998).

6 Medrano, H., Escalona, J. M., Bota, J., Gulías, J. & Flexas, J. Regulation of photosynthesis of C3 plants in response to progressive drought: stomatal conductance as a reference parameter. *Ann Bot* **89**, 895–905 (2002).

7 Socias, X., Correia, M., Chaves, M. & Medrano, H. The role of abscisic acid and water relations in drought responses of subterranean clover. *J Exp Bot* **48**, 1281–1288 (1997).

8 Chaves, M., Flexas, J. & Pinheiro, C. Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. *Ann Bot* **103**, 551-560 (2009).

9 Pinheiro, C. & Chaves, M. Photosynthesis and drought: can we make metabolic connections from available data. *J Exp Bot* **103**, 869–882 (2011).

10 Chaves, M. M., Maroco, J. P. & Pereira, J. S. Understanding plant responses to drought-from genes to the whole plant. *Funct Plant Biol* **30**, 239-264, doi:10.1071/FP02076 (2003).

11 Azad, K. & Kaminskyj, S. A fungal endophyte strategy for mitigating the effect of salt and drought stress on plant growth. *Symbios* **68**, 73-78, doi:10.1007/s13199-015-0370-y (2016).

12 Bae, H. *et al.* The beneficial endophyte *Trichoderma hamatum* isolate DIS 219b promotes growth and delays the onset of the drought response in Theobroma cacao. *J Exp Bot* **60**, 3279-3295, doi:10.1093/jxb/erp165 (2009).

13 Khan, A. L., Shin, J. H., Jung, H. Y. & Lee, I. J. Regulations of capsaicin synthesis in *Capsicum annuum* L. by *Penicillium resedanum* LK6 during drought conditions. *Sci. Hortic.* **175**, 167-173, doi:10.1016/j.scienta.2014.06.008 (2014).

14 Redman, R. S. *et al.* Increased fitness of rice plants to abiotic stress via habitat adapted symbiosis: a strategy for mitigating impacts of climate change. *PLoS ONE* **6**, e14823, doi:https://doi.org/10.1371/journal.pone.0014823 (2011).

15 Sun, C. A. *et al.* Piriformospora indica confers drought tolerance in Chinese cabbage leaves by stimulating antioxidant enzymes, the expression of drought-related genes and the plastid-localized CAS protein. *J Plant Physiol* **167**, 1009-1017, doi:10.1016/j.jplph.2010.02.013 (2010).

16 Bayat, F., Mirlohi, A. & Khodambashi, M. Effects of endophytic fungi on some drought tolerance mechanisms of tall fescue in a hydroponics culture. *Russ J Plant Physiol* **56**, 510-516, doi:10.1134/s1021443709040104 (2009).

17 Cong, G. Q., Yin, C. L., He, B. I., Li, L. & Gao, K. X. Effect of the endophytic fungus *Chaetomium globosum* ND35 on the growth and resistance to drought of winter wheat at the seedling stage under water stress. *Shengtai Xuebao/ Acta Ecologica Sinica* **35**, 6120-6128, doi:10.5846/stxb201401200153 (2015).

18 Elmi, A. A. & West, C. P. Endophyte infection effects on stomatal conductance, osmotic adjustment and drought recovery of Tall fescue. *New Phytol.* **131**, 61-67, doi:10.1111/j.1469-8137.1995.tb03055.x (1995).

19 Khan, A. L. *et al.* Co-synergism of endophyte *Penicillium resedanum* LK6 with salicylic acid helped *Capsicum annuum* in biomass recovery and osmotic stress mitigation. *BMC Microbiol.* **13**, 13, doi:10.1186/1471-2180-13-51 (2013).

20 Nagabhyru, P., Dinkins, R. D., Wood, C. L., Bacon, C. W. & Schardl, C. L. Tall fescue endophyte effects on tolerance to water-deficit stress. *BMC Plant Biol.* **13**, 17, doi:10.1186/1471-2229-13-127 (2013).

21 Morse, L. J., Day, T. A. & Faeth, S. H. Effect of Neotyphodium endophyte infection on growth and leaf gas exchange of Arizona fescue under contrasting water availability regimes. *Environ Exp Bot* **48**, 257-268, doi:10.1016/s0098-8472(02)00042-4 (2002).

22 Sherameti, I., Tripathi, S., Varma, A. & Oelmuller, R. The root-colonizing endophyte Pirifomospora indica confers drought tolerance in *Arabidopsis* by stimulating the expression of drought stress-related genes in leaves. *Mol. Plant-Microbe Interact.* **21**, 799-807, doi:10.1094/mpmi-21-6-0799 (2008).

23 Baltruschat, H. *et al.* Salt tolerance of barley induced by the root endophyte *Piriformospora indica* is associated with a strong increase in antioxidants. *The New phytologist* **180**, 501–510, doi:10.1111/j.1469-8137.2008.02583.x. (2008).

24 Vadassery, J. *et al.* A cell wall extract from the endophytic fungus *Piriformospora indica* promotes growth of Arabidopsis seedlings and induces intracellular calcium elevation in roots. *Plant J* **59**, 193–206 (2009).

25 Ghabooli, M. *et al.* Proteomics study reveals the molecular mechanisms underlying water stress tolerance induced by Piriformospora indica in barley. *J Proteomics* **94**, 289-301, doi:10.1016/j.jprot.2013.09.017 (2013).

26 Guler, N. S., Pehlivan, N., Karaoglu, S. A., Guzel, S. & Bozdeveci, A. *Trichoderma atroviride* ID20G inoculation ameliorates drought stress-induced damages by improving antioxidant defence in maize seedlings. *Acta Physiol Plant* **38**, doi:10.1007/s11738-016-2153-3 (2016).

27 Ren, A. *et al.* Benefits of a fungal endophyte in *Leymus chinensis* depend more on water than on nutrient availability. *Environ Exp Bot* **108**, 71-78, doi:10.1016/j.envexpbot.2013.11.019 (2014).

28 Vazquez-de-Aldana, B. R., Garcia-Ciudad, A., Garcia-Criado, B., Vicente-Tavera, S. & Zabalgogeazcoa, I. Fungal endophyte (*Epichloe festucae*) alters the nutrient content of Festuca rubra regardless of water availability. *PLoS ONE* **8**, 14, doi:10.1371/journal.pone.0084539 (2013).

29 Odokonyero, K., Acuna, T. B., Cardoso, J. A., Jimenez, J. D. & Rao, I. M. Fungal endophyte association with *Brachiaria* grasses and its influence on plant water status, total non-structural carbohydrates and biomass production under drought stress. *Plant Soil* **409**, 273-282, doi:10.1007/s11104-016-2947-5 (2016).

30 Ren, A. Z. & Clayy, K. Impact of a horizontally transmitted endophyte, *Balansia henningsiana*, on growth and drought tolerance of *Panicum rigidulum*. *Int J Plant Sci* **170**, 599-608, doi:10.1086/597786 (2009).
31 Rodriguez, R. J. *et al.* Stress tolerance in plants via habitat-adapted symbiosis. *ISME J.* **2**, 404-416, doi:10.1038/ismej.2007.106 (2008).
32 Worchel, E. R., Giauque, H. E. & Kivlin, S. N. Fungal symbionts alter plant drought response. *Microb Ecol* **65**, 671-678 (2013).
33 Metawin: statistical software for meta-analysis. version 2 (Sinauer Associates, Sunderland, Massachusetts, USA, 2000).
34 Borenstein, M., Hedges, L. V., Higgins, J. P. T. & Rothstein, H. R. *Introduction to meta-analysis*. (Chichester, England: Wiley, 2009).
35 Newsham, K. K. A meta-analysis of plant responses to dark septate root endophytes. *New Phytol.* **190**, 783–793 (2011).
36 Leuchtmann, A., Bacon, C. W., Schardl, C. L., White, J. F. & Tadych, M. Nomenclatural realignment of *Neotyphodium* species with genus *Epichloë*. *Mycol* **106**, 202–215, doi:10.3852/13-251. ISSN 0027-5514 (2014).
37 Johnson, N. C., Graham, J. H. & Smith, F. A. Functioning of mycorrhizal associations along the mutualism–parasitism continuum. *New Phytol.* **135**, 575–585 (1997).
38 De Battista, J. P., Bacon, C. W., Severson, R., Plattner, R. D. & Bouton, J. H. Indole acetic acid production by the fungal endophyte of tall fescue. *Agron J* **82**, 878–880 (1990).
39 Malinowski, D. P. & Belesky, D. P. Adaptation of endophyte-infected cool-season grasses to environmental stresses: mechanisms of drought and mineral stress tolerance. *Crop Sci* **40** (2000).
40 Harman, G. E. Myths and dogmas of biocontrol. Changes in perceptions derived from research on *Trichoderma harzianum* T-22. *Plant Dis* **84**, 377-393 (2000).
41 Harman, G. E., Howell, C. R., Viterbo, A., Chet, I. & Lorito, M. *Trichoderma* species – opportunistic, avirulent plant symbionts. *Nature Reviews Microbiology* **2**, 43-56 (2004).
42 Deshmukh, P. S., Sairam, R. K. & Shukla, D. S. Measurement of ion leakage as a screening technique for drought resistance in wheat genotypes. *Indian Journal of Plant Physiology* **35** 89e91 (1991).
43 Dionisio-Sese, M. L. & Tobita, S. Antioxidant responses of rice seedlings to salinity stress. *Plant Sci.* **135** 1e9. (1998).
44 Premchandra, G. S., Saneoka, H. & Ogata, S. Cell membrane stability an indicator of drought tolerance as affected by applied nitrogen in soybean. *J. AGRIC. SCI.* **115** 1332e1343 (1990).
45 Mittler, R. Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci* **7**, 405–410 (2002).
46 Chepsergon, J., Mwanburi, L. & Kassim, M. K. Mechanism of drought tolerance in plants using *Trichoderma* spp. . *Int J Sci Res* **3**, 1592–1595 (2014).
47 Lilley, J. M., Ludlow, M. M., McCouch, S. R. & O'Toole, J. C. Locating QTL for osmotic adjustment and dehydration tolerance in rice. *J Exp Bot* **47**, 1427–1436 (1996).
48 Dixon, D. P., Lapthorn, A. & Edwards, R. Plant glutathione transferases. *Genome Biol* **3** reviews3004 (2002).
49 Kaiser, W. M., Kaiser, G., Schöner, S. & Neimanis, S. Photosynthesis under osmotic stress. Differential recovery of photosynthetic activities of stroma enzymes, intact chloroplasts and leaf slices after exposure to high solute concentrations. *Planta* **153**, 430e435. (1981).
50 Del Rio, D., Stewart, A. J. & Pellegrini, N. A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. *Nutr Metabol Cardiovasc Dis* **15**, 316–328 (2005).

51 Pryor, W. A. & Stanley, J. P. A suggested mechanism for the production of malonaldehyde during the autoxidation of polyunsaturated fatty acids. Nonenzymatic production of prostaglandin endoperoxides during autoxidation. *J Org Chem* **40**, 3615–3617 (1975).
52 Harman, G. E. Overview of mechanisms and uses of *Trichoderma* spp. *Phytopathology* **96**, 190–194 (2006).
53 Khan, M. H. & Panda, S. K. Alterations in root lipid peroxidation and antioxidative responses in two rice cultivars under NaCl salinity stress. *Acta Physiol Plant* **30**, 81e89 (2008).
54 Mastouri, F., Bjorkman, T. & Harman, G. E. *Trichoderma harzianum* enhances antioxidant defense of tomato seedlings and resistance to water deficit. *Mol Plant-Microb Inter* **25**, 1264–1271 (2012).
55 Briat, J. F., Curie, C. & Gaymard, F. Iron utilization and metabolism in plants. *Curr Opin Plant Biol* **10**, 276–282 (2007).
56 Brautigam, K. *et al.* Dynamic plastid redox signals integrate gene expression and metabolism to induce distinct metabolic states in photosynthetic acclimation in *Arabidopsis*. *The Plant cell* **21**, 2715–2732. (2009).
57 Muhlenbock, P. *et al.* Chloroplast signaling and LESION SIMULATING DISEASE1 regulate crosstalk between light acclimation and immunity in Arabidopsis. *The Plant cell* **20**, 2339–2356. (2008).
58 Yoshiba, Y., Kiyosue, T., Nakashima, K., Yamaguchi-Shinozaki, K. & Shinozaki, K. Regulation of levels of proline as an osmolyte in plants under water stress. *Plant Cell Physiol* **38**, 1095-1102 (1997).
59 Dickman, M. B. & Chen, C. B. Proline suppresses apoptosis in the fungal pathogen *Colletotrichum trifolii*. *PNAS* **102**, 3459-3464 (2005).
60 Aspinall, D. & Paleg, L. G. in *The Physiology and Biochemistry of Drought Resistance in Plants* (eds L. G. Paleg & D. Aspinall) 205–241 (Academic Press, 1981).
61 Gzik, A. Accumulation of proline and pattern of a-amino acids in sugar beet plants in response to osmotic, water and salt stress. *Environ Exp Bot* **36**, 29-38 (1996).
62 Verbruggen, N. & Hermans, C. Proline accumulation in plants, a review. *Amino Acids* **35**, 753-759 (2008).
63 Ashraf, M. & Foolad, M. A. Improving plant abiotic-stress resistance by exogenous application of osmoprotectants glycine betaine and proline. *Environ Exp Bot* **59**, 206-216 (2007).
64 Field, A. P. & Gillett, R. How to do a meta-analysis. *Brit J Math Stat Psy* **63**, 665-694 (2010).
65 Vohnı´k, M., Albrechtova´, J. & Vosa´tka, M. The inoculation with Oidiodendron maius and Phialocephala fortinii alters phosphorus andnitrogen uptake, foliar C:N ratio and root biomass distribution in Rhododendron cv. Azurro. *Symbios* **40**, 87–96 (2005).
66 WebPlotDigitizer Version: 3.11 (2011).
67 Gurevitch, J. & Hedges, L. Statistical issues in ecological metaanalyses. *Ecol* **80**, 1142–1149 (1999).
68 Lajeunesse, M. J. & Forbes, M. R. Variable reporting and quantitative reviews: acomparison of three meta-analytical techniques. *Ecol. Lett.* **6**, 448-454, doi:10.1046/j.1461-0248.2003.00448.x (2003).
69 Hoeksema, J. D. *et al.* A meta-analysis of context-dependency in plant response to inoculation with mycorrhizal fungi. *Ecol. Lett.* **13**, 394-407 (2010).
70 Holmgren, M., Gómez-Aparicio, L. Q., J. L. & Valladares, F. Non-linear effects of drought under shade-reconciling physiological and ecological models in plant communities. *Oecologia* **169**, 293-305, doi:10.1007/s00442- 011-2196-5 (2012).

71 Mayerhofer, M. S. K., G. & Harper, K. A. The effects of fungal root endophytes on plant growth. *Mycorrhiza* **23**, 199-128, doi:10.1007/s00572- 012-0456-9 (2013).
72 McGrath, J. M. & Lobell, D. B. Reduction of transpiration and altered nutrient allocation contribute to nutrient decline of crops grown in elevated CO2 concentrations. *Plant Cell Environ* **36**, 697-705, doi:10.1111/pce.12007 (2013).
73 Veresoglou, S. D., Menexes, G. & Rillig, M. C. Do arbuscular mycor- rhizal fungi affect the allometric partition of host plant biomass to shoots and roots? A meta-analysis of studies from1990 to 2010. *Mycorrhiza* **22**, 227-235, doi:10.1007/s00572-011-0398-7 (2012).
74 Kontopantelis, E. & Reeves, D. MetaEasy: a meta-analysis add-in for microsoft excel. *J Stat Soft* **30**, 1-25 (2009).
75 DerSimonian, R. & Laird, N. Meta-analysis in clinical trials. *Control Clin Trials* **7**, 177-188 (1986).
76 Brockwell, S. E. & Gordon, I. R. A comparison of statistical methods for meta-analysis. *Stat Med* **20**, 825-840 (2001).
77 Higgins, J. P. T. & Thompson, S. G. Quantifying heterogeneity in a meta- analysis. *Stat Med* **21**, 1539–1558, doi:10.1002/sim.1186 (2002).
78 Huedo-Medina, T. B. S.-M., J. , Marín-Martínez, F. & Botella, J. Assessing heterogeneity in meta-analysis: Q statistic or I2 index? *Psychol Methods* **11**, 193-206, doi:10.1037/1082-989X.11.2.193 (2006).
79 Higgins, J. P., Thompson, S. G., Deeks, J. J. & Altman, D. G. Measuring inconsistency in meta-analyses. *Brit Med J* **327**, 557-560 (2003).

Contributions

Developing concept, database search, data analysis and interpretation and drafting the article: K. M. G. D; Critical revision of the article: M. G. K. and S. J.W. All authors approved the final version of the manuscript.

Competing Interests

The authors declare that they have no competing interests.

Supplementary file S1: Publications used for meta-analysis in the present study [1-67]

1 Amalric, C., Sallanon, H., Monnet, F., Hitmi, A. & Coudret, A. Gas exchange and chlorophyll fluorescence in symbiotic and non-symbiotic ryegrass under water stress. *Photosynthetica* **37**, 107-112, doi:10.1023/a:1007027131613 (1999).
2 Assuero, S. G. *et al.* Endophyte infection accelerates morpho-physiological responses to water deficit in tall fescue. *New Zealand J Agric Res* **49**, 359-370 (2006).
3 Azad, K. & Kaminskyj, S. A fungal endophyte strategy for mitigating the effect of salt and drought stress on plant growth. *Symbios* **68**, 73-78, doi:10.1007/s13199-015-0370-y (2016).
4 Bae, H. *et al.* The beneficial endophyte Trichoderma hamatum isolate DIS 219b promotes growth and delays the onset of the drought response in Theobroma cacao. *J Exp Bot* **60**, 3279-3295, doi:10.1093/jxb/erp165 (2009).

5 Barker, D. J., Hume, D. E. & Quigley, P. E. *Negligible physiological responses to water deficit in endophyte-infected and uninfected perennial ryegrass*. (Plenum Press Div Plenum Publishing Corp, 1997).

6 Bayat, F., Mirlohi, A. & Khodambashi, M. Effects of endophytic fungi on some drought tolerance mechanisms of tall fescue in a hydroponics culture. *Russ J Plant Physiol* **56**, 510-516, doi:10.1134/s1021443709040104 (2009).

7 Briggs, L., Crush, J., Ouyang, L. & Sprosen, J. Neotyphodium endophyte strain and superoxide dismutase activity in perennial ryegrass plants under water deficit. *Acta Physiol Plant* **35**, 1513-1520, doi:10.1007/s11738-012-1192-7 (2013).

8 Buck, G. W., West, C. P. & Elbersen, H. W. *Endophyte effect on drought tolerance in diverse Festuca species*. (Plenum Press Div Plenum Publishing Corp, 1997).

9 Cheplick, G. P. Recovery from drought stress in Lolium perenne (Poaceae): Are fungal endophytes detrimental. *Am. J. Bot.* **91**, 1960-1968, doi:10.3732/ajb.91.12.1960 (2004).

10 Cheplick, G. P., Perera, A. & Koulouris, K. Effect of drought on the growth of Lolium perenne genotypes with and without fungal endophytes. *Funct Ecol* **14**, 657-667, doi:10.1046/j.1365-2435.2000.00466.x (2000).

11 Cong, G. Q., Yin, C. L., He, B. I., Li, L. & Gao, K. X. Effect of the endophytic fungus *Chaetomium globosum* ND35 on the growth and resistance to drought of winter wheat at the seedling stage under water stress. *Shengtai Xuebao/ Acta Ecologica Sinica* **35**, 6120-6128, doi:10.5846/stxb201401200153 (2015).

12 Donoso, E. P., Bustamante, R. O., Caru, M. & Niemeyer, H. M. Water deficit as a driver of the mutualistic relationship between the fungus *Trichoderma harzianum* and two wheat genotypes. *Appl. Environ. Microbiol.* **74**, 1412-1417, doi:10.1128/aem.02013-07 (2008).

13 Elbersen, H. W. & West, C. P. Growth and water relations of field-grown tall fescue as influenced by drought and endophyte. *Grass Forage Sc* **51**, 333-342, doi:10.1111/j.1365-2494.1996.tb02068.x (1996).

14 Elbersen, H. W. & West, C. P. *Endophyte effects on growth and water relations of tall fescue*. (Plenum Press Div Plenum Publishing Corp, 1997).

15 Elmi, A. A. & West, C. P. Endophyte infection effects on stomatal conductance, osmotic adjustment and drought recovery of Tall fescue. *New Phytol.* **131**, 61-67, doi:10.1111/j.1469-8137.1995.tb03055.x (1995).

16 Ghabooli, M. *et al.* Proteomics study reveals the molecular mechanisms underlying water stress tolerance induced by Piriformospora indica in barley. *J Proteomics* **94**, 289-301, doi:10.1016/j.jprot.2013.09.017 (2013).

17 Gibert, A., Volaire, F., Barre, P. & Hazard, L. A fungal endophyte reinforces population adaptive differentiation in its host grass species. *New Phytol.* **194**, 561-571, doi:10.1111/j.1469-8137.2012.04073.x (2012).

18 Guler, N. S., Pehlivan, N., Karaoglu, S. A., Guzel, S. & Bozdeveci, A. Trichoderma atroviride ID20G inoculation ameliorates drought stress-induced damages by improving antioxidant defence in maize seedlings. *Acta Physiol Plant* **38**, doi:10.1007/s11738-016-2153-3 (2016).

19 Hahn, H. *et al.* Neotyphodium fungal endophytes confer physiological protection to perennial ryegrass (*Lolium perenne* L.) subjected to a water deficit. *Environ Exp Bot* **63**, 183-199, doi:10.1016/j.envexpbot.2007.10.021 (2008).

20 Hall, S. L., McCulley, R. L., Barney, R. J. & Phillips, T. D. Does Fungal Endophyte Infection Improve Tall Fescue's Growth Response to Fire and Water Limitation? *PLoS ONE* **9**, 9, doi:10.1371/journal.pone.0086904 (2014).

21 Hamilton, C. E. & Bauerle, T. L. A new currency for mutualism? Fungal endophytes alter antioxidant activity in hosts responding to drought. *Fungal Div* **54**, 39-49, doi:10.1007/s13225-012-0156-y (2012).

22 He L, M. C., Jones S S, and Hatier J H B. Productivity in simulated drought and post-drought recovery of eight ryegrass cultivars and a tall fescue cultivar with and without Epichloë endophyte. *Crop Pasture Sci*, doi:http://dx.doi.org/10.1071/CP16208 (2017).

23 Hesse, U. *et al.* Effects of Neotyphodium endophytes on growth, reproduction and drought-stress tolerance of three *Lolium perenne* L. genotypes. *Grass Forage Sc* **58**, 407-415, doi:10.1111/j.1365-2494.2003.00393.x (2003).

24 Hesse, U. *et al.* Influence of water supply and endophyte infection (*Neotyphodium* spp.) on vegetative and reproductive growth of two Lolium perenne L. genotypes. *Eur. J. Agron.* **22**, 45-54, doi:10.1016/j.eja.2003.12.002 (2005).

25 Hill, N. S., Pachon, J. G. & Bacon, C. W. *Acremonium coenophialum*-mediated short- and long-term drought acclimation in tall fescue. *Crop Sci* **36**, 665-672 (1996).

26 Hubbard M, G. J. a. V. V. Fungal endophytes enhance wheat heat and drought tolerance in terms of grain yield and second-generation seed viability. *J. Appl. Microbiol.* **116**, 09--122, doi:doi:10.1111/jam.12311 (2013).

27 Jia, T., Shymanovich, T., Gao, Y. B. & Faeth, S. H. Plant population and genotype effects override the effects of Epichloe endophyte species on growth and drought stress response of *Achnatherum robustum* plants in two natural grass populations. *J. Plant Ecol.* **8**, 633-641, doi:10.1093/jpe/rtv004 (2015).

28 Kane, K. H. Effects of endophyte infection on drought stress tolerance of Lolium perenne accessions from the Mediterranean region. *Environ Exp Bot* **71**, 337-344, doi:10.1016/j.envexpbot.2011.01.002 (2011).

29 Kannadan, S. & Rudgers, J. A. Endophyte symbiosis benefits a rare grass under low water availability. *Funct Ecol* **22**, 706-713, doi:10.1111/j.1365-2435.2008.01395.x (2008).

30 Khan, A. L., Shin, J. H., Jung, H. Y. & Lee, I. J. Regulations of capsaicin synthesis in *Capsicum annuum* L. by *Penicillium resedanum* LK6 during drought conditions. *Sci. Hortic.* **175**, 167-173, doi:10.1016/j.scienta.2014.06.008 (2014).

31 Khan, A. L. *et al.* Co-synergism of endophyte *Penicillium resedanum* LK6 with salicylic acid helped *Capsicum annuum* in biomass recovery and osmotic stress mitigation. *BMC Microbiol.* **13**, 13, doi:10.1186/1471-2180-13-51 (2013).

32 Khan, A. L., Waqas, M. & Lee, I. J. Resilience of *Penicillium resedanum* LK6 and exogenous gibberellin in improving *Capsicum annuum* growth under abiotic stresses. *J. Plant Res.* **128**, 259-268, doi:10.1007/s10265-014-0688-1 (2015).

33 Malinowski, D., Leuchtmann, A., Schmidt, D. & Nosberger, J. Growth and water status in meadow fescue is affected by *Neotyphodium* and *Phialophora* species endophytes. *Agron J* **89**, 673-678 (1997).

34 Malinowski, D., Leuchtmann, A., Schmidt, D. & Nosberger, J. Symbiosis with *Neotyphodium uncinatum* endophyte may increase the competitive ability of meadow fescue. *Agron J* **89**, 833-839 (1997).

35 Malinowski, D. P., Zuo, H., Kramp, B. A., Muir, J. P. & Pinchak, W. E. Obligatory summer-dormant cool-season perennial grasses for semiarid environments of the southern Great Plains. *Agron J* **97**, 147-154 (2005).

36 Mastouri, F., Bjorkman, T. & Harman, G. E. *Trichoderma harzianum* Enhances Antioxidant Defense of Tomato Seedlings and Resistance to Water Deficit. *Mol. Plant-Microbe Interact.* **25**, 1264-1271, doi:10.1094/Mpmi-09-11-0240 (2012).

37 Miranda, M. I., Omacini, M. & Chaneton, E. J. Environmental context of endophyte symbioses: interacting effects of water stress and insect herbivory. *Int J Plant Sci* **172**, 499-508, doi:10.1086/658921 (2011).
38 Molina-Montenegro, M. A. *et al.* Root-endophytes improve the ecophysiological performance and production of an agricultural species under drought condition. *Aob Plants* **8**, 11, doi:10.1093/aobpla/plw062 (2016).
39 Morse, L. J., Day, T. A. & Faeth, S. H. Effect of *Neotyphodium* endophyte infection on growth and leaf gas exchange of Arizona fescue under contrasting water availability regimes. *Environ Exp Bot* **48**, 257-268, doi:10.1016/s0098-8472(02)00042-4 (2002).
40 Murphy, B. R., Nieto, L. M., Doohan, F. M. & Hodkinson, T. R. Fungal endophytes enhance agronomically important traits in severely drought-stressed barley. *J. Agron. Crop. Sci.* **201**, 419-427, doi:10.1111/jac.12139 (2015).
41 Nagabhyru, P., Dinkins, R. D., Wood, C. L., Bacon, C. W. & Schardl, C. L. Tall fescue endophyte effects on tolerance to water-deficit stress. *BMC Plant Biol.* **13**, 17, doi:10.1186/1471-2229-13-127 (2013).
42 Oberhofer, M., Gusewell, S. & Leuchtmann, A. Effects of natural hybrid and non-hybrid Epichloe endophytes on the response of Hordelymus europaeus to drought stress. *New Phytol.* **201**, 242-253, doi:10.1111/nph.12496 (2014).
43 Odokonyero, K., Acuna, T. B., Cardoso, J. A., Jimenez, J. D. & Rao, I. M. Fungal endophyte association with Brachiaria grasses and its influence on plant water status, total non-structural carbohydrates and biomass production under drought stress. *Plant Soil* **409**, 273-282, doi:10.1007/s11104-016-2947-5 (2016).
44 Oliveira, J. A., Rottinghaus, G. E., Collar, J. & Castro, P. Perennial ryegrass endophytes in Galicia, Northwest Spain. *J. AGRIC. SCI.* **129**, 173-177, doi:10.1017/s0021859697004711 (1997).
45 Pandey, V. *et al.* Dose-dependent response of *Trichoderma harzianum* in improving drought tolerance in rice genotypes. *Planta* **243**, 1251-1264, doi:10.1007/s00425-016-2482-x (2016).
46 RAHMAN, M. H. S., W. R. MATTHEW, C. SABREEN, S. OKUBO, A AND ISLAM K. R. Response of diploid perennial ryegrass to fungal endophyte AR29 infections under water stress. *Commun. Soil Sci. Plant Anal.* **46**, 845–860, doi:10.1080/00103624.2015.1011747 (2015).
47 Ravel, C., Courty, C., Coudret, A. & Charmet, G. Beneficial effects of *Neotyphodium lolii* on the growth and the water status in perennial ryegrass cultivated under nitrogen deficiency or drought stress. *AGRONOMIE* **17**, 173-181, doi:10.1051/agro:19970304 (1997).
48 Ren, A., Gao, Y., Wang, W. & Wang, J. Photosynthetic pigments and photosynthetic products of endophyte-infected and endophyte-free *Lolium perenne* L. under drought stress conditions. *Front. Biol. China* **1**, 168-173, doi:10.1007/s11515-006-0025-8 (2006).
49 Ren, A. *et al.* Benefits of a fungal endophyte in Leymus chinensis depend more on water than on nutrient availability. *Environ Exp Bot* **108**, 71-78, doi:10.1016/j.envexpbot.2013.11.019 (2014).
50 Ren, A. Z. & Clayy, K. Tmpact of a horizontally transmitted endophyte, Balansia henningsiana, on growth and drought tolerance of Panicum rigidulum. *Int J Plant Sci* **170**, 599-608, doi:10.1086/597786 (2009).
51 Ren, A. Z. *et al.* Benefits of a symbiotic association with endophytic fungi are subject to water and nutrient availability in Achnatherum sibiricum. *Plant Soil* **346**, 363-373, doi:10.1007/s11104-011-0824-9 (2011).
52 Richardson, M. D., Chapman, G. W., Hoveland, C. S. & Bacon, C. W. Sugar alcohols in endophyte-infected tall fescue under drought. *Crop Sci* **32**, 1060-1061 (1992).

53 Richardson, M. D., Hoveland, C. S. & Bacon, C. W. Photosynthesis and stomatal conductance of symbiotic and nonsymbiotic tall fescue. *Crop Sci* **33**, 145-149 (1993).
54 Rudgers, J. A. & Swafford, A. L. Benefits of a fungal endophyte in Elymus virginicus decline under drought stress. *Basic Appl. Ecol.* **10**, 43-51, doi:10.1016/j.baae.2007.12.004 (2009).
55 Sherameti, I., Tripathi, S., Varma, A. & Oelmuller, R. The root-colonizing endophyte Pirifomospora indica confers drought tolerance in Arabidopsis by stimulating the expression of drought stress-related genes in leaves. *Mol. Plant-Microbe Interact.* **21**, 799-807, doi:10.1094/mpmi-21-6-0799 (2008).
56 Shukla, N., Awasthi, R. P., Rawat, L. & Kumar, J. Biochemical and physiological responses of rice (Oryza sativa L.) as influenced by Trichoderma harzianum under drought stress. *Plant Physiol. Biochem.* **54**, 78-88, doi:10.1016/j.plaphy.2012.02.001 (2012).
57 Sun, C. A. *et al.* Piriformospora indica confers drought tolerance in Chinese cabbage leaves by stimulating antioxidant enzymes, the expression of drought-related genes and the plastid-localized CAS protein. *J Plant Physiol* **167**, 1009-1017, doi:10.1016/j.jplph.2010.02.013 (2010).
58 Swarthout, D. *et al.* Measures of leaf-level water-use efficiency in drought stressed endophyte infected and non-infected tall fescue grasses. *Environ Exp Bot* **66**, 88-93, doi:10.1016/j.envexpbot.2008.12.002 (2009).
59 Tian, Z., Huang, B. & Belanger, F. C. Effects of *Epichloë festucae* fungal endophyte infection on drought and heat stress responses of strong creeping red fescue. *J. Am. Soc. Hortic. Sci.* **140**, 257-264 (2015).
60 Vazquez-de-Aldana, B. R., Garcia-Ciudad, A., Garcia-Criado, B., Vicente-Tavera, S. & Zabalgogeazcoa, I. Fungal Endophyte (Epichloe festucae) Alters the Nutrient Content of Festuca rubra Regardless of Water Availability. *PLoS ONE* **8**, 14, doi:10.1371/journal.pone.0084539 (2013).
61 Waqas, M. *et al.* Endophytic Fungi Produce Gibberellins and Indoleacetic Acid and Promotes Host-Plant Growth during Stress. *Molecules* **17**, 10754-10773, doi:10.3390/molecules170910754 (2012).
62 West, C. P., Carson, R. D., Guerber, C. A. & De Los Reyes, B. in *Molecular Breeding for The Genetic Improvement of Forage Crops and Turf: Proceedings of the 4th International Symposium on The Molecular Breeding of Forage and Turf, a Satellite Workshop of the XXth International Grassland Congress, July 2005, Aberystwyth, Wales* 217 (Wageningen Academic Publishers, 2005).
63 West, C. P., Izekor, E., Turner, K. E. & Elmi, A. A. Endophyte effects on growth and persistence of tall fescue along a water-supply gradient. *Agron J* **85**, 264-270 (1993).
64 Wu, L. J. *et al.* Endophytic benefit for a competitive host is neutralized by increasing ratios of infected plants. *Acta Oecol.-Int. J. Ecol.* **70**, 112-120, doi:10.1016/j.actao.2015.12.009 (2016).
65 Yang, T., Ma, S. & Dai, C. C. Drought degree constrains the beneficial effects of a fungal endophyte on Atractylodes lancea. *J. Appl. Microbiol.* **117**, 1435-1449, doi:10.1111/jam.12615 (2014).
66 Zhang, Q., Gong, M., Yuan, J., Hou, Y., Zhang, H., Wang, Y., and Hou, X. Dark septate endophyte improves drought tolerance in sorghum. *International Journal of Agriculture & Biology* **19**, 53–60, doi:10.17957/IJAB/15.0241 (2017).
67 Zhang, Y. P. & Nan, Z. B. Growth and anti-oxidative systems changes in *Elymus dahuricus* is affected by Neotyphodium endophyte under contrasting water availability. *J. Agron. Crop. Sci.* **193**, 377-386, doi:10.1111/j.1439-037X.2007.00279.x (2007).