

**ENDOPHYTES: A POTENT SEED BIO-PRIMING
AGENTS FOR ABIOTIC STRESS MITIGATION IN
SELECTED CROPS**

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**DEPARTMENT OF SEED SCIENCE AND TECHNOLOGY
UNIVERSITY OF AGRICULTURAL SCIENCES
BANGALORE**

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for the award of the degree of

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*Affectionately dedicated to
my beloved Parents,
my Sister and
my respected Guide*

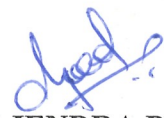


**DEPARTMENT OF SEED SCIENCE AND TECHNOLOGY
UNIVERSITY OF AGRICULTURAL SCIENCES
BANGALORE**

CERTIFICATE

This is to certify that the thesis entitled “**ENDOPHYTES: A POTENT SEED BIO-PRIMING AGENTS FOR ABIOTIC STRESS MITIGATION IN SELECTED CROPS**” submitted by **Ms. ROOPASHREE, B., ID. No. PALB 8105** in partial fulfilment of the requirements for the award of the degree of **DOCTOR OF PHILOSOPHY** in **SEED SCIENCE AND TECHNOLOGY** to the University of Agricultural Sciences, Bangalore, is a record of *bona-fide* research work carried out by her during the period of her study in this University, under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles.

BANGALORE
MARCH, 2022


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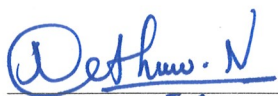
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ENDOPHYTES: A POTENT SEED BIO-PRIMING AGENTS FOR ABIOTIC STRESS MITIGATION IN SELECTED CROPS

ROOPASHREE, B.

ABSTRACT

Drought is a major limiting factor influencing plant growth and development. In recent years there have been several studies to demonstrate the use of endophytes in mitigating stress tolerances. Endophytes include symptomless bacteria and fungi that are ubiquitously present in the intercellular spaces of the host plants. Endophytes isolated from extreme habitats are able to colonize the non-host plants and impart stress tolerance response. Against this background area of knowledge, the present study was taken up to study the role of selected 8 endophytes on early seedling growth of maize, green gram, soybean, and brinjal. Among 8 fungal endophytes, K-23, LAS-6 and P-37 had shown increased seedling growth of pre-germinated maize seeds both under control and PEG-8000 induced drought stress conditions. Not only in the seedling level but also in the greenhouse conditions these selected endophytes (K-23, LAS-6 and P-37) have been reported a significant increase in the plant height, stem girth, leaf area, root volume, photosynthetic rate, chlorophyll content, antioxidant enzyme activity, and yield content when plants were subjected to drought stress. In order to unravel the molecular mechanism, maize plants inoculated with K-23 were subjected to comparative transcriptome analysis. It revealed that genes involved in the photosynthetic pathway, synthesis of secondary metabolites, starch and sucrose metabolism were upregulated in endophyte enriched seedlings when subjected to drought stress. This study set as proof of concept to demonstrate endophytes adapted to extreme habitats can be effectively used to modulate non-host plant responses to abiotic stresses such as drought.

March, 2022

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Major Advisor

**ಅಂತರ್ ಸಹಜೀವಿಗಳು(ಎಂಡೋಫೈಟ್ಸ್): ಆಯ್ದ ಬೆಳೆಗಳಲ್ಲಿ ಪ್ರಬಲ ಜೈವಿಕ ಬೀಜೋಪಚಾರ
ಸೂಕ್ಷ್ಮಾಣುಗಳ ಸಹಯೋಗದಿಂದ ಅಜೈವಿಕ ಒತ್ತಡಗಳ ಉಪಶಮನತೆ**

ರೂಪಶ್ರೀ, ಬಿ.

ಪ್ರಬಂಧದ ಸಾರಾಂಶ

ಬರಗಾಲವು ಸಸ್ಯಗಳ ಬೆಳವಣಿಗೆ ಹಾಗೂ ಅಭಿವೃದ್ಧಿಯ ಮೇಲೆ ಪ್ರಭಾವ ಬೀರುವ ಪ್ರಮುಖ ಅಂಶವಾಗಿದೆ. ಇತ್ತೀಚಿನ ವರ್ಷಗಳಲ್ಲಿ ಒತ್ತಡ ಸಹಿಷ್ಣುತೆಯನ್ನು ಉಪಶಮನ ಮಾಡಲು ಅಂತರ್ ಸಹಜೀವಿಗಳ ಬಳಕೆಯನ್ನು ಪ್ರಮಾಣೀಕರಿಸಲು ಹಲವು ಅಧ್ಯಯನಗಳು ನಡೆದಿವೆ. ಅಂತರ್ ಸಹಜೀವಿಗಳು ರೋಗಲಕ್ಷಣಗಳಿಲ್ಲದ ಬ್ಯಾಕ್ಟೀರಿಯಾ ಮತ್ತು ಶಿಲೀಂಧ್ರಗಳನ್ನು ಒಳಗೊಂಡಿರುತ್ತವೆ ಹಾಗೂ ಅವು ಸಸ್ಯಗಳ ಅಂತರಕೋಶದ ಭಾಗಗಳಲ್ಲಿ ಸರ್ವತ್ರವಾಗಿರುತ್ತದೆ. ವಿಪರೀತ ಆವಾಸ ಸ್ಥಾನಗಳಿಂದ ಪ್ರತ್ಯೇಕಿಸಲಾದ ಅಂತರ್ ಸಹಜೀವಿಗಳು ಬೇರೆ ಸಸ್ಯಗಳನ್ನು ವಸಾಹತುವನ್ನಾಗಿ ಮಾಡಲು ಹಾಗೂ ಒತ್ತಡ ಸಹಿಷ್ಣುತೆಯ ಪ್ರತಿಕ್ರಿಯೆಯನ್ನು ನೀಡಲು ಸಾಧ್ಯವಾಗುತ್ತದೆ. ಈ ಮಾಹಿತಿಯ ಹಿನ್ನೆಲೆಯೊಂದಿಗೆ ಮೆಕ್ಸಿಕೋಳ, ಹೆಸರು ಕಾಳು, ಸೋಯಾ ಅವರೆ ಹಾಗೂ ಬದನೆಕಾಯಿ ಬೆಳೆಗಳಲ್ಲಿ ಆರಂಭಿಕ ಸಸ್ಯ ಬೆಳವಣಿಗೆಯ ಮೇಲೆ ಆಯ್ದ ಎಂಟು ಅಂತರ್ ಸಹಜೀವಿಗಳ ಪಾತ್ರವನ್ನು ತಿಳಿಯಲು ಪ್ರಸ್ತುತ ಅಧ್ಯಯನವನ್ನು ತೆಗೆದುಕೊಳ್ಳಲಾಯಿತು. ಎಂಟು ಶಿಲೀಂಧ್ರ ಅಂತರ್ ಸಹಜೀವಿಗಳಲ್ಲಿ ಇವು ನಿಯಂತ್ರಣ ಹಾಗೂ PEG-8000 ಪ್ರಚೋದಿತ ಬರ ಒತ್ತಡ ಮೊಳಕೆಯೊಡೆದ ಮೆಕ್ಸಿಕೋಳದಲ್ಲಿ ಸಸಿ ಬೆಳವಣಿಗೆಯನ್ನು ಹೆಚ್ಚಿಸಿವೆ. ಸಸಿ ಬೆಳವಣಿಗೆಯಲ್ಲಿ ಮಾತ್ರವಲ್ಲದೆ, ಆಯ್ದ ಮಾಡಿದ ಈ ಅಂತರ್ ಸಹಜೀವಿ(ಕೆ-23, ಎಲ್.ಎ.ಎಸ್-6 ಹಾಗೂ ಪಿ-37) ಉಪಚರಿತ ಗಿಡಗಳು ಪ್ರಚೋದಿತ ಬರ ಒತ್ತಡಕ್ಕೆ ಒಳಗಾದರೂ ಗಿಡದ ಎತ್ತರ, ಕಾಂಡದ ಸುತ್ತಳತೆ, ಎಲೆಯ ವಿಸ್ತೀರ್ಣ, ಬೇರುಗಳ ಪ್ರಮಾಣ, ದ್ಯುತಿಸಂಶ್ಲೇಷಕ ಪ್ರಮಾಣ, ಕ್ಲೋರೊಫಿಲ್ ಅಂಶ, ಉತ್ಕರ್ಷಣ ನಿರೋಧಕ ಕಿಣ್ವ ಚಟುವಟಿಕೆ ಮತ್ತು ಇಳುವರಿ ಪ್ರಮಾಣವನ್ನು ಹಸಿರುಮನೆ ವಾತಾವರಣದಲ್ಲೂ ಗಮನಾರ್ಹವಾಗಿ ಹೆಚ್ಚಿರುವುದು ಕಂಡುಬಂದಿದೆ. ಆಣ್ವಿಕ ಕಾರ್ಯವಿಧಾನವನ್ನು ಬಿಚ್ಚಿಡಲು, ಕೆ-23 ಯೊಂದಿಗೆ ಉಪಚರಿಸಲ್ಪಟ್ಟ ಮೆಕ್ಸಿಕೋಳದ ಸಸ್ಯಗಳನ್ನು ತುಲನಾತ್ಮಕ ಪ್ರತಿಲೇಖನ ವಿಶ್ಲೇಷಣೆಗೆ ಒಳಪಡಿಸಲಾಯಿತು. ಬರ ಒತ್ತಡದಲ್ಲಿ ಅಂತರ್ ಸಹಜೀವಿಗಳಿಂದ ಸಮೃದ್ಧವಾದ ಸಸ್ಯಗಳಲ್ಲಿ ದ್ಯುತಿಸಂಶ್ಲೇಷಕ ಮಾರ್ಗ, ದ್ವಿತೀಯಕ ಚಯಾಪಚಯ ಕ್ರಿಯೆಯ ಸಂಶ್ಲೇಷಣೆ, ಪಿಷ್ಟ ಮತ್ತು ಸುಕ್ರೋಸ್ ಚಯಾಪಚಯ ಕ್ರಿಯೆಯಲ್ಲಿ ಒಳಗೊಂಡಿರುವ ಅನುವಂಶಿಕ ಧಾತುಗಳು ಸಮೃದ್ಧವಾಗಿರುವುದು ಕಂಡುಬಂದಿದೆ. ಒಟ್ಟಿನಲ್ಲಿ, ಸಸ್ಯಗಳಲ್ಲಿರುವ ಅಂತರ್ ಸಹಜೀವಿಗಳನ್ನು ಅಧಿಕ ಅಜೈವಿಕ ಒತ್ತಡಗಳಿರುವ ಪ್ರದೇಶಗಳಲ್ಲಿ ಬೆಳೆದಿರುವ ಸಸ್ಯಗಳಿಂದ ಬೇರ್ಪಡಿಸಿ ಬೇರೆ ಸಸ್ಯಗಳಿಗೆ ಹಾಕುವ ಮೂಲಕ ಸಸ್ಯದ ಬೆಳವಣಿಗೆ ಮತ್ತು ಅಜೈವಿಕ ಒತ್ತಡಗಳಿಗೆ ಪ್ರತಿರೋಧಕ ಶಕ್ತಿಯನ್ನು ನೀಡಲು ಸಹಕರಿಸುತ್ತದೆ ಎನ್ನುವುದು ಪ್ರಸ್ತುತ ಅಧ್ಯಯನವು ಸಾಬೀತುಪಡಿಸಿದೆ.

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LIST OF ABBREVIATIONS

DAS	:	Days after sowing
SCMR	:	SPAD chlorophyll meter reading
°C	:	Degree Celsius
RWC	:	Relative water content
ROS	:	Reactive oxygen species
CAT	:	Catalase
PPO	:	Polyphenol oxidase
cm	:	Centimeter
MDA	:	Malondialdehyde
SEM	:	Scanning electron microscope
<i>et al.</i>	:	Co-Workers
IAA	:	Indole acetic acid
GA	:	Gibberellic acid
ABA	:	Absciscic acid
SA	:	Salicylic acid
JA	:	Jasmonic acid
g	:	Gram
kg	:	Kilogram
mg	:	Milligram
LSU	:	Large subunit
ITS	:	Internal transcribed spacer
<i>TUB2</i>	:	Beta-tubulin
<i>i.e.,</i>	:	That is
LAS-6	:	<i>Chaetomium madrasense</i>
SF-5	:	<i>Fusarium equiseti</i>
V4-J	:	<i>Fusarium equiseti</i>
K-23	:	<i>Fusarium incarnatum</i>
K-26	:	<i>Fusarium redolens</i>
P-37	:	<i>Ulocladium sp.</i>
P-10	:	<i>Fusarium solani</i>
PJ-9	:	<i>Fusarium equiseti</i>
SEm±	:	Standard error of mean
vs	:	Versus
NS	:	Non-significant

I INTRODUCTION

Over the last several decades, the cumulative impact of human life on our planet, notably the industrial revolution, has resulted in a progressive increase in greenhouse gas production, contributing to global warming. Consequently, the climate is changing dramatically, leading to a rise in the frequency and intensity of droughts, as well as other abiotic stresses (Lobell and Gourdj, 2012; Anderson and Song, 2020; Borrelli *et al.*, 2020). At the same time, crisis to feed the budding world population, which will be more than 9.7 billion by 2050, is augmented by restraining issues like spatial allocation of land for agriculture and further consolidated by abiotic stress (Castaneda *et al.*, 2016). In India, out of the 159.7 million hectares of agricultural land (Himani, 2014), 42% is under drought (Gogoi and Tripathi, 2019; Ahluwalia *et al.*, 2021). It would be difficult to sustain increasing crop yields under drought (Lehmann and Rillig, 2014), as the quantity of fresh water for agricultural usage is dwindling (Mazdiyasni and AghaKouchak, 2015; Gray *et al.*, 2016; Bigot *et al.*, 2018). Therefore, it is critical to move the paradigm towards sustainable agriculture and find answers to concerns like water shortage and its influence on food security (Kaushal, 2019).

Drought is a well-studied abiotic stress factor with significant ecological and agronomic consequences (Feller and Vaseva, 2014). When soil water availability is limited to critical levels, plants can no longer grow or function normally (Akula and Ravishankar, 2011). Drought affects plants at practically every stage, either directly or indirectly (Golldack *et al.*, 2014; Anjum *et al.*, 2017). It affects seed germination, which results in late emergence, and poor seedling establishment (Yan, 2015; Steiner *et al.*, 2017). Drought stress has a detrimental influence on plant growth and development, due to abnormal physiological processes such as loss of turgidity, rate of carbon assimilation, gaseous exchange, and oxidative damage due to accelerated production of reactive oxygen species (ROS) (Hussain *et al.*, 2018), all of which affect leaf development, enzymatic activity, ion absorption (Todaka *et al.*, 2017). Under severe stress, reduction in leaf relative water content (RWC) affects stomatal conductance and net photosynthetic rate (Ullah *et al.*, 2017). Plant productivity was reduced by 50 to 73 percent under drought stress, depending

on the growth stage and intensity of the stress period (Berry *et al.*, 2013). Many of these processes are governed by a set of genes and pathways and are regulated differently.

Several studies have looked at, how plants react to drought stress at the molecular and physiologic levels. Modern agro-biotechnologies, such as introducing new genes or modifying the expression levels of existing genes, are being used to test plants for drought tolerance (Lu *et al.*, 2013). In conjunction with natural resource management, drought-resistant varieties have been developed by plant breeding and genetic engineering approaches to improve water use efficiency and productivity (Bailey-Serres *et al.*, 2019; Mittler and Blumwald, 2010). However, because of the intricacy of abiotic stress tolerance systems, developing new tolerant cultivars is a lengthy process, and genetically modified plants are not well accepted in some parts of the world (Fahad *et al.*, 2014; Hoang *et al.*, 2015). Therefore, the plant-biotic method, which incorporates microbes, has emerged as an alternative method for sustainable agriculture in recent years. The role of symbiotic microorganisms on phenotypic adjustment of plants to environmental stresses has emerged attention (Dhanyalakshmi *et al.*, 2019; Acuna-Rodriguez *et al.*, 2020).

Microorganisms have played a critical part in agriculture's long-term sustainability (Vaxevanidou *et al.*, 2015). There is longstanding evidence that microorganisms like nitrogen-fixing microbes and arbuscular mycorrhiza fungi promote plant growth (Berendsen *et al.*, 2012; Santoyo *et al.*, 2016). Microbes (bacteria, fungi, and actinomycetes) that live intracellularly or intercellularly inside plants without causing diseases are known as endophytes (Nair and Padmavathy, 2014). The ubiquitous effect of these endophytes on plant health and growth is poorly understood; they improve stress tolerance, disease resistance, nutrient availability (Coutinho *et al.*, 2015; Yuan *et al.*, 2017). Endophytic fungi also produce phytohormones to maintain cytosolic ion homeostasis to overcome stress (Hamayun *et al.*, 2017).

Endophytes appear to be a realistic choice in this situation, having successfully coped with drought in the past. Whenever, the seeds and seedlings are confronted with hazards (Bever *et al.*, 2015), the seed endophytes are the primary inoculum for plants (Barret *et al.*, 2015), which significantly impact plants' ability to combat this plethora

of biotic and abiotic stressors. Plant adaptive behavior was improved by inoculating endophytes, which enhanced nutrient intake, photosynthetic activity, chlorophyll levels, root growth, hydration status, antioxidant enzymes, phytohormone signaling, and soluble sugars. Under drought conditions, it also boosts stress-responsive genes secondary metabolites, and it lowers the malondialdehyde levels in plants (Gagné-Bourque *et al.*, 2015; Chen *et al.*, 2017; Jayakumar *et al.*, 2020).

The endophytes are noteworthy for their ability to colonize without apparent symptoms and provide habitat-adapted fitness advantages to genetically distant hosts, both monocots and eudicots (Rodriguez *et al.*, 2008). Endophytes also can transfer habitat-specific stress tolerance to plants through a process known as Habitat Adapted Symbiosis (Rodriguez *et al.*, 2004). Plants in symbiosis with endophytic fungi can improve its growth and physiological status (Gamalero *et al.*, 2009) under stressful environments. It has been hypothesized that host fungal endophytes provide plant fitness to extreme habitats through intergenomic epigenetic mechanisms to allow the plant to tolerate specific stresses (Rodriguez *et al.*, 2008). According to some reports, plants adapted to harsh habitats typically harbor endophytes that are highly tolerant to extreme conditions through mutualistic association (Manasa *et al.*, 2020; Sangamesh *et al.*, 2018).

Endophytes have been shown to stimulate the growth of diverse plants (Ma *et al.*, 2011). In specific situations, the ability of endophytes to encourage plant growth depends on the host's genotype (Kim *et al.*, 2012). In contrast, many endophytes can have a broad host range, as shown by *Burkholderia phytofirmans* PsJN, isolated from onion roots, promotes the growth of *Arabidopsis thaliana*, grape, maize, potato, switch-grass, tomato, and wheat (Sessitsch *et al.*, 2005; Sheibani-Tezerji *et al.*, 2015) making them a powerful tool in agriculture. If properly developed, this type of endophytic symbiotic technology could be valuable for mitigating plants' abiotic stress, such as drought. Towards this end, scientists worldwide are exploring novel endophytes found in extreme habitats, that could be used to confer symbiotic fitness to plants by imparting habitat-adapted symbiotic properties (Sampangi-Ramaiah *et al.*, 2020).

Here, in this study, an attempt has been made to modulate plant developmental process under drought stress using a novel seed priming technology comprising fungal endophytes that form symbiotic associations with a diverse group of crop plants like maize, green gram, soybean, and brinjal, especially those with agronomical interest and which are generally grown under rainfed conditions by conferring significant levels of drought tolerance. Seed priming with the use of fungal endophytes appears as more beneficial, eco-friendly, and stable due to better colonization adaptability and suitability under abiotic stress conditions. The present study aims to explore the potential ability of diverse fungal endophytes in seed priming to achieve the goals of sustainable agriculture and to overcome limitations associated with field applications along with uncovering molecular mechanisms involved. In light of this, the following objectives were framed:

1. To examine the role of selected fungal endophytes in imparting drought tolerance in maize, soybean, green gram, and brinjal.
2. Validation of hits obtained from objective 1 and understanding the physiological and biochemical mechanisms involved in conferring drought stress tolerance in greenhouse conditions.
3. To assess the performance of best crop-endophytes combination at the field level.

II REVIEW OF LITERATURE

In the wake of changing climatic conditions, plants are frequently exposed to a wide range of biotic (bacteria, fungi, and virus attacks) and abiotic (extreme temperatures, drought, flooding, high salinity, pesticides, heavy metals, excessive light, and ultraviolet (UV) radiation) stresses at various stages of their development, all of which have negative effects on productivity and survival (Bostock *et al.*, 2014; Petrov *et al.*, 2015). Abiotic stresses which are environmental perturbations act negatively on the growth and development of plants. Drought is one of the most devastating abiotic stresses, affecting the growth and productivity of the majority of cultivated crops, particularly in arid and semiarid environments (Bodner *et al.*, 2015; Etesami and Maheshwari, 2018). Given this critical problem, both conventional breeding and biotechnological approaches are being used to generate drought-tolerant crop plants. However, they are costly affair and time consuming. Exploration into the use of microbes, notably endophytic fungi, in boosting plant growth and yield as well as modulating plant responses to abiotic stress has received a lot of attention in recent years (Chitnis *et al.*, 2020). Microorganisms form a mutualistic relationship with the host plant and serve as biofertilizers and biocontrol agents, promoting the plant's growth, health, and production (Vishwakarma *et al.*, 2020). The literature pertaining to the latter issues on addressing the underlying mechanisms that endophytes are used to regulate plant development and drought tolerance are reviewed and presented in this chapter.

2.1 Drought stress and its impact on plant phenology and growth forms

Drought stress is a severe problem in both arid and semiarid landscape restoration. Drought has been classified as (i) meteorological drought, which is usually caused by a long-term deficit of rainfall; (ii) hydrological drought, begin by a shortfall in river/reservoirs flow; (iii) pedological drought, ascribed to a limitation in soil-water storage, particularly in the soil solum; (iv) agronomic drought, which is a major concern, being induced by a shortage of plant-available water to maintain the physiological needs of evapotranspiration during the growing period (Mishra and Singh, 2010). Droughts of all types, especially pedological and agronomic droughts, have a negative impact on seedling

establishment and crop stand. In 2021, according to the Drought Early Warning System (DEWS), a real-time drought monitoring platform, over a fifth of India's land area *i.e.*, 21.06 percent is facing drought, which is 62 percent more than the area under drought during the same period last year, which was 7.86 percent.

Drought is a major limiting factor for plant productivity (Karl *et al.*, 2009). Water makes up 80–95 percent of the fresh biomass of the plant body, and it plays an important role in various physiological activities, including plant growth, development, and metabolism (Brodersen *et al.*, 2019). Because, both the severity and duration of the stress are significant, drought-induced yield losses are likely to outnumber all other causes (Ma *et al.*, 2020). It reduces leaf size, leaf area, photosynthetic pigments, photosynthetic efficiency, stem extension, and root proliferation, as well as interrupting plant water relations and limiting water efficiency (Fathi and Tari, 2016; Wu *et al.*, 2008).

The early phase of drought stress causes a decrease in shoot growth while root growth is maintained, resulting in a higher root/shoot ratio. The accumulation of salts and ions in the upper layers of the soil under moderate or severe drought conditions causes osmotic stress and ion toxicity in plants (Vasques *et al.*, 2016). The turgor pressure of plant cells diminishes as the degree of drought stress increases, causing plant cell walls to wrinkle and loosen. Drought-induced stomatal closure limits CO₂ uptake, resulting in reduced gas exchange, CO₂ assimilation, and photosynthetic machinery impairment (Zargar *et al.*, 2017). At cellular and molecular level, plants exposed to drought stress produce reactive oxygen species (ROS) such as superoxide anion radicals (O₂⁻), hydrogen peroxide (H₂O₂), hydroxyl radicals (OH), alkoxy radicals (RO), and singlet oxygen (¹O₂), which causes oxidative stress and impair cell function through lipid peroxidation, membrane deterioration, and protein, lipid, and nucleic acid degradation (Apel and Hirt, 2004; Das and Roychoudhury, 2014). It also enhances ethylene biosynthesis, which drives growth inhibition and causes premature leaf senescence (Dubois *et al.*, 2018). Drought stress led to reduced seed germination and seedling establishment in a wide range of crops, including maize (Liu *et al.*, 2015a), pea (Okçu *et al.*, 2005), rice (Manickavelu *et al.*, 2006), and alfalfa (Zeid and Shedeed, 2006). Other research has found that drought stress inhibits growth and reduces crop yields in barley (Samarah, 2005), wheat (Rampino *et al.*, 2006),

rice (Lafitte *et al.*, 2007), and maize (Anjum *et al.*, 2017). Overall, the plant's response to drought is multifaceted, with effects ranging from the biochemical to the whole plant level (Osakabe *et al.*, 2014).

2.2 How do plants sense and respond to drought?

Due to their static nature, plants sense external or internal stimuli and fine-tune their physiological and developmental processes to generate a reaction and adapt to the environment. Plants have specific receptors that detect stress (Zhu, 2016). While dealing with drought stress, it's critical to distinguish primary stress signals from secondary stress signals caused by a shortage of water. Drought generates hyperosmotic stress, often known as osmotic stress, and the osmosensor proteins sense a water shortage through osmosensing activities and activate downstream signaling to trigger a stress response (Figure 2.1). Cells detect osmotic stress by losing turgor as a result of cell wall loosening, followed by an increase in abscisic acid (ABA) concentration (Zeevaart and Creelman, 1988).

Plants respond to drought stress by emitting a range of biological signals at the right times and at the right speed to survive (Takahashi and Shinozaki, 2019). Short-term responses to prevent water loss through transpiration from guard cells, as well as long-term adaptations to gain stress resistance at the whole-plant level, necessitate a diversity of cellular and molecular regulatory mechanisms in plants (Nakashima *et al.*, 2014). Because it is multidimensional stress, it causes a wide range of physiological, biochemical, and molecular reactions in plants. An increase in osmoregulatory molecules, which stabilize cell membranes and maintain cell turgor, is one of the physiological responses that plants use when they are stressed due to lack of water (Fang and Xiong, 2015). Proline and soluble sugars, for example, are osmoprotective compounds that accumulate in response to many forms of environmental stress, such as drought (Moustakas *et al.*, 2011), and help to mitigate the effects of oxidative stress (Labudda, 2013). Hydraulic signals, electric currents, calcium waves, reactive oxygen species (ROS), mRNAs, and phytohormone movements all play a role in drought stress responses (Kudla *et al.*, 2018; Yang *et al.*, 2021). Recent studies have discovered unique roles of hormone-like peptides as signaling molecules that mediate drought stress responses (Takahashi *et al.*, 2018).

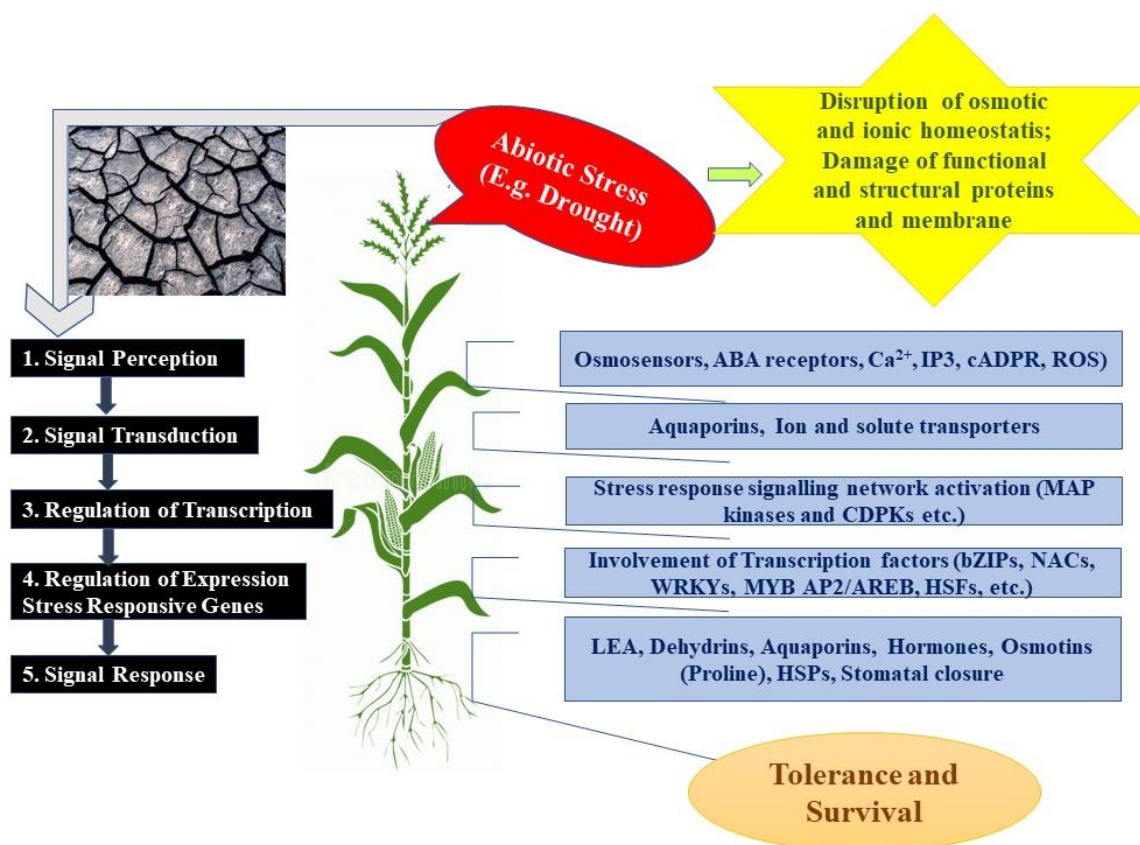


Figure 2.1: Response of plants to drought stress.

(Adapted from Lamaoui *et al.*, 2018; Zenda *et al.*, 2019; Yang *et al.*, 2021).

At a molecular level, plants perceive water stress signals via membrane-localized stress sensors and receptors such as abscisic acid (ABA), Ca²⁺, inositol-1, 4, 5-triphosphate (IP3), cyclic adenosine 5'-diphosphate ribose (cADPR), ROS, etc., and the perceived stimulus is then relayed to intracellular compartments through secondary messengers especially calcium ions. Further, these calcium ions activate appropriate signaling pathways to transduce downstream signals. In all signaling pathways, MAPK (mitogen-activated protein kinases) and CDPK (calcium-dependent protein kinases) act as potent signal transducers that initiate various transcription networks (bZIPs, NACs, WRKYs, MYB AP2/AREB, HSFs, etc.). The transcription factors regulate the transcription of different stress-related proteins, such as proline (pro), glycine betaine (GB), soluble sugar (SS), late embryogenesis abundant (LEA) proteins, aquaporin (AQP), heat shock proteins (HSPs), and dehydrins, which in turn act as chaperones to provide drought tolerance (Kaur

and Asthir, 2017; Yang *et al.*, 2021). Several chromatin regulators have recently been discovered to play a role in the regulation of stress-responsive gene networks under drought stress (Kim *et al.*, 2015).

2.3 Mechanisms and approaches to overcome drought in plants

Different adaptive mechanisms that make plants more tolerant to the adverse effects of drought stress have been developed through evolution (Batool *et al.*, 2020). Thus, plants' responses to drought stress vary from plant level up to molecular. Four types of morphological and physiological response strategies to drought stress are present, *i.e.*, tolerance, avoidance, escape, and recovery (Fang and Xiong, 2015). Tolerance is defined as the plant's ability to resist dehydration using osmoprotectants (Luo *et al.*, 2018). Avoidance is based on the undisturbed occurrence of physiological processes (such as stomata regulation, root system development). Escape is an adjustment of the plant's life cycle by shortening the life cycle to avoid drought stress. Recovery is the ability of a plant to restart growth after exposure to extreme drought stress (Manavalan *et al.*, 2009).

The use of best management practices related to sowing time, plant population, plant genotype, soil, and nutrient management can help to reduce grain yield losses in field crops subjected to drought stress (Parry *et al.*, 2005). However, the use of transgenic plants with drought-tolerant events is perhaps the drought stress mitigation approach most heavily publicized and the one receiving more attention at present. Several efforts like breeding, molecular and genomic approaches are being undertaken to develop drought-tolerant plants through usual conventional breeding methods (Oliveira *et al.*, 2020), with the focus to improve water use efficiency, stomatal conductance, osmotic adjustments (Naeem *et al.*, 2020).

In the face of climate change, certain future adaptations can be predicted by observing the functioning of microbes in extreme environments. In addition, in natural adverse ecosystems, except for the ability to form spores, microorganisms support plant growth and protect them from drought stress (Table 2.1), simultaneously providing an optimal environment for the development of plants tissues (Prajakta *et al.*, 2019). Among microorganisms, fungi can be much more drought-tolerant than bacteria (Treseder *et al.*,

Table 2.1: Endophytes assisted drought tolerance in plants

Endophytes	Host plant	Plant part used for isolation	Inoculated plant/s	Beneficial features related to drought tolerance	References
<i>Sphingomonas</i>	<i>Tephrosia apollinea</i>	Leaves	<i>Glycine max</i>	Increased plant biomass (dry), glutathione, glutamine, photosynthetic pigments, glycine, and proline	Asaf <i>et al.</i> , 2017
<i>Burkholderia phytofirmans</i> and <i>Enterobacter</i> sp.	<i>Allium cepa</i>	Roots	<i>Zea mays</i>	Leaf area, shoot and root biomass, photosynthesis, chlorophyll content, and photochemical efficiency increased	Naveed <i>et al.</i> , 2014
<i>Rhizophagus irregularis</i>	<i>Zea mays</i>	Roots	<i>Zea mays</i>	Higher root biomass enhanced stomatal conductance, reduced oxidative damage, and enhanced hydraulic conductivity	Quiroga <i>et al.</i> , 2017
<i>Embellisia chlamydospore</i> , <i>Cladosporium oxysporum</i> , and <i>Paraphoma</i> sp.	<i>Hedysarum scoparium</i>	Root	<i>Glycyrrhiza uralensis</i> and <i>Zea mays</i>	Increased root biomass and root: shoot ratio	Li <i>et al.</i> , 2019
<i>Periconia macrospinoso</i> , <i>Neocamarosporium chichastianum</i> , and <i>Neocamarosporium goegapense</i>	<i>Seidlitzia rosmarinus</i> , <i>Zygophyllum eichwaldii</i> , and <i>Haloxylon ammodendron</i>	Root	<i>Cucumis sativus</i> L. and <i>Solanum lycopersicum</i> L.	Increase in proline content and antioxidant enzymatic activities as well as an increase in growth parameters and chlorophyll content	Moghaddam <i>et al.</i> , 2021

Endophytes	Host plant	Plant part used for isolation	Inoculated plant/s	Beneficial features related to drought tolerance	References
<i>Nectria haematococca</i>	<i>Chrysanthemum indicum</i> L.	Root	<i>Solanum lycopersicum</i> L.	Significantly improved all the plant growth parameters like the plant height, stem girth, leaf characteristics, plant biomass, and proline accumulation of drought-stressed tomato plants.	Valli and Muthukumar, 2018
<i>Pantoea agglomerans</i>	<i>Alhagi sparsifolia</i> Shap. (Leguminosae)	Leaves	<i>Triticum aestivum</i>	Increased accumulation of soluble sugars, decreased accumulation of malondialdehyde (MDA) and decreased degradation of chlorophyll in leaves of drought-stressed wheat.	Chen <i>et al.</i> , 2017
<i>Trichoderma atroviride</i>	<i>Camellia sinensis</i>	Leaves	<i>Zea mays</i>	ROS scavenging enzymes SOD, CAT, APX, and GR showed elevated activity and lowered H ₂ O ₂ production in inoculated seedlings under drought stress.	Guler <i>et al.</i> , 2016
<i>Paraconiothyrium</i> strains, <i>Embellisia chlamydospore</i> , and <i>Phialophora</i> sp.	<i>Gymnocarpus przewalskii</i>	Roots	<i>Ammopiptanthus mongolicus</i>	Exhibited significantly positive effects on plant branch number, potassium, and calcium content.	Li <i>et al.</i> , 2018
<i>Ampelomyces</i> sp.	<i>Pyrrhopappus carolinianus</i>	Stem and Root	<i>Solanum lycopersicum</i> L.	Enhanced growth and yield under optimal growth conditions and drought conditions	Morsy <i>et al.</i> , 2020

2018) due to several mechanisms to overcome drought stress, including osmolytes and thick cell walls (Treseder and Lennon, 2015).

Given the above, endophytic mediation is a promising approach in improving drought tolerance in a shorter time as compared to classical breeding programs. Thus, much more has to be done from this point of view to establish the real value of the endophytes in conferring drought tolerance. For this goal, environmentally-controlled experiments need to be validated in long-term field experiments, thereby reducing the real advantage between the endophytic approaches over the classical breeding.

2.4 Seed/plant as Meta-organism

A seed is a latent embryonic plant that has been supplied with nutrients to support early seedling growth and is protected by a hardened outer covering. Seeds also include a colony of endosymbionts that perform several important functions for developing seedlings and seedlings are less likely to survive without these symbiotic microbes (Verma and White, 2019). Mycovitality (Vujanovic and Vujanovic, 2007) refers to symbiotic connections between seeds and endophytic fungi, and it is a major and ongoing study field (Vujanovic *et al.*, 2000; Selosse *et al.*, 2011). Seeds from a wide range of plants, including monocotyledons, dicotyledons, and herbaceous plants, have been found to contain diverse types of endophytic microbes (Truyens *et al.*, 2015; Parsa *et al.*, 2016). The seed microbiome provides an immediate source of microbial aid for seedling establishment, which is especially significant for invasive plant species, when finding a compatible microbiome partner is difficult (Hodgson *et al.*, 2014). Seed-borne bacterial and fungal endophytes have a significant impact on seed germination under unfavorable environmental conditions (Shearin *et al.*, 2018). Seed endophytes are mostly mobilized via vascular connections or vertical transmission (Hodgson *et al.*, 2014). They have been shown to have direct and indirect mechanisms for improving plant growth and development, as well as increasing plant tolerance to biotic and abiotic stresses (Santoyo *et al.*, 2016).

Plants can be thought of as complex multi-genomic creature (meta-organisms) made up of the plant, its microbiome, and a network of interspecific interactions (Thijs *et*

al., 2016). The rhizosphere (microorganisms linked with the root and soil), endosphere (endophytic organisms), and phyllosphere, make up the microbiome (organisms that live on the surface of the aerial parts). Endophytes can invade plant tissues at any time during their life cycle. Plants are connected with two types of fungal symbionts: (a) endophytic fungi, which live and associate completely inside plant tissues (leaf, stem, and root), and (b) mycorrhizal fungi, which live only in roots and extend out into the rhizosphere. Mutualism, commensalism, and parasitism are all examples of symbiotic lifestyles in fungal symbionts. Drought and metal tolerance (Lewis, 1985), disease resistance, thermo-tolerance, growth improvement (Marks and Clay, 1990; Varma *et al.*, 1999), herbivore resistance, and higher nutrient uptake are all possible benefits of mutualistic symbioses. Commensalism symbioses have no beneficial or negative effects on hosts, whereas Parasitic symbioses, on the other hand, have a detrimental impact on host fitness by reducing growth rate and fecundity, or by triggering disease symptoms that eventually lead to death (Lewis, 1985; Marks and Clay, 1990).

2.5 An overview of endophytic microorganisms

Researchers believe that endophytic microorganisms existed 400 million years ago when higher plants first appeared on the planet and that they may have aided plant evolution (Sun *et al.*, 2012b). Exanthemata, or outgrowths in wheat leaves, were the first endophytes to be characterized (Unger, 1833). Leveille (1846) identified these outgrowths in wheat leaves as fungal structures, referring to them as "endophytic fungi". However, the term "endophytes" was initially coined by de Bary in 1866 to refer to all microbes found inside living, healthy plants. Mutualistic symbiosis was first documented in the Paleozoic era in the roots of the fossil tree *Amyelon radicans*, indicating that plant-fungal interaction existed at the time (Bacon and Hill, 1996). Endophytic connections may have changed dramatically since then.

Researchers have proposed several definitions of endophytes. Endophytes are bacteria, fungi, actinomycetes, and algae that spend their entire or part of their life cycle in the symplast or apoplast region of healthy plant tissues without causing disease symptoms (Kharwar *et al.*, 2014). Endophyte establishment is defined by Ernst *et al.* (2003) as a

symbiotic interaction between plants and fungi. Endophyte mutualism is divided into two types: constitutive endophytic mutualism and inducible endophytic mutualism. In constitutive endophytic mutualism, endophytes can develop in diseased plants, and can also be vertically transmitted via seeds to the next generation of the host. On the other hand, in inducible endophytic mutualism, the interior of the host tissues is severely colonized with localized infections which can evolve by numerous fungi (Carroll, 1988).

Based on their symbiotic characteristics, fungal endophytes (FE) are divided into four groups (Rodriguez *et al.*, 2009). Class 1 endophytes are clavicipitaceous fungi that live in the roots of some cool-season grasses and are spread vertically through their seeds. Many plants' shoots, roots, and rhizomes are colonized extensively by Class 2 endophytes, which are transported both vertically and horizontally. Class 3 endophytes have a wide host range and only colonize the shoot in a limited way; they are transferred horizontally. Endophytes of class 4 are localized to the roots and are also horizontally transferred. Endophytes have a close relationship with their plant partners and may have co-evolved with them, resulting in a variety of plant growth benefits (Chitnis *et al.*, 2020). The relationship is referred to be symbiotic because endophytic fungi benefit their hosts in exchange for nutrients obtained from the host plants. Colonization can transfer these effects to a wide range of crop species, making them extremely helpful in agriculture (Marquez *et al.*, 2007; Rodriguez *et al.*, 2008; Redman *et al.*, 2011).

Thousands of beneficial endophytes may be existing but unexplored due to lack of study focus in this subject. Several of these may be irreversibly lost due to environmental contamination, deforestation, habitat fragmentation, and biodiversity losses before their significance are discovered (Kandalepas *et al.*, 2015). As a result, it is necessary to investigate the reservoir of these biological molecules for drought stress mitigation.

2.6 Habitat adapted symbiosis of endophytic fungi

"Habitat adapted symbiosis" refers to the association of plants with endophytes (fungi or bacteria) that aid in conferring plants to adapt to given climatic conditions in a habitat-specific manner, more especially by class II non-claviceptaceous endophytes from plants adapted to geothermal soils, coastal beaches and agricultural fields (Rodriquez *et*

al., 2008). Cross-inoculation of endophytic fungi isolated from plants in harsh environments, which conferred tolerance in non-host and sensitive plant species, confirms this approach (Rodriguez *et al.*, 2009; Redman *et al.*, 2011).

The capacity of endophytes to colonize non-host plants and impart habitat-specific stress tolerance shows that stress tolerance is the result of symbiotic interaction. In high-stress environments, this habitat-specific phenomenon provides an inter-genomic epigenetic mechanism for plant adaptation and survival (Redman *et al.*, 2002; Rodriguez *et al.*, 2008). Endophyte interactions in high-stress habitats have shown that at least certain fungal endophytes can provide host plants with habitat-specific stress resistance (Lata *et al.*, 2018). Drought and salinity stress tolerance (Yuan *et al.*, 2016) and thermo-tolerance (Sangamesh *et al.*, 2018) is conferred on non-host plants by fungal endophytes from desert plants.

2.7 Isolation of endophytes

Over the last few decades, it has been clear that endophytes can be isolated from any plant (Hardoim *et al.*, 2015; Suryanarayanan *et al.*, 2018; Giauque *et al.*, 2019). These studies revealed many characteristics of endophytes, including their widespread distribution, sustained presence in plants, non-pathogenic nature (Rodriguez *et al.*, 2009), locating them as candidates with high potential for crop improvement. Although the taxonomy of endophytes is diverse, only a few species have been isolated, identified, and characterized to date (Hawksworth, 2001).

2.8 Seed bio-priming with endophytes

The method of inoculation has an impact on endophyte colonization and activity; thus, its importance should not be neglected (Afzal *et al.*, 2013). Even though comparable approaches were used in different tests by the same research organizations, there is no uniform methodology for inoculation throughout the literature (Rho *et al.*, 2018). Seed inoculation and soil inoculation are the two most commonly used approaches in research (Zhao *et al.*, 2016; Mohanty *et al.*, 2017). Seed inoculation is a technique in which researchers co-cultivate prepared liquid inoculum and introduce it to host plants when they

are still in the seed or seedling stage. Soil inoculation, on the other hand, is directly introducing inoculum into root media or pots where host plants are grown. Pruned-root dip (Bressan and Borges, 2004), spot inoculation (Clemow *et al.*, 2011), and foliar spray (Olivares *et al.*, 2015) are all used in the same way. In support of the preceding assertion, a meta-analysis indicated that seed inoculation is a widely utilized approach for endophyte inoculation (Tufail *et al.*, 2021). This kind of inoculation is a relatively effective strategy for introducing endophytes into the soil (Deaker *et al.*, 2004), especially in stressed conditions. Mycorrhizal fungal seed bio-priming has been shown to improve seed germination, seedling survival, and seedling establishment time (Prasad *et al.*, 2016). As a result, the inoculation method chosen is determined by the convenience of the experiment, and to look at how endophytes colonize plants and the plant-endophyte interaction.

2.9 Molecular characterization of endophytes

The morphological method of identification of endophytes might be useful for many endophytes (Verma *et al.*, 2014). Due to its failure to sporulate in culture, a considerable number of isolates are categorized as *Mycelia sterilia*. Many attempts have been made to improve *Mycelia sterilia* sporulation in culture by altering the nutrient composition of the medium, but none have proven effective. Because of the lack of literature, even some spore-producing fungi are difficult to classify into appropriate taxonomic groups. Furthermore, some taxonomists consider that morphological characteristics are insufficient to differentiate some taxa or are not reliable taxonomic features (Sun and Guo, 2012a). As a result, molecular approaches may be the best alternative for identifying unidentified endophytic microbes. Isolation of total genomic DNA from the host plant, PCR amplification of conserved DNA (rDNA) sequences using specific or universal primers, sequencing of PCR products, BLAST options in GenBank or other DNA databanks, retrieval of related sequences from the databank, and construction of phylogenetic trees using molecular software are all required for molecular identification. Because some portions of nucleic acid sequences are particularly stable and conserved across a vast spectrum of organisms, genotype-based identification of microbes is thought to be more reliable (Lindahl *et al.*, 2013). In recent years, a more precise assessment of the identification and diversity of fungi has been achieved using molecular markers including

the internal transcribed spacer (ITS) region of nuclear ribosomal DNA, LSU, β -tubulin2 (*TUB2*), and γ -actin (*ACT*) (White *et al.*, 1990; Vilgalys and Hester, 1990; Woudenberg *et al.*, 2009; Carbone and Kohn, 1999). Manasa *et al.* (2020) used forward (ITS1) and reverse (ITS4) primers to identify the fungal endophytes by amplifying the 28S ribosomal region of the internal transcribed spacer (ITS) of the genomic DNA.

2.10 Approaches for validation of endophytes

Use of test crops

a. Maize (*Zea mays* L.): is one of the world's leading cereal crops in terms of production (Dowswell, 2019). Maize cultivation is practiced by fifteen million farmers in India. It is grown on 9.72 million hectares in India, with a yield of 28.64 million tons and productivity of 2945 kilograms per hectare (Anonymous, 2020). Karnataka is a leading producer, accounting for 13.84 percent of total maize production, or 3.73 million tonnes, on an area of 1.40 million hectares with a productivity of 2839 kilograms per hectare. By 2022, India would demand 45 million metric tons of maize. However, just 15% of maize planted land is irrigated, and water scarcity has posed a challenge to production because in many countries it is grown in locations where rainfall is 300–500mm, which is close to or below the critical level for a good yield (Hanjra and Qureshi, 2010). A 40 percent water reduction resulted in a 39.3 percent fall in maize yield (Daryanto *et al.*, 2016; Dicko *et al.*, 2018). It has a significant impact on the physiological, biochemical, and molecular aspects of the plant (Usmani *et al.*, 2020). Drought causes reduced leaf development, cellular desiccation, free radical generation, enzymatic activity disturbances, membrane damage, lipid peroxidation, carbon assimilation, protein degradation, and photosynthesis (Hussain *et al.*, 2019; Narayan *et al.*, 2021). Drought stress during the vegetative period can cause maize to develop at a slower rate, extend the vegetative growth stage, redirect roots, and change carbohydrate distribution. Short-term water shortages have been observed to result in dry weight losses of 28-32 percent during the vegetative development stage and 66-93 percent during the tasselling and ear formation stages, respectively (Cakir, 2004). Long-term drought (21 days) during the pre-flowering stage has also been proven to decrease leaf size and internodes, delay tassel and silk emergence and result in yield losses of 15 to 25% (Nesmith and Ritchie,

1992). Drought stress for five days around pollination causes improper embryo development and drop in kernel number (Zinselmeier *et al.*, 1999). The photosynthesis of the maize ear leaf contributes significantly to the growth of biomass. Five or six leaves near and above the ear produce the majority of the photosynthate for kernel yield. Drought stress causes a significant decline in photosynthetic rate, limiting growth by diminishing the size of the "source" in plants (Liu *et al.*, 2015b).

b. Mung bean [*Vigna radiata* (L.) R.Wilczek]: is India's third-largest pulse crop, accounting for almost 16% of the country's total pulse area. It is primarily grown in Asian countries, although it has spread to portions of Africa, Australia, and South America (Pratap *et al.*, 2020). It is planted on around 4.5 million hectares in India, yielding 2.5 million tonnes with a productivity of 548 kilograms per hectare and accounting for 10% of total pulse production. Karnataka produces 1.43 million tonnes on a land area of 4.21 million hectares with a yield of 339 kilograms per hectare (Anonymous, 2021). It has a strong root system architecture that is actively involved in symbiotically fixing atmospheric nitrogen into the soil (approximately 58–109 kg/ha) (Lindström and Mousavi, 2020). Despite being a valuable crop, mungbean yield has remained flat due to drought stress (Nair and Schreinemachers, 2020), which affects all phases of crop growth (Pratap *et al.*, 2020). It is more sensitive throughout the reproductive period, which encompasses a sequence of events from anthesis to maturity. Drought stress in mungbean impacts seed germination and seedling establishment in the early stages, resulting in low crop growth due to reduced cell division and cell elongation (Hussain *et al.*, 2008). According to Zare *et al.* (2013), drought stress reduced mungbean yields by 51 percent to 85.50 percent. In comparison to other main crops, progress in generating stress-tolerant cultivars of mungbean through genetics and plant breeding has been slow (Cheng and Tian, 2011). This is due to the lack of genetic diversity in cultivated mung beans. After all, plant breeding yielded only a few parental lines, necessitating the use of non-conventional methods (like endophytes treatment) to improve drought tolerance in the mungbean.

c. Soybean (*Glycine max* (L.) Merr.): is the world's most widely cultivated edible oil, protein, and feedstock crop. It is grown on around 12.09 million hectares in India, with

a total production of 11.22 million tonnes and a productivity of 928 kilograms per hectare. Karnataka produces 0.36 million tonnes on 0.32 million hectares with a productivity of 1137 kilograms per hectare (Anonymous, 2020). Soybean plants are more sensitive to drought stress during the seed filling stage than a vegetative period because various energy substances such as amino acids and sugars are rapidly transported, synthesized, and stored in seeds during the seed filling stage, which determines seed size, weight, and composition as well as final yield (Farooq *et al.*, 2017; Zhou *et al.*, 2019). Drought stress reduces photosynthetic carbon assimilation capacity and has a significant impact on soybean seed weight (Du *et al.*, 2020). Due to a lack of water, drought-stressed soybeans have smaller leaves and reduced height by 4.3 percent (Navari-Izzo *et al.*, 1990; Hao *et al.*, 2013, Mak *et al.*, 2014). Drought stress at the vegetative stage, in particular, lowered plant height more than drought stress at the R2 (full flowering), R4 (complete pod formation), and R6 (full seed filling) phases (Li *et al.*, 2013; Maleki *et al.*, 2013). Many soybean accessions have exhibited reduced root lengths and dry biomass accumulation during drought circumstances (Thu *et al.*, 2014). Drought alters root architecture (root depth, root branching density, and root angle), as well as the partitioning of root to shoot biomass as root mass increases (Franco *et al.*, 2011; Fenta *et al.*, 2014).

d. Brinjal (*Solanum melongena* L.): a member of the Solanaceae family and belonging to the Solanum L. genus, is grown in tropical and subtropical climates around the world (Hirakawa *et al.*, 2014, Taher *et al.*, 2017). It is grown on around 0.723 million hectares in India, yielding a total of 12.345 million tonnes with a productivity of 17.5 metric tons per hectare. Karnataka produces 0.43 million tonnes on 0.017 million hectares with a 25.34 metric ton per hectare productivity (Glance, 2018). Throughout their whole biological cycle, eggplants are extremely vulnerable to abiotic stresses. They are quite susceptible to water deficits, as they require a large amount of water for growth and development (Fu *et al.*, 2013). Drought stress in the brinjal crop limits its growth, development, and yield. Drought has been demonstrated to have a negative impact on *S. melongena* plant height, total dry weight, and fruit yield (Rodan *et al.*, 2020; Plazas *et al.*, 2019). When eggplant exposed to various drought stress durations, it showed a

reduction in transpiration rate, stomatal conductance, and photosynthetic rate (Sarker *et al.*, 2005). Because eggplant is one of the most important crops for the Mediterranean region, and climate change will increase the frequency and intensity of combined stress, addressing climate-resilient vegetable issues is critical.

2.11 Response of crop systems and fungal endophytes to Polyethylene glycol induced water stress

In vitro assays most typically involve the introduction of stress using osmotic agents such as polyethylene glycol (PEG-6000), which is chemically inert, non-ionic, and cell impermeable due to its high molecular weight, effectively simulating drought without causing any deleterious effects (Khakwani *et al.*, 2011). PEG is a better chemical to use to cause water stress (Kaur *et al.*, 1998). Many research has employed polyethylene glycol (PEG) as an abiotic stress inducer to select drought-tolerant and sensitive germplasm (Jatoi *et al.*, 2014). PEG induces an artificial water deficit, affecting water intake, turgor pressure, and cell division, all of which lead to a reduction in shoot and root growth. This conclusion was consistent with the findings of Khakwani *et al.* (2011), who found that under PEG-induced stress, shoot and root lengths of wheat cultivars were significantly reduced compared to the control.

Polyethylene glycol (PEG) has been used to intentionally create drought stress in a variety of crops, including mungbean (Saima *et al.*, 2018), soybean (Vijay *et al.*, 2018), maize (Khodarahmpour, 2011), and brinjal (Siaga *et al.*, 2016). In several crop plants, an increase in PEG concentration resulted in a drop in seedling vigor and germination percentage. Dutta and Bera, (2008) used PEG to evaluate 15 mungbean genotypes and found that the seedling's drought tolerance index was a very consistent parameter for screening genotypes for drought responsiveness. Bibi *et al.* (2010) found that drought stress causes a considerable reduction in taproot length and diameter. Aslam *et al.* (2013) evaluated 17 mungbean genotypes for drought tolerance at the seedling stage, finding that early growth was the best selection factor for tagging drought tolerant/sensitive genotypes.

For measuring the reaction of fungus to water stress, several approaches involving PEG exist. Polyethylene glycol is nontoxic and is not metabolized by fungi, according to

research (Mexal & Reid, 1973). Most fungi produce compatible solutes to cope with water stress. Jennings and Burke, (1990) characterized it as chemicals, that can vary their concentration in the cell in response to changes in external water potential, allowing the cell to retain turgidity while not influencing enzyme activity. They accumulate in the mycelium before being translocated to the conidia during conidiation. The amount of sugar alcohols accumulated by mycelia changes quantitatively but not qualitatively when fungus grow under water stress (Pfyffer and Rast, 1988). PEG-induced matric water stress tolerance (-0.16 MPa to -2.96 MPa) for ericoid endophytes of Epacridaceae hosts was investigated by Hutton *et al.* (1996), who described three growth response types: Type I: generally limited growth after 5 weeks, Type II: maximum growth at control with decreasing growth as matric water stress increased, and Type III: maximum growth when matric water stress is present. Chen *et al.* (2003) investigated ericoid mycorrhizal (ERM) fungi for PEG-induced water stress tolerance (-0.05 MPa to - 2.24 MPa) and found that ERM endophytes function under water stress.

2.12 Plants-Endophyte crosstalk

The plant is a complex ecosystem with a versatile communal life because they are heavily populated by pro-and eukaryotic microorganisms and represents a biological system. This system exists as an information processing entity with complex processes of communication, occurring throughout the individual plant. The plant cellular information processing network constitutes the foundation for processes like growth, defense, and adaptation to the environment. The associated microorganisms and their investment in information conditioning are often ignored. Endophytes as plant partners are an indispensable integrative part of the plant system.

Microbiota is comprised of a diverse endophytic microorganism that plays a function in plant defense and assists the plant system survive in stressful situations (Kozyrovska, 2013). In the context of environmental influences, the plant regulates its interactions with beneficial and pathogenic microorganisms, and the outcome of these interactions is determined by the interacting partners and their surroundings. Individual plants have distinct microbiomes, and changes in plant-associated microbiome architecture

are mostly attributable to genetic control by the host. Vertically-transferred core microbial species, on the other hand, evolve with the plant host species (Johnston-Monje and Raizada, 2011).

First, endophytic microbes have a wide range of impacts on the functioning of a plant micro-ecosystem and may influence plant responses to numerous environmental changes, including climate change (Friesen *et al.*, 2011; Bodenhausen *et al.*, 2013). Second, plants and microbes both produce a wide range of identical metabolites with similar precursors. For example, a universal precursor for isoprenoids (carotenoids, quinones, hormones, and secondary metabolites that aid in plant defense and communication) can be produced in two ways, and both are found in eubacteria and archaea (Kirby and Keasling, 2009). Howitz and Sinclair, (2008) developed the xenohormesis hypothesis, which says that stress-induced chemicals from plants can be sensed by microbes, which are capable of producing identical secondary metabolites. Plants and microbes, according to this theory, have homologous gene clusters (which are likely horizontally transmitted between domains) that might be cross-activated by plant hosts or endophytes in an emergency (e. g. abiotic stress). Third, endophytes, like other plant-associated microbes, have certain tools for interacting with plant hosts. Finally, it has been demonstrated in numerous laboratories that indigenous endophytic microbial communities detect external cues, altering community structure in response to biotic and abiotic stimuli affecting the plant system (Ardanov *et al.*, 2012; Thomas *et al.*, 2008).

2.13 Endophyte Services: What are they doing for the plants?

Due to an early plant-fungus association that has occurred during evolution, endophytes have been trended as an eco-friendly biotic approach for promoting plant growth. Waqas *et al.* (2012a) found that endophytic fungal isolates (*Chrysosporium pseudomerarium*, *Aspergillus fumigatus*, and *Paecilomyces* sp.) improved seed germination, vigor index, shoot and root length by breaking down cellulose and providing carbon to seedlings. Murphy *et al.* (2017) found that using endophytic fungus to treat barley and oat seeds boosted seed germination and subsequent seedling growth. Under normal and stressful conditions, seed priming with beneficial fungi and bacteria can significantly

improve seed germination and emergence, seedling establishment, crop growth, and yield parameters (Prasad *et al.*, 2020; Ayesha *et al.*, 2021).

The combination of the endophytic fungus *P. indica* with a nitrogen fixer and a phosphorous solubilizer significantly increased finger millet growth, yield, and quality (Arunkumar and Shivaprakash, 2017). Inoculation of endophytic strain *P. indica* on *Brassica campestris* spp. *chinensis* L. increased plant growth, carotenoids, antioxidant pathways, phenolic acid, and flavonoids (Khalid *et al.*, 2017). *Trichoderma harzianum* increased yield, chlorophyll content, starch content, nucleic acid content, total protein content, phytohormone content in maize plants (Akladios and Abbas, 2014) and showed a significant increase in the plant height, photosynthetic rate, chlorophyll content, stomatal conductance, and also on the tiller and panicle number in rice (Doni *et al.*, 2018). *P. liquidambari* forms a symbiotic relationship with rice, assisting in nitrogen accumulation and uptake, as well as significantly increasing the growth and yield of rice (Yang *et al.*, 2014).

Rehman *et al.* (2018) investigated an endophytic *Pseudomonas* sp. strain that increased wheat growth and zinc status following seed priming. Despite the fact that the author claimed that seed priming increased the yield, whereas, soil and foliar application boosted protein content, zinc concentration in the aleurone layer, endosperm, and total grain. Inoculation with *P. indica* also increased photosystem and electron transfer chain activity, as well as the accumulation of proteins that protect primary metabolism, photorespiration, transporters, energy regulation, and autophagy. In an experiment with *Brassica oleracea* var. *acephala* (kale), Poveda *et al.* (2020) reported that aerial biomass of plants infected with *Fusarium* sp. and *Acrocalymma* sp. was doubled than that of control plants. Morsy *et al.*, (2020) isolated fungal endophytes from plants of high salt areas and drought-stressed habitats that confer abiotic stress tolerance to tomato plants. In comparison to non-symbiotic tomato plants, endophytes treatment provided salinity stress resistance and increased root biomass and fruit yield in both pot and field situations. In some cases, endophytic fungal enrichment resulted in a lower magnitude of growth. Reduction in growth in response to endophytic colonization can be seen as weak parasitism (Mandyam *et al.*, 2013), or as induction of host resistance resulting in the allocation of

carbon to the production of expensive defense compounds rather than to vegetative growth (Aime *et al.*, 2013).

2.14 Endophyte elicitation of drought tolerance

Endophytes are essentially involved in the growth and development of host plants, either directly or indirectly (Figure 2.2), by secreting various growth-promoting attributes such as phytohormone synthesis, nutrient acquisition, siderophore production, antibiotic phosphate solubilization, starch hydrolysis, and mitigating various biotic and abiotic conditions (Shade *et al.*, 2017). One of the best-known illustrations of endophytic fungi conferring stress tolerance to hosts was found in *Piriformospora indica*. Upon inoculation of Arabidopsis and Chinese cabbage with *P. indica*, derived from *Prosopis juliflora* roots from the Thar desert, the plants displayed drought tolerance by expressing drought-stress-related genes (Sun *et al.*, 2010). Endophyte-inoculated plants germinated faster in the second generation than endophyte-free plants, indicating that endophytes have the potential to improve wheat tolerance to heat and drought stress and witnessed increased plant height, average seed weight (ASW), total seed weight (TSW), and water-use efficiency (WUE) in endophytes inoculated wheat plants in the pot experiment (Hubbard *et al.*, 2014). Similarly, Bodhankar *et al.* (2019) investigated the effects of pre-treating maize seeds with the endophytic isolates *Corynebacterium hansenii* and *Bacillus subtilis*, which increase maize growth and physiology under drought stress following inoculation.

In comparison to uninoculated plants, drought-stressed plants inoculated with endophytes showed significant improvements in root and shoot fresh weight, photosynthetic rate, and stomatal conductance. Increased production of osmolytes such as sugars (fructans, glucose, and sucrose) and amino acids was reported as biochemical reactions (glutamic acid and asparagine). Under drought stress, endophyte-inoculated plants have a high concentration of gamma-aminobutyric acid (GABA). Similarly, Chen *et al.* (2017) found that inoculating wheat seedlings with the endophytic strain *Pantoea alhagi* isolated from *Alhagi sparsifolia* increased their growth under drought conditions and that the endophyte-treated plants had higher soluble sugar accumulation and lower malondialdehyde concentrations.

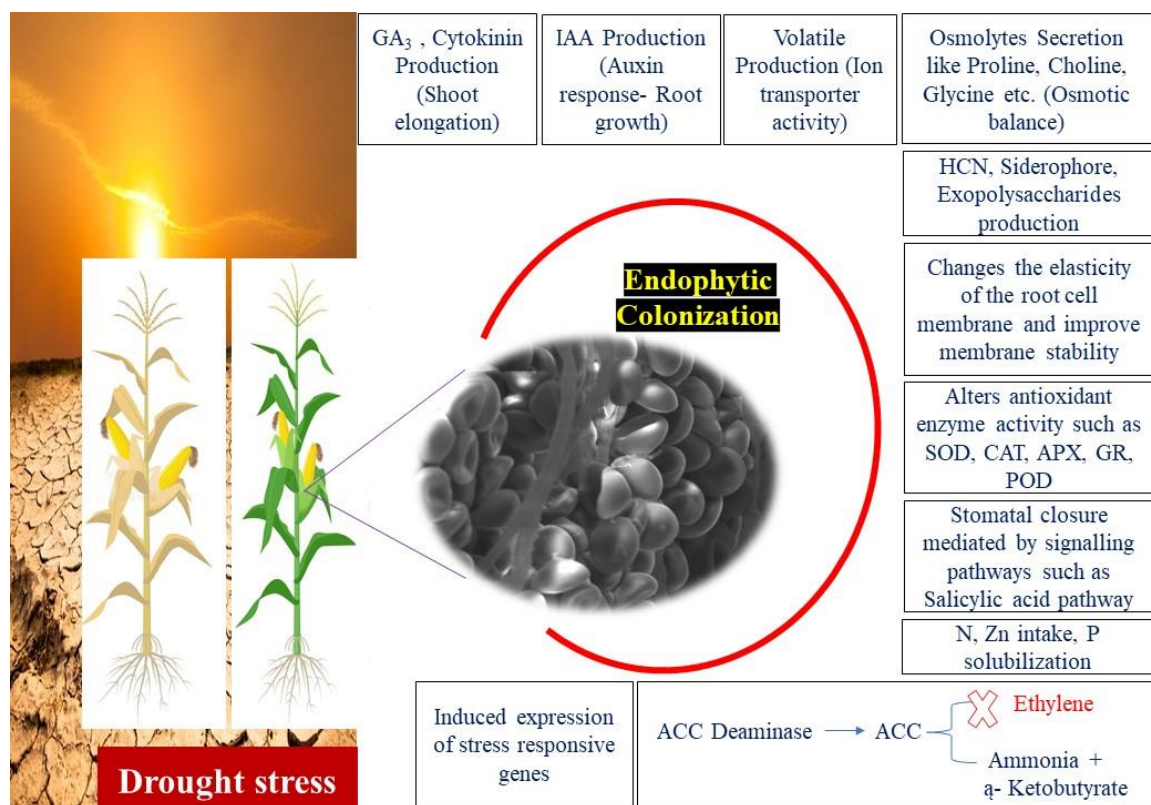


Figure 2.2: Endophyte mediated drought tolerance in plants
(Adapted from Vurukonda *et al.*, 2016; Ullah *et al.*, 2019; Verma *et al.*, 2021)

Piriformospora indica colonization readjusts plant metabolites and proteome to increase stress adaption and also maintains the presence of aquaporins in drought-stressed plants (Ghaffari *et al.*, 2019). Sandhya *et al.* (2017) found that some endophytic bacterial strains obtained from diverse crops have growth promotion capacity as well as drought tolerance up to (1.02) matric potential. Drought stress was alleviated in the grass *Brachypodium distachyon* with the help of an endophytic bacteria *Bacillus subtilis* B26, which also increased stress-responsive genes (Gagne-Bourque *et al.*, 2015).

a) Hormonal regulation

Phytohormones are widely known for their function in regulating plants' normal physiological and metabolic behavior under stress conditions through adaptive responses. The most prevalent are auxin (IAA), cytokinin (CK), gibberellin (GA), ethylene, and abscisic acid (ABA). Additional phytohormones that may influence plant growth under

normal or stress conditions include salicylic acid (SA), jasmonic acid (JA), nitric oxide (NO), nitrogen dioxide (NO₂), strigolactone (SL), and brassinosteroids (BR). Under drought stress, the hormones auxin and cytokinin increase stomatal opening, whereas ABA and ethylene regulation causes stomatal closure (Khan *et al.*, 2020).

Endophytic microbes produce hormones or stimulate the production of native plant hormones, influencing plant development and signaling (Pinski *et al.*, 2019). Endophytic microbial strains produce the phytohormones ethylene, IAA, GA, and cytokinins, which affect the growth of host plant cells and tissues directly or indirectly (Verma *et al.*, 2017). Endophyte-produced phytohormones are thought to drive plant cell development to induce nutrient release to the endophytes (Gupta *et al.*, 2019). Waqas *et al.* (2017) found that inoculating soybeans with phytohormone secreting *Galactomyces geotrichum* endophyte increased macronutrient absorption. The auxin transport capability of a strain of *Pseudomonas*, improved the architecture of the Arabidopsis root system (Zamioudis *et al.*, 2015). Auxin synthesis by strains of *Pseudomonas* sp. and *Pantoea dispersa* isolated from rice seeds was reported (Verma *et al.*, 2017), after inoculating rice seedlings with these microbes improved root and root hair growth. In comparison to control, Hamayun *et al.* (2009) observed that the culture filtrate of the endophyte *Aspergillus fumigatus*, dramatically improved seed germination and increased plant growth in soybean plants. GA₃, GA₄, and GA₇, as well as inactive GA₇, GA₁₉, and GA₂₄ secreted by *A. fumigatus*, aided soybean plant growth. Endophytic bacterial strain *Bacillus amyloliquefaciens* from rice seeds produced gibberellins (GAs) which help in boosting host physiology (Shahzad *et al.*, 2016), and produced markedly more salicylic acid, and had lower levels of endogenous abscisic acid. IAA and GA produced by *Sphingomonas* sp. LK11 increased tomato seedling growth (Khan *et al.*, 2014). Similarly, *S. mutabilis* strain IA1 from the Saharan desert region produced IAA and GA, which aided wheat seedling growth (Toumatia *et al.*, 2016). Endophytes that can produce GA have a positive impact on the growth and production of many agricultural plants. For example, under dry conditions, the GA-secreting rhizobacterium *Pseudomonas putida* H-2-3 boosted soybean growth (Kang *et al.*, 2014). Endomicrobiota auxin production stimulates ACC synthase, increasing ACC concentration, which activates ACC deaminase (Argueso *et al.*, 2007). The *Limonium*

sinense produced ACC deaminase, which enhanced overall plant development when exposed to salinity (Qin *et al.*, 2014).

ABA protects plants from abiotic stresses by regulating multiple stress-responsive genes and preventing water loss through stomatal closure (Zhang *et al.*, 2006). It has previously been reported that salt stress causes an increase in ABA content in plants and that fungal interaction with plants can regulate ABA production (Porcel *et al.*, 2012). Khan *et al.* (2011) found that salt-injured soybean plants colonized by *Penicillium minioluteum* had lower ABA levels than untreated plants. Thus, the association with endophytic fungi reduces ABA levels (Khan *et al.*, 2015a). In many plants, jasmonic acid (JA) induces the biosynthesis of defense-related proteins and protective secondary metabolites in response to abiotic stress (Shinozaki and Yamaguchi-Shinozaki, 2007), whereas salicylic acid (SA) is a signaling molecule that aids in stomatal behavior, respiration, and the generation of antioxidants (Sharma *et al.*, 1996; Raskin, 1992). Endophyte infection reduces stress by lowering abscisic acid levels, changing jasmonic acid levels, and increasing salicylic acid levels (Waqas *et al.*, 2012b). Induced systemic resistance to abiotic stressors is increased by the symbiotic interaction of fungi and plants, and high endogenous SA can buffer the harmful consequences of ROS buildup (Khan *et al.*, 2013). As a result, it is possible to conclude that increasing phytohormone production through the use of endophytic bacteria is a viable strategy for achieving sustainable agriculture under stressful conditions (Vaishnav *et al.*, 2019).

b) Siderophore production

Under iron limiting conditions, endophytic microorganisms have a greater ability to produce organic acid and siderophores (low molecular weight iron-chelating chemicals) (Ahmed and Holmstrom, 2014). According to certain studies, endophytes have a stronger affinity for Fe^{3+} ions than phytosiderophores. Plant hosts that aren't infected with microorganisms have high levels of iron deficiency (Carvalhais *et al.*, 2013). In the root endophytic microbiota of rice plants, Sessitsch *et al.* (2012) found considerable overexpression of genes associated with siderophore synthesis, reception, and iron storage. Apart from delivering iron to hosts, siderophores are thought to play an important function

in biocontrol by quenching the bioavailable iron, denying phytopathogens access (Aznar and Dellagi, 2015).

c) Phosphate solubilization

Phosphorus is a necessary mineral for plant growth, yet due to its chemistry, it may be relatively unavailable to plants. The majority of phosphorus in soil comes in the form of phosphates, which are mainly metal complexes that bind them to minerals or organic materials. To maximize crop productivity, synthetic P fertilizers must be applied to such soils. Synthetic fertilizers, on the other hand, have several negative consequences on both biotic and abiotic ecosystem components. Exploiting local microorganisms, particularly those capable of solubilizing insoluble nutrients in the soil, is an alternate technique for reducing the usage of synthetic fertilizers. As a result, inorganic phosphate solubilization is a crucial mechanism in which plant-associated bacteria and fungi promote plant growth.

Fungi are more efficient in solubilizing bound nutrients than other soil microorganisms, (Pradhan and Sukla, 2006). Gupta *et al.* (2007) observed that fungal isolates solubilized $\text{Ca}_3(\text{PO}_4)_2$ and rock phosphate more efficiently than bacteria. *P. indica* plays a crucial role in P transfer to plants, especially in P-deficient environments (Kumar *et al.*, 2011). Furthermore, Vassilev *et al.* (2006) found that *Aspergillus* and *Penicillium* effectively solubilize rock phosphate, and fungal association improves phosphorous uptake capacity by plants (Alvarez *et al.*, 2009), indicating that phosphate-solubilizing microorganisms are beneficial for long-term agriculture (Khan *et al.*, 2007). *Curvularia geniculata*, a dark septate endophyte isolated from *Parthenium hysterophorus* L., has the potential to solubilize multiple phosphorus (P) sources [AlPO_4 , FePO_4 , and $\text{Ca}_3(\text{PO}_4)_2$] and produce indole acetic acid when inoculated into pigeon pea (*Cajanus cajan*) plants. The plants grew significantly faster than non-inoculated control plants, suggesting that *C. geniculata* enhances plant development through phosphorus solubilization and phytohormone production (Priyadharsini and Muthukumar, 2017).

d) Starch hydrolysis

Amylases are starch degrading enzymes that catalyze the hydrolysis of polysaccharide internal glycosidic linkages. The majority of amylases come from soil fungi like *Aspergillus*, *Penicillium*, and *Rhizopus* (Pandey *et al.*, 2000). There are limited publications on endophytic fungus amylases, which are mostly studied for beneficial secondary metabolites with various bioactivities. Endophytic fungus *Gibberella pulicaris*, *Acremonium* sp., *Synnematosus* sp., and *Nodulisporium* sp. produced raw starch degrading enzymes (Marlida *et al.*, 2000). Maria *et al.* (2005) found that a few endophytic isolates from the mangrove angiosperm *Acanthus ilicifolius* L. and the mangrove fern *Acrostichum aureum* L. produced amylase.

e) Photosynthetic capacity

The chlorophyll content has a direct effect on the photosynthetic rate because it is the principal pigment for photosynthesis in plants. Soil Plant Analysis Development (SPAD) measurements determine the relative chlorophyll content of leaf tissue by measuring the transmittance of tissues in the red and infrared regions (Bussotti and Pollastrini, 2015). They are a simple, quick, and non-destructive method for determining chlorophyll content in leaves (Li *et al.*, 2009). PSII photosynthetic activities are positively correlated with chlorophyll content, especially under water-stressed situations. Meng *et al.* (2001) discovered that, within specific limits, the chlorophyll content was positively connected to the leaf net photosynthetic rate. The delay in lowering photosynthetic activity by infected endophytes corresponded to a delay in chlorophyll breakdown. *F. acuminatum* significantly increased SPAD values under drought stress conditions (He *et al.*, 2019; Aghai *et al.*, 2019).

Photosynthesis is one of the most important physiological processes in plants that are most vulnerable to water deficit and is directly related to the accumulation of biomass (Rozpdek *et al.*, 2015). Liang *et al.*, 2001 reported that under extreme drought, the net photosynthetic rate of E+ plants was considerably higher than that of E- plants in *Lolium perenne* L. Furthermore, Morse *et al.*, 2007 showed that under extreme drought stress in *Festuca*, the net photosynthetic rate, stomatal conductance, and transpiration rate

of E+ plants were considerably higher than those of E- plants. The photosynthetic rate, transpiration rate, and stomatal conductance were all higher in the endophyte-treated *P. edulis* than in the control group, demonstrating that endophyte might improve plant photosynthesis and thereby promote growth (Yuan *et al.*, 2018). Xu *et al.*, 2021 studied that endophyte infection improved all photosynthetic indicators of *F. sinensis* under drought stress, except for intercellular carbon dioxide concentration. These findings suggest that endophytes can improve *F. sinensis* drought resistance by improving photosynthesis, which is consistent with the finding that endophytes can alter the host plant's strategy and enhance the metabolism and accumulation of substances in host plants to adapt to water shortages (Xia *et al.*, 2018).

f) Water relations and relative membrane permeability (RMP)

In many crops, leaf relative water content (RWC) is a key indicator of water stress and drought resistance (Jolly *et al.*, 2014). It explains how water is used to keep cells hydrated when there is a drought. Plant water status is improved by endophyte association, which increases RWC (Odokonyero *et al.*, 2016). Under drought conditions, endophyte association in *Brachiaria* cultivars (particularly cv. Tully) increases cellular apoptosis susceptibility proteins (Sun *et al.*, 2010) which stimulates aerenchyma formation (*i.e.*, intercellular gas-filled lacunae) in the leaf sheath and root tissues, where it holds apoplastic water and allows for water conservation in plant cells, contributing to higher values of leaf RWC (Touchette *et al.*, 2014; Cardoso *et al.*, 2014; Cardoso *et al.*, 2013). Drought reduced relative water content in both inoculated and un-inoculated plants (Naveed *et al.*, 2014), although endophyte inoculation significantly increased relative water content by 30% compared to un-inoculated controls. This could be due to the infected plants developing a more effective root system and reducing the inhibitory effect of drought on roots (Dodd *et al.*, 2004).

In drought-stressed E+ plants, higher RWC indicates less cell wall damage and a greater ability of cells to absorb and save water than in drought-stressed E- plants (Malinowski and Belesky, 2000). As a result, the endophytic connection may aid in the preservation of cell wall structure under drought. Endophytes may cause alterations in the

host cell wall by upregulating genes involved in the production of hemicellulose, cell wall modifiers, and cell wall proteins (Dupont *et al.*, 2015). When compared to normal irrigation, drought stress accelerated relative membrane permeability (RMP) in both inoculated and uninoculated seedlings, however, endophyte inoculation helped seedlings retain the RMP and reduced leaf damage by 43% compared to uninoculated seedlings (Naveed *et al.*, 2014). A positive correlation between drought stress sensitivity and membrane damage (EL) was observed by Vardharajula *et al.* (2011) and Sandhya *et al.* (2010).

g) Antioxidant Regulation by endophytes under drought stress

Plants naturally produce reactive oxygen species (ROS) as a result of their normal metabolic activity (Foyer and Noctor, 2016). Under normal conditions, ROS work as signaling molecules and help to maintain symbiosis with intracellular symbiotic organisms (White *et al.*, 2018). However, plants require ROS scavengers like antioxidant amino acids and enzymes to maintain homeostasis. Excess ROS and oxidative damage to nucleotides, proteins, and lipids, as well as cell death, resulting from a disruption of the equilibrium between ROS formation and scavenging (Rehman *et al.*, 2018). Furthermore, depending on the kind of cell, the level of stress, and the ROS gene network, each cell compartment has its own ROS homeostasis or signaling pathways (Dietz *et al.*, 2016).

Endophytic microbe colonization increases plant levels of antioxidant enzymes such catalase (CAT), superoxide dismutase (SOD), peroxidase (POD), and ascorbate peroxidase (APX) (Lata *et al.*, 2018) or non-enzymatic antioxidant compounds like ascorbate (AsA), glutathione (GSH) and carotenoids (Prasad *et al.*, 2013). Endophytes stimulate the production of antioxidants to counteract a range of free radicals which are necessary for normal cellular function. Osmolyte production maintains the sodium-potassium ratio, which nullifies the osmotic effect generated by stress conditions (Barnawal *et al.*, 2016). Endophyte inoculations dramatically reduce accumulated ROS in the plant cell (Redman *et al.*, 2011), via activating antioxidant enzymes. Under saline conditions, lower levels of CAT, APX, and GR were noticed in barley root tissues, but when *Piriformospora indica* colonized the barley roots, the antioxidant enzymes and

ascorbic acid levels increased (Baltruschat *et al.*, 2008), due to which hydrogen peroxide, superoxide, and lipid peroxidation were considerably reduced (Sadeghi *et al.*, 2020).

Drought promotes plant chlorophyll degradation, MDA accumulation, and tissue water loss, all of which cause photosynthesis to decrease (Xiang *et al.*, 2018). Plants accumulate proline in response to drought stress, which reduces tissue and cell damage (Sun *et al.*, 2010; Hasanuzzaman *et al.*, 2012; He *et al.*, 2017). Accumulation of proline is thought to protect membranes and proteins from ROS damage and to aid in osmotic adjustment in abiotic stress situations (Anjum *et al.*, 2011; Ullah *et al.*, 2019). Endophytes improve the drought resilience of plants by actively accumulating proline in the host tissue and lowering MDA levels (Hahn *et al.*, 2008; Nagabhyru *et al.*, 2013). *P. tabulaeformis* seedlings treated with endophyte accumulated less MDA, broke down less chlorophyll, and enhanced proline accumulation than seedlings treated with other treatments (Zhou *et al.*, 2021). Furthermore, under stress conditions, rice infected with *B. amyloliquefaciens* considerably increased growth as well as levels of some critical antioxidant amino acids as cysteine, aspartic acid, glutamic acid, phenylalanine, and proline.

h) Prospecting stress-responsive genes to understand the relevance of endophytes under drought stress

Plants can sense environmental changes, generate and transmit perceptual signals, and regulate their physiology by controlling the expression of many regulatory and functional genes in a complex and integrated manner (Rasheed *et al.*, 2016). Plants can change the expression of different genes in response to drought. These genes can be classified into two broad classes based on their potential roles (Shinozaki and Yamaguchi-Shinozaki, 2007). The first group includes transcription factors (TFs), phytohormones, phosphatases, and protein kinases, as well as other genes involved in transcriptional control and signaling cascades. Heat shock proteins (HSPs), dehydrins, senescence-related genes, membrane protectants, osmoprotectants, transporters, antioxidants, and other genes are among the second set of genes that protect plant cells from stress. Many plant species under stress have been studied using transcriptome analysis to assess expression levels and gene architecture (Fracasso *et al.*, 2016; Haider *et al.*, 2017). It's been used to find genes that

control the intricate interactions and metabolic processes that occur when plants are stressed by drought (Zhao *et al.*, 2018). The endophytic association may help maintain cell wall structure under drought stress by up-regulating cell wall-associated genes that manufacture hemicellulose, cell wall modifiers, and cell wall proteins in the host cell wall (Dupont *et al.*, 2015).

The transcriptomics technique demonstrated that when endophyte *B. phytofirmans* PsJN was inoculated into potato plants, transcripts involved in cellular homeostasis, transcriptional regulation, and ROS detoxification were enhanced under drought stress. This shows that endophytes can detect physiological changes in plants and adjust gene expression accordingly (Sheibani-Tezerji *et al.*, 2015). Plants colonized by an ACC deaminase-producing strain *Enterobacter cloacae* had increased transcripts of genes involved in cell division and proliferation (Hontzeas *et al.*, 2004). In plants colonized by *P. fluorescens*, upregulation of auxin response genes and downregulation of ethylene response genes were found (Wang *et al.*, 2005).

Under drought stress, maize seedlings treated with *Pseudomonas putida* strain FBKV2 demonstrated upregulation of genes involved in β -alanine and choline biosynthesis, heat shock proteins, and late embryogenesis abundant (LEA) proteins, all of which could play a role in drought tolerance (SkZ *et al.*, 2018). *P. indica* colonization of soybean plants stimulated genes involved in the phenylpropanoid and lignin pathways, both of which are known to play a role in oxidative stress tolerance (Bajaj *et al.*, 2018). *P. chlororaphis* colonized plants of *Arabidopsis thaliana*, showed increased salicylic acid regulated genes, and ethylene response genes (Cho *et al.*, 2011). In pepper plants, inoculated with *Bacillus licheniformis* under drought stress, six differently expressed stress proteins were discovered (Lim and Kim, 2013). In wheat, inoculated with *Bacillus amyloliquefaciens* and *Azospirillum brasilense*, stress-related genes APX1, SAMS1, and HSP17.8 were induced (Kasim *et al.*, 2013). In *Paenibacillus polymyxa* inoculated *A. thaliana*, increased mRNA transcripts of a drought response gene, EARLY RESPONSE TO DEHYDRATION 15 (ERD15), improved drought tolerance (Timmusk and Wagner, 1999).

2.15 Crop growth modulation using fungal endophytes under field conditions

The balanced interactions of endophytes with their host plants during the entire symbiotic phase allow for improved adaptation to environmental changes (Rosenberg and Zilber-Rosenberg, 2018). However, the effectiveness of these microbes at the field level is highly dependent on their performance, which necessitates additional research into the barriers to effective product development.

Interestingly, there is a vast amount of research on beneficial microbes for plants that may be utilized to guide the screening process, develop best practices for validation, and uncover some problems that may prevent benefits from being transferred from greenhouse to field. Numerous publications support the beneficial effects of fungal endophytes on plant development and performance in adverse conditions (Gundel *et al.*, 2013). In the United States, Australia, and New Zealand, *Epichloe* (Class 1) endophyte strains have been employed to boost the productivity of forage grasses in the field and the robustness of turf grasses (Young *et al.*, 2014; Kauppinen *et al.*, 2016). Endophyte-mediated plant trait improvement provided roughly \$200 million per year to the New Zealand economy (Johnson *et al.*, 2013). *Trichoderma* sp. which lives on the roots of stressed plants improves yields by activating metabolic processes that convert the hazardous reactive oxygen species produced during stress into less toxic chemicals (Harman *et al.*, 2021). *Fusarium equiseti* increased *T. subterraneum* herbage yield in the greenhouse, while *B. spectabilis* improved *T. subterraneum* forage quality by reducing fiber content and *P. pratensis* fodder quality by increasing crude protein. *S. intermedia* increased Ca, Cu, Mn, Pb, Tl, and Zn mineral uptake in sub clover, while *M. hiemalis* increased K and Sr uptake in Kentucky bluegrass, demonstrating the potential of fungal endophytes to improve herbage productivity and nutritional value of fodder (Garcia-Latorre *et al.*, 2021). Despite the immense potential of endophytes in the treatment of abiotic stress, field-rational tests are few. As a result, more study is needed to address these constraints and characterize how plant-endophyte interactions function in the field.

Conclusion: Drought is one of the foremost serious abiotic stresses experienced worldwide. As a result, endophytes have the potential to be used as a protective component in agricultural operations under extreme climatic conditions, as well as regulating plant physiological stress responses. Exploiting endophytes to increase agricultural productivity is a fascinating prospect, whereas, manipulation of the endophytic microbiota for enhancing host development and productivity, on the other hand, has remained a difficult task. Understanding how beneficial endophytes are recruited, optimized, and maintained within the host at different growth phases will require more research. Even though much work has to be done, endophytes may soon act as partial or complete alternatives for chemical fertilizers.

III MATERIAL AND METHODS

The main goal of the experiment was to examine the role of a few selected endophytic fungi in modulating plant growth and development under drought stress. This chapter describes the details of materials and methods used to conduct various experiments to meet the objectives of the study. *In-vitro* experiments were conducted at the Department of Seed Science and Technology, University of Agricultural Sciences (UAS), Gandhi Krishi Vignana Kendra (GKVK), Bengaluru, and Department of Crop Physiology, UAS, GKVK, Bengaluru. Pot and field experiments were conducted at NSP, UAS, GKVK, Bengaluru. The details of the experimental methodology are described below.

3.1 Source of endophytic fungal isolates

Endophytic fungi from habitat-adapted plants have been collected and preserved at School of Ecology and Conservation Laboratory, Department of Crop Physiology, UASB, GKVK, Bengaluru. A few fungal endophytes from the library were partially characterized for their potential to impart abiotic stress tolerance in earlier studies (Jagadeesh, 2014; Arya Sunil, 2019; Sowmyashree, 2019; Yashaswini, 2019). For this study, eight fungal endophytes (Table 3.1) were chosen and revived on fresh potato dextrose agar medium. These endophytes were first isolated from various plant species that grew in diverse parts of India like Kargil (J&K), Pangong Tso, Tamil Nadu, Karnataka, Kerala, and Rajasthan.

Table 3.1. Sites in cold deserts of the Himalayas from where plants were sampled for endophytic fungal isolation

Sl. No.	Sample Code	Location	Latitude (° N)	Longitude (° E)	Altitude (AMSL)	Plant part/ Host
1	K-23	Kargil (J&K)	34°34'22"	76°7'57"	2750	Wild plant
2	K-26	Kargil (J&K)	34°34'22"	76°7'57"	2750	Wild plant
3	P-10	Pangong Tso	33°43'2.74"	78°53'29.08"	4250	Wild plant
4	P-37	Pangong Tso	33°43'2.74"	78°53'29.08"	4250	Wild plant
5	SF-5	Tamil Nadu	11.1271° N	78.6569° E	-	<i>Suaeda filiformis</i>
6	PJ-9	Karnataka	15.3173° N	75.7139° E	-	<i>Protis juliflora</i>
7	V4-J	Pokkali	9.9667°N	76.3168°E	-	<i>Oryza sativa</i>
8	LAS-6	Thar desert	27.4695° N	70.6217° E	-	<i>Lasiurus scindicus</i>

3.2 Crop systems

For the study, diversified crop systems such as maize (*Zea mays* L.) were obtained from National seed project, UAS, GKVK, Bengaluru. Green gram (*Vigna radiata* (L.) R.Wilczek) were obtained from Krishi Vigyan Kendra (KVK), Chamarajanagar. Soybean (*Glycine max* (L.) Merr.) were obtained from Zonal Agricultural Research Station (ZARS), All India Coordinated Research Project (AICRP) on soybean, UAS, GKVK, Bengaluru, and brinjal (*Solanum melongena* L.) from Indian Institute of Horticulture Research (IIHR), Hesaragatta, Bengaluru (Table 3.2).

Table 3.2: List of crop systems along with genotypes used in the present study

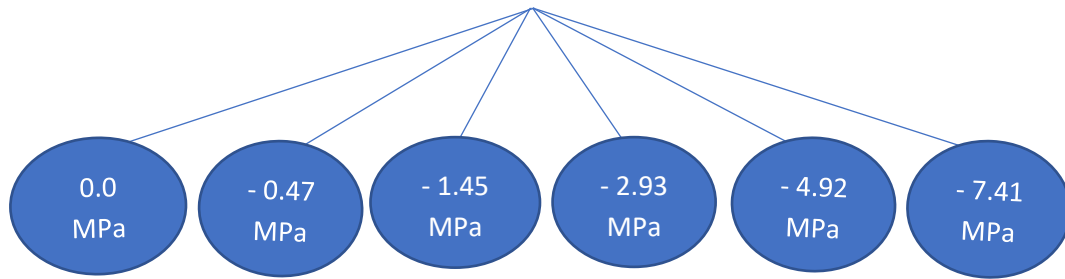
Sl. No.	Crop systems	Genotypes
1	Maize (<i>Zea mays</i> L.)	NAC-6002 (composite) and MAH 14-5 (hybrid)
2	Green gram (<i>Vigna radiata</i> (L.) R.Wilczek)	BGS-9 and KKM-3
3	Soybean (<i>Glycine max</i> (L.) Merr.)	JS-335 and KBS-23
4	Brinjal (<i>Solanum melongena</i> L.)	Arka Harshitha and Arka Unnathi

3.3 Polyethylene glycol (MW- 8000) induced screening for drought-sensitive genotypes of maize, green gram, soybean, and brinjal

Seed samples of all genotypes were surface sterilized using 70 percent ethanol for 30 seconds followed by sodium hypochlorite solution (3 percent) for three minutes and repeatedly washed with sterile water (Arnold *et al.*, 2000). The method followed for standardization was the paper towel method with four replications for each treatment. Different concentrations (5%, 10%, 15%, 20%, 25%) of PEG-8000 solution were prepared in sterile distilled water (Muddarsu and Manivannan, 2017). These concentrations are corresponding to the osmotic potential of (-0.47 MPa, -1.45 MPa, -2.93 MPa, -4.92 MPa, -7.41 MPa) respectively. Paper towel was soaked in the solution; excess solution was removed before exposing the seeds. Twenty-five seeds of each genotype were placed on the paper towels and incubated at 27°C in the growth chamber. Seedling root and shoot



Representative images of surface sterilized pre-germinated seeds of two genotypes each belonging to maize, green gram, soybean and brinjal



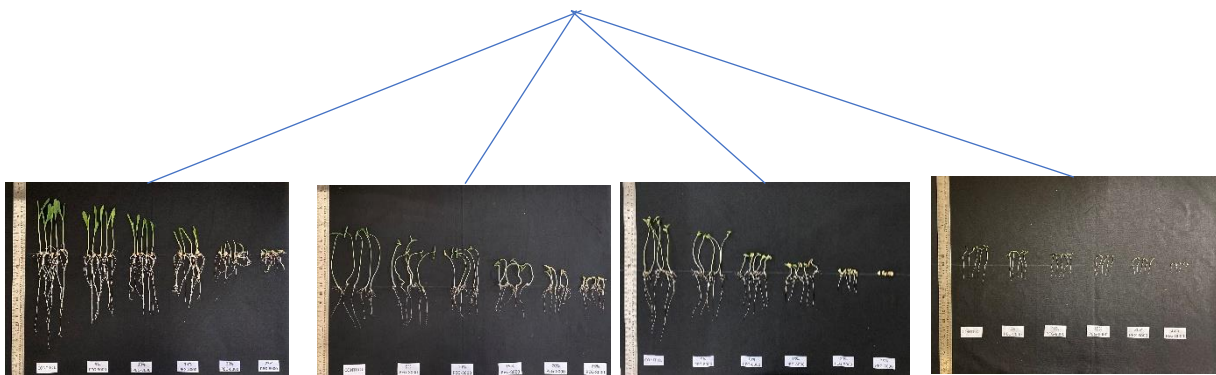
Drought stress: Different concentrations of PEG-8000 prepared to treat the above seeds



Seedling root and shoot length was recorded at final count of respective crop



LC₅₀ Value of PEG-8000 was calculated by Probit analysis and drought sensitive varieties/hybrid were identified



MAH 14-5

BGS-9

KBS-23

Arka Harshitha

Plate 3.1: Polyethylene glycol (MW- 8000) induced screening for drought sensitive genotypes of maize, green gram, soybean and brinjal

length were recorded at the final count day of the respective crop (Muddarsu and Manivannan, 2017). LC₅₀ value was calculated by Probit analysis using statistical software IBM SPSS statistics 20 (<https://www.ibm.com/in-en/analytics/spss-statistics-software>). The LC₅₀ was observed for all genotypes, and drought-sensitive genotypes from each crop system were selected and were used as a benchmark to screen different endophytes (Plate 3.1).

3.4 Pure culture response of fungal endophytes to matric induced water stress

The experiment was used as the first conformational test to verify that the selected endophytes can grow in drought stress conditions. Potato dextrose agar (PDA) agar plug to potato dextrose amended broth (PDB) was defined as the primary experiment. The combination reduces the impact of osmotic potential by utilizing chemically defined media and provides time for fungi to adjust to the environment. One 5 mm agar plug containing actively growing *mycelium* were placed into an autoclaved 250 ml Erlenmeyer flask that contained 100 ml of Potato Dextrose Broth and the targeted amount of Polyethylene glycol (PEG-8000) according to the protocol of Michel (1983): $\Psi = 1.22[\text{PEG}]^2T - 134[\text{PEG}]^2 - 4.4[\text{PEG}]$. Each isolate was assayed at control (PDB without PEG), -0.47 MPa, -1.45 MPa, -2.93 MPa, -4.92 MPa, -7.41 MPa. The Erlenmeyer flask was covered with sterilized tin foil, sealed with parafilm, placed in a shaker chamber (90 rpm at 27°C) for seven days. Whatman 8 filter paper was weighed to four decimal places, and mycelium was filtered from the broth. The filter paper was rinsed with distilled water to remove any residual broth. The washed filter paper with mycelium was placed in an oven for 12 hours before obtaining the final weight. Reported fungal growth weight is the final weight of the mycelium plus filter paper minus the original weight of the filter paper. Growth types were assessed as outlined in Hutton *et al.* (1996).

3.5 Morphological characterization of endophytic fungi

Fungal isolates characterized were subjected to morphological studies. The five-day-old endophytic fungal culture grown on PDA was mounted on a glass slide and stained with Bromo cresol purple/cotton blue to stain the fungal mycelia/spore/conidia and covered with coverslips. The slide was observed under the fluorescence microscope. Colony

characteristics like conidiophore morphology and conidia (color, size, shape) were observed. Other attributes like colony appearance (color, texture, etc.), margin characteristics, and depth of growth into the medium were observed using standard identification manuals (Barnett, 1960; Booth, 1971; Domsch and Gams, 1972; Ellis, 1976; Sutton, 1980; Arx, 1981).

3.6 Molecular identification of the selected fungal endophytes

3.6.1 Isolation of genomic DNA from endophytic fungi

Endophytic fungal isolates were grown on PDA media for five days at 25°C. The above fungal isolates were sub-cultured on potato dextrose broth (PDB) 100 ml and allowed to grow for five days at 25°C. The mycelial mat was filtered, stored at -80°C for DNA isolation. DNA was isolated using mycelial mat by a modified method of Lacap *et al.* (2003). Mycelial mat (100 mg) was macerated in pestle and mortar using liquid nitrogen along with a pinch of Poly Vinyl Pyridine (PVP) and extraction buffer (Working concentration: 2 X CTAB, 100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, pH 8.0 and 1% SDS). The mixture was transferred to 2 ml Eppendorf tube and β -mercaptoethanol (10 μ l) was added, vortexed (2 min), tubes were incubated in a water bath at 65°C for 30 min. The mixture was cooled and centrifuged (12,000 rpm for 10 min) and the supernatant was transferred to fresh 2 ml Eppendorf tubes to which 500 μ l of 12:24:1 (phenol: chloroform: isoamyl alcohol) was added and centrifuged at 12,000 rpm for 10 min. The supernatant obtained was transferred into 1.5 ml Eppendorf tubes and was suspended in chilled ethanol (70 %) and incubated at -20°C overnight. The samples were cooled to 4°C and centrifuged (12,000 rpm at 4°C for 10 min), ethanol was discarded, and the obtained pellet was air-dried until the smell of ethanol was removed. The pellet was dissolved in sterile water. To remove the RNA, 2 μ l of RNase (10 mg/mL) was added to the DNA solution and incubated at 37°C in a water bath for 30 min. The obtained DNA was fractionated on 0.8% agarose and visualized following staining with ethidium bromide and quantified using NanoDrop. A working solution of 50 ng/ μ L concentration was prepared and stored at 4°C for further use.

3.6.2 Amplification and sequencing

Internal Transcribed Spacer (ITS) region

Molecular identification of endophytic fungi was done by amplifying the 28S r ribosomal region of Internal Transcribed Spacer (ITS). ITS primers amplify the non-coding regions present between the conserved regions of 18S, 5.8S, and 28S rRNA genes thus helping to distinguish the fungi at the genus or species level. Forward and reverse primers, ITS1 and ITS4, respectively (White *et al.*, 1990) were used to amplify the highly variable ITS1 and ITS2 sequences surrounding the 5.8S-coding sequence and situated between the Small Subunit-coding sequence (SSU) and the Large Subunit-coding sequence (LSU) of the ribosomal operon. Polymerase chain reaction (PCR) was carried out to amplify the genomic DNA of endophytic fungus. All the constituents in the reaction were mixed and amplification of fungal (ITS1-5.8S-ITS2) ribosomal region was carried using thermocycler with the following PCR specifications, initial denaturation of 94 °C for 10 min followed by 35 cycles with denaturation of 94 °C for 1 min, the annealing temperature of 58 °C for 1 min, extension for 1 min at 72 °C and final extension at 72 °C for 10 min. The resulting amplified PCR product of expected size was resolved on the agarose gel (1 % (w/v)) prepared in 1X TAE (Tris-acetate-EDTA) buffer, stained with ethidium bromide, and documented under UV light in a trans-illuminator (Plate 3.2). Sequence details of ITS primers are listed in Appendix 1. The amplified DNA products was sequenced by Eurofins Genomics India Pvt. Ltd., Bengaluru – 560048. The sequence data received from the company was analyzed for homology using NCBI GenBank.

PCR components (25 µl)

- Master mix (2X)- 12.5 µl
- Forward primers (10 pmol)- 1.25 µl
- Reverse primers (10 pmol)- 1.25 µl
- Template DNA (~50 ng /µl) – 2.5 µl
- Sterile distilled H₂O – 7.5 µl

3.6.3 Sequence analysis and homology search

The fungal nucleotide sequences were used as queries to search the closest matches in the nucleotide database Basic Local Alignment Search Tool (BLASTn) at the NCBI GenBank (www.ncbi.nlm.nih.gov). Based on the maximum homology and total score, names were assigned to fungal endophytes using the criterion described by Higgins *et al.* (2007). Genus and species of the database match were accepted wherever the identity was $\geq 97\%$ and only the genus was accepted to fungal endophytes when the identity of fungal sequences was $\leq 96\%$ (Arnold, 2007; Higgins *et al.*, 2007).

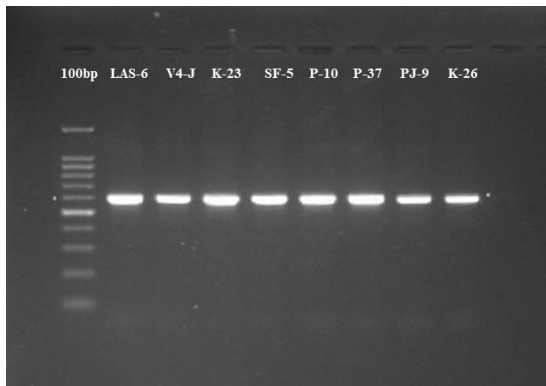
3.6.4 Phylogenetic analysis

Phylogenetic analysis was performed to know the relationship between identified species with the sequence of the same species deposited in the NCBI GenBank. The sequences were aligned using ClustalW incorporated in MEGA11 software (Kumar *et al.*, 2018), and the alignments were manually adjusted. Phylogenetic relatedness among the fungal species obtained from different habitat plants was determined by employing a UPGMA method (Sneath and Sokal, 1973) with 1000 bootstrap replications (Felsenstein, 1985).

3.7 Screening of endophytes for plant growth-promoting attributes

3.7.1 Phosphate solubilization assay

Eight fungal isolates obtained from pure cultures were preliminarily screened for their potential to solubilize TCP as insoluble inorganic phosphate sources on Pikovskaya (PVK) agar. One litre (1L) of PVK agar contained the following (g/L): 0.5g $(\text{NH}_4)_2\text{SO}_4$, 0.1g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02g NaCl, 0.02g KCl, 0.003g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.003g $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 5g $\text{Ca}_3(\text{PO}_4)_2$, 10g glucose, 0.5g yeast extract, 15g agar and 1000mL distilled water (Pikovskaya, 1948). The medium was autoclaved at 121°C for 15 min. Sterilized PVK agar was poured into sterilized Petri plates. Fungal mycelium plugs of each isolate grown on PDA at 28°C for 7 days were cut from the edges of each actively growing colony using a sterile cork borer (5 mm^3). Fungal mycelium plugs were then placed on Petri plates containing PVK agar supplemented with 0.5% TCP for 7 days at 28°C . Plugs of sterile



Ethidium bromide-stained agarose gel showing PCR amplified product of ITS rDNA gene of all selected eight endophytes



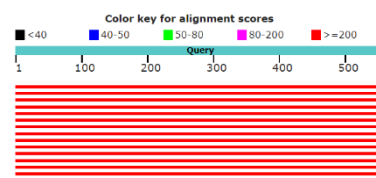
Ethidium bromide-stained agarose gel showing PCR amplified product of ITS rDNA gene, Tubulin and LSU of three finalized endophytes (K-23, LAS-6 and P-37)

TGAGGGTGTGGTTTGCTGGCAACAGCGTCCGCCCAAGTATTTTTTCACCCATGCTTTTTG
CGCACTTTTTGTTTCCTGGGCGAGTTTCGCTCGCCACCAGGACCCAACCATAAACCTTTTT
TTATGCAGTTGCAATCAGCGTCAGTATAATAATTCAATTTATTAANAACCTTCAACAACGG
ATCTCTTGGTTCTGGCATCGATGAAGAAGCGAGCGAAATGCGATACGTAGTGTGAATTGC
AGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGG
CATGCCTGTTGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTGGGCGTCTTTTTG
TCTCTCCCTTGTGGGGGAGACTCGCCTTAAACGATTGGCAGCCGACCTACTGGTTTT
CGGAGCGCAGCACAAATTTGCGCCTTCCAATCCACGGGGCGGCATCCAGCAAGCCTTTGT
TTTCTATAACAAATCCACATTTTGACCTCGGATCAGGTAGGGATACCCGCTGAACCTTAAG
CATATCAATAAGCGGAGGAA

Nucleotide Sequence



BLAST



Sequences producing significant alignments:

Select All None Selected 0

Alignments Download Link Clipboard Graphics Distance from results

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Connorsia capsicola clone HCC7912462.1 internal transcribed spacer 1 partial sequence, 5.8S ribosomal RNA gene and internal transcribed spacer 2 complete sequence, and large subunit ribosome	1011	1011	100%	0.0	100.00%	M527460.1
Setosphaeria rostrata genomic DNA sequence contains 18S rDNA gene, ITS1, 5.8S rDNA gene, ITS2 and 28S rDNA gene, strain BSRP 15114	1011	1011	100%	0.0	100.00%	LT387843.1
Setosphaeria rostrata genomic DNA sequence contains 18S rDNA gene, ITS1, 5.8S rDNA gene, ITS2 and 28S rDNA gene, strain BSRP 15274	1011	1011	100%	0.0	100.00%	LT387840.1
Setosphaeria rostrata genomic DNA sequence contains 18S rDNA gene, ITS1, 5.8S rDNA gene, ITS2 and 28S rDNA gene, strain F368 11773	1011	1011	100%	0.0	100.00%	LT387834.1
Setosphaeria rostrata genomic DNA sequence contains 18S rDNA gene, ITS1, 5.8S rDNA gene, ITS2 and 28S rDNA gene, strain BSRP 115432	1011	1011	100%	0.0	100.00%	LT387824.1
Setosphaeria rostrata genomic DNA sequence contains 18S rDNA gene, ITS1, 5.8S rDNA gene, ITS2 and 28S rDNA gene, strain BSRP 115550	1011	1011	100%	0.0	100.00%	LT387822.1

Searching for closest match from GenBank database

Plate 3.2: Schematic illustration of steps involved in the molecular identification of endophytic fungal isolates

PDA were used as controls. Three replicates were tested for each fungal isolate. The ability of the endophytes to solubilize insoluble phosphorus and form a clear halo zone around them was considered as a positive result for phosphorus solubilization potential.

3.7.2 Detection of the phytohormone IAA production by endophytes

The phytohormone IAA being indole compound was detected by following the method delineated by Gordon and Weber, 1951. The endophytes were cultivated in 50 mL potato dextrose broth supplemented with L-tryptophan (2 mg mL⁻¹) at 30 °C for 4 days in the dark. Later the broth was filtered through Whatman No. 1 filter paper and centrifuged at 10,000 rpm for 10 min at 4°C to get clean culture filtrate. The 4 mL of freshly prepared Salkowski reagent (50 ml of 35% perchloric acid and 1 ml of 0.5 M ferric chloride) was added to an aliquot of 2 mL of clean culture filtrate and incubated in dark for 30 min. The absorbance readings were recorded at 530 nm in Spectrophotometer (Multiskan Thermo scientific). The pink to red color was considered positive for IAA production. Three replications were maintained (Plate 3.3). The standard curve was constructed by using different concentrations of IAA viz., 5, 10, 20, 30, and 40 µg ml⁻¹. The concentration of IAA in the culture filtrate was determined by substituting the OD value as Y value in the slope formula and determining the IAA concentration *i.e.*, X value.

3.7.3 Amylase production by fungal endophytes

The fungal isolates were screened for amylolytic activity by a starch hydrolysis test on a starch agar plate (Aneja, 2007). A spot inoculation of fungal endophytes was made onto the plates in triplicate under aseptic condition and incubated at 30°C for 5 days. After incubation, Lugol's iodine solution was flooded with a dropper for 30 seconds on the starch agar plate. The presence of blue color around the growth indicates a negative result and a clear zone of hydrolysis around the growth indicates a positive result.

3.7.4 Siderophore estimation assay

Fungal endophytes were checked for siderophore producing ability by universal CAS assay (Schwyn and Neilands, 1987). Before starting the experiment, glassware was rinsed with 3 mol/l hydrochloric acid (HCl) to remove iron and subsequently washed in

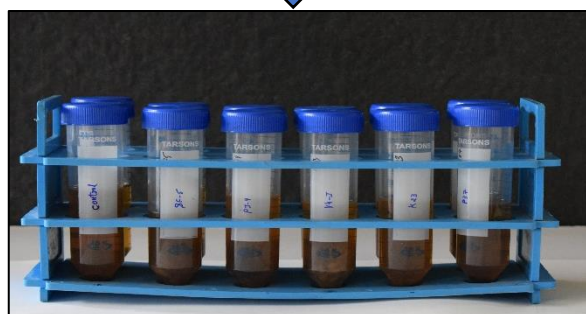
deionized water (Cabaj and Kosakowska, 2009). For the qualitative method, CAS reagent was prepared as per Schwyn and Neilands, 1987. Briefly, 121 mg CAS was dissolved in 100 ml distilled water and 20 ml of 1 mM ferric chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) solution prepared in 10 mM HCl. This solution was added to 20 ml hexadecyl trimethyl ammonium bromide (HDTMA) solution under stirring. HDTMA solution was prepared by mixing 729 mg HDTMA in 400 ml distilled water. The CAS-HDTMA solution was sterilized. CAS agar plates were prepared by mixing 100 ml CAS reagent in 900 ml sterilized potato dextrose agar medium. Endophytes were spot inoculated on each plate. An un-inoculated plate was taken as control. After inoculation, plates were incubated at 28 °C for 5-7 days and observed for the formation of an orange zone around the fungus (Louden *et al.*, 2011).

3.8 Identifying the best crop-endophyte combination for drought stress tolerance under *in-vitro* conditions

Drought-sensitive genotypes in each crop system (MAH 14-5, BGS-9, KBS-23, and Arka Harshitha of maize, green gram, soybean, and brinjal respectively) were obtained from the previous experiment were used to screen fungal endophytes for drought stress tolerance. Seeds were surface sterilized as described previously (section 3.3) and pre-germinated on sterile moist blotters. Five-day-old fungal colony culture was used for inoculum preparation and uniform pre-germinated seeds were treated with mycelial suspension (10^6 spores/ml) for 3 hrs (Dhingra and Sinclair, 1993; Zhang *et al.*, 2014). The fungal load in the suspension was calculated using a haemocytometer. The corresponding control was treated with sterile distilled water. Then pre-germinated seeds were subjected to drought stress by placing them on a sterile paper towel amended with PEG-8000 (at LC_{50} dose) with the corresponding control (Plate 3.4). The seedlings were incubated at 27°C in the growth chamber. Four replications for each treatment were maintained and each replication comprised of twenty-five seedlings. Root and shoot lengths were recorded on the final count after incubation (Sangamesh *et al.*, 2018). The treatment (T) details are as follows.

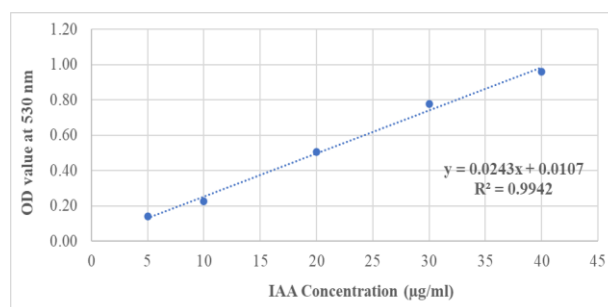
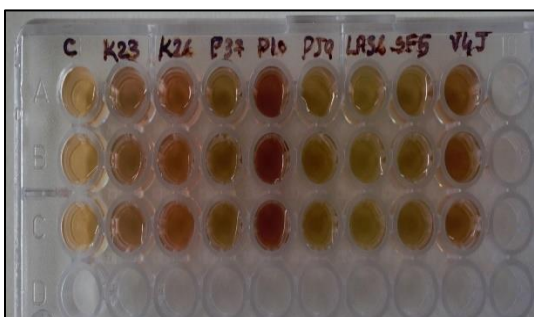
The endophytes were cultivated in 50 mL potato dextrose broth supplemented with L-tryptophan (2 mg mL^{-1}) at 30°C for 4 days in dark

Broth was filtered through Whatman No. 1 filter paper and centrifuged at 10,000 rpm for 10 min at 4°C



4 ml of Salkowski reagent (50 ml of 35% perchloric acid and 1 ml of 0.5 M ferric chloride) + 2 mL of culture filtrate and incubated in dark for 30 min

Pink to red colour was considered positive for IAA production and IAA was confirmed by all isolates



Absorbance at 530 nm was taken and plotted them against standard graph

Standard graph

Plate 3.3: Detection of the phytohormone IAA production by endophytes

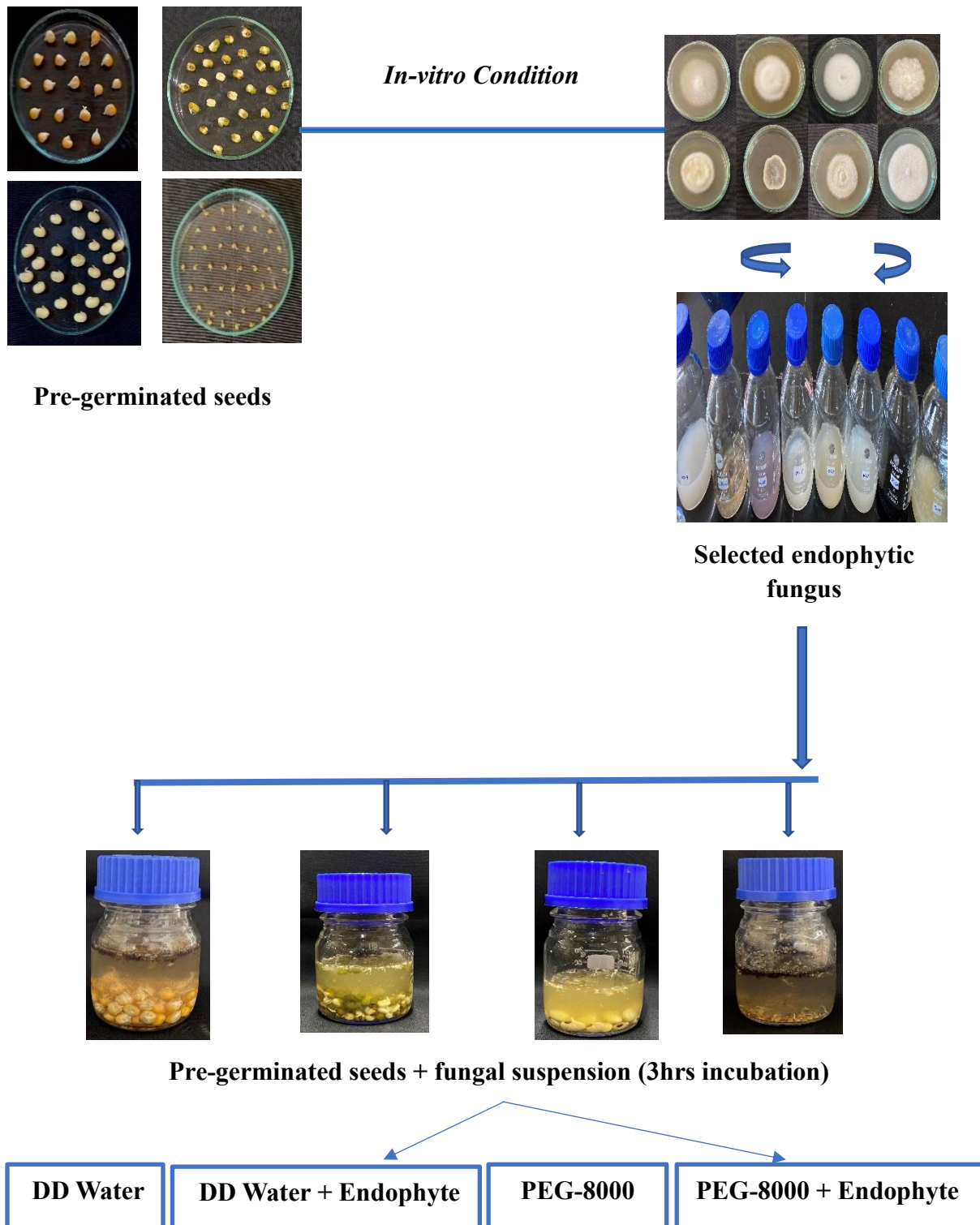


Plate 3.4: (*In-vitro*) scenario showing seed enrichment with endophytes for evaluating drought stress mitigation

Treatments
T ₁ : Control (Sterile water)
T ₂ : Control + Endophytic fungus
T ₃ : PEG-8000 (LC ₅₀ value of respective genotypes)
T ₄ : PEG-8000 (LC ₅₀ value of respective genotypes) + Endophytic fungus

The endophyte-treated seedlings from each treatment were taken for re-isolation of treated endophytes. The root, shoot, leaves are cut into segments of approximately 0.5 cm² randomly using sterile blades. The segments were surface sterilized by treating with 70 percent ethanol for 1 minute followed by sodium hypochlorite solution (1 %) for 1 minute and repeatedly washed with sterile water. The surface-sterilized segments were incubated on Petri plates with PDA media amended with streptomycin (200 µg/ml) at 28°C until fungal growth emerged at the cut ends. The re-isolated endophytic fungi were compared with their pure culture and morphologically identified.

3.9 Evaluation of best crop-endophytes combinations for their ability to impart drought stress tolerance under greenhouse conditions

To evaluate the best crop-endophytes combination *i.e.*, maize with K-23, LAS-6, and P-37 for drought stress tolerance, a pot experiment was carried out in the greenhouse conditions at NSP, UAS, GKVK, Bengaluru.

3.9.1 Determination of Field capacity of the soil

Field Capacity is the amount of soil moisture or water content held in the soil after excess water has drained away. There are different models and/or approaches to calculate the soil available water (SAW) for plants. The field capacity was determined by the Gravimetric method as described by Earl, 2003. Empty plastic plots were weighed using a weighing balance and the weight was used as an empty pot value (W). All the pots were filled with the potting mixture containing soil: sand: FYM (2:1:1) and their weight was used as a dry weight value (W_D). These pots were saturated with water during evening hours to minimize the evaporation loss of water and the excess was allowed to drain. The

weight of saturated soil was taken in the early morning and this weight was used as a wet weight value (W_w). The field capacity was calculated as follows:

Weight of an empty plastic pot = W

Weight of Plastic pot + potting mixture (sand, soil, FYM) = W_D

Weight of Plastic pot + saturated soil = W_w

Field capacity (100 %) = $(W_w - W) - (W_D - W)$

Similarly, 75 % and 50 % Field capacity value was calculated.

3.9.2 Surface sterilization and pre germination of Maize seeds

Maize (*Zea mays* L.) seeds of hybrid MAH 14-5 were surface sterilized as discussed earlier (section 3.3). The surface-sterilized seeds were kept for pre-germination on sterile blotter paper in an incubator at 28 °C.

3.9.3 Preparation of fungal suspension and inoculation of fungal endophytes to pre-germinated maize seeds

Five days old fungal colonies of selected fungal isolates (K-23, LAS-6, and P-37) grown on PDA medium were collected along with mycelium using a hairbrush in sterile distilled water. The inoculum quantity was estimated and pre-germinated seeds were treated with mycelial suspension as discussed earlier in section 3.8. For control, pre-germinated seeds were treated with sterile water and sown in pots. Single plant per pot was maintained. All pots were watered equally to maintain 100% field capacity until the 45th day. At the seedling stage, re-isolation of endophytes from inoculated seedlings was done as mentioned in section 3.8 (Plate 3.5).

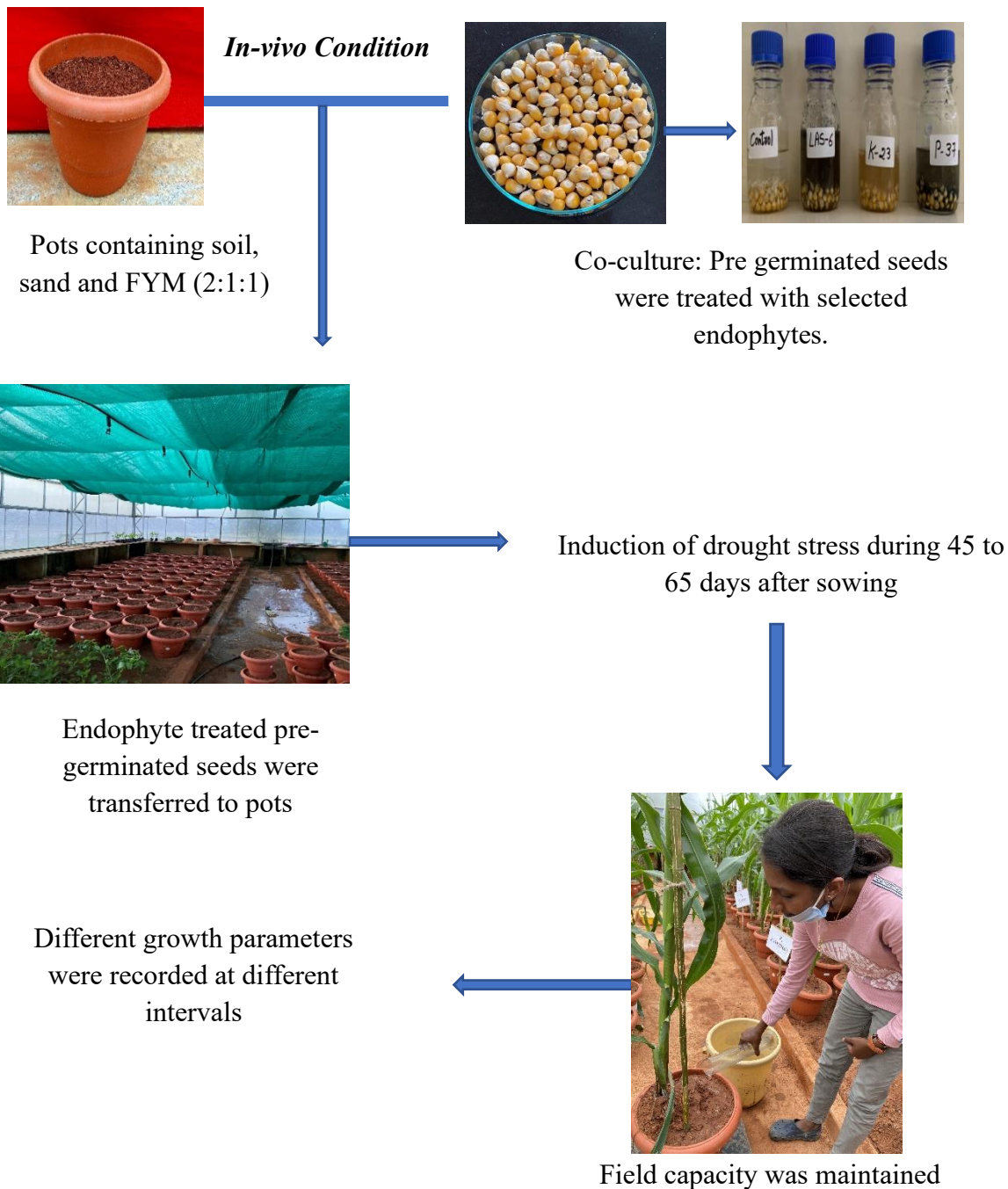


Plate 3.5: Diagram illustrating the method followed to evaluate endophytic enriched maize seeds to different soil field capacity under greenhouse (*in-vivo*) condition

The treatment (T) details are as follows:

T ₁	Field capacity (100%)
T ₂	Field capacity (100%) + LAS-6
T ₃	Field capacity (100%) + K-23
T ₄	Field capacity (100%) + P-37
T ₅	Field capacity (75%)
T ₆	Field capacity (75%) + LAS-6
T ₇	Field capacity (75%) + K-23
T ₈	Field capacity (75%) + P-37
T ₉	Field capacity (50%)
T ₁₀	Field capacity (50%) + LAS-6
T ₁₁	Field capacity (50%) + K-23
T ₁₂	Field capacity (50%) + P-37

3.9.4 Drought stress imposition by gravimetric approach.

Maize is sensitive to moisture stress during tasselling, silking, and early grain filling stage *i.e.*, 45 to 65 days after sowing, which can have the greatest impact on yield potential due to desiccation of the silks and pollen grains, which can result in poor pollination (Sah *et al.*, 2020). So, here the drought stress was imposed from 45 to 65 days after sowing by withholding water until the required soil field capacity (75% and 50%) was reached. Here, the required level of field capacity was maintained by the gravimetric approach (Karaba *et al.*, 2007). There were 12 treatments with 20 replications each. All pots were watered equally up to 100 % field capacity until the 40th day and later on, field capacity was reduced in respective treatments for 5 days in order to create drought stress at 45th day. From 45 to 65 days after sowing, the treatments were watered to their respective field capacity daily. Drought stress was given to plants by adding water of 75 % and 50 % field capacity in respective pots. All pots were weighed daily in the morning to

know the water loss through transpiration and the value was noted. The amount of water to be added to maintain the field capacity was calculated and added to their respective pots using a measuring cylinder.

3.9.5 Observations recorded in Maize crop

Measurement of biometric parameters

3.9.5.1 Plant height (cm)

Plant height was measured at four stages viz., 30, 45, 65 days after sowing (DAS) and at maturity. Plant height was measured from the soil surface to the apex of the top most young leaf of the plant and expressed in centimeters.

3.9.5.2 Number of leaves per plant

The numbers of fully opened leaves were recorded at 30, 45, 65 DAS, and maturity.

3.9.5.3 Stem girth (cm)

Stem girth (cm) was measured using the Vernier Caliper, at 30, 45, and 65 days after sowing; it was measured at 10 cm above the ground level.

3.9.5.4 Leaf area (cm²)

Three replications or plants from each treatment were randomly selected at 30, 45, 65 DAS and used to measure leaf area, root length, root volume, and plant dry weight. Here, the leaves were separated from the plant and were passed through the leaf area meter immediately (WD3 Win DIAS Leaf Image Analysis System, Delta-T services, UK) to compute the total leaf area of a plant.

3.9.5.5 Root length (cm)

Roots were separated from the harvested plants and root lengths were recorded at 30, 45, 65 DAS and maturity. It's expressed in centimeters (cm/plant).

3.9.5.6 Root volume (cm³)

The root volume was recorded at 30, 45, 65 DAS and maturity by the water displacement method. A measuring cylinder of 500 ml was filled up to 300 ml with water. Root was dipped in water and the volume change was recorded as the root volume and expressed in cubic centimeter per plant (cm³/plant).

3.9.5.7 Plant dry weight (g)

After measuring leaf area, root length, and root volume, those plants were allowed to dry in a hot air oven at 60°C for two days and the dry weight was recorded in grams (g/plant)

Measurement of physiological parameters of plants

3.9.5.8 SPAD chlorophyll meter reading (SCMR)

The relative leaf chlorophyll content of individual plants was measured using the hand-held dual-wavelength meter (SPAD 502, Chlorophyll meter, Minolta Camera Co., Ltd., Japan). The observations were recorded from a fully expanded fourth leaf from the top of the plants at three stages (30, 45, and 65 DAS). Three readings were recorded at each stage from each plant and averaged.

3.9.5.9 Measurement of gas exchange parameters using IRGA

Gas exchange parameters such as CO₂ Assimilation rate A (μmol m⁻²s⁻¹), stomatal conductance g_s (mmol m⁻²s⁻¹), and Transpiration T (mol m⁻²s⁻¹) were measured in control and endophytes treated plants at 30 days after sowing, using a portable photosynthetic system, Infrared gas analyzer-IRGA (LI-COR-Inc. Lincoln, Nebraska, USA). Flag leaf was clamped to the leaf chamber, observations were recorded after A, g_s, T values reached a steady-state level (Nataraja and Jacob, 1999). The measurements were made at an ambient CO₂ concentration of 360 μmol mol⁻¹ and PPFD of 1400 μmol m⁻²s⁻¹, Licor light source, and a chamber temperature of 28 °C (Morgan and Lecain, 1991).

3.9.5.10 Leaf relative water content (RWC)

Relative water content was calculated by measuring the fresh weight, turgid weight, and dry weight of the known number of leaf discs from treatment plants at 55 days after sowing. Fully expanded leaves were excised and fresh weight was recorded. Later, the leaf discs were soaked in distilled water for 5 hours, and then the turgid weight was taken. The samples were kept in an oven at 80°C for 2 days. The dry weight of the sample was documented (Ali and Ashraf, 2011). The RWC of the treatment leaves was calculated using the following formula and expressed as a percentage (Plate 3.6).

$$\text{RWC (\%)} = [(\text{FW} - \text{DW}) / (\text{TW} - \text{DW})] \times 100$$

Where, FW: Fresh weight, DW: Dry weight and TW: Turgid weight.

3.9.5.11 Estimation of chlorophyll stability index (CSI)

Leaf samples were collected from control and endophytes treated plants under normal and stressed conditions at 55 days after sowing, washed in distilled water, and used for estimation of chlorophyll pigments. Leaf samples (100 mg) were cut into equal segments and immersed in 3 ml of Acetone (80%): DMSO (1:1 v/v) mixture. The samples were incubated in the dark overnight for leaching of chlorophyll pigments. Absorbance was recorded at 645 and 663 nm using a UV-visible spectrophotometer. Chlorophyll a, chlorophyll b, and total chlorophyll were calculated as described by Mafakheri *et al.* (2010) and expressed as mg/g of fresh weight (Plate 3.6).

$$\text{Chlorophyll a} = [\{12.7 (\text{A}_{663}) - 2.69 (\text{A}_{645})\} \times \text{volume/ weight}] \times 1000$$

$$\text{Chlorophyll b} = [\{22.9 (\text{A}_{645}) - 4.68 (\text{A}_{663})\} \times \text{volume/ weight}] \times 1000$$

$$\text{Total Chlorophyll (mg/g of fresh weight)} = [\text{Chlorophyll a} + \text{Chlorophyll b}].$$

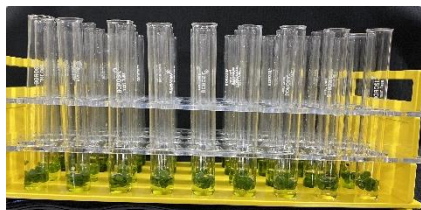
Measurement of biochemical parameters of plants

3.9.5.12 Estimation of enzyme activity

Leaf samples were collected from all treatments, *i.e.*, without stress and drought-stressed plants (endophyte treated and control) at 55 days after sowing. The samples were



Collection of leaf bits
(100mg) from all treatments



The leaf bits were incubated
in acetone (80%): DMSO (1:1
v/v) mixture in dark for
overnight.



Chlorophyll was extracted
from leaf bits in acetone
DMSO mixture



The absorbance was recorded
at 645 and 663nm

**Estimation of chlorophyll
content**



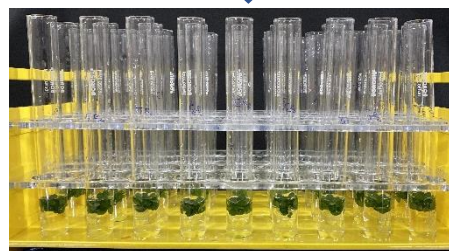
Leaf samples were collected



Leaf discs were made of
uniform size, Fresh weight
(FW) was recorded



The leaf discs were
transferred into 10 ml water,
turgid weight (TW) recorded
after 5 hours



Leaf discs were dried in a
hot air oven for 2 days @
80°C, dry weight (DW)
recorded



$$RWC (\%) = \frac{FW - DW}{TW - DW} \times 100$$

**Estimation of relative
water content**

Plate 3.6: Diagram illustrating the salient method followed in estimating chlorophyll content and relative water content

labeled, frozen in liquid nitrogen, and stored at -80 °C for the processing of enzyme assays (catalase, peroxidase, polyphenol oxidase).

Sample preparation

Leaf tissue (0.5 g) was ground to a fine powder with mortar and pestle using liquid nitrogen. Potassium phosphate buffer (2 ml), (50 mM; pH 7.0) containing EDTA (1 mM), and soluble PVP (1 %) was added and homogenized. The homogenate was transferred to 2ml Eppendorf tubes and centrifuged (Eppendorf, India) at 12,000 rpm for 15 min at 4 °C and the supernatant was carefully transferred to fresh tubes and used for estimation of antioxidant enzymes, viz., catalase (CAT), peroxidase (POX), and polyphenol oxidase using Multiskan Thermo scientific spectrophotometer. Three independent replications of enzyme extract were maintained for enzyme assays.

a) Measurement of catalase activity

The enzyme activity was assayed according to (Masia, 1998) with minor modifications. The assay mixture of catalase activity contained 165 µl of 0.05 M Phosphate buffer pH (7.0), with 20 µl of 0.3% H₂O₂ and 15 µl of enzyme extract. The change in absorbance was recorded at 240 nm for 6 minutes at an interval of every minute with a Multiskan Thermo scientific spectrophotometer. The results were expressed as a change in Units/gm fresh weight.

$$\text{Catalase activity (Units/gm FW)} = \frac{\text{OD change/min} \times \text{Factor}}{\text{The volume of a sample}} \times \frac{\text{Total volume}}{\text{Wt. of sample}}$$

Factor: 1 OD change/min=187.528 Units of enzyme

b) Measurement of polyphenol oxidase activity

Assay of PolyPhenol oxidase activity was carried out by the procedure described by Kumar and Khan, 1982. The assay mixture of polyphenol oxidase contained 144 µl of 0.05 M Phosphate buffer pH (7.0), with 6 µl of 1.25% pyrogallol and 50 µl of enzyme extract. The change in absorbance was recorded at 450 nm for 6 minutes at an interval of

every minute with a Multiskan Thermo scientific spectrophotometer. The results were expressed as a change in Units/gm fresh weight.

$$\text{Polyphenol oxidase activity (Units/gm FW)} = \frac{\text{OD change/min}}{\text{The volume of a sample}} \times \frac{\text{Total volume}}{\text{Wt. of sample}}$$

3.9.5.13 Extraction and estimation of proline

Proline was estimated using the method described by Bates *et al.* (1973). Leaf samples were collected from all treatments, *i.e.*, without stress and drought-stressed plants (endophyte treated and control) at 55 days after sowing. The proline content was expressed as $\mu\text{moles g}^{-1}$ FW.

Reagents:

- Acid ninhydrin: Ninhydrin (1.25 g) was dissolved in 30 ml of glacial acetic acid and 20 ml of 6 M phosphoric acid with agitation.
- Sulphosalicylic acid (3% w/v): Sulphosalicylic acid (7.5 g) was dissolved in distilled water and the volume made to 250 ml.
- Stock solution of L-proline (100 mM): Standard L-proline (1.15 g) was dissolved in distilled water and the final volume made to 100 ml.
- Working standard solution of L-proline (1 mM): From the stock solution, 1 ml was again diluted to 100 ml with distilled water. This diluted working solution contained 1 μmole of proline per ml.

Leaf samples (0.5 g) were homogenized with 10ml of 3% (w/v) aqueous solution of sulphosalicylic acid and the homogenate filtered through Whatman No. 2 filter paper. A suitable volume of the filtrate (2 ml) was reacted with equal volumes of acid ninhydrin (2 ml) and glacial acetic acid (2 ml) and boiled for 30 min at 100°C and the reaction was terminated by placing the tubes in ice. Chromophore was extracted using toluene (4 ml) and absorbance was read at 520 nm keeping toluene as a blank. The proline concentration was determined from the standard curve and calculated on a fresh weight basis (Plate 3.7).

Calibration of a standard curve:

In a triplicate set of test tubes, the working standard solution of proline was pipetted as 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 μ l having the corresponding proline concentration of 0 to 100 μ moles/ml. The volume was made to 1 ml with distilled water. The colour was developed as described above. The absorbance was recorded at 520 nm and a standard graph of absorbance against the concentration of proline was plotted (Figure 3).

Amount of proline in extracts:

$$\text{Proline } (\mu\text{moles/g tissue}) = \frac{\mu\text{gm of proline/ml} \times \text{ml of toluene} \times \text{ml of salicylic acid}}{115.5 \times \text{sample taken (g)}}$$

Where 115.5 is the molecular weight of proline,

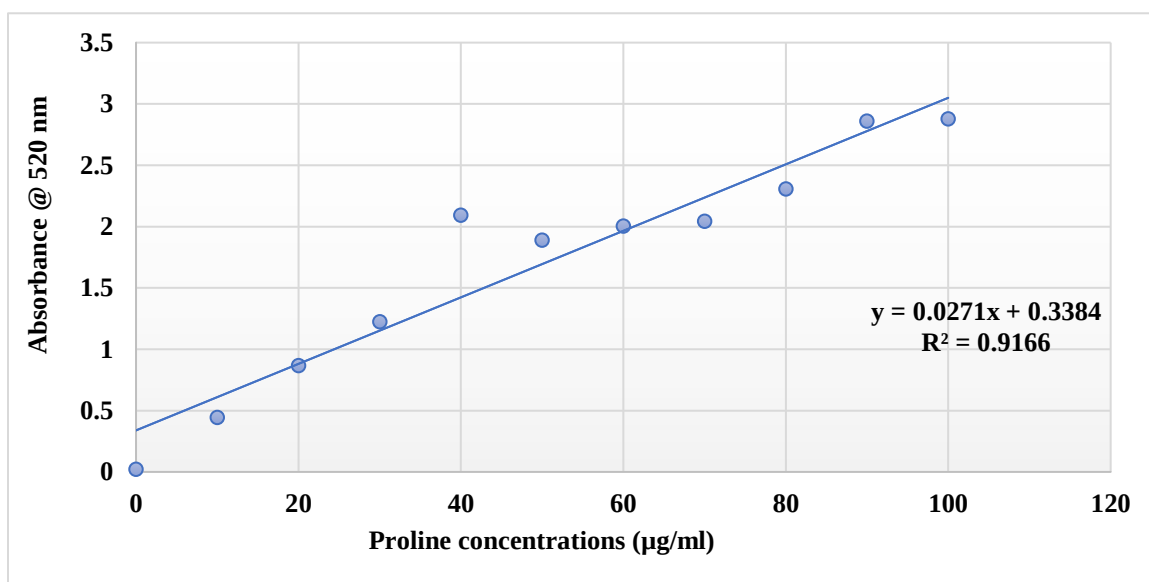


Figure 3.1: Proline standard curve

3.9.5.14 Measurement of malondialdehyde (MDA) content

Malondialdehyde is a product of the peroxidation of unsaturated fatty acids in phospholipids and is responsible for cell membrane damage. Lipid peroxidation was estimated from the accumulated MDA (Dhindsa *et al.*, 1981). Leaf samples were collected from all treatments, *i.e.*, without stress and drought-stressed plants (endophyte treated and

control) at 55 days after sowing. Leaf tissue (0.2 g) was homogenized in 4 ml of 0.1% (w/v) trichloroacetic acid (TCA) and the homogenate was centrifuged at 12,000 rpm for 10 min at room temperature. The supernatant was mixed with an equal volume of 0.5% thiobarbituric acid (TBA) in 20% (w/v) TCA. The mixture was boiled (30 min at 95 °C) and then quickly cooled on ice, followed by centrifugation for 10 min at 10,000 rpm. The absorbance of the supernatant was measured at 532 nm and corrected for nonspecific turbidity by subtracting the absorbance at 600 nm. The level of lipid peroxidation was expressed in nmol/g fresh weight (Plate 3.7).

Measurement of yield parameters of plants

3.9.5.15 Days to silking

Days to silking were measured as the number of days taken from the date of sowing to the date when silks were emerging from the ear of the individual plant.

3.9.5.16 Days to anthesis

The number of days taken from the date of sowing to the date of the first pollen shed in each plant was recorded as days to anthesis.

3.9.5.17 Tassel length (cm)

Tassel length was measured from the lowest branch point to the tip of the tassel in centimeters.

3.9.5.18 Cob length (cm)

After harvesting, the cob was dehusked and the length of the cob was measured from the base to the tip of the cob in centimeters.

3.9.5.19 Cob diameter (cm)

After dehusking and drying the main cob, cob diameter was measured at the middle of the main cob and expressed in centimeters.

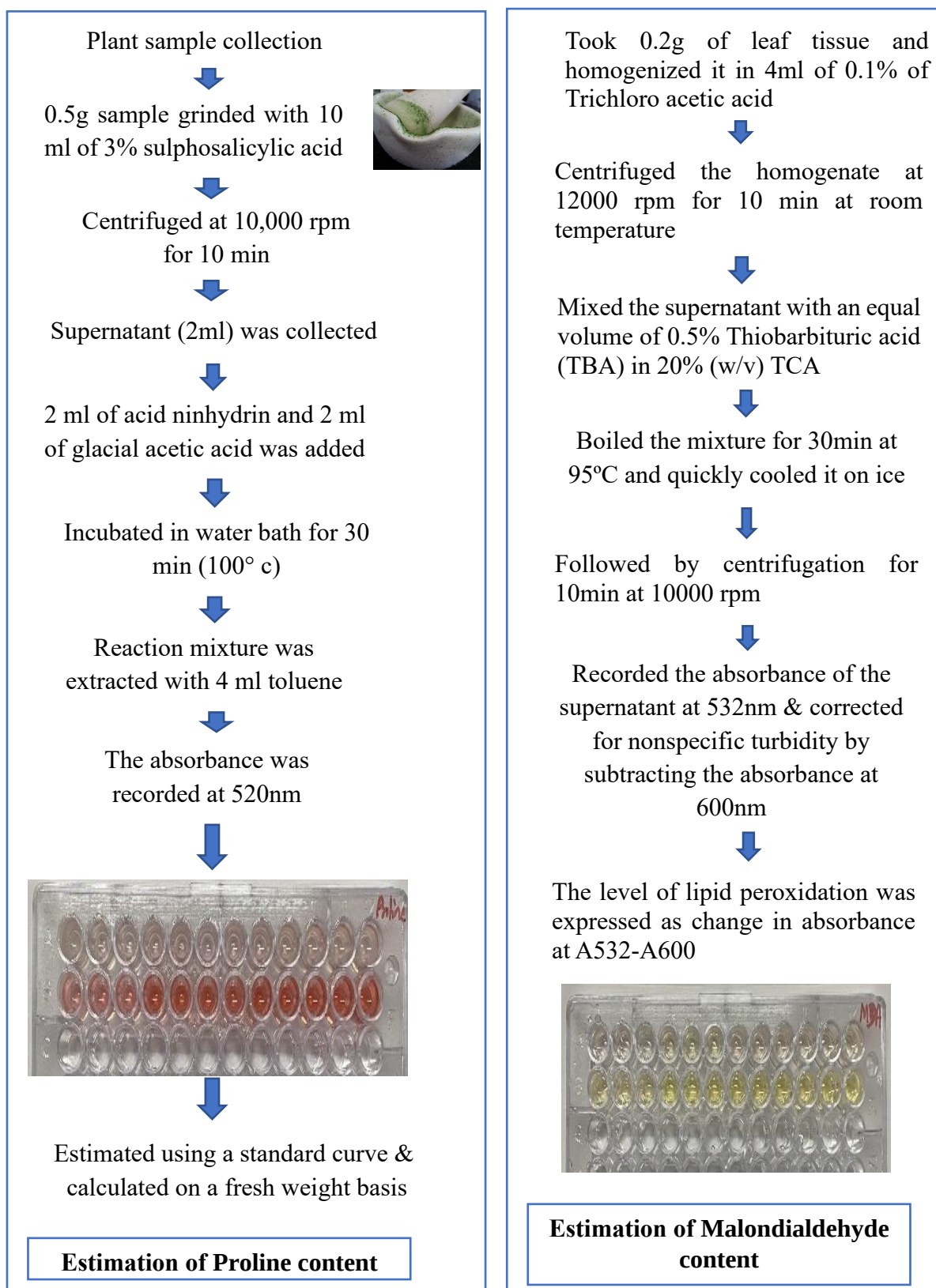


Plate 3.7: Diagram illustrating the salient method followed in estimating Proline and Malondialdehyde content

3.9.5.20 Cob weight (g)

After harvesting, the cob was dehusked and the weight of the dried main cob of an individual plant was taken and recorded in grams.

3.9.5.21 Number of kernel rows per cob

The total number of kernels bearing rows in the main cob was counted and recorded as a number of kernel rows per cob.

3.9.5.22 Number of kernels per row

The number of kernels on the longest row in the main cob was counted and recorded as a number of kernels per row.

3.9.5.23 Kernel row length (cm)

The kernel row length of each cob of the individual plant was measured in centimeters from top to the base of the longest kernel row.

3.9.5.24 Test weight (g)

The weight of randomly selected one hundred well-filled kernels from each plant was recorded as test weight and expressed in grams. If the number of kernels present in a plant was less than 100, the weight of all the kernels was recorded and converted to test weight.

3.9.5.25 Grain yield per plant (g)

The weight of the total number of kernels produced from all the cobs of the individual plant was recorded as grain yield per plant and expressed in grams.

3.10 Assessing the performance of the best crop-endophyte combination in the field conditions

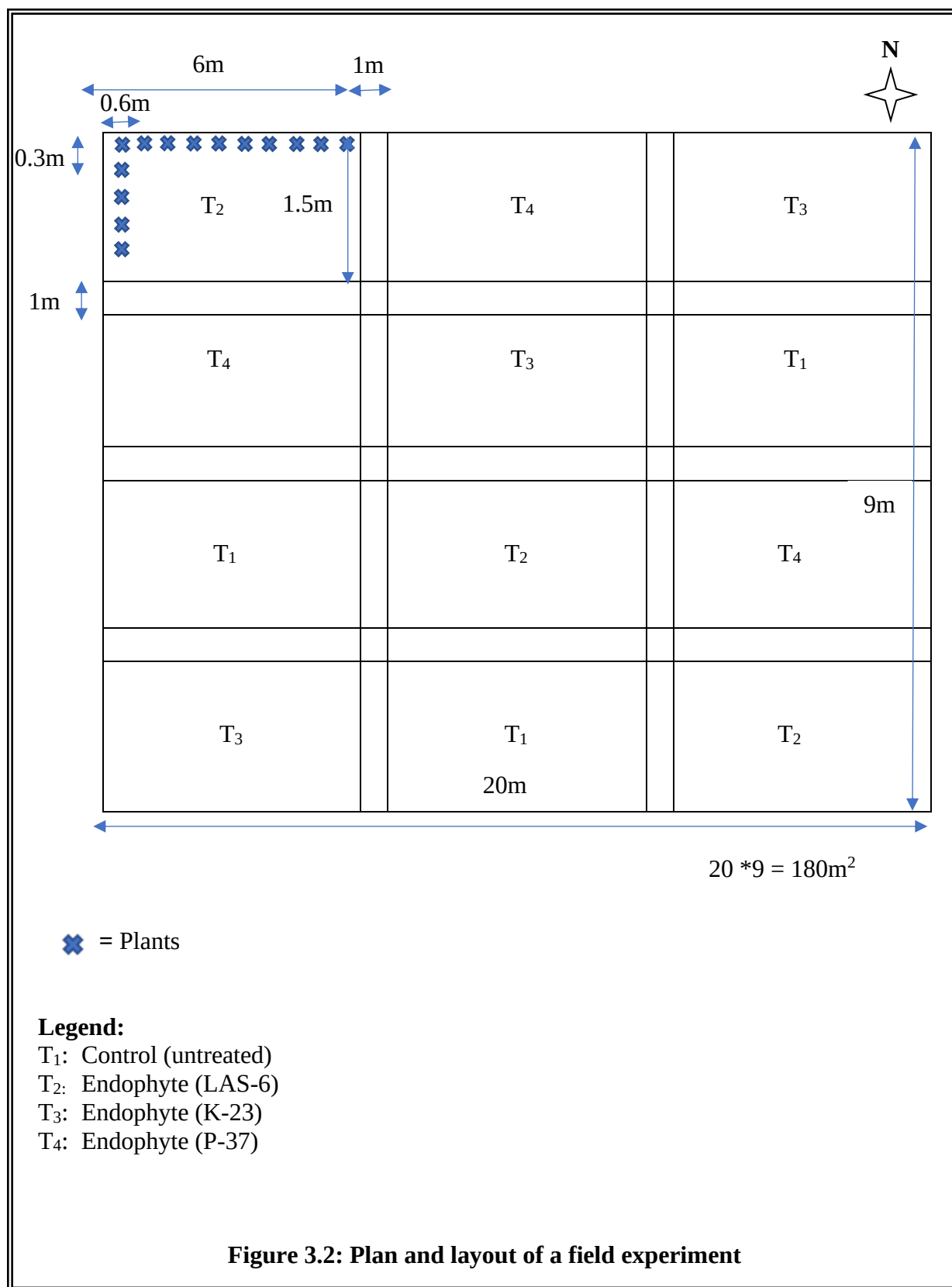
Selected three endophytes *i.e.*, LAS-6, K-23, and P-37 were evaluated in Maize (MAH 14-5) under field conditions in NSP, UAS, GKVK, Bengaluru. The location is situated between 13° 05" North latitude and 77° 34" East longitude at an altitude of 924 m

above mean sea level. The annual rainfall ranges from 528 mm to 1374.4 mm with a mean of 915.8 mm and the location comes under agro-climatic zone V (Eastern dry zone) of Karnataka. The soil of the experimental site was red sandy clay loam with slightly alkaline (pH 6.3) and the electrical conductivity was normal (0.322 dSm^{-1} at 25°C). The actual weather data as well as normal weather data viz., total rainfall, temperature (minimum and maximum), evaporation, mean daily evaporation, relative humidity, and mean daily sunshine hours during the growth period of the crop (March 2021- June 2021) were recorded and represented in the appendix- II and III.

3.10.1 Experimental details

Maize seeds were surface sterilized as shown in (section 3.3) and kept for pre-germination. Then these seeds were treated with selected three endophytes (K-23, LAS-6 and P-37) as shown in (section 3.8). After treatment, the pre-germinated seeds were sown in two experimental plots, each having four treatments with three replications in randomized block design. The experimental plot was brought to a fine tilth by deep ploughing and repeated harrowings. The required numbers of plots (24 plots) were laid out with small bunds. The seed rows were marked and drawn at a distance of 60 cm apart in each plot. The individual plot size is 9m^2 having 10 lines with 5 plants each. Plots received all recommended agricultural practices as irrigation and fertilization in the field. The pre-germinated seeds were sown by manual dibbling at the congenial field condition in the *summer* season on 02-03-2021 during the year 2020-21. The seeds were hand-dibbled at five cm deep in the earlier marked rows at 30 cm intra row spacing. At the seedling stage, re-isolation of endophytes from inoculated seedlings was done as shown in section 3.8.

The necessary aftercare operations such as hand weeding, thinning, inter cultivation, and plant protection measures were carried out as and when required to maintain a good and healthy seed crop. The crop was also given protective irrigation depending upon the soil moisture condition. Timely plant protection measures were taken to control pests and diseases as and when required. The crop was harvested when cobs started drying, the color of leaves turned pale yellow. The seeds were separated from cobs, then cleaned and dried in the shade until the seed moisture content reduced up to 11-12 percent.



3.10.2 Design and plan of the layout

The field experiment was laid out by simple RBD having two plots with four treatments and three replications each. The experimental plan of one plot is depicted in Figure. 3.2 and another plot represent the same.

3.10.3 Observations recorded

Measurement of biometric and physiological parameters

3.10.3.1 Plant height (cm)

The plant height was recorded as mentioned in section 3.9.5.1.

3.10.3.2 Number of leaves per plant

The numbers of fully opened leaves were recorded as mentioned in section 3.9.5.2.

3.10.3.3 Stem girth (cm)

Stem girth was measured as mentioned in section 3.9.5.3

3.10.3.4 Leaf area (cm²)

The leaf area was measured as mentioned in section 3.9.5.4.

3.10.3.5 SPAD chlorophyll meter reading (SCMR)

SPAD was measured as mentioned in section 3.9.5.8.

3.10.3.6 Measurement of gas exchange parameters using IRGA

Gas exchange parameters using IRGA were measured as mentioned in section 3.9.5.9.

Measurement of yield parameters of plants

3.10.3.7 Days to silking

The days to silking were recorded as mentioned in section 3.9.5.15.

3.10.3.8 Days to anthesis

The days to anthesis were recorded as mentioned in section 3.9.5.16.

3.10.3.9 Cob length (cm)

The length of the cob was measured as mentioned in section 3.9.5.18.

3.10.3.10 Cob diameter (cm)

The cob diameter was measured as mentioned in section 3.9.5.19.

3.10.3.11 Cob weight (g)

The cob weight was measured as mentioned in section 3.9.5.20.

3.10.3.12 Number of kernel rows per cob

The total number of kernels bearing rows was measured as mentioned in section 3.9.5.21.

3.10.3.13 Number of kernels per row

The number of kernels per row was measured as mentioned in section 3.9.5.22.

3.10.3.14 Kernel row length (cm)

The kernel row length was measured as mentioned in section 3.9.5.23.

3.10.3.15 Test weight (g)

The test weight was measured as mentioned in section 3.9.5.24.

3.10.3.16 Grain yield per plant (g)

The grain yield per plant was measured as mentioned in section 3.9.5.25.

3.11 Scanning electron microscope (SEM)

The three endophytes (K-23, LAS-6, and P-37) which performed well under *in vitro*, pot, and field experiments were used for this study. The samples were analysed at

Central Instrumentation Facility, UAS, GKVK, Bengaluru. Mycelial discs (5 mm dia) of five-day-old endophytic fungal culture grown on nylon membrane in PDA plates were cut and placed in perforated capsules and fixed in un-buffered aqueous 3 % glutaraldehyde in phosphate buffer for 4 hrs (Samson *et al.*, 1979). The mycelial discs were rinsed thrice in water for 5 min each and postfixed with 1 % osmium tetroxide (OsO₄) for 2 hrs and washed with water as mentioned above. The samples were dehydrated with a series of ethanol concentrations: 30, 50, 70, 80, 90, and 100 %, and later transferred to liquid CO₂ during critical point drying. The specimens were mounted on stubs using a double sticky carbon tape coated with gold in a sputter-coater (5nm thickness). Finally, the morphology was examined under SEM in SE2 mode (ZEISS; EVO18).

3.12 Amplification of Large subunit (LSU) and partial β -tubulin II (*TUB2*) region

The three endophytes (K-23, LAS-6 and P-37) which performed well under *in-vitro*, pot and field experiments were further molecularly identified using several well-known primer pairs that amplify: sections of the nuclear ribosomal RNA gene large subunit (LSU, D1–D2 domains of 26/28S) and partial β -tubulin II (*TUB2*) (Appendix I). Isolation of genomic DNA was performed as mentioned in section 3.6.1. PCR reaction was set as mentioned in section 3.6.2 and Touchdown PCR was performed where an initial denaturation at 95 °C for 5 min was followed by 10 cycles of 45 s at 95 °C, 45 s starting at 68 °C and dropping by 1 °C per cycle until a temperature of 58 °C was reached and a 1 min extension at 72 °C. The initial 10 cycles were then followed by 35 cycles of 45 s at 95 °C, 45 s at 58 °C, and 1 min at 72 °C. A final extension at 72 °C for 5 min completed the PCR. Sequence details of all primers are listed in Appendix 1 (Plate 3.2). The amplified DNA of all primers set was sequenced by Eurofins Genomics India Pvt. Ltd., Bengaluru – 560048. The sequence data received from the company was analyzed for homology using NCBI GenBank as mentioned in section 3.6.3 and phylogenetic analysis was done as per section 3.6.4.

3.13 Phytohormone analysis by LC-MS/MS

The phytohormone analysis was conducted at Friedrich Schiller University, Jena, Germany. The treatment details were: T₁: Control (Sterile water), T₂: Control + Endophyte

(K-23), T₃: PEG-8000 (-2.79 MPa) and T₄: PEG-8000 (-2.79 MPa) + Endophyte (K-23). About 250 mg of fresh maize tissue were extracted and homogenized in 1.0 ml methanol containing 40 ng D4-SA (Santa Cruz Biotechnology, USA), 40 ng D6-ABA (Toronto Research Chemicals, Toronto, Canada), and D5-indole acetic acid (D5-IAA, OlChemIm s.r.o., Olomouc, Czech Republic) as internal standards. Samples were agitated on a horizontal shaker at room temperature for 30 min and centrifuged at 13,000 rpm for 10 min at 10 °C and the supernatant was collected for analysis by LC-MS/MS.

Table 3.3: Details of the analysis of phytohormones by LC-MS/MS [HPLC 1260 (Agilent Technologies)-QTRAP6500 (SCIEX)] in negative ionization mode

Q1	Q3	RT (min)	Compound	Internal std	RF	DP	CE
263.00	153.20	6.4	ABA	D6-ABA	1.0	-20	-22
136.93	93.00	5.9	SA	D4-SA	1.0	-20	-24
345.00	143.00	4.8	GA3	D4-SA	7.6	-20	-36
173.85	129.90	6.0	IAA	D5-IAA	-	-20	-14

Phytohormone analysis was performed by LC-MS/MS on an Agilent 1260 series HPLC system (Agilent Technologies) coupled to a tandem mass spectrometer QTRAP 6500 (SCIEX, Darmstadt, Germany). Chromatographic separation was achieved on a Zorbax Eclipse XDB-C18 column (50 x 4.6 mm, 1.8 µm, Agilent Technologies). Water containing 0.05% formic acid and acetonitrile were employed as mobile phases A and B, respectively. The elution profile was: 0-0.5 min, 5% B; 0.5-9.5 min, 5-58% B; 9.5-9.52 min, 58-100% B; 9.52-11.0 min, 100% B and 11.01-14.0, min 5% B. Flow rate was kept at 1.1 ml/min and the column temperature was maintained at 25°C. The mass spectrometer was equipped with a Turbo spray ion source operated in negative ionization mode. The ion spray voltage was maintained at -4,200 eV. The turbo gas temperature was set at 620°C. Nebulizing gas was set at 60 psi, curtain gas at 40 psi, heating gas at 60 psi, and collision gas was set to “medium”. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode in negative ionization mode, details of the instrument parameters and response factors for quantification can be found in Table 3.3. For quantification of

GA3, the internal D4-SA standard was used applying an experimentally-determined response factor of 7.6.

3.14 Transcriptome study

In light of results from laboratory, pot, and field experiments, a plan was developed to perform transcriptomic analysis using endophyte (K-23) and maize under normal/unstressed and drought stress conditions. Here, maize seeds were surface sterilized as shown in (section 3.3) and kept for pre-germination. Then these seeds were treated with endophyte as shown in (section 3.8). The experiment was conducted in sterile glass plates covered with glass jars. Then pre-germinated seeds were subjected to drought stress by placing them on sterile blotters amended with PEG-8000 (-2.79 MPa). The seedlings were incubated at 27°C in the growth chamber. Five replications for each treatment were maintained and each replication comprised of ten seedlings. Root and shoot samples were harvested separately after 7 days for RNA isolation.

The harvested samples were immediately frozen in liquid nitrogen and kept at -80 °C until RNA extraction. Shoot and root samples of different treatments collected in triplicates were used for RNA isolation using Trizol reagent with the following procedure (Plate 3.8).

The treatment (T) details are as follows.

Treatments
T ₁ : Control (Sterile water)
T ₂ : Control + Endophyte (K-23)
T ₃ : PEG-8000 (-2.79 MPa)
T ₄ : PEG-8000 (-2.79 MPa) + Endophyte (K-23)

RNA isolation from plant tissue

Protocol followed:

1. Maximum of 100 mg stored (-80 °C) maize tissue (shoot and root separately) of different treatments was taken and immediately tissues were placed in liquid nitrogen and ground thoroughly.
2. Tissue powder was transferred to 2ml Eppendorf tube (Liquid nitrogen was allowed to evaporate, but not allowed the tissue to get thawed) and added 1ml of Trizol reagent and kept in room temperature for 5 min.
3. It was mixed thoroughly by pipetting and kept in ice for 15 min. Centrifuged at 10000 rpm for 5 min at 4°C and transferred liquid phase to new Eppendorf tube.
4. 200µl of chloroform (chilled) was added and vortexed for 30 seconds followed by centrifugation at 13000 rpm for 15 min at 4°C. Pipetted aqueous phase to new Eppendorf tube and added 200µl of chloroform and centrifuged at 13000 rpm for 15 min at 4°C.
5. Later aqueous phase was pipetted to 1.5ml Eppendorf tube and 500µl of ice-cold isopropanol was added and incubated at -20°C for overnight.
6. Centrifuged at 13000 rpm for 15 min at 4°C and pellet formed was washed by 70% ethanol (twice) at 13000 rpm for 15 min at 4°C and air dried the pellet in ice for 30 min.
7. Finally, pellet was dissolved in nuclease free water (50µl).
8. The quality of the RNA was analyzed by loading 3 µl of the sample on 1.3% agarose gel prepared in 87.5 ml of 10X MOPS buffer and 12.5 ml of fresh formaldehyde (37%). Estimated the RNA concentration by measuring its absorbance at 260 nm. Purified RNA samples were stored at – 80 °C until submission for sequencing.

The RNA extracted was outsourced to Molsys for transcriptome sequencing and analysis. Extracted sample RNA quality was checked on Qubit 4.0 fluorometer (Thermofisher #Q33238) using RNA HS assay kit (Thermofisher #Q32851) following manufacturer's protocol. To measure the purity of the extraction, measured the

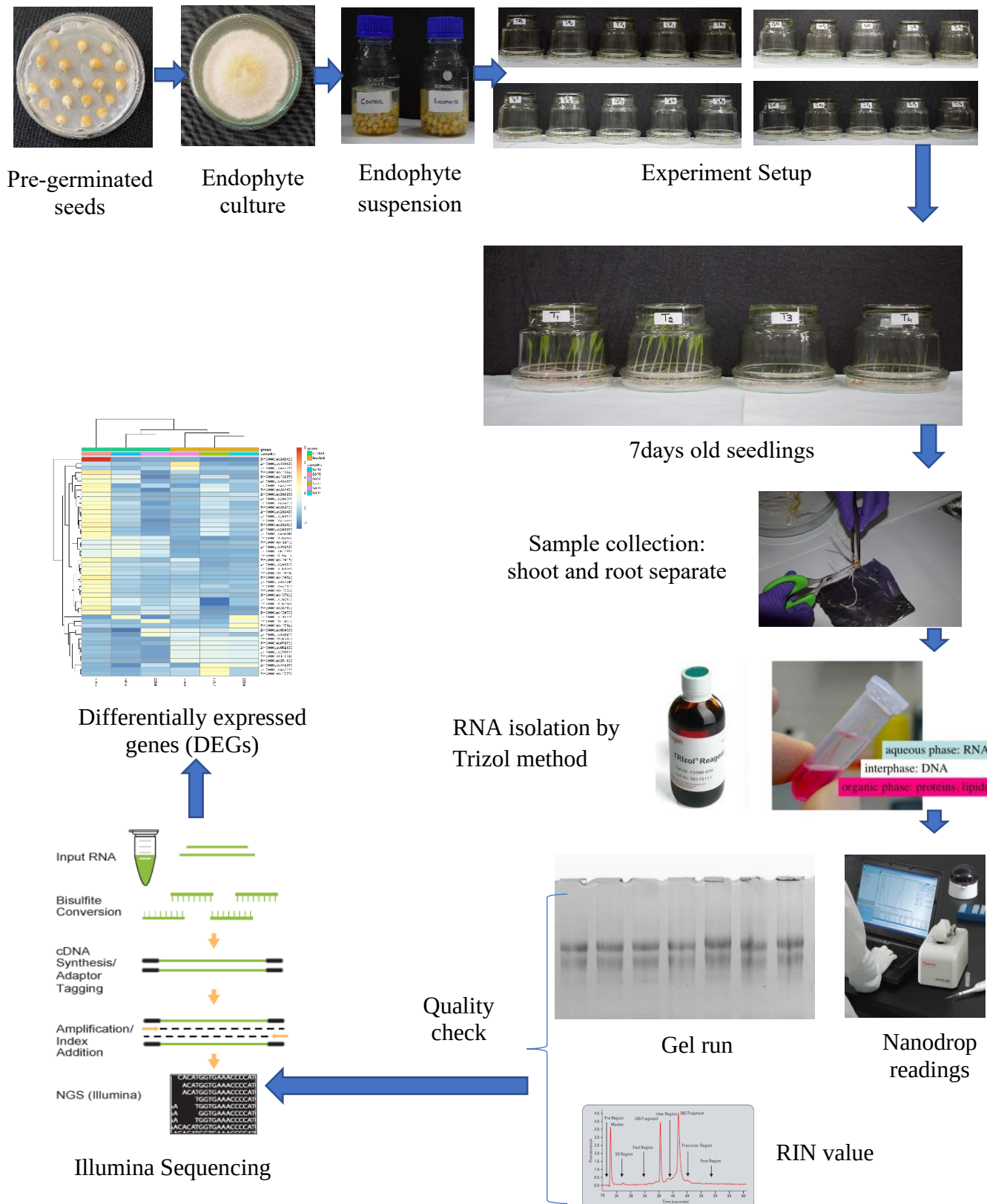


Plate 3.8: Diagram illustrating the transcriptomic profiling of maize (*Zea mays* L.) seedlings in response to endophyte inoculation under drought stress

concentration on Nanodrop 1000. Finally, to obtain RIN values, RNA was checked on the TapeStation using HS RNA screen tape (Agilent).

Library preparation and QC: Final libraries were quantified using Qubit 4.0 fluorometer (ThermoFisher #Q33238) using a DNA HS assay kit (ThermoFisher #Q32851) following the manufacturer's protocol. To identify the insert size of the library, it was queried on TapeStation 4150 (Agilent) utilizing high sensitive D1000 screentapes (Agilent # 5067- 5582) following manufacturers' protocol.

Transcriptome sequencing was done using Illumina HiSeq. Quality assessment of the raw fast reads of the sample was performed using FastQC v.0.11.9 (Andrews, 2010). The raw fast reads were pre-processed using Fastp v.0.20.1 (Chen *et al.*, 2018), followed by quality re-assessment using FastQC. The *Zea Mays* genome was indexed using hisat2-build (Kim *et al.*, 2015). Processed reads were mapped to the indexed genome using Hisat2. The Aligned reads from individual samples were quantified using feature count v.0.46.1 (Liao *et al.*, 2014) to obtain gene counts. These gene counts were used as inputs to DESeq2 (Love *et al.*, 2014) for differential expression estimation. Differentially expressed genes protein sequences were extracted from the maize protein file and subjected to Diamond blastp for similarity search against nrdb and used for annotation using blast2go (Conesa and Gotz, 2008). Differentially expressed gene ids were subjected to agriGO online tool for Gene Ontology (GO) analysis to generate GO terms. The blast2go GO output is subjected to the WEGO (Ye *et al.*, 2018) online tool to get GO bar plots. These protein sequences were used for KOBAS (Bu *et al.*, 2021) for Gene set enrichment analysis.

3.16 Statistical analysis

The experiments were carried out in a completely randomized design in the laboratory and greenhouse experiments, whereas randomized complete block design in field conditions. Standard errors were calculated for each parameter in each treatment. Most statistical analyses were performed in the R statistical environment and Phyton. Statistical comparisons were done by one-way ANOVA tests followed by a post-hoc Tukey analysis; the same letters above the graph are statistically indistinguishable at 95 % confidence.

IV RESULTS AND DISCUSSION

Climate change has decimated crop productivity worldwide, threatening global food security. Droughts occur more frequently because of decreased precipitation, which reduces crop yields by disrupting plant growth, physiology, and reproduction (Joshi *et al.*, 2016). However, a great deal of progress has been made regarding crop improvement in hostile environments and the use of microbial technology and genetic engineering to meet the population's increasing food and nutrition needs.

Due to its ability to provide an alternative to the conventional methods that have not been very successful in reducing abiotic stresses in plants, endophytes have drawn increasing attention as a tool to alter plant responses to abiotic stress in recent years. In addition, endophyte symbiotic technology provides a more ecological approach than genetic engineering. Due to such associations, there is mounting evidence that plants will be more resilient to climate change and global warming. Accordingly, it has been suggested that plants could be protected from abiotic stress by using endophytes (Yuan *et al.*, 2016; Xie *et al.*, 2017).

This study was conducted to test whether endophytic fungi isolated from plants growing in stressful environments could confer drought tolerance in non-host plants. This chapter outlines the outcomes of the current investigation.

4.1 Polyethylene glycol (MW-8000) induced screening for drought-sensitive genotypes of maize, green gram, soybean, and brinjal

In-vitro screening for LC₅₀ value involved the induction of physiological drought using agents such as polyethylene glycol (PEG 8000). Different concentrations, such as - 0.47 MPa, -1.45 MPa, -2.93 MPa, -4.92 MPa, and -7.41 MPa, and control was used. For this study, two genotypes each of maize (MAH 14-5, NAC-6002), green gram (BGS-9, KKM-3), soybean (JS-335, KBS-23), and brinjal (Arka Harshitha, Arka Unnathi) were evaluated for growth response under different PEG concentrations.

In all the genotypes of crop systems examined, the seedling growth was significantly affected as PEG 8000 induced stress increased. The genotypes MAH 14-5, BGS-9, KBS-23, and Arka Harshitha from maize, green gram, soybean, and brinjal showed significantly lower growth than the other genotypes. Therefore, these were selected as sensitive genotypes to screen for drought stress tolerance upon endophytes treatment. Here, the results were expressed as absolute growth under different levels of drought stress and control. Thus, -2.79 MPa, -2.57 MPa, -1.28 MPa, and -3.38MPa of PEG 8000 is the most differentiating concentration for assessing maize (MAH 14-5), green gram (BGS-9), soybean (KBS-23), and brinjal (Arka Harshitha), respectively against drought tolerance in the early stages of crop growth. These are considered as sub-lethal concentrations to evaluate seedlings treated with different fungal isolates for stress tolerance (Plate 4.1, Figure 4.1.1 and Figure 4.1.2).

Polyethylene glycol (PEG), a neutral polymer with high water solubility, has become the most often utilized osmoticum in drought-stress response research to imitate reductions in soil water potential. PEG with a high molecular weight (8000 or higher) cannot penetrate the plant cell wall pores, creating conditions that are similar to the effect of reduced matric potentials, resulting in water loss from both the protoplast and the cell wall, as well as the collapse of the entire cell, including the wall, with minimal metabolic intervention (Iglesias *et al.*, 2018). As a result, PEG solutions have proven to be the most practical option for imitating drought in short-term tests. It decreases turgor pressure and affects cell division, ultimately reducing shoot and root growth. This result was consistent with the findings of Ghosh *et al.* (2020), who demonstrated that under PEG-induced stress, shoot and root length of wheat cultivars significantly reduced compared to controls. Similarly, Razavizadeh *et al.*, 2019 evaluated the thyme seeds under different concentrations (0, 2, 4, 6, and 8%) of PEG. Four weeks after PEG-induced water stress, a significant decrease in seedling growth was seen. Similarly, using PEG, eight chilli cultivars screened for drought stress showed decreased root and shoot length with increased concentrations (Muddarsu and Manivannan, 2017).

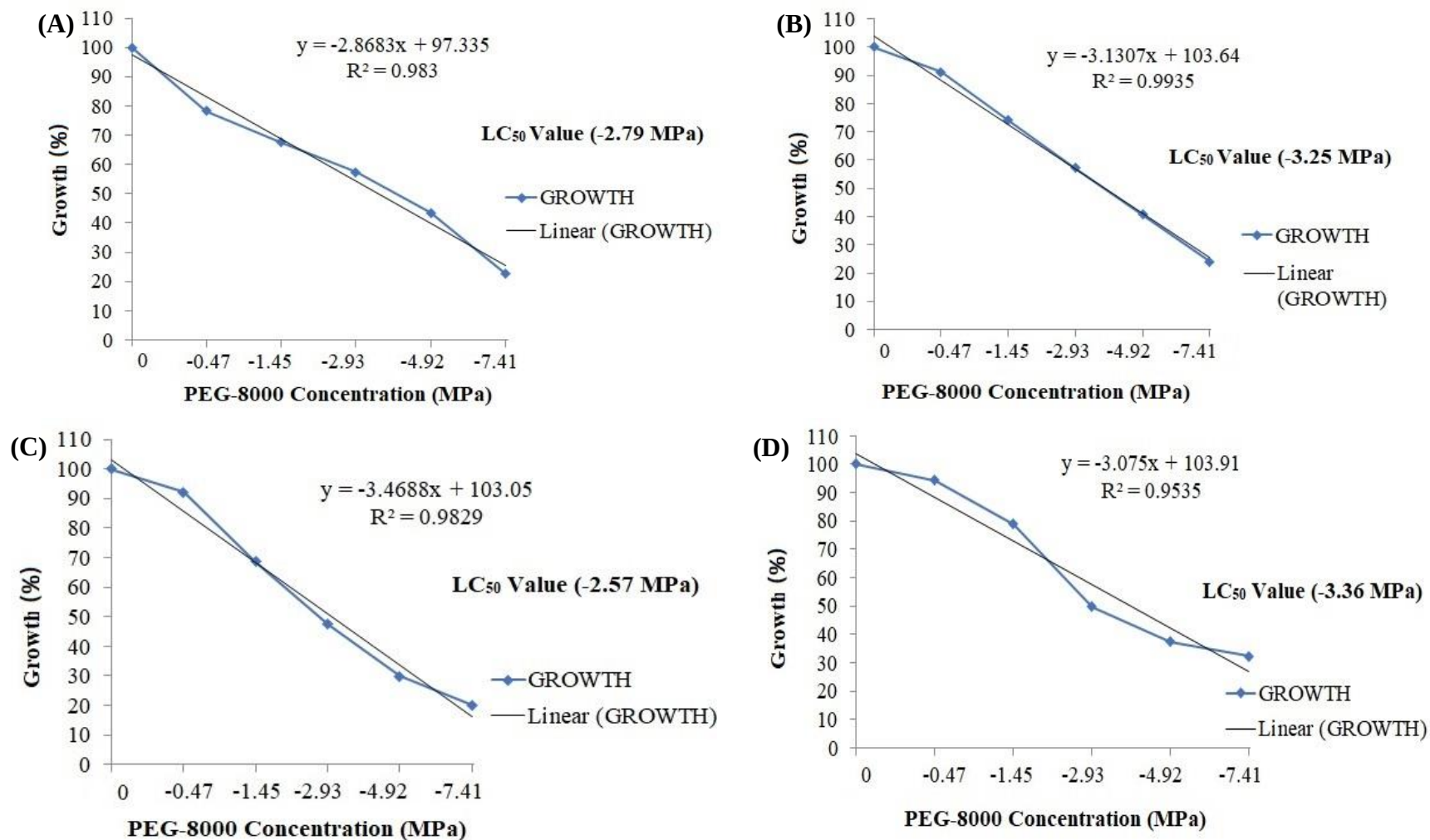


Figure 4.1.1: Effect of different concentrations of PEG-8000 on seedling growth of maize (A) MAH 14-5 (B) NAC-6002, and green gram (C) BGS-9 (D) KKM-3

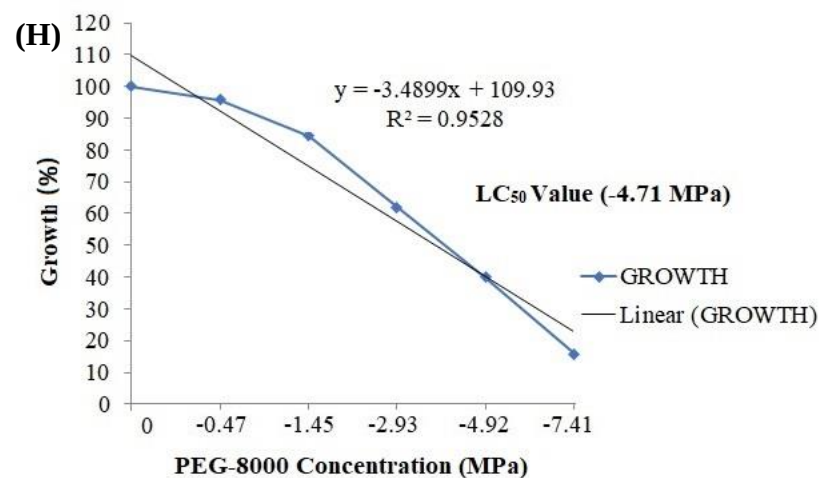
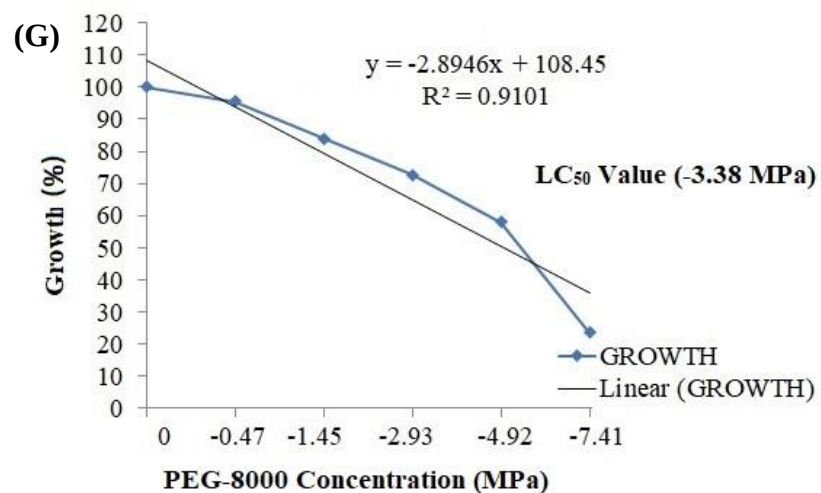
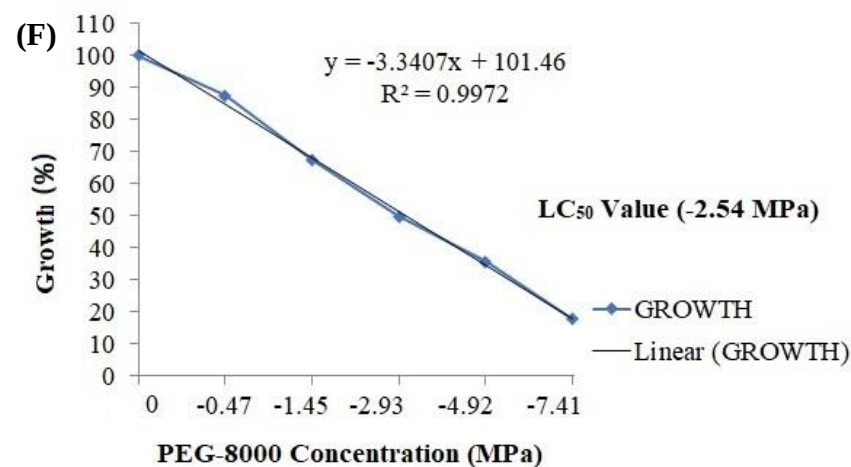
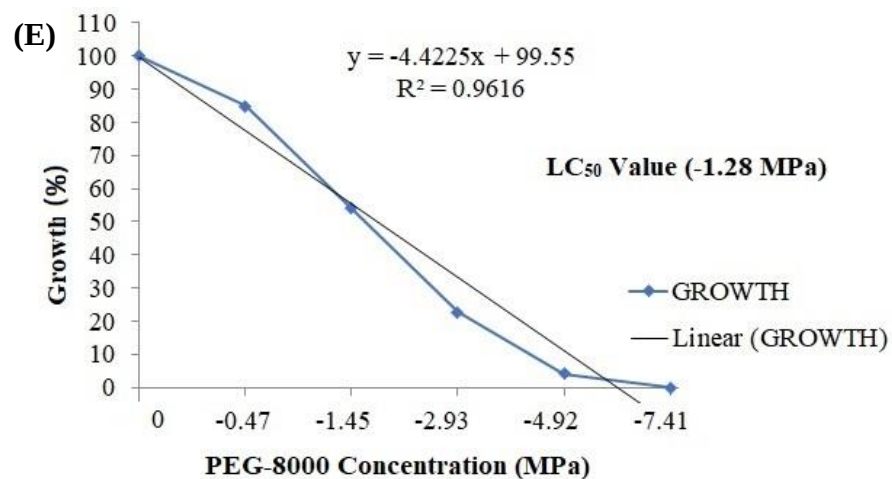


Figure 4.1.2: Effect of different concentrations of PEG-8000 on seedling growth of soybean (E) KBS-23, (F) JS-335 and brinjal (G) Arka Harshitha (H) Arka Unnathi

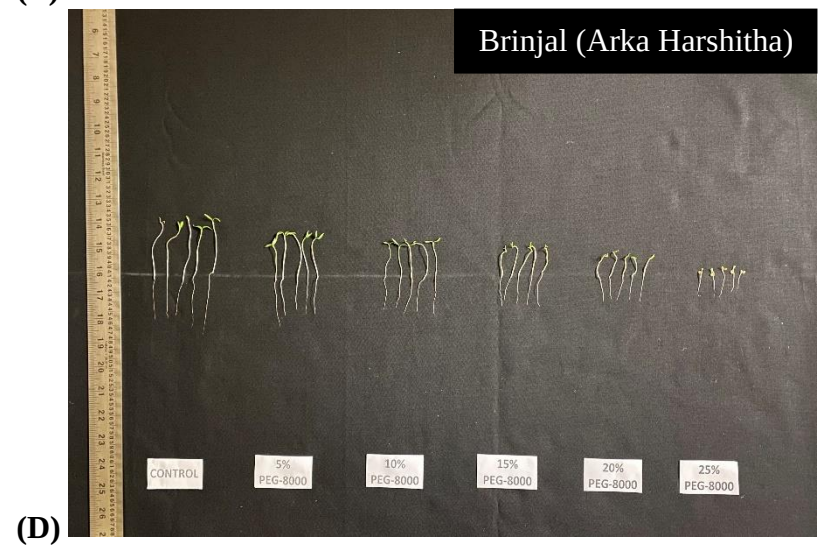
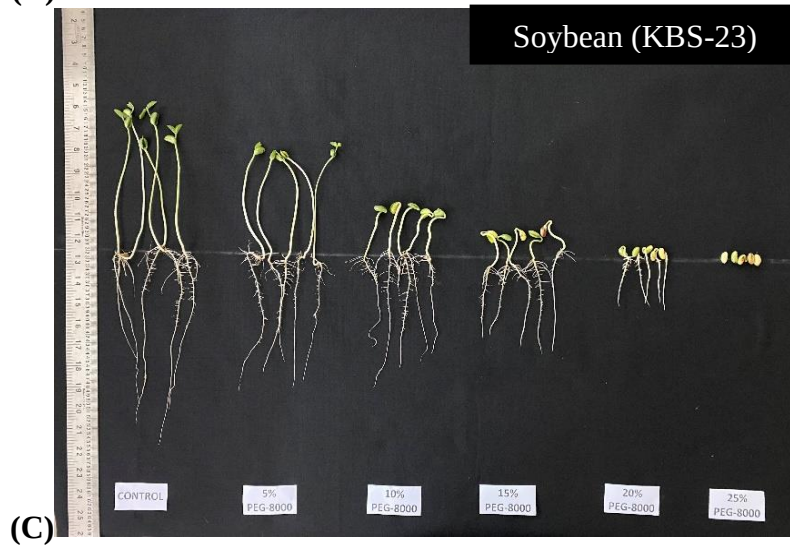
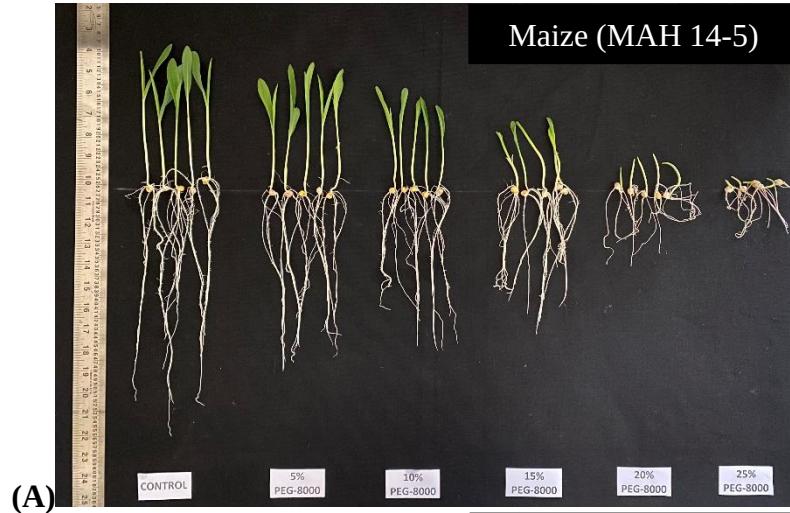


Plate 4.1: Effect of PEG-8000 concentration on seedling length of drought-sensitive genotypes of maize (A) MAH 14-5, green gram (B) BGS-9, soybean (c) KBS-23, and brinjal (d) Arka Harshitha

4.2 Pure culture response of fungal endophytes to matric-induced water stress

The response of fungi to matric-induced water stress can be assessed by various approaches using Polyethylene glycol. PEG is not metabolized by fungi (Mexal and Reid, 1973). In the present study, all fungal endophytes increased biomass at -7.41 MPa, when compared to the control (without PEG-8000), indicating a Type III response to matric water stress, as defined by Hutton *et al.* (1996) and represented in Table 4.1 and Figure 4.2. The most notable effect was an increase in overall growth as measured by weight.

Most fungi produce compatible solutes to help them cope with water stress. Jennings and Burke (1990) classified these as compounds that can vary their concentration in the cell in response to changes in external water potential, retaining turgidity while having no influence on enzyme activity. They build up in the mycelium first, then migrate to the conidia during conidiation. In addition to this, the accumulation of sugar alcohols by mycelia takes place when fungi grow under water stress (Pfyffer and Rast, 1988). It alters quantitatively with different levels of stress conditions. PEG-induced matric water stress tolerance (-0.16 MPa to -2.96 MPa) for ericoid endophytes of Epacridaceae hosts was investigated by Hutton *et al.* (1996), who identified three types of growth responses: Type I: after five weeks, generally negligible growth, Type II: maximum growth at control with decreasing growth as matric water stress increases, and Type III: maximum growth under some degree of matric water stress. Raudabaugh *et al.* (2011) investigated endophytic isolates for their ability to grow on matric modified media (PEG-8000) at ca. 0 MPa, -5 MPa, -10 MPa, -15 MPa, -20 MPa and found that the endophytes produced greater biomass (mg) from -10 MPa to -20 MPa.

4.3 Morphological characterization of endophytic fungi

The morphological characterization of fungal isolates showed that all sporulating fungi had spores with varying morphology. The colony of fungal culture on PDA plates and morphotype of endophytic fungal isolates are represented in (Plate 4.2 and 4.3). Standard identification manuals were used to characterize the morphology of fungal isolates (Barnett, 1960; Booth, 1971; Domsch and Gams 1972, 1980; Ellis, 1976; Sutton, 1980; Arx, 1981).

Table 4.1: Fungal isolates growth response to matric water stress (PEG-8000) at 7 days (Inoculum on PDA agar transferred to the broth)

Fungal isolates \ PEG-8000 Concentration	Type of growth response	Average dry weight (g) at different water potential:						
		Control	-0.47 MPa	-1.45 MPa	-2.93 MPa	-4.92 MPa	-7.41 MPa	CD (P<0.05)
LAS-6	III	0.09 ± 0.002 ^a	0.36 ± 0.003 ^b	0.52 ± 0.004 ^c	0.80 ± 0.004 ^d	1.00 ± 0.026 ^e	1.40 ± 0.008 ^f	0.035
SF-5	III	0.05 ± 0.001 ^a	0.15 ± 0.005 ^b	0.55 ± 0.019 ^c	0.98 ± 0.019 ^d	1.99 ± 0.029 ^e	2.49 ± 0.042 ^f	0.073
V4-J	III	0.07 ± 0.002 ^a	0.28 ± 0.010 ^b	0.48 ± 0.013 ^c	0.73 ± 0.030 ^d	1.03 ± 0.033 ^e	1.32 ± 0.026 ^f	0.068
K-23	III	0.10 ± 0.003 ^a	0.26 ± 0.001 ^b	0.54 ± 0.007 ^c	0.74 ± 0.004 ^d	1.05 ± 0.030 ^e	1.51 ± 0.013 ^f	0.043
K-26	III	0.07 ± 0.001 ^a	0.29 ± 0.006 ^b	0.80 ± 0.018 ^c	1.03 ± 0.026 ^d	1.54 ± 0.028 ^e	2.66 ± 0.041 ^f	0.074
PJ-9	III	0.07 ± 0.001 ^a	0.38 ± 0.010 ^b	0.58 ± 0.026 ^c	1.11 ± 0.048 ^d	1.32 ± 0.029 ^e	1.76 ± 0.034 ^f	0.090
P-10	III	0.11 ± 0.001 ^a	0.23 ± 0.008 ^a	0.72 ± 0.032 ^b	1.15 ± 0.032 ^c	1.39 ± 0.038 ^d	1.48 ± 0.040 ^e	0.091
P-37	III	0.10 ± 0.003 ^a	0.26 ± 0.013 ^b	0.46 ± 0.018 ^c	0.81 ± 0.009 ^d	1.08 ± 0.025 ^e	1.38 ± 0.015 ^F	0.048

Note: Means with the same superscript in a row do not differ significantly as per Duncan Multiple Range Test (DMRT). Values represented are mean ±SE (n=3).

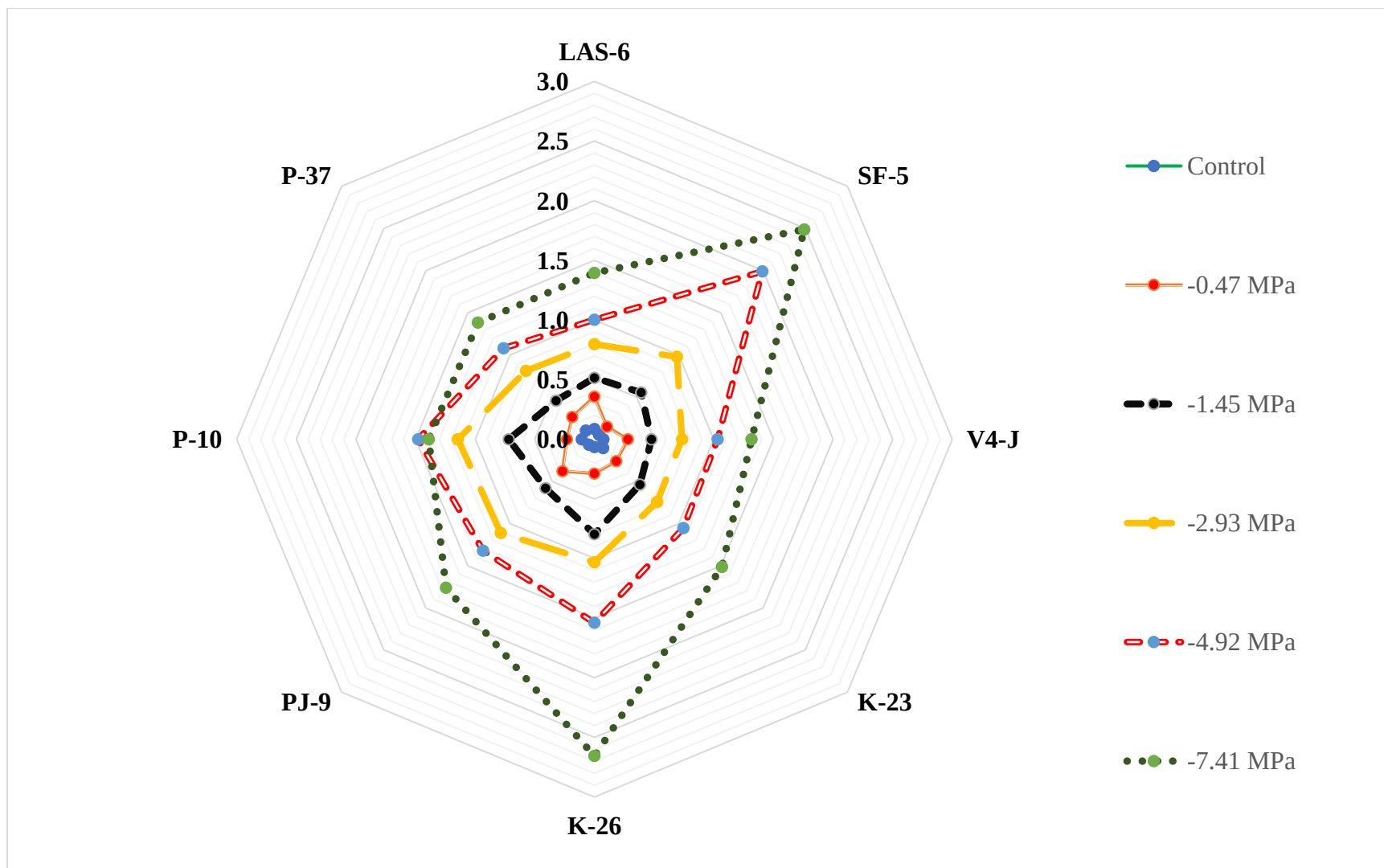


Figure 4.2: Growth response of fungal endophytes to PEG-8000 induced water stress

K-23 had either micro or macroconidia. Microconidia were single-celled, hyaline, nonseptate, ovoid. Macroconidia were four to five septate, slightly curved, tapered at the apex and found similar to *F. incarnatum*.

K-26 had flat mycelium and produced white with very light pink-purple aerial mycelium. The reverse side of the colonies was light yellowish-brown to aurantium. Microconidia were oval to cylindrical with 0 to 1 septa and were produced on monophialides and found similar to *F. redolens*.

P-10 had colonies that were white and cottony and, on the underside, they were reddish-brown. The colony was loose, slimy, and sporadic. It was growing rapidly on PDA. The conidiophores branched into thin, elongated monophialides that produce conidia. The macroconidia were slightly curved, hyaline, broad, and had 3-4 septa. Microconidia were oval or cylindrical, hyaline, smooth, and lack septa and looked like *F. solani*.

P-37 resemble those of the genus *Alternaria*. The colonies were velvety with coloration ranging from olivaceous brown to black. Conidia can range from obovoid to short ellipsoidal, with colors of golden brown to blackish brown, roughened with 1–5 oblique or longitudinal septa and 1–5 lateral septa. Conidiophores were pale brown, erect, and multicelled and was matching with *Ulocladium* sp.

SF-5, PJ-9 and V4-J produced white colonies which gradually turned to light cream color at agar base. Microscopic observations revealed that mycelium was smooth, branched, cylindrical, and septate. Conidiophores were cylindrical, short, simple, septate. Microconidia were round to oval in shape, hyaline, 0–1 septate and macroconidia were curved with short apices pedicellate basal cells, hyaline, 3–4 septate. Clamydospores were produced terminally, singly or in chains, nearly spherical and hyaline. Their characters were matching with *F. equiseti*.

LAS-6 had an olivaceous to the grey-green color colony while the under surface was tinged with yellow. Asci emerged from the ascomata and were club-shaped and ascospores were lemon-shaped. The ascospores were broadly limoniform, and triangle-

shaped in a side view, brown when mature and was matching with *Chaetomium madrasense*.

4.4 Molecular characterization of endophytic fungi

Based on morphological and molecular features, microorganisms can be identified up to species level. However, classification based solely on morphological characteristics is often inadequate, resulting in ambiguity. As the genes encoding 16S rRNA in prokaryotes and 18S rRNA/ITS in eukaryotes are widely distributed, functionally constant, sufficiently conserved, and of appropriate length, they can provide a picture of evolution covering all living microbes (Franco-Duarte *et al.*, 2019).

All eight endophytic fungal isolates obtained were subjected to molecular characterization by amplifying the rDNA region using Internal Transcriber Sequence (ITS) primers. The ITS region fragment is composed of ITS1, 5.8S rDNA, and ITS2. The size usually ranges from 450 to 750 bp (Op De Beeck *et al.*, 2014). The ITS region sequences are widely used in the molecular taxonomy of eukaryotes because they are, small in size, presence of highly conserved sequences, easy to detect even from small quantities of DNA due to the high copy number of the rRNA clusters and a high degree of variation even between closely related species (Madigan *et al.*, 2009). The amplified product was sequenced and compared with the NCBI database using the nucleotide BLAST search to identify the closest published sequences in GenBank. The results of the BLAST search for ITS region (Fungal identity, query length, query coverage, identity, and closest NCBI gene bank number) are listed in Table 4.2. The sequences obtained for fungal isolates had similarities ranging from 98 to 100%. Many sequences had similarities above 99 % and were assigned species identity. Among eight endophytes, the genus *Fusarium* was found to be dominant.

Therefore, in the present study eight fungal isolates were identified based on the homology of their sequences. The ITS partial gene sequence of the K-23 fungal isolate with 503 base pair (bp) showed 99.4 percent homology with *Fusarium incarnatum* available in the NCBI database. The phylogenetic tree constructed with other *Fusarium* species available in the NCBI GenBank with the sequences of identified organism,

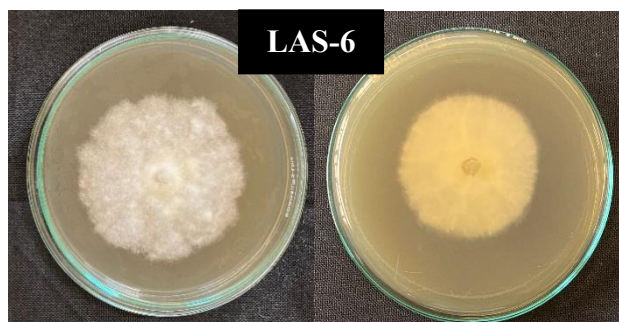
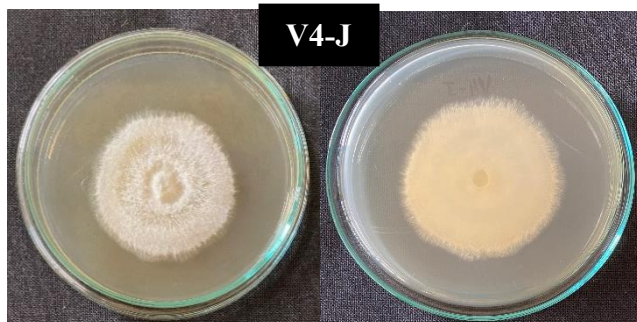
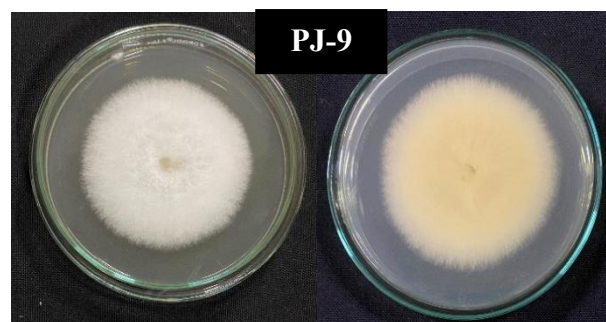
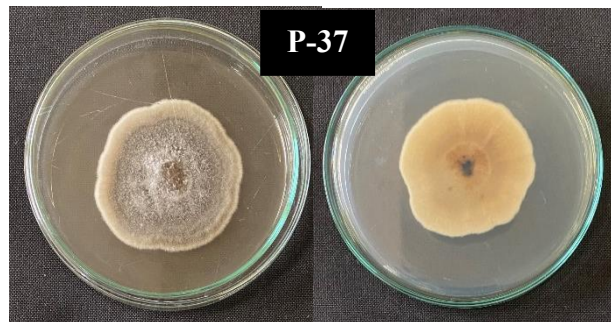
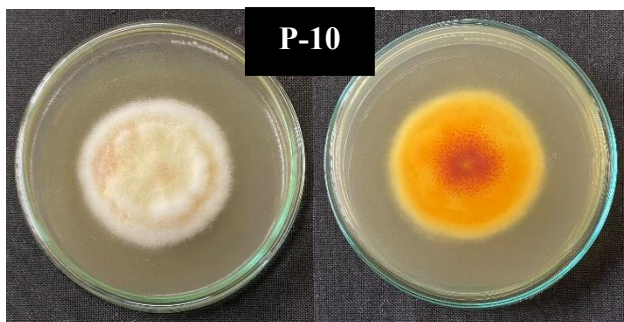
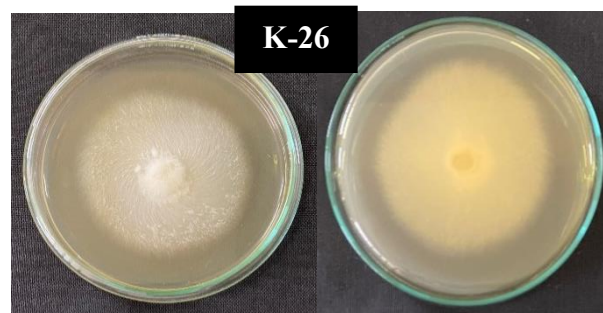
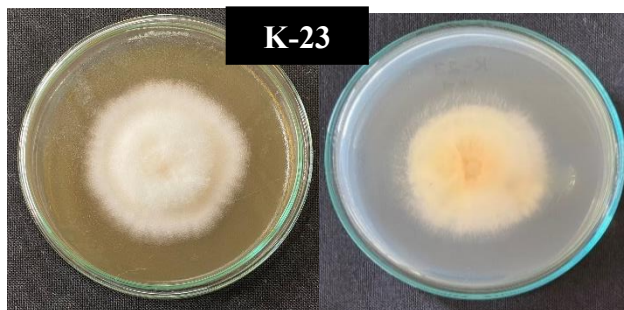


Plate 4.2: Fungal endophytes used in the study (front and reverse view), and its colony characteristics

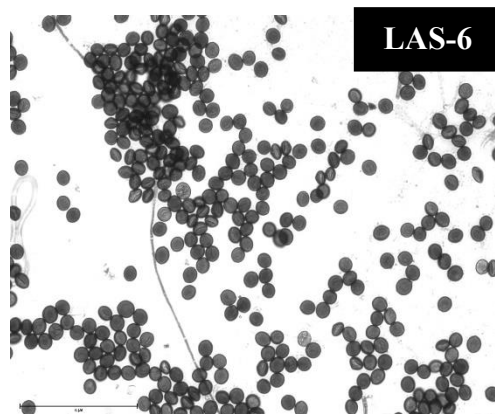
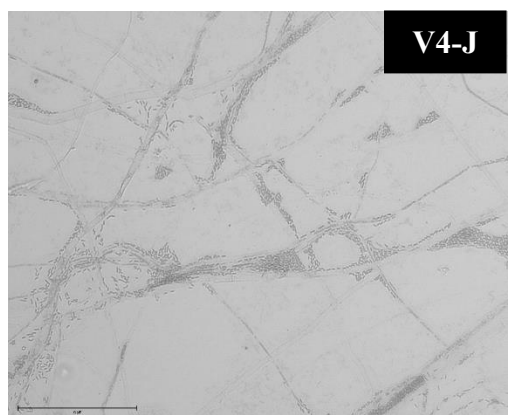
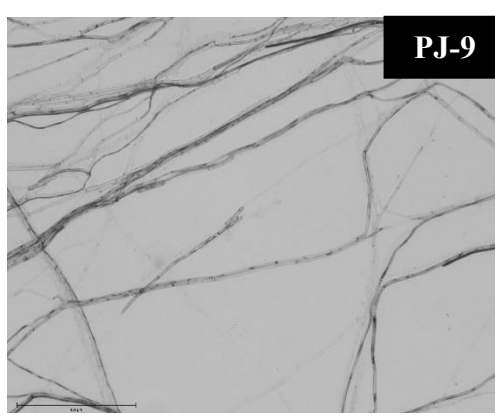
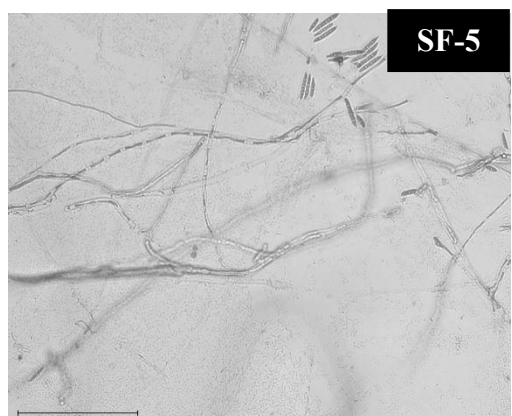
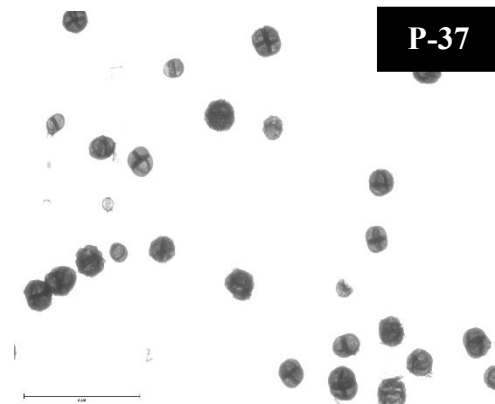
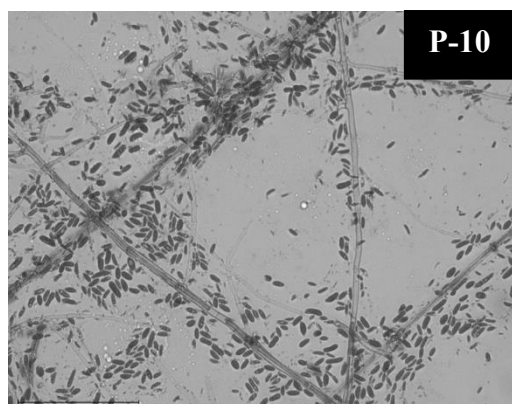
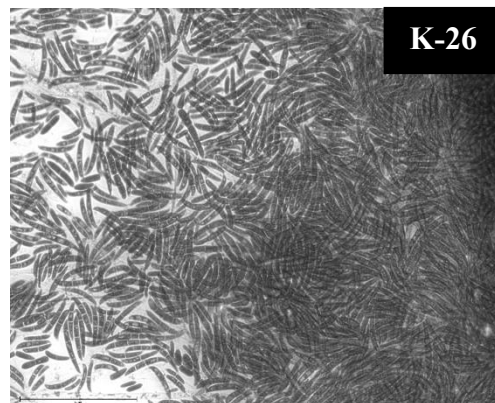


Plate 4.3: Morphological characteristics (spore, conidia and mycelial pattern) of fungal endophytes

however showed that it is closely related to *Fusarium incarnatum* isolate PKS9 (MT032390.1). Therefore, the fungus K-23 was identified as *Fusarium incarnatum*.

The ITS partial gene sequence of the K-26 fungal isolate with 525 base pair (bp) showed 99.62 percent homology with *Fusarium redolens* available in the NCBI database. However, the phylogenetic tree constructed with other *Fusarium* species available in the NCBI GenBank with the sequences of identified organism showed that it is closely related to *Fusarium redolens* strain AT03-6 (MT563397.1). Therefore, the fungus K-26 was identified as *Fusarium redolens*.

The ITS partial gene sequence of the P-10 fungal isolate with 534 base pair (bp) showed 99.63 percent homology with *Fusarium solani* available in the NCBI database. However, the phylogenetic tree constructed with other *Fusarium* species available in the NCBI GenBank with the sequences of identified organism showed that it is closely related to *Fusarium solani* isolate ColPat-359 (MK968891.1). Therefore, the fungus P-10 was identified as *Fusarium solani*.

The ITS partial gene sequence of the P-37 fungal isolate with 496 base pair (bp) showed 99 percent homology with *Ulocladium* sp. available in the NCBI database. However, the phylogenetic tree constructed with other *Ulocladium* and *Alternaria* species available in the NCBI GenBank with the sequences of identified organism showed that it is closely related to *Ulocladium* sp. ACR-D5 (KJ160501.1). Hence, the fungus P-37 was identified as *Ulocladium* sp.

The ITS partial gene sequence of the SF-5, PJ-9 and V4-J fungal isolate with 484, 492, 483 base pair (bp) showed 99.79, 99.8, and 99.59 percent homology with *Fusarium equiseti* available in the NCBI database and the phylogenetic tree constructed with other *Fusarium* species available in the NCBI GenBank with the sequences of identified organism showed that they are closely related to *Fusarium equiseti* strain CB33-2 (MT558602.1), *Fusarium equiseti* isolate s077 (HQ649908.1) and *Fusarium equiseti* isolate 31 (MW812253.1) respectively. Therefore, the fungus SF-5, PJ-9, and V4-J were identified as *Fusarium equiseti*.

Table 4.2: Molecular identification based on sequencing of ITS region of eight endophytic fungal isolates

Sample Code	Query length (bp)	Blast search result	Query cover (%)	Identity (%)	Homologue
K-23	503	<i>Fusarium incarnatum</i>	100	99.40	MT032390.1
K-26	525	<i>Fusarium redolens</i>	100	99.62	MT563397.1
P-10	534	<i>Fusarium solani</i>	100	99.63	MK968891.1
P-37	496	<i>Ulocladium</i> sp.	99	99.00	KJ160501.1
SF-5	484	<i>Fusarium equiseti</i>	100	99.79	MT558602.1
PJ-9	492	<i>Fusarium equiseti</i>	100	99.80	HQ649908.1
V4-J	483	<i>Fusarium equiseti</i>	100	99.59	MW812253.1
LAS-6	512	<i>Chaetomium madrasense</i>	100	99.80	MH864195.1

The ITS partial gene sequence of the LAS-6 fungal isolate with 512 base pair (bp) showed 99.8 percent homology with *Chaetomium madrasense* available in the NCBI data base and the phylogenetic tree constructed with other *Chaetomium* species available in the NCBI GenBank with the sequences of identified organism showed that it is closely related to *Chaetomium madrasense* strain CBS 126663 (MH864195.1). Therefore, the fungus LAS-6 was identified as *Chaetomium madrasense* (Table 4.2, Figure 4.3).

Fungal barcode sequences are found within the ubiquitous, multicopy ribosomal RNA gene. Within this, the internal transcribed spacer (ITS) region has been accepted as a universal barcode for fungi (Schoch *et al.*, 2012). With the most clearly defined gap between inter and intra-specific variation, the ITS region has the highest probability of successful identification for the widest range of fungi (Badotti *et al.*, 2017).

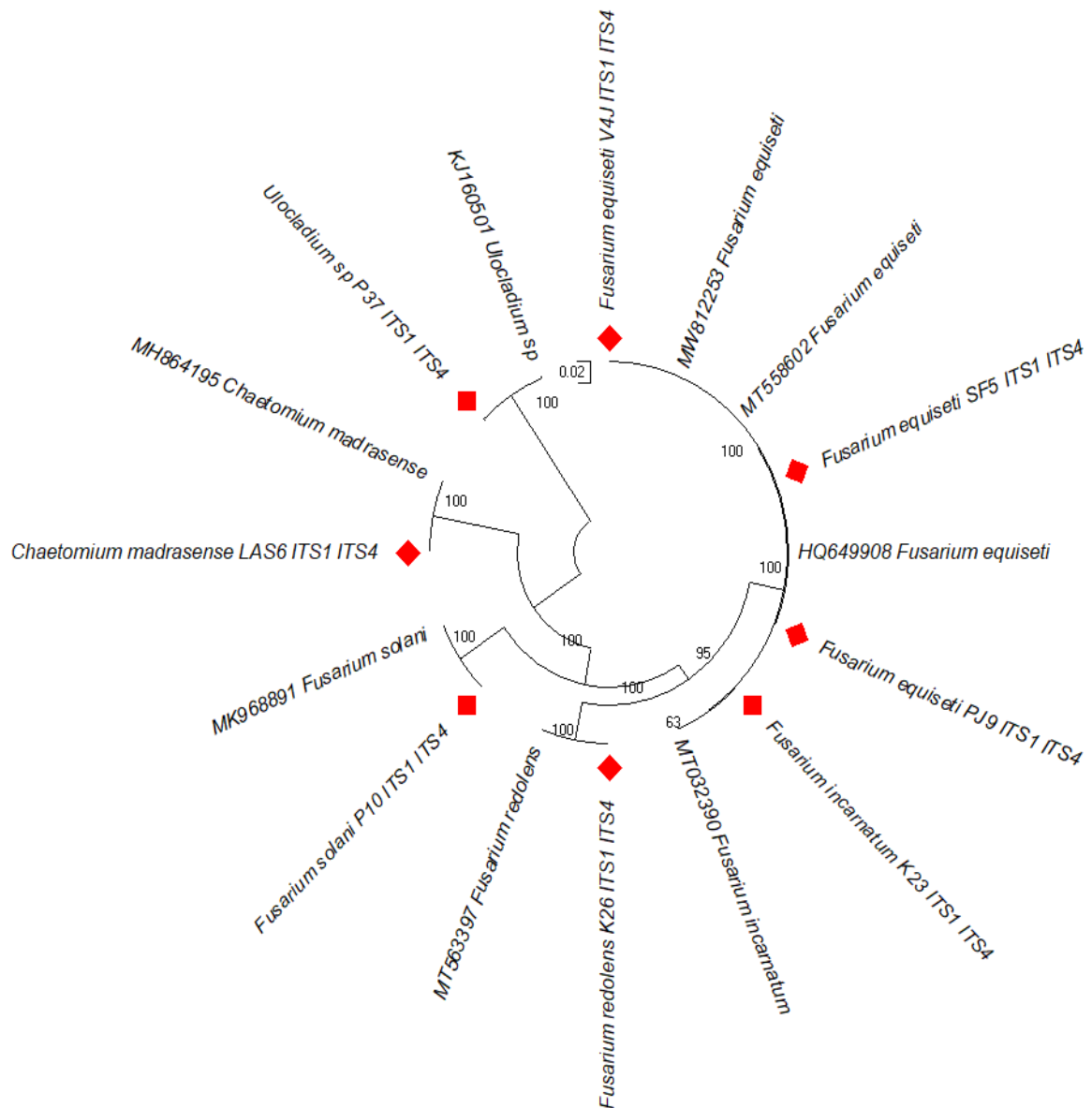


Figure 4.3: UPGMA phylogenetic consensus tree inferred from rDNA ITS Sequences of eight fungal isolates along with their ITS reference sequence from NCBI. The tree was constructed with Bootstrap values of 1000 replicates. The isolate whose sequences have been submitted in NCBI GenBank are highlighted with red colour symbol.

4.5 Screening of endophytes for plant growth-promoting attributes

(a) Phosphate solubilization assay

Qualitative estimation of P-solubilization in fungal strains can be done by measuring the zone of solubilization on Pikovskaya's agar medium containing an insoluble P-source (Chauhan *et al.*, 2017). The phosphate solubilizing ability of each fungal isolate was analyzed. Except for LAS-6 (*Chaetomium madrasense*), all other fungal isolates were capable of forming halo zones around fungal colonies and thus confirming phosphate solubilization (Table 4.3 and Plate 4.4). The development of halo zones around the fungal colonies may be due to the production of organic acids, which will acidify the cell into the surrounding environment and causes the release of mineral ions P by substitution of H⁺ cations bound to phosphate (Park *et al.*, 2009). This scenario will make phosphate to be available to plants. Thus, inorganic phosphate solubilization is one of the major mechanisms of plant growth promotion by plant-associated fungi (Varga *et al.*, 2020).

(b) Siderophore production

Endophytes produce siderophores which are low-molecular-weight (< 1000 Da), Fe (III)-specific ligands that can chelate iron compounds with high affinity. The siderophore producing potential of all eight fungal isolates were examined. Except SF-5, V4-J, and LAS-6 all other isolates (K-23, K-26, P-10, P-37, and PJ-9) produced siderophores forming orange zone around mycelium as evidenced by positive reaction in CAS agar plate test (Table 4.3 and Plate 4.5). These compounds facilitate iron uptake in a variety of environments thus promoting plant growth (Verma *et al.*, 2011; Zhang *et al.*, 2011).

(c) Amylase activity

The endophytic fungal isolates were screened for amylase activity. Among eight isolates, P-10, K-26, SF-5, P-37, and V4-J have given positive results as they have hydrolyzed starch by the production of amylase in starch agar medium, thus forming clear zones around fungal mycelium (Table 4.3 and Plate 4.6). The production of extracellular enzymes like amylase plays a significant role in the decay of organic matter and plant

growth promotion. Many plant beneficial fungi produce extracellular enzymes through an indirect mechanism for plant growth promotion (Gupta *et al.*, 2015). The production of hydrolytic enzymes by endophytes emerges as important for colonization of plant roots and movement of fungi into the interior of plants rendering help in plant growth (El-Deeb *et al.*, 2012; Choubane *et al.*, 2016).

(d) Quantitate assay for indole acetic acid (IAA) production by fungal endophytes

The ability to biosynthesize IAA was observed in all fungal isolates (Table 4.3 and Plate 3.3). Quantitatively IAA production in presence of L-Tryptophan ranged from 13.36 ± 0.15 to 39.43 ± 1.05 $\mu\text{g/ml}$. Isolate P-10 showed the highest production of IAA of 39.43 ± 1.05 $\mu\text{g ml}^{-1}$ followed by K-26 (26.03 ± 0.30 $\mu\text{g ml}^{-1}$) and V4-J (24.40 ± 1.35 $\mu\text{g ml}^{-1}$). It is speculated that the endophyte production of these molecules could benefit the plant through induction of some changes in its morphology, especially in roots, or increasing its length to absorb more water or its length to explore the soil in depth especially in harsh environments (Hazarika *et al.*, 2021).

Table 4.3: Plant growth promotion activities of the fungal endophytes under *in-vitro* conditions

Fungal isolates	Phosphate solubilization	Siderophore production	Amylase activity	IAA production ($\mu\text{g/ml}$)
K-23	+	+	-	16.56 ± 0.26
K-26	+	+	+	26.03 ± 0.30
P-10	+	+	+	39.43 ± 1.05
P-37	+	+	+	18.03 ± 0.41
SF-5	+	-	+	16.46 ± 0.51
PJ-9	+	+	-	16.90 ± 0.72
V4-J	+	-	+	24.40 ± 1.35
LAS-6	-	-	-	13.36 ± 0.15

Note:- implies no halo zone indicates no enzyme activity; + implies the formation of halo zone indicates enzyme activity; values show the average of triplicates mean $\pm$ SD.

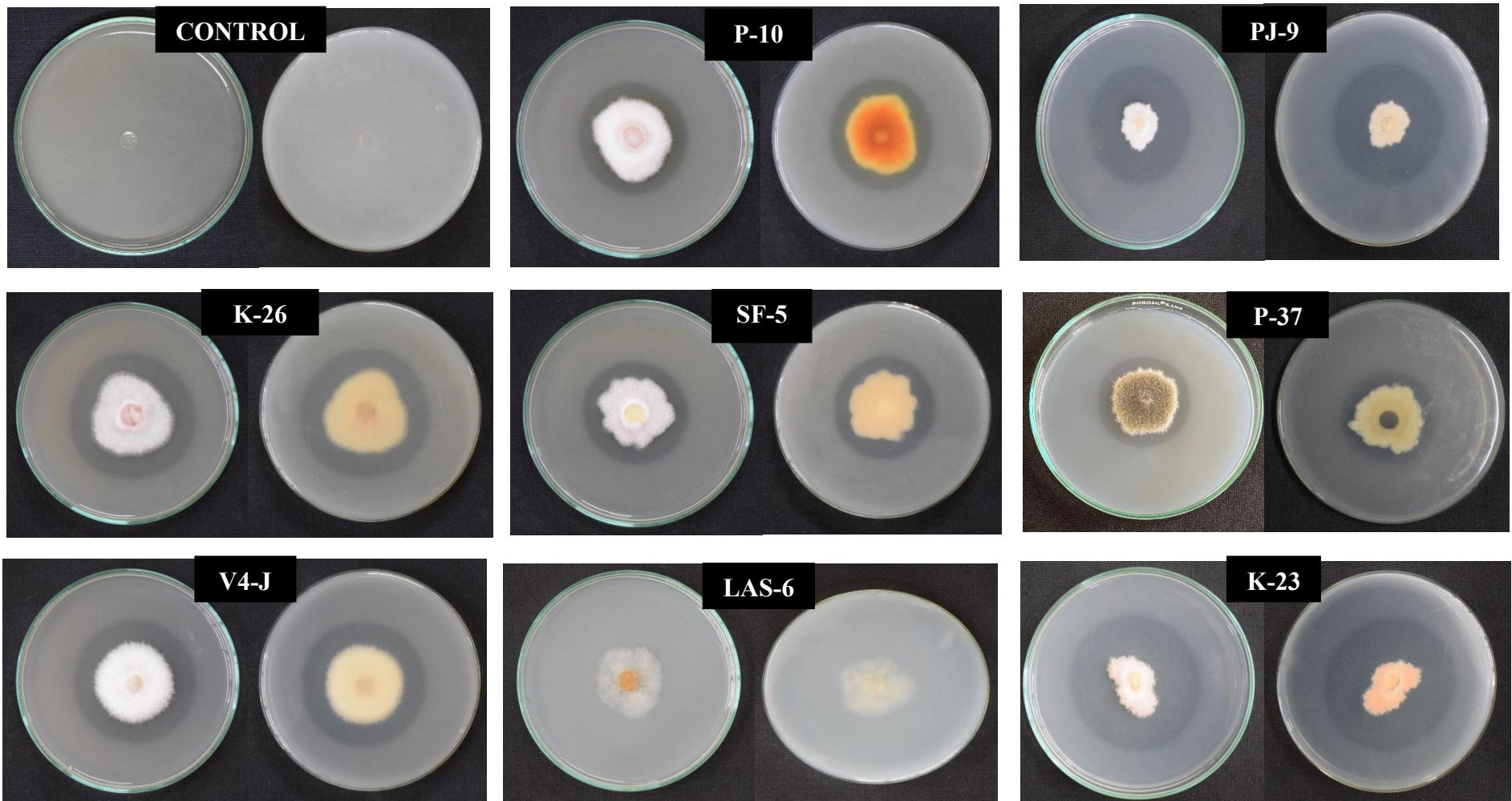


Plate 4.4: The phosphate solubilization activity of endophytic fungi on Pikovskaya's media forming halo zones around mycelial growth

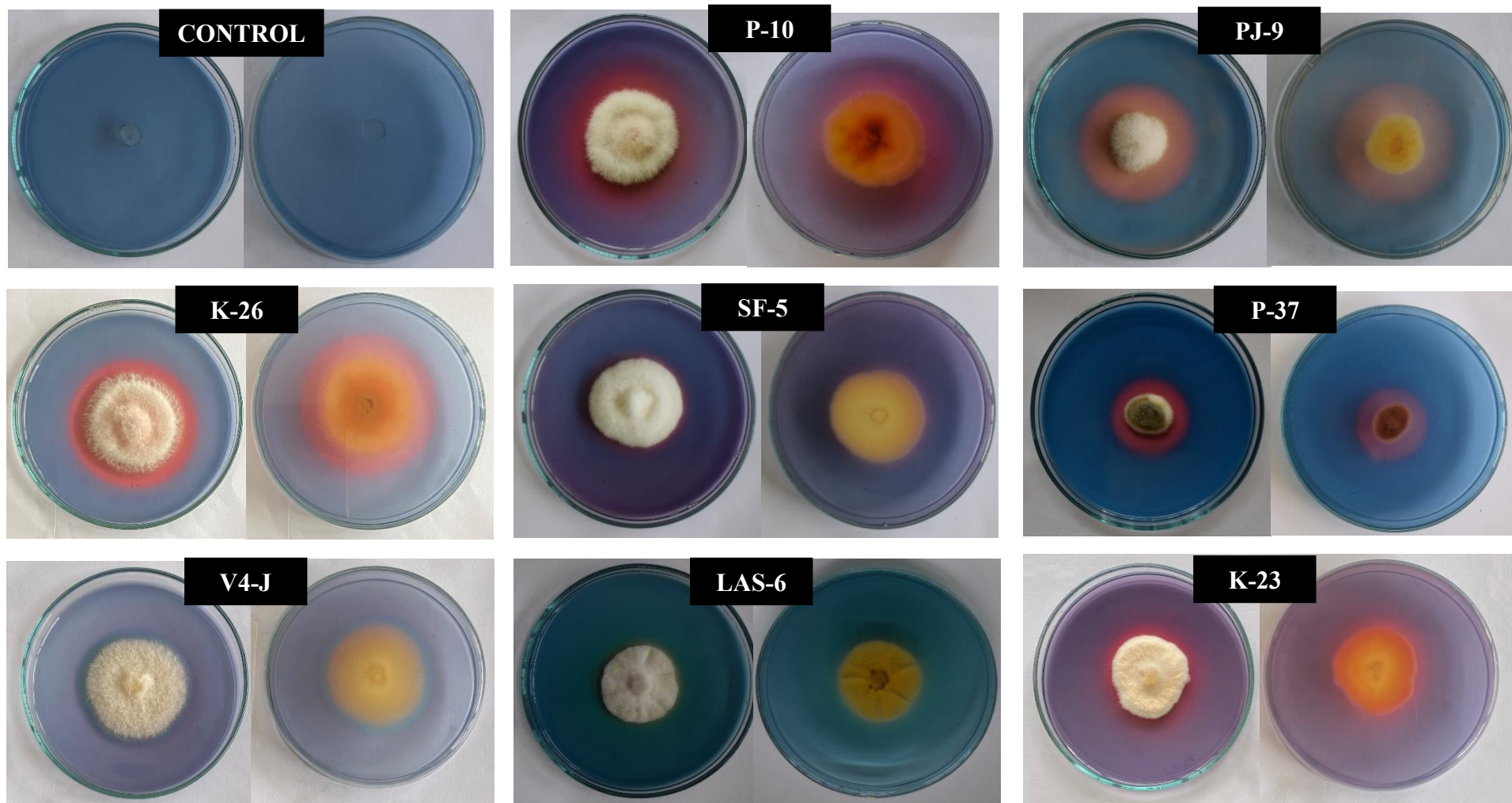


Plate 4.5: Siderophore production activity by fungal isolates forming orange halo zones through FePO_4 solubilization around mycelial growth

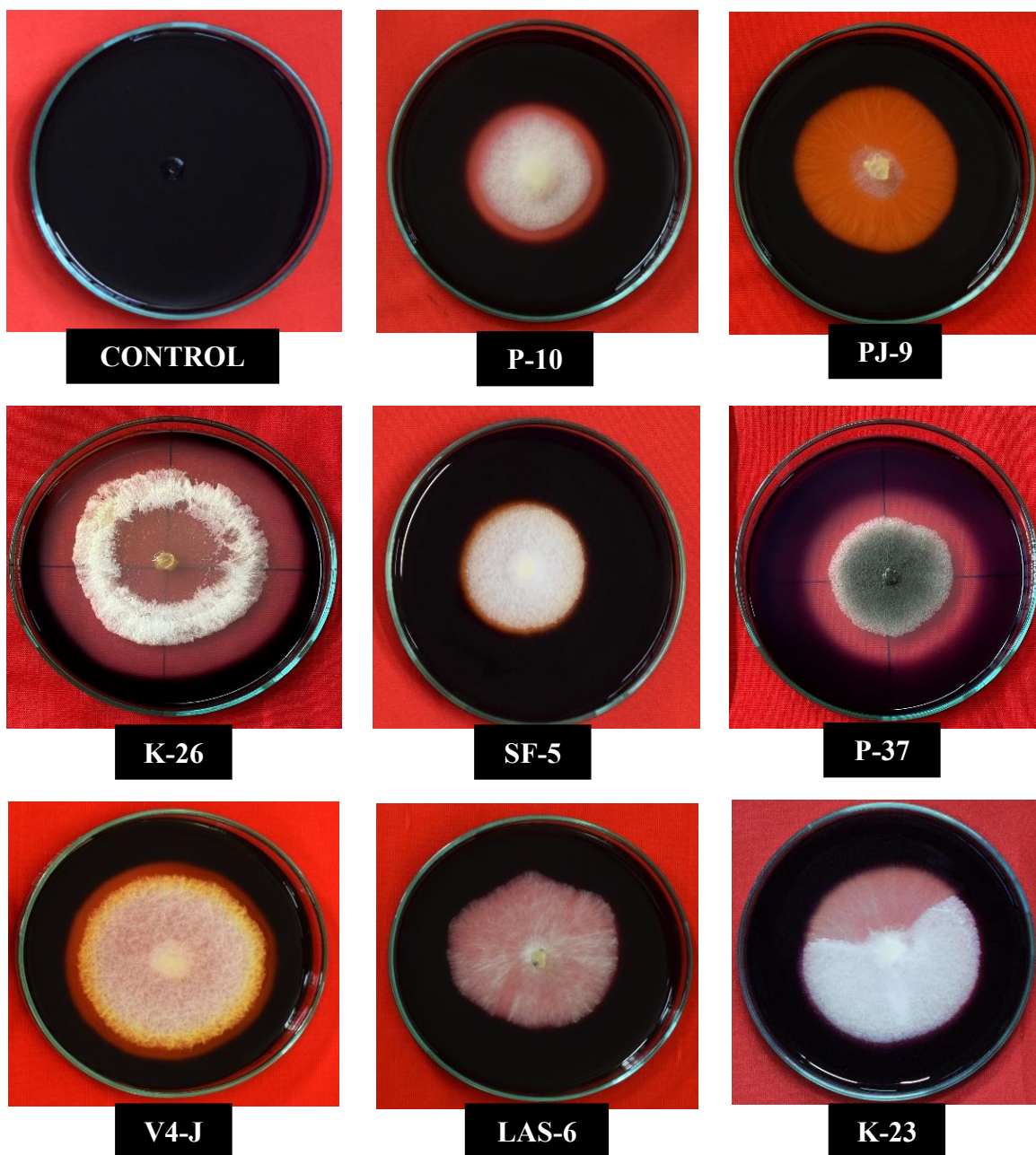


Plate 4.6: Amylase activity of fungal isolates showing zone of starch hydrolysis

4.6 Identifying the best crop-endophyte combinations for drought stress tolerance under *in-vitro* conditions

All eight fungal isolates obtained from the culture collections were used to evaluate their ability to modulate early seedling growth in normal and drought stress conditions in drought-sensitive genotypes of each crop system such as maize (MAH 14-5), green gram (BGS-9), soybean (KBS-23) and brinjal (Arka Harshitha). For inducing drought stress condition, the pre-germinated seeds of maize (MAH 14-5), green gram (BGS-9), soybean (KBS-23), and brinjal (Arka Harshitha) were subjected to LC₅₀, -2.79 MPa, -2.57 MPa, -1.28 MPa, and -3.38 MPa of PEG-8000 respectively (Figure 4.1.1 and Figure 4.1.2). All selected eight endophytes showed significantly increased seedling length in maize (MAH 14-5) under unstressed conditions and under PEG-8000 induced drought stress conditions, all endophytes significantly increased seedling length except, PJ-9 (*Fusarium equiseti*) (Figure 4.4.1). Whereas, green gram enriched with LAS-6 (*Chaetomium madrasense*), SF-5 (*Fusarium equiseti*), K-26 (*Fusarium redolens*), and V4-J (*Fusarium equiseti*) significantly enhanced seedling growth under normal as well as PEG-8000 induced drought stress condition compared to control (Figure 4.4.2). Under drought stress conditions, the soybean pre-germinated seeds enriched with LAS-6 (*Chaetomium madrasense*), V4-J (*Fusarium equiseti*), K-23 (*Fusarium incarnatum*), and P-37 (*Ulocladium* sp.) showed significantly increased seedling growth, but under unstressed conditions, only V4-J (*Fusarium equiseti*) performed better (Figure 4.4.3). Yet, in brinjal, there was no significant difference between control and fungal endophytes treated plants (Figure 4.4.4).

However, the effect of an endophyte on a particular plant host may be variable (Bastías *et al.*, 2021) depending on the endophytic species, the host genotype, and the environmental conditions (Ahlholm *et al.*, 2002). Here, in the present study, each endophytic isolate behaved differently in each crop system studied. This could be due to the differences in plant-endophyte interactions and each host system may have unique signaling molecules or mechanisms. Among the different interactions studied between endophytes and crop systems, the three fungal isolates viz., K-23 (*Fusarium incarnatum*), LAS-6 (*Chaetomium madrasense*), and P-37 (*Ulocladium* sp.) were performing better in maize (MAH 14-5), so this was considered as the best crop-endophyte combinations (Plate

4.7) to study in greenhouse and field conditions. In order to confirm the colonization of these three endophytes, re-isolation from different tissues (root, shoot, and leaves) of inoculated maize (MAH 14-5) seedlings was done under both normal and drought stress conditions. Mycelia of these three endophytes emerged after 3 days of incubation from the cut ends of leaf, stem, and root of endophyte-treated seedlings (Plate 4.8.1 and 4.8.2). This was confirmed by morphological comparison with their respective mother culture grown on PDA. Colony characters and fruiting bodies of all the three isolates were found similar to the respective endophytes. However, further molecular characterization is required for precise confirmation.

Here, the endophytes modulate various essential functions in plants by directly affecting growth, development, and tolerance to stress conditions (Wani *et al.*, 2015; Molina-Montenegro *et al.*, 2016; Acuna-Rodriguez *et al.*, 2020). Mechanisms employed by endophytes include production of various chemical metabolites (e.g., plant growth hormones, secondary compounds) and modulation of host gene expression and other secondary metabolic pathways (Bacon and White, 2016; Deepika *et al.*, 2016; Ramos *et al.*, 2018; He *et al.*, 2021). Similar results were also reported by Sangamesh *et al.* (2018), where the root length of IR-64 paddy seedlings treated with LAS-4 (*Aspergillus* sp.) and SAP-3 (*Aspergillus* sp.) fungal endophytes significantly increased compared to non-treated control plants under PEG induced drought stress. Khan *et al.* (2015b) isolated endophytic fungi *Fusarium tricinctum* RSF-4L from leaves of *Solanum nigrum*. The culture filtrate of the above fungus enhanced the growth attributes of Dongjin rice plants. Subsequently, gas chromatography/mass spectrometry analyses revealed that RSF-4L produced indole acetic acid. This indicates that endophytes synthesize bioactive compounds that could play important roles in promoting plant growth. Recent studies have demonstrated that certain endophytes promote host plant growth through the synthesis of phytohormones such as indole-3-acetic acid (IAA), gibberellins (GAs), and cytokinins (You *et al.*, 2013; Khan *et al.*, 2017). Under drought conditions, upregulation of free glucose, fructose, trehalose, sugar alcohols, proline, and glutamic acid were detected in shoots and roots of tall fescue colonized by *Neotyphodium coenophialum* compared to control (Nagabhyru *et al.*, 2013).

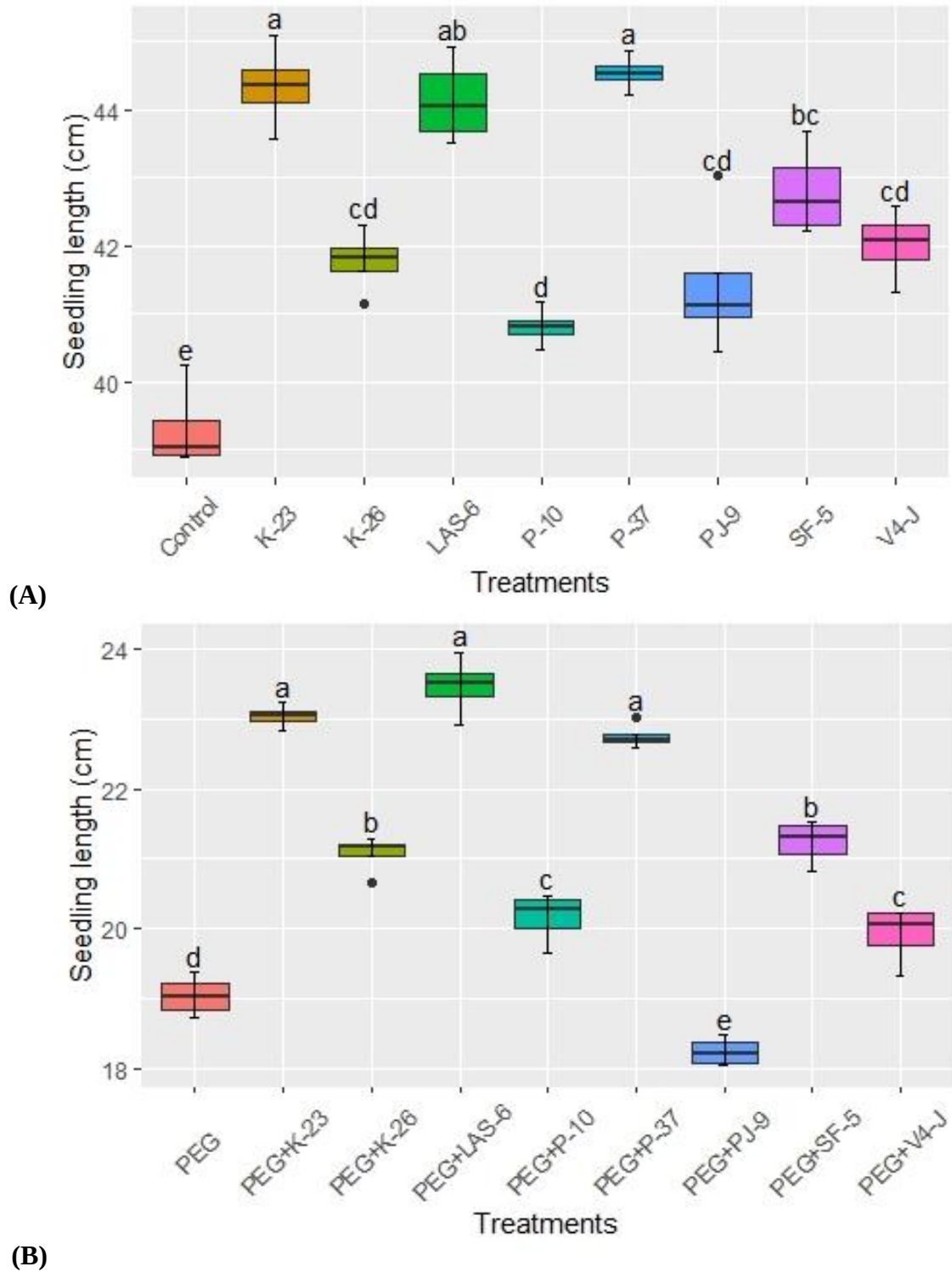
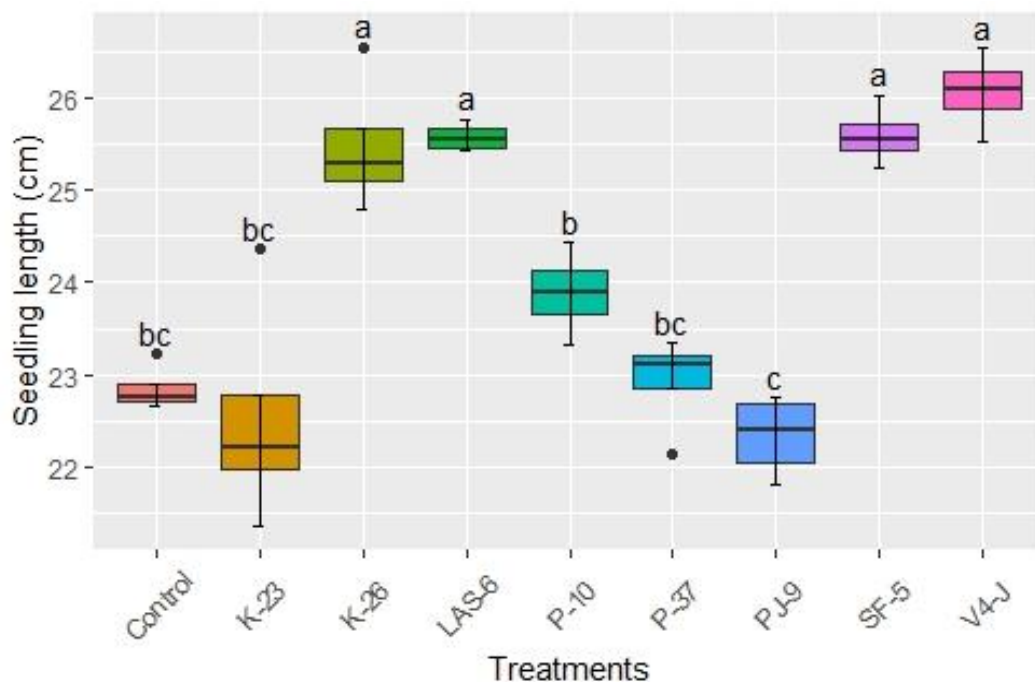
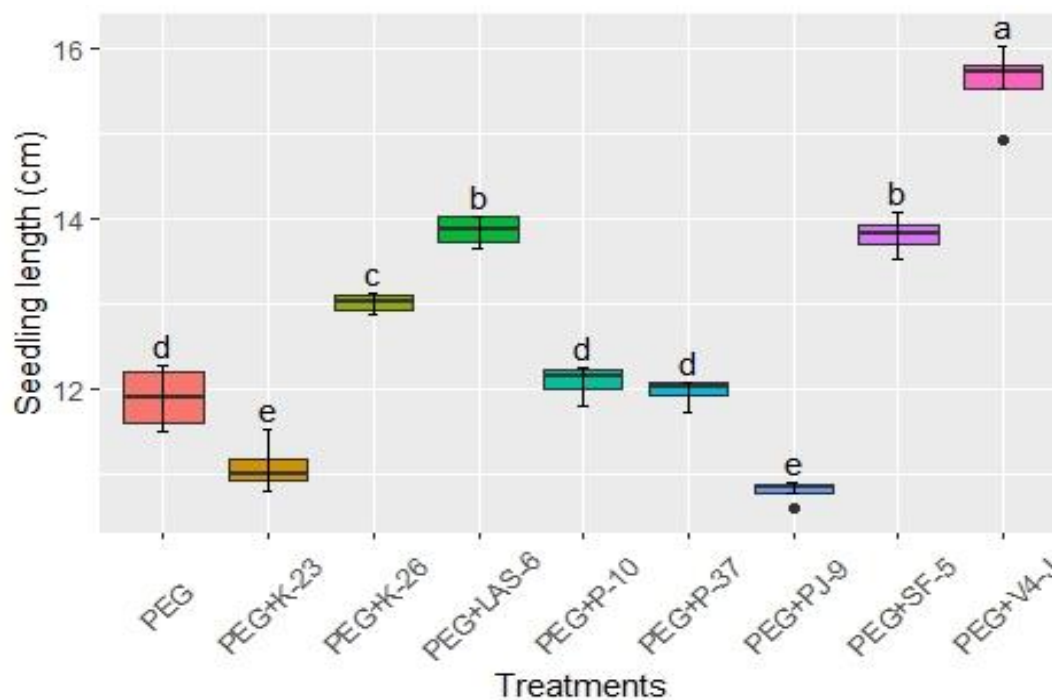


Figure 4.4.1: Seedling length of maize (MAH 14-5) colonized with different fungal isolates under (A) unstressed and (B) PEG-8000 induced drought stress conditions. Mean $\pm$ SE (n=40) with dissimilar alphabets are significantly different at Tukey's $p \leq 0.05$. Lines over boxplots indicate standard error over mean.

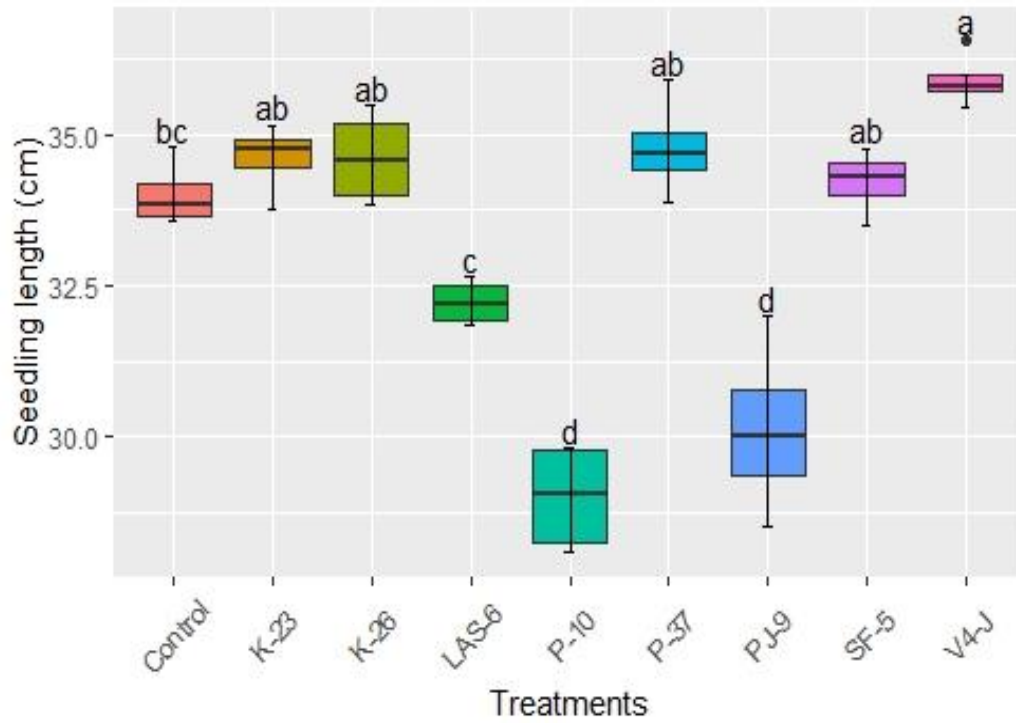


(A)

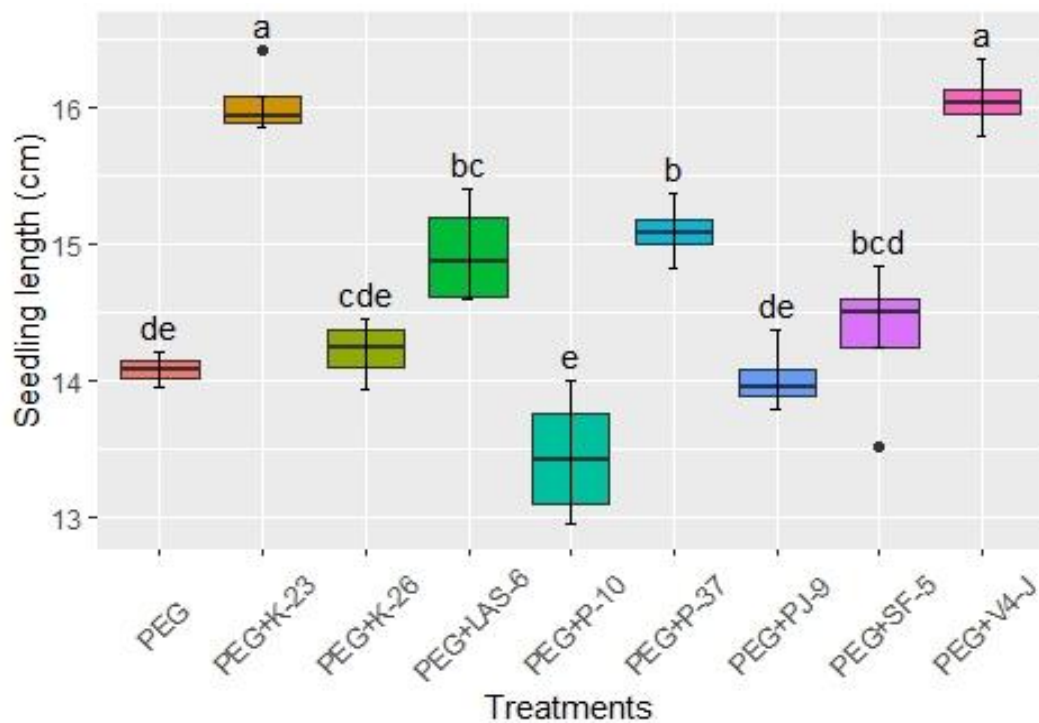


(B)

Figure 4.4.2: Seedling length of green gram (BGS-9) colonized with different fungal isolates under (A) unstressed and (B) PEG-8000 induced drought stress conditions. Mean $\pm$ SE (n=40) with dissimilar alphabets are significantly different at Tukey's $p \leq 0.05$. Lines over boxplots indicate standard error over mean.

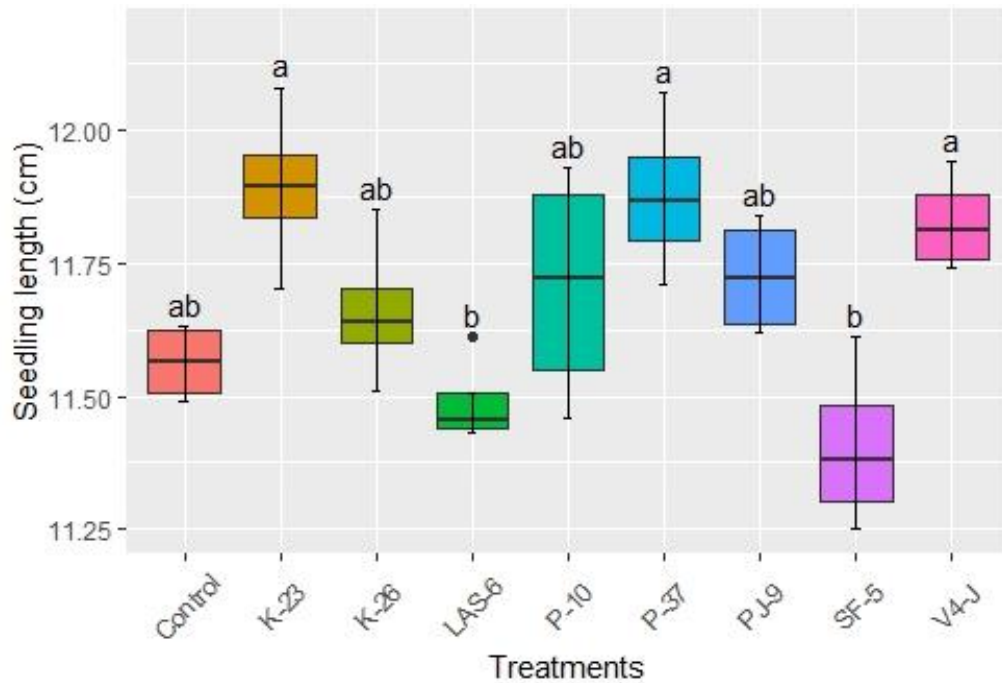


(A)

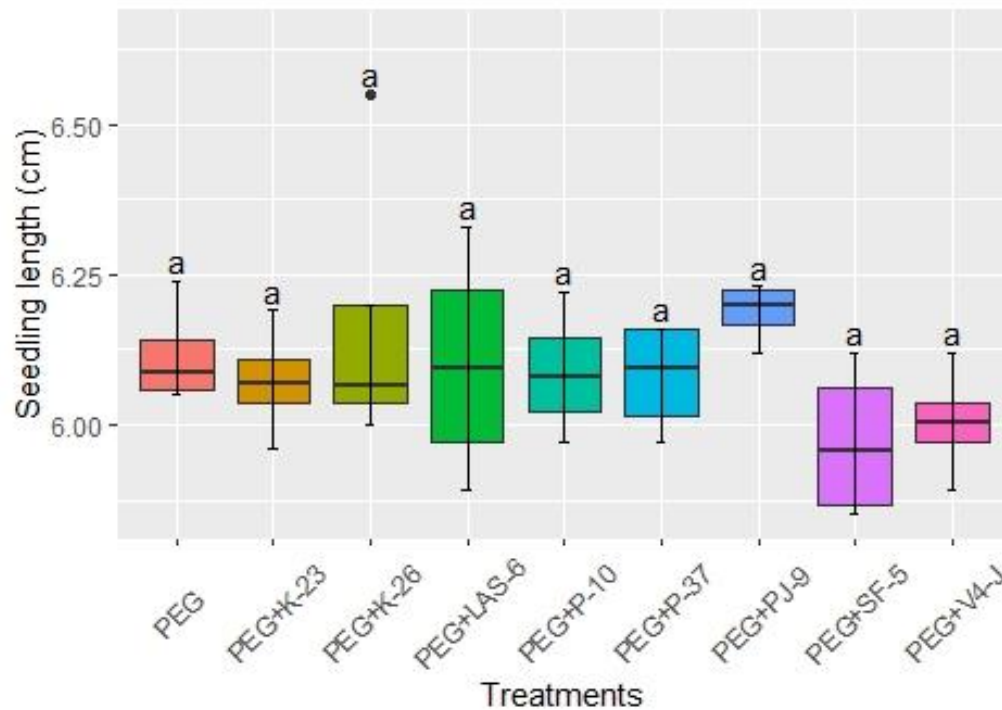


(B)

Figure 4.4.3: Seedling length of soybean (KBS-23) colonized with different fungal isolates under (A) unstressed and (B) PEG-8000 induced drought stress conditions. Mean $\pm$ SE (n=40) with dissimilar alphabets are significantly different at Tukey's $p \leq 0.05$. Lines over boxplots indicate standard error over mean.



(A)



(B)

Figure 4.4.4: Seedling length of brinjal (Arka Harshitha) colonized with different fungal isolates under (A) unstressed and (B) PEG-8000 induced drought stress conditions. Mean $\pm$ SE (n=40) with dissimilar alphabets are significantly different at Tukey's $p \leq 0.05$. Lines over boxplots indicate standard error over mean.

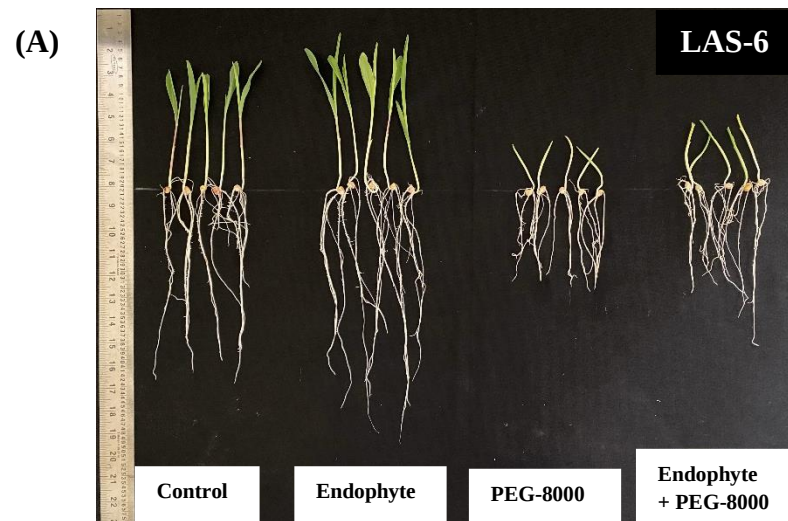


Plate 4.7: Performance of best crop-endophyte combination under unstressed and PEG-8000 induced drought stress after 7 days of incubation. Maize (MAH 14-5) treated with (A) LAS-6, (B) P-37, and (C) K-23

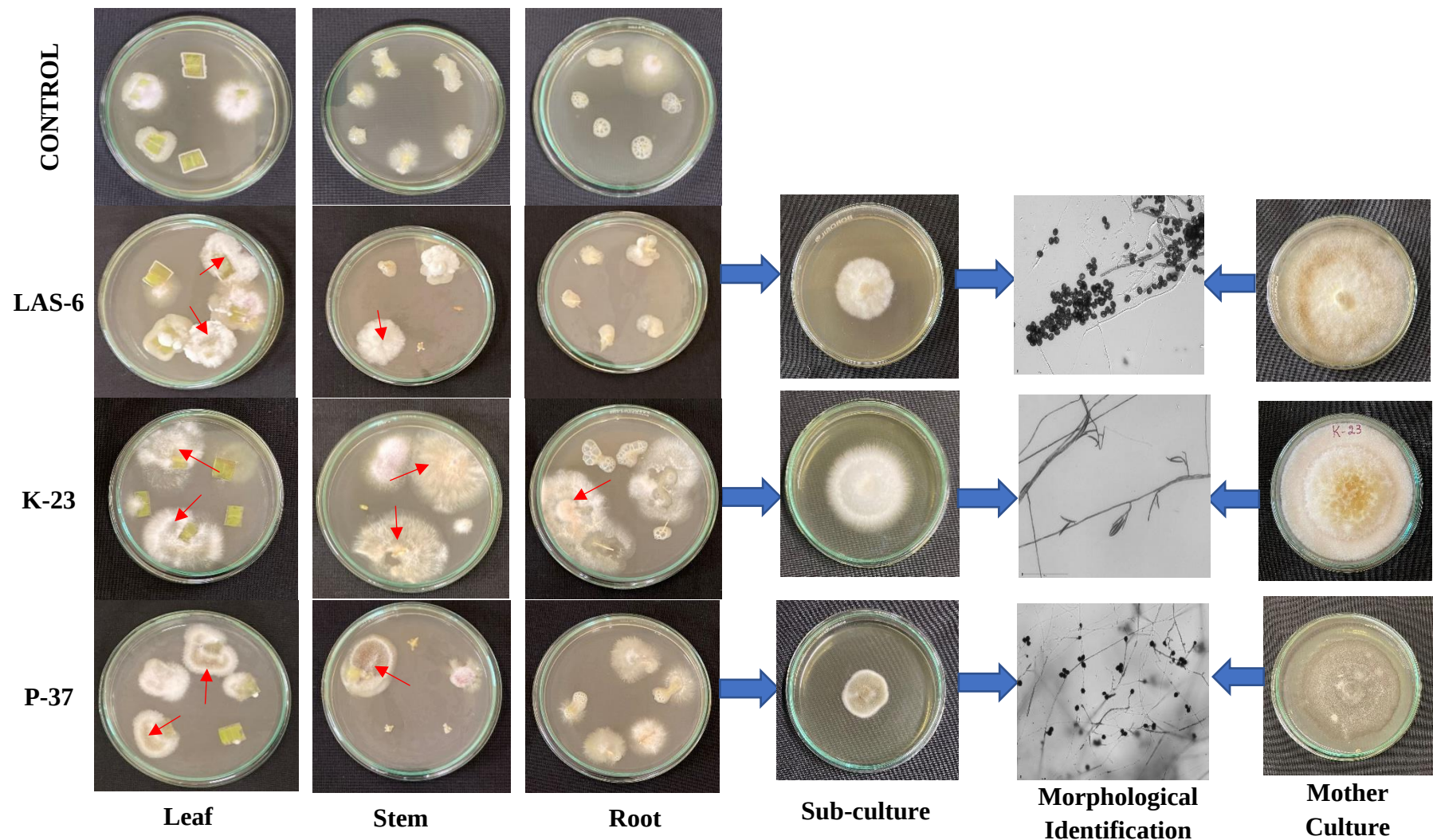


Plate 4.8.1: Colonization of endophytic fungi in leaf, shoot, and roots of endophyte treated plants under unstressed condition (*In-vitro*) and morphological comparison of re-isolated endophytes with mother culture

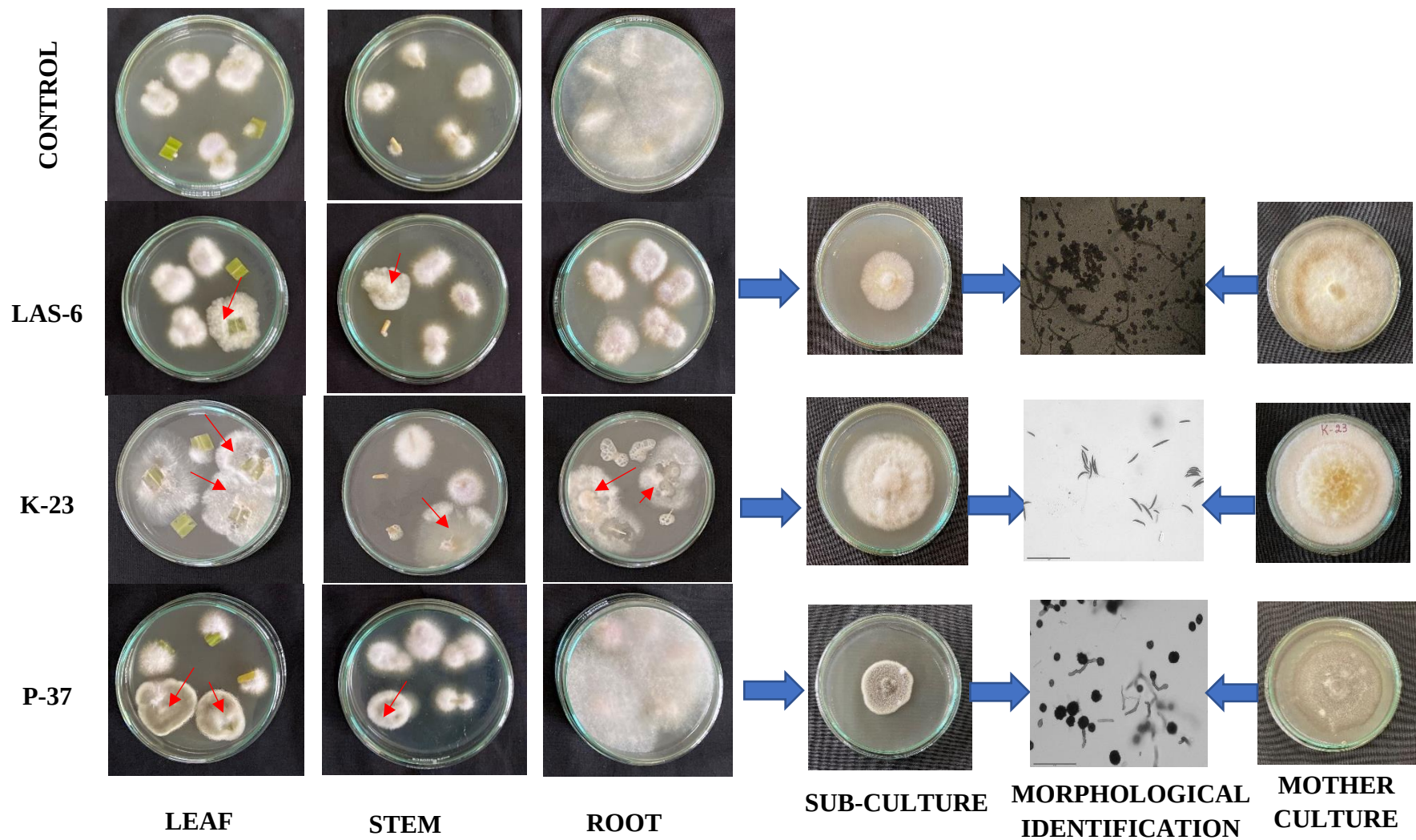


Plate 4.8.2: Colonization of endophytic fungi in leaf, shoot and roots of endophyte treated plants under PEG-8000 induced drought stress condition (*In-vitro*) and morphological comparison of re-isolated endophytes with mother culture

Increased levels of proline, soluble sugar, and catalase (CAT) were observed in wheat colonized by endophyte *Chaetomium globosum* under water stress (Cong *et al.*, 2015).

Similarly, it has been noticed that enrichment of pre-germinated seeds with a few fungal endophytes resulted in a lower magnitude of growth which significantly reduced seedling growth and behaved differently under normal and drought stress conditions. Plant growth responses in this experiment could be explained by various reasons, the most obvious of which is their inability to colonize phylogenetically distant species of plants. It could also be due to a lack of effective signaling between the endophyte and the plant, which is particularly true when the endophyte is put into a non-host system. Reduced growth in response to endophytic colonization can be interpreted as weak parasitism (Mandyam *et al.*, 2013) or as induction of host resistance, with carbon being allocated to the production of defence compounds rather than to vegetative growth (Aime *et al.*, 2013).

4.7 Evaluation of best crop-endophytes combinations for their ability to impart drought stress tolerance under greenhouse conditions

4.7.1 Determination of field capacity

The volume of soil moisture or water content retained in the soil after surplus water has been drained away and the rate of downward movement has reduced is known as field capacity (FC). It's a useful indicator of a soil's water-holding ability. Soil water has the potential to alter not only the physical condition of the soil but also the chemical and biological conditions, such as plant nutrient intake and soil organism activity. As a result, in agricultural systems, soil water management is critical. The field capacity of the potting mixture utilized in the pot experiment was assessed in this investigation.

Weight of an empty plastic pot (Capacity – 25 kg) (W) = 0.593 kg

Weight of Plastic pot + potting mixture (sand, soil, FYM) = W_D = 18 kg

Weight of Plastic pot + saturated soil = W_W = 22.442 kg

Field capacity (100 %) = $(W_W - W) - (W_D - W)$ = 4.442 kg = 4442 ml

Similarly, 75 % Field capacity value = 3331.8 ml of water, Pot weight = 21.331 kg and 50 % Field capacity value = 2221.2 ml of water, Pot weight = 20.221 kg.

Therefore, for 100 % FC 4442 ml, 75 % FC 3331.8 ml, 50 % FC 2221.2 ml of water was added initially and maintained daily by weighing the pot from 45 to 65 DAS.

These three water regimes, namely control (100 % Field Capacity), moderate (75 % Field Capacity), and severe (50 % Field Capacity) stress conditions, were maintained from 45 to 65 DAS, for evaluating the best crop-endophytes combination at the pot level by considering the work done by Sah *et al.* (2020), where drought stress during the pre-flowering and grain filling stages immensely affects plant performance due to imprecise traits function. Drought stress reduced grain yield per hectare by increasing flowering days, days to maturity, anthesis silk interval, decreasing leaf number, abnormal expression of secondary stress-responsive characteristics, and loss of normal root architecture.

4.7.2 In-vivo evaluation of selected endophytes on maize growth and physiology at 30 DAS

To evaluate the selected endophytes on maize under greenhouse conditions at 30 DAS, three endophytic fungi LAS-6 (*C. madrasense*), K-23 (*F. incarnatum*), and P-37 (*Ulocladium sp.*) inoculated maize seeds along with non-inoculated seeds were sown in pots under 100% field capacity. The growth attributes like, plant height, number of leaves, stem girth, leaf area, root length, root volume, plant dry biomass and physiological traits like SPAD chlorophyll meter reading (SCMR), photosynthetic rate, stomatal conductance, transpiration rate, total chlorophyll were recorded. The phenotype of plants (shoot and root) grown in pots at 30 DAS under 100% field capacity with endophytes treatments are represented in Plate 4.10 and confirmation of colonization was done by re-isolation from leaf and shoot of treated plants and represented in Plate 4.9.1 and 4.9.2. Endophytes treated plants performed better compared to control in soil conditions.

Growth parameters

Under 100% field capacity, plant height varied from 86.44 to 95.88 cm among the treatments. LAS-6 (*C. madrasense*) and K-23 (*F. incarnatum*) inoculated plants showed a

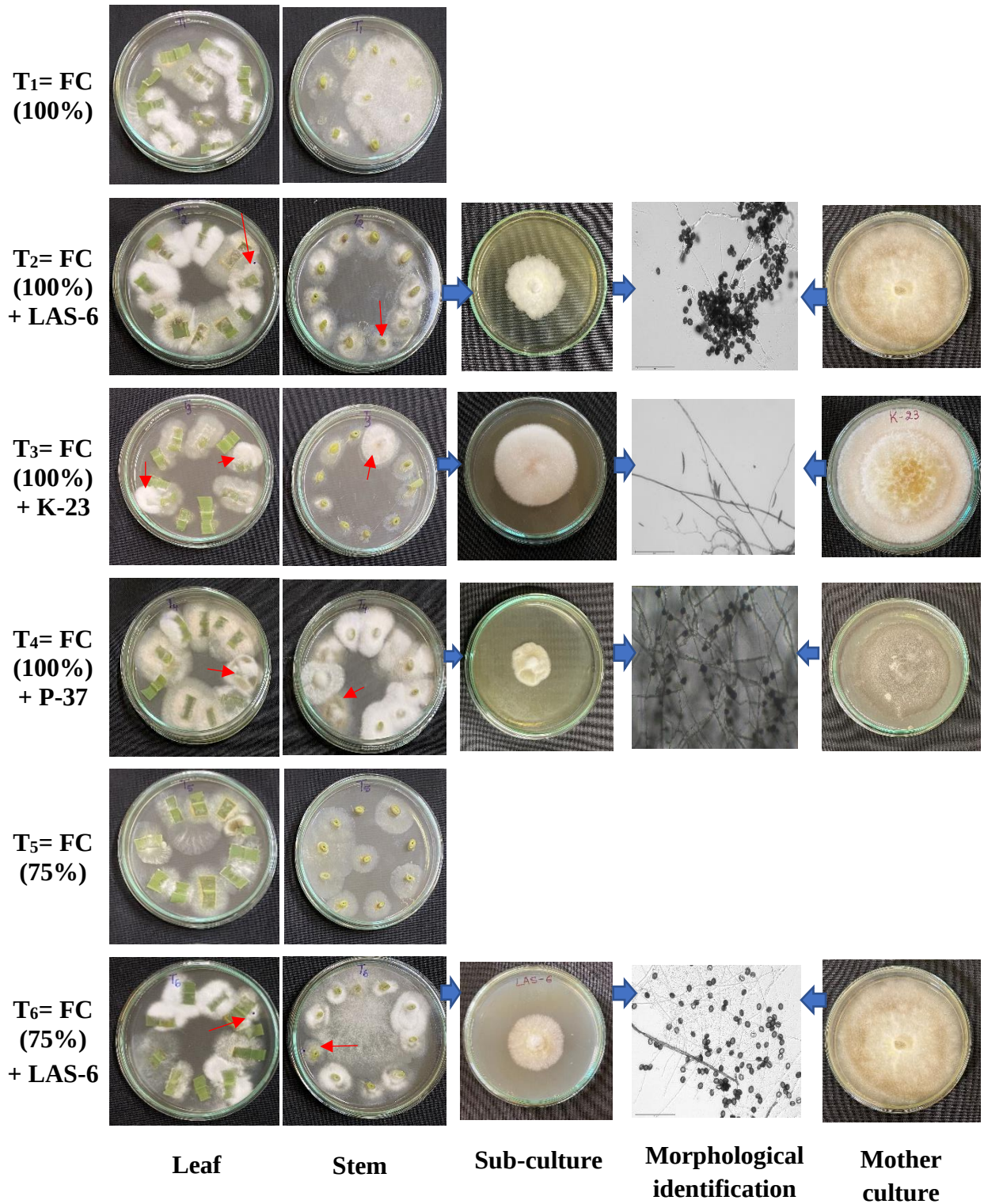


Plate 4.9.1a: Colonization of endophytic fungi in leaf and shoot of treated plants sown in pots and morphological comparison of re-isolated endophytes with mother culture

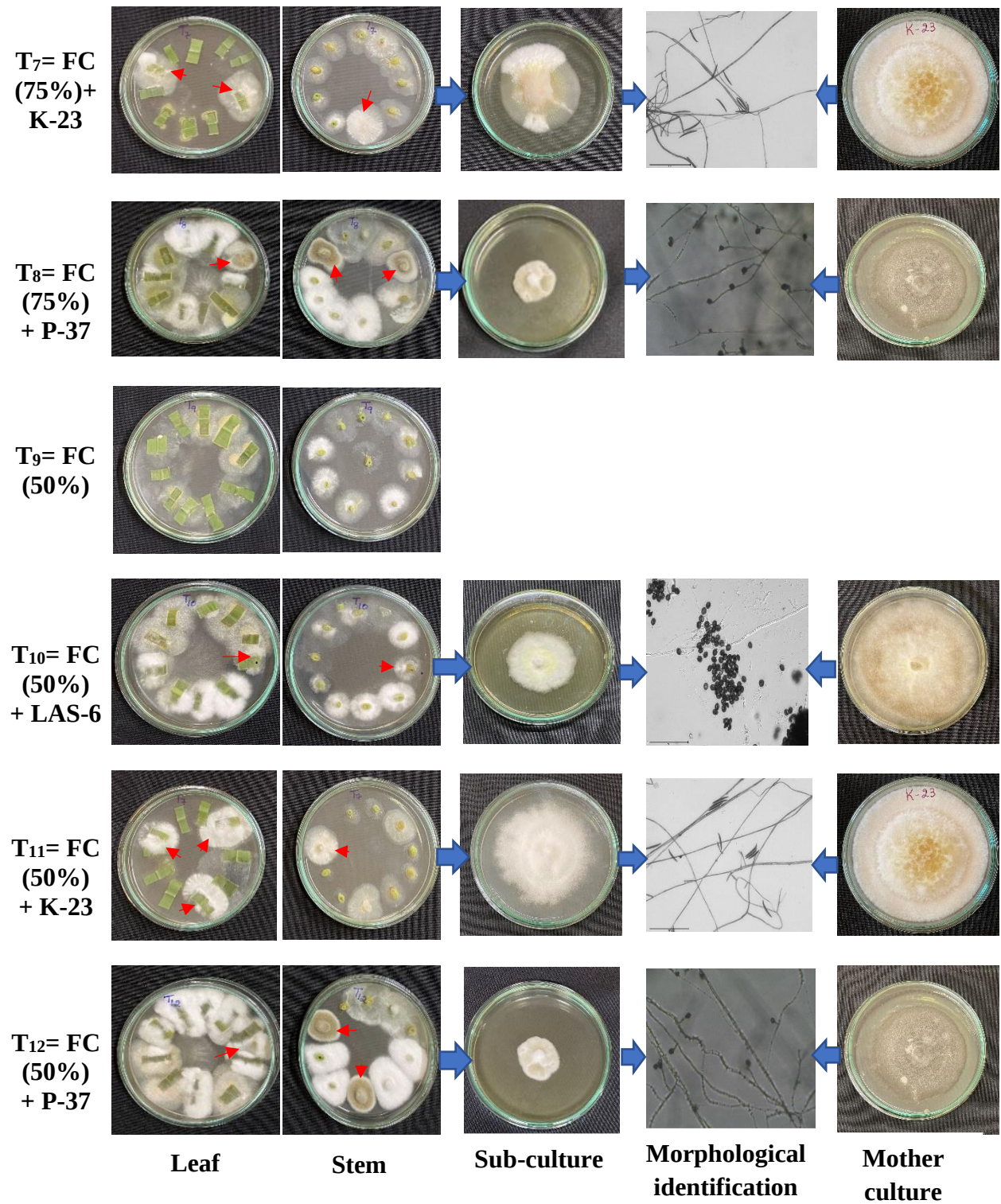


Plate 4.9.1b: Colonization of endophytic fungi in leaf and shoot of treated plants sown in pots and morphological comparison of re-isolated endophytes with mother culture

(A)



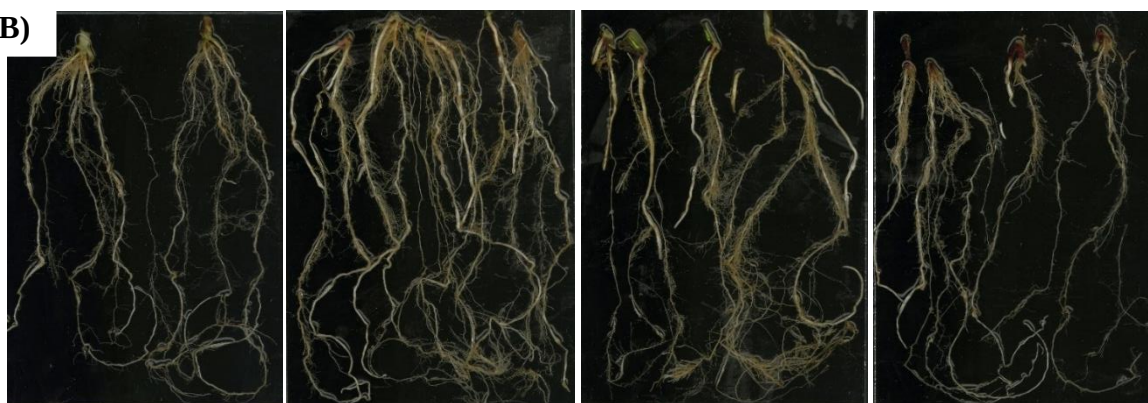
T₁ (Control)

T₂ (LAS-6)

T₃ (K-23)

T₄ (P-37)

(B)



T₁ (Control)

T₂ (LAS-6)

T₃ (K-23)

T₄ (P-37)

Plate 4.10: Phenotype of non-inoculated and endophytes inoculated plants of maize at 30 DAS (A) Shoot traits and (B) Root traits

statistically significant increase in plant height 95.88 cm and 95.45 cm respectively, compared to control (86.44 cm). While P-37 (*Ulocladium sp.*) treated plants had a marginal increase (92.40 cm) in plant height (Figure 4.5.1). In contrast, there was no significant difference in number of leaves among the treatments (Figure 4.5.1). Stem girth was significantly high in endophyte inoculated plants, LAS-6 (1.70 cm), K-23 (1.74 cm) and P-37 (1.66 cm) compared to control (1.35 cm). In addition, endophytes inoculation increased the leaf area, where K-23 (3408.87 cm²) inoculated plants showed significant increase in leaf area compared to control (2685.98 cm²), but LAS-6 (3301.23 cm²) and P-37 (3193.06 cm²) showed marginal increase (Figure 4.5.2).

Root volume was more in endophyte inoculated plants than non-inoculated plants. Among the treatments, LAS-6 (73.3 cm³) and K-23 (70.0 cm³) showed significant increase compared to control (46.6 cm³), whereas P-37 (63.3 cm³) showed marginal increase. But, increase in root length was not statistically significant among endophytes inoculated and control plants. Plant dry biomass was more in endophyte inoculated plants than non-inoculated plants. LAS-6 (24.5 g) and K-23 (23.3 g) showed statistically significant increase in plant dry biomass compared to control (20.6 g) and, P-37 (22.6 g) inoculated plants were on par with control plants (Figure 4.5.2).

In total, LAS-6 and K-23 inoculated plants performed better in greenhouse conditions at 30 days after sowing than non-inoculated plants. But, a marginal increase in some parameters was observed in P-37 inoculated plants. The growth promotion may be attributed to the increase in photosynthetic efficiency upon endophytes treatment (Figure 4.5.3). However, in recent studies, it was revealed that plant growth promotion due to the secretion of phytohormones, such as indole-3-acetic acid (IAA), gibberellins, cytokinin, and secondary metabolites by the endophytic fungi (Waqas *et al.*, 2015; Ismail *et al.*, 2021) and partly owing to the fact that endophytes could have enhanced the host's uptake of nutritional elements such as nitrogen and phosphorus from the soil (García-Latorre *et al.*, 2021), and also endophytes might have genes that assist to produce some signalling molecule for growth promotion of plants (Ali *et al.*, 2014b).

Similar effect on plant growth due to endophytic treatment has been reported previously in various plant species under *in-vivo* conditions. The positive plant growth promotion is also evidenced by hormone producing fungal isolates like *Penicillium chrysogenum*, *P. crustosum*, *Phoma glomerata*, *Pestalotiopsis neglecta*, *Williopsis saturnus* in different crop systems (Fu *et al.*, 2015; Fouda *et al.*, 2015; Hassan, 2017; Waqas *et al.*, 2012b). The symbiotically induced growth response was observed in tomato, cucumber plants when treated with *Trichoderma harzianum*, *Penicillium janthinellum*, *Exophiala* sp. (Mastouri *et al.*, 2010; Khan *et al.*, 2013).

Physiological parameters

Photosynthetic parameters recorded in endophyte inoculated and non-inoculated plants showed that the photosynthesis rate was increased upon endophyte treatment. Under 100% field capacity, LAS-6 and K-23 showed a significant increase (Figure 4.5.3) among the treatments, in photosynthetic assimilation rate, stomatal conductance, and transpiration rate compared to non-inoculated plants. P-37 inoculated plants showed a marginal increase in these parameters. SCMR and total chlorophyll obtained also showed a similar trend with respect to photosynthetic parameters in plants inoculated with endophytes. In total, endophyte-treated plants had high photosynthetic assimilation rates due to higher stomatal conductance, transpiration rate, and higher chlorophyll content maintained by endophyte-inoculated plants.

The increase in photosynthetic efficiency of plants obtained in this study is in agreement with the increased photosynthetic rate, chlorophyll content, stomatal conductance of maize plants treated with endophyte *Trichoderma harzianum* (Akladios and Abbas, 2014; Doni *et al.*, 2019). Furthermore, Morse *et al.*, 2007 showed that in *Festuca*, the net photosynthetic rate, stomatal conductance, and transpiration rate of E+ plants were considerably higher than those of E- plants. The photosynthetic rate, transpiration rate, and stomatal conductance were all higher in the endophyte-treated *P. edulis* than in the control group, demonstrating that endophyte might improve plant photosynthesis and thereby promote growth (Yuan *et al.*, 2018). Xu *et al.*, 2021 demonstrated that endophyte infection improved all photosynthetic indicators of

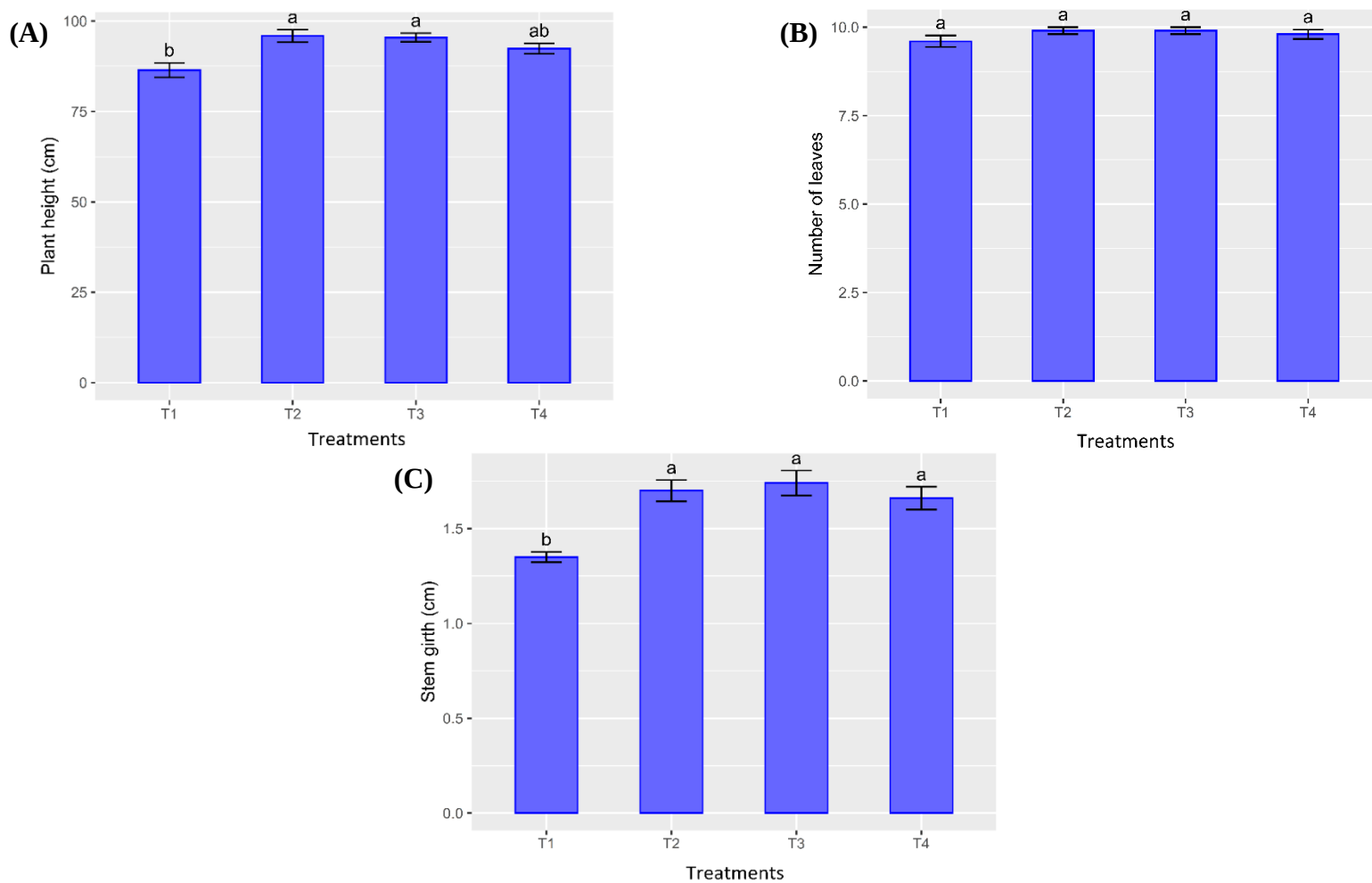


Figure 4.5.1: Effect of selected endophytic fungi on growth parameters of maize at 30 DAS, (A) Plant height, (B) Number of leaves and (C) Stem girth. Values represented are mean $\pm$ SE(n=10) and with dissimilar alphabets are significantly different at Tukey's $p \leq 0.05$. Lines over bar plots indicate standard error over mean.

Note: T₁= FC 100%, T₂= FC 100% + LAS-6, T₃= FC 100% + K-23, T₄= FC 100% + P-37, where FC = Field capacity

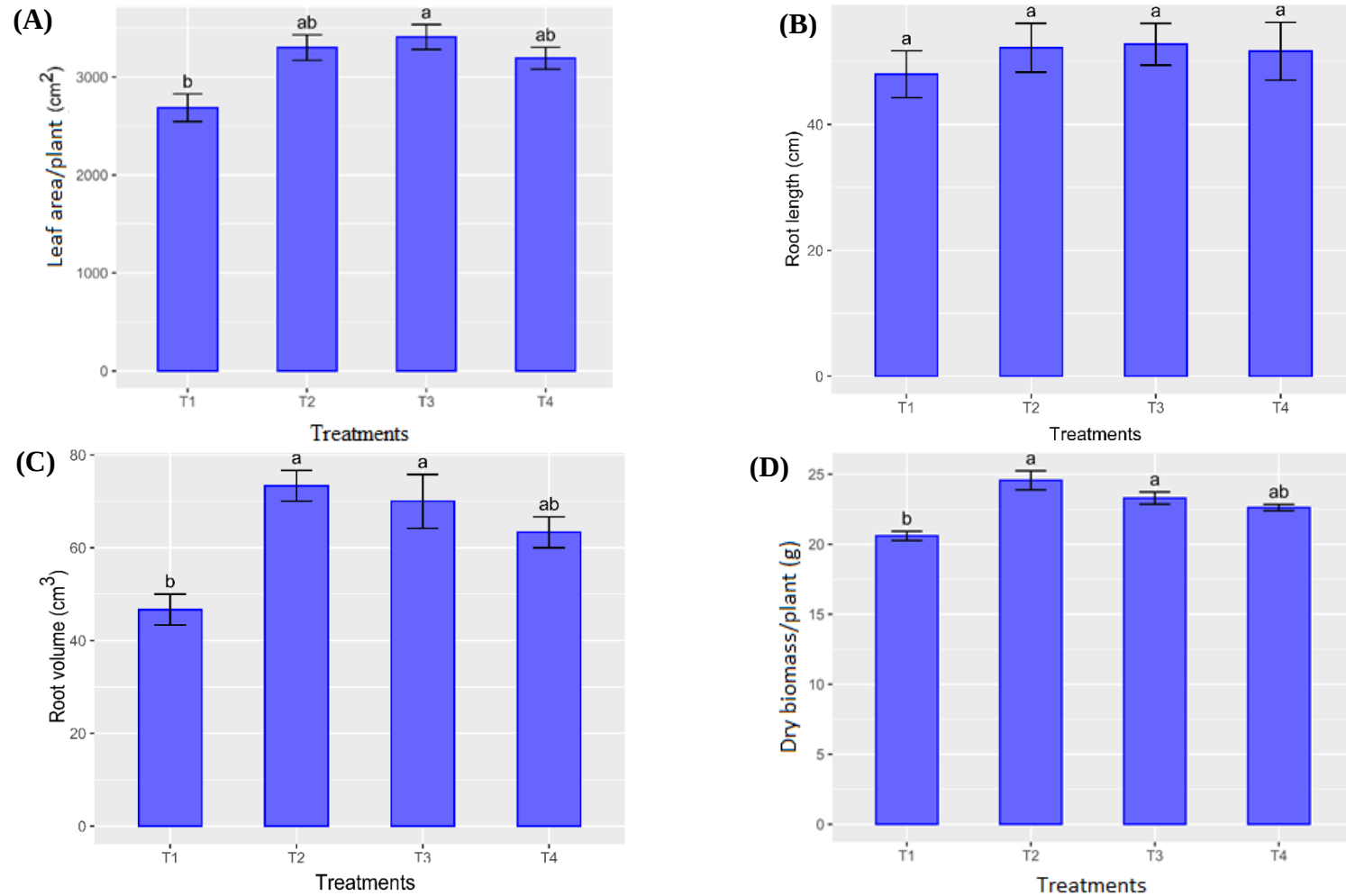


Figure 4.5.2: Effect of selected endophytic fungi on growth parameters of maize at 30 DAS, (A) Leaf area, (B) Root length, (C) Root volume and (D) Dry biomass. Values represented are mean±SE (n=3) and with dissimilar alphabets are significantly different at Tukey's $p \leq 0.05$. Lines over bar plots indicate standard error over mean.

Note: T₁= FC 100%, T₂= FC 100% + LAS-6, T₃= FC 100% + K-23, T₄= FC 100% + P-37, where FC = Field capacity

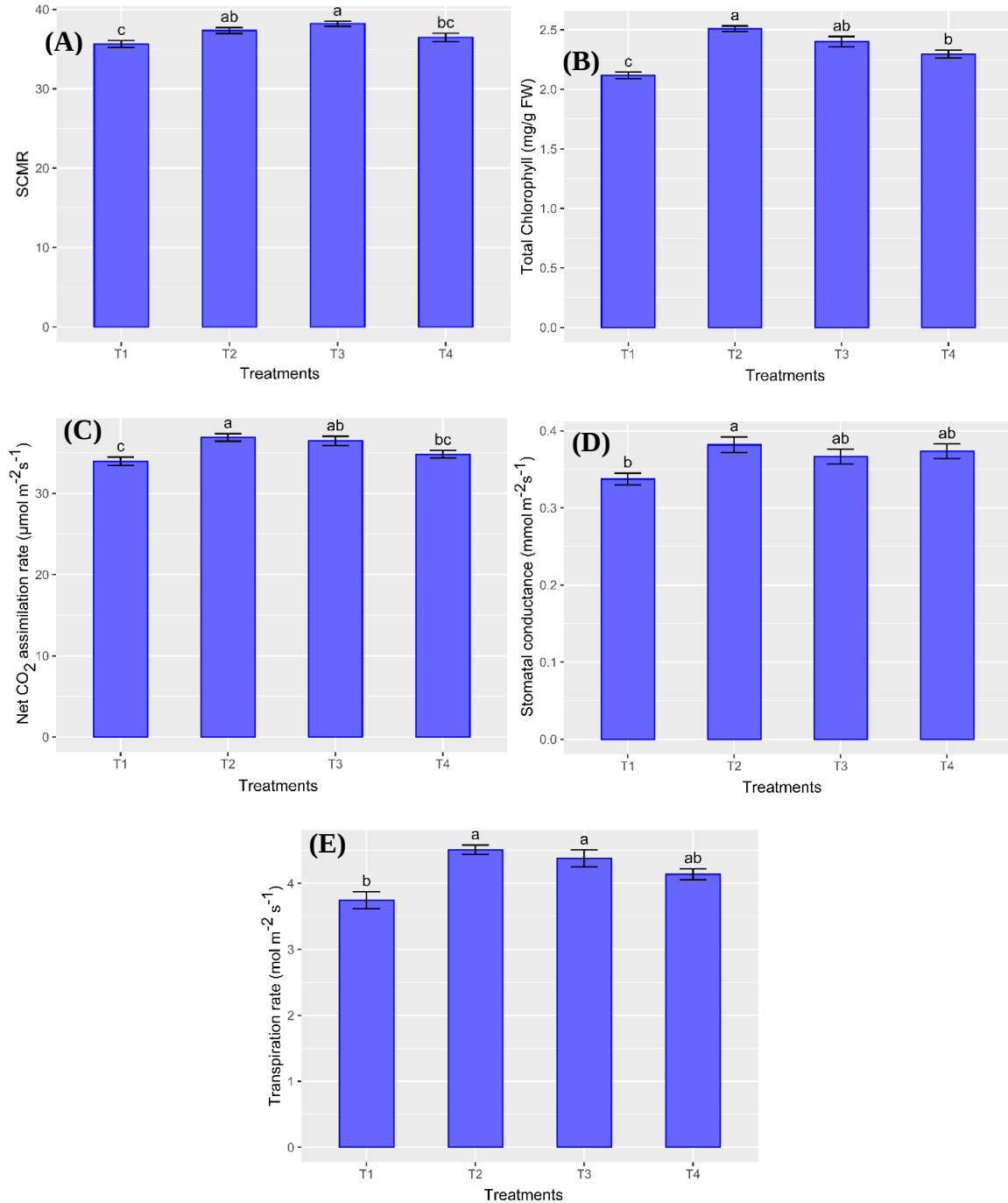


Figure 4.5.3: Effect of selected endophytic fungi on physiological parameters of maize at 30 DAS, (A) SCMR, (B) Total chlorophyll, (C) Photosynthetic rate, (D) Stomatal conductance and (E) Transpiration rate. Values represented are mean $\pm$ SE(n=10) and with dissimilar alphabets are significantly different at Tukey's $p \leq 0.05$. Lines over bar plots indicate standard error over mean.

Note: T₁= FC 100%, T₂= FC 100% + LAS-6, T₃= FC 100% + K-23, T₄= FC 100% + P-37, where FC = Field capacity

F. sinensis under drought stress, except for intercellular carbon dioxide concentration. These findings suggest that endophytes can improve plant growth by improving photosynthesis, which is consistent with the finding that endophytes can alter the host plant's strategy and enhance metabolism (Xia *et al.*, 2018).

4.7.3 In-vivo evaluation of selected endophytes for drought stress tolerance in maize

To evaluate the selected endophytes LAS-6 (*Chaetomium madrasense*), K-23 (*F. incarnatum*), and P-37 (*Ulocladium* sp.) for drought stress mitigation in maize, three water regimes, namely control (100 % Field Capacity), moderate (75 % Field Capacity), and severe (50 % Field Capacity) stress conditions were maintained at a critical stage of the crop, *i.e.*, from 45 to 65 DAS.

Growth parameters

The observations of growth attributes like, plant height, number of leaves, stem girth, leaf area, root length, root volume and plant dry biomass were taken at 45 and 65 DAS. The phenotype of plants (shoot and root) grown in pots at 45 and 65 DAS under 100, 75 and 50% field capacity with endophytes treatments are represented in Plate 4.11 and 4.12 respectively. Plant height was recorded at 45th and 65th day intervals after sowing. At 45 DAS, there was no significant difference in plant height between 100 % FC (135.51 cm), 75 % FC (133.91 cm), and 50 % FC (131.97 cm). However, there was a significant increase in plant height of endophytes inoculated plants compared to non-inoculated plants. At 65 DAS, there was a difference in plant height between 100, 75 and 50 % FC and also significant difference between endophytes inoculated and uninoculated plants. The plants treated with an endophytic fungus, K-23 showed significant increase in plant height under all three field capacities, compared to the non-inoculated plants. LAS-6 performed better under 75% and 50% FC, and P-37 showed significant increase in plant height at 50% FC with respect to untreated plants (Figure 4.6.1).

By contrast, there was no significant difference in the number of leaves among plants that were under 100 % FC, 75 % FC, and 50 % FC, as well as between plants that were inoculated with and those that were not inoculated with endophytes at 45 and 65 DAS

(Figure 4.6.1). Stem girth did not differ significantly among different field capacities at 45 DAS, however, for 65 DAS, there was a significant decrease in stem girth at 50% FC (2.12 cm) compared to 75% FC (2.53 cm) and 100% FC (2.63 cm). At 45 as well as 65 days after sowing, K-23, LAS-6 and P-37 inoculated plants had a significantly higher stem girth than non-inoculated plants (Figure 4.6.2).

A significant increase in leaf area was observed in endophyte treated plants compared to control, despite there being no difference between plants with 100 % FC (4835.03 cm²), 75 % FC (4811.93 cm²), and 50 % FC (4803.98 cm²) at 45 DAS. However, at 65 DAS, there was a significant decrease in leaf area at 75% FC (6163.84 cm²) and 50% FC (5662.64 cm²) compared with 100% FC (6493.56 cm²). In contrast to plants not treated with endophytes, those treated with endophytes showed a significant increase in leaf area irrespective of the different field capacities. The three selected endophytes (K-23, LAS-6, and P-37) showed a significant increase in leaf area in comparison with untreated plants both at 45 and 65 DAS (Figure 4.6.2).

Despite different field capacities, as well as endophyte-treated versus non-treated plants, root length did not differ significantly in both conditions at 45 and 65 DAS (Figure 4.6.3). Whereas the root volume did not differ significantly among different field capacities at 45 DAS, however, for 65 DAS, there was a significant decrease in root volume at 50% FC (113.33 cm³) and 75% FC (130.00 cm³) compared to 100% FC (150.00 cm³). At 45 as well as 65 days after sowing, K-23, LAS-6 and P-37 inoculated plants had a significantly higher root volume than non-inoculated plants (Figure 4.6.3).

At 45 DAS, plant dry biomass did not differ between different field capacities, but at 65 DAS, plant dry biomass decreased significantly at 50% FC (78.23 g) and 75% FC (88.21 g) as compared to 100% FC (96.21 g). However, compared to untreated plants, K-23, LAS-6, and P-37 inoculated plants demonstrated a substantial increase in plant dry biomass under 100, 75 and 50% FC at 45 and 65 DAS (Figure 4.6.4).

The plant growth is affected at any stage of crop growth due to drought stress. Drought stress induces a drastic reduction in growth of above and below ground parts of

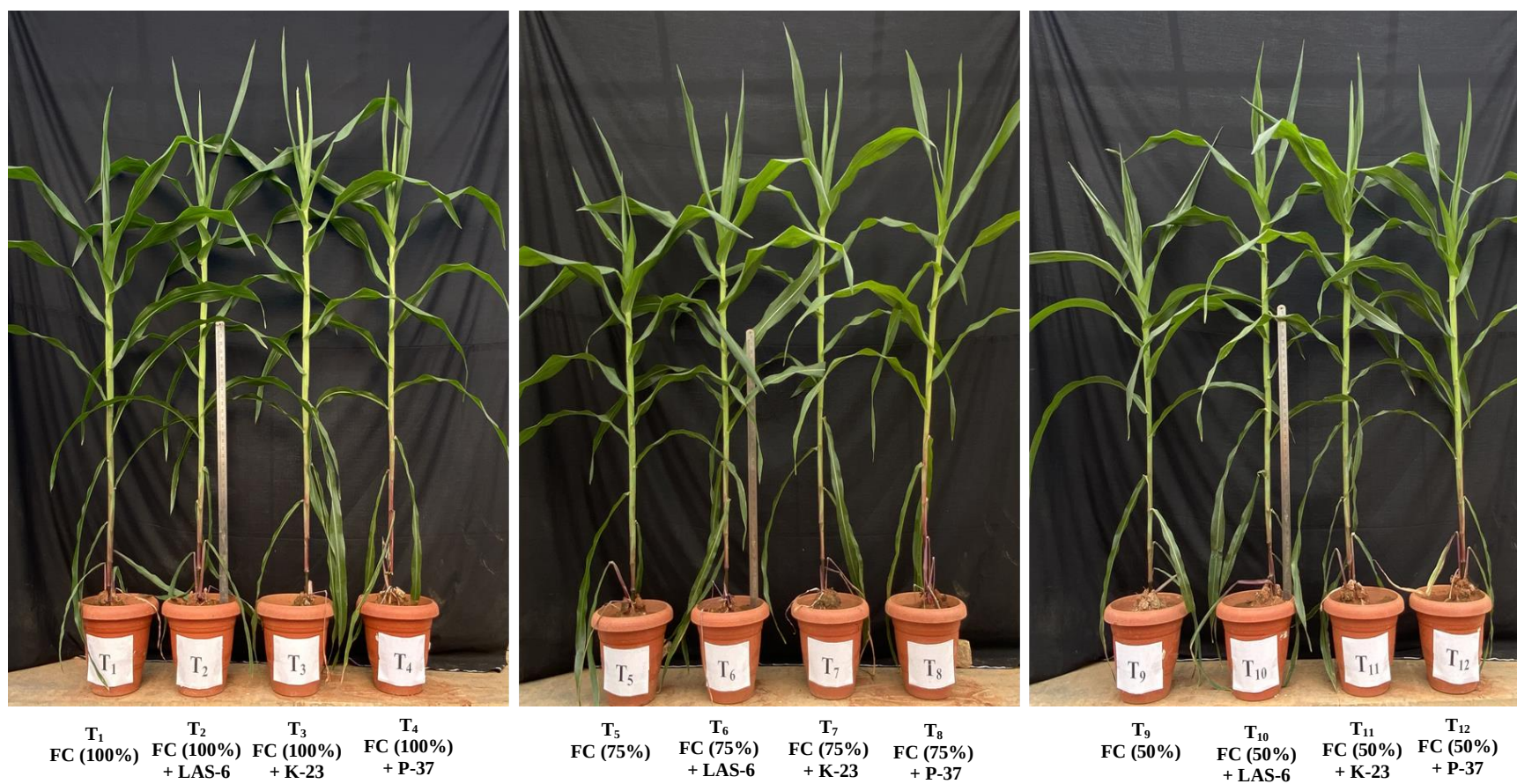


Plate 4.11: Phenotype of non-inoculated and endophytes inoculated plants of maize under different field capacities at 45 DAS

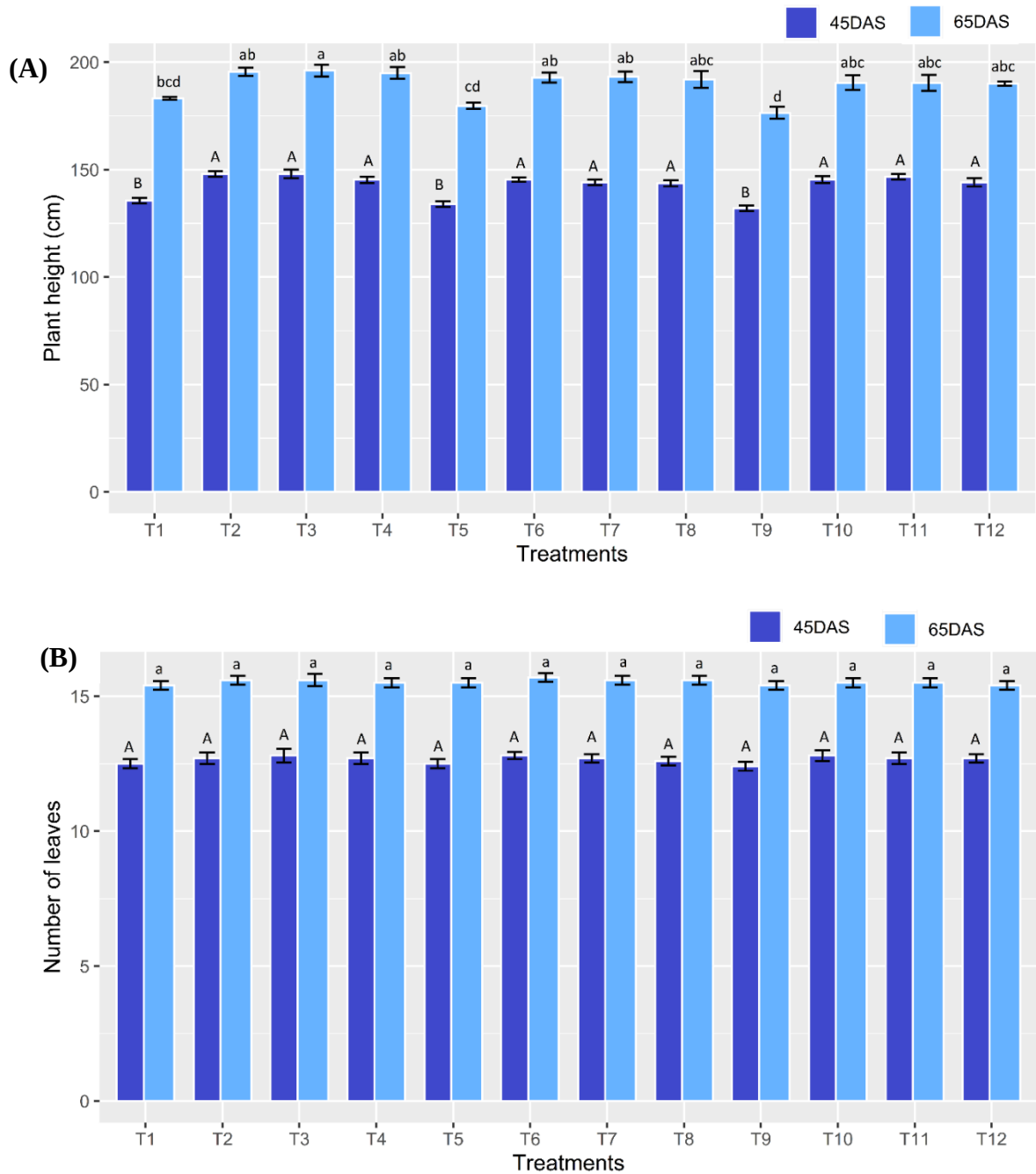


Figure 4.6.1: Effect of selected endophytic fungi on growth parameters of maize under different field capacities at 45 and 65 DAS, (A) Plant height, (B) Number of leaves. Values represented are mean $\pm$ SE(n=10) and with dissimilar alphabets are significantly different at Tukey's $p \leq 0.05$. Lines over bar plots indicate standard error over mean.

Note: T₁= FC 100%, T₂= FC 100% + LAS-6, T₃= FC 100% + K-23, T₄= FC 100% + P-37, T₅= FC 75%, T₆= FC 75% + LAS-6, T₇= FC 75% + K-23, T₈= FC 75% + P-37, T₉= FC 50%, T₁₀= FC 50% + LAS-6, T₁₁= FC 50% + K-23, T₁₂= FC 50% + P-37, where FC = Field capacity

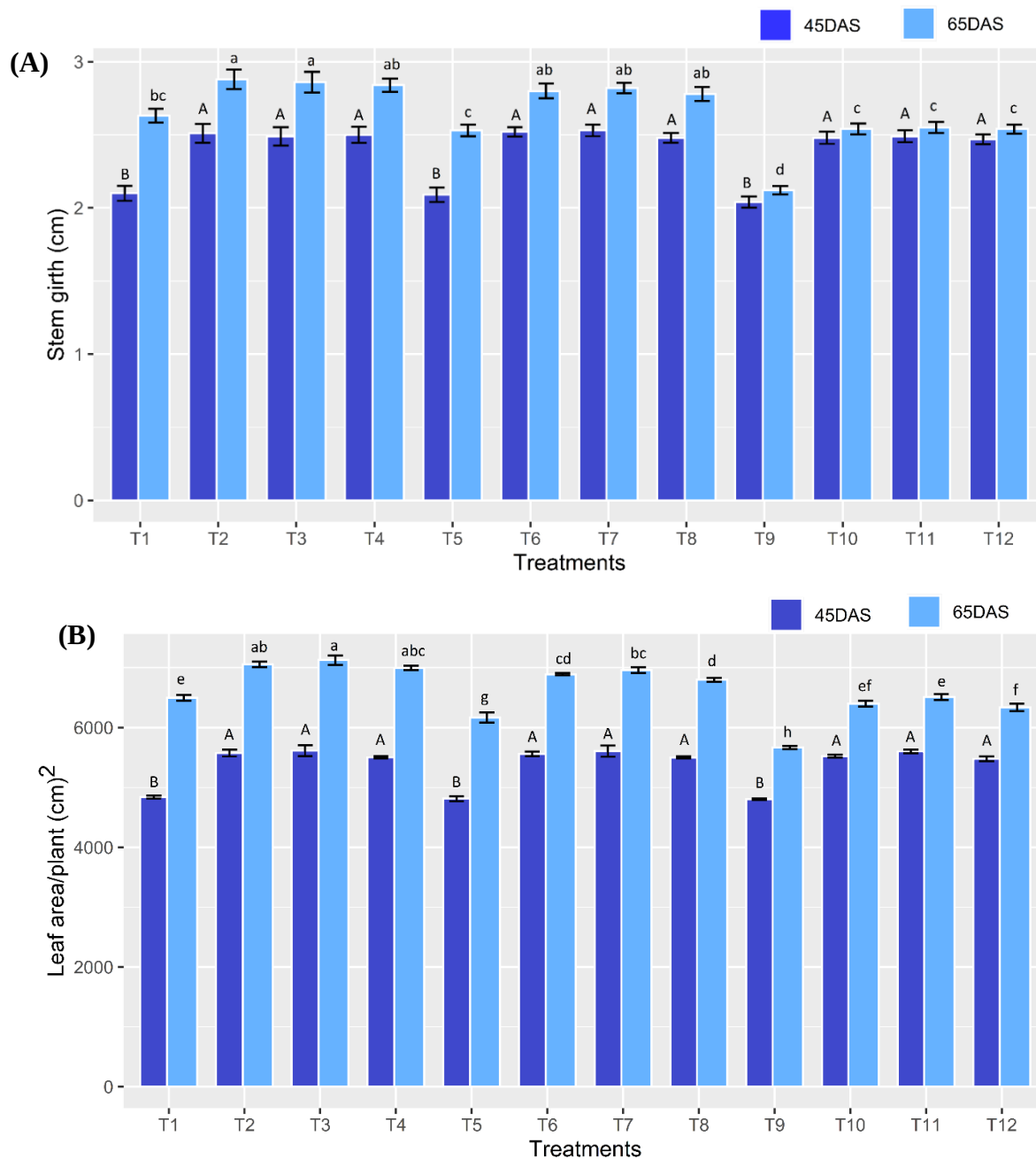


Figure 4.6.2: Effect of selected endophytic fungi on growth parameters of maize under different field capacities at 45 and 65 DAS, (A) Stem girth, (B) Leaf area. Values represented are mean $\pm$ SE(n=10) for stem girth and mean $\pm$ SE(n=3) for leaf area and with dissimilar alphabets are significantly different at Tukey's $p \leq 0.05$. Lines over bar plots indicate standard error over mean.

Note: T₁= FC 100%, T₂= FC 100% + LAS-6, T₃= FC 100% + K-23, T₄= FC 100% + P-37, T₅= FC 75%, T₆= FC 75% + LAS-6, T₇= FC 75% + K-23, T₈= FC 75% + P-37, T₉= FC 50%, T₁₀= FC 50% + LAS-6, T₁₁= FC 50% + K-23, T₁₂= FC 50% + P-37, where FC = Field capacity

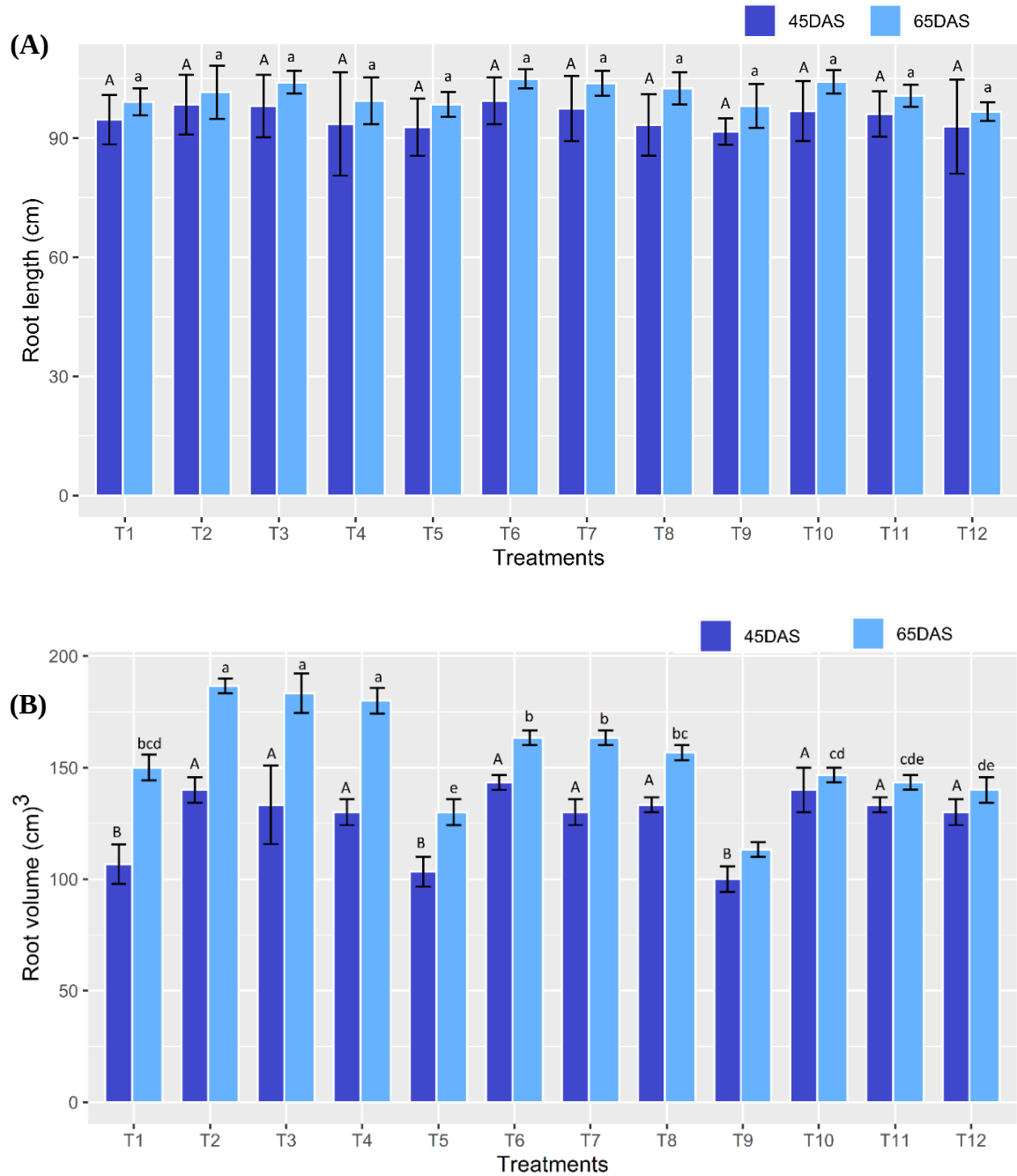


Figure 4.6.3: Effect of selected endophytic fungi on growth parameters of maize under different field capacities at 45 and 65 DAS, (A) Root length, (B) Root volume. Values represented are mean±SE(n=3) and with dissimilar alphabets are significantly different at Tukey's $p \leq 0.05$. Lines over bar plots indicate standard error over mean.

Note: T₁= FC 100%, T₂= FC 100% + LAS-6, T₃= FC 100% + K-23, T₄= FC 100% + P-37, T₅= FC 75%, T₆= FC 75% + LAS-6, T₇= FC 75% + K-23, T₈= FC 75% + P-37, T₉= FC 50%, T₁₀= FC 50% + LAS-6, T₁₁= FC 50% + K-23, T₁₂= FC 50% + P-37, where FC = Field capacity

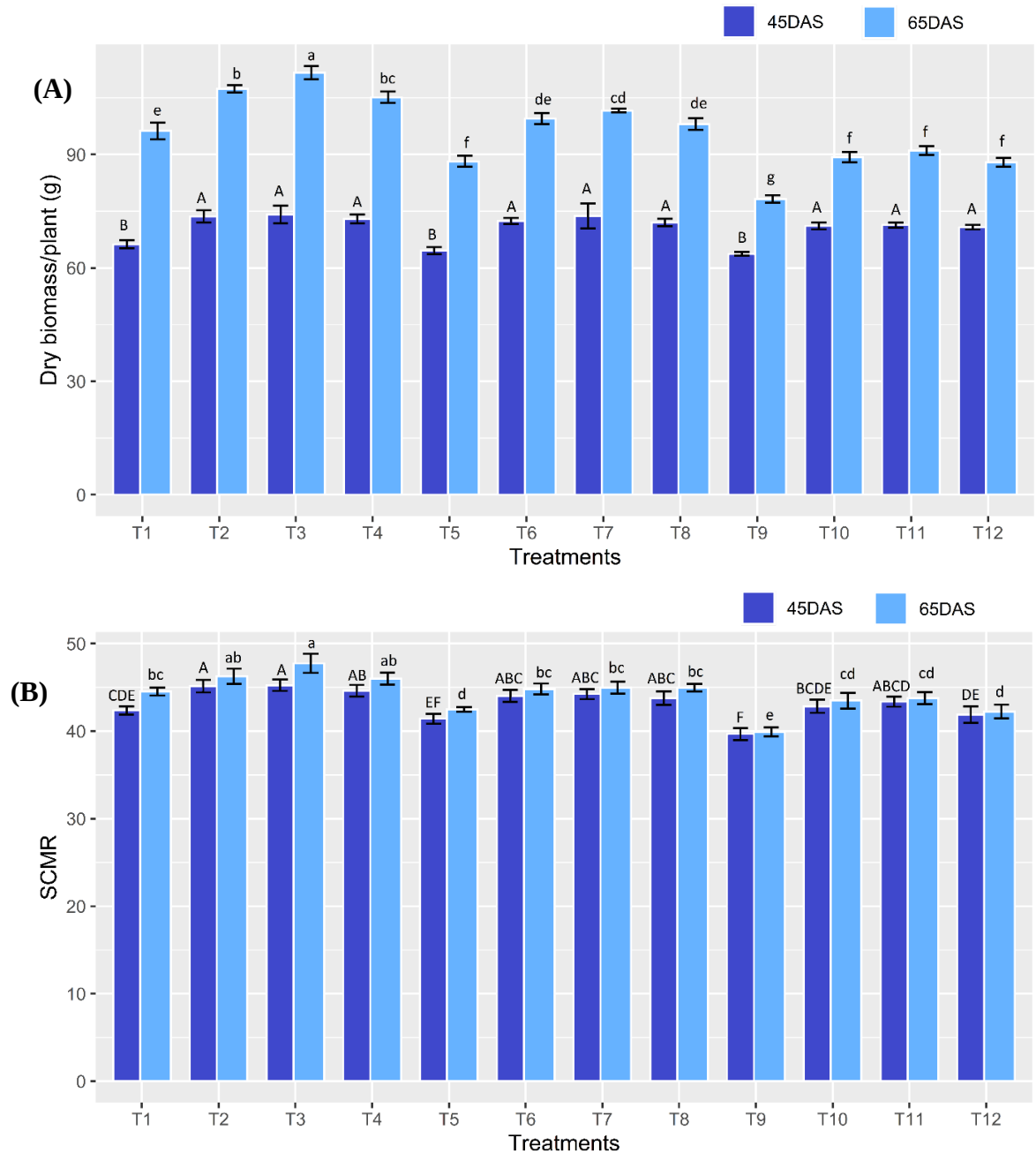


Figure 4.6.4: Effect of selected endophytic fungi on (A) Plant dry biomass and (B) SCMR of maize under different field capacities at 45 and 65 DAS. Values represented are mean $\pm$ SE(n=3) for Plant dry biomass and mean $\pm$ SE(n=10) for SCMR, and with dissimilar alphabets are significantly different at Tukey's $p \leq 0.05$. Lines over bar plots indicate standard error over mean.

Note: T₁= FC 100%, T₂= FC 100% + LAS-6, T₃= FC 100% + K-23, T₄= FC 100% + P-37, T₅= FC 75%, T₆= FC 75% + LAS-6, T₇= FC 75% + K-23, T₈= FC 75% + P-37, T₉= FC 50%, T₁₀= FC 50% + LAS-6, T₁₁= FC 50% + K-23, T₁₂= FC 50% + P-37, where FC = Field capacity

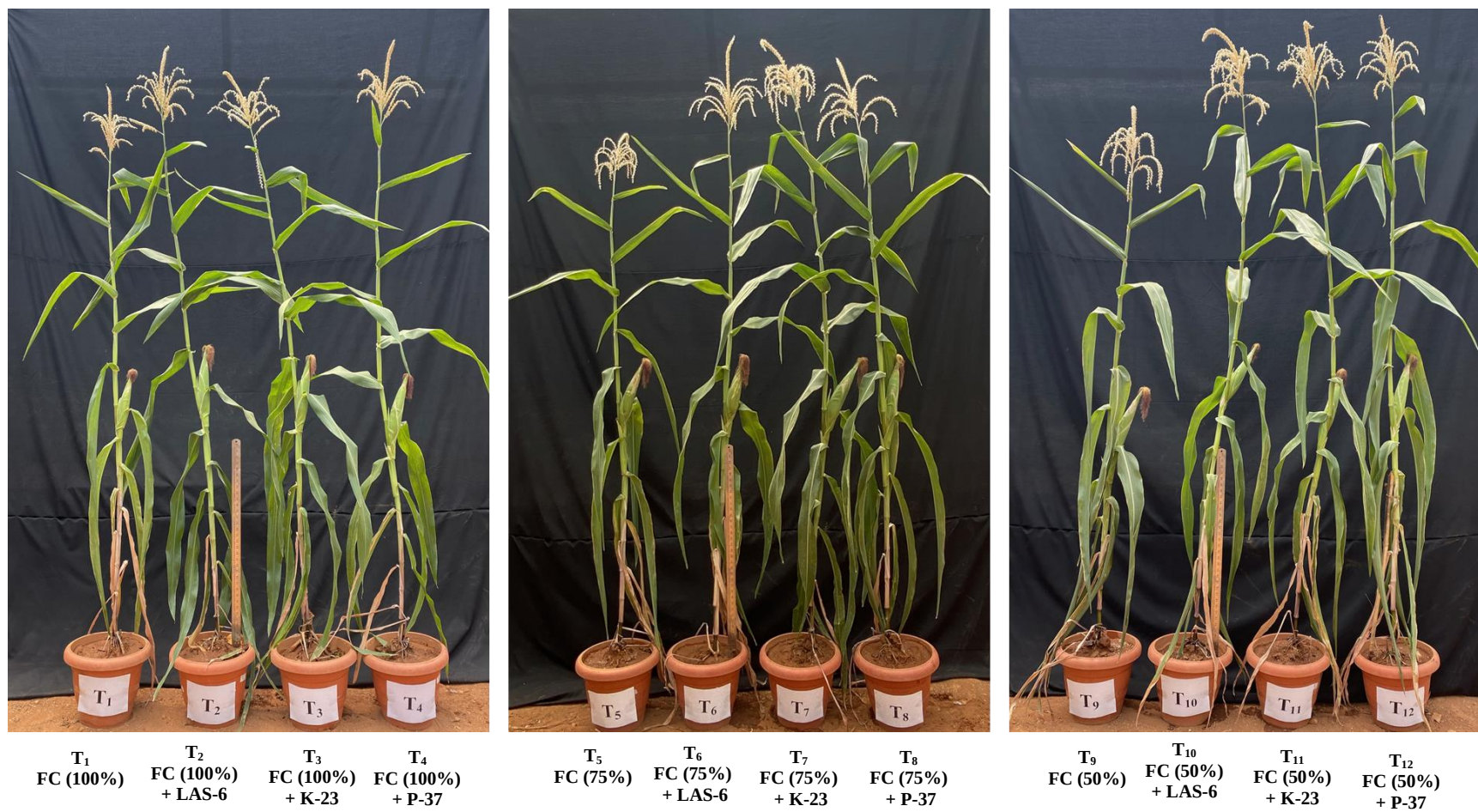


Plate 4.12: Phenotype of non-inoculated and endophytes inoculated plants of maize under different field capacities at 65 DAS

maize plant. Initially, exposure of plants to drought stress induces osmotic stress inhibiting cell elongation, cell division, leaf area expansion, which results in reduced plant height, stem girth, shoot and root dry weight (Sah *et al.*, 2020; Wang *et al.*, 2019; Khan *et al.*, 2015c; Shen *et al.*, 2019).

The enhanced plant growth under drought stress due to endophytes treatment shows that, endophytes from adverse habitat can impart drought stress tolerance to maize genotype in a habitat-specific manner through symbiotic association. These endophytes can regulate plant physiological activities against drought stress by releasing and/or enhancing the capability of the host plant to produce phytohormones (gibberellins, auxin, cytokinin) and secondary metabolites (phenols and flavonoids) (Khan *et al.*, 2014; Rajamanikyam *et al.*, 2017), which under drought stress, inhibit H₂O₂ production, antagonize ethylene-based abscission and promote overall plant growth (Afzal *et al.*, 2019). Similarly, it controls ABA levels for normal stomatal functioning to minimize water losses. Also, it can regulate stress-responsive genes to mediate the plant defense system against stress damages (Ganie *et al.*, 2021). Several studies on the beneficial role of endophytic microbes in enhancing drought stress tolerance in a variety of crop plants like wheat (Chen *et al.*, 2017), licorice (He *et al.*, 2019), rice (Saddique *et al.*, 2018), pepper (Vigani *et al.*, 2019), and others (Chourasiya *et al.*, 2018) have been studied. Similarly, maize plants inoculated with *Bacillus* sp. developed protection against drought stress by reducing the activity of the antioxidant enzymes APX and Glutathione peroxidase (GPX) (Vardharajula *et al.*, 2011). Soybean plants inoculated with gibberellins secreting rhizobacterium *P. putida* H-2-3 improved plant growth under drought conditions (Sang-Mo *et al.*, 2014). Production of ABA and gibberellins by *Azospirillum lipoferum* alleviated drought stress in maize plants (Cohen *et al.*, 2009). Endophyte-inoculated plants showed a 27.9–75% increase in plant fresh weight under the well-watered condition and a 40–77% increase in plant dry weight as compared to (uninoculated) controls under the drought stress condition in soybean (Dubey *et al.*, 2021).

Physiological parameters

The observations of physiological parameters like, SCMR were recorded at 45 and 64 DAS, whereas relative water content and total chlorophyll were recorded at the mid-period of drought stress, *i.e.*, 55 DAS.

At 45 DAS, there was a marginal decrease in SCMR at 50 and 75% FC compared to 100% FC. But at 65 DAS, SCMR decreased significantly at 50% FC (39.92) and 75% FC (42.48) as compared to 100% FC (44.53). However, compared to untreated plants, K-23, LAS-6, and P-37 inoculated plants demonstrated a substantial increase in SCMR under 100, 75, and 50% FC at 45 and 65 DAS (Figure 4.6.4). Chlorophyll content was measured at the mid-period of drought stress induction (55 DAS). The decrease in chlorophyll content was observed when plants were subjected to 50 and 75% FC. But the endophytes treated plants had maintained higher chlorophyll content than the non-inoculated plants suggesting that endophytes can improve chlorophyll stability under drought stress (Figure 4.6.5). Endophytes treated plants had a marginal increase in relative water content (RWC) levels under 100% FC compared to non-inoculated plants. At 75 and 50% FC, RWC levels were found to decrease in non-inoculated plants, whereas, endophytes treated plants maintained higher RWC levels (Figure 4.6.5).

The extent of drought stress on plants reflects directly on the above-ground phenotype of the plant. Photosynthesis is one of the most important physiological processes in plants that are most vulnerable to water deficit and is directly related to the accumulation of biomass (Rozpdek *et al.*, 2015), and in many crops, leaf relative water content (RWC) is a key indicator of water stress (Jolly *et al.*, 2014). So, the extent of drought stress in plants can be analyzed by measuring various physiological parameters like SCMR, chlorophyll, and relative water content.

Relative water content (RWC) of plants reflects the water status of the plant. A substantial amount of water in plants is crucial for maintaining the osmotic levels in a plant cell, nutrient uptake, and to survive under drought stress conditions (Soltys-Kalina *et al.*, 2016). Increased levels of RWC in endophytes treated plants at 75 and 50% FC (Figure 4.6.5) shows that endophytes help in maintaining higher plant water status required for

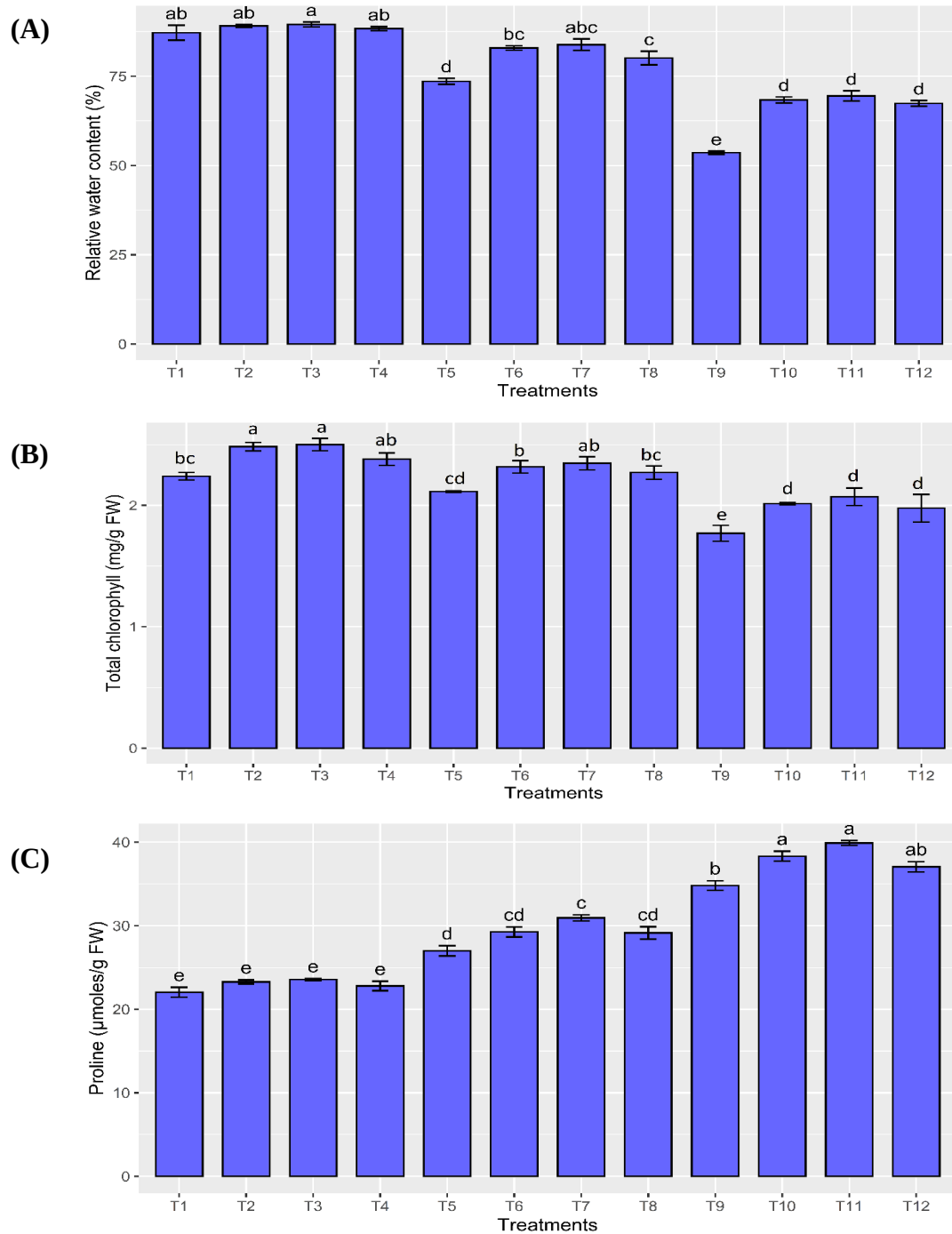


Figure 4.6.5: Effect of selected endophytic fungi on (A) Relative water content, (B) Total chlorophyll and (C) Proline content of maize under different field capacities at 55 DAS. Values represented are mean $\pm$ SE(n=3) and with dissimilar alphabets are significantly different at Tukey's $p \leq 0.05$. Lines over bar plots indicate standard error over mean.

Note: T₁= FC 100%, T₂= FC 100% + LAS-6, T₃= FC 100% + K-23, T₄= FC 100% + P-37, T₅= FC 75%, T₆= FC 75% + LAS-6, T₇= FC 75% + K-23, T₈= FC 75% + P-37, T₉= FC 50%, T₁₀= FC 50% + LAS-6, T₁₁= FC 50% + K-23, T₁₂= FC 50% + P-37, where FC = Field capacity

physiological activities in turn promoting plant growth and development (Odokonyero *et al.*, 2016). An increase in RWC, under drought stress by various endophytes, has been reported. Under drought conditions, endophyte association in *Brachiaria* cultivars (particularly cv. Tully) increases cellular apoptosis susceptibility proteins (Sun *et al.*, 2010) which stimulates aerenchyma formation (*i.e.*, intercellular gas-filled lacunae) in leaf sheath and root tissues, where it holds apoplastic water and allows for water conservation in plant cells, contributing to higher values of leaf RWC (Touchette *et al.*, 2014; Cardoso *et al.*, 2014; Cardoso *et al.*, 2013). Drought reduced relative water content in both inoculated and un-inoculated plants (Naveed *et al.*, 2014), although endophyte inoculation significantly increased relative water content by 30% compared to un-inoculated controls in maize plants.

Drought promotes plant chlorophyll degradation (Xiang *et al.*, 2018), which has a direct effect on photosynthetic rate as it is the principal pigment for photosynthesis in plants and the drought tolerance ability of plants directly depends on the chlorophyll stability index (Kapoor *et al.*, 2020). The increase in chlorophyll content and SCMR values under drought stress in endophyte treated plants observed in this study (Figure 4.6.4 and 4.6.5) are in support of earlier reports when inoculated with endophytic fungi under drought stress (He *et al.*, 2019; Aghai *et al.*, 2019; Yuan *et al.*, 2018). Drought stress hampers the plant's photosynthetic efficiency by affecting the Photosystem, PSII (Siddique *et al.*, 2016). However, this effect can be reduced to some extent in association with endophytes helping the plant to maintain optimal plant growth under drought stress (Odokonyero *et al.*, 2016; He *et al.*, 2019; Aghai *et al.*, 2019; Xu *et al.*, 2021). The increased rate of photosynthesis was also recorded from the drought-stressed maize plants colonized by endophytes (Siddique *et al.*, 2022).

Biochemical parameters

To evaluate the triggering of antioxidative defense response against drought stress in maize plants upon endophyte treatment, a series of plant defense-related enzymatic assays like Catalase (CAT), Polyphenol oxidase (PPO), and estimation of non-enzymatic molecules like Proline was performed. Malondialdehyde content was quantified as an

indicator of lipid peroxidation. Leaves of 55 days old plants were sampled from different field capacity conditions of endophyte inoculated and non-inoculated plants.

ROS scavenging small metabolites such as, proline maintains redox balance in cells during oxidative damage (Roychoudhury *et al.*, 2012). Endophyte's treatment enhances proline concentration that usually favors ROS scavenging in the plants (Shavalikohshori *et al.*, 2020). In our present study, we measured proline from endophytes inoculated and non-inoculated plants under 100, 75, and 50% FC. Under 100% FC, there was no significant difference between endophytes treated and control plants, whereas under 75% FC, K-23 showed a significant increase in proline content compared to untreated plants, and under 50% FC, LAS-6, and K-23 showed significant increase and P-37 showed a marginal increase in proline with respect to untreated plants (Figure 4.6.5). Proline is an excellent osmolyte that helps in the stabilization of sub-cellular macromolecules such as proteins and cell membranes. Besides, it is involved in scavenging free radicals, balancing redox homeostasis and signaling, thereby assisting plants to cope under stress conditions (Ullah *et al.*, 2019). Shukla *et al.* (2015) reported that priming of *Triticum aestivum* L. with *Trichoderma harzianum* improved drought stress tolerance by mediating enhanced synthesis and accumulation of proline, thereby conferring drought stress tolerance.

An increase in ROS levels leads to elevated levels of lipid peroxidation in cells resulting in membrane damage (Juan *et al.*, 2021). The extent of membrane damage can be estimated by measuring the malondialdehyde (MDA) which serves as an indicator of the extent of oxidative damage in stressed plants. MDA levels increased significantly under 50% FC compared to 75 and 100% FC in non-inoculated plants. But, in endophytes inoculated plants, there was a significant reduction in MDA levels under 50 and 75% FC compared to non-inoculated plants (Figure 4.6.6). Endophytes improve the drought resilience of plants by actively accumulating proline in the host tissue and lowering MDA levels (Hahn *et al.*, 2008; Nagabhyru *et al.*, 2013). *P. tabulaeformis* seedlings treated with endophyte accumulated less MDA, broke down less chlorophyll, and enhanced proline accumulation than seedlings treated with other treatments (Zhou *et al.*, 2021). Furthermore, under stress conditions, rice infected with *B. amyloliquefaciens* considerably increased growth as well as levels of some critical antioxidant amino acid as proline.

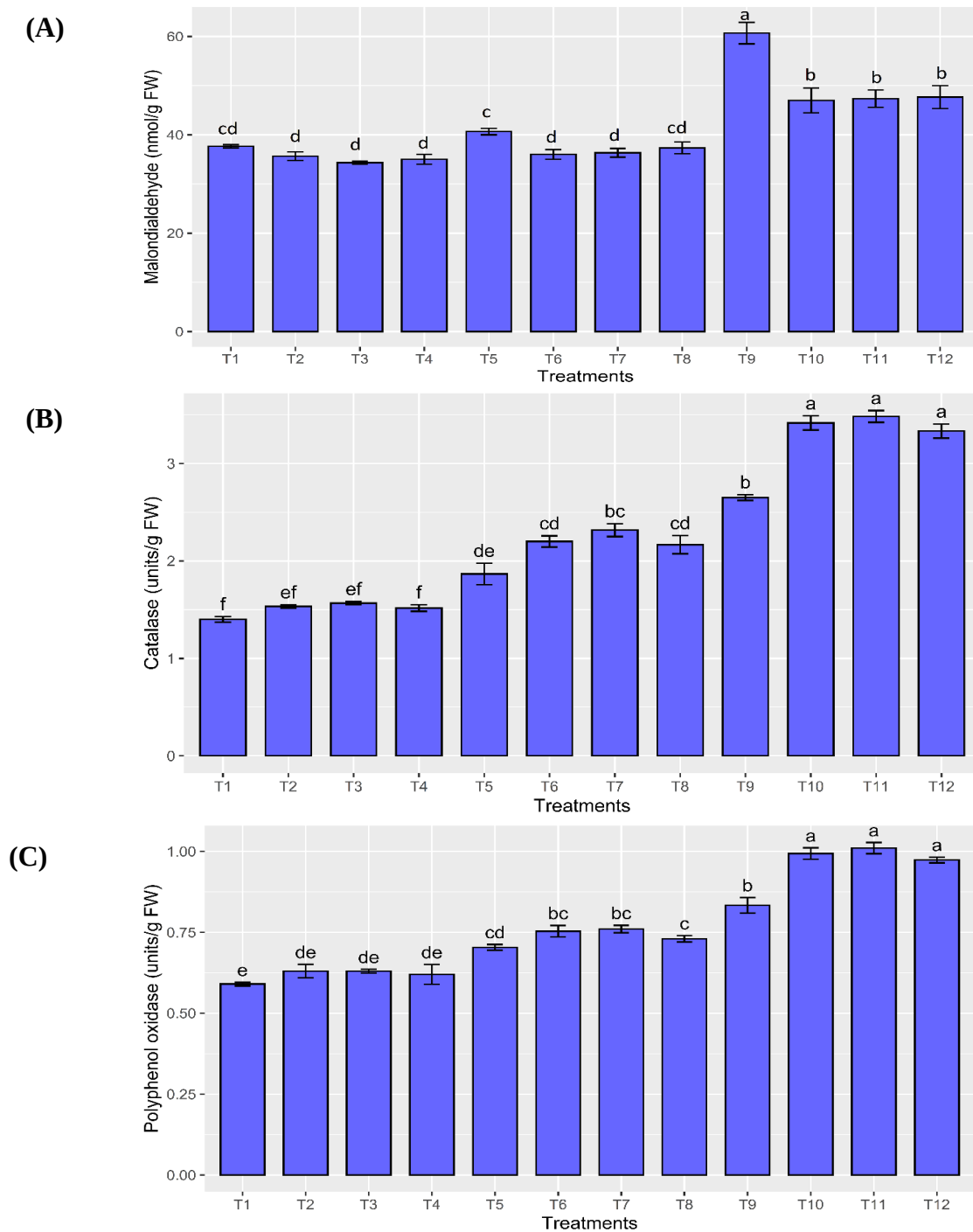


Figure 4.6.6: Effect of selected endophytic fungi on (A) Malondialdehyde content, (B) Catalase activity and (C) Polyphenol oxidase content of maize under different field capacities at 55 DAS. Values represented are mean $\pm$ SE (n=3) and with dissimilar alphabets are significantly different at Tukey's $p \leq 0.05$. Lines over bar plots indicate standard error.

Note: T₁= FC 100%, T₂= FC 100% + LAS-6, T₃= FC 100% + K-23, T₄= FC 100% + P-37, T₅= FC 75%, T₆= FC 75% + LAS-6, T₇= FC 75% + K-23, T₈= FC 75% + P-37, T₉= FC 50%, T₁₀= FC 50% + LAS-6, T₁₁= FC 50% + K-23, T₁₂= FC 50% + P-37, where FC = Field capacity

In the present study, it was observed that the catalase enzyme activity was significantly high in endophytes (K-23, LAS-6, and P-37) inoculated plants under 50% FC compared to non-inoculated plants. Under 75% FC, K-23 treated plants showed a significant increase in CAT enzyme, whereas LAS-6 and P-37 showed a marginal increase over the non-inoculated plants (Figure 4.6.6). Similarly, polyphenol oxidase activity was also found to be high in endophytes (K-23, LAS-6, and P-37) treated plants compared to untreated plants under 50% FC condition. It was observed that polyphenol oxidase activity did not change in endophytes treated plants under 75% and 100% FC compared to 50% FC (Figure 4.6.6). Endophytic microbe colonization increases plant levels of antioxidant enzymes such catalase (CAT), superoxide dismutase (SOD), peroxidase (POD), and polyphenol oxidase (PPO) (Lata *et al.*, 2018). Endophytes stimulate the production of antioxidants to counteract a range of free radicals which are necessary for normal cellular function. Catalase (CAT) acts as a cellular sink of H_2O_2 and catalyzes its disproportionation into H_2O and O_2 . The increased activity of CAT and PPO shows the effective scavenging of H_2O_2 generated in endophytes treated plants under drought stress. This indicates that endophytes in the symbiotic association are assumed to upregulate the activity of CAT and PPO to scavenge ROS, thus helping plants to survive under drought stress. Similar results have been obtained in various crop plants under drought stress in endophytic fungal treatments (Sadeghi *et al.*, 2020; Siddique *et al.*, 2022; Zhou *et al.*, 2021).

Yield related parameters

Yield and yield-related parameters like days to anthesis, days to silking, tassel length, cob length, cob diameter, cob weight, number of kernel rows per cob, number of kernels per row, kernel row length, test weight, and grain yield per plant were recorded in endophyte inoculated and non-inoculated plants grown under different field capacities.

The plants which were not inoculated with endophytes took more days for anthesis and silking under 50% FC compared to 75 and 100% FC. Whereas, the plants which were inoculated with endophytes took fewer days for anthesis and silking compared to non-inoculated plants under different field capacity regimes (Figure 4.6.7). The tassel length did not vary significantly with different field capacity regimes and also between endophyte

inoculated and non-inoculated plants (Figure 4.6.7). Cob length of non-inoculated control plants was significantly lower at 50% FC compared to 75 and 100% FC. Whereas, under different field capacity conditions, those plants which were inoculated with endophytes showed a significant increase in cob length compared to non-inoculated plants (Figure 4.6.8, Plate 4.13). The cob diameter of non-inoculated plants significantly decreased under 50% FC compared to 75 and 100% FC, but there was no significant difference between endophyte treated and untreated plants across different field capacity conditions (Figure 4.6.8). Cob weight was reduced with decreased soil moisture at 50% FC without endophyte inoculation while the plants with endophyte showed an increase in cob weight. Among the endophytes, K-23 inoculated plants showed a significant increase in cob weight in different field capacity conditions (Figure 4.6.8).

The number of kernel rows per cob did not differ due to drought stress at 50% FC, and also endophyte inoculation did not affect the kernel rows in cob (Figure 4.6.9). Whereas, the number of kernels per row was significantly reduced in non-inoculated plants under drought stress at 50% FC compared to 75 and 100% FC. But the plants which were treated with endophytes showed a significant increase in the number of kernels per row in all three field capacity conditions and among the endophytes, K-23 performed better (Figure 4.6.9). Kernel row length was drastically decreased under drought stress at 50% FC condition. But the endophytes treated plants showed a significant increase in kernel row length at 50% FC as well as under 75 and 100% FC conditions compared to non-inoculated plants (Figure 4.6.9).

All these above attributes corresponded to the yield of the plant. Under 75 and 50% FC, the test weight of seeds was significantly reduced in non-inoculated plants. While the plants inoculated with endophytes showed an increase in test weight of seeds compared to non-inoculated plants under different field capacity conditions (Figure 4.6.10). Grain yield per plant was drastically reduced under drought stress (50% FC) in untreated plants, however, those plants treated with endophytes showed a significant increase in grain yield compared to untreated plants in 100, 75, and 50% FC conditions (Figure 4.6.10). Thus, an increase in seed yield upon endophytes treatment could be due to an increase in net photosynthetic rate (Figure 4.5.3) and the rate of photoassimilate remobilization from

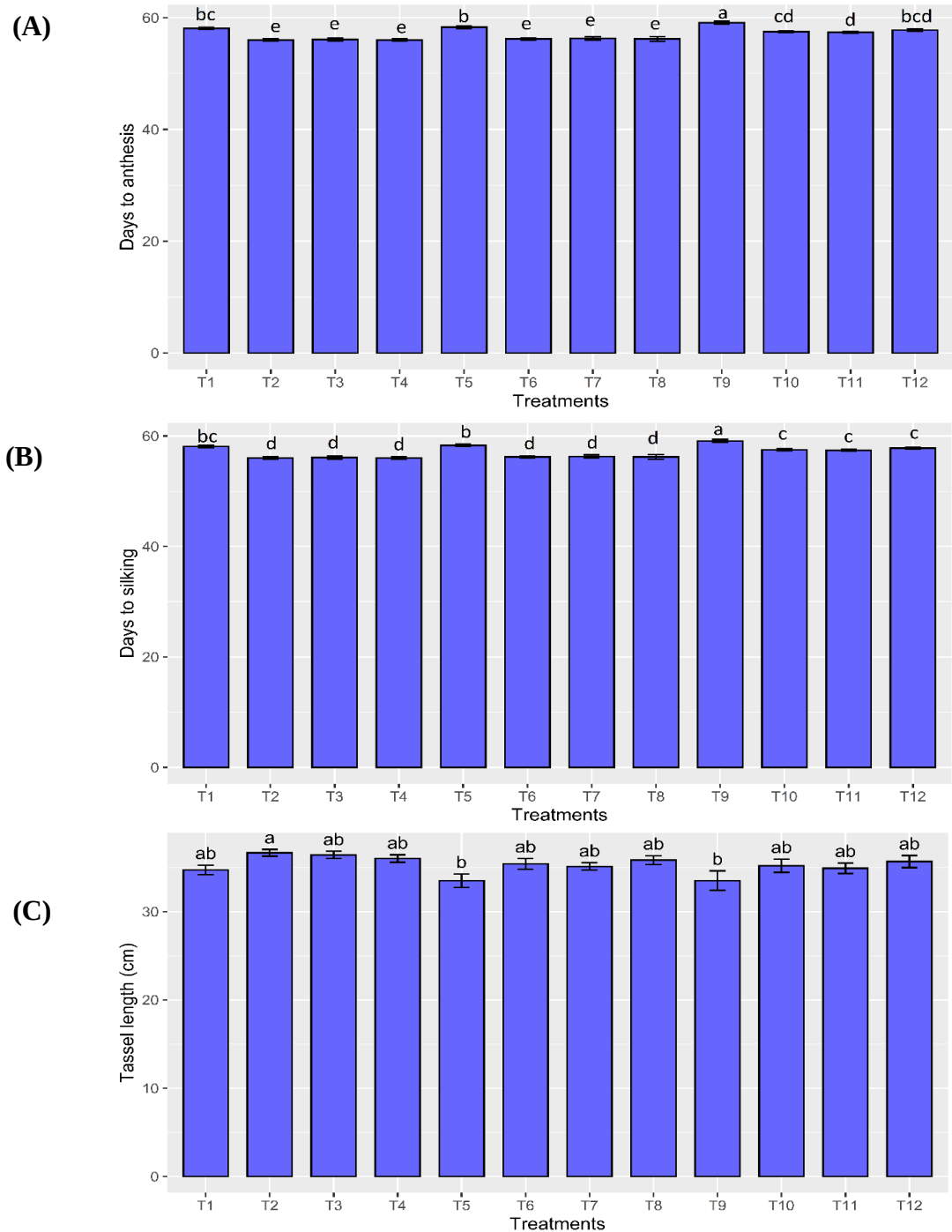


Figure 4.6.7: Effect of selected endophytic fungi on (A) Days to anthesis, (B) Days to silking and (C) Tassel length of maize under different field capacities. Values represented are mean $\pm$ SE(n=10) and with dissimilar alphabets are significantly different at Tukey's $p \leq 0.05$. Lines over bar plots indicate standard error over mean.

Note: T₁= FC 100%, T₂= FC 100% + LAS-6, T₃= FC 100% + K-23, T₄= FC 100% + P-37, T₅= FC 75%, T₆= FC 75% + LAS-6, T₇= FC 75% + K-23, T₈= FC 75% + P-37, T₉= FC 50%, T₁₀= FC 50% + LAS-6, T₁₁= FC 50% + K-23, T₁₂= FC 50% + P-37, where FC = Field capacity

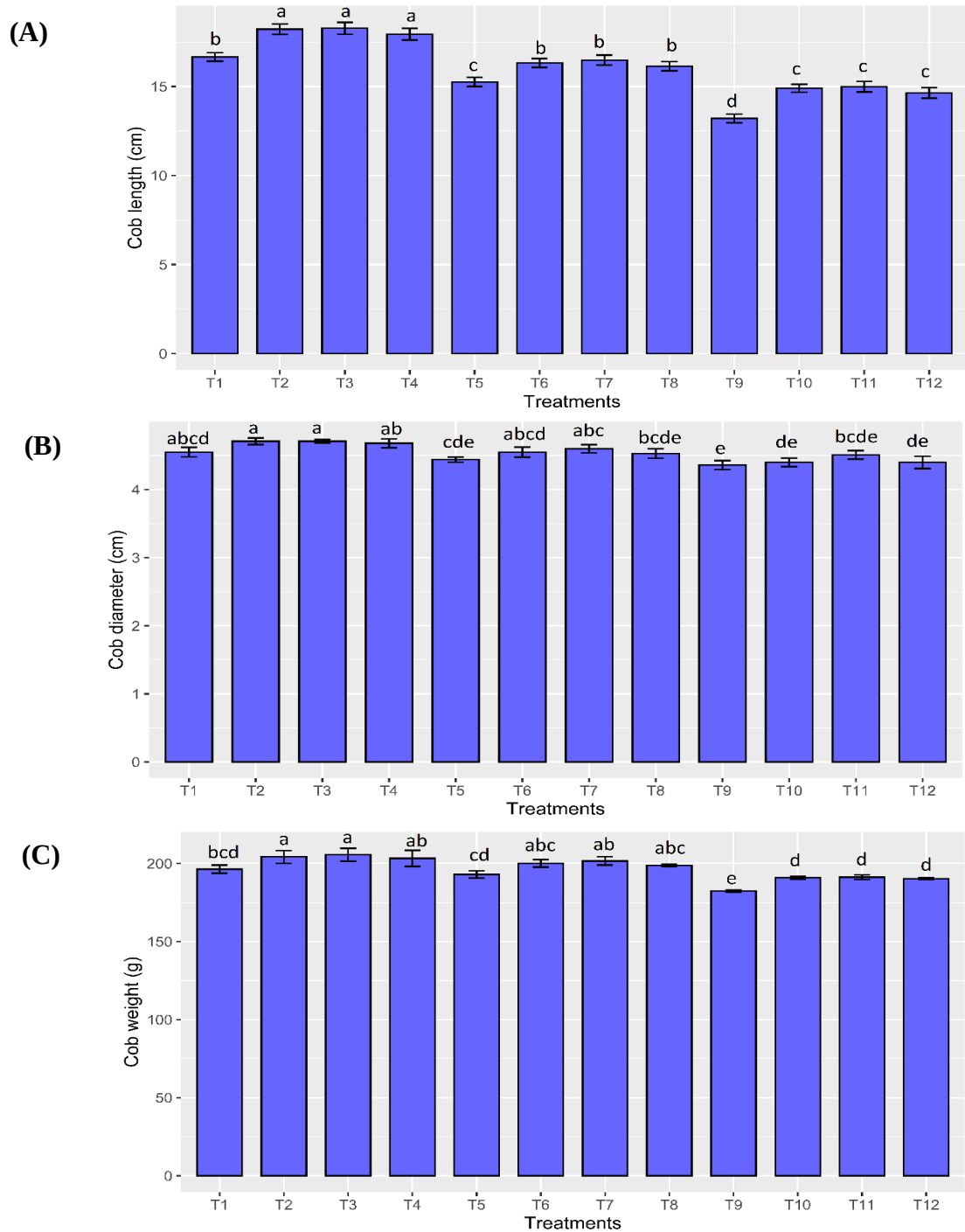


Figure 4.6.8: Effect of selected endophytic fungi on (A) Cob length, (B) Cob diameter and (C) Cob weight of maize under different field capacities. Values represented are mean $\pm$ SE(n=10) and with dissimilar alphabets are significantly different at Tukey's $p \leq 0.05$. Lines over bar plots indicate standard error over mean.

Note: T₁= FC 100%, T₂= FC 100% + LAS-6, T₃= FC 100% + K-23, T₄= FC 100% + P-37, T₅= FC 75%, T₆= FC 75% + LAS-6, T₇= FC 75% + K-23, T₈= FC 75% + P-37, T₉= FC 50%, T₁₀= FC 50% + LAS-6, T₁₁= FC 50% + K-23, T₁₂= FC 50% + P-37, where FC = Field capacity

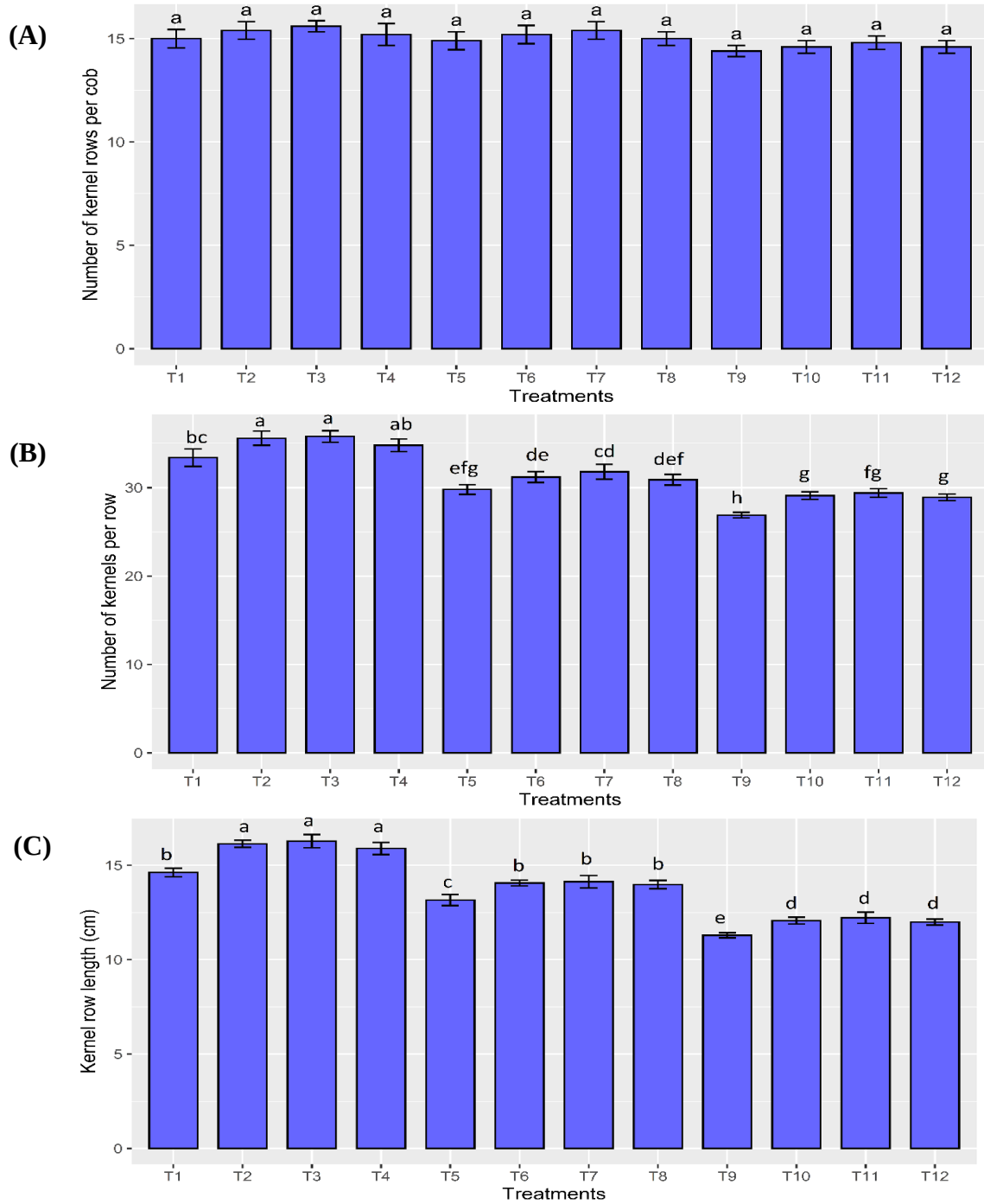


Figure 4.6.9: Effect of selected endophytic fungi on (A) Number of kernel rows per cob, (B) Number of kernels per row and (C) Kernel row length of maize under different field capacities. Values represented are mean $\pm$ SE(n=10) and with dissimilar alphabets are significantly different at Tukey's $p \leq 0.05$. Lines over bar plots indicate standard error over mean.

Note: T₁= FC 100%, T₂= FC 100% + LAS-6, T₃= FC 100% + K-23, T₄= FC 100% + P-37, T₅= FC 75%, T₆= FC 75% + LAS-6, T₇= FC 75% + K-23, T₈= FC 75% + P-37, T₉= FC 50%, T₁₀= FC 50% + LAS-6, T₁₁= FC 50% + K-23, T₁₂= FC 50% + P-37, where FC = Field capacity

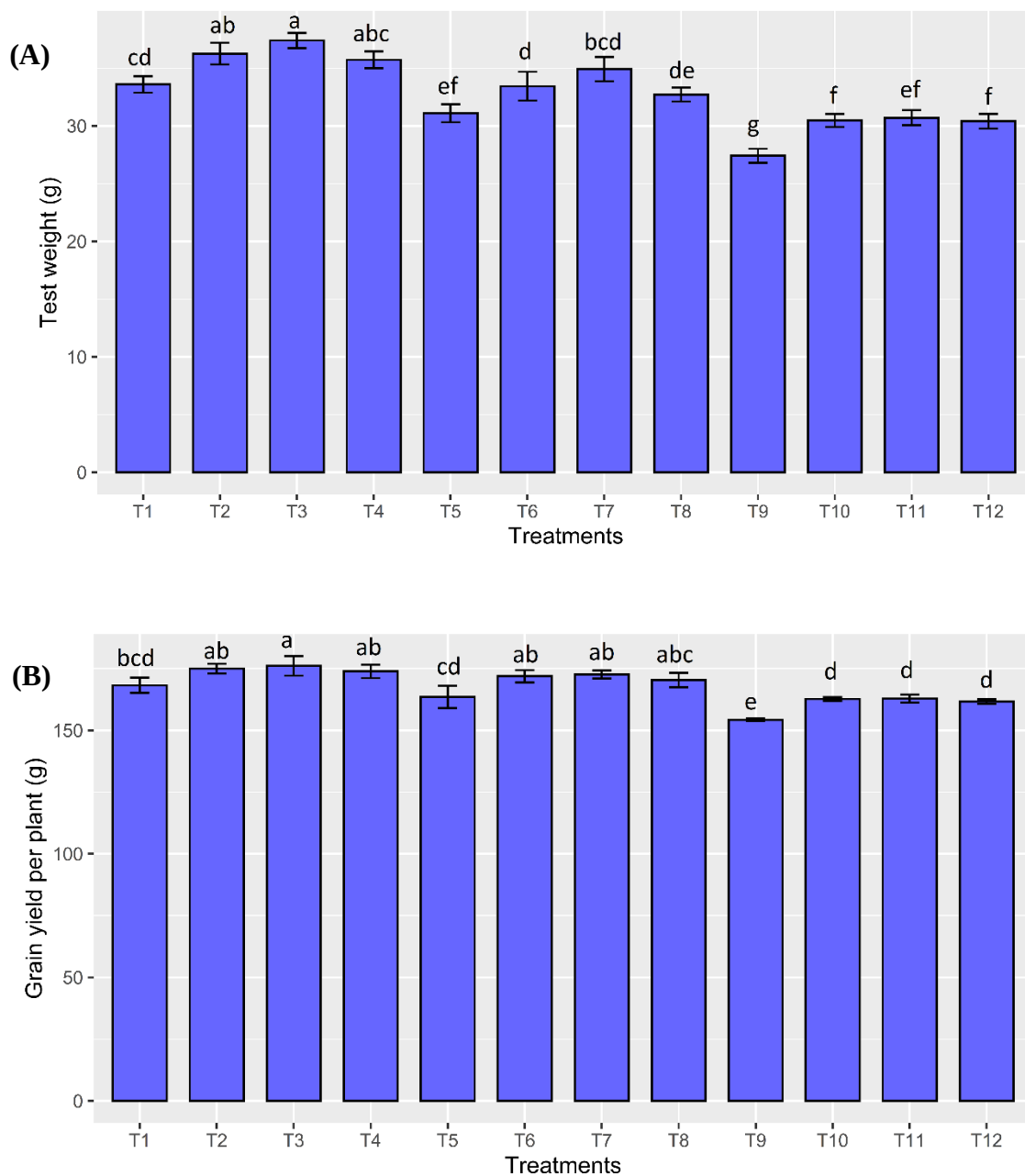


Figure 4.6.10: Effect of selected endophytic fungi on (A) Test weight, and (B) Grain yield per plant of maize under different field capacities. Values represented are mean $\pm$ SE(n=10) and with dissimilar alphabets are significantly different at Tukey's $p \leq 0.05$. Lines over bar plots indicate standard error over mean.

Note: T₁= FC 100%, T₂= FC 100% + LAS-6, T₃= FC 100% + K-23, T₄= FC 100% + P-37, T₅= FC 75%, T₆= FC 75% + LAS-6, T₇= FC 75% + K-23, T₈= FC 75% + P-37, T₉= FC 50%, T₁₀= FC 50% + LAS-6, T₁₁= FC 50% + K-23, T₁₂= FC 50% + P-37, where FC = Field capacity

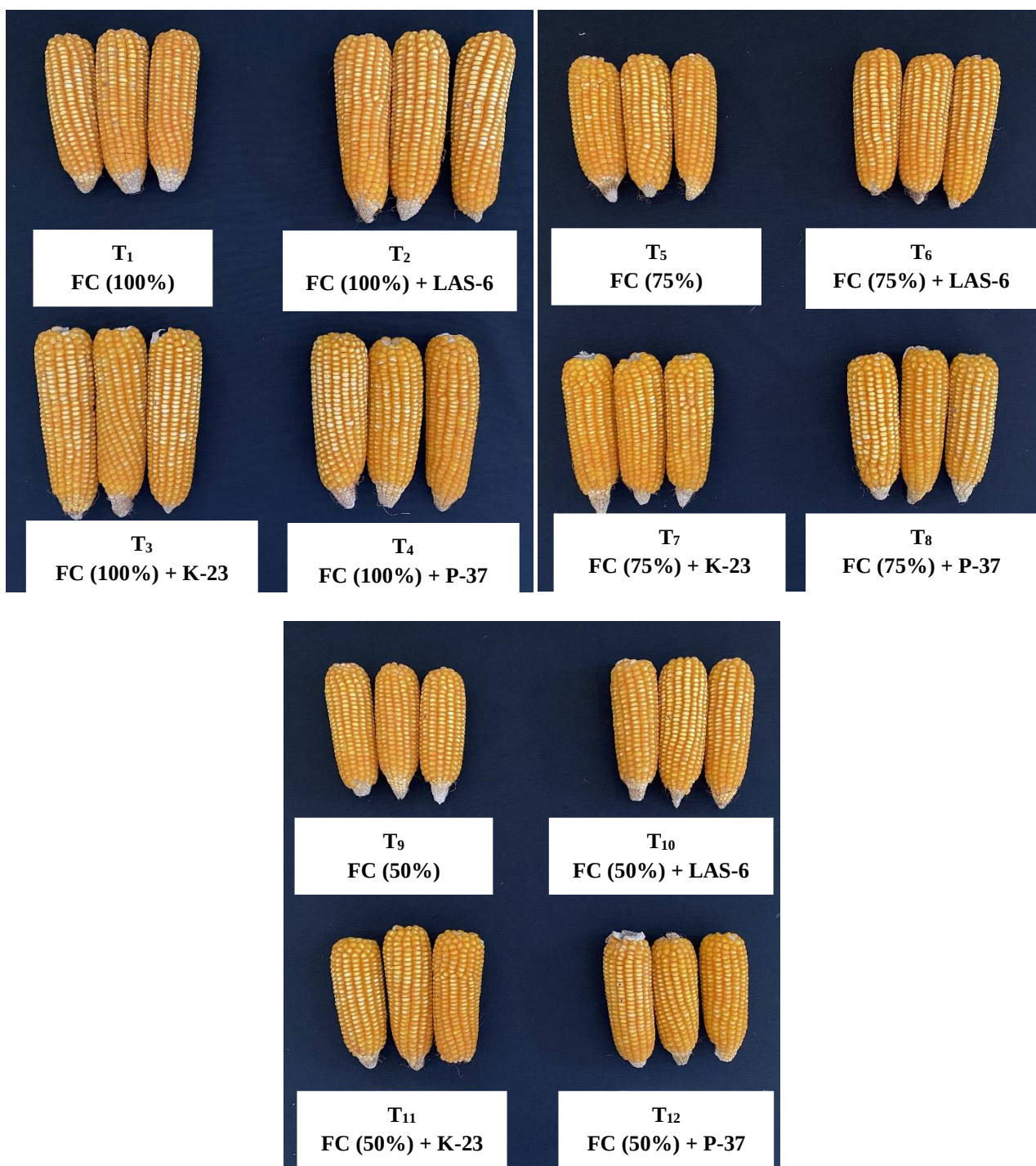


Plate 4.13: Phenotype of cobs from non-inoculated and endophytes inoculated plants of maize under different field capacities

source to sink. This study provides proof of concept that endophytes inoculation can improve plant growth and yield under drought stress in a habitat-specific manner through symbiotic association with plants.

Drought is a major limiting factor for plant productivity (Karl *et al.*, 2009) as water makes up 80–95 percent of the fresh biomass of the plant body, and it plays an important role in various physiological activities, including plant growth, development, and metabolism (Brodersen *et al.*, 2019). Because both the severity and duration of the stress are significant, drought-induced yield losses are likely to outnumber all other causes (Ma *et al.*, 2020). A 40 percent water reduction results in a 39.3 percent fall in maize yield (Daryanto *et al.*, 2016; Dicko *et al.*, 2018). Maize is sensitive to drought stress during the pre-flowering and grain filling stages, which immensely affects plant performance and yield due to impaired functions.

In the present study, the drought stress was imposed on plants from 45 to 65 days after sowing, which is a critical stage for the maize crop. Hence the decline in growth parameters like plant height, leaf area, stem girth, root volume, and dry biomass was observed at 65 DAS, which is directly related to yield and yield components. Some results have been documented on yield and yield components of endophytes treated plants under drought stress. Hubbard *et al.* (2014) showed an increase in average seed weight (ASW), total seed weight (TSW) of wheat under drought stress by cross infecting with endophytic fungi. Inoculation with *V. paradoxus* 5C-2 improved growth, yield, and water-use efficiency of droughted peas (Belimov *et al.*, 2009). Hubbard *et al.* (2014) reported that wheat seeds treated with six Ascomycota fungi enhanced growth under drought stress conditions.

4.8 A field assessment of selected endophytes to bridge a technological gap

The experiment was carried out in a field condition to evaluate the selected endophytes LAS-6 (*C.madrasense*), K-23 (*F.incarnatum*), and P-37 (*Ulocladium sp.*) on growth, physiology, and yield of maize genotype (MAH 14-5). Pre-germinated seeds were treated with the above-mentioned endophytes, whereas control was treated with water.

Endophytes treated pre-germinated seeds along with non-inoculated ones were sown in the field (Figure 3.2).

Growth parameters

Biometric parameters like plant height, number of leaves, stem girth, leaf area, and shoot biomass were recorded at 30, 45, and 65 DAS (Figure 4.7.1, 4.7.2, 4.7.3 and Plate 4.15). At 30 DAS, plant height (cm) varied between 78.71 to 86.47 cm/plant among treatments, with highest in P-37 (86.47 cm), LAS-6 (86.34 cm) and K-23 (85.75 cm) compared to control (78.71 cm). The number of leaves did not vary significantly among the treatments. Stem girth varied between 1.66 to 1.95 cm/plant, with significant increase in P-37 (1.99 cm), K-23 (1.98 cm) and LAS-6 (1.95 cm) compared to control (1.66 cm). Leaf area varied between 2838.27 cm² to 3438.51 cm²/plant with significant increase in K-23 (3438.51 cm²), P-37 (3398.88 cm²) followed by LAS-6 (3359.82 cm²) compared to control (2838 cm²). Shoot biomass varied between 18.59 to 22.32 g/plant with a significant increase in K-23 (22.32 g) followed by LAS-6 (21.73 g) and P-37 (21.17 g) compared to control (18.59 g).

At 45 DAS, plant height (cm) varied between 117.80 to 130.58 cm/plant among treatments, with highest in LAS-6 (130.58 cm), P-37 (129.18 cm) and K-23 (128.73 cm) compared to control (117.80 cm). The number of leaves did not vary significantly among the treatments. Stem girth varied between 2.22 to 2.49 cm/plant, with a significant increase in LAS-6 (2.49 cm), P-37 (2.46 cm), and K-23 (2.45 cm) compared to control (2.22 cm). Leaf area varied between 4989.00 cm² to 5492.82 cm²/plant with significant increase in P-37 (5492.82 cm²), K-23 (5415.88 cm²) followed by LAS-6 (5357.62 cm²) compared to control (4989.00 cm²). Shoot biomass varied between 52.16 to 61.66 g/plant with a significant increase in LAS-6 (61.66 g) followed by K-23 (61.06 g) and P-37 (58.83 g) compared to control (52.16 g).

At 65 DAS, plant height (cm) varied between 164.35 to 174.13 cm/plant among treatments, with highest in LAS-6 (174.13 cm), P-37 (173.72 cm) and K-23 (172.59 cm) compared to control (164.35 cm). The number of leaves did not vary significantly among the treatments. Stem girth varied between 2.25 to 2.78 cm/plant, with a significant increase

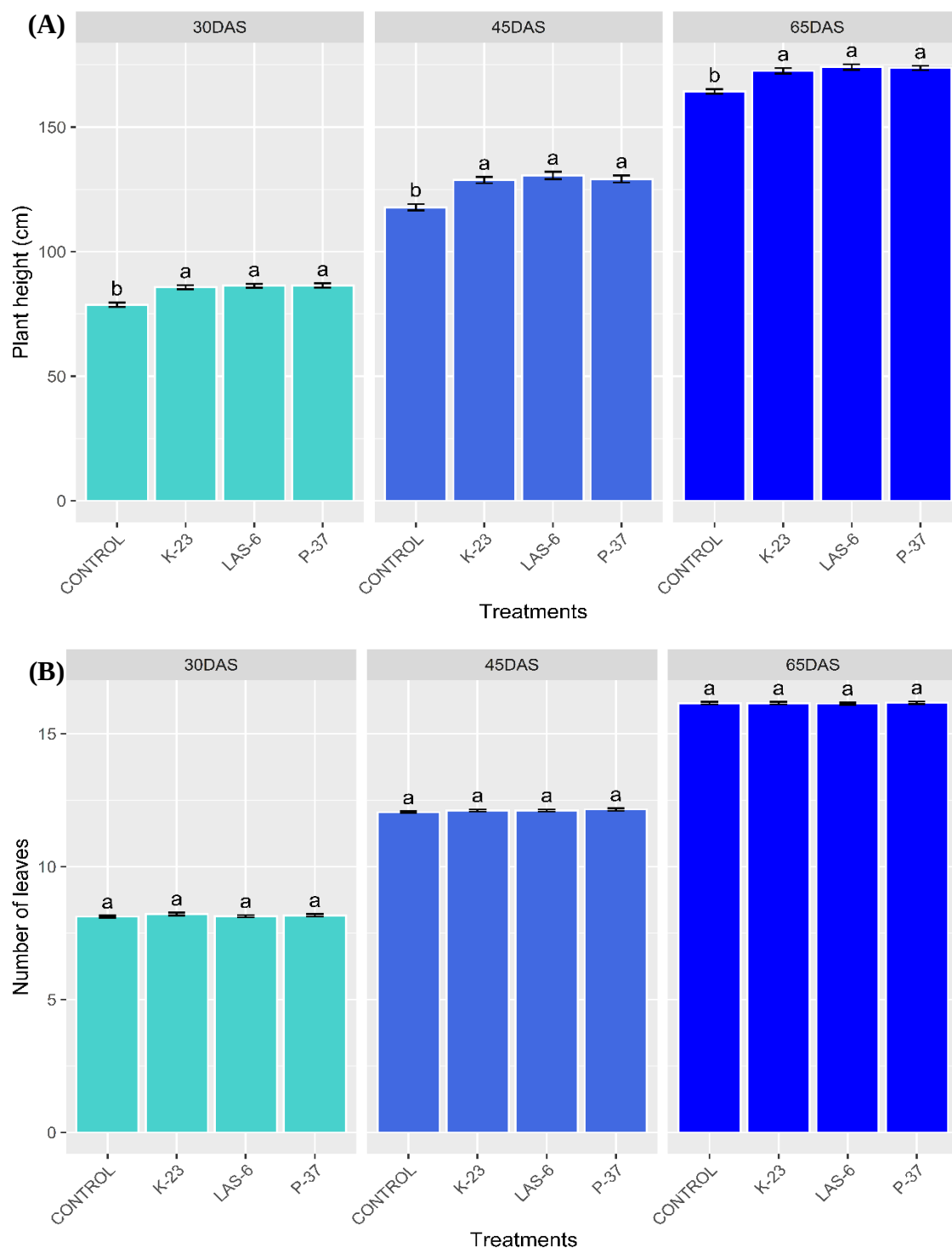


Figure 4.7.1: Effect of selected endophytic fungi on growth parameters of maize under field condition (A) Plant height, (B) Number of leaves. Values represented are mean $\pm$ SE(n=60) and with dissimilar alphabets are significantly different at Tukey's $p \leq 0.05$. Lines over bar plots indicate standard error over mean.

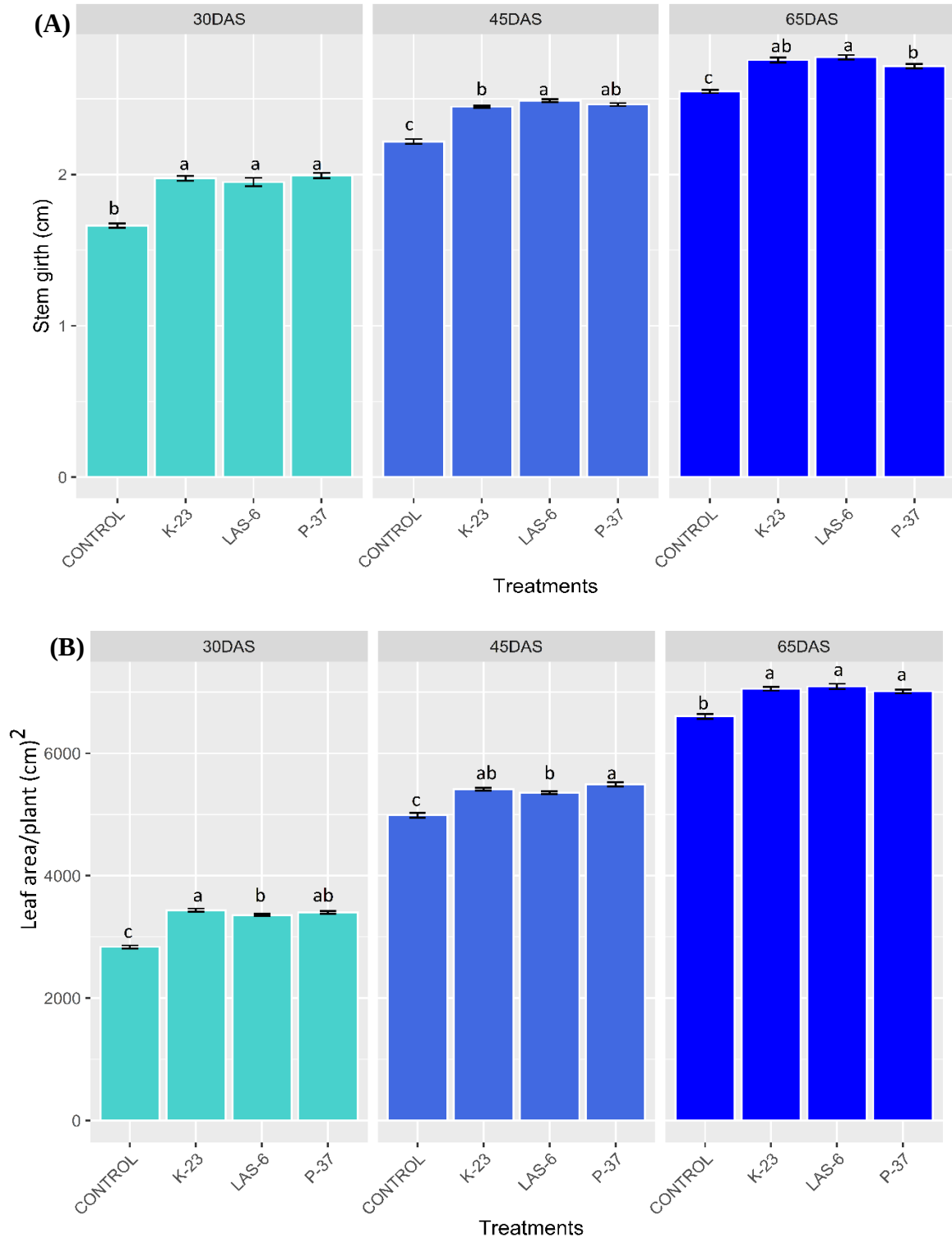


Figure 4.7.2: Effect of selected endophytic fungi on growth parameters of maize under field condition (A) Stem girth and (B) Leaf area. Values represented are mean $\pm$ SE(n=60) for stem girth and mean $\pm$ SE(n=18) for leaf area and with dissimilar alphabets are significantly different at Tukey's $p\leq 0.05$. Lines over bar plots indicate standard error over mean.

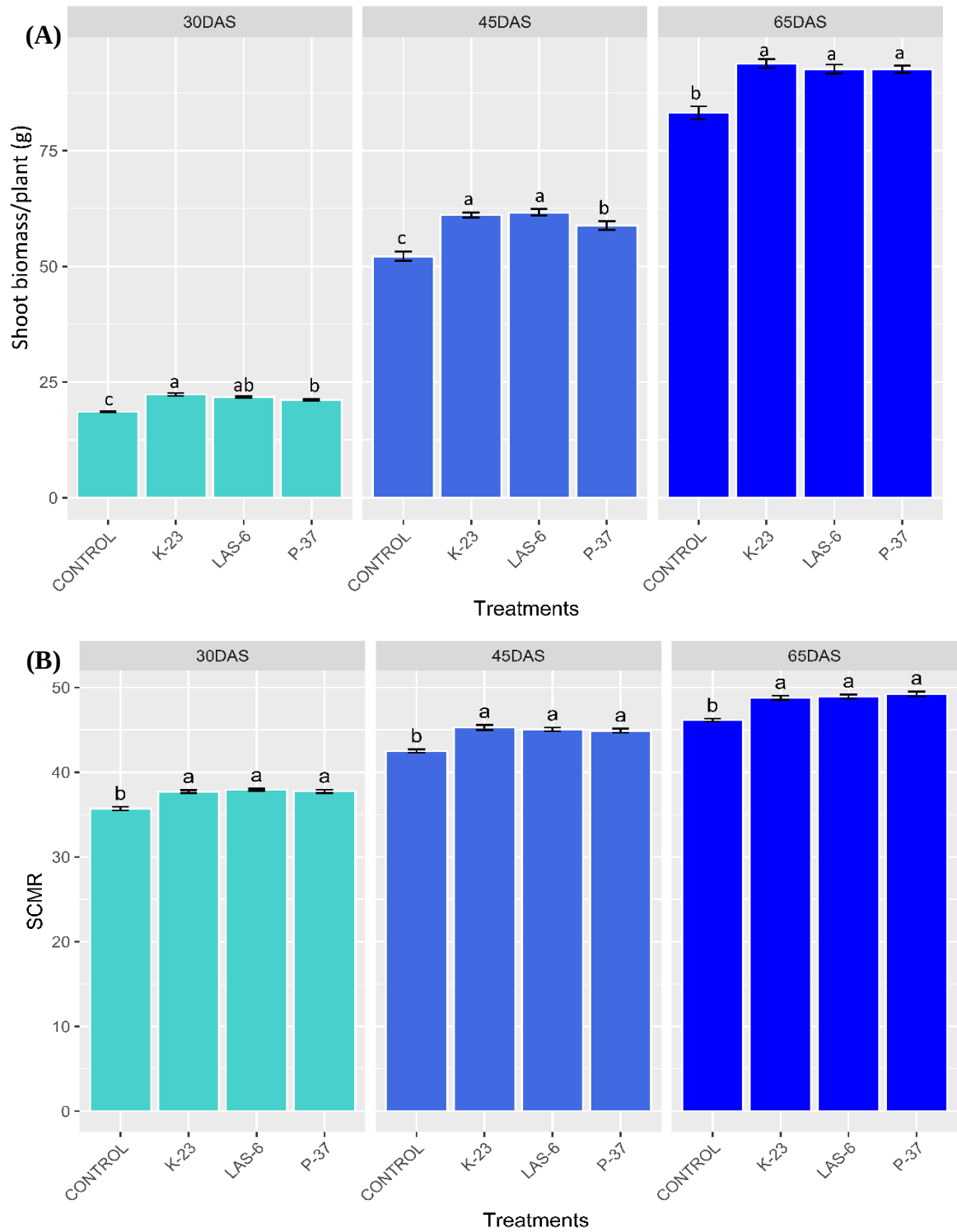


Figure 4.7.3: Effect of selected endophytic fungi on (A) Shoot biomass and (B) SCMR of maize under field condition. Values represented are mean $\pm$ SE(n=18) for Plant dry biomass and mean $\pm$ SE(n=60) for SCMR, and with dissimilar alphabets are significantly different at Tukey's $p \leq 0.05$. Lines over bar plots indicate standard error over mean.

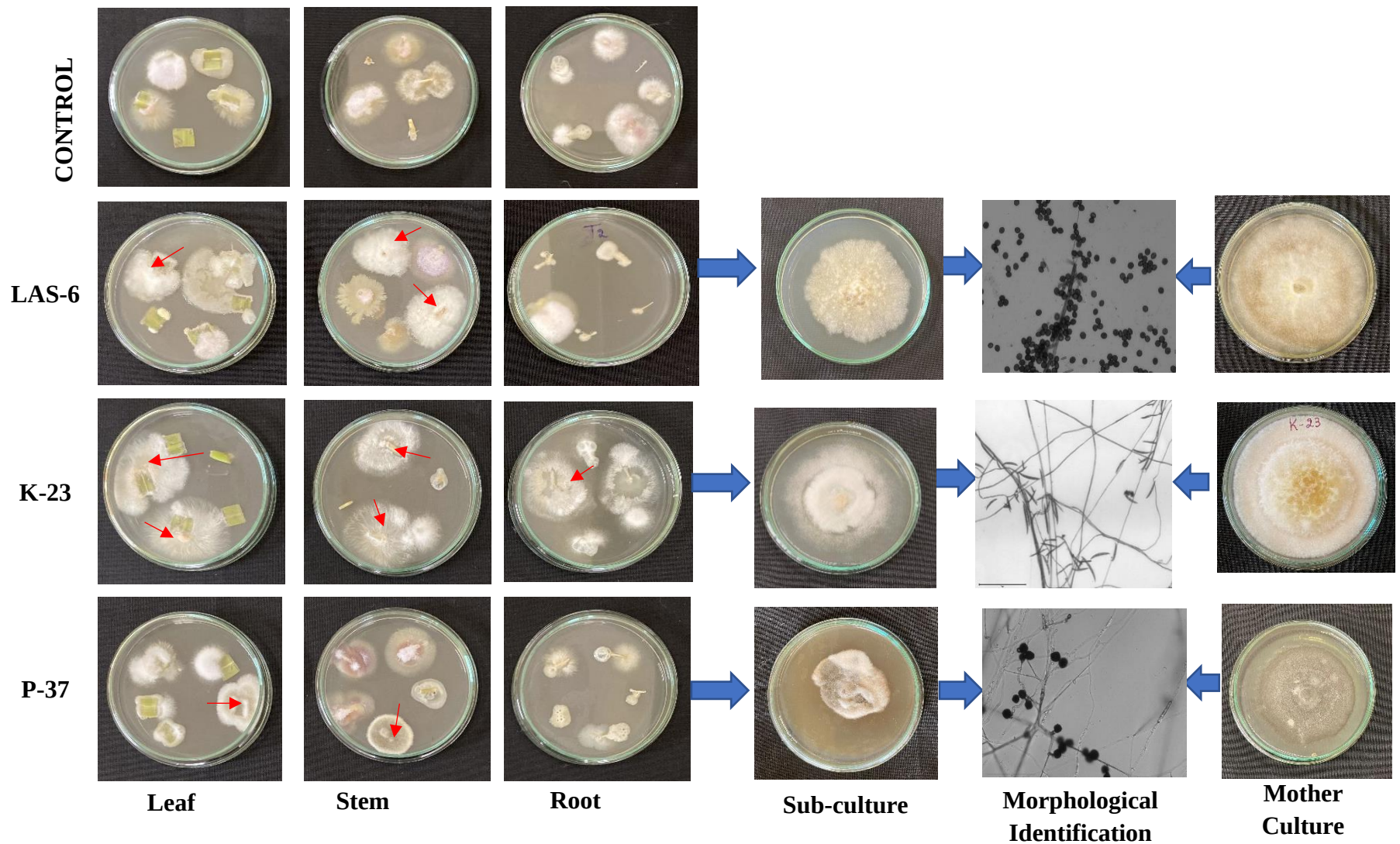


Plate 4.14: Colonization of endophytic fungi in leaf, shoot and roots of endophyte treated plants in field condition and morphological comparison of re-isolated endophytes with mother culture

in LAS-6 (2.78 cm), K-23 (2.76 cm), and P-37 (2.72 cm) compared to control (2.55 cm). Leaf area varied between 6605.24 cm² to 7091.25 cm²/plant with significant increase in LAS-6 (7091.25 cm²), K-23 (7054.56 cm²) followed by P-37 (7010.65 cm²) compared to control (6605.24 cm²). Shoot biomass varied between 83.17 to 93.77 g/plant with a significant increase in K-23 (93.77 g) followed by LAS-6 (92.63 g) and P-37 (92.59 g) compared to control (83.17 g). This is represented in Figures 4.7.1, 4.7.2 and 4.7.3.

The growth promotion may be attributed to the increase in photosynthetic efficiency upon endophytes treatment (Figure 4.7.4). However, in recent studies, it was revealed that plant growth promotion due to the secretion of phytohormones, such as indole-3-acetic acid (IAA), gibberellins, cytokinin, and secondary metabolites by the endophytic fungi (Waqas *et al.*, 2015; Ismail *et al.*, 2021) and partly owing to the fact that endophytes could have enhanced the host's uptake of nutritional elements such as nitrogen and phosphorus from the soil (García-Latorre *et al.*, 2021), and also endophytes might have genes that assist to produce some signalling molecule for growth promotion of plants (Ali *et al.*, 2014a).

A similar effect on plant growth due to endophytic treatment has been reported previously in various plant species under *in-vivo* conditions. The positive plant growth promotion is also evidenced by hormone-producing fungal isolates like *Penicillium chrysogenum*, *P. crustosum*, *Phoma glomerata*, *Pestalotiopsis neglecta*, *Williopsis saturnus* in different crop systems (Fu *et al.*, 2015; Fouda *et al.*, 2015; Hassan, 2017; Waqas *et al.*, 2012b). The symbiotically induced growth response was observed in tomato, cucumber plants when treated with *Trichoderma harzianum*, *Penicillium janthinellum*, *Exophiala* sp. (Mastouri *et al.*, 2010; Khan *et al.*, 2013).

Physiological parameters

Physiological parameters like SPAD chlorophyll meter reading (SCMR) was taken at 30, 45, and 65 DAS (Figure 4.7.3), and photosynthetic rate, stomatal conductance, transpiration rate were recorded at 30 DAS (Figure 4.7.4). At 30, 45, and 65 DAS, the SCMR was significantly increased in endophyte-treated plants compared to non-inoculated plants.

Under field conditions, LAS-6, K-23, and P-37 showed a significant increase (Figure 4.7.4) in photosynthetic assimilation rate, stomatal conductance, and transpiration rate compared to non-inoculated plants. SCMR obtained at 30 DAS also showed a similar trend with respect to photosynthetic parameters in plants inoculated with endophytes. In total, endophyte-treated plants had high photosynthetic assimilation rates due to higher stomatal conductance, transpiration rate, and higher chlorophyll content maintained by endophyte-inoculated plants.

The increase in photosynthetic efficiency of plants obtained in this study is in agreement with the increased photosynthetic rate, chlorophyll content, stomatal conductance of maize plants treated with endophyte *Trichoderma harzianum* (Akladios and Abbas, 2014; Doni *et al.*, 2019). Furthermore, Morse *et al.*, 2007 showed that in *Festuca*, the net photosynthetic rate, stomatal conductance, and transpiration rate of E+ plants were considerably higher than those of E- plants. Xu *et al.*, 2021 demonstrated that endophyte infection improved all photosynthetic indicators of *F. sinensis* under drought stress, except for intercellular carbon dioxide concentration. These findings suggest that endophytes can improve plant growth by improving photosynthesis, which is consistent with the finding that endophytes can alter the host plant's strategy and enhance metabolism (Xia *et al.*, 2018).

Yield parameters

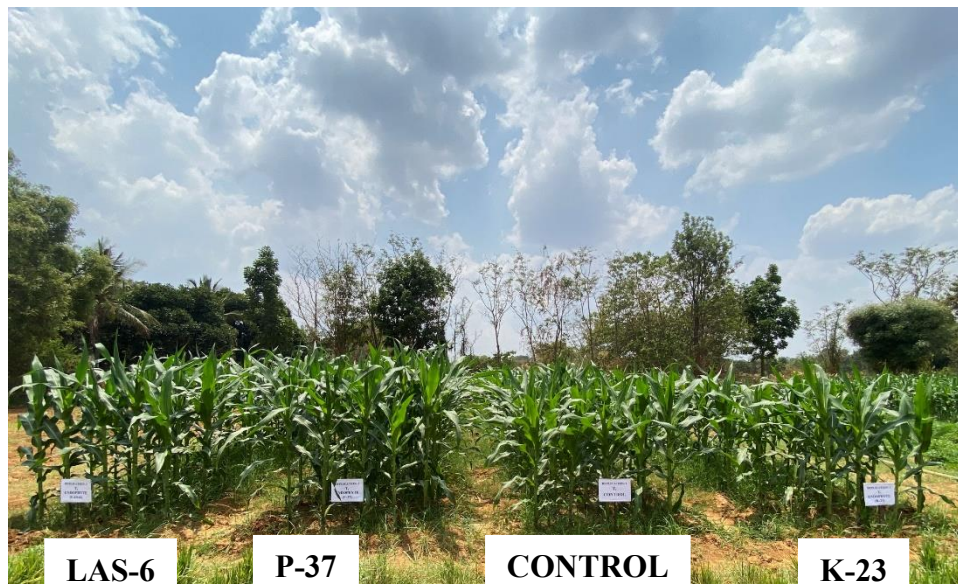
Yield and yield-related parameters like days to anthesis, days to silking, cob length, cob diameter, cob weight, number of kernel rows per cob, number of kernels per row, kernel row length, test weight, and grain yield per plant were recorded in endophyte inoculated and non-inoculated plants grown under field conditions.

The plants which were not inoculated with endophytes took more days for anthesis (58.75 days) and silking (59.88 days) under field conditions. Whereas the plants inoculated with endophytes, K-23 (55.60, 56.73 days), P-37 (55.78, 56.97 days), and LAS-6 (55.88, 56.95 days) took fewer days for anthesis and silking respectively (Figure 4.7.5). The cob length of plants that were inoculated with endophytes K-23 (20.01 cm), LAS-6 (19.52 cm), and P-37 (19.33 cm) showed a significant increase compared to non-inoculated plants

(A)



(B)



(C)

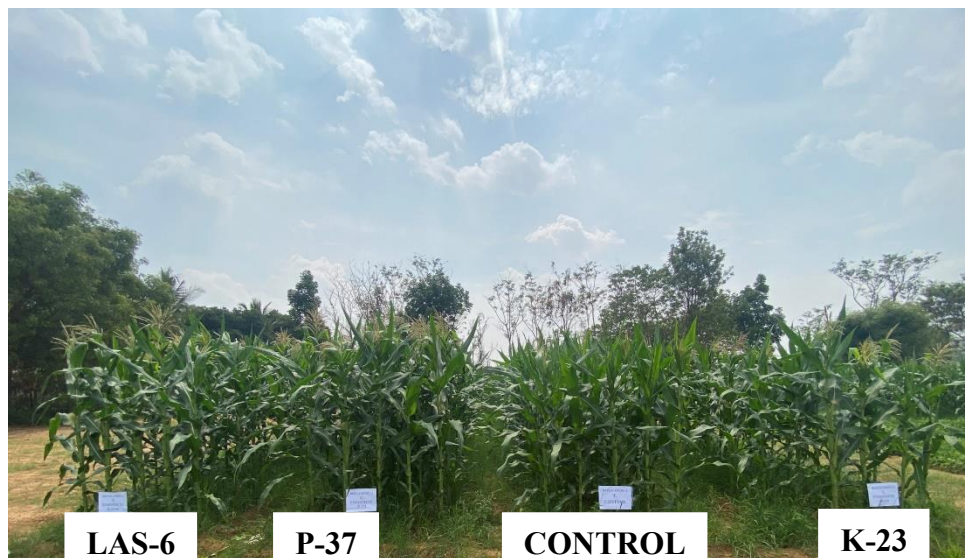


Plate 4.15: Phenotype of non-inoculated and endophytes inoculated plants of maize at (A) 30 DAS, (B) 45 DAS and (C) tasselling and silking stage under field condition

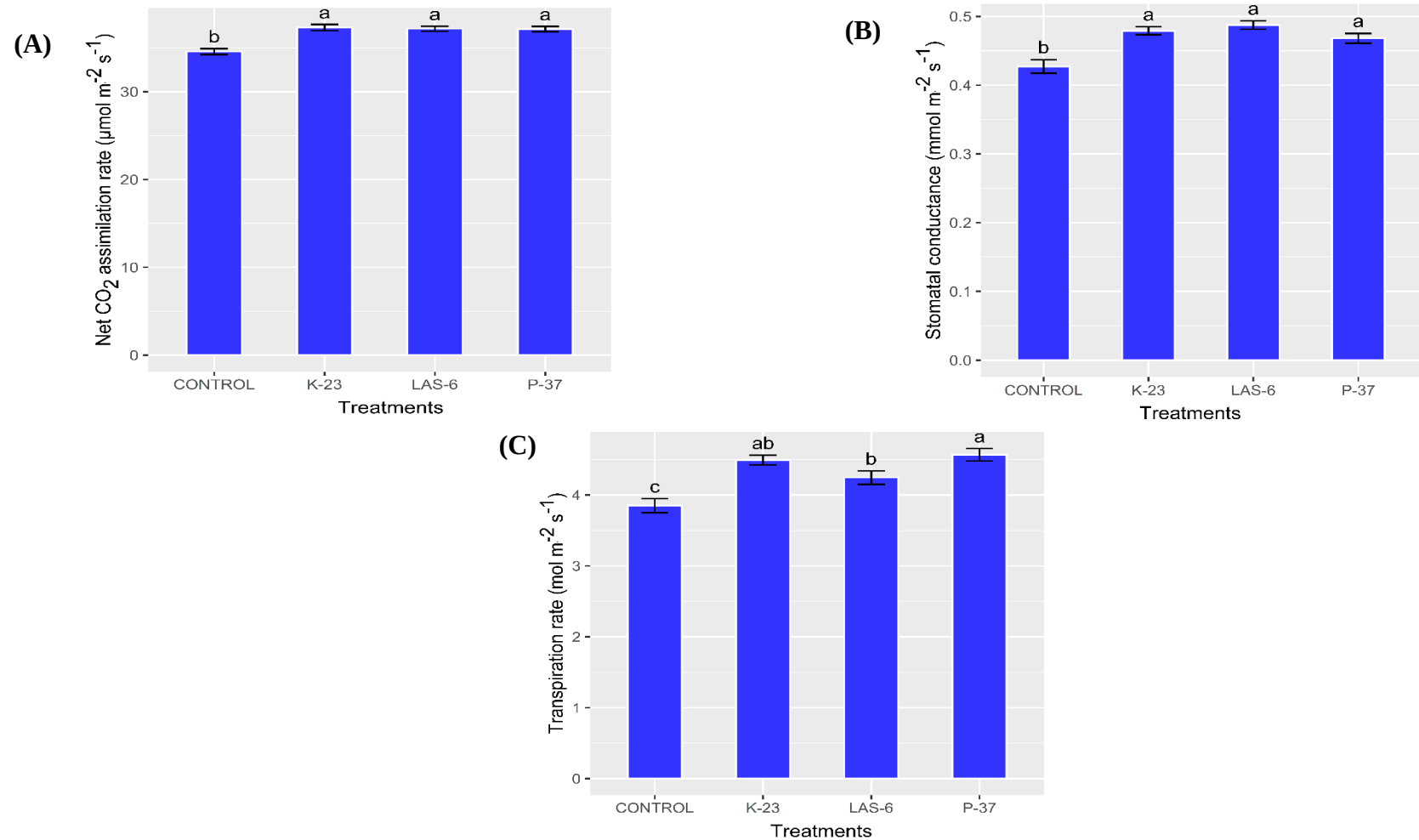


Figure 4.7.4: Effect of selected endophytic fungi on physiological parameters of maize under field condition (A) Photosynthetic rate, (B) Stomatal conductance, and (C) Transpiration rate. Values represented are mean $\pm$ SE(n=30) and with dissimilar alphabets are significantly different at Tukey's $p \leq 0.05$. Lines over bar plots indicate standard error over mean.

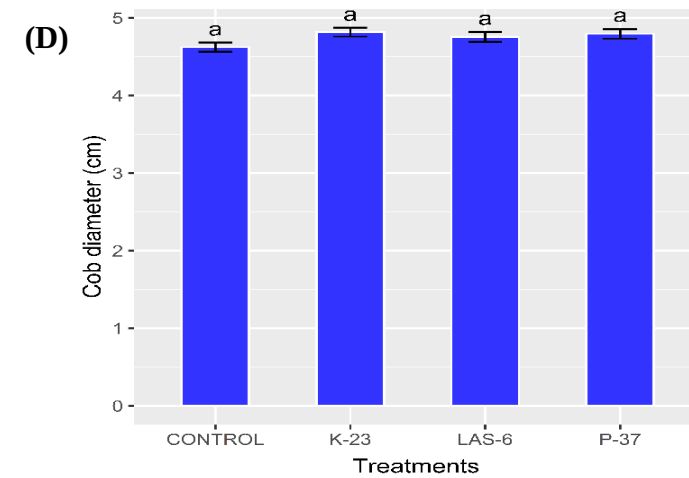
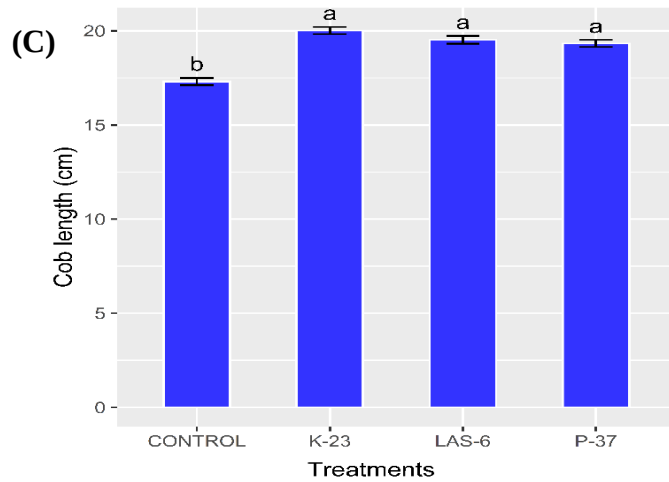
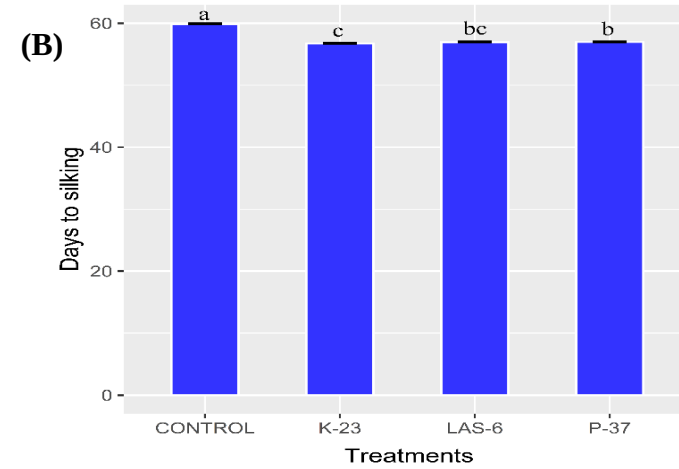
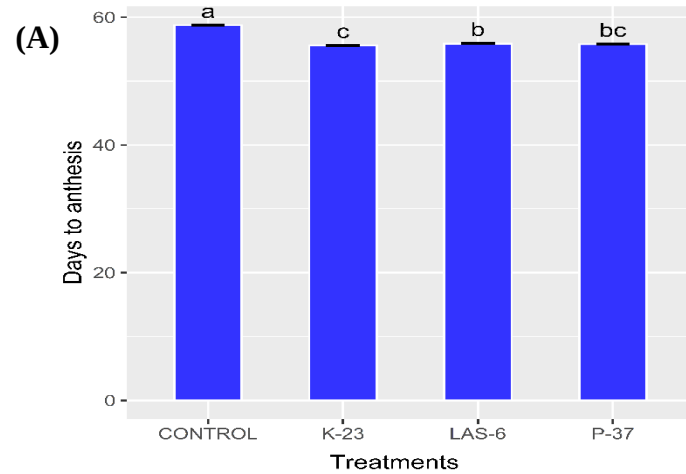


Figure 4.7.5: Effect of selected endophytic fungi on (A) Days to anthesis, (B) Days to silking, (C) Cob length and (D) Cob diameter of maize under field condition. Values represented are mean $\pm$ SE(n=60) and with dissimilar alphabets are significantly different at Tukey's $p \leq 0.05$. Lines over bar plots indicate standard error over mean.

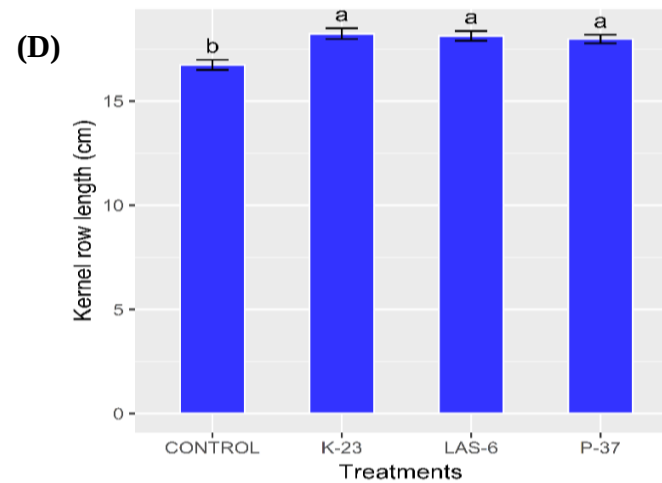
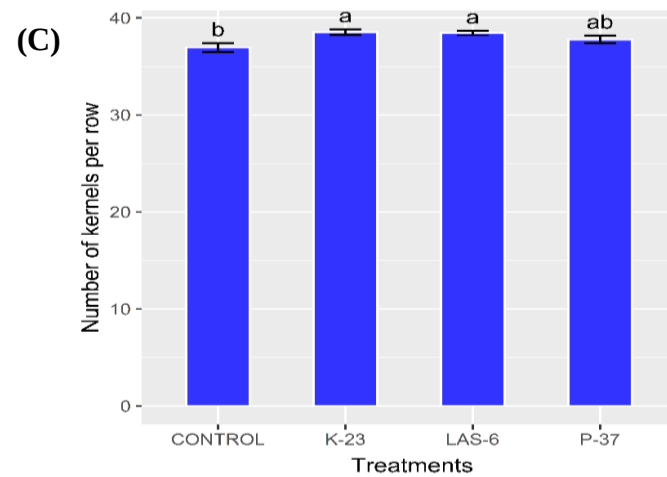
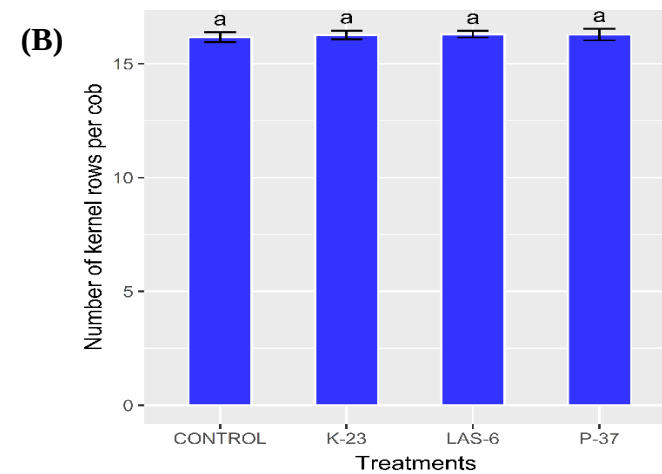
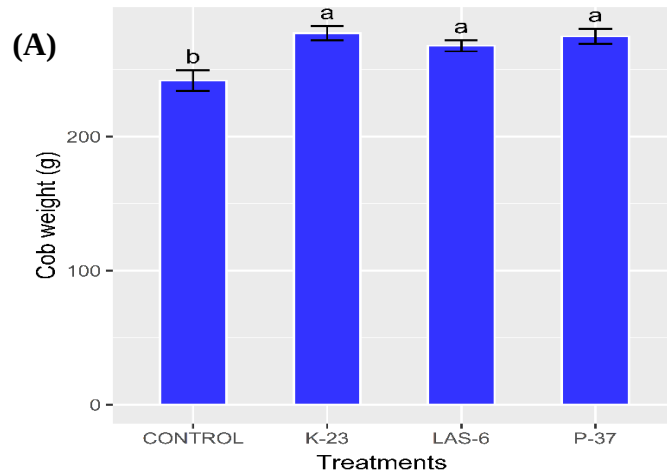


Figure 4.7.6: Effect of selected endophytic fungi on (A) Cob weight, (B) Number of kernel rows per cob, (C) Number of kernels per row and (D) Kernel row length of maize under field condition. Values represented are mean $\pm$ SE(n=60) and with dissimilar alphabets are significantly different at Tukey's $p \leq 0.05$. Lines over bar plots indicate standard error over mean.

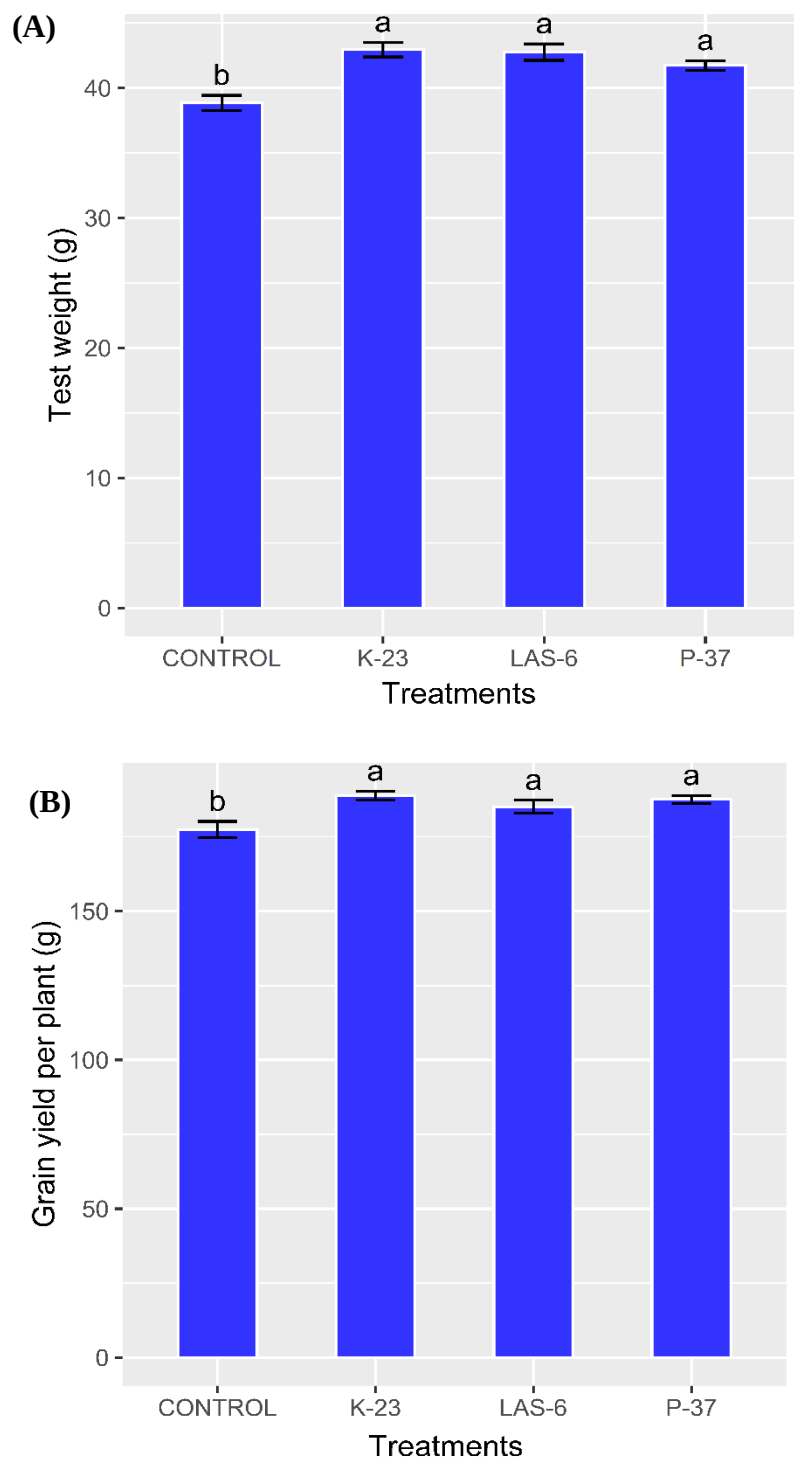


Figure 4.7.7: Effect of selected endophytic fungi on (A) Test weight, (B) Grain yield per plant of maize under field condition. Values represented are mean $\pm$ SE(n=60) and with dissimilar alphabets are significantly different at Tukey's $p \leq 0.05$. Lines over bar plots indicate standard error over mean.



Plate 4.16: Phenotype of cobs from non-inoculated and endophytes inoculated plants of maize under field condition

(17.30 cm), which is given in Figure 4.7.5 and Plate 4.16. Endophyte inoculation did not change the cob diameter significantly (Figure 4.7.5). The cob weight of plants that were inoculated with endophytes K-23 (277.18 g), P-37 (274.87 g), and LAS-6 (267.85 g) showed a significant increase compared to non-inoculated plants (241.88 g) and given in Figure 4.7.6. The number of kernel rows per cob did not differ due to endophyte inoculation (Figure 4.7.6). Whereas the number of kernels per row was significantly increased in the endophytes K-23 (38.53), LAS-6 (38.45), and P-37 (37.78) treated plants compared to control (36.95). The kernel row length was highest in K-23 (18.24 cm), LAS-6 (18.14 cm), and P-37 (17.98 cm) treated plants and lowest in non-inoculated plants (16.74 cm), which is represented in Figure 4.7.6.

All these above attributes corresponded to the yield of the plant. The plants inoculated with endophytes K-23 (42.95 g), LAS-6 (42.76 g), and P-37 (41.73 g) showed an increase in the test weight of seeds than the non-inoculated plants (38.85 g) under field conditions (Figure 4.7.7). Grain yield per plant was significantly high from endophytes K-23 (188.85 g), LAS-6 (185.15 g), and P-37 (187.59 g) treated plants compared to untreated plants (177.50 g) in field conditions (Figure 4.7.7). Thus, increase in seed yield upon endophytes treatment could be due to an increase in net photosynthetic rate (Figure 4.7.4) and the rate of photoassimilate remobilization from source to sink. This study provides proof of concept that endophytes inoculation can improve plant growth and yield under field conditions in a habitat-specific manner through symbiotic association with plants.

Plants face different stresses under field conditions due to the drastic and rapid global climate changes, which can alter the morphological, physiological, and molecular responses that disturb normal plant productivity. Plants attain various common and different physiological and molecular protective approaches for tolerance under field stresses. In this study, the possible cross-talk between plants and endophytes to increase yield was studied under field conditions. The increase in growth parameters like plant height, leaf area, stem girth, and dry biomass was observed at 30, 45, and 65 DAS, which is directly related to yield and yield components. Despite the immense potential of endophytes in increasing crop yield, field-rational tests are few. Some results have been documented on yield and yield components of endophytes treated plants under field

conditions. In the United States, Australia, and New Zealand, *Epichloe* (Class 1) endophyte strains have been employed to boost the productivity of forage grasses in the field and the robustness of turf grasses (Young *et al.*, 2014; Kauppinen *et al.*, 2016). *Trichoderma* sp. which lives on the roots of stressed plants improves yields by activating metabolic processes that convert the hazardous reactive oxygen species produced during stress into less toxic chemicals (Harman *et al.*, 2021).

4.9 Characterization of the best performing endophytes

(a) Morphological identification using Scanning electron microscope (SEM)

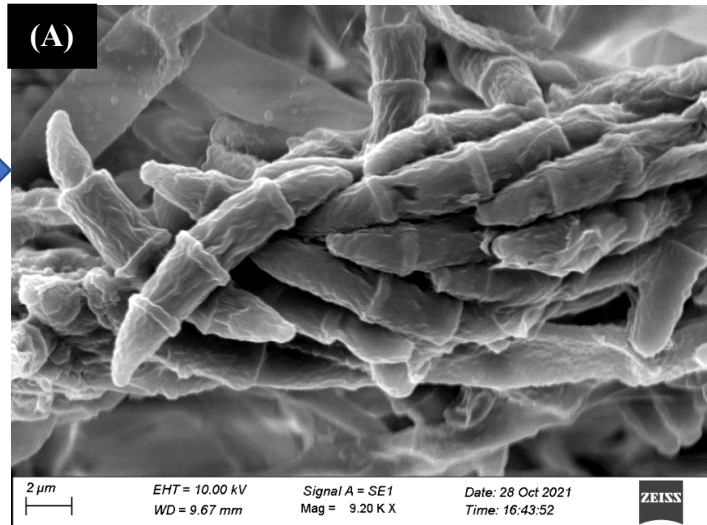
The three endophytes (K-23, LAS-6 and P-37) which performed best under *in-vitro*, greenhouse, and field experiments were further morphologically characterized using a scanning electron microscope (SEM). In K-23 (*F. incarnatum*) macroconidia were seen wide, straight to slightly curved, 3 to 5 septate, and abundant in sporodochia. SEM image of LAS-6 (*C. madrasense*) confirmed the presence of whole perithecia with ostiolar pores. The hairs under SEM of entangled ascospores were flexuous and coiled for effective dispersal. The ascospores were smooth, oval in shape with the presence of a single apical germ pore. The single whole ascomata showed a tuft of slightly curved ascomatal hairs of different lengths. Ahamed *et al.* (2005) also reported that SEM image of ascospore were lemon-shaped and with apical papillae at both ends indicating the presence of germ pores. SEM image of P-37 (*Ulocladium* sp.) showed that spores are multi-celled due to their transverse and longitudinal septation, have an obovoid (narrowest at the base) rough-walled shape, and most of them become verrucose. Spores appeared with uniform, knobby surfaces, and with a bud at one end (Plate 4.17).

(b) Molecular identification using large subunit (LSU) and partial β -tubulin II (*TUB2*) region

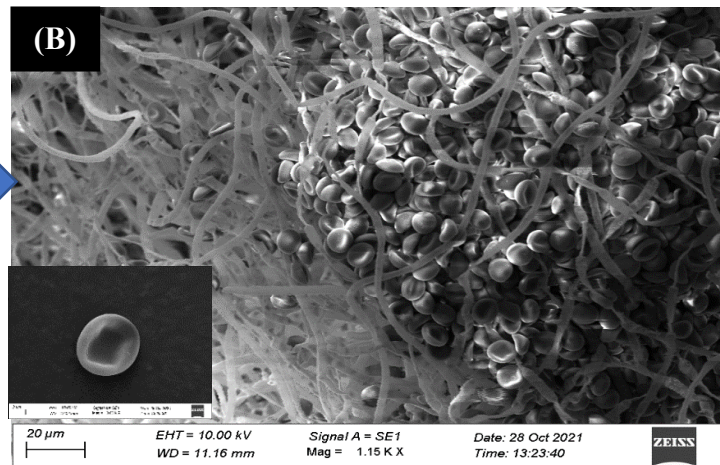
The ribosomal targets, especially the large-subunit RNA gene (D1-D2 region) and partial β -tubulin II (*TUB2*) region have been shown particular promise for the molecular identification of some fungi. Therefore, an assessment was done on these regions for the identification of the three best performing endophytes. The LSU gene region sequence of K-23, LAS-6 and P-37 fungal isolates with 852, 848, 860 base pair (bp) showed 99.7, 99.8



K-23



LAS-6



P-37

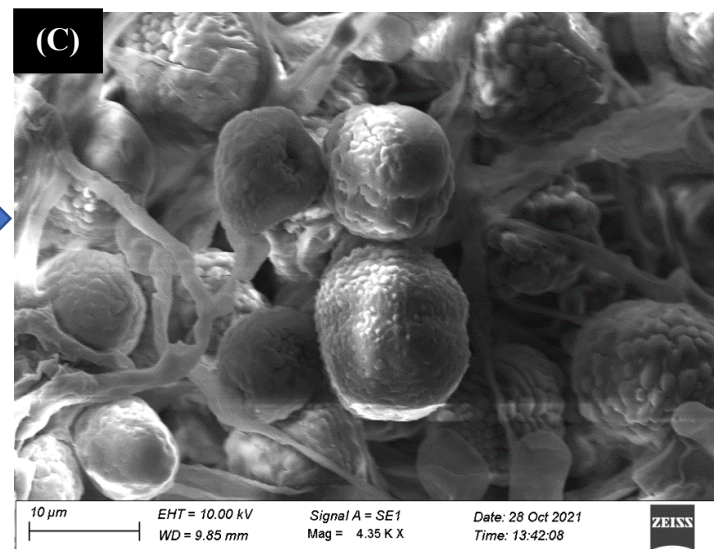


Plate 4.17: SEM image of spores from three fungal isolates, (A) *Fusarium incarnatum*, (B) *Chaetomium madrasense*, (C) *Ulocladium* sp.

and 100 percent homology with *Fusarium incarnatum*, *Chaetomium madrasense* and *Ulocladium sp.* respectively, in the NCBI data base (Table 4.4, Figure 4.8). The partial β - tubulin II (*TUB2*) region sequence of K-23, LAS-6 and P-37 fungal isolates with 288, 426, 271 base pair (bp) showed 100, 99.7 and 98.46 percent homology with *Fusarium incarnatum*, *Chaetomium madrasense* and *Ulocladium sp.* respectively, in the NCBI data base (Table 4.5, Figure 4.8). The phylogenetic tree constructed with respective species available in the NCBI GenBank with the sequences of identified organisms showed the close relatedness.

Table 4.4: Molecular identification based on sequencing of LSU region of best three endophytic fungal isolates

Sl. No.	Sample Code	Query length	Blast search result	Query cover (%)	Identity (%)	Homologue
1	K-23	852	<i>Fusarium incarnatum</i>	100	99.7	MH877332.1
2	LAS-6	848	<i>Chaetomium madrasense</i>	100	99.8	KX958072.1
3	P-37	860	<i>Ulocladium sp.</i>	99	100	MH875428.1

Table 4.5: Molecular identification based on sequencing of *TUB2* region of best three endophytic fungal isolates

Sl. No.	Sample Code	Query length	Blast search result	Query cover (%)	Identity (%)	Homologue
1	K-23	288	<i>Fusarium incarnatum</i>	100	100	MK752398.1
2	LAS-6	436	<i>Chaetomium madrasense</i>	100	99.7	JN256175.1
3	P-37	271	<i>Ulocladium sp.</i>	95	98.46	JF417689.1

In most fungi, the LSU gene area has two hypervariable sections, D1 and D2, bordered by comparatively conserved sequence regions (Hibbett *et al.*, 2011). This arrangement allows LSU gene sequences to be aligned for phylogenetic analysis. For

fungal phylogeny and taxonomy placement, the LSU region has been extensively used (Opik *et al.*, 2010). Despite the fact that the ITS region serves as a good "bar code" for environmental diversity research, the level of sequence heterogeneity in this region prevents reliable sequence alignment. The LSU gene serves as a molecular marker for the phylogenetic placement of new fungal lineages discovered through environmental surveys, as well as the investigation of basal fungal lineages. Among protein-coding markers, beta-tubulin (*TUB2*) have been most commonly used for inferring phylogenetic relationships among fungi (Schoch *et al.*, 2009 and Zhao *et al.*, 2014) and identifying fungal species.

4.10 Effect of K-23 (*F. incarnatum*) on endogenous hormone levels of maize under PEG-8000 induced drought stress

Phytohormones are widely known for their function in regulating plants' normal physiological and metabolic behaviour under stress conditions through adaptive responses. The most prevalent are auxin (IAA), gibberellin (GA), abscisic acid (ABA) and salicylic acid (SA). Endophytes are essentially involved in the growth and development of host plants, either directly or indirectly by secreting various phytohormones or stimulate the production of native plant hormones, influencing plant development and signaling (Pinski *et al.*, 2019) that helps in mitigating various abiotic conditions (Shade *et al.*, 2017).

The endophyte treated plants were shown to have high production of IAA and GA under unstressed and PEG-8000 induced drought stress condition. IAA was increased in K-23 treated plants (97.92, 22.77 ng/g FW) than in untreated plants (58.65, 19.04 ng/g FW) under unstressed and drought stress situation respectively. GA concentration was significantly increased from 260.95 ng/g FW (without K-23) to 570.95 ng/g FW (with K-23) in control conditions and from 64.43 ng/g FW (without K-23) to 135.63 ng/g FW (with K-23) at drought stress condition. Whereas, ABA levels was significantly decreased in K-23 inoculated plants (24.20, 37.54 ng/g FW) compared to non-inoculated plants (39.28, 64.10 ng/g FW) under unstressed and PEG-8000 induced drought stress respectively. The salicylic acid was found to be significantly increased in K-23 inoculated plants (4.04 ng/g FW) compared to non-inoculated plants (12.29 ng/g FW) in unstressed conditions, but in PEG-8000 induced drought stress condition, the salicylic acid was shown to have a

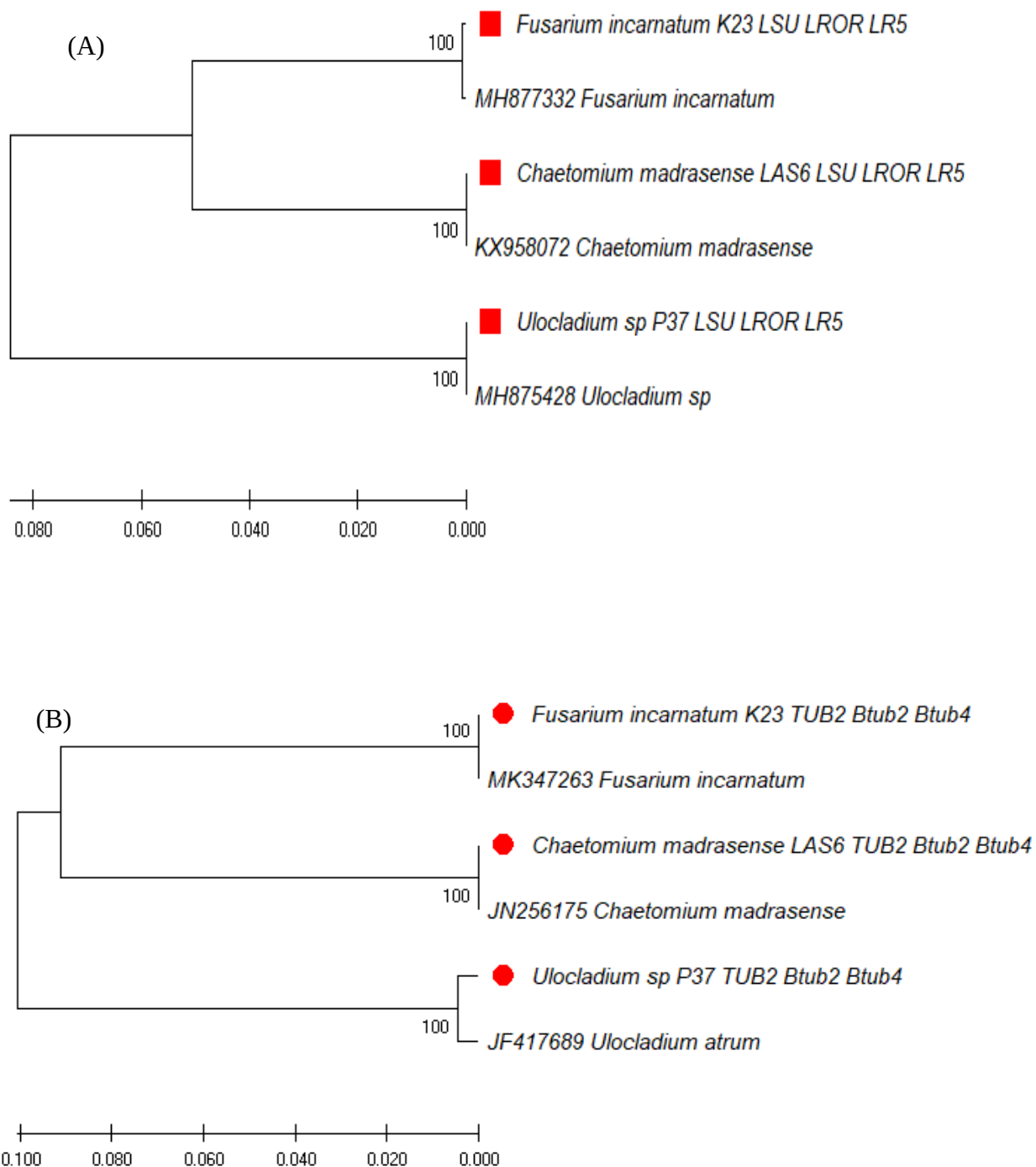


Figure 4.8: UPGMA phylogenetic consensus tree inferred from (A) LSU region (B) *TUB2* region sequences of best forming three fungal isolates (K-23, LAS-6 and P-37) along with their respective reference sequence from NCBI. The tree was constructed with Bootstrap values of 1000 replicates. The isolate whose sequences have been submitted in NCBI GenBank are highlighted with red colour symbol.

marginal increase in K-23 treated plants (70.50 ng/g FW) compared to untreated plants (60.43 ng/g FW) and given in figure 4.9.

Similar results were seen in endophytic bacterial strain *Bacillus amyloliquefaciens* produced gibberellins (GAs) which help in boosting host physiology (Shahzad *et al.*, 2016), and produced markedly more salicylic acid, and had lower levels of endogenous abscisic acid. IAA and GA produced by *Sphingomonas* sp. LK11 increased tomato seedling growth (Khan *et al.*, 2014). It has previously been reported that salt stress causes an increase in ABA content in plants and that fungal interaction with plants can regulate ABA production (Porcel *et al.*, 2012). Khan *et al.* (2011) found that salt-injured soybean plants colonized by *Penicillium minioluteum* had lower ABA levels than untreated plants. Thus, the association with endophytic fungi reduces ABA levels (Khan *et al.*, 2015a). Endophyte infection reduces stress by lowering abscisic acid levels and increasing salicylic acid levels (Waqas *et al.*, 2012b). Induced systemic resistance to abiotic stressors is increased by the symbiotic interaction of fungi and plants, and high endogenous SA can buffer the harmful consequences of ROS buildup (Khan *et al.*, 2013). As a result, it is possible to say that increasing phytohormone production through the use of fungal endophytes is a viable strategy for achieving sustainable agriculture under stressful conditions (Vaishnav *et al.*, 2019).

4.11 Transcriptomic analysis

To study the underlying molecular mechanisms involved in maize (MAH 14-5) upon inoculation with endophyte K-23 (*F. incarnatum*), RNA sequencing was carried out with different treatment combinations *i.e.*, Endophyte (K-23) vs Control and PEG-8000 + Endophyte (K-23) vs PEG-8000.

Endophyte (K-23) vs Control

From the RNA Seq data, a total of 1543 genes were differentially expressed. Among them, 773 genes were upregulated and 770 genes were downregulated (Figure 4.10.1.).

These DEGs were later subjected to GO terms, where most of the genes had belonged to biological functioning followed by molecular processes and cellular functioning processes. Within the GO term- ‘Biological function’, most of the genes were encoded to the localization process, genes involved in conferring abiotic stress tolerance, metabolic process, biological regulation, nitrogen metabolism, and cellular process genes (Figure 4.10.2). Similarly, within the GO term- ‘Molecular function’, genes encoding various transcription factors that go and bind to DNA and genes that enhance transcription factor activity were observed. Under the GO term- ‘Cellular components’ various protein complexes that belong to cell membrane and organelles were significantly enriched (Figure 4.10.2).

Bubble plot analysis for the upregulated DEGs has shown that genes involved in the biosynthesis of secondary metabolites, metabolic pathways, MAPK signaling, hormone signaling genes were enriched (Figure 4.10.3). Further to study the various pathway that is interacting, MapMan analysis was performed (Figure 4.10.4). Genes involved in the synthesis of several antioxidants such as ascorbate, glutathione followed by light reaction pathway, maintenance of tetrapyrrole structure of chlorophyll, various amino acids, and genes encoding carbohydrate metabolisms were expressed.

In the present study, maize plants enriched with K-23 endophyte have been shown to differentially regulate genes involved in secondary metabolites, hormones, MAPK signaling genes, antioxidant biosynthesis, and carbohydrate metabolism genes. Secondary metabolites as such are not directly involved in plant growth and development, however, they prepare the plants well in advance to adapt to biotic and abiotic stress (Khare *et al.*, 2020). Ogbe *et al.* (2020) has shown that medicinal plants that were enriched with endophyte were reported to have more amount of secondary metabolites accumulation when compared to control plants.

As shown in Plate 3.8, endophyte enriched plants have shown significantly higher seedling growth when compared to control. Interestingly, from the transcriptome data (Figure 4.10.3) endophyte enriched plants were shown to upregulate genes involved in the biosynthesis of growth-promoting hormones such as auxin, GA, and cytokinins. In the past

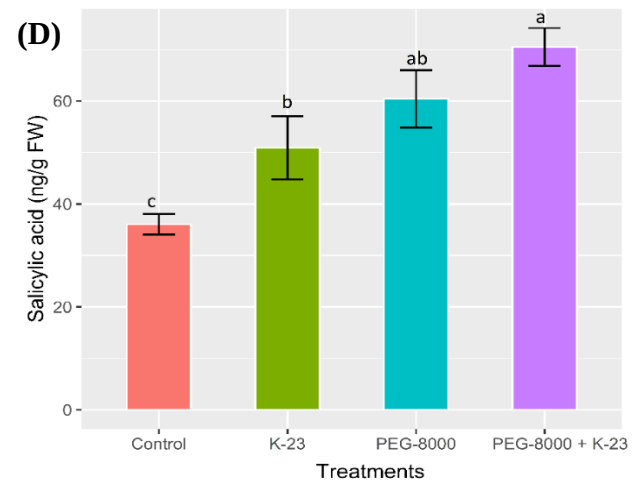
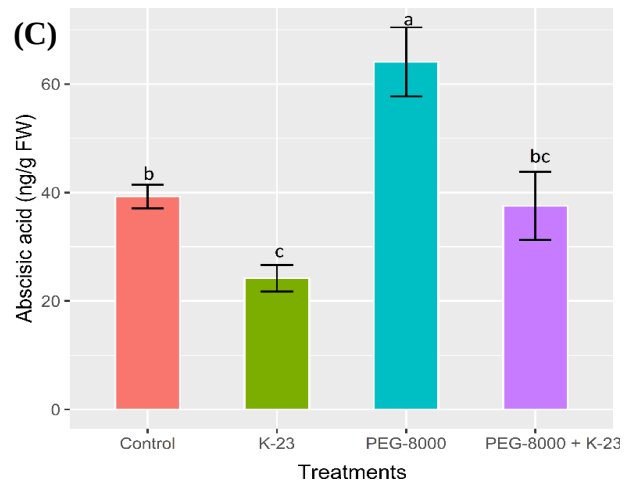
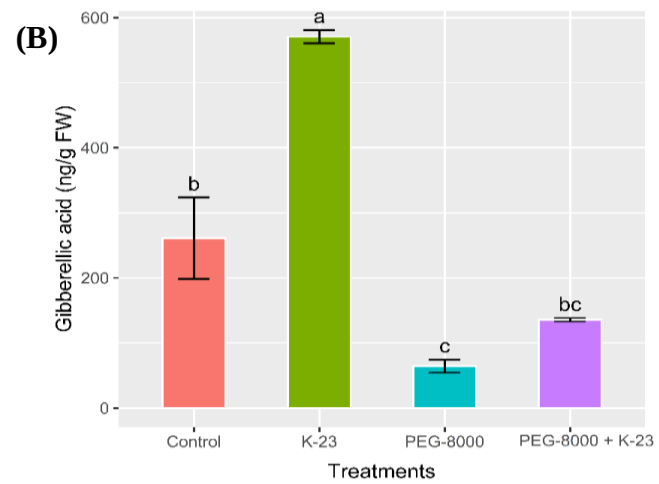
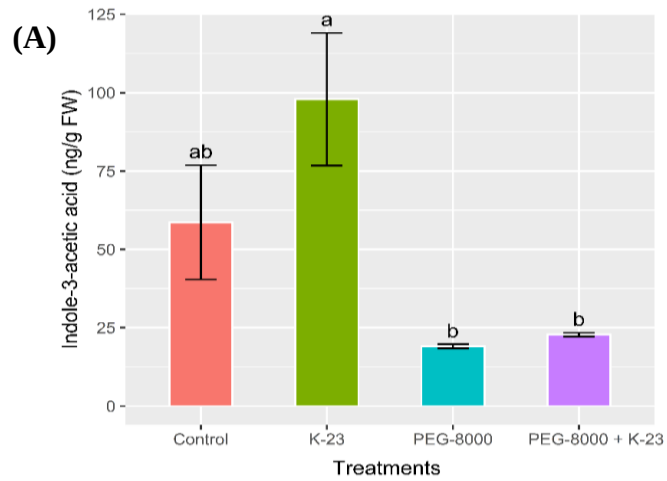


Figure 4.9: Effect of K-23 (*F. incarnatum*) on endogenous hormone levels of maize under PEG-8000 induced drought stress.

Note: (A) Indole-3-acetic acid, (B) Gibberellic acid, (C) Absciscic acid and (D) Salicylic acid

few years' endophytes have been gaining much importance due to their ability to modulate growth hormones. For instance, Zhao *et al.* (2021) has shown that transcriptome analysis of *Achnatherum inebrians* plants enriched with *Epichloe* endophyte have accumulated more amount of growth hormones such as GA and cytokinin. Mitogen-activated protein kinases are the signaling molecules that transduce and activate phosphorylation of downstream-regulated genes such as hormone biosynthesis and signaling genes (Lin *et al.*, 2021). K-23 endophyte has also been shown to upregulate several antioxidant genes such as glutathione, ascorbate. Endophytes enriched plants have been reported to enhance the antioxidants to reduce the amount of reactive oxygen molecules generation and thereby reduce oxidative stress damage (Hamilton *et al.*, 2012).

PEG-8000 + Endophyte (K23) and PEG-8000

When endophyte enriched and non-enriched seedlings were subjected to PEG stress, a total of 558 DEGs were expressed. Among them, 258 genes showed increased transcript abundance and 300 genes showed decreased transcript abundance (Figure 4.10.1).

Comparative gene ontology (GO) terms encoding cellular functioning followed by biological processes and molecular functioning processes were expressed. Within the GO term- 'Cellular function', most of the genes belonging to the protein-containing complex, organellar genes were expressed (Figure 4.10.2). Similarly, within the GO term- 'Biological components' various cellular metabolic processes were enriched (Figure 4.10.2).

Bubble plot analysis for the upregulated DEGs has shown that genes involved in photosynthesis, biosynthesis of secondary metabolites, metabolic pathways, carbon fixation and metabolism, starch and sucrose metabolism genes were enriched (Figure 4.10.3). Further to study the various pathway that is interacting, MapMan analysis was performed (Figure 4.10.5). Genes involved in the synthesis of light reaction pathways and maintenance of tetrapyrrole structure of chlorophyll, carbohydrate metabolisms, lipid synthesis, Krebs cycle, Nitrate metabolism were expressed.

K-23 endophyte has not only promoted seedling growth under control conditions but also has imparted drought stress tolerance to maize seedlings. RNA seq analysis of endophyte enriched and non-enriched maize seedling under drought stress has shown that genes involved in the catabolism of sucrose, starch were expressed. Sucrose and starch serve as precursor molecules of glucose to synthesize osmolytes such as trehalose (disaccharide) and mannitol (sugar alcohol). During stress conditions endophyte enriched plants tend to accumulate more amount of osmolyte and thereby reducing the water potential of the plants compared to its surrounding. Anzhi *et al.* (2004) have shown that endophyte enriched lolium plants were shown to have enhanced relative water content, soluble sugars, and starch content when compared to endophyte-free plants. Under drought stress, maize seedlings treated with *Pseudomonas putida* strain FBKV2 demonstrated upregulation of genes involved in β -alanine and choline biosynthesis, heat shock proteins, and late embryogenesis abundant (LEA) proteins, all of which could play a role in drought tolerance (SkZ *et al.*, 2018).

These results indicate that K-23 helps plants not only in basic growth promotion but also modulate their ability to combat drought stress in maize seedlings. Therefore, endophytes could serve as an alternative approach for imparting drought stress tolerance in the field level.

Salient findings from the research:

- Endophytes isolated from plants adapted to extreme habitats were not only able to colonize agriculturally important crops but also imparted growth promotion and drought tolerance. Out of eight fungal endophytes, three (K-23, LAS-6, and P-37) of them performed better by inducing growth promotion in maize (MAH 14-5).
- Selected three fungal endophytes (K-23, LAS-6, and P-37) were able to successfully colonize and promote maize growth even under field conditions.
- Endophyte (K-23) conferred drought tolerance to its host plant by the upregulation of stress-responsive genes and synthesis of phytohormones in addition to the production of antioxidant enzymes. These precise mechanisms through which such effects are manifested were highlighted by transcriptomics and phytohormone analysis.

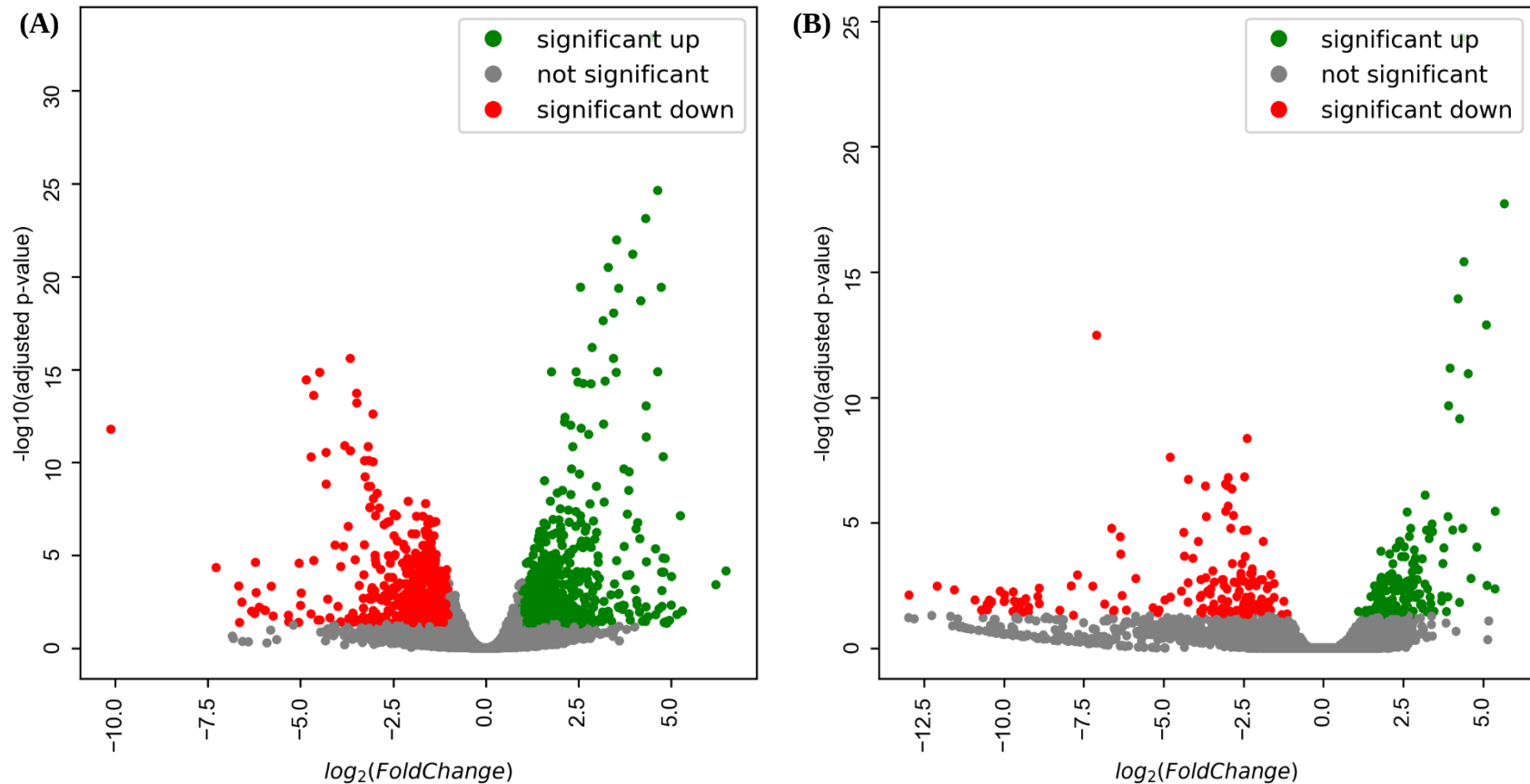


Figure 4.10.1: Volcano plot for differentially expressed genes, (A) K-23 (*F. incarnatum*) vs Control and (B) PEG-8000 + K-23 (*F. incarnatum*) vs PEG-8000.

The x-axis shows the $\log_2$ fold change in gene expression between different treatments, and the y-axis shows the statistical significance of the differences. Significantly up and downregulated genes are highlighted in green and red colors, respectively. Genes did not express differently between the treatment group and control group are in grey.

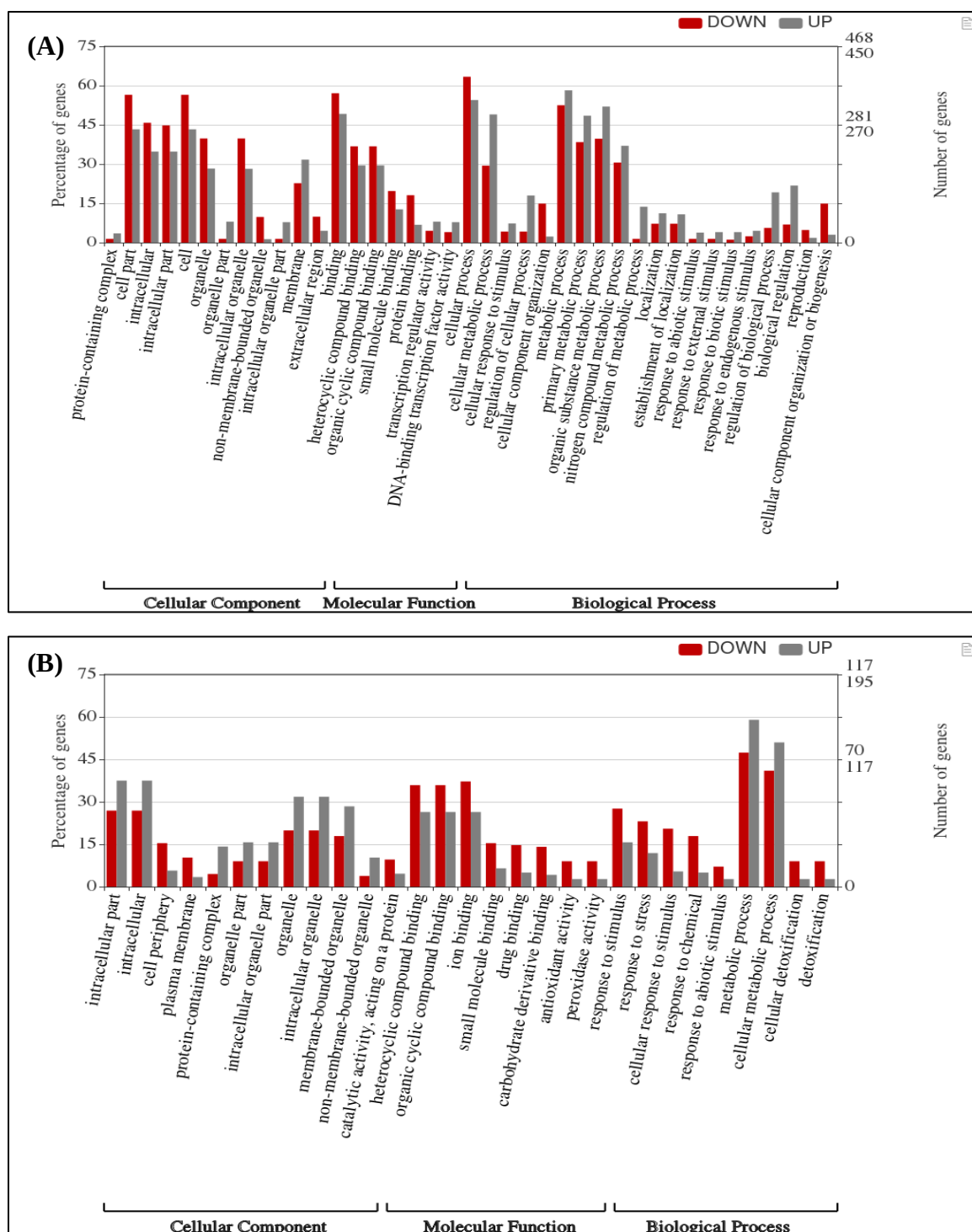


Figure 4.10.2: Gene ontology functional classification, (A) K-23 (*F. incarnatum*) vs Control and (B) PEG-8000 + K-23 (*F. incarnatum*) vs PEG-8000.

X-axis shows user selected GO terms; y-axis shows the percentages of genes (number of a particular gene divided by total gene number). Different groups are used to represent distinct biological process, cellular component, and molecular function.

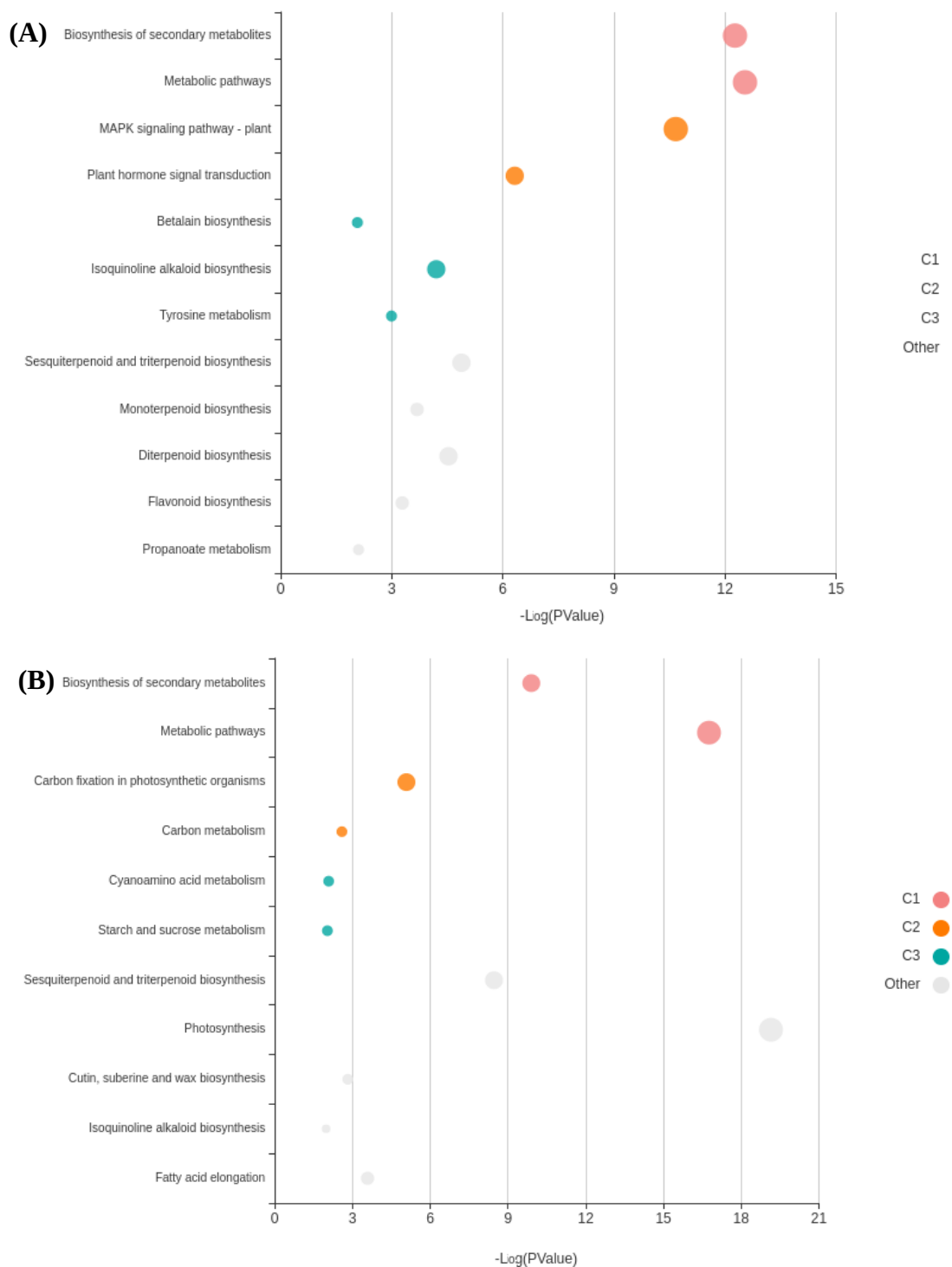


Figure 4.10.3: Bubble plot analysis of upregulated genes. (A) K-23 (*F. incarnatum*) vs Control and (B) PEG-8000 + K-23 (*F. incarnatum*) vs PEG-8000.

X-axis represent log P-values of differentially expressed genes and Y-axis shows enriched functional pathways. Each bubble represents the various pathways involved, and the size of the bubble represents the number of genes corresponds to that particular function. C1, C2 and C3 represents colour of the bubbles clustered on the basis of common function.

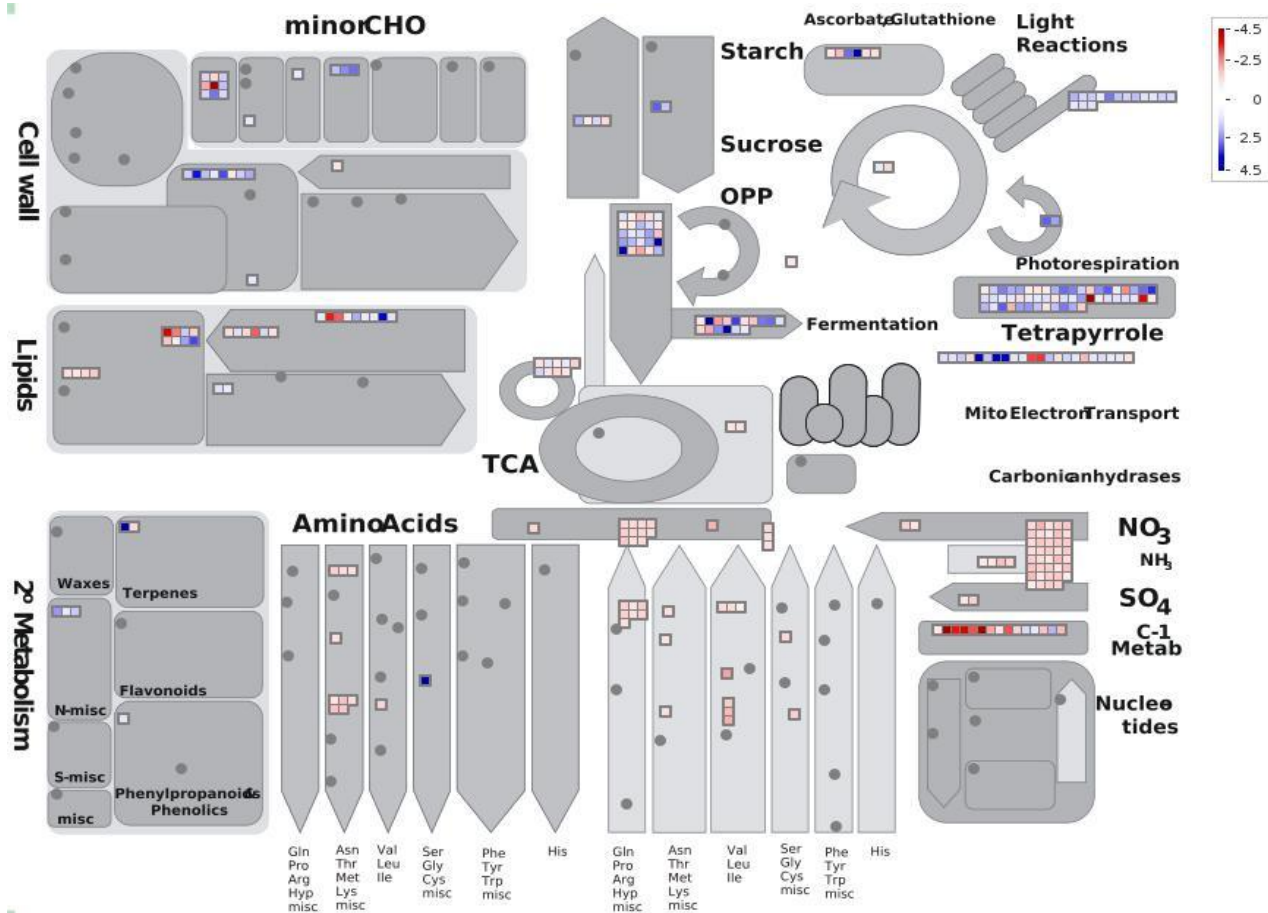


Figure 4.10.4: MapMan overview of differentially expressed transcripts involved in different metabolic processes under unstress condition in K-23 (*F. incarnatum*) treatment.

The images were obtained using MapMan software, showing different functional categories that passed the cutoff (less than 0.05 Q value and greater than 2-fold change) for differential expression. Red stands for downregulation, and blue stands for upregulation.

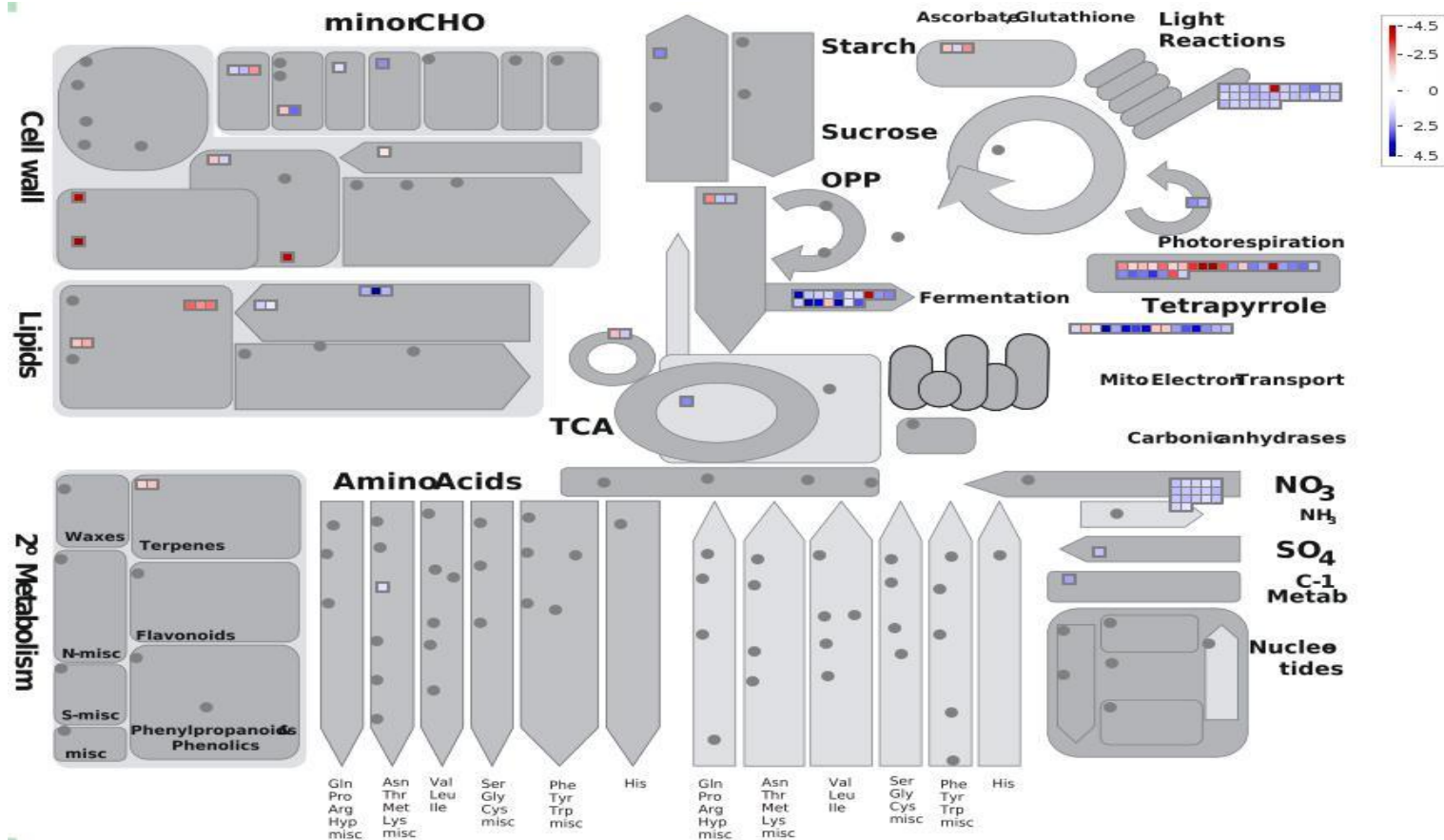


Figure 4.10.5: MapMan overview of differentially expressed transcripts involved in different metabolic processes under PEG-8000 induced drought stress condition in K-23 (*F. incarnatum*) treatment.

The images were obtained using MapMan software, showing different functional categories that passed the cutoff (less than 0.05 Q value and greater than 2-fold change) for differential expression. Red stands for downregulation, and blue stands for upregulation.

VI SUMMARY

Major cultivable crops like maize, green gram, soybean, and brinjal are rainfed, whose production decreases with increased drought stress. Plants in the course of evolution, due to their immobile nature have developed adaptive processes to some extent to mitigate drought stress conditions, however plants cells cannot maintain homeostasis due to the failure of metabolic pathways upon a certain threshold of stress factors (Tuzhikov *et al.*, 2011; Bostock *et al.*, 2014; Petrov *et al.*, 2015). In light of this, different measures are being adopted. A number of agronomically significant traits have been introduced by conventional breeding or through contemporary genetic engineering methods to make drought-tolerant plants. Although the role of plant breeders in combating food crises through developing improved and resistant crop varieties cannot be ignored. Recent advances in plant sciences suggest that microbial dynamic equilibrium with plant systems is vital for the germination, growth, and reproductive phases of the plant life cycle. In addition, increasing seed vigor, one of the primary factors that determine growth, development, and production, using beneficial endophytes, ought to be of paramount importance.

Whereas the use of microbes like fungal endophytes has been exploited in the recent past to mitigate different stresses that a plant is facing by providing drought tolerance and aid in nutrient availability and uptake (Lata *et al.*, 2018; Prajakta *et al.*, 2019). It is hypothesized that the plants adapted to extreme habitats harbor endophytes tolerant to high-stress conditions through symbiotic association. Such endophytes would help the plant confer drought stress tolerance in non-host plants in a habitat-specific manner. With this hypothesis, the present study explored to prospect the endophytic fungi from extreme habitat-adapted plants and evaluate their role in imparting drought stress tolerance in maize, green gram, soybean, and brinjal. It was intended to screen, identify and evaluate the selected endophytic fungi to mitigate drought stress in these crops. Below are the outcomes of the current study:

The Polyethylene glycol (MW-8000) induced screening for drought-sensitive genotypes of crops was carried out. Among two genotypes each of maize (MAH 14-5,

NAC-6002), green gram (BGS-9, KKM-3), soybean (JS-335, KBS-23), and brinjal (Arka Harshitha, Arka Unnathi), the genotypes MAH 14-5, BGS-9, KBS-23, and Arka Harshitha from each crop system showed significantly reduced growth than the other genotypes under drought stress conditions and -2.79 MPa, -2.57 MPa, -1.28 MPa, and -3.38 MPa of PEG-8000 was considered as the most differentiating (sub-lethal) concentrations for screening maize (MAH 14-5), green gram (BGS-9), soybean (KBS-23), and brinjal (Arka Harshitha) treated with fungal isolates against drought stress tolerance respectively.

All selected eight fungal endophytes were morphologically and molecularly characterized. Molecular characterization for the ITS region of rDNA was carried out. Where K-23, K-26, P-10, P-37, LAS-6 belonged to *Fusarium incarnatum*, *Fusarium redolens*, *Fusarium solani*, *Ulocladium* sp., *Chaetomium madrasense* respectively, and SF-5, PJ-9, V4-J belonged to *Fusarium equiseti*. Further screening of endophytes for plant growth-promoting attributes like phosphate solubilization, siderophore production, amylase activity, indole acetic acid (IAA) production was analyzed and confirmed that endophytes were showing positive reactions to certain activities tested.

Eight endophytes were screened for their ability to mitigate drought stress using maize, green gram, soybean, and brinjal. Of which three endophytes (K-23, LAS-6, and P-37) performed better in maize (MAH 14-5) by enhancing seedling length both under control and PEG-8000 induced drought stress (-2.79 MPa) conditions compared to other treatment combinations.

These three fungal endophytes were further evaluated under greenhouse conditions in maize (MAH 14-5). Three different levels of field capacity (100, 75, and 50 % FC) were imposed from 45 to 65 days after sowing (DAS) to measure the moisture stress gravimetrically. The growth parameters of maize were recorded before inducing drought stress at 30 DAS. In general, the endophytes treated plants showed increased plant height, stem girth, leaf area, root volume, plant dry biomass compared to untreated plants under 100% FC. In addition, the physiological parameters such as SCMR, net CO₂ assimilation rate, stomatal conductance, transpiration rate and total chlorophyll content were also significantly high in endophyte treated plants. Further, the growth and physiological

parameters were recorded after inducing drought stress at 45 and 65 DAS. Under drought stress conditions, the endophytes treated plants showed increased plant height, stem girth, leaf area, root volume, plant dry biomass compared to untreated plants under 100, 75, and 50 % FC. Further, endophytic fungi were able to maintain high SCMR, total chlorophyll, leaf water status indicating the essence of the endophytes for mitigating drought stress in maize crop. Endophytic fungi-treated plants maintained lower MDA content and had higher activity of ROS scavenging enzymes like catalase, polyphenol oxidase, and proline. The yield and yield-related parameters were increased in endophyte inoculated plants compared to uninoculated plants.

Under field experiment, three fungal endophytes K-23, LAS-6, and P-37 were evaluated for their ability to promote plant growth and development. Observations recorded at 30, 45 and 65 DAS showed that endophytes treatment significantly increased plant height, stem girth, leaf area, shoot biomass, and SCMR. Further endophytic fungi were able to maintain increased stomatal conductance, transpiration rate resulting in high assimilation rates in field conditions at 30DAS. The number of days taken for anthesis and silking were less in endophyte treated plants, whereas the endophyte inoculated plants had increased cob length, cob weight, number of kernels per row, kernel row length, all of which corresponded to the yield of the plant. These three fungal endophytes (K-23, LAS-6, and P-37) were reconfirmed their presence in the tissue of the inoculated plants by re-isolating on PDA medium and observing their morphological similarity under *in-vitro*, greenhouse, and field experiments.

In addition, best performing fungal endophytes K-23, LAS-6 and P-37 were again morphologically characterized through SEM and molecularly confirmed using large subunit (LSU) and partial β -tubulin II (*TUB2*) region. They found homology with *Fusarium incarnatum*, *Chaetomium madrasense*, and *Ulocladium sp.* respectively.

Hormone profiling revealed that the ABA content was reduced in endophyte (K-23) treated plants under stress. IAA, GA, and SA concentration increased under control and stress conditions in treatments with endophyte (K-23). These results suggest that the

endophyte K-23 served as a good candidate in ameliorating drought stress in the maize system.

RNA-Seq analysis of K-23 endophyte enriched maize seedlings have shown to upregulate secondary metabolite genes and hormones biosynthesis and MAPK signaling pathways that are involved in growth promotion were expressed. Similarly, when subjected to drought stress, endophyte enriched plants have upregulated the genes involved in conferring salt stress tolerance such as antioxidant pathway genes, starch, and sucrose biosynthesis genes and genes involved in maintaining the structural integrity of tetrapyrrole were involved.

The study has opened up a good possibility of using fungal endophytes for the improvement of plant growth under stressful and non-stress conditions. Endophyte enrichment can be used as a technology to mitigate drought stress.

Future line of work

- The possibility of microbial consortia using endophytes with beneficial microbes will be an added advantage for improved crop growth and yield.
- The molecular mechanism involved in drought tolerance of selected fungi must be explored by multi-omics approaches like genomics, proteomics, metabolomics, and phenomics.
- Genomics-assisted breeding using beneficial microbiomes (enhanced microbial activities and microbiome characteristics) can be exploited in the future for crop improvement.
- Whole-genome sequencing (WGS) can be used in future studies to reveal the molecular mechanism of the interaction between microbe and plant.

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APPENDIX I

List of primers used for molecular identification of fungal endophytes

- **Nucleotide sequence of ITS primers:**

ITS1(Internal transcribed spacer-forward): 5' TCCGTAGGTGAACCTGCGG 3'

ITS4 (Internal transcribed spacer-reverse): 5' TCCTCCGCTTATTGATATGC 3'

- **Nucleotide sequence of LSU primers:**

LROR (forward): 5'ACCCGCTGAACTTAAGC 3'

LR5 (reverse): 5'TCCTGAGGGAAACTTCG 3'

- **Nucleotide sequence of *TUB2* primers:**

Btub2Fd (forward): 5'GTBCACCTYCARACCGGYCARTG 3'

Btub4Rd (reverse): 5'CCRGAYTGRCCRAARACRAAGTTGTC 3'

APPENDIX II

Physical and chemical properties of the soil of the experimental site

Sl. No.	Particulars of analysis	Values	Method adopted
I	Physical properties		
1	Sand (%)	46.30	Hydrometer method (Piper, 2002)
2	Silt (%)	19.60	
3	Clay (%)	34.10	
4	Bulk density (mg m^{-3})	1.28	Clod Method (Piper, 2002)
II	Chemical properties		
1	pH (1:2.5)	6.3	pH meter method (Jackson, 1973)
2	Electrical conductivity (dS m^{-1})	0.32	Conductivity bridge (Jackson, 1973)
3	Organic carbon (g kg^{-1})	0.41	Wet oxidation method (Lindsay and Norvell, 1978)
4	Available nitrogen (kg ha^{-1})	143.46	Alkaline permanganate method (Subbaiah and Asija, 1956)
5	Available phosphorus (kg ha^{-1})	27.16	Olsen's method (Jackson, 1973)
6	Available potassium (kg ha^{-1})	374.16	Flame photometry (Jackson, 1973)
7	Available sulphur (kg ha^{-1})	26.94	Spectrophotometer (Jackson, 1973)
8	Available iron (mg kg^{-1})	3.18	Atomic absorption spectrophotometer method (Lindsay and Norvell, 1978)
9	Available copper (mg kg^{-1})	5.87	
10	Available manganese (mg kg^{-1})	8.76	
11	Available zinc (mg kg^{-1})	0.73	

APPENDIX III

Mean monthly actual meteorological data March 2021-June 2021 during crop growth period at NSP, GKVK, Bengaluru

Year	Month	Total Rainfall (mm)	Mean Relative humidity (%)	Mean Temperature (°C)		Sunshine (hours /day)	Open Pan Evaporation
				Maximum	Minimum		
2021	March	0.0	37.0	32.5	16.9	9.1	7.8
2021	April	67.0	38.0	33.5	19.8	7.4	6.8
2021	May	19.4	43.0	31.5	20.6	6.6	5.4
2021	June	117.6	53.0	29.2	19.5	5.2	4.5
Mean		51	42.75	31.67	19.2	7.07	6.12

Drought Tolerant Endophytic Fungi for Enhancing Early Seedling Growth under Stressful Conditions in Selected Crops

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ABSTRACT

Plants have adapted a successful functional symbiosis mechanism to overcome extreme environment and for continuation of life cycle. Endophyticism is one such symbiosis which is now gaining tremendous pace in sustainable agricultural research strategy as an alternative for chemical free agriculture. An attempt was made to evaluate the potential of endophytes in alleviating drought stress and promoting early seedling growth in green gram, soybean and brinjal. Initially, endophytic fungal isolates were assayed for their ability to grow on matrix modified media (PEG-8000) at ca. 0 MPa, -0.47 MPa, -1.45 MPa, -2.93 MPa, -4.92 MPa, -7.41 MPa. The nature of the response was revealed to be positive, as biomass(g) of endophytes was in proportional to induced drought stress. Then, the seedling growth of all crop systems was gauged in above mentioned PEG-8000 concentrations and LC₅₀ (reduction in seedling length) was computed to be -2.57 MPa, -1.28 MPa, -3.38 MPa of PEG-8000 for green gram (BGS 9), soybean (KBS 23) and brinjal (Arka Harshitha), respectively. By reckoning LC₅₀, fungal endophytes were screened for growth promotion and drought stress alleviation in above-mentioned crop systems. Green gram (BGS 9) enriched with *Fusarium* sp. (SF-5, K-26, V4-J), *Chaetomium* sp. (LAS 6) and soybean (KBS 23) enriched with *Fusarium* sp. (V4-J) significantly enhanced seedling growth under normal as well as PEG-8000 induced drought stress condition. In brinjal (Arka Harshitha), there was no significant difference between endophyte treated and untreated seedlings. Our results suggest that host responses to colonization were variable and depended on the host ecotype which is controlled by variability in the host and endophyte genotypes.

Keywords : Endophytes, PEG-8000, Drought stress, Seedling growth

PLANT-microbe interactions are among the vital processes that are not only essential for the survival of both the partners but also important in the functioning of the agricultural system. Among the plant interacting microbes, endophytic fungi have gained marvelous attention of plant biologists due to their mutualistic interactions with the host crops (Miransari, 2011). Whereas, drought stress is among the most destructive abiotic stresses that increased in intensity over the past decades and is expected to cause serious problems in more than 50 per cent of the arable lands by 2050 (Kasim *et al.*, 2013). Plants perceive and respond to stress. Along with plants, the endophytes also perceive signals and transduce, which later leads to the expression of genes encoding proteins that are involved in providing drought tolerance. Several

mechanisms have been proposed for endophytes-mediated drought stress tolerance in plants (Estrada *et al.*, 2013), some of them include phytohormonal activity, synthesis of volatile compounds, antioxidant defense, accumulation of osmolytes and regulation of diverse types of genes and alteration in root morphology (Fig. 1). The term Induced Systemic Tolerance (IST) has been coined to accommodate the microbial-induced physical and chemical changes in plants, which result in enhanced tolerance to abiotic stresses (Yang *et al.*, 2009). An amalgamation of seed priming with the application of plant beneficial fungi and bacteria can significantly improve seed germination and emergence, seedling establishment, crop growth and yield parameters under normal and stress conditions (Prasad *et al.*,

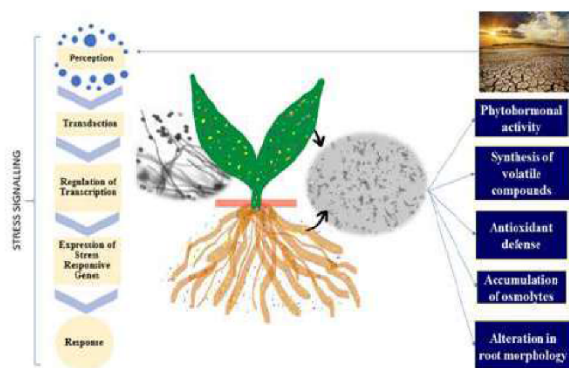


Fig. 1: Portraying potential mechanisms of endophytes in drought stress mitigation

2020). In this framework, the study was aimed to assess whether drought-tolerant endophytes promote seedling growth under normal and drought stress conditions in diverse crop systems like soybean, green gram and brinjal.

MATERIAL AND METHODS

The study was conducted at the Department of Seed Science and Technology, University of Agricultural Sciences, GKVK, Bengaluru, Karnataka.

Source of Endophytic Fungi

In a previous study, endophytes-maintained in School of Ecology and Conservation Laboratory, Department of Crop Physiology, University of Agricultural Sciences (UAS-B), Gandhi Krishi Vignana Kendra (GKVK), Bengaluru have been partially characterized for their ability to impart abiotic stress tolerance. Eight fungal endophytes isolated (Table 1) were selected for the study and rejuvenated on fresh Potato dextrose agar medium.

Pure Culture Response of Fungal Endophytes to Matric induced Water Stress

The experiment was used as the first conformational test in order to verify that the selected endophytes are able to grow in drought stress condition. PDA agar plug to PD amended broth is defined as the primary experiment. The combination reduces the impact of osmotic potential by utilizing chemically defined media

and provides time for fungi to adjust to the environment. One 5 mm agar plugs containing actively growing mycelium were placed into an autoclaved 250 ml Erlenmeyer flask that contained 100 ml of Potato Dextrose Broth and the targeted amount of Polyethylene glycol (PEG-8000) according to the protocol of Michel (1983): $\Psi = 1.22 [\text{PEG}]^2 T - 134 [\text{PEG}]^2 - 4.4 [\text{PEG}]$. Each isolate was assayed at control (PDB without PEG), -0.47 MPa, -1.45 MPa, -2.93 MPa, -4.92 MPa, -7.41 MPa. The Erlenmeyer flask was covered with sterilized tin foil, sealed with Parafilm, placed in a shaker chamber (90 rpm at 27 °C) for seven days. Whatman filter paper was weighed to four decimal places and mycelium was filtered from the broth. The filter paper was rinsed with distilled water to remove any residual broth. The washed filter paper with mycelium was then placed in an oven for 12 hours, prior to obtaining the final weight. Reported fungal growth weight is the final weight of the mycelium plus filter paper minus the original weight of the filter paper. Growth types were assessed as outlined in Hutton *et al.* (1996).

In Vitro Evaluation of Green gram, Soybean and Brinjal Genotypes for Drought Stress

The seeds of drought-sensitive green gram [*Vigna radiata* (L.) Wilczek] genotype (BGS 9), soybean [*Glycine max* (L.) Merrill] genotype (KBS 23) and brinjal (*Solanum melongena* L.) genotype (Arka Harshitha) were collected and surface-sterilized by following the standard protocol (Arnold *et al.*, 2000). The method followed for standardization was the paper towel method with four replications for each treatment. Different levels of osmotic stress (-0.47 MPa, -1.45 MPa, -2.93 MPa, -4.92 MPa, -7.41 MPa) was created using different concentrations of polyethylene glycol (PEG-8000). Paper towel was soaked in the solution, excess solution was removed before exposing the seeds. Twenty-five seeds of each genotype were placed on the paper towel and incubated at 27 °C in the growth chamber. Seedling root and shoot length were recorded at the final count of the respective crop (Muddarsu and Manivannan, 2017). LC_{50} value was calculated by Probit analysis using statistical software

IBM SPSS statistics 20 (<https://www.ibm.com/in-en/analytics/spss-statistics-software>).

Screening of Fungal Endophytes for Drought Stress Tolerance under *in-vitro* Conditions

Seeds of drought-sensitive genotypes, BGS 9, KBS 23, Arka Harshitha of green gram, soybean and brinjal, respectively were surface sterilized as described previously and pre-germinated on sterile moist blotters. Five-day-old fungal colony culture was used for inoculum preparation and uniform pre-germinated seeds were treated with mycelial suspension (10^6 spores / ml) for 3 hrs (Zhang *et al.*, 2014). The concentration of mycelial suspension was calculated using hemocytometer. The corresponding control was treated with sterile distilled water. Then pre-germinated seeds were subjected to drought stress by placing them on sterile paper towel amended with PEG-8000 (at LC_{50} dose) with the corresponding control. The seedlings were incubated at 27 °C in the growth chamber. Four replications for each treatment was maintained and each replication comprised of twenty-five seedlings. Root and shoot lengths were recorded on the final count after incubation (Sangamesh *et al.*, 2017). The colonization ability of endophytes in treated seedlings was determined by re-isolation of the endophytes by preparing small segments of the shoot and root tissue using the standardized protocol (Schulz *et al.*, 1993). The segments were surface sterilized as described above and inoculated on PDA and incubated at 28 ± 2 °C for

five days. The fungal colonies emerging out of the tissue segments were identified based on the morphological characteristics of the mother culture. The treatment details are, T_1 (Control), T_2 (Endophytes), T_3 (PEG-8000) and T_4 (PEG-8000 + Endophytes).

RESULTS AND DISCUSSION

Pure Culture Response of Fungal Endophytes to Matric Induced Water Stress

Several methods utilizing PEG exist for evaluating the response of fungi to matric-induced water stress. Polyethylene glycol has been reported to be nontoxic and not metabolized by fungi (Mexal & Reid, 1973). All fungal endophytes (Table 1) increased biomass(g) at -7.41 MPa compared to control (without PEG-8000) which demonstrates a Type III growth response and significant change was in the increase of over-all growth, as determined by weight (Fig. 2). To overcome water stress, most fungi produce compatible solutes as compounds that are able to change in concentration in the cell in response to a change in external water potential, thus maintaining turgidity while having no significant effect on enzyme activity (Jennings and Burke, 1990). Hutton *et al.* (1996) examined PEG-induced, matric water stress tolerance (-0.16 MPa to -2.96 MPa) for ericoid endophytes of Epacridaceae hosts and described three growth response types: Type I: overall minimal growth after five weeks, Type II: maximum growth at

TABLE 1
Sites all over India from where plants were sampled for endophytic fungal isolation

Sample Code	Location	Latitude (°N)	Longitude (°E)	Altitude (MSL)	Plant part/ Host
K-23	Kargil (J&K)	34°34'22"	76°7' 57"	2750	Leaf
K-26	Kargil (J&K)	34°34'22"	76°7' 57"	2750	Leaf
P-10	Pangong Tso	33°43' 2.74"	78°53' 29.08"	4250	Stem
P-37	Pangong Tso	33°43' 2.74"	78°53' 29.08"	4250	-
SF-5	Tamil Nadu	11.1271° N	78.6569° E	-	<i>Suaeda filiformis</i>
PJ-9	Karnataka	15.3173° N	75.7139° E	-	<i>Protis juliflora</i>
V4-J	Pokkali	9.9667° N	76.3168° E	-	Paddy (<i>Vytilla-4</i>)
LAS-6	Thar desert	27.4695° N	70.6217° E	-	<i>Lasiurus scindicus</i>

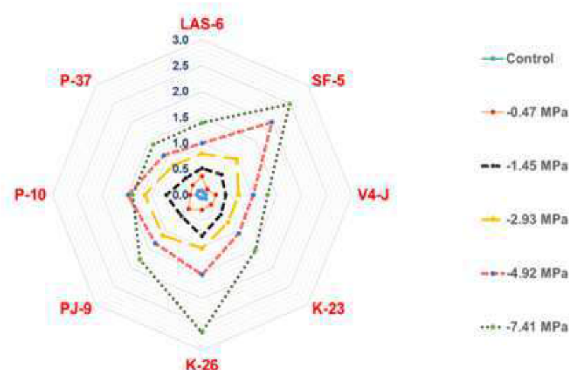


Fig. 2: Growth response of fungal endophytes to different matrix induced water stress (PEG-8000) at 7 days on shake culture. Inoculum on PDA agar transferred to broth.

control with decreased growth as matrix water stress increased and Type III: maximum growth under least degree of matrix water stress. Chen *et al.* (2003) examined ericoid mycorrhizal (ERM) fungi for PEG induced water stress tolerance (-0.05 MPa to -2.24 MPa) and concluded that ERM endophytes function under water stress conditions. In axenic culture, the endophytic fungi from four saline habitat adapted plants showed tolerance to NaCl concentrations as high as 1.0 M. These results suggest that endophytes may also acquire tolerance to abiotic stresses, just as expressed by their respective host plants (Manasa *et al.*, 2015).

In-vitro Evaluation of Green gram, Soybean and Brinjal Genotypes under PEG-Induced Stress

Polyethylene glycol, a non-toxic osmotic agent, is widely used to induce osmotic stress which reduces water potential. In the present study, in all three

crops tested, the seedling growth was significantly affected as PEG-8000 concentration increased (Fig. 3). Results were expressed as absolute growth under different levels of drought stress along with control. The analysis indicated that osmotic potential of -2.57 MPa, -1.28 MPa and -3.38 MPa was the most differentiating concentration for assessing the tolerance levels in green gram (BGS 9), soybean (KBS 23) and brinjal (Arka Harshitha), respectively. The stress level was considered as sub-lethal concentrations to evaluate seedlings treated with different fungal isolates for stress tolerance. PEG induces artificial water deficit which affects water uptake, decreases turgor pressure and reduces cell division that eventually causes reduction in shoot and root growth (Khakwani *et al.*, 2011).

Evaluation of Endophytic Fungal Isolates for Early Seedling Growth under Normal and Drought Stress Conditions in Crop System

All eight fungal isolates obtained from the culture collections were used to evaluate their ability to modulate early seedling growth in normal and drought stress conditions in crop systems such as green gram (BGS 9), soybean (KBS 23) and brinjal (Arka Harshitha). Green gram enriched with *Fusarium* sp. (SF-5, K-26, V4-J), *Chaetomium* sp. (LAS-6) and soybean enriched with *Fusarium* sp. (V4-J) significantly enhanced seedling growth under normal as well as PEG-8000 induced drought stress conditions. Yet in brinjal, there was no significant difference between endophytes treated and untreated seedlings (Table 2 and 3). Each endophytic isolate behaved differently in each crop system studied. This

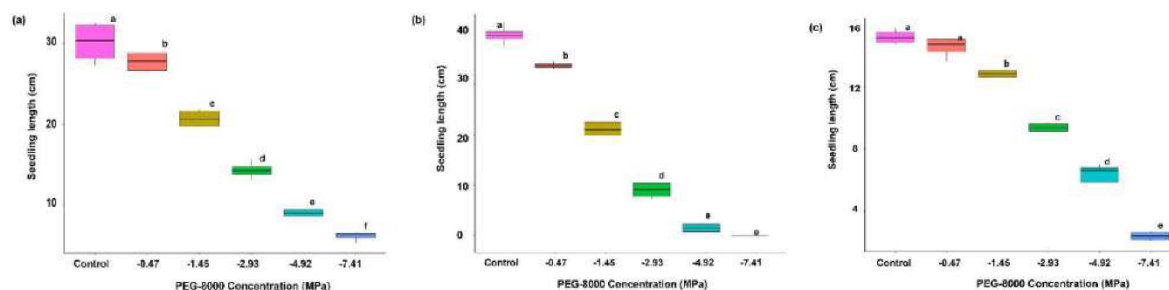


Fig. 3: Effect of PEG-8000 concentration on seedling length (cm) of (a) Green gram (BGS 9), (b) Soybean (KBS 23), (c) Brinjal (Arka Harshitha). Different letters indicate significantly different values ($P < 0.05$)

TABLE 2
Effect of endophytic fungi on seedling length of green gram, soybean and brinjal under normal condition

Treatments	Normal condition [Seedling length (cm)]		
	Green gram (BGS 9)	Soybean (KBS 23)	Brinjal (Arka Harshitha)
Control	22.86 ± 0.26 ^c	34.01 ± 0.27 ^b	11.56 ± 0.13
LAS-6	25.57 ± 0.28 ^a	32.23 ± 0.31 ^c	11.49 ± 0.12
SF-5	25.59 ± 0.31 ^a	34.22 ± 0.30 ^b	11.41 ± 0.14
K-26	25.48 ± 0.37 ^a	34.62 ± 0.39 ^b	11.66 ± 0.16
PJ-9	22.33 ± 0.30 ^c	30.13 ± 0.31 ^d	11.73 ± 0.14
V4-J	26.06 ± 0.29 ^a	35.92 ± 0.31 ^a	11.83 ± 0.16
K-23	22.54 ± 0.31 ^c	34.59 ± 0.31 ^b	11.89 ± 0.18
P-10	23.88 ± 0.32 ^b	28.99 ± 0.31 ^e	11.71 ± 0.13
P-37	22.93 ± 0.28 ^c	34.79 ± 0.38 ^b	11.88 ± 0.18
F-Test (p=0.05)	*	*	NS
C.D(0.05)	0.84	0.89	-
S.Em±	0.3	0.32	0.15

Note: Means with same superscript in a column do not differ significantly as per Duncan Multiple Range Test (DMRT).
Values represented are mean ± SE (n=40).

TABLE 3
Effect of endophytic fungi on seedling growth of green gram, soybean and brinjal under drought stress condition

Treatments	Drought stress condition [Seedling length (cm)]		
	Green gram (BGS 9)	Soybean (KBS 23)	Brinjal (Arka Harshitha)
PEG-8000	11.89 ± 0.16 ^d	14.08 ± 0.11 ^c	6.12 ± 0.08
PEG-8000 + LAS-6	13.85 ± 0.17 ^b	14.94 ± 0.19 ^b	6.10 ± 0.09
PEG-8000 + SF-5	13.81 ± 0.15 ^b	14.33 ± 0.13 ^c	5.98 ± 0.07
PEG-8000 + K-26	13.01 ± 0.17 ^c	14.22 ± 0.07 ^c	6.17 ± 0.08
PEG-8000 + PJ-9	10.80 ± 0.14 ^e	14.01 ± 0.10 ^c	6.19 ± 0.08
PEG-8000 + V4-J	15.60 ± 0.16 ^a	16.05 ± 0.10 ^a	6.01 ± 0.09
PEG-8000 + K-23	11.08 ± 0.15 ^e	16.03 ± 0.08 ^a	6.07 ± 0.09
PEG-8000 + P-10	12.08 ± 0.15 ^d	13.44 ± 0.14 ^d	6.09 ± 0.09
PEG-8000 + P-37	11.96 ± 0.15 ^d	15.09 ± 0.10 ^b	6.08 ± 0.09
F-Test (p=0.05)	*	*	NS
C.D(0.05)	0.43	0.33	-
S.Em±	0.16	0.11	0.08

Note: Means with same superscript in a column do not differ significantly as per Duncan Multiple Range Test (DMRT).
Values represented are mean ± SE (n=40)

could be due to the differences in plant-endophyte interactions and each host system may have unique signaling molecules or mechanisms. In some cases, endophytic fungal enrichment resulted in a lower magnitude of growth. Reduction in growth in response to endophytic colonization can be seen as weak parasitism (Mandyam *et al.*, 2013) or as induction of host resistance resulting in the allocation of carbon to the production of expensive defense compounds rather than to vegetative growth (Aime *et al.*, 2013). Positive effects could be due to the production of plant growth hormones (You *et al.*, 2013) by the endophytes. Obledo *et al.* (2003) isolated *Fusarium oxysporum* from *Agave- victoria reginae* Moore. *In vitro* raised *Agave- victoria reginae* inoculated with the *F. oxysporum* showed a higher photosynthetic efficiency compared to uninoculated plants. Khan *et al.* (2015) isolated endophytic fungi *Fusarium tricinctum* RSF-4L from leaves of *Solanum nigrum*. The culture filtrate of the above fungus enhanced the growth attributes of Dongjin rice plants. Subsequently, gas chromatography / mass spectrometry analyses revealed that RSF-4L produced indole acetic acid. This indicates that endophytes synthesize bioactive compounds that could play important roles in promoting plant growth. Recent studies have demonstrated that certain endophytes promote host plant growth through the synthesis of phytohormones such as indole-3-acetic acid, gibberellins and cytokinins (You *et al.*, 2013 and Khan *et al.*, 2017).

The study demonstrates the importance of endophytes from plants that are adapted to extreme habitats and their importance towards the improvement of crop growth in stressed conditions. It would be interesting to examine the underlying physiological and molecular basis of such cross-adaptations to make the endophyte-based strategy more robust as an alternative to conventional crop improvement programs.

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