Developing a quantum dot nanotracker for endophytic bacteria translocation in plants

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A mini-thesis submitted in partial fulfilment of the requirements for the degree of Magister Scientiae in the Department of Biotechnology,

University of the Western Cape.

December 2017

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ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisor **Dr. Marshall Keyster** for his scientific expertise and guidance for this project and for making me a part of his laboratory. I am thankful for the opportunity he has given me to develop myself as a scientist.

I would also like to express my gratitude to my brilliant colleagues **Dr. Arun Gokul, Junaid Mia** and **Ruo Mou Wu** for transferring their scientific expertise to me. Thanks to my amazing colleague and friend **Zaahira Omar** for her insight, patience, guidance and willingness to assist and most importantly for being alongside me through this chapter of my life. A special thanks to **Lauren Swartz** and **Shannen Keyser** for assisting me with the florescence microscope.

I would like to convey my gratitude to the EBL laboratory for always assisting when needed and making me feel at home. My gratitude also extends to **Prof Onani** and **Zuraan Paulse** for assisting me in the chemical aspect of my project and to all who was involved in the Nanoscience Program. I am extremely grateful for being a part of such a prestige program and a special thanks to **Miss Valencia** for always being my go to person.

I would like to thank my family and friends for their support and love especially **Nuha Haroun** and **Aa'ishah Brenner**. Thank you to my parents **Tabassum Fakie** and **Zakwaan Essop** for allowing me the opportunity to follow my dreams. And to my loving boyfriend **Fuad Johnstone** for being my strength and motivation through it all, I am forever grateful for your endless love. I would also like to praise the **Almighty** for without Him, none of this would be possible.

Finally I would like to thank **DST** for the funding that was granted to me thus allowing me to further pursue my academic career.

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
Ca	Calcium
Cd	Cadmium
CdTe	Cadmium Telluride
CQD	Carbon Quantum dots
Cu	Copper
dH_2O	Distilled water
Fe	Iron
GFP	Green fluorescent protein
IAA	Indole-3-acetic acid
ICP-OES	Inductively coupled plasma optical emission spectrometry
K	Potassium
LB	Luria Broth
Mg	Magnesium
Mn	Manganese
Mo	Molybdenum
MPA	Mercaptopropionic acid
Ni	Nickle
nm	Nanometer
OD	Optical density
P	Phosphorous
PBS	Phosphate buffered saline
PGPB	Plant growth promoting bacteria
PGPR	Plant growth promoting rhizobacteria
PL	Photo luminesces
QDs	Quantum dots
TEM	Transmission electron microscopy
UV-Vis	Ultraviolet visible spectroscopy
Zn	Zinc

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KEYWORDS

Endophytes

Quantum dots

Nanotechnology

Brassica napus L

Translocation

CdTe QDs

Indole Acetic Acid

Fluorescence

Bioimaging

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ABSTRACT

Endophytes are bacteria that have been shown in previous studies to promote plant growth without eliciting any sign of infection or plant disease, as well as promote bio-fortification and has thus gained much attention in agriculture. However, due to the limited confirmatory visual evidence of the *in planta* presence of bacteria, the significance of this plant-endophyte interaction has not been comprehensively understood. The aim of this study was to synthesise quantum dots to track the translocation of endophytes in a plant. Conventional methods track endophytes by transformation of green fluorescent protein which has the problem of fluorophore bleaching and blinking reducing detectability. Quantum dots (QDs) offer distinct advantages over organic dyes and fluorescent proteins for biological imaging applications because of their brightness, photostability, tunability and their extremely small size ensures little to no interference with the normal functions of the bacterium. This project required the synthesis of cadmium telluride QDs to be capped with mercaptopropionic acid thus making them biocompatible and the subsequent characterization techniques were performed. CdTe and Carbon QDs were transformed into the endophytic bacteria and were thereafter used to treat *Brassica napus* L plants. Physiological and biochemical assays were done to detect the effect of QDs on the plant and bacteria, respectively. Furthermore, ICP-MS was used to determine the concentration of macro and micronutrients within different organs of each plant, and the relative uptake and translocation of the endophytes were imaged using the IVIS Lumina II imager by tracking QDs taken up by the endophyte. Results obtained in this study showed successful transformation of endophytic bacteria with QDs in a dose dependant manner and the cellular uptake increased as the QD concentration increased. The growth of the endophytic bacteria was slightly inhibited but not arrested by the presence of QDs and deduced that QDs could be used for tracking purposes. Biochemical activities tested suggest that the QDs at low concentrations had no detrimental effects on the bacteria and at certain concentrations elevated both IAA production and phosphate solubilization. This coincided with the plant morphology and biomass when treated with transformed QD endophytic bacteria. Thus these results confirm the use of QDs as a tracking tool for endophytic bacteria. The IVIS Lumina bioimaging system revealed successful translocation of endophytes in the roots, stems and leaves using QDs as the fluorescent

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signal. Thus this study concludes that QDs can be used as an effective and efficient tool as a nanotracker to detect the translocation of endophytic bacteria, and possibly other bacteria, within plants.

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CHAPTER 1

Literature Review

1.1. Introduction

In 2004 it was predicted that in approximately 50 years, the human population is to increase by 50%, before reaching a maximum of 9 to 10 billion (United Nations, 2004). However, much more than a 50% increase in food production will be required to meet the needs and expectations of this population for two principal reasons (Rothstein, 2007). Firstly, there's an increase in consumption of more animal products which is increasing the demand for the primary grain and oil seed crops. Secondly, the use of crops for industrial products, particularly ethanol from starch, to meet increasing energy demand is also growing at a rapid pace. Crop yields are further impacted by climate change and the diseases that affects them (Barbetti *et al*., 2012). The demand for food, feed, and feedstocks for bioenergy and biofactory plants will increase proportionally due to population growth, prosperity, and bioeconomic growth. It will be necessary to raise biomass production and economic yield per unit of land, not only under optimum growing conditions, but even more under conditions constrained by climate, water availability and soil quality. Globally, food demand will increase by 50% and the area of cultivated land by 10% by 2030 (Spiertz, 2013). Adaptive and proactive food systems are needed with cross-level, cross-scale, and cross-sector investments and the use of frontier technologies to attain food security. Numerous authors have suggested that increasing crop yields, rather than clearing more land for food production, is the most sustainable path for food security according to a review done by Ray *et al.* (2013). Moreover, crop yield growth has been shown as an effective tool in reducing global poverty and undernourishment, as farmers themselves constitute the vast majority of the poor and the undernourished (Ray *et al*., 2013). It is necessary to maintain that high productivity however, it is important to alter as little as possible to the environment. Clearly there is a need to head for more environmentally sustainable agricultural practises while maintaining ecosystems and biodiversity. One potential way to decrease negative environmental impact resulting from continued use of chemical fertilizers, herbicides and pesticides is the use of plant growth-promoting rhizobacteria (PGPR) (Pérez-Montano *et al*., 2014).

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1.2. Endophytes

Endophytes are conventionally defined as bacterial or fungal microorganisms that colonize healthy plant tissue intercellularly and/or intracellularly without causing any apparent symptoms of disease (Gaiero *et al*., 2013). They are ubiquitous, colonize all plants, and have been isolated from almost all plants examined thus far. Their association can be obligative or facultative and causes no harm to their host plants (Nair and Padmavathy, 2014). Plants strictly limit the growth of endophytes and these endophytes use many mechanisms to gradually adapt to their living environments. In order to maintain stable symbiosis, endophytes produce several compounds that promote growth of plants and help them adapt better to the environment. Endophytic bacteria have been implicated in supplying biologically fixed nitrogen in non-legumes, and these associations can increase the nitrogen economy of a crop, thus reducing the requirement for nitrogen fertilizers (Pérez-Montano *et al*., 2014). Bacterial endophytes have also been shown to prevent disease development through endophyte-mediated *de novo* synthesis of compounds and fungitoxic metabolites (Pérez-Montano *et al*., 2014). Benefits to plants from host–PGPR interactions have been shown to include plant health and growth, suppress disease-causing microbes and accelerate nutrient availability and assimilation (Gaiero *et al.,* 2013; Nair and Padmavathy, 2014; Pérez-Montano *et al*., 2014).

Plant-growth-promoting bacterial endophytes (PGPBEs) facilitate plant growth via three interrelated mechanisms namely phytostimulation, biofertilization, and biocontrol (Gaiero *et al.,* 2014). Phytostimulation is the direct promotion of plant growth through the production of phytohormones. Endophytic bacteria produces a wide range of phytohormones such as auxins, cytokines and gibberellic acids. Plants require sixteen essential elements including carbon, hydrogen, nitrogen, oxygen and phosphorus. These essential elements are available to plants for their growth and development in chemical form, which they obtain from the atmosphere, soil, water and organic matter. Endophytes also play an important role in the uptake of these nutrients. They elicit different modes of action in *Festuca arundinacea* adaptation to phosphorous deficiency and induce increased uptake of nitrogen. The promotion of plant growth by increasing the accessibility or supply of major nutrients is termed biofertilization (Nair and Padmavathy, 2014). A well-studied form of biofertilization

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is nitrogen fixation, which is the conversion of atmospheric nitrogen to ammonia. The promotion of plant growth through protection from phytopathogens is known as biocontrol. Several mechanisms may be involved, including the production of siderophores or antibiotics (Gaiero *et al.,* 2014). These different modes of plant growth promotion by endophytic bacteria can be seen in Figure 1.

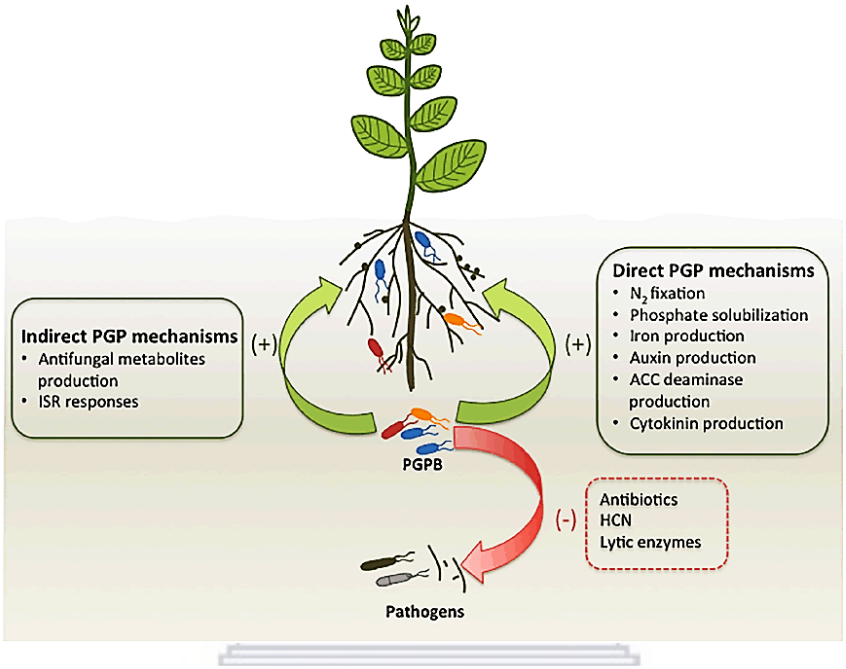

Figure 1. PGPB's direct and indirect mechanisms for plant growth promotion adapted from (Premachandra *et al*. 2016).

Agricultural production currently depends on the large-scale use of chemical fertilizers. These fertilizers have become essential components of modern agriculture because they provide essential plant nutrients such as nitrogen, phosphorus and potassium. However, the overuse of fertilizers can cause unanticipated negative environmental impacts (De Souza *et al.,* 2015). To achieve maximum benefits in terms of fertilizer savings and better growth, the PGPB-based inoculation technology should be utilized along with appropriate levels of fertilization. Moreover, the use of efficient inoculants can be considered an important strategy for sustainable management and for reducing environmental problems by decreasing the use of chemical fertilizers. The success and efficiency of PGPB as inoculants for agricultural crops are influenced by various factors, among them is the ability of these bacteria to colonize plant roots, the exudation by plant roots and the soil health.

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1.3. Endophyte colonization

Endophytes reside entirely within plant tissues and may grow within roots, stems and/or leaves, emerging to sporulate at plant or host-tissue senescence (Hardoim *et al.,* 2015). In general, two major groups of endophytic fungi have been recognized previously, reflecting differences in evolutionary relatedness, taxonomy, plant hosts, and ecological functions as seen in Table 1. The clavicipitaceous endophytes (C-endophytes), which infect some grasses; and the non-clavicipitaceous endophytes (NC-endophytes), which can be recovered from asymptomatic tissues of nonvascular plants, ferns and allies, conifers and angiosperms (Rodrigues *et al.,* 2009). Successful colonization by endophytes depends on many variables, including plant tissue type, plant genotype, the microbial taxon and strain type, and biotic and abiotic environmental conditions. Different colonization strategies have been described for clavicipitaceous and non-clavicipitaceous endophytes.

Table 1. Symbiotic criteria used to characterize fungal endophytic classes adapted from (Rodriques *et al.,* 2009)

	Clavicipitaceous	Non-clavicipitaceous		
Criteria	Class 1	Class 2	Class 3	Class 4
Host range	Narrow	Broad	Broad	Broad
Tissue(s) colonized	Shoot and rhizome	Shoot, root and rhizome	Shoot	Root
In planta colonization	Extensive	Extensive	Limited	Extensive
In planta biodiversity	Low	Low	High	Unknown
Transmission	Vertical and horizontal	Vertical and horizontal	Horizontal	Horizontal
Fitness benefits*	NHA	NHA and HA	NHA	NHA

*Nonhabitat-adapted (NHA) benefits such as drought tolerance and growth enhancement are common among endophytes regardless of the habitat of origin. Habitat-adapted (HA) benefits result from habitat-specific selective pressures such as pH, temperature and salinity.

Endophyte distribution within plants depends on a combination of its ability to colonize and the allocation of plant resources. Root endophytes often colonize and penetrate the epidermis at sites of lateral root emergence, below the root hair zone, and in root cracks (Gaiero *et al.,* 2014). These colonizers are capable of establishing populations both inter- and intracellularly. After initial colonization, some endophytes can move to other areas of the plant by entering the vascular tissues and spreading systemically as seen in Figure 2. The second factor influencing distribution is the allocation of resources throughout the plant. Different plant tissues can harbour compositionally distinct endophyte communities.

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The entire plant colonization process is collectively regulated by certain bacterial traits known as colonization traits (Chaturvedi *et al.,* 2016). In the interactive colonization processes, communication between the plant and bacterium (and *vice versa*) plays a key role. Bacterial root colonization often starts when the bacteria recognizes specific compounds in the root exudates. Plants produce exudates from roots to interact with microorganisms for their own ecological and evolutionary benefit. Flavonoids are considered as important compounds in plant–microbe communications. Bacteria respond to these exudates by showing chemotactic movement towards them. The apparent specificity in these interactions could relate to bacterial nutritional requirements and, in each of these cases, chemotaxis towards specific resources could determine the specificity of the interaction. The most common mode of entry of endophytic bacteria into plant tissues is through primary and lateral root cracks, and diverse tissue wounds occurring as a result of plant growth. Root wounds allow the leakage of plant metabolites and thus they become sites that attract bacteria (Sessitsch *et al*., 2005). Other sites through which endophytes enter plants include stomata (particularly on leaves and young stems) lenticels (which usually are present in the periderm of stems and roots) and germinating radicles (Scott *et al.,* 1996 and Gagné *et al.,* 1987). Bacteria can also enter via emergence of lateral roots or root hair cells (Hallmann *et al.*, 1997).

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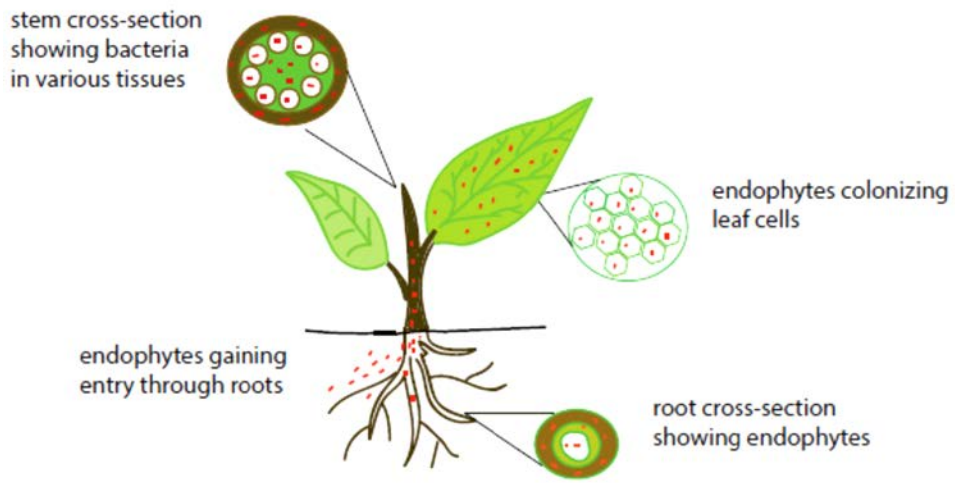

Figure 2. Diagram depicting the entry of endophytes in roots and its spread throughout the plant adapted from (Chaturvedi *et al.*, 2016).

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1.4. Significance of endophyte research

Discovery of new solutions for the establishment of sustainable agricultural is essential that may avoid the heavy use of fertilizers and pesticides as a reliance of productivity booster. Plant associative beneficial microbes are expected to harness their contribution in integrated pest management schemes over the coming decades (Jain and Pundir, 2017). There is global ever growing demand for implanting ecologically compatible and eco-friendly practices in agriculture, capable of providing adequate solutions for improving agriculture productivity. Improvement of endophyte resources could bring us a variety of benefits, such as novel and effective bioactive compounds that cannot be synthesized by chemical reactions (Waqas *et al.,* 2017). For this, there should be a better understanding about endophytes, their significance and their roles that they play. Understanding the biology of plants and their microbial ecology becomes an important factor. As evidenced by an increasing number of publications on endophytes in recent years, many studies have been performed on evaluating their colonization pattern of vegetative tissues as well as their effects on plant growth. Endophytes colonize all plant organs, and they have been isolated from the roots, stems, and leaves as previously mentioned. The occurrence of endophytes in specific tissues supports the view that they have a particular role in host plant tissues (Zhou *et al.,* 2015). For example, endophytes found in plant roots help with water uptake, nutrient acquisition, and plant growth promotion, while those found in the leaves and stems strengthen plant defence mechanisms (Khan *et al*., 2014; Waqas *et al.,* 2015; Hartley *et al.,* 2015; Zhou *et al.,* 2015). Table 2 shows a list of endophytic bacteria isolated from different organs. These publications indirectly suggest their importance to the hosts and to the environment at different sites in the plant. The locations of endophytes in different plant compartments are disputable, but powerful image analyses can provide information about the exact co-localization within plant tissue (Hardoim *et al.,* 2015).

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Table 2. Endophytic bacteria isolated from different organs of different plant species

Organism Species	Plant species	Plant organ	Reference
Galactomyces geotrichum	*Glycine max*	Roots	Waqas *et al.,* 2017
Cronobacter sakazakii	*Agave tequilana*	leaves	Martínez-Rodríguez *et al.,* 2014
Psuedomonas resinovorans	*Gynura procumbens*	Leaves	Bhore *et al.,* 2010
Acenitobacter calcoaceticus	*Gynura procumbens*	Leaves	Bhore *et al.,* 2010
Bacillus atrophaeus	*Stanleya pinnata*	Stems	Sura-de Jong *et al.,* 2015
Pantoea agglomerans	*Stanleya pinnata*	Roots	Sura-de Jong *et al.,* 2015
Pseudomonas koreensis	*Stanleya pinnata*	Roots	Sura-de Jong *et al.,* 2015
Bacillus atrophaeus	*Stanleya pinnata*	Leaves	Sura-de Jong *et al.,* 2015
Advenella kashmirensis	*Astragalus bisulcatus*	Roots	Sura-de Jong *et al.,* 2015
Herbaspirillum seropedicae	*Oryza officinalis*	Stems	Zakria *et al.,* 2007

Endophytes could be used for their various applications in boosting plant growth and development and can make vital contribution in agricultural biotechnology as seen in Figure 3. The ability of diverse bacterial endophytes to promote plant-growth occurs as a consequence of either direct or indirect mechanisms (Chaturvedi *et al.*, 2016). Direct promotion of plant growth occurs when a bacterium either facilitates the acquisition of essential nutrients or modulates of level of hormones within a plant. Nutrient acquisition facilitated by PGPB typically includes nitrogen, phosphorus and iron. Modulation of hormone levels may entail PGPB synthesizing one or more of the phytohormones auxin, cytokinin and gibberellin. In addition, some PGPB can lower levels of the phytohormone ethylene by synthesizing an enzyme, 1-aminocyclopropane-1-carboxylate (ACC) deaminase which cleaves the compound ACC, the immediate precursor of ethylene in all higher plants. Indirect promotion of plant growth occurs when a PGPB decreases the damage to plants following infection with a phytopathogen including some soil fungi and bacteria (Santoyo *et al.,* 2016). This usually occurs by the inhibition of the pathogen by the PGPB. Thus the endophytic bacteria are known to contribute to the host plants growth and development by producing plant growth regulators. The bacterial endophytes are also known to increase host plants resistance to plant pathogens and parasites, to promote biological nitrogen fixation, and to

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produce antibiotics (Bhore *et al.*, 2010). Importance of endophytic bacteria can clearly been seen in Table 3 showing different products that have been derived or produced from various endophytic bacteria with activities such as anticancer and antifungal properties.

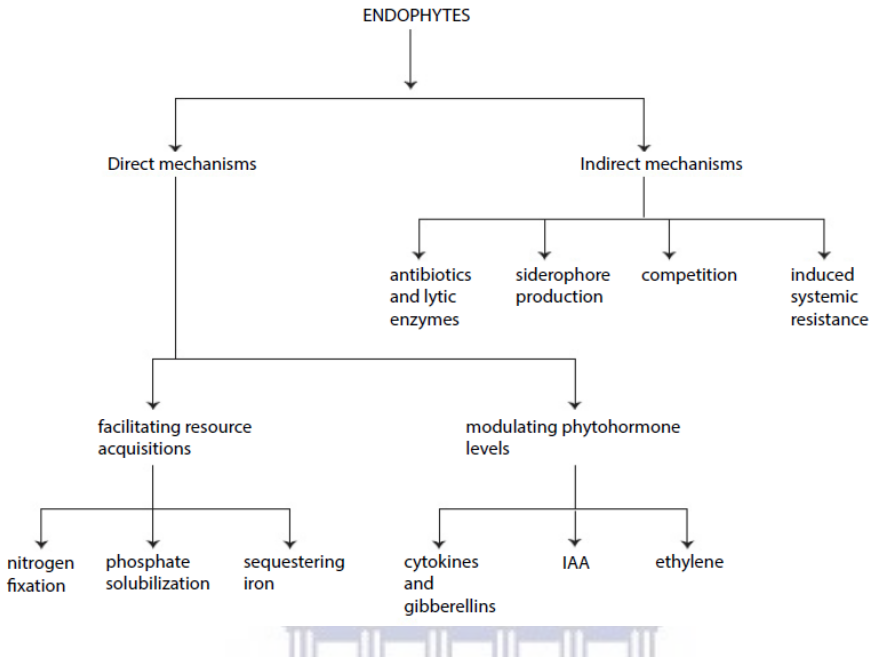

Figure 3. Outline of various mechanisms carried out by endophytes to promote plant growth adapted from (Chaturvedi *et al.*, 2016).

Table 3. Natural products derived or produced from various endophytic bacteria adapted from (Ryan *et al.*, 2008).

Organism	Plant association	Activity	Reference
Taxomyces andreanae	*Taxus brevifolia*	Anticancer	Strobel *et al.* (1993)
Pseudomonas viridiflava	Grass	Antimicrobial	Miller *et al.* (1998)
Streptomyces griseus	*Kandelia candel*	Antimicrobial	Guan *et al.* (2005)
Streptomyces NRRL 30562	*Kennedia nigriscans*	Antibiotic Antimalarial	Castillo *et al.* (2002)
Streptomyces NRRL 30566	*Grevillea pteridifolia*	Antibiotic	Castillo *et al.* (2003)
Serratia marcescens	*Rhyncholacis penicillata*	Antifungal	Strobel *et al.* (2004)

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The best strategy for the application of endophytes in agricultural systems is not yet known. The most obvious approach is to add inoculants to the soil or as seed dressings. There are reports of this approach being successful for sugar cane (Silva *et al.*, 2012). An alternative approach is to amend the agricultural system to encourage the indigenous community to respond and aid host plant growth and defence, although this requires a better understanding of the soil microbiome. The high rates of inorganic fertilizers currently added to crops circumvent the need for a healthy microbiome to aid nutrient acquisition, and so it follows that lower fertilizer rates will enable the selection of enhanced beneficial interactions with endophytes (Le Cocq *et al.*, 2017).

1.5. Nanotechnology and its unique properties for *in vivo* imaging

1.5.1. Nanotechnology

The definition of Nanotechnology is the design, fabrication and application of nanostructures or nanomaterials, as well as the fundamental understanding of the relationships between physical properties or phenomena and material dimensions (Kroll *et al.,* 2007). Nanotechnology is a new field or a new scientific domain which deals with materials or structures typically ranging in size of 0.1 to 100 nm in which one nanometer is 10^{-9} meter (Nikalje, 2015). On a nanometer scale, materials or structures may possess new physical properties or exhibit new physical phenomena similar to quantum mechanics such as electrical conductance, chemical reactivity, magnetism, optical effects and physical strength compared to their bulk materials. Nanotechnology has an extremely broad range of potential applications from nanoscale electronics and optics, to nanobiological systems and nanomedicine, to new materials (Kroll *et al.,* 2007). It therefore requires formation of and contributions by multidisciplinary teams of physicists, chemists, materials scientists, engineers, molecular biologists, pharmacologists and others to work together on (i) synthesis and processing of nanomaterials and nanostructures, (ii) understanding the physical properties related to the nanometer scale, (iii) design and fabrication of nano-devices or devices with nanomaterials as building blocks, and (iv) design and construction of novel tools for characterization of nanostructures and nanomaterials. Synthesis and processing of nanomaterials and nanostructures are the essential aspect of nanotechnology. Studies on new physical properties and applications of nanomaterials and nanostructures are possible

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only when nanostructured materials are made available with desired size, morphology, crystal and microstructure and chemical composition. Nanoparticles are generally defined as engineered structures with at least one dimension less than 100 nm (Nikalje, 2015). In recent years, an increasing number of products composed of these tiny structures have entered the market. The number of consumer products using nanotechnology has grown from 212 to 609 since PEN launched the world's first online inventory of manufacturer-identified nanotech goods in March 2006 (Parlini, 2008). Health and fitness items, which includes cosmetics and sunscreens, represent 60 percent of inventory products. Nanoparticles also offer an extraordinary opportunity for applications in pharmacology and medicine. Additionally, they have been applied in bioimaging (Gun'ko, 2016).

1.5.2 Quantum dots

Quantum dots (QDs) are the nanomaterial of interest for the application of bioimaging. QDs are nanometer-scale semiconductor crystals composed of groups II–VI or III–V elements (Jamieson *et al.,* 2007). Quantum confinement effects give rise to unique optical, electronical, photochemical and photophysical properties in QDs. Their greater brightness, solid photostability and unique capabilities for multiplexing, combined with their intrinsic symmetric and narrow emission bands, have made QDs far better substitutes for organic dyes in existing imaging and diagnostic assays (Xiao *et al.,* 2010). Many new techniques have been developed during the last decade, utilizing the unique photophysical properties of QDs, for *in vitro* biomolecular profiling of cancer biomarkers, *in vivo* tumour imaging, and dual-functionality tumour-targeted imaging and drug delivery (Fang *et al.,* 2012; Hild *et al*., 2008; Lidke *et al.,* 2007 and Zrazhevskiy *et al*., 2010).

These semiconducting nanoparticles are often composed of cadmium selenide or cadmium telluride (CdTe) cores, encased in zinc sulphide shells. When exposed to ultra violet (UV) light, QDs fluoresce in bright, pure, and precise colours of almost any wavelength of visible light. They can be covered with protective polymer coatings and embedded with receptor molecules, often carboxyl or amino groups that allow the QDs to be conjugated with other compounds which includes antibodies (Whiteside *et al.,* 2012). In this way, QDs can be used to track the movement of diverse compounds at minute spatial scales within microbes and

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soil. However, the applications of QDs in biological systems requires them to be made water soluble and less toxic (Zhu *et al.,* 2013). Therefore, numerous methods have been developed for creating hydrophilic QDs. One effective route is to exchange the hydrophobic layer of organic solvent with hydrophilic ligands such as thiol-containing molecules and peptides, which was designated as “cap exchange”. Hydrophilic molecules such as dihydrolipoic acid (DHLA) and mercaptopropionic acid (MPA) are often used as the stabilizers for the synthesis of hydrophilic QDs as can be seen in Figure 4.

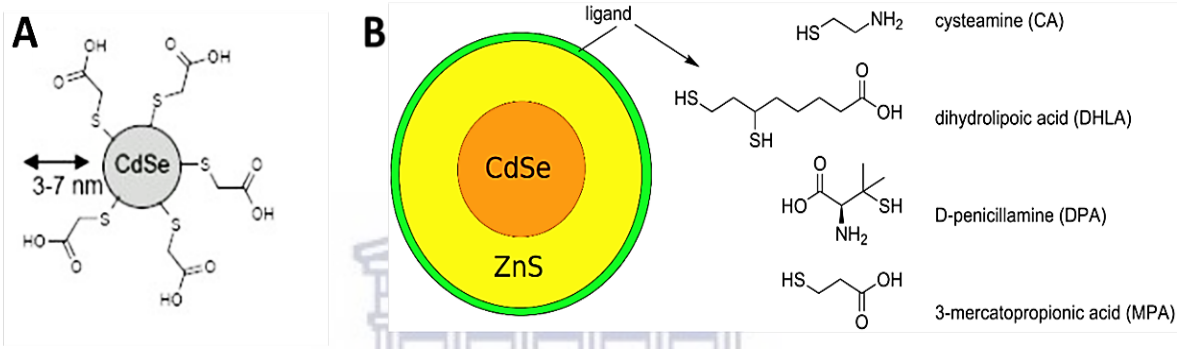

Figure 4. Chemical composition of the most commonly used QDs in biological applications. A) CdSe QDs in which solubilisation is accomplished by self-assembly of an alkanethiol (such as mercaptoacetic acid, shown) whose –SH bonds directly to the semiconductor, leaving the carboxylate group free to interact with aqueous solution. B) core-shell CdSe/znS QD with different ligands. Figures adapted from (Breus *et al.,* 2016; Whiteside *et al.,* 2012).

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Every nanoparticle needs to be characterised following their synthesis to determine if the objective has been achieved. Nanometer scaled confinement of electrons in quantum dot structures influences and determines their optical properties in an essential way (Bimberg *et al.,* 1999; Wang and Zunger, 1997). Shape, size and strain field of single QDs as well as the quality, density, and homogeneity of equisized and equishaped dot arrangements are important features of QDs (Rizvi *et al.,* 2010). These features control the optical properties, the emission and absorption of light, the lasing efficiency, and other optoelectronic device properties as can be seen in Figure 5. Their emission colour depends on their size, chemical composition and surface chemistry, and can be tuned from the ultraviolet to the visible and near-infrared (NIR) wavelengths (Rizvi *et al.,* 2010).

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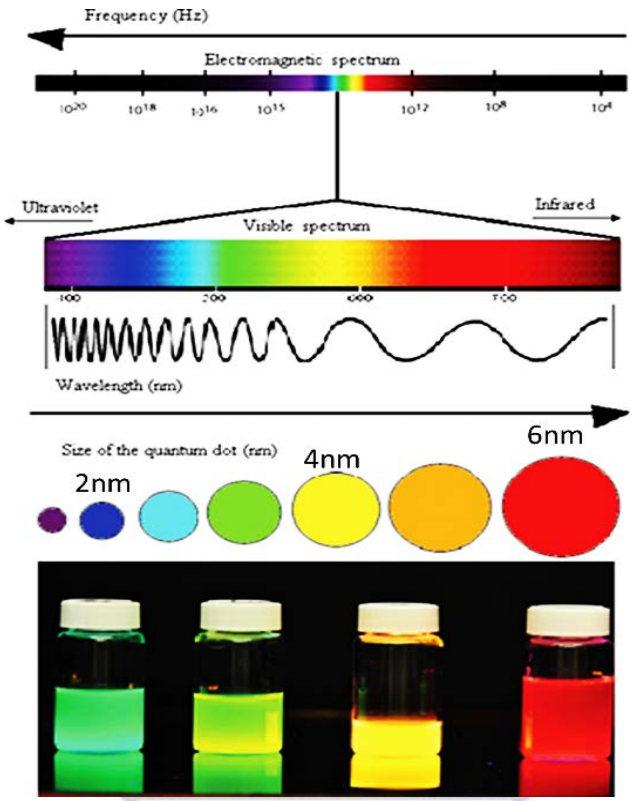

Figure 5. Size tuneable emission spectra of quantum dots and their relative size adapted from (Rizvi *et al.,* 2010).

1.5.3. Carbon quantum dots

Over the last several years metal-based QD bioimaging methodologies have been explored, however, with the increase in QD studies there's an increase in their toxicity concerns for their intrinsically toxic elements like cadmium (Zhu *et al.,* 2014). Unlike metal-based QDs, Carbon quantum dots (CQDs) are constituted by intrinsically non-toxic element, carbon, which makes them a particularly useful and promising bio-analytical tool (Wang and Hu, 2014). Toxicity studies have been conducted by various research groups, and CQDs appear to have low toxicity *in vitro* and *in vivo* indicating their excellent biocompatibility. Carbon is a black material, and is generally considered to have low solubility in water and weak fluorescence however, at the nanoscale, carbon has high solubility and is very fluorescent. Compared to traditional semiconductor quantum dots and organic dyes, photoluminescent CQDs are superior in terms of robust chemical inertness, facile modification and has high resistance to photobleaching. CQDs also have outstanding electronic properties as electron donors and acceptors, causing chemiluminescence and electrochemical luminescence, thus allowing them to have potential applications in optronics, catalysis and sensors.

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The structure of CDs consist of sp^2/sp^3 carbon and oxygen/nitrogen based groups or polymeric aggregations (Zhu *et al.,* 2014). CDs mainly contain graphene quantum dots (GQDs), carbon nanodots (CNDs) and polymer dots (PDs). The GQDs possess a single or few layers of graphene and connected chemical groups on the edge. CNDs are always spherical and they are divided into carbon nanoparticles without crystal lattice while CQDs have an obvious crystal lattice. The PDs is aggregated or cross-linked polymer, which is prepared from linear polymer or monomers (Zhu *et al.,* 2014).

1.5.4. Nanoparticle characterisation instrumentation

Photoluminescence spectroscopy (PL) is a contactless, non-destructive method of probing the electronic structure of materials (Liqiang, *et al.,* 2006). The high sensitivity of this technique provides the potential to identify extremely low concentrations of intentional and unintentional impurities that can strongly affect material quality and device performance. It can also determine the quantitative expression of fluorescence efficiency known as the quantum yield which is the fraction of excited state molecules returning to the ground state by fluorescence. It also determines chemical structures, size and electronic bandgap.

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Transmission electron microscope (TEM) is often used to characterize quantum dots by providing the size and morphology of the nanoparticle (Ma *et al.,* 2006). Electrons are focused through a sample and can thus give the internal structure of the nanoparticle. TEM provides topographical, morphological, compositional and crystalline information. One can study small details in the cell or different materials down to near atomic levels thus making it the perfect imaging instrument for characterizing nanoparticles.

1.6. Quantum dots compared to the conventional fluorescence dye

In a previous study, organic dyes and fluorescent proteins, such as Texas Red, Cy3 and enhanced green fluorescent protein (EGFP), were used to observe GLUT4 translocation in live mammalian cells (Qu *et al.,* 2011). These studies only observed particular segments of GLUT4 traffic due to their rapid photobleaching and relative weak fluorescent signal against strong

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autoflourescence background. Labelling endophytes with any of these organic dyes would result in a similar outcome. QDs coupled with biorecognition molecules such as streptavidin, peptides, proteins, and DNA, overcome the limitations that conventional dyes suffer from, and provide useful alternatives for long-term multicolour cellular, molecular, and *in vivo* imaging (Chan *et al*., 2002; Dahan *et al.,* 2001 and Jaiswal *et al.,* 2004). The broad emission spectra of conventional dyes may overlap and this limits the number of fluorescent probes that can be tagged to biomolecules for simultaneous imaging in a single experiment. QDs have narrow emission spectra that can be controlled by altering the size, composition and surface coatings of the dots. Hence, multiple QDs emitting different colours can be excited by a single wavelength of light, making them ideal for multiplexed imaging. Thus, labelling of endophytes with QDs can provide a new insight into endophyte translocation mechanisms.

A study previously done by Qu and his colleagues (2011) compared the fluorescence images of GLUT4-QDs with GLUT4-EGFP, which is a fluorescence protein widely used in the study of GLUT4 translocation (Qu *et al.,* 2011). The fluorescence intensity of QDs was brighter than EGFP even though the exposure time of EGFP was twice that of QDs. A single fluorescence particle of QD can be detected easier than that of EGFP. The QDs signal intensity was six times stronger than background intensity, whereas the EGFP signal intensity was three and a half times stronger than background intensity. The ratio of QDs signal intensity and background intensity was significantly higher than the ratio of EGFP signal intensity and background intensity. As a result, it is easier to locate GLUT4 in live L6 cells by labelling with QDs. These results strongly suggests that QDs are more suitable for observing intracellular GLUT4 translocation than fluorescent proteins in live mammalian cells proving the significance of QDs. Organic dyes have a fast decay rate (<5 ns) whereas for QDs its slow (30-100 ns); this slow decay rate of QDs is valuable in overcoming autofluorescence or background tissues, hence improving signal to noise ratio (Rizvi *et al*., 2010).

Organic dyes have low photobleaching threshold and usually bleaches within a few minutes on exposure to light. The photobleaching threshold of QDs are high thus making them extremely photostable due to their inorganic core, which is resistant to metabolic degradation and can maintain high brightness even after undergoing repeated cycles of excitation and fluorescence for hours (Rizvi *et al.,* 2010). Hence, they can be used for long

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term monitoring and cell-tracking studies. In fluorescent images, single QD could be detected easily. Qu *et al*. observed single GLUT4-QD in three-dimensional and investigate the detailed behaviour of the dynamics of GLUT4. This method can reveal the entire dynamic process of GLUT4 translocation in live L6 cells in real time, which previous studies cannot do. Furthermore, temporal resolution can be improved by reducing spatial resolution and expose time to make the results more accurate.

1.7. Conclusion

The demand for food, feed, and feedstocks for bioenergy and biofactory plants will increase proportionally due to population growth, prosperity, and bioeconomic growth. Globally, food demand and cultivated land will increase exponentially. Increasing crop yields, rather than clearing more land for food production, is the most sustainable path for food security. Endophytes maintain stable symbiosis with plants by producing phytohormones that promote plant growth and help them adapt better to the environment. They also suppress disease-causing microbes and accelerate nutrient availability and assimilation. We must then head for more environmentally sustainable agricultural practices while maintaining ecosystems and biodiversity. Endophytes are the potential solution to decrease negative environmental impact resulting from continued use of chemical fertilizers, herbicides and pesticides. The green fluorescent protein (GFP) gene marker has been widely used to visualize and track the colonization patterns of bacterial strains within inoculated host plants. It provides a unique and visual phenotype for studying the population dynamics of microorganisms within plant tissues. QDs are nanometer sized fluorescent semiconductor crystals with unique photochemical and photophysical properties. Their greater brightness, solid photostability and unique capabilities for multiplexing, combined with their intrinsic symmetric and narrow emission bands, suggests that QDs could be better substitutes for organic dyes in existing assays.

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1.8. Justification

The use of efficient inoculants can be considered an important strategy for sustainable management and for reducing environmental problems by decreasing the use of chemical fertilizers. Inoculum efficiency both for crop yield enhancement and for disease control is majorly dependent on the bacteria's ability to colonize certain tissue within the plant. Therefore, a better understanding of the endophytic bacterial colonization patterns and the survival of introduced inocula both in the rhizosphere and *in planta* is a critical prerequisite for the development of effective ways to deliver and manage inocula. The locations of endophytes in different plant compartments are disputable, but powerful image analyses can provide information about the exact co-localization within plant tissues and about physical contacts between different microbial groups. The occurrence of endophytes in specific tissues supports the view that they have a particular role in host plant tissues. Thus tracking the localization of endophytic bacteria in plants would provide insight of their particular role in the plant and better our understanding of endophyte-plant associations.

Conventional methods track endophytes by transformation of green fluorescent protein (GFP) which has the problem of fluorophore bleaching and blinking reducing detectability. Quantum dots offer distinct advantages over organic dyes and fluorescent proteins for biological imaging applications because of their brightness, photostability, and tenability and their extremely small size ensures little to no interference with the normal functions of the bacterium.

1.9. Aims and objectives

- Synthesize MPA capped CdTe Quantum dots.
- Transform endophytic bacteria with CdTe QDs and Carbon QDs respectively.
- Find optimum concentration of QDs for transformation efficiency.
- Test the biochemical activities of the transformed endophytes – IAA production and Phosphate solubilization.
- Test the nutrient uptake of plants treated with transformed endophytes by inductively coupled plasma optical emission spectrometry (ICP-OES).

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- These QD particles will essentially be used to visualise the uptake and translocation of endophytes in plants using the IVIS Lumina bioimaging system.

1.10. Highlights

- Water soluble MPA- capped CdTe QDs were successfully synthesized with the average size of 5nm.
- CdTe QDs have an excitation and emission wavelength of 420 nm and 550nm - 720 nm respectively.
- Carbon QDs have an excitation and emission wavelength of 320 nm and 410nm - 510 nm respectively.
- Fluorescence microscopy revealed successful transformation of endophytic bacteria with both Carbon and CdTe QDs.
- The concentration of 0,7 µM and 1,0 µM CdTe QDs and 150 µM Carbon QDs seemed to be the best for sufficient bacterial uptake.
- Cellular uptake of QDs were dose dependant: an increase in concentration lead to an increase in bacterial uptake.
- Bacterial growth curves showed that the QDs doesn't inhibit the bacteria and can be used for tracking purposes.
- Endophytic bacteria possessed good phosphate solubilization activity in the absence or presence of CdTe and Carbon QDs.
- Even at relatively high concentrations of CdTe and Carbon QDs, the bacterial sample is still able to produce IAA even if its production is slightly inhibited.
- All the samples tested have more than 70% germination indicating that the QDs are tolerated by the plant and can be thus used as a tracking device for bacteria.
- Images generated by the IVIS Lumina imaging system revealed a similar translocation pattern of endophytic bacteria in Garnet for both CdTe QD and Carbon QD samples.
- These QD particles were successful in its use to visualise the uptake and translocation of endophytes in plants using the IVIS Lumina bioimaging system.
- This is the first study to report the use of QDs to track the translocation of endophytic bacteria in plants.

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CHAPTER 2

Methods and materials

2.1. Synthesis of MPA capped CdTe quantum dots

The MPA capped CdTe QDs used in this study was synthesized according to previous studies, however, modifications were made (Chomoucka *et al.,* 2013; Yan *et al.,* 2010). The first step of synthesis involved the preparation of NaHTe by dissolving 2 mM of sodium borohydride ($NaBH_4$) and 0.08 mM tellurium (Te) in 20 ml of deionized water (dH_2O). This solution was heated at 80 °C for 30 minutes, which resulted in a colour change from clear to purple. For the subsequent synthesis of CdTe, 5 ml of the NaHTe solution was used. Another solution was prepared containing 0.8 mM cadmium chloride ($CdCl_2$) and 1.2 mM 3-mercaptopropionic acid (MPA) dissolved in 30 ml of deionized water at pH 11.7, forming the Cd/MPA precursor. This solution was heated to 100 °C under inert atmospheric conditions; however, prior to reaching the final temperature, the 5 ml of NaHTe solution was injected at 80 °C. The reaction was maintained at 100 °C for 2 hours allowing the growth of CdTe nanocrystals and a colour change from purple to yellow was observed. Thereafter the solution was rapidly cooled down on ice and stored in dark conditions at room temperature. The concentration of the quantum dots was determined using a formulae developed by Peng *et al*. (2003).

2.2. Carbon quantum dots

Carbon QDs were bought from Sigma-Aldrich, code: 900414. The sample came in 10 ml dispersed in water with a 0.2% concentration. It has a quantum efficiency of >5% with an emission wavelength of 450-550 nm according to its packaging.

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2.2. Characterization of quantum dots

2.2.1. Ultraviolet visible spectroscopy (UV-vis) and Photoluminescence (PL) spectroscopy

Initial analysis of both MPA capped CdTe and Carbon QDs were performed using UV-vis and PL spectroscopy. UV absorption spectra was measured at room temperature using the Thermo Nicolet evolution 100. PL emission spectra was measured at room temperature using Horiba NanoLog. Data was plotted into the software OriginPro 2017 to obtain graphs.

2.2.2. Transmission Electron Microscopy (TEM)

Subsequently, transmission electron microscopy studies were performed using the Hitachi H800, operated at 200 kV to determine the size of quantum dots. The software ImageJ was used to manually determine the size of the nanoparticles.

2.3. Synthesizing chemically competent endophytes

Endophytic bacteria were isolated from the *Echium plantagenium* plant and isolated Y2 strain endophytic bacterial colonies were given as a gift from a fellow colleague. The endophytic bacteria used in this study were made chemically competent according to previous studies, however, modifications were made (Swift and Baneyx, 2015). 500 ml of Luria Broth (LB) was prepared and autoclaved at 121 °C for 20 minutes, after the LB was cooled it was transferred to an autoclaved litre conical flask and inoculated with selected isolated bacterial colonies. The inoculated LB was incubated at 37 °C in the orbital shaker incubator at 200 rpm until cell growth reached mid logarithmic phase (optical density at 600 nm = 0.4 - 0.6). Cells were sedimented by centrifugation at 8,000 rpms for 8 minutes. Supernatant was discarded and the pellet resuspended in 100 ml of 100 mM ice cold calcium chloride ($CaCl_2$) and then left on ice for 30 minutes. Following a 30 minute incubation on ice, the bacterial culture was centrifuged at 8,000 rpms for 8 minutes, supernatant discarded and pellet resuspended into 12.5 ml of 1.0 mM cold $CaCl_2$ and held on ice overnight. Thereafter glycerol stocks were made

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by adding glycerol to a 10% (v/v) final concentration and aliquots (500 µl) were stored at -80 °C for future use.

2.4. Cellular uptake of quantum dots

For uptake experiments, the method used was according to previous studies, however, modifications were made (Swift and Baneyx, 2015). Competent cells were thawed at room temperature, washed twice with 900 µl 1X PBS (137 mM NaCl; 2.7 mM KCl; 4.3 mM Na_2HPO_4; 1.47 mM KH_2PO_4; adjusted to a final pH of 7.4 made up in 1L dH_2O) with intervening cycles of centrifugation at 4,000 rpm for 5 minutes in a micro centrifuge, and resuspended in the same saline buffer. CdTe QDs (250 µl for a dose of 0.5 µM, 0.7 µM and 1 µM) were added and the mixture was incubated on ice for 30 minutes. Thereafter it was heat shocked at 42 °C for 90 seconds and immediately placed on ice for 2 minutes. 3 ml of LB broth was added to the sample and incubated at 37 °C overnight on an orbital shaker. Cells were washed twice with 1X PBS to remove unincorporated QDs and then resuspended in 1X PBS buffer. The same was done for the uptake of CQDs, using the concentrations 50 µM, 100 µM and 150 µM of CQD. Control endophytic cells followed the same procedure of PBS washes and stored in 1X PBS.

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2.5. Bacterial growth curves

500 µl transformed endophyte culture (obtained in Section 2.4) was inoculated into 30 ml LB broth (500 ml autoclaved flask) and grown at 37 °C on a shaker to reach an OD of 1.0 at 600 nm. The same was done for the untransformed endophyte culture (control). Once OD was reached, the samples were diluted to an OD of 0.1 at 600 nm respectively using fresh sterile LB. From there on, the OD was taken every hour of both transformed and untransformed endophytic culture until the OD reached a plateau. This was done in triplicates for both CdTe and CQD transformed endophyte samples.

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2.6. Fluorescence microscopy

The transformed endophyte culture (obtained in Section 2.4) was inoculated on a micro slide and heat fixed. The glass slide was flooded with 1:1000 DAPI (4', 6-diamido- 2-phenylindole) stain for 10 minutes in the dark. The slide was then washed twice with 1X PBS and the coverslip was added. The slides contained a control (endophytes only) and the experimental (different concentrations of CdTe and CQD transformed endophytic sample per slide). The slides were viewed using a Nikon 59 Eclipse 50i fluorescent microscope available at the MBS department, UWC. Each slide was viewed under the 20X magnification using the appropriate filter sets such as the triband fluorescent filter. This filter reads various nanometres of wavelengths thus allowing multiple colours to fluoresce at once.

2.7. Test for the activity of transformed endophytes

2.7.1. Phosphate solubilization

10 µl of bacterial broth obtained in Section 2.4 were inoculated on phosphate plates (Yeast extract 0.05%, Dextrose 1%, Calcium phosphate 0.5%, Ammonium sulphate 0.05%, Potassium chloride 0.200%, Magnesium sulphate 0.01%, Manganese sulphate 0.00001%, Ferrous sulphate 0.00001%, Agar 1.5%) and incubated at 30 °C for 7 days and observed for the formation of halos (clear zones) around the isolates. Each plate was split in 4, thus 4 samples were inoculated per plate.

2.7.2. Indole acetic acid (IAA) production

IAA production was measured by a colorimetric test using Van Urk Salkowski. 30 mls of Yeast Extract Manitol (YEM) (Yeast extract 0.1%, Mannitol 1%, Dipotassium phosphate 0.05%, Magnesium sulphate 0.02%, Sodium chloride 0.01%) was inoculated with 1 ml culture (obtained in Section 2.4) and added with 0.1% tryptophan and without tryptophan (control) and allowed to grow for 4 days at 37 °C in an orbital shaker. The aliquot (2 ml) was centrifuged at 13,000 rpm for 10 minutes and the supernatant was mixed in a 2:1 ratio with Salkowski

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reagent (0.5 M $FeCl_3$ and 35% perchloric acid) and incubated for 30 minutes in the dark at room temperature. A standard curve was made by using YEM and a known concentration of IAA ranging from 0-100 ug/ml. The OD was read at 530 nm. Each sample was done in triplicates for both the control and experimental.

2.8. Surface sterilization of canola seeds (*Brassica napus* L)

Canola seeds (*Brassica napus* L) were surface sterilized using 3.5% hyperchlorate bleach, 70% ethanol and autoclaved dH_2O washes to get rid of any contaminating epiphytes. The samples were then plated using pour plate technique after the last wash to check for successful surface sterilization using Reasoner's 2A agar (R2A). The plates were incubated at 30 °C for 10 days.

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2.9. Plant germination and growth trials

The transformed endophyte culture (obtained in Section 2.4.) was inoculate in 30 ml LB broth and grown overnight on a shaker at 37 °C. Surface sterilized canola seeds were soaked in LB broths containing the transformed endophyte for 30 minutes and left on a shaker at room temperature (OD of culture was the same for all inoculants). Two controls were used, one with endophytes only and the other contained only sterile LB broth. Seeds were removed from broth and planted 2cm deep in a soil mixture of 2:1 (soil: silica sand) and then pre-treated with 100 ml dH_2O. The planted seeds were covered in 2 ml of the respective culture all at the same OD. All the treatments and controls were replicated 5 times therefore 3 seeds per cup and 5 cups were used per isolate making it a total of 15 seeds per sample. The plants received 80 ml water every 3rd day. Germination was performed in the greenhouse set at 20 ^{o}C with natural lighting. Seeds were monitored for germination percentage and physiological growth differences over a 6 week period. Plants were harvested on day 42 of growth. Dry weights were taken of the roots and shoots (in triplicates per sample). The rest of the plant material were grounded up for ICP analysis.

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2.10. Macro and micro element quantification using ICP-OES

The root and shoot samples for ICP analysis were grounded into fine powder using liquid Nitrogen. 200 mg of ground tissue was transferred to an Eppendorf tube and mixed with 65% Nitric acid. Tubes were placed at 90 °C for 3 hours/until sample was dissolved. Using a syringe 9 ml of 2% Nitric acid was taken up and 1 ml of sample and then filtered into a greiner tube ready for ICP analysis. The samples were analysed for macronutrients Ca, Mg, K, P and micronutrients Fe, Zn, Mo, Mn, Cu, Ni and the presence of Cd by axially viewed inductively coupled plasma optical emission spectrometry (ICP-OES, Varian Inc) available at the Chemistry department, UWC.

2.11. *In vivo* imaging using the IVIS Lumina Bioimaging system

Bioimaging of QDs within the plants was done by using the IVIS® Lumina II imaging system and the Living Image software version 3.0 (Caliper Life Science) available at Stellenbosch University. Two filter sets were used, and the results were subsequently overlaid by the system software to generate the images. For the CdTe QD samples the GFP filter set was used which included an emission filter (515 - 575 nm), excitation filter (445 - 490 nm) and a background filter (410 - 440 nm). The second filter used was the Cy5.5 filter set which also included an emission filter (695 - 770 nm), excitation filter (615 - 665 nm) and a background filter (580 - 610 nm). For the carbon QD sample, only the GFP filter set was used as it had the closest wavelength range to CQDs excitation and emission wavelength. The acquisition of the images were performed with the following system parameters: subject height 0.5 cm, field of view 12.5 cm, lamp level high and an automatic exposure time (0.5- 60 seconds). Each plant was examined for the roots, stems and leaves. Each sample was repeated 6 times.

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CHAPTER 3

Results

3.1. Characterization of quantum dots

The initial characterization of Carbon QDs and water-soluble MPA capped CdTe semiconducting nanoparticles was done using a UV lamp to detect fluorescence of quantum dots synthesized. The brightly reddish/pink fluorescent sample (Figure 1A) and greenish/yellow fluorescent sample (Figure 1B) indicates the successful synthesis of CdTe QDs and CQDs respectively. The second QD characterization technique used was the ultraviolet-visible spectroscopy and the photoluminescence spectroscopy to obtain the QD excitation wavelength which was at 420 nm for MPA-CdTe (Figure 2A). This subsequently yielded the emission wavelength of 730 nm (Figure 2B). The PL spectra revealed Full Width Half Maximums (FWHM) of 130 nm. The Excitation wavelength of CQDs was at 320 nm (Figure 3A) which subsequently yielded an emission wavelength of 410 -510 nm (Figure 3B) with a FWHM of 140 nm. Subsequent analysis of QD samples were done to determine the size of the nanoparticles using TEM. The image obtained (Figure 4A) displays polydispersed MPA-CdTe nanoparticles, with some showing a tendency to agglomerate. The particles are spherical in shape and have an average diameter ranging from 2-6 nm. The image obtained (Figure 4B) displays polydispersed carbon nanoparticles, with some showing tendency to agglomerate. The particles are spherical in shape and have an average diameter ranging from 3-5 nm.

According to the work of Peng and co-workers (2003), the extinction coefficient (ε) of the MPA-CdTe QDs can be determined by the equation as follows: $\text{Ɛ} = 10043\ (D)^{2.12}$. Wherein D (nm) is the diameter or size of a given nanocrystals sample, λ is the wavelength of the first absorption peak (from low energy) of the corresponding sample and ε is the extinction coefficient of the corresponding sample (Yu *et al.,* 2003). In the present experiments, the diameter (D) of the MPA-CdTe QD was determined using HRTEM as 5 nm. The concentration of the MPA-CdTe QDs was determined as 2.035×10^{-6} M (2.035 µM) using the Beer–Lambert

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law A=ƐbC. The concentration of CQDs had a known concentration of 0.2 % (2mg/ml) when purchased by Sigma.

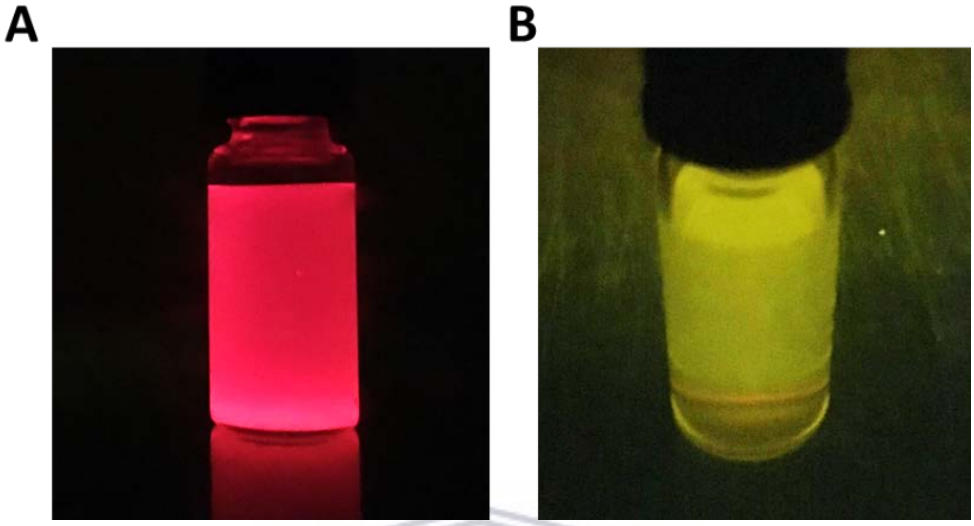

Figure 1. Fluorescence of QDs synthesized under UV radiation. A) MPA-CdTe QD fluorescing reddish/pink in colour. B) Carbon QD fluorescing yellow/greenish in colour.

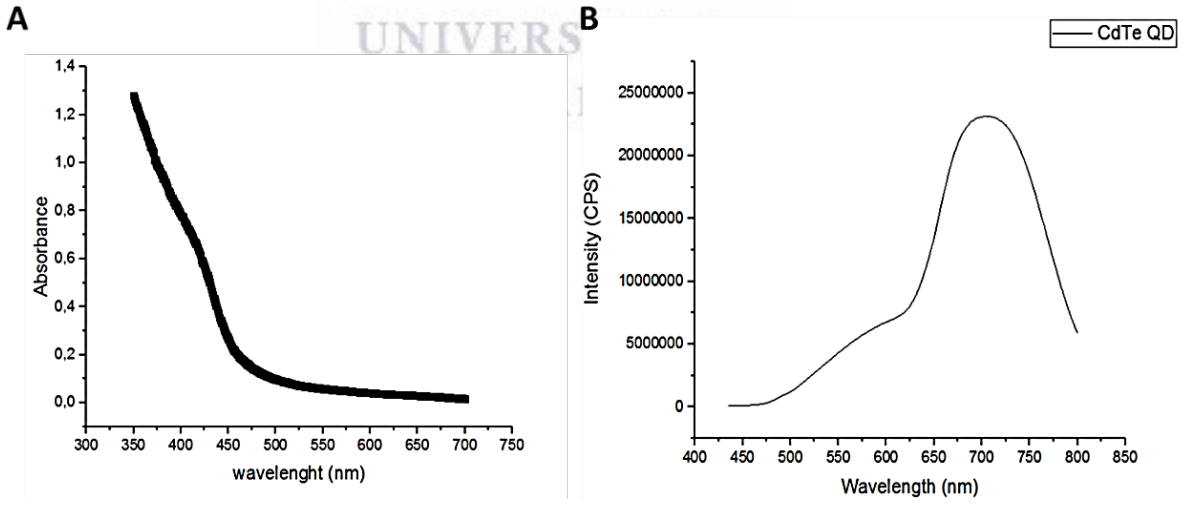

Figure 2. **UV-vis and PL Spectra of MPA-CdTe QD sample.** A) UV-vis Spectra of MPA-CdTe QD. Samples were excited at 420 nm obtained with an OD of 0,63. B) PL spectra of MPA-CdTe. Emission wavelength of 720 nm being measured from an optical path length of 5nm.

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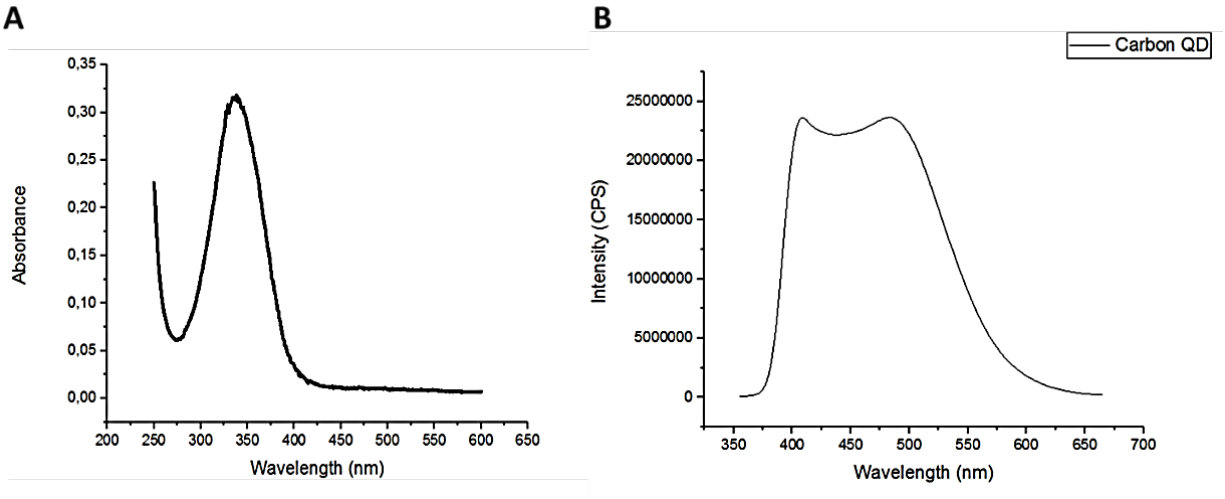

Figure 3. **UV-vis and PL Spectra of carbon QD sample**. A) UV-vis Spectra of Carbon QD. Samples were excited at 320nm obtained with an OD of 0,33. B) PL spectra of Carbon QD. Emission wavelength of 410-510 nm being measured from an optical path length of 5nm.

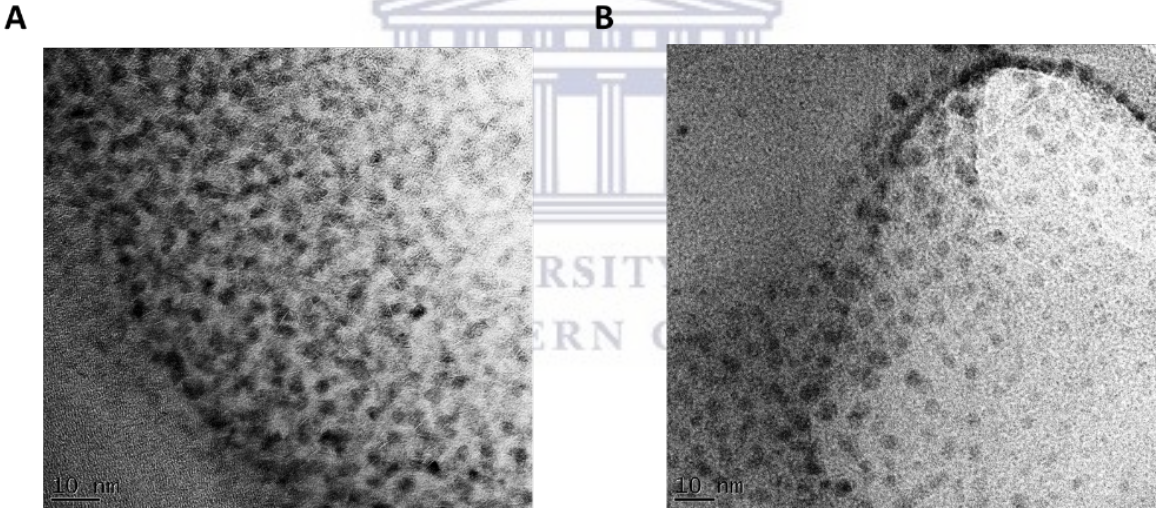

Figure 4. HRTEM micrograph of quantum dots. A) MPA-CdTe QD TEM micrograph. B) Carbon QD micrograph. Scale bar of 10 nm.

3.2. Cellular uptake of quantum dots

In order to show that the endophytes were transformed with the MPA-CdTe quantum dots, the bacterial culture was washed with saline buffer to remove any untransformed MPA-CdTe quantum dots. The resulting pellet was the transformed culture as seen in Figure 5A. The transformed bacterial pellet containing MPA-CdTe QDs has a much deeper yellow pigment

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(tubes 2-4) compared to the untransformed control (tube 1) which indicates successful transformation (Figure 5A). The pellet was resuspended in saline buffer and Figure 5B shows the fluorescence of the resulting culture under UV radiation. This image shows a clear increase in fluorescence as the concentration of CdTe QD increases (tubes 2-4). The same was done for the CQDs. The resulting overnight culture was washed with saline buffer to remove untransformed CQDs. Figure 5C shows the resulting pellets starting from endophytes control (tube 1), following in increasing concentration of CQD (tube 2-4). The pellet in tube 4 had the deepest yellow indicating high CQD uptake. Figure 5D shows the pellets resuspended in saline buffer under UV radiation. The fluorescent yellow indicates presence of quantum dots thus indicating the successful uptake of CQD under various concentrations (tube 2-4).

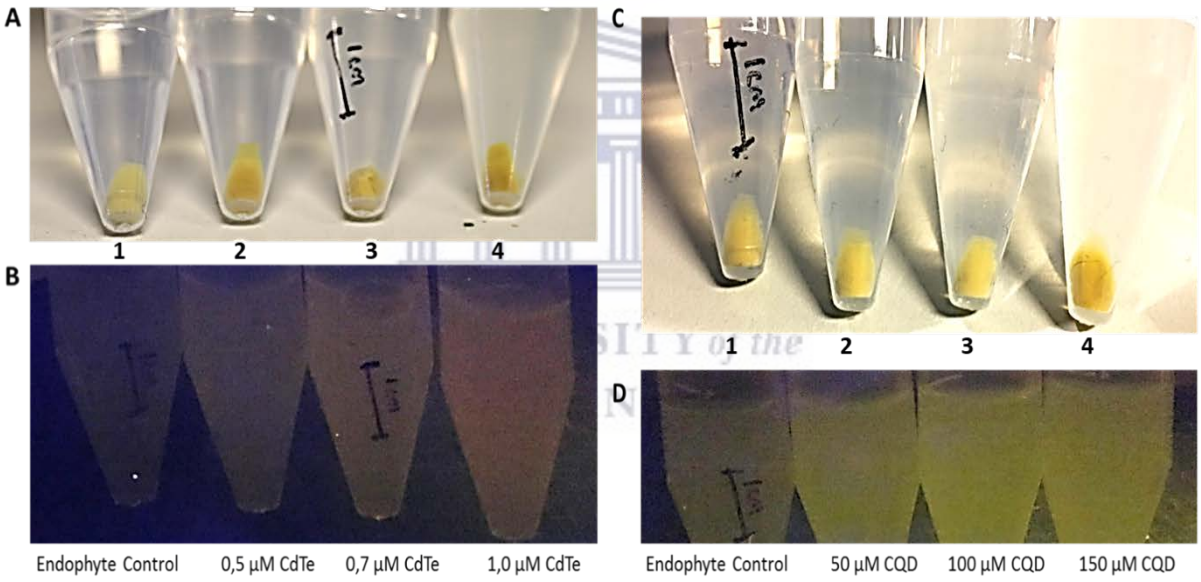

Figure 5. **Cellular uptake of quantum dots in endophytes**. A) Resulting pellet of CdTe QD uptake in endophytes following centrifugation. B) Pellet resuspended in saline buffer and viewed under UV radiation. C) Resulting pellet of Carbon QD uptake in endophytes following centrifugation. D) Pellet resuspended in saline buffer and viewed under UV radiation.

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3.3. Bacterial growth curves

The bacterial growth of the transformed bacteria was compared to that of the untransformed bacteria to detect how the QDs effect their growth pattern. The pellets obtained in Figure 5A and C were grown in LB overnight thereafter the OD was brought down to 0.1 and read at every hour at a 600 nm wavelength. As seen in Figure 6A for CdTe QDs growth curve, there was exponential growth from the first hour till the second hour for all four bacterial cultures. There is no real change in growth pattern of all the transformed bacterial cultures compared to the control (untransformed). The CdTe QDs samples does show a slight difference in their growth patterns compared to that of the control however, according the error bars there is no real significant change. The lag phase is short and the slope is not very steep. Thus these results do look promising as it shows that the MPA-CdTe QDs does not completely inhibit the bacteria's ability to grow and can be used for tracking purposes.

The same experimental procedure was done for samples containing CQDs. As seen in Figure 6B there is exponential growth from the first hour till the second (corresponding to that of the CdTe growth pattern). The same outcome can be seen with CQDs as CdTe QDs. There is no significant difference in growth patterns of the control compared to the transformed bacterial culture. Although QDs slows down the bacteria's rate of growth it does not inhibit its ability to tolerate the presence of the QDs and are still able to grow in its presence.

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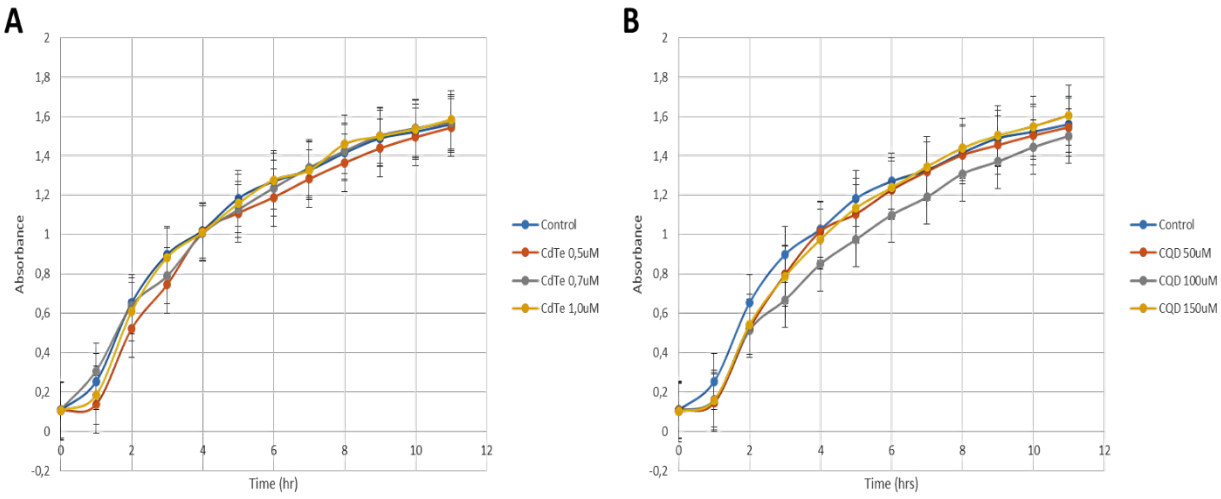

Figure 6. Growth curve of bacterial culture. A) Transformed endophyte culture with MPA capped CdTe QDs. Blue – Endophyte control, Orange- CdTe conc. 0.5 µM, Grey- 0,7 µM and Yellow – 1 µM. All samples were done in triplicates and OD taken at a 600 nm wavelength. B) Transformed endophyte culture with

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Carbon QDs. Blue – Endophyte control, Orange- CQD conc. 50 µM, Grey- 100 µM and Yellow – 150 µM. OD was taken at a 600 nm wavelength and data represents the mean ±SD (n=3). Means are not significantly different ($p \leq 0.05$).

3.4. Fluorescence microscopy and the determination of optimum transformation concentration

In order to determine cellular uptake of QDs in bacteria, fluorescence microscopy was performed. This visual identification of transformation also indicates the optimum concentration required for efficient bacterial transformation. The pellets obtained in Figure 5A was resuspended in saline buffer. The bacterial culture was heat fixed on microscope slides and stained with DAPI. DAPI is a fluorescent dye that stains the nucleus of cells. Figure 7A is the control (only endophytes), the presence of blue stain indicates the presence of bacteria and absence of QDs which fluoresces a red colour. Figure 7B – 7D shows the blue fluorescent dots indicating the presence of endophytes as well as red fluorescence which is that of the MPA-CdTe QDs. These images gives clear indication that the transformation of endophytes with MPA capped CdTe QDs were successful. Figure 7D shows an overwhelming amount of red and this is due to the increased concentration of CdTe in the sample. Thus for plant trials Figure 7C and D (0,7 µM and 1,0 µM) was used as it had the highest CdTe QD bacterial transformation.

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The same was done for the carbon QD samples (Figure 5C). The bacterial culture was heat fixed on microscope slides and stained with DAPI and then washed with saline buffer. Figure 8A is the control (only endophytes), the presence of blue stain indicates the presence of bacteria and absence of QDs also corresponding to the exact same sample in Figure 7A. Figure 8B – 8D shows the blue fluorescent dots indicating the presence of endophytes as well as yellow fluorescence which is that of the carbon QDs. These images gives clear indication that the transformation of endophytes with carbon QDs were successful. Figure 8D shows an overwhelming amount of yellow and this is due to the increased concentration of CQD in the sample. Thus for plant trials Figure 8D (150 µM) was used as it had the highest Carbon QD bacterial transformation.

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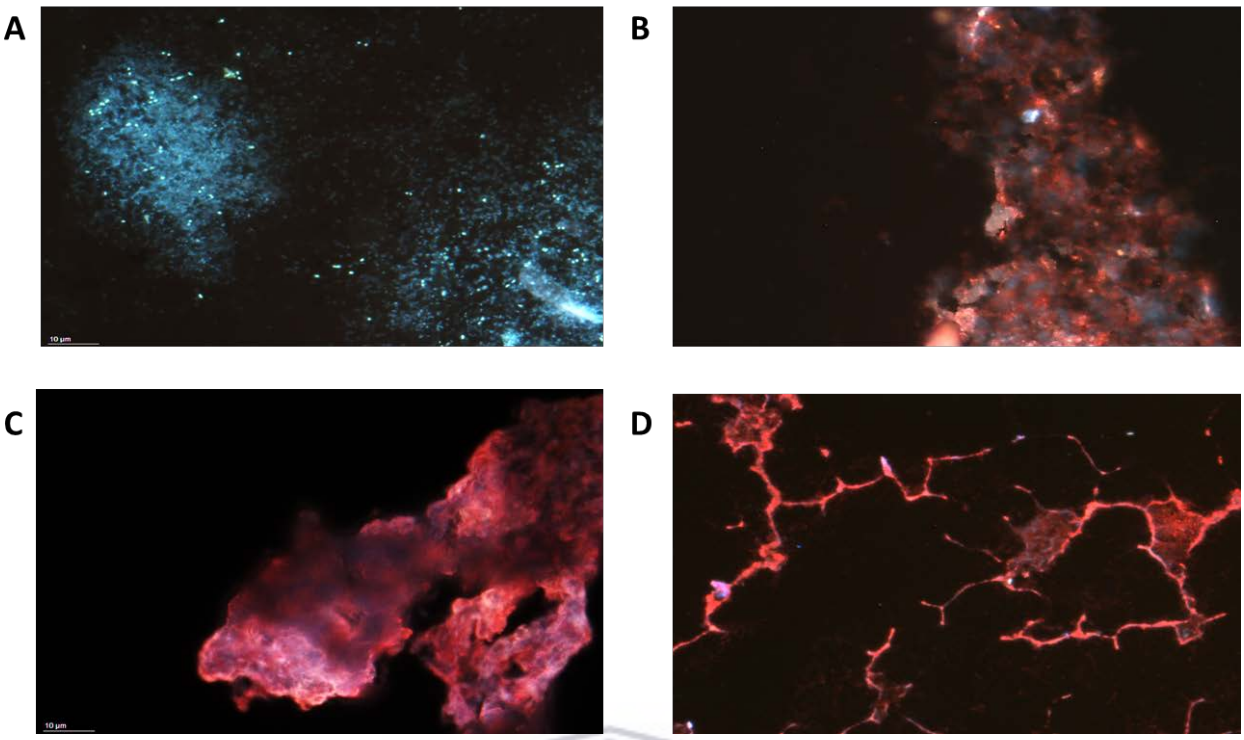

Figure 7. Fluorescent micrographs of the endophyte bacteria transformed with CdTe QDs. A) The control (only endophytes). B) CdTe conc. 0.5 µM. C) CdTe conc. 0.7 µM. D) CdTe conc. 1.0 µM. All slides were done in triplicates and each image has a scale bar of 10 µm obtained under 20X magnification. Images were taken several hours following transformation.

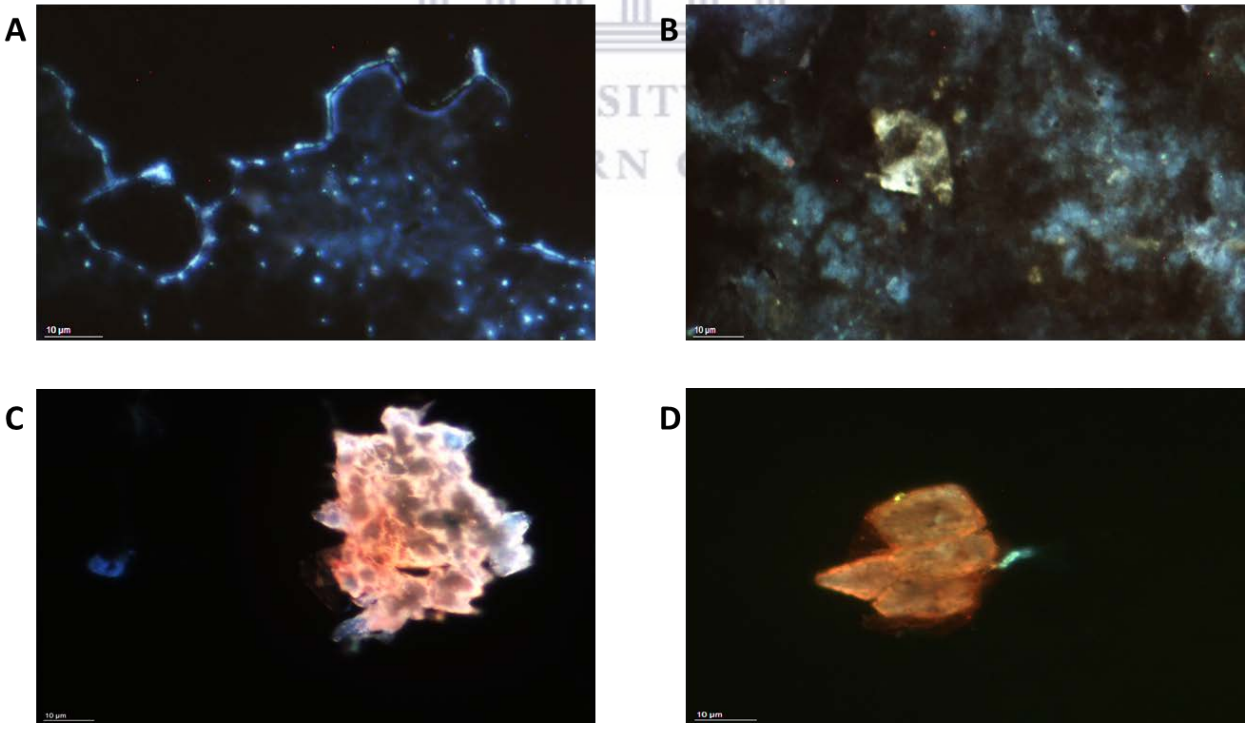

Figure 8. Fluorescent micrographs of the endophyte bacteria transformed with carbon QDs. A) The control (only endophytes). B) CQD conc. 50 µM. C) CQD conc. 100 µM. D) CQD conc. 150 µM. All slides were done in triplicates and each image has a scale bar of 10 µm obtained under 20X magnification. Images were taken several hours following transformation.

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3.5. The activity of transformed endophytes

3.5.1. Phosphate solubilization

The QDs transformed in bacteria were tested for phosphate (P) solubilization as it is the second major nutrient required for plant growth. Phosphate solubilizing bacteria and fungi play an important role in P bioavailability. The insoluble and inaccessible forms of P are hydrolysed to soluble and available forms through the process of solubilization (inorganic P) and mineralization (organic P). In Figure 9A, halos can be seen that were formed around the CdTe bacterial isolates on the phosphate plates, indicating that the endophytes are able to grow on the media and solubilize phosphate. Therefore, these findings strongly suggests that this endophytic bacteria strain may play a role in the regulation of phosphate and that role is not affected when transformed with CdTe QDs. The carbon QDs were also tested for phosphate solubilization and as seen in Figure 9B, there are halos present for each concentration however, as the QD concentration increases the halo size decreases.

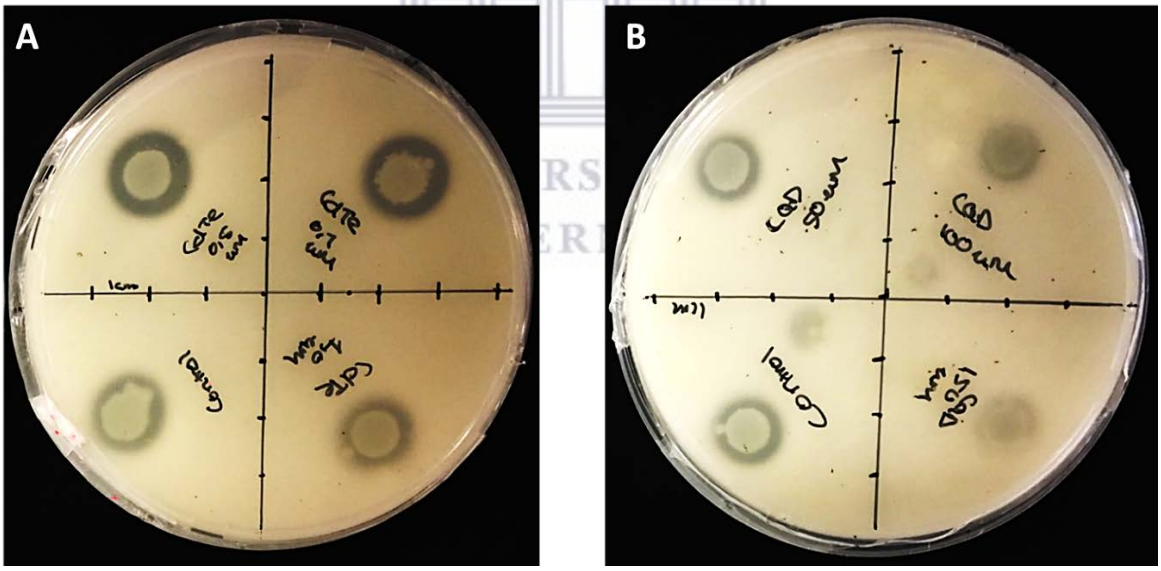

Figure 9. Phosphate solubilization test of A) CdTe QDs transformed bacteria under various concentrations and B) Carbon QDs transformed bacteria of various concentrations. Plates were done in triplicates.

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3.5.2. IAA production

Indole acetic acid (IAA) production was tested to determine if QDs affect the biochemical activities of endophytes. The IAA production of the endophyte strain (control) was significantly greater than the 1,0 µM CdTe transformed sample, with the control having 68 µg/ml IAA production and the 1,0 µM CdTe bacterial sample having an IAA production of 16 µg/ml. When compared to the rest of the samples tested, this sample seems to be the most affected in IAA production as seen in Figure 10 A & B. The 0,7 µM CdTe transformed sample outcompeted the control in IAA production and produced IAA close to the maximum of the standard curve (79 µg/ml). The 150 µM Carbon QD transformed sample produced less IAA (51 µg/ml) than the control.

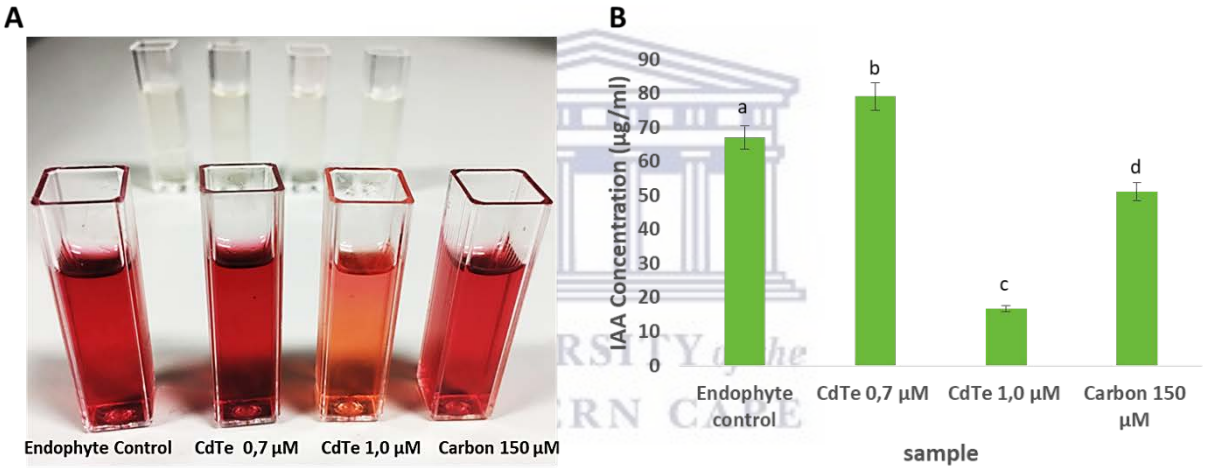

Figure 10. Indole acetic acid production. A) Colorimetric visualisation of samples. B) IAA concentrations obtained for each sample by reading the OD at 530 nm and using a standard curve made by using Yeast Extract Mannitol (YEM) and a known concentration of IAA ranging from 0-100 ug/ml. Data represents the mean ±SD (n=3). Means with different letters are significantly different from each other ($p \leq 0.05$). All cultures were used with the same OD.

3.6. Plant germination and growth trials

3.6.1. Germination percentage

In order to assess the effect of treatment with QD transformed bacteria on the *Brassica napus* L plant, the morphology, germination and biomass of the QD treated and untreated control plants were compared. The growth percentage was recorded at the end of 42 days in greenhouse. The LB control had 67% germination whereas the endophyte control had 87%

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germination. The CdTe 0,7 µM transformed endophytic sample had 93% germination and the 1,0 µM had 86% germination. The CQD transformed bacterial culture showed 80% germination. All the transformed bacterial samples have more than 70% germination indicating that the QDs are tolerated by the plant.

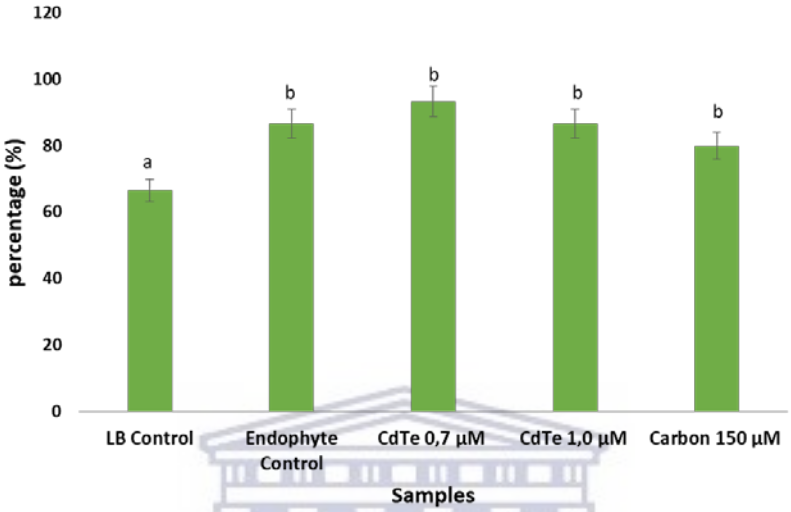

Figure 11. Germination percentage of *Brassica napus* L after 42 days with and without treatment. 15 treated seeds planted per treatment, 3 seeds per cup. Means with different letters are significantly different from each other ($p \leq 0.05$).

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3.6.2. Root and shoots length

The morphology of the canola plants following 42 days of growth can be seen in Figure 12. The endophyte control and all plants treated with QD transformed bacteria showed an increase in roots and shoots compared to the LB control indicating the positive effects of the PGPB bacteria. The sample with 0,7 µM CdTe QD showed the best plant growth compared to the rest of the samples tested, having the thickest and longest roots as well as being the only isolate that flowered within the 6 weeks. CdTe 1,0 µM QD sample showed an increase in roots and shoot length compared to both the controls. The Carbon 150 µM QD sample showed similar plant growth compared to the endophyte control with slightly longer and thicker roots. Due to bad lighting, leaf colour appears pale however, there was no change in leaf colour for all plants treated.

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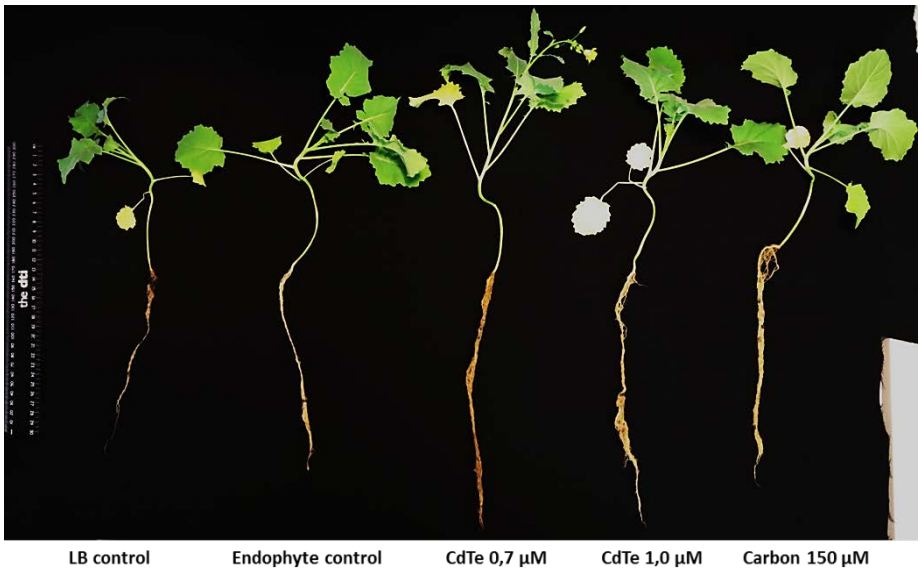

Figure 12. Image of *Brassica napus* L seedlings after 42 days in greenhouse.

3.6.3. Dry weights of roots and shoots

There is a similar pattern between the weight of roots and that of their respective shoots for all samples (Figure 13 A & B). The plants treated with endophytic bacteria only showed an increase in biomass for both roots (48%) and shoots (25%) when compared to the LB control plants. The bacteria transformed with CdTe 0,7 µM QD treated plants had the highest biomass compared to the rest of the samples, followed by the CdTe 1,0 µM QD treated plants. The transformed CdTe 0,7 µM QD treated plants showed a 52% increase in root biomass and a 23% increase in the shoots compared to the endophyte control. The transformed CdTe 1,0 µM QD treated plants showed a 37,5% increase in root biomass and a 16,7% increase in the shoots compared to the endophyte control. The root biomass of the Carbon QD treated plants had an increase of 20% compared to the endophyte control treated plants, however, there is a similarity between their shoots with the endophyte control only having a 3% increase in the shoots. The biomass obtained for each sample correlates to the size and morphology of the plants obtained in Figure 12.

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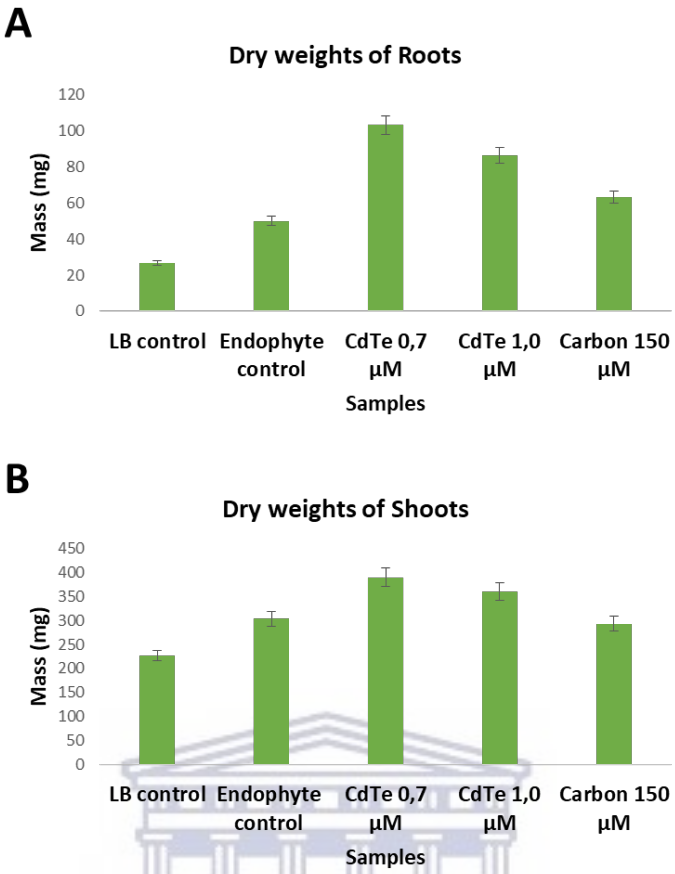

Figure 13. Dry weight of *Brassica napus* L plants after 42 days. A) Dry weights of roots. B) Dry weights of shoots. Data represents the mean ±SD (n=3), means with different letters are significantly different from each other ($p \leq 0.05$).

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3.7. ICP analysis of nutrient uptake

Macro and micronutrient analyses of the LB control and treated plants were done using ICP analysis to detect the presence of the macronutrients (Mg, Ca, K, P) and micronutrients (Fe, Zn, Mn, Mo, Cu, Ni). All nutrients analysed were significantly higher in plants treated with endophytes compared to the LB control treated plant. The 1,0 µM CdTe bacterial treated plants seemed to be the most affected in the roots however, increased in the shoots when compared to the LB control. This endophytic strain has superior nutrient acquisition capabilities as all plants treated with endophytes in the presence and absence of QDs showed an increase in nutrients, especially in the shoots. This higher accumulation of macro and micronutrients observed from the endophyte treated plants could correlate to the increased dry weight and biomass of the treated plant. The untransformed endophyte treated plants

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showed the best regulation overall, showing a significant increase in all macro elements tested, as well as in microelements such as Cu with an increase of 50%, Ca was up by 84% and Fe showed a 27% increase in the roots compared to the LB control. The 0,7 µM CdTe transformed bacterial treated plants showed the highest increase in roots by the elements Zn which increased by 43%, Ni by 72% and a 15% increase in K and in the shoots the highest increase in an element was Mo by 31%, Fe by 19% and Ca by 15% compared to that of the LB control. The CQD transformed bacterial treated plants showed an increase of Mg uptake by 31% and a 26% increase in K in the roots. In the shoots there was a 97-100% increase in Ni for all plants treated with endophytes in the presence or absence of QDs and a 31-38% increase in P. The highest amount of Cd present was in the 1,0 µM CdTe bacterial treated plants with an increase in 63% in the roots and 52% in the shoots followed by 0,7 µM with 61% in the roots and 40% in the shoots compared to the LB control.

Table 1. Inductively coupled plasma optical emission spectrometry (ICP-OES) of samples roots. Macronutrients represented in blue, micronutrients in pink and Cadmium in purple. Colour scale from green (increase in uptake) to red (decrease in uptake) and yellow (no change). Mean ± standard deviation, $n = 3$; values followed by same letter in a row were not significantly different ($p \leq 0.05$).

Elements	LB Control	Endophyte Control	0,7 µM CdTe	1,0 µM CdTe	150 µM Carbon
Ca	141,55^{a}	260,81^{b}	173,30^{c}	141,60^{a}	185,76^{c}
Mg	32,69^{a}	56,87^{b}	40,23^{a}	36,93^{a}	47,32^{a}
K	264,63^{a}	301,55^{b}	312,67^{b}	212,03^{c}	355,81^{d}
P	48,12^{a}	55,92^{a}	52,74^{a}	35,51^{b}	54,55^{a}
Fe	11,70^{a}	16,03^{b}	12,31^{a}	6,64^{c}	10,83^{a}
Zn	1,5863^{a}	2,6620^{b}	2,7635^{b}	1,6858^{a}	1,7747^{a}
Mn	1,5158^{a}	2,2665^{b}	1,5233^{a}	1,1815^{c}	1,3043^{c}
Cu	0,3910^{a}	0,7767^{b}	0,5095^{a}	0,4265^{c}	0,5524^{a}
Mo	0,01320^{a}	0,03341^{b}	0,02720^{c}	0,01485^{a}	0,02000^{d}
Ni	0,00882^{a}	0,01589^{b}	0,03199^{c}	0,01051^{d}	0,01661^{b}
Cd	0,00308^{a}	0,00167^{b}	0,00799^{c}	0,00854^{c}	0,00204^{b}

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Table 2. Inductively coupled plasma optical emission spectrometry (ICP-OES) of samples shoots. Macronutrients represented in blue, micronutrients in pink and Cadmium in purple. Colour scale from green (increase in uptake) to red (decrease in uptake) and yellow (no change). Mean ± standard deviation, $n = 3$; values followed by same letter in a row were not significantly different ($p \leq 0.05$).

Elements	LB Control	Endophyte control	0,7 µM CdTe	1,0 µM CdTe	150 µM Carbon
Ca	135,83^{a}	147,93^{a}	159,12^{a}	148,99^{a}	158,06^{a}
Mg	34,15^{a}	41,38^{a}	37,86^{a}	36,21^{a}	39,46^{a}
K	587,40^{a}	698,61^{a}	659,56^{a}	645,27^{a}	715,20^{a}
P	36,49^{a}	54,10^{b}	55,73^{b}	53,29^{b}	58,99^{b}
Fe	0,9721^{a}	0,9871^{a}	1,2033^{b}	0,6112^{c}	0,8902^{a}
Zn	1,0805^{a}	1,3112^{b}	1,2990^{b}	1,2496^{b}	1,3369^{b}
Mn	0,7654^{a}	0,9497^{b}	0,9186^{b}	0,9470^{b}	0,9975^{b}
Cu	0,2830^{a}	0,2945^{a}	0,3005^{a}	0,2735^{a}	0,3181^{a}
Mo	0,02247^{a}	0,02116^{a}	0,03292^{b}	0,02889^{c}	0,02818^{c}
Ni	0,00047^{a}	0,03039^{b}	0,02617^{c}	0,03071^{b}	0,01590^{d}
Cd	0,00164^{a}	0,00108^{a}	0,00272^{b}	0,00345^{b}	0,00144^{a}

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3.8. Bioimaging of endophytic translocation in plants via QDs

In order to assess whether the transformation of endophytic bacteria with QDs leaded to translocation in the plant, the untransformed bacteria were used as a positive control. Treatment of *Brassica napus* L (Garnet) with the untransformed bacteria showed a similar trend with no QD uptake in both the shoots and roots under both filter sets (Figure 14 A & B; Figure 15 A & B). There is a difference in fluorescence signal when comparing the control to the transformed CdTe QD treatments (Figure 14 C –F). The uptake of these QDs in the roots suggests a widespread form of distribution, however, their relative intensities suggest that Garnet has more nanoparticles emitting light in the roots when compared to the shoots. A similar dispersion pattern is displayed within the roots and shoots of the transformed CQD treatment (Figure 15), however, the intensity of light emitted is higher for CQD treatment than that of CdTe QD treatments. When the plants were treated with the transformed bacteria containing the QDs, a complete visual track of the translocation and dispersion patterns can be seen. In the roots of both QD treated plants, the same form of distribution is displayed; with the QDs emitting light throughout the length of the root. However, a

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difference in the intensity of light between the roots and shoots suggest that *Brassica napus* L retains a larger portion of the particles, and thus bacteria, within the roots.

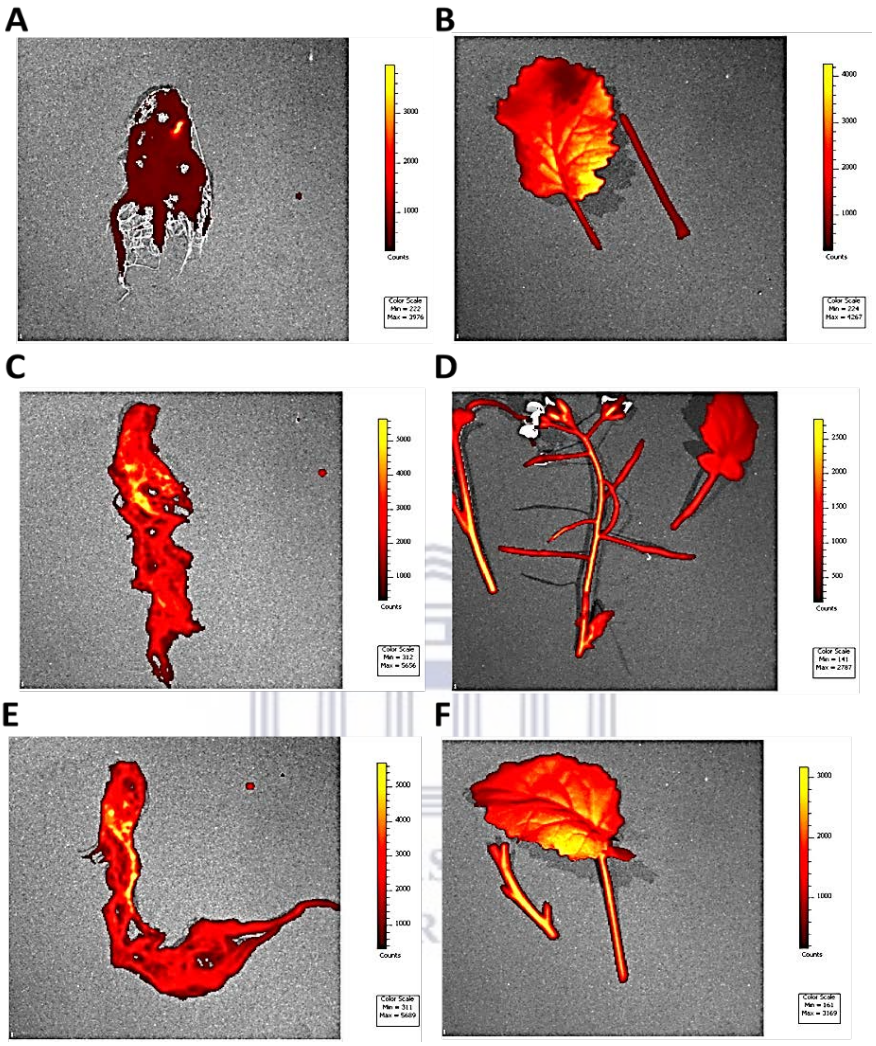

Figure 14. Imaging of the translocation of untransformed (control - A & B) and transformed endophytic bacteria with 0,7 µM CdTe QDs (C & D) and 1,0 µM CdTe QDs (E & F) within the roots and shoots of *Brassica napus* L plants. Colour bars indicate the relative intensity of light emitted. Each image is a representative of a set of 6 images.

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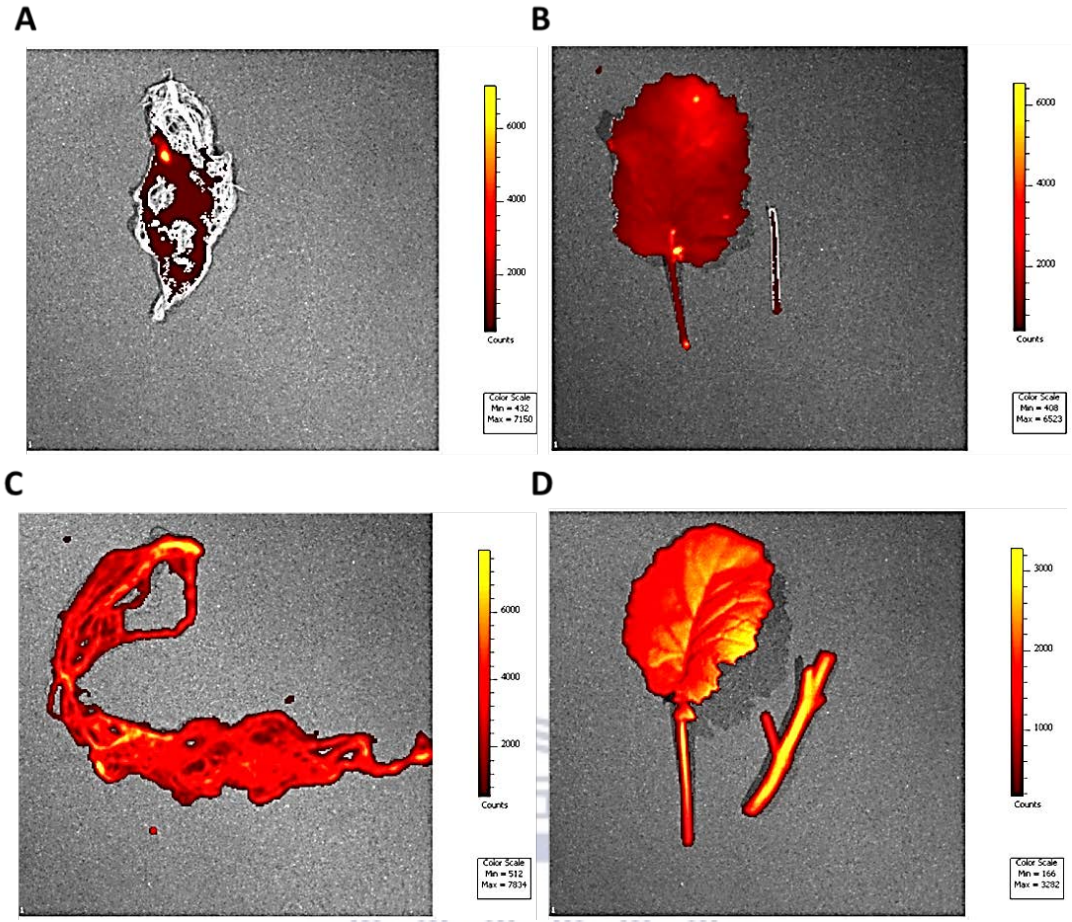

Figure 15. Imaging of the translocation of untransformed (control - A & B) and transformed endophytic bacteria with 150 µM Carbon QDs (C & D) within the roots and shoots of *Brassica napus* L plants. Colour bars indicate the relative intensity of light emitted. Each image is a representative of a set of 6 images.

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CHAPTER 4

Discussion

Endophytes are bacterial or fungal microorganisms that colonize healthy plant tissue intracellularly and/or intercellularly without causing any apparent symptoms of disease (Gaiero *et al.,* 2013). The benefits to plants from host–PGPR interactions have been shown to include improved plant health and growth, suppression of disease-causing microbes, and accelerated nutrient availability and assimilation (Nair and Padmavathy, 2014). Endophyte distribution within plants depends on a combination of its ability to colonize and the allocation of plant resources. Different plant tissues can harbour compositionally distinct endophytic communities (Zhou *et al*., 2015). Endophytes colonize all plant organs, and they have been isolated from the roots, stems, and leaves. The occurrence of endophytes in specific tissues supports the view that they have a particular role in host plant tissues (Khan *et al.*, 2014; Waqas *et al*., 2015 and Hartley *et al*., 2015). This study aims to incorporate nanotechnology in order to provide insight into the translocation and localization of endophytes within the plants. This aims to be the first study done to use a new nanotracking tool in order to provide information on endophyte translocation and colonization.

Water-soluble CdTe QDs were synthesised, which contained MPA as the capping ligand. MPA was used as the protective polymer making the QDs less toxic and biocompatible thus creating hydrophilic QDs as previously mentioned (Section 1.5.2). These semiconducting nanoparticles are composed of cadmium telluride cores and when exposed to UV light the QDs fluoresce in bright, pure, and precise colours of almost any wavelength of visible light. The CdTe QDs synthesized in this study fluoresced a bright pinkish/red colour under UV radiation, thus indicating the successful synthesis of quantum dots (Figure 1A). The excitation wavelength of these molecules were measured at 420 nm and the subsequent emission spectrum displayed a PL peak at 550 nm and 720 nm (Figure 2). This data coincided with that described by Yan *et al* (2010); which revealed a similar emission wavelength for MPA-capped CdTe QDs. It also coincided with the pink colour it fluoresces under UV radiation which indicates high emission wavelength as described by Rizvi *et al* (2010) (Figure 5. Section 1.5.2). There are two emission peaks in the PL spectra and this is likely due to the nanoparticles in

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the sample having a wide size distribution. Thus at different sizes there will be different emission wavelengths. QDs have a broad excitation spectra, their peak emission wavelength is independent of the wavelength of excitation light (Rizvi *et al.,* 2010). This means that variable-sized QDs can be excited by a single wavelength of light, as long as this wavelength is shorter than the absorption onset. This property finds application in multiplexed imaging where a number of different-sized QDs with discrete emission peaks and hence different colours can be excited by a single wavelength of light.

Images provided via TEM showed polydispersed MPA-CdTe QDs with some showing signs of aggregation (Figure 4A). A large size distribution is evident by the images, ranging from 3-6 nm; this can be confirmed by the broad FWHM (130 nm) of the PL curves (Duan *et al.,* 2009). The TEM image obtained coincides with that of the PL spectra (Figure 2B). The software ImageJ was used to manually determine the size range of the nanoparticles synthesized in this study, with the average size and most abundant size being 5 nm corresponds with the colour it exhibits under UV. A study by Shen *et a*l (2013) suggests that broad size distribution and relatively small surface/volume ratio of the particles greatly decreases the quantum yield. These factors are controllable by altering the parameters of synthesis such as the reflux time (Shen *et al.,* 2013). However, for the purpose of this study, the previously described synthesis of QDs was sufficient for its intended use.

Unlike metal-based QDs, carbon quantum dots (CQDs) are composed of an intrinsically non-toxic element, carbon, which makes them a particularly useful and promising bio-analytical tool. For this reason CQDs were used to compare it to the traditional semiconductor quantum dots. The CQDs fluoresced a bright yellow colour under the UV lamp (Figure 1B). The excitation wavelength of these QDs were measured at 320 nm and the subsequent emission spectrum displayed a PL peak at 410nm - 510 nm (Figure 3) which corresponds with that described by Wang and Hu (2014). It also corresponds with the yellow colour it fluoresces under UV radiation which indicates high emission wavelength, but not as high as CdTe as described by Rizvi *et al* (2010) (Figure 5. Section 1.5.2). There are two emission peaks in the PL spectra and this is due to the nanoparticles in the sample not being uniform in size. Images provided via TEM showed QD polydispersity with a size range of 3-5 nm. The TEM image obtained corresponds with PL spectra (Figure 3B). The software ImageJ was used to manually determine the size range of the nanoparticles synthesized in this study, with the average size

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and most abundant size being 4 nm corresponding with the colour it exhibits under UV (Rizvi *et al.,* 2010). The CQDs were bought from Sigma-Aldrich and the data obtained in this study corresponds with that provided literature product information.

For the transformation of bacteria with QDs, different concentrations of both CdTe and CQD nanoparticles were incubated with mid-exponential phase endophytic cells overnight. In agreement with the results of Wenhua and co-workers (2004) fluorescent material colocalized with sedimented cells if they were first made chemically competent by incubation with calcium chloride at low temperature (Wenhua *et al.,* 2004). This treatment transiently affects the integrity of the outer membrane and is routinely used for introducing naked DNA into cells, although the precise mechanisms at play remains unknown (Swift and Baneyx, 2015). QD uptake can already be seen by the deep yellow pellet in the transformed samples (tube 2-4) compared to the light yellow obtained in the control (tube 1) as seen in Figure 5A. Not unexpectedly, QD uptake was dose-dependent and a linear increase in cell fluorescence under UV radiation was observed when the CdTe QD concentration was increased from 0,5 to 1 µg/ml (Figure 5B). There is low QD uptake in the 0,5 µM concentration due to its low concentration thus indicating that as concentration of the QD increases so does the fluorescence. It can be deduced that chemical disruption of the outer membrane is required for the uptake of QDs and uptake of QDs by endophytic cells were successful. The same experimental procedure was followed for the CQDs. Figure 5C shows the resulting pellets with the deepest yellow pellet observed for the highest CQD concentration (tube 4) suggesting the highest uptake of CQD at that concentration. Figure 5D shows the pellets resuspended in saline buffer and then placed under UV radiation. The fluorescent yellow indicates the presence of CQDs thus indicating the successful uptake of CQD of various concentrations (tube 2-4). This was the first visual evidence indicating successful transformation of bacteria by QDs (Figure 5).

Toxicity of the quantum dots was investigated by examining the bacterial growth patterns of the transformed endophytic bacteria compared to the untransformed endophytic control. Exponential growth from the first hour till the second hour was observed for all bacterial cultures (Figure 6A & B). The transformed bacterial CdTe samples shows very little difference in their growth patterns compared to that of the untransformed control. The presence of MPA-CdTe QDs does not completely inhibit the ability of the bacteria to grow suggesting that

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the bacteria is able to tolerate the presence of CdTe QDs even if its growth rate is slightly slowed down.

The same experimental procedure was followed for bacterial samples containing CQDs. Growth of the endophytic bacteria was slightly inhibited but not arrested by the presence of CQDs. The metal based QD transformed bacteria seems to be thriving compared to the CQD transformed bacteria as the CdTe bacterial growth pattern is less affected compared to the CQDs against the controls. The reasonable explanation for these results—no noticeable effect upon bacterial growth rate upon exposure to CdTe QDs over the range of QD concentrations studied—is the low concentration of CdTe (compared to the high concentration of CQD) and the CdTe being capped with MPA thus making the CdTe more biologically compatible compared to the naked CQDs. The results obtained for the bacterial growth pattern looks promising as it shows that the QDs does not arrest the bacteria's ability to grow and can be used for tracking purposes as the bacteria is able to tolerate the presence of QDs. In future studies LIVE/DEAD assays needs to be performed to further explain the results obtained for the bacterial growth curves which will distinguish between bacteria with intact and damaged cell membranes thus giving insight into membrane integrity.

Florescence microscopy was then used to confirm that the QDs were in fact taken up by the endophytic bacteria and to determine the optimum concentration required for efficient bacterial transformation. QDs are useful for tracking particle uptake and movement within a variety of organisms however, fluorescence will only be detected when intact core-QDs are present, as the breakdown components are very unlikely to reconstitute into fluorescent QDs within plants (Mancini *et al*., 2008; Koo *et al*., 2015). The acquired fluorescent micrographs (Figure 7 & 8) demonstrates the cellular uptake of QDs which coincides with the bacterial pellets obtained and placed under UV radiation earlier in the study (Figure 5 B & D). DAPI is a commonly used fluorescent dye for bacterial enumeration or cell counting that binds strongly to A-T rich regions in DNA and is relatively unreactive with inert, non-biological matter, thus making it useful in differentiating between biotic and abiotic components in a sample (Kapuscinski, 1995). The absorption maximum is at a wavelength of 358 nm (UV) and its emission maximum is at 461 nm thus DAPI-stained cells appear blue under a fluorescent microscope. The DAPI stain is therefore used to show the presence of the control against the quantum dots by staining to the nucleus of the endophytic cells. Fluorescence colours

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exhibited for CdTe and CQDs obtained under the fluorescent microscope coincides with the colours it exhibits under UV (Figure 1), with CdTe being red/pink and CQD being yellow. The untransformed endophytic control (Figure 7A) shows the presence of what appears to be blue fluorescent rod shapes of bacteria with no red fluorescence (absence of QDs) indicating the presence of untransformed endophytes. In Figure 7B-D the red florescence emitted from the CdTe QDs increases as concentration of QD increases from 0,5 - 1,0 µM. This confirms the conclusion drawn from Figure 5 which shows the increase in fluorescence from the bacterial pellet under UV radiation as concentration of QD increases. The 1,0 µM CdTe transformed bacterial sample has the highest cellular uptake as shown (Figure 7D) indicating the highest bacterial transformation compared to the 0,5 µM and 0,7 µM CdTe bacterial sample. Thus as concentration of QD increases so does the cellular uptake. The control in Figure 8A corresponds to the control of Figure 7A showing no QD contamination and the clear blue fluorescence of the DAPI stain in the endophytes. As the concentration of CQD increases so does their cellular uptake (Figure 8 B-D) with a decrease in untransformed endophytes. The 150 µM CQD sample has the highest cellular uptake indicating the highest bacterial transformation. Thus for plant trials, the bacterium transformed with 0,7 µM and 1,0 µM CdTe QD and 150 µM CQD were used as it had the highest bacterial transformation and yields the brightest florescence.

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There is a significance difference in QD concentration used for the transformation between CdTe QDs (0,5 - 1,0 µM) and CQDs (50 - 150 µM) and there are three main reasons behind this which can now be explained. Firstly, the CdTe QDs emits a brighter fluorescence signal compared to CQDs (Figure 1), thus if one were to significantly dilute the CQDs it would result in a drastic reduction in its fluorescence compared to the CdTe QD. Secondly, using CdTe QDs at any higher concentrations would not be wise as it is a heavy metal and at very high concentrations would be extremely toxic to the cells and its environment whereas carbon at high concentrations are not as toxic as CdTe QDs would've been. Lastly, it was shown (Figure 7 and 8) that there is a very high uptake of QDs by the bacteria at just 1,0 µM CdTe whereas at 50 µM of CQDs there is very low QD uptake by the bacteria. Only at 150 µM CQD concentration could high uptake be seen, similar to that of 1,0 µM CdTe QDs. Thus the concentration of CQDs at high concentrations are required for good bacterial transformation and low concentrations of CdTe is sufficient for good bacterial transformation.

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Phosphorus is the second most important nutrient for plants, after nitrogen (Oteino *et al.,* 2015). Phosphorous compounds are abundant in agricultural soil and exist as mineral salts or incorporated into organic compounds which is in an insoluble form. Plants require approximately 30 µmol/l of phosphorus for maximum productivity, but only about 1 µmol/l is available in many soils. Therefore, the unavailability of phosphorus in many soils has been recognized as a major growth limiting factor in agricultural and horticultural systems. The promising alternative to chemical fertilizers and pesticides is the use of PGPB inoculants as live microbial biofertilizers. One of the major mechanisms of plant growth promotion by plant associated bacteria is the ability of the bacteria to solubilize phosphate. This involves bacteria releasing organic acids into the soil which solubilize the phosphate complexes converting them into ortho-phosphate which is available for plant uptake and utilization (Oteino *et al.,* 2015). The biochemical activity of transformed bacterial QD samples were compared to that of the untransformed endophytic control to determine if the presence of QDs would have any detrimental effects on the bacteria. Thus the transformed bacteria were tested for phosphate solubilization to detect if the presence of QDs inhibits the bacteria's ability to solubilize phosphate. The presence of halos, which can be seen as the clear zones formed around the bacterial isolates on the phosphate plates (Figure 9A & B), indicates that the endophytes were able to grow on the media and solubilize phosphate. Therefore, these results suggests that this endophytic bacterial strain may play a role in the regulation of phosphate and that role is not affected when transformed with QDs. For both QDs tested, there are halos present for each concentration however, as the concentration increases the clear zone around the isolates decreases in size but does not arrest the bacteria's ability to solubilize phosphate. The availability of soil microorganisms to convert insoluble forms of phosphorus to a soluble form is an important trait in plant growth promoting bacteria for increasing yields (Oteino *et al.,* 2015). The results described here suggests that the endophytic bacteria strain tested possess good phosphate solubilization activity in the absence or presence of CdTe and CQDs.

Phytohormones such as gibberellins, cytokinins, jasmonic acid, abscisic acid, ethylene and indole-3-acetic acid (IAA) are synthesized by diverse plant-associated microbes (Patel and Patel, 2014). Auxins are a group of indole ring compounds which have the ability to improve plant growth by stimulating cell elongation, root initiation, seed germination and seedling

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growth (El-Tarabily *et al.,* 2008). IAA plays a vital role in plant development and at the same time its additional supply can support the host in stress conditions such as drought. Many bacteria isolated from the rhizosphere have the capacity to synthesize IAA *in vitro* in the presence or absence of physiological precursors such as tryptophan. In plants, there are four Trp-dependent pathways that have been proposed: indole-3-acetamide (IAM), indole-3-pyruvic acid (IPA), tryptamine (TRA), and indole-3-acetaldoxime pathways. Although different plant species might use specific strategies or modifications to optimize synthetic pathways, plants would be expected to share evolutionarily conserved core mechanisms for IAA biosynthesis (Fu *et al.,* 2015). The release of L-tryptophan in root exudates may result in its conversion into IAA by rhizosphere microbes. Numerous environmental factors, including pH value and temperature, can influence IAA biosynthesis as well as vitamins and amino acids present. In this study the untransformed endophytic control strain produced high concentrations of IAA of 68 µg/ml (Figure 10 A & B). In a study done by Mohite (2013) it was shown that high production of IAA around 50-63 µg/ml was produced by rhizosphere microorganism after 4 days which correlates with this endophytic strain. In the transformed bacterial culture containing 0,7 µM CdTe QDs, the IAA production increased to 79 µg/ml however, when the concentration of CdTe increased to 1,0 µM the IAA production decreased to 16 µg/ml. This drop in IAA production can clearly be seen in the change of colour from a deep red to a light pink (Figure 10 A). CdTe is a heavy metal and these types of metals at high concentrations become toxic for biochemical activities such as IAA production (Carlos *et al.,* 2016). Bacteria, including PGPB, have different mechanisms that can immobilize, mobilize or modify heavy metals, thus increasing their endurance to heavy metals. IAA auxin microbial synthesis is usually present in 80% of rhizosphere isolated microorganisms, isolated bacteria have the possibility to produce IAA in the presence of heavy metals. This kind of synthesis has been involved in the plant growth and development processes and in their defence strategies. Thus even at relatively high concentrations the CdTe bacterial sample is still able to produce IAA even if its production is inhibited.

The IAA production for the transformed bacterial culture with 150 µM CQD is lower than that of the control with a concentration of 51 µg/ml. However, it does not take away the ability of the endophyte to produce IAA but slightly inhibits it. IAA production by plant growth promoting rhizobacteria can vary among different species and it is also influenced by culture

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condition, growth stage and substrate ability (Mirza *et al.,* 2001). In addition, carbon and nitrogen sources have been proven as essential factors influencing bacterial and fungal IAA production. Earlier studies regarding Rhizobium strains vary in their utilization and production of IAA with different carbon sources. The Rhizobium strains 12, 16 and 18 require sucrose and Rhizobium strain 13 and Rhizobium sp. from *Cajanus cajan* require glucose for maximum production of IAA (Datta and Basu 2000; Shridevi and Konada 2007). However, white-rot fungus *Lentinus sajor-caju* showed increase production in glucose-containing medium but a substantial decrease in IAA biosynthesis when using sucrose medium (Yurekli et al. 2003).

For plant trials the *Brassica napus* L plant was used. The optimal temperature for growth and development is just over 20 °C, growth range between 12 °C and 30 °C. In this study, plants were grown at 20 °C with the same conditions. The plants were treated with LB (control), endophytic bacteria (positive control), transformed endophytic bacteria containing 0,7 µM CdTe QDs, 1,0 µM CdTe QDs and 150 µM CQDs respectively, all having the same OD when inoculated. Following 42 days of plant growth, the LB control had 68% germination and the endophyte control had 87% germination (Figure 11). This increase in germination percentage clearly gives rise to evidence that this endophytic strain increases seedling germination. The CdTe 0,7 µM transformed endophytic sample had 93% germination and the 1,0 µM had 86% germination. The 0,7 µM CdTe sample outcompetes the endophyte control and this correlates to the IAA production in which it produced a higher IAA concentration compared to that of the endophytic control (Figure 10). As stated earlier, IAA production can stimulate seed germination by affecting the activity of enzymes. The CQD transformed bacterial culture showed an 80% germination. All endophyte treated plants (untransformed and transformed bacteria) showed an increase in germination percentage compared to the LB control, further confirming previous studies that endophytes increase seedling germination (Hubbard *et al*., 2012; Paguia and Valentino, 2016 and Walitang *et al.,* 2017). All the samples have more than 70% germination indicating that the QDs are tolerated by the plant and can thus be used as a tracking device for bacteria.

In order to assess the effect of treatment with QDs on the *Brassica napus* L, the roots and shoots of the QD treated and untreated control plants were compared. The growth parameters of both roots and shoots appeared to be positively affected by treatment with

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both CdTe and CQDs. There is a clear change in plant morphology of the QD treated plants compared to both the controls. It can be seen that the plants treated with transformed or untransformed endophytes have an increase in both root and shoot length (Figure 12) and their biomass (Figure 13) when compared to the LB control. There is a 25% increase in shoot biomass and a 48% increase in root biomass of the endophyte control compared to that of the LB control. These results correlate with Long *et al.* (2008) who confirms that endophytic strains isolated showed a change in the root growth of *S. nigrum* which is correlated to the production of IAA and ACC deaminase. This can be seen when comparing the length and biomass of roots and shoots of the 0,7 µM CdTe transformed bacterial treated plants with a 52% increase in root biomass and a 23% increase in the shoots compared to the endophyte control. The IAA production of the CdTe 0,7 µM QD sample was the highest compared to the rest of the samples (Figure 10) thus suggesting that the production of IAA by plant-associated bacteria stimulates root and shoot development of their host plants. This was the only plant that began budding after 42 days (Figure 12) indicating that IAA could be one of many factors which speeds up germination and promotes plant growth (Long *et al.,* 2008). Although 1,0 µM CdTe transformed bacterial treated plants showed relatively low IAA production (16 µg/ml), it seemed to be efficient in promoting plant growth under metal stress. The CdTe 1,0 µM treated plants had the lowest IAA production compared to the rest of the samples however, its root and shoot length and biomass (Figure 12 & 13) was still higher than the endophyte control. Bacterial IAA at low levels has been previously implicated in promoting primary root elongation through cell division (Gravel *et al.,* 2007). In a previous study done by Clarke and Brennan (2012), they showed the effect of cadmium (Cd) on the growth and development of tobacco plants. They found at low levels of Cd concentration there was an increase in shoot elongation and the leaf numbers increased. It is not unusual to find instances where small amounts of Cd stimulates certain growth parameters (Kuboi *et al.,* 1986). An increase in dry weight of lettuce shoots was reported by Turner (1973) when exposed to Cd. In such cases, it was assumed that Cd was enhancing or suppressing the uptake of another nutrient rather than acting as an essential element (John and Van Laerhoven, 1976). It was also suggested that stimulations in growth may be due to increased turgor pressure and stomatal aperture induced by low levels of Cd (Kirkham, 1978). Even though the IAA production obtained by this endophytic strain when transformed with 1,0 µM CdTe QD is significantly inhibited it still produced a higher range of IAA compared to that of

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bacteria with no growth promoting properties. Thus even though the CdTe QD at high concentration inhibits the production of IAA in this bacteria strain, it does not arrest the bacteria's ability to produce IAA.

The CQD treated plants show a similar morphology to that of the endophyte control with the exception of CQD treated plants having thicker roots. This correlates to the 20% increase in root biomass and no significant difference in shoot biomass or morphology between the two. There is an overall increase in root length and biomass of QD treated plants compared to that of the controls (Figure 12 & 13) however, no significant increase in their shoots with the exception of the CdTe 0,7 µM QD treated plants. There is a clear correlation between the plants morphology and their respective biomass. Thus, plants inoculated with endophytes presented a significantly higher root/shoot ratio than the LB control, indicating that with the help of endophytic bacterium, plants can acquire nutrients more efficiently from soils for plant biomass production, especially for roots. Moreover, the length and biomass of root and shoot in media absence of QDs were lower than that in media with the presence of QDs. A possible explanation might be that the endophytic bacteria could exert more efficient functions that help plants to cope with adverse environmental stress (Rajkumar *et al.,* 2009). Another possible reason for the increase in morphology, biomass and IAA production in transformed CdTe QD bacterial treated samples could be due to the CdTe QDs being MPA capped. As explained previously the MPA cap makes the QDs biocompatible and as shown in Figure 4 of Section 1.5.2, the MPA capped CdTe QDs have carboxyl groups exposed. This MPA cap could be causing some pathway to be activated or it could be protecting the plant from avoiding Cd metal ions being released from the CdTe QD.

Plant-derived foods are known to be an important source of proteins and dietary minerals, especially in developing countries. Presently more than three million people worldwide suffer from micronutrient malnutrition, and biofortification or increasing the bioavailable nutrients such as Zinc and Iron in staple crops is considered a feasible option (Rana *et al*., 2012). Among the strategies aimed towards biofortification of mineral micronutrients, enhancement of physiological processes such as uptake from the rhizosphere can be mediated through PGPR. Some of the PGPB bacteria species are widely used in agriculture to increase seeds' production as well as yield and in disease control. PGPB bacteria increases

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plant growth and development directly by regulating the physiology of plants through synthesis of plant hormones and indirectly by increasing the plants access to the soil's nutrients and minerals (Delshadi *et al.,* 2017). In a study done by Delshadi *et al*. (2017), it was shown that the PGPB bacteria studied increased plant dry weight and yield due to an effect on increased nutrient uptake. Thus plant growth promotion can be seen in an increase in plant dry weight, which can be credited to the acquisition of nutrients necessary for the growth of plants. Many previous research has being conducted which has shown that the PGPB bacteria are able to facilitate the acquisition nutrients, such as phosphorous, iron, zinc, potassium, manganese, magnesium, calcium and nitrogen to increase plant growth (Delshadi *et al.,* 2017; James, 2000; Subramanian *et al*., 2014; Rajkumar and Freitas, 2009). In this study ICP analysis were conducted to determine the amounts of macronutrients which included Potassium (K), Phosphorous (P), Calcium (Ca) and Magnesium (Mg) and the micronutrients analysed included Zinc (Zn), Copper (Cu), Iron (Fe), Manganese (Mn), Nickle (Ni) and Molybdenum (Mo). Much of the increase in dry weight observed from plants treated with endophytes when compared to the LB control treated plants can be due to the increase in nutrient uptake (Figure 12 & 13).

The nutrient uptake in roots and shoots of the plants that were inoculated with the untransformed endophyte was much higher compared to plants that was inoculated with CdTe and carbon transformed endophytes with the exception of certain elements. The macro elements Ca, Mg, K and P which were analysed all has an important role to play in the development of plants. In contrast to other macronutrients, a high proportion of Ca is found as a structural component in cell walls playing a major role in the formation of the cell wall membrane and its plasticity (Silva and Uchida, 2000). In the roots alone, there was an 84% increase in Ca of the endophyte control compared to that of the LB control (Table 1). When comparing the 0,7 µM CdTe transformed bacterial treated plants, there was an 18% increase compared to the LB control and a 24% increase in Carbon treated plants. However, in the shoots 0,7 µM CdTe bacterial treated plants showed the highest increase in Ca (15%) compared to the LB control. Root extension, shoot elongation and pollen growth are all dependent on adequate Ca (Carter and Webster, 1979). The secretion of mucilage by root caps which help root tips penetrate through soil also needs sufficient Ca. Downward root growth (gravitropic response) relies on adequate Ca in the root caps. Thus the increase in Ca

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from plants treated with endophytes compared to the LB control could assist in its increase in plant biomass (Silva and Uchida, 2000).

Potassium (K) plays a vital role in the translocation of essential nutrients, water and other substances from the roots through the stem to the leaves (Silva and Uchida, 2000). K regulates the opening and closing of stomata therefore regulating the uptake of CO_2 thus enhancing photosynthesis. Therefore a lack of K results in chlorosis and stunted growth. K is vital for plant growth because K is known to be an enzyme activator that promotes metabolism. As seen in Figure 12 there is no yellowing of the leaves of any of the plants thus indicating that there is sufficient amounts of K present. There was a 15% increase in plants treated with 0,7 µM CdTe and a 25% increase in CQD treated plants when compared to the LB control of the roots (Table 1) even outcompeting the endophyte control. Adequate amounts of K can enhance the total dry mass accumulation of crop plants under stress in comparison to lower K concentrations (Wang *et al.,* 2013). This increase in K for all plants treated with endophytic bacteria in the presence or absence of QDs might be attributable to stomatal regulation by K^+ and corresponding higher rates of photosynthesis. Furthermore, K is also essential for the translocation of photoassimilates in root growth. In a previous study done by Lindhauer (1985), it was reported that fine K nutrition not only increased plant total dry mass and leaf area, but also improved the water retention in plant tissues under drought stress.

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Phosphorus (P) functions in the plant as a structural element and also in energy transfer. The structural components that rely on P include nucleic acids and phospholipids, which are important membrane constituents (Silva and Uchida, 2000). Starch synthesis and nutrient uptake requires ATP which is the principle phosphate energy compound. Energy produced during respiration and photosynthesis is captured by these phosphate compounds, which are then transported to areas that are building plant tissue (Thakur *et al*., 2014). However, this nutrient is one of the most limited resource because it exists mostly as apatite which the plant cannot utilize. A good PGPB bacterial strain have phosphate solubilization properties as previously mentioned. However, there seems to be sufficient amounts of P present in the LB control. Previous research done by Bolland (1997) and Bailey and Grant (1993) suggests that canola is highly efficient at recovering P from soil and fertilizer sources. Canola has the ability to exude hydrogen ions and organic compounds such as citric and malic acids, which

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acidify the root zone and allow mineral P to become plant available (Hinsinger, 2001; Hocking, 2001). In the shoots there was a 33% increase in P for plants treated with untransformed endophytes, a 35% increase for 0,7 µM CdTe bacteria treated plants, 32% increase for 1,0 µM treated plants and a 38% increase for Carbon treated plants when compared to the LB control (Table 2). The increase in the amount of P taken up by the plant will increase its growth, aid in early root development and growth thereby helping to establish seedlings quickly, it also aids in flower initiation, and seed and fruit development (Silva and Uchida, 2000). An increase in P gives rapid and vigorous start to plants, strengthens straw, decreases lodging tendency and brings about early maturity of crops (Thakur *et al.,* 2014). A previous study done by Niedziela (2015) reported that smaller leaves and daughter-bulbs that weigh less were symptoms of P deficiency as well as delayed flowering (Niedziela *et al.,* 2015). This therefore further suggests the ability of this endophytic strain to solubilize phosphate.

Various nutrients are essential for photosynthesis such as Mg which is as a major constituent of the chlorophyll molecule, Fe which is essential in the synthesis and maintenance of chlorophyll in plants, Mn which is involved in the oxidation-reduction process in photosynthesis and Zn which helps the plant produce chlorophyll (Silva and Uchida, 2000). The 0,7 µM CdTe transformed bacterial treated plants showed the highest increase in roots by the elements Zn which increased by 43%, the CQD transformed bacterial treated plants showed an increase of Mg uptake by 31% when compared to the LB control. The untransformed bacteria showed a 27% increase in Fe and 33% increase in Mn in the roots when compared to the LB control. None of the plants treated showed any signs of chlorosis thus indicating sufficient amounts of these nutrients present. Overall all these nutrients increased in the roots but mostly in the shoots of plants treated with endophytes (transformed and untransformed) when compared to the LB control (Table 1 & 2).

Nickle (Ni) is a micronutrient that is required by both higher and lower plants in very small amounts and plays a part in seed germination, iron uptake, nitrogen fixation and reproductive growth (Brown *et al*., 1987a). In the shoots there is 97-99% increase in Ni when comparing the plants treated with endophytes to the LB control. There is also a 45% increase in plants treated with untransformed endophytes and a 73% increase in plants treated with 0,7 µM CdTe bacterial culture compared to the LB control of the roots. This increase in Ni

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could play a role in the increase in germination percentage achieved by the respective treated plants (Figure 11). The earliest report of a growth response to Ni additions under controlled experimental conditions indicated that Ni deficiency has a wide range of effects on plant growth and metabolism. These include effects on plant growth, plant senescence, N metabolism and Fe uptake (Brown *et al*., 1987b). In a previous study done by Brown *et al.* (1987a), they reported a decrease in germination percentage in plants grown without Ni additions to their growth media whereas plants grown with Ni in their nutrient solutions produced grain with germination percentages in excess of 95%. This result therefore suggests the positive role that endophytes have on nutrient uptake which could further explain the increase in germination percentage for plants treated with endophytes especially for that of 0,7 µM CdTe.

Copper (Cu) increased in both roots and shoots compared to the LB control with 1,0 µM CdTe being slightly inhibited in the shoots. The Cu increased for 0,7 µM CdTe and 150 µM Carbon treated plants compared to that of the endophyte control in the roots by 23% and 29% respectively (Table 1). Cu is essential in several plant enzyme systems involved in photosynthesis and is part of the chloroplast protein plastocyanin, which forms part of the electron transport chain (Silva and Uchida, 2000). Cu also increases plant growth. Deficiency in Cu results in interveinal chlorosis shortly after emergence, wilting leaves and delayed flowering with a shortened flowering stem. Zn is required in the synthesis of tryptophan, which in turn is necessary for the formation of indole acetic acid (IAA) in plants and plays role in RNA and protein synthesis (Hafeez *et al*., 2013). Zn also enhances budding and its deficiency results in chlorosis and inhibited stem elongation (Bloodnick, 2017). The highest increase in Zn is for 0,7 µM CdTe treated plants (43%) followed by the untransformed endophyte treated plants (40%) when compared to the LB control plants roots (Table 1). Zn deficiency results in the new leaves being smaller in size, reduced flowering and budding shortened internodes. This high increase in Zn for 0,7 µM treated plants could play a role in its high increase seen for IAA production (Figure 10) and assist in the early budding as it was the only plant that began budding after 6 weeks (Figure 12).

In this study, PGPB inoculation led to an enhancement in the Zn, Fe and Cu content of canola plants, indicating a possible role in improving the translocation of micronutrients (Rana *et al.,* 2012). Several genes have been implicated in translocation of metal micronutrients into the

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xylem or across the root-shoot junction, including FRD3, FPN1, HMA2, HMA4, HMA5, and MTP3 (Waters and Sankaran 2011). Increased flux into shoots could possibly provide additional micronutrients for seed biofortification, and may activate native homeostasis mechanisms that increase root uptake capacity to keep pace. Once the nutrients are taken up into the root symplast, nutrients must be released into the dead xylem elements for their long-distance transport, with the transpiration stream to the shoot (Sondergaard *et al*., 2004). Clarkson proposed that ion secretion into the xylem vessels occurs across the plasma membrane of the living cells bordering the xylem consisting of pericycle cells and xylem parenchyma (Clarkson, 1993). Many transporters have been localized to the root pericycle, and in several cases it has been shown that they are involved in xylem loading of nutrients. Some examples, such as the SKOR, BOR1, and SOS1 transporters, are illustrative. The *SKOR* gene encodes an outward-rectifying K^+ channel in the plasma membrane that is expressed in the root pericycle and stelar parenchyma cells (Gaymard *et al.*, 1998). Its disruption by a transferred DNA results in strongly decreased K^+ translocation toward the shoots. The plasma membrane-localized Zn^{2+} pumps HMA2 and HMA4 are present in vascular tissues (Hussain *et al.*, 2004). In a double mutant, where both genes encoding these pumps are disrupted, Zn^{2+} accumulate in the root, whereas shoots show Zn^{2+} deficiency symptoms. This would suggest that these pumps are indeed involved in xylem loading of Zn^{2+} in the root. Therefore for future work, different ion transporters and genes expressed involved in nutrient uptake needs to be identified to further explain the increase of some nutrients from the roots to the shoots.

It could be speculated that the observed plant growth promotion might have been caused by enhanced plant mineral uptake, hormonal stimulation and improved plant water relationship associated with the colonization by this endophytic strain. Results from the present study clearly shows that this specific endophytic isolate holds great growth promoting potential, such as superior nutrient acquisition capabilities, increased phosphorus solubilization and was able to releases high levels of IAA. Even in the presence of QDs the endophyte was still able to improve nutrient acquisition when compared to the LB control. However, upon transformation most of the nutrient profiles decreased when compared to the untransformed endophyte.

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Each type of plant is unique and has an optimum nutrient range as well as a minimum requirement level. Below this minimum level, plants start to show nutrient deficiency symptoms (Silva and Uchida, 2000). No plants treated showed any signs of nutrient deficiency thus even when treated with QDs, the plant was still able to take up nutrients efficiently and this could be due to the presence of the endophytes and the MPA cap around the CdTe QDs. The increased mineral content in all plants treated with endophytes could be attributed to the enhancement of the root growth and development due to the positive influence of microbial activity. Therefore, the results of this study suggest that inoculation with promising bacterial strains can enhance macro and micronutrient content as well as plant yield, and improve soil quality by enhancing microbial activity in the rhizosphere region. Such strains represent promising candidates for inclusion in integrated nutrient management practices for canola crop.

IVIS Lumina II imaging system is a highly flexible and versatile equipment routinely used for quantitative and non-invasive pre-clinical imaging of lab animals (Daniel *et al*., 2013). This device has an increased fluorescent transmission extending into the near infrared (NIR) range with full transmission through 900 nm. This new illumination technology in combination with the company's patented Compute Pure Spectrum (CPS) algorithm ensures spectral unmixing for sensitive multispectral imaging to monitor multiple biological events occurring in the same organism (Guatham *et al.,* 2014). This bioimaging system proved to be a useful tool for semi-quantitative measurement and spatial distribution of QDs translocation in the shoots and roots of *B.napus* L. Detection and quantification of the nanoparticle is hampered by auto-fluorescence of plant tissues, which is mainly due to chlorophyll (Stephan *et al*., 2011). However, interference by autofluorescence of plant tissues can often be reduced or eliminated by specific optical filters thus in this study the untransformed bacteria treated plants were used as a positive control. The filter settings and system software significantly reduced background fluorescence by chlorophyll in the leaves. When comparing the transformed and untransformed bacteria clear translocation of the endophytic bacteria within the plant can be seen (Figure 14 & 15). The untransformed endophytic bacteria (control) had low fluorescence in their roots and shoots compared to the CdTe QDs transformed endophytic bacteria. The fluorescence of the roots and shoots of CdTe 0,7 µM treated sample and that of the CdTe 1,0 µM treated sample was similar in intensity and

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distribution. For both roots (Figure 14 C & E) there is a clear increase in fluorescence from a bright red to yellow colour indicating the increase in fluorescence signal at that point in the roots when compared to the control containing dark red fluorescence thus no QDs present (Figure 14A). This shows that the roots contain most of the endophytic bacteria as it has the strongest fluorescent signal compared to the shoots. In a previous study done by Compant *et al.* (2005), the PsJN endophytic strain tagged to GFP colonized the root interior and thereafter spread to the stems and leaves. Other studies have also detected endophytes within aerial plant parts, including stems, leaves, and flowers (Lamb *et al.,* 1996; Misaghi and Donndelinger, 1990; Reiter *et al*., 2002; Sessitsch *et al.,* 2002). It has been suggested that bacteria can be transported in xylem vessels through the transpiration stream or by colonizing intercellular spaces from roots to aerial parts (Elbeltagy *et al.*, 2001; Gyaneshwar *et al.,* 2001; Hurek *et al.,* 1994; James *et al.,* 2004; Shishido *et al*., 1992). The translocation of the endophytic bacteria can clearly be seen moving from the roots into the stems and then the leaves (Figure 14 D & F). The bright fluorescent yellow colour present in the stems and leaves of the transformed bacteria treated plants and the absence of it in the untransformed bacteria treated plants (Figure 14B) indicates the presence of endophytic bacteria in those regions of the plant. For the CQDs, different filter sets were used due its lower emission wavelength compared to that of CdTe QDs. The exact same pattern was seen when analysing the transformed bacteria with Carbon QD treated plants (Figure 15). This pattern of uptake and translocation was similarly displayed by CdTe QDs. Thus these images generated by the IVIS Lumina imaging system showed the successful use of using QDs for tracking the translocation of bacteria within a plant. This imaging system proved to be successful in tracing the uptake and translocation of QDs within *B. napus* L; more importantly allowing visualization of endophytic translocation, in which the QDs were essentially taken up by.

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CONCLUSION AND FUTURE PROSPECTS

While fluorescent dyes and proteins have been applied broadly to problems in biological sciences, some of their inherent weaknesses have significantly limit the potential of related fluorescence techniques. QDs are appealing alternatives to conventional fluorophores due to their superior optical properties and have the potential to meet some of these challenges in biotechnology. The first part of this study required the QDs to be transformed into the endophytic bacteria. QD uptake was initially determined by the transformed bacterial cultures fluorescence under a UV lamp. Subsequent analysis was done using a fluorescence microscope which confirmed successful transformation of endophytes with QDs. The images obtained from the fluorescence microscope revealed that the QD into bacteria (uptake) was dose-dependent therefore as the concentration of the QD increased so did the cellular uptake. The second part of the study involved toxicity studies of the untransformed bacteria compared to that of the transformed bacteria to determine the possible effects QDs could pose on the bacteria's growth and biochemical activities. The QDs showed no detrimental effect on the IAA production of the bacteria, even increasing it at a certain concentration. When the plants were treated with QD transformed bacteria it yielded greater plant biomass and showed an increase in seed germination and root and shoot elongation when compared to untransformed bacteria. All these factors indicate that the bacteria and the plant are able tolerated the presence of CQDs and MPA-capped CdTe and at low concentrations, can be used as a new nanotracker tool. The final part of this study proved that QDs has the potential to be an effective tool for the visualization of the translocation of endophytic bacteria within plants. This was noticeable from the acquired images captured by the IVIS Lumina bioimaging system indicating the successful translocation of the bacteria through the roots to the stems and leaves of *Brassica napus* L using QDs as the fluorescence signal. The practical use of nanoparticles such as QDs were perfectly displayed in this study. Its unique physical, chemical and optical properties make them a useful tool for targeting and imaging several other molecules as well.

To validate and justify the biochemical results observed in this study, future work entails more comprehensive analysis. This will be done by subjecting the transformed bacteria containing QDs to cell death assays, LIVE/DEAD membrane integrity assays, siderophores and

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ACC deaminase assays etc. to further understand and explain the effects QDs could have on the bacteria and plant. Toxicity of nanoparticles is a major concern and the lack of studies in this area of research requires intensive studies to be done thus determining the effect of QDs on the bacteria is an important criteria for future work. Confocal microscopy is another instrument that could be used in future work to determine the translocation of bacteria within a plant using QDs as the signalling molecule. The bacterial strain in this study needs to be identified using 16S DNA sequencing to determine its genus and species name.

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