The Application of a Novel Microbial Sensor on Tomato (*Solanum lycopersicum* L.) Growth Monitoring

by

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ABSTRACT

Precision agriculture (PA) integrating information technology arouses broad interests and has been extensively studied to increase crop production and quality. Sensor probe technology, as one of the PA technologies, provides people with accurate real-time data, which has become an essential part of precision agriculture.

Herein a novel microbial sensor probe (MiProbE) is applied to monitor and study the growth of tomatoes (*Solanum lycopersicum* L.) in real-time at germination and seedling stages. The result showed the raw Miprobe signals present day/night cycles. Alginate-coated probes effectively avoided signal response failure and were more sensitive to the treatments than uncoated probes. The probe signals from successfully germinated tomato seeds and non-germinated seeds were different, and the signal curve of the probe was closely related to the growth conditions of tomato seedlings. Specifically, the rising period of the probe signals coincided with the normal growth period of tomato seedlings. All probes exhibited sudden increases in signal strength after nutrient treatments; however, subsequent probe signals behaved differently: algae extract-treated probe signals maintained a high strength after the treatments; chemical fertilizer-treated probe signals decreased earlier after the treatments; chemical fertilizers and algae extract-treated probe signals also maintained a higher strength after the treatments. Moreover, the relationship between ash-free dry weight and the signal curve indicated that the signal strength positively correlates with the dry weight, although other biological activities can affect the probe signal at the same time. Further study is still needed to investigate the relationship between plant biomass and Miprobe signal.

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CHAPTER 1

INTRODUCTION

In the past few decades, the greenhouse effect, environmental pollution, and food security have attracted people's attention. Precision agriculture (PA) is a farming management concept that optimizes yield and quality, increases profitability, reduces environmental impacts and inputs (Balafoutis et al., 2017; Kumar & Ilango, 2018; Stafford, 2000). Managing the spatial and temporal crop and soil variability within a field and providing access to a large amount of data can help make management decisions. (Stafford 2000; Fountas et al. 2005; Reichardt and Jürgens 2009; Aubert et al. 2012; Shibusawa, 1998; Clasen 2016). The rapid development of PA is mainly due to the wide application of several technologies, including Global Positioning System (GPS), geographic information system (GIS), miniaturized computer components, automatic control, sensors, advanced information processing, and telecommunications (Gibbons, 2000).

Many types of probes have been applied to collect various field data. A near-infrared (NIR) soil sensor can be used to predict soil organic matter, moisture contents of surface and subsurface soils by measuring soil spectral reflectance within the waveband of 1600nm to 2600 nm (Hummel et al., 2001). NIR sensors can also predict nitrogen content, but it needs strict pretreatment of soil samples (Nie et al., 2017). Soil electrical conductivity (EC) sensor has been proven to effectively detect soil water holding capacity in non-saline soils (Lund et al., 2000). Electrochemical sensing combined with an ion-selective membrane can measure the soil nutrient parameters such as nitrate, phosphate, potassium, but the accuracy is a big problem in long-term and continuous measurements (Ali et al., 2020). Numerous researchers and manufacturers have attempted to develop soil sensors to

measure mechanical, physical, and chemical soil properties (Adamchuk et al., 2004). However, few probes are designed to collect plant physiological data in real-time.

In agriculture monitoring, it is essential to collect spatialized information about growing conditions of the vegetated land (Atzberger, 2013). Admittedly, remote sensing can significantly contribute to gathering the information of crop biomass and yield, plant stress, and crop disease over large areas (Atzberger, 2013; Kastens et al., 2005; Zhang et al., 2005; Balint et al., 2011; Yuan et al., 2017). However, there are still many problems in remote sensing. For crop yield forecasting, crop-specific masks are always needed, and several difficulties are encountered when such masking is applied to different crops and/or multiple years of imagery (Becker et al., 2010; Atzberger, 2013). For crop disease and pest monitoring, remote sensing can only conduct at the leaf, canopy, and field levels (Yuan et al., 2017). Another weakness of remote sensing is that it cannot provide precise data over small areas due to its limitation on resolution. Therefore, providing information on plant roots, covering small areas, is needed to make up remote sensing drawbacks.

In the development of biofertilizers and breeding, researchers usually face a heavy workload on screening. Meanwhile, there are no available tools that allow researchers to rapidly discover the advantages of biofertilizers or varieties (e.g., several hours) instead of a few months or an entire season. For instance, algae extracts have been found to have bio-stimulating effects that can improve plant growth, nutrient use efficiency, tolerance to abiotic stresses, and product quality in limited quantities (Calvo et al., 2014; Chiaiese et al., 2018; Du Jardin 2015; Rouphael and Colla 2018; Parrado et al., 2007). Many studies have shown that the bio-stimulating effects vary depending on the different algal strains, different types of crops, timing, and doses (Barone et al., 2018; Farid et al., 2019; Layek et

al., 2018; Mutale-joan et al., 2020; Nie et al., 2017; Saadaoui et al., 2019; Trivedi et al., 2017). Therefore, a quick and effective screening method is in high demand in the future.

The connection between plant roots and rhizosphere microorganisms is strong and closely related to plant physiological activities. The rhizosphere is a natural reservoir for numerous organisms and is recognized as one of the most complicated ecosystems (Hinsinger & Marschner, 2006; Pierret et al., 2007; Jones & Hinsinger, 2008; Hinsinger et al., 2009; Raaijmakers et al., 2009). It has been clarified that some important symbiotic microorganisms can reduce the incidence of plant diseases, promote nutrient utilization of host plants (Spence et al. 2014), and resist non-biological stress (Friesen et al. 2011; Zelicourt et al. 2013). In return, plants provide carbon sources for microbial growth activities and community composition through rhizosphere sedimentation (Mendes et al., 2013). Soil microorganisms have been proven to improve plant growth by transforming and translating essential nutrients in the soil and making them available to plants (Richardson, 2001; Gyaneshwar et al., 2002; Khan et al., 2007; Richardson et al., 2009). Toju et al. (2018) found that plants can establish beneficial connections with some microorganisms in the rhizosphere to reduce the damage caused by drought. Cook et al. (1995) found that plants actively recruit beneficial soil microorganisms in their rhizospheres to counteract pathogen assault. Vurukonda et al. (2016) also concluded that plant growth-promoting rhizobacteria improve plant nutrition and induce host plants to develop resistance to biotic and abiotic stresses by dissolving minerals, secreting plant growth regulators, inhibiting pathogenic microorganisms and self- metabolites. Nitrification and denitrification are essential activities of soil microorganisms in the nitrogen cycles (Hayatsu et al., 2008). Plant and microorganisms may compete for nitrogen

when nitrogen is limited in the environment (Hodge et al., 2000; Liu et al., 2016; Kaye& Hart, 1997). Because of a close relationship between plant roots and rhizosphere microbial activities, the activities of rhizosphere microorganisms will affect the health and growth of plants. Thus, while activities such as photosynthesis and respiration may be used to monitor plants directly, indirect monitoring through rhizosphere microorganisms' activities may also hold possibilities.

The Miprobe is a potentiometric sensor, which is similar to pH probes. However, the biofilm theoretically functions as a selective membrane rather than a glass membrane. The biofilm mediates the voltage of the sensors, which results in a composite signal of multiple environmental parameters. Many environmental parameters such as redox, nutrient, and humidity will impact the measurements in the soil environment. Hence, normalization of the raw data is always needed to eliminate those environmental impacts. The Miprobe needs a reference electrode; the Ag/AgCl electrode of the ORP probe is used as the reference in this study. Like the ORP probes, Miprobe is designed to monitor changes in a system rather than determine their absolute value.

In this study, the microbial sensor (MiProbE™) was tested to monitor tomato (*Solanum lycopersicum* L.) seedlings' growth and biomass accumulation. Tomato seeds were selected for this study, because of their small size and ease of cultivation. A control experiment was conducted first to investigate the performance of Miprobe in a sterile environment. This control experiment also aimed at observing the germination of seeds and root activities. A second experiment was carried out in a non-sterile environment. Two types of tomato seeds were prepared (alginate-coated and uncoated seeds) to evaluate the effects of alginate on the Microbe signals. Three different nutrient combinations were

applied to tomato seedlings to evaluate the performance of Miprobe on monitoring nutrient acquisition by tomato seedlings. The third experiment was to evaluate the ability of Miprobe for tomato biomass monitoring.

CHAPTER 2

EVALUATION OF MIPROBE PERFORMANCE IN A LOW-MICROBIAL ABUNDANCE ENVIRONMENT

Abstract

The purpose of the experiment was to investigate the probe's performance in a sterile or low-microbial abundance environment. All experimental materials and tools were initially either surface-sterilized or autoclaved. The result showed that the probe signal was unstable before tomato (*Solanum lycopersicum* L.) seed germination and became stable after. Raw Miprobe signal is negatively correlated with ORP and water level and does not work well in low microbial abundance environment. The normalized probe signals increased during the normal growth period of tomato seedlings. Two weeks after tomato germination, tomato seedlings developed leaf chlorosis symptoms, while the probe signal showed a downward trend. Therefore, the probe signal can identify the germination of seeds, and the signal strength may be related to plant growth condition.

2.1 Introduction

The MiProbE™ microbial sensor is a novel microbial sensor probe technology, combined with a Microbial Signal Processor™ and cloud-based analytics platform to monitor the microbial biofilm response in real-time (MiProbE™ Technology, n.d.). This sensor technology also works when biofilm fouling prevents reliable OPR (Oxygen Reduction Potential) or DO (Dissolved Oxygen) sensor readings because the biofilm is its active surface for the sensor probe, which means it does not need maintenance (MiProbE™

Technology, n.d.). The sensor probe can work independently or cooperate with other sensors for the optimized process (MiProbE^{TM} Technology, n.d.).

Alginate is a naturally occurring anionic polymer typically obtained from brown seaweed. It has been extensively investigated and used for many biomedical applications due to its biocompatibility, low toxicity, relatively low cost, and mild gelation by adding divalent cations such as Ca^{2+} (Gombotz & Wee 1998). Alginate hydrogels are three-dimensionally cross-linked networks composed of hydrophilic polymers with high water content and commonly used in biomedicine, including wound healing, drug delivery, and tissue engineering applications. (Yong & Mooney, 2012). A combination of aqueous alginate solution with ionic cross-linking agents is the most common method to prepare hydrogel (Park & Mooney, 2012). Calcium chloride ($CaCl_2$) is one of the most frequently used agents to cross-link alginate ionically. Encapsulation of living cells in a polymeric gel is a well-established technology in a broad and increasing range of different applications (Park and Chang, 2000). Many studies have demonstrated that alginate hydrogel is an ideal matrix for bacteria growth it can be used as an inoculant carrier for soil bacteria (Fenice et al., 2000; Zohar-Perez et al., 2002; Bashan, 1986; Young et al., 2006).

Here, alginate hydrogel was used to fix tomato (*Solanum lycopersicum* L.) seeds to the Miprobe directly, though the effects of seedling roots and the abiotic environment on the Miprobe signals were previously unknown. It is essential to know how the Miprobe, as a microbial sensor, reacts in the absence or low abundance of microbes, however. Thus, four main hypotheses were postulated: 1) that soil microbe and tomato activities could be observed; 2) that germination vs. non-germination can be distinguished; 3) that seeds vs. seedlings can be distinguished; and 4) that seedling growth conditions can be distinguished.

2.2. Materials and Methods

Preparation of alginate

3.3 g of sodium alginate (Alginic acid sodium salt from brown algae, BioReagent, suitable for immobilization of microorganisms, Sigma-Aldrich) and 4.3 g of MOPS were dissolved in 225 ml water. Then the suspension was stirred at 180 rpm and 95 ℃ until it is completely dissolved. The dissolved gases during the stirring process in the alginate solution were removed with a vacuum pump. Then, the alginate solution was sterilized at 121 ℃ for 30 min. $CaCl_2$ solution was prepared by dissolving 2.49 g $CaCl_2$ and 4.7 g of MOPS in 225 ml deionized water (DI water). The solution was autoclaved at 121 ℃ for 45 min.

Alginate coating of seeds

The seeds of tomato ‘Golden Jubilee’ (Marde Ross & Company) were surface sterilized with 30% bleach for 15min, then washed 3 times with sterile water. Coating of seeds was carried out in a sterile laminar flow hood. The alginate was added dropwise with the aid of a 10-ml sterile syringe on the tomato seeds and the probe surface. The coated seeds and probes were then boned together, soaked in 0.1 M $CaCl_2$ solution to form the alginate hydrogel (Fig. 2.1.). The excess Ca^{2+} ions were washed out with sterile tap water after 3 min of the formation of alginate hydrogel.

Fig. 2.1 - Coated Miprobe and Tomato Seed.

Miprobe lab instrumentation

MiProbe Gen2 electrodes (Fig. 2.1) and Raspberry Pi 4 Compute Module (Fig. 2.2) were adopted in this experiment. The Miprobe Gen2 is able to work in soils and aqueous environments, and the graphite, titanium, carbon fiber–based sensing electrodes benefit the outdoor/long-term deployments and soil application of the probe. The Gen2 Miprobe uses ORP (oxygen reduction potential) as a reference. The configuration of Raspberry Pi 4 is as follows:

- eMMC-based OS storage
- Quad-Core 1.5ghz 64bit ARMv8 SoC
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- Supports PCIe-based ML/AI Accelerators

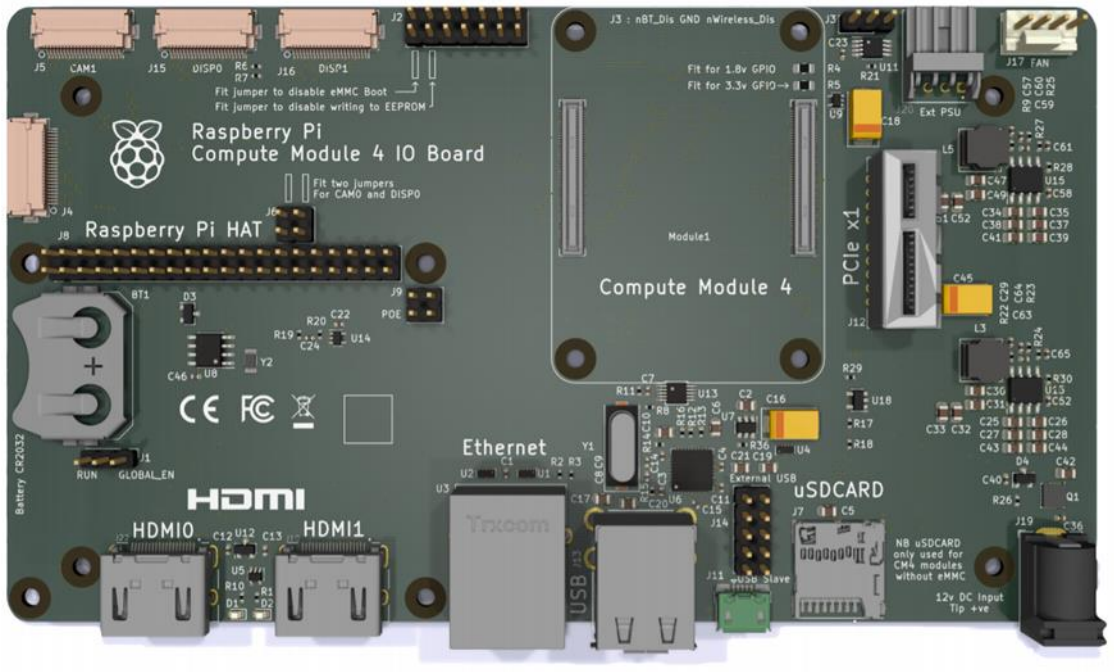

Fig. 2.2 Raspberry Pi 4 Compute Module

MiProbe Lab Instrumentation (Fig. 2.3) utilizes a cross-platform (Linux/Mac/Windows) Python SDK for automatically logging data off the USB/Serial interface. This allows automation of data logging from each sensor connected to the host device (usually a Raspberry Pi 3B or 4 running Linux). The Python SDK provides a demo logging tool and libraries for automating complex experiments to a NoSQL-based Cloud Database (DynamoDB).

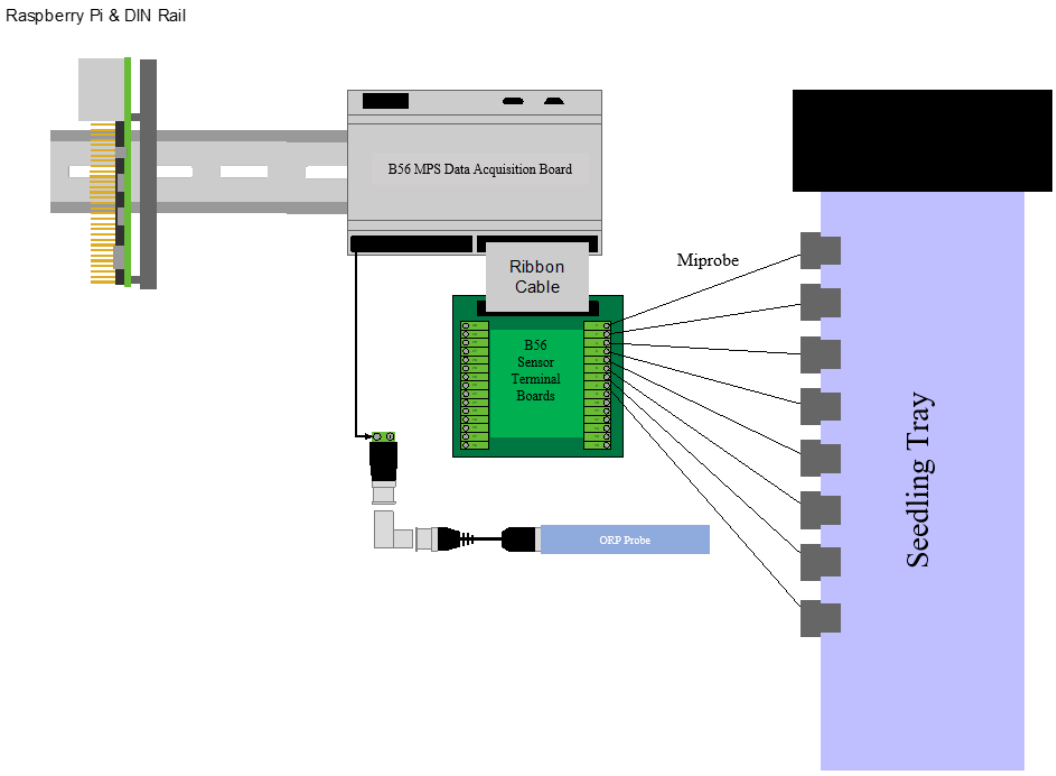

Fig. 2.3 Miprobe and Other Components

Experimental design

The experiment was started on Oct. 26th and lasted for 35 days. A total of 30 tomato seeds along with probes were started in a sterile environment. Specifically, tomato seeds were surface sterilized with 30% bleach, the seedling tray was surface sterilized with 75% ethanol, while soil, alginate, tap water, and tools (such as tweezers and syringes) were autoclaved for 45 mins at 121 °C. A completely randomized positional experimental design was adopted in this experiment (Salkind, 2010). The seeds were embedded in root riot cubes (Hydrodynamics International Inc., USA) 1cm deep. The tray was cultured in a dark room, and light was provided by two Giixer 1000W LED grow lights (NOVA SILK ROAD SARL, France) in full- spectrum for 24 hours per day. The seedling cubes were put in

starter trays and soaked in water, and sterile tap water was added every two days to keep the water level at 2 cm (Fig. 2.4). The air temperature during the experiment was 22 ℃ and humidity was 27 %.

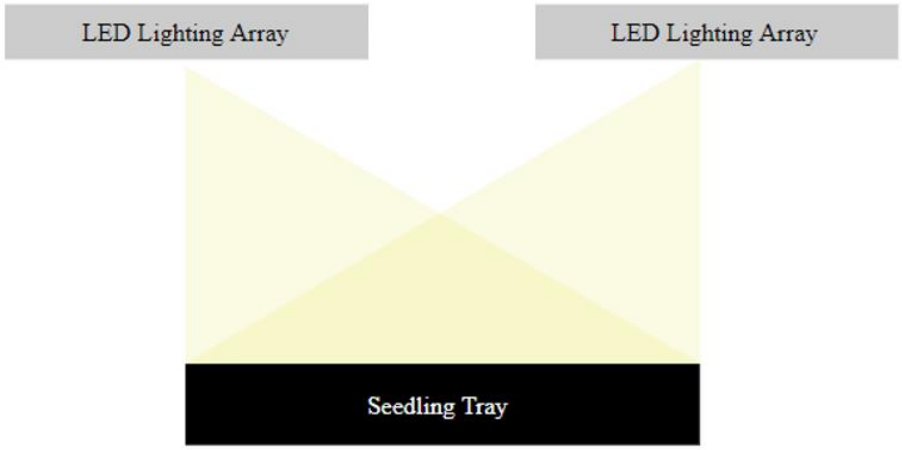

Fig. 2.4 Experimental Setup

Normalization of raw Miprobe signal

Normalization is aimed to improve visualization. The normalized data was obtained by subtracting the average of non-germinating seed probe signals from other probe signals.

2.3. Results and Discussion

The data between day 6 and day 8 was lost due to the disconnection of the Wi-Fi network, because the Raspberry Pi cannot automatically restart after the disconnection. The N04 probe was broken in this experiment, so it was excluded from the dataset. The seeds that did not germinate include N18, N21, N12, N16, N08, N07, and N10. The germination rate was 76.67%.

In the first week of the experiment (day 0 to day 8), probe signals had bigger fluctuations, and the correlation between probe signals in this period was low (Fig. 2.5) compared with the r^2 correlation between probe signals in the rest of the experiment, which is higher than 0.9 (Fig. 2.5). The Miprobe signals are based on ORP Ag/AgCl reference, so the ORP probe (N54) is also closely correlated with Miprobe signals. The signal fluctuation may be caused by low microbial abundance in the environment.

The raw Miprobe data is shown in Fig. 2.7. As the figure shows, ORP is negatively correlated to the Miprobe signal. The water level was low on day 12 and was replenished at 2 PM on that day. After the water was replenished, the ORP showed an increasing trend while the Miprobe signal decreased. Therefore, it can be concluded that the Miprobe signal is negatively correlated to the ORP and water level. It can also be found that the raw Miprobe signal showed more significant fluctuations in the first week of the experiment. The signal fluctuations may relate to low microbial abundance in the environment. As a result of environmental disturbance, it is necessary to improve visualization of the Miprobe signal by normalization.

The average of normalized data is shown in Fig. 2.8. The average of normalized data also presented fluctuations from day 0 to day 5 (SD = ±2.56), compared to the signal from day 9 to day 14 (SD = ±1.05) (Fig. 2.8). It can be estimated that the probe signals tend to have fewer fluctuations around day 6 (the stabilization period is about 7 days). There was an increase from day 6 to day 16, while the signal trend started to drop after day 16. Network fluctuations may cause the spikes on day 11, 14, 25, and 30. There was no recorded human disturbance on these days.

The first recorded tomato seeds germination date was on day 4, and most of the tomato seedlings germinated on day 8 (Fig. 2.6a and Fig 2.6b). On the other hand, the probe signals started to stabilize from day 6. The probe signals may have stabilized because of the gathering of microbes in the rhizosphere. Therefore, the coincidence of seed germination and probe signal stabilization demonstrated that the Miprobe is able to identify seed germination.

Observation showed that tomato seedlings grow normally before day 17. Slightly yellowing on old leaves was observed after day 17, and nitrogen deficiency and/or 24-hours photoperiod may account for it (Fig. 2.6c). Observations on day 27 showed that tomato seedlings developed severe symptoms of leaf chlorosis. Specifically, the new leaves turned yellow, the old leaves withered, and the growth of new leaves is hindered (Fig. 2.6d). The new leaves started to wither on day 30 (Fig. 2.6e), and only one single leaf was left on some plants on day 35. The Miprobe signal rose from day 6 to day 17, and the slope is higher before day 10, which coincides with the normal growing period of tomato seedlings. The probe signal started to drop after day 17 and became stable after day 30. Nutrient deficiency and/or 24-hours photoperiod may cause the decrease in signal after day 17, and the stable signal strength may represent a stable condition of the seedlings (Fig. 2.8).

In summary, the probe signal appears correlated to tomato seedling growth conditions (Fig. 2.8). Normalized probe signals increased during the growth period of tomato seedlings (day 4 to day 11). Two weeks after tomato germination, tomato seedlings developed nutrient deficiency symptoms (day 10 to day 17), while the probe signal showed a downward trend. In future experiments, other growth parameters such as dry weight, leaf number, and chlorophyll concentration are needed to pair with the probe signal.

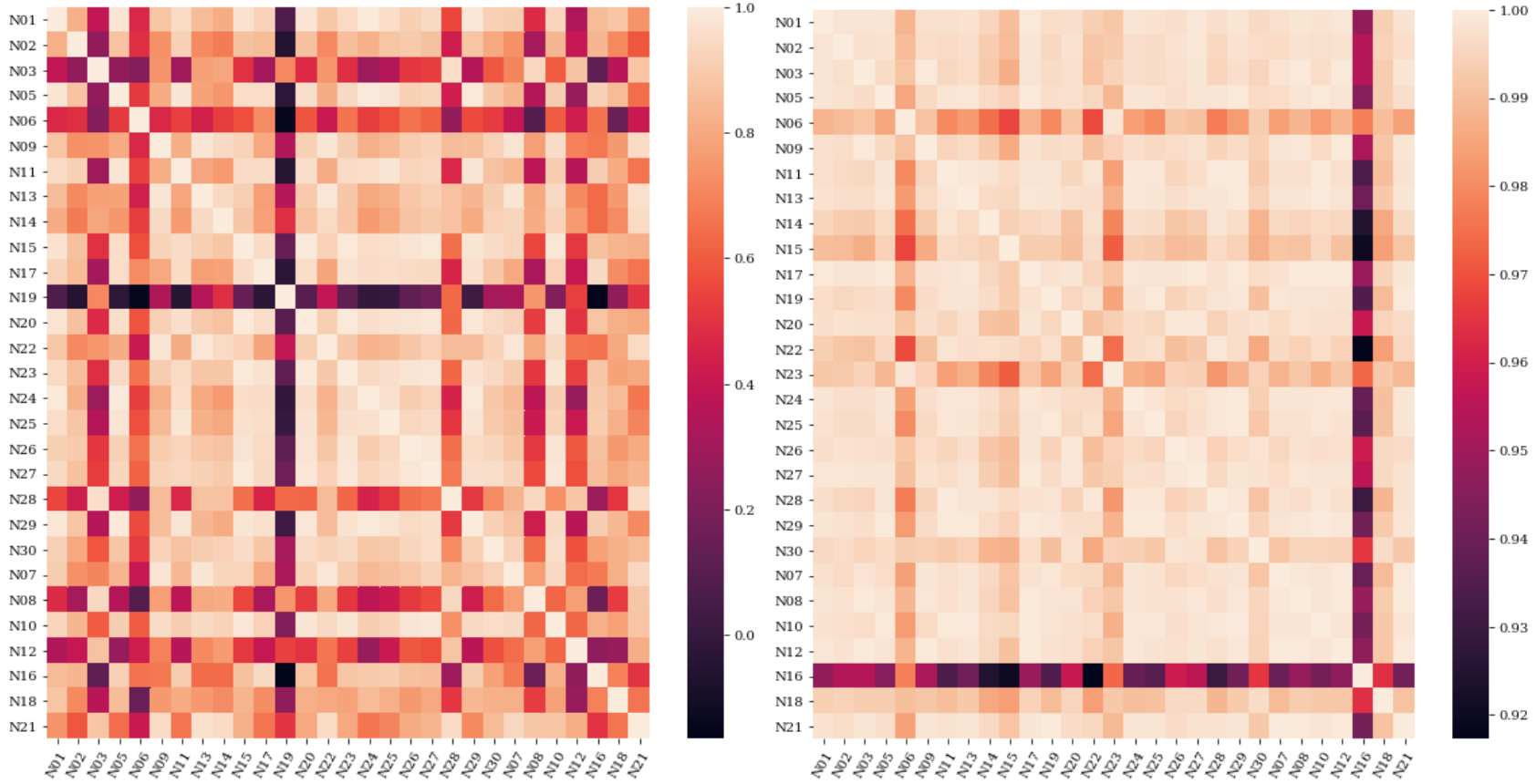

Fig. 2.5 - Correlation Between All Probes. Left: Day 0 to Day 8. Right: Day 8 to Day 35. N04 probe was broken in this experiment, so it was excluded from the dataset.

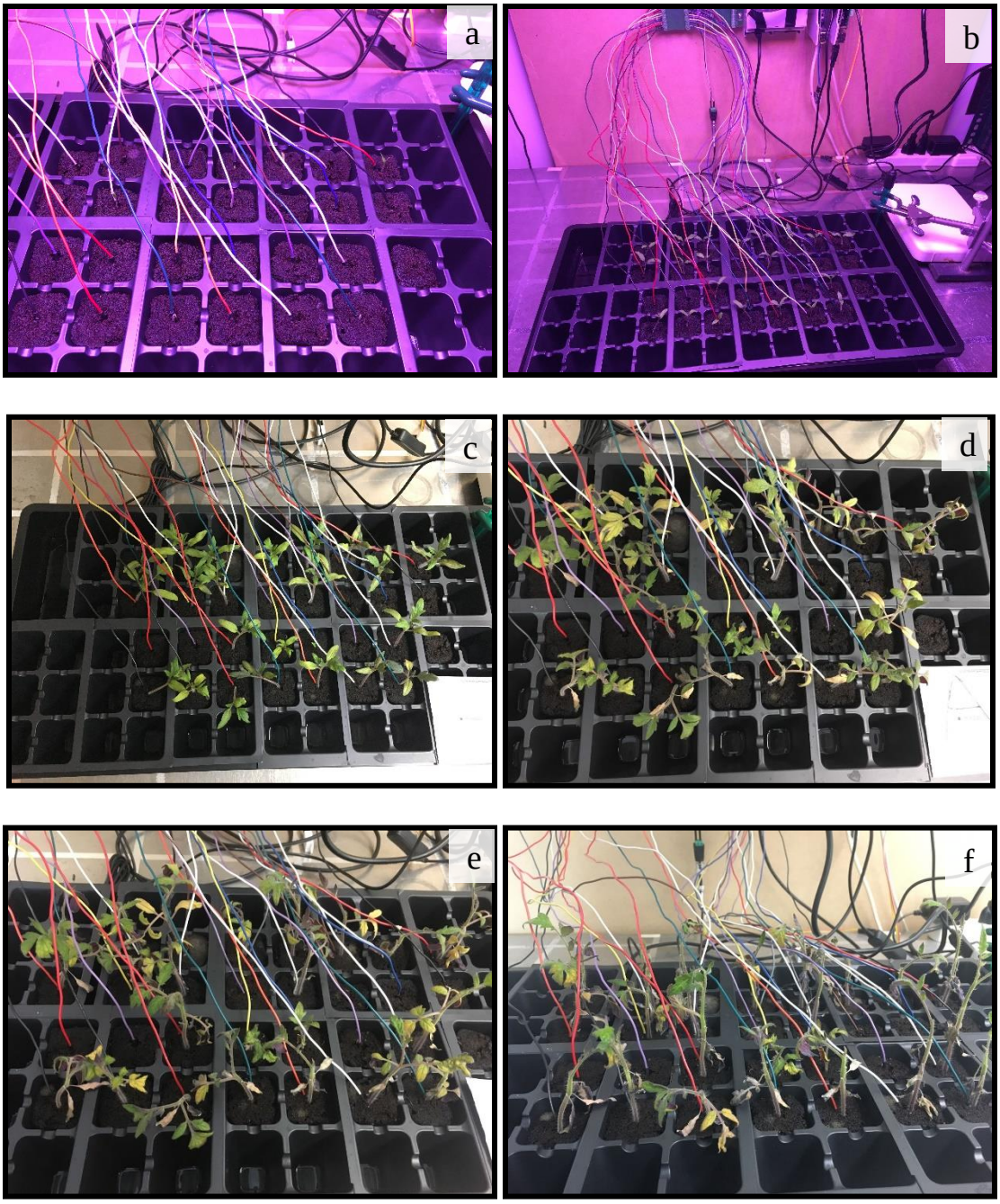

Fig. 2.6 Seedlings Growth Conditions. **a**-Day 4, **b**-Day 8, **c**-Day 17, **d**-Day 27, **e**-Day 30, **f**-Day 35.

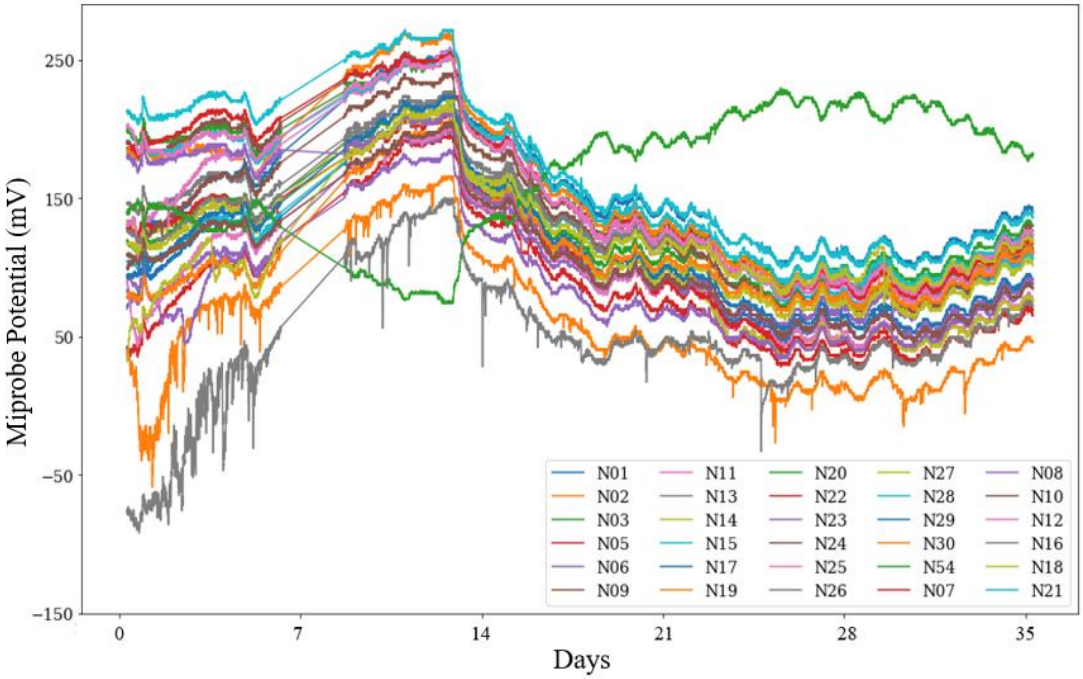

Fig. 2.7 – Raw Miprobe signals. The data between day 6 and day 8 was lost due to the disconnection of the Wi-Fi network. N04 probe was excluded from the dataset because it was found to be broken. N54 (green curve) represents ORP probe signal. Water level was found to be low on day 12 and it was replenished at 2 PM on that day.

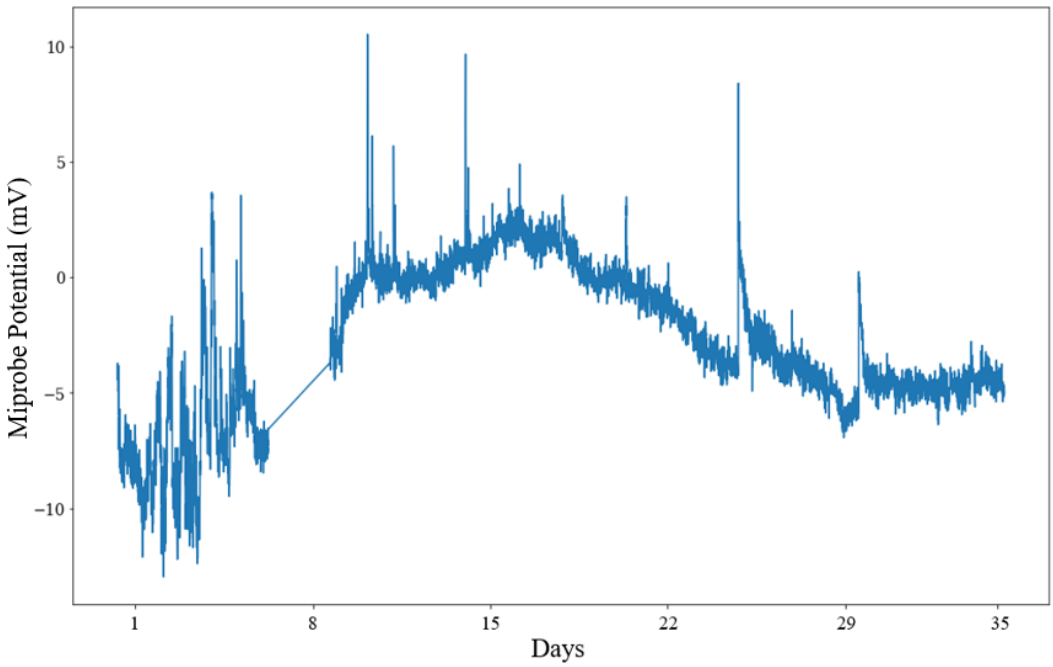

Fig. 2.8 – Average of Normalized Miprobe Potential. Normalized data was obtained by subtracting non-germinating seeds probe signals from living seedlings probe signals. The data between day 6 and day 8 was lost due to the disconnection of the Wi-Fi network.

2.4 Conclusion

The germination of plant seeds is accompanied by the establishment of root-related microbial communities, which will significantly affect the probe signal. Miprobes need a stabilization period (the initial 7 days in this experiment) to represent plant and/or microbial activities, because the probe needs a biofilm to be formed on its surface. The probe signal stabilization is closely related to the germination of the tomato seeds. Raw Miprobe signal is prone to environmental disturbance, such as ORP, water level, and microbial abundance. Other environmental parameters such as nutrient, soil type, and photoperiod remain unknown. Normalized data indicate that Miprobe can identify living seedlings and non-germinating seeds. The preliminary result showed that tomato growth conditions might

affect the Miprobe signal. More experiments are needed to investigate what activities the Miprobe is responding to specifically.

CHAPTER 3

EVALUATION OF MIPROBE PERFORMANCE IN THE ABSORPTION OF NUTRIENTS BY TOMATO SEEDLINGS

Abstract

The experiment investigated the effects of three different fertilizer combinations on tomato seedlings and probes. Meanwhile, the effects of alginate coating on the stability of the probe signal were also tested. Results showed that all probe signals exhibit spikes after the three different treatments. The treatment of algae extract had no significant effect on tomato seedlings' biomass under the conditions tested, but the probe signals maintained a high strength after the first dose of the treatment. The probe signals after chemical fertilizer treatment showed a downward trend after the first dose of the treatment. However, chemical fertilizer and algae extract-treated probes can still maintain a high signal strength after the first dose of treatment. The probe without an alginate coating is prone to have signal fluctuations and less sensitive to nutrient treatments. Alginate coated probes can significantly reduce signal response failures, and coated probes have more sensitive responses when applying treatments. It can be concluded that the biomass may not be the only reason for the signal increase, and the Miprobe signal is significantly affected by fertilizer addition. Further study is needed to investigate the relationship between biomass and the probe signals.

3.1. Introduction

Crop nutrient deficiency is widespread and difficult to diagnose in the early stage of agricultural production (Graham, 2008). The nutrient deficiency symptoms are variable

and are mainly dependent on well-trained experts and visual observation. When these symptoms become obvious (e.g., leaves yellowing, shed off, wither), yield loss is inevitable. In traditional agriculture, farmers usually overuse fertilizer to guarantee crop yield (Wu et al., 2018). Such sort of rough management mode causes the waste of mineral resources and environmental problems such as eutrophication. Precision agriculture relies on soil analysis to guide fertilization. Soil analysis is an effective method to obtain soil nutrient status; however, it is time-consuming and not economical.

In this chapter, Miprobe response to nutrients and plant biomass is explored. Meanwhile, the effects of alginate coating in Miprobe signal stabilization are further evaluated and discussed in this chapter. Miprobe is expected to be a practical tool for quick screening soil fertility and diagnosing plants stresses.

3.2. Materials and Methods

Cultivation and harvesting of *Chlorella vulgaris*

The cultivation method is based on Garcia-Gonzalez & Sommerfeld, 2016. The Microalga *Chlorella vulgaris* was cultivated at the Arizona State University, Arizona Center for Algae Technology, and Innovation (AzCATI). Seven 4’ x 48’ production row panel photobioreactors with standard BG-11 culture medium were adopted (Stanier et., al 1971), bubbled with air mixed with 1% carbon dioxide for algae cultivation. The biomass was harvested on day 14 by centrifugation and then frozen until further process. The frozen biomass was thawed at 4 ℃ for 24 hours, spread onto ten metal trays to a thickness of 1.5 cm, and then dried at -40 ℃ for about 48 hours with Freeze-dryer (Millrock Max Series).

The biomass was collected and stored at 4 ℃ for further use (Garcia-Gonzalez & Sommerfeld, 2016).

Preparation of *Chlorella vulgaris* extract

The preparation method is based on Mutale-joan et al., 2020. 0.3 g dry *Chlorella vulgaris* biomass was hydrolysed in 20 ml of 0.2 M sulfuric acid. The mixtures were heated at 95 ℃ for 2 hours, stir continuously, and mixed with sonic for 10 minutes every 30 minutes. The slurry was then sterilized at 121 ℃, cooled to room temperature, and centrifuged at 4000 rpm for 10 min at 4 ℃ (Mutale-joan et al., 2020). The supernatant was balanced to pH 7 using NaOH and stored at 4℃.

Preparation of alginate

3.3 g of sodium alginate (Alginic acid sodium salt from brown algae, BioReagent, suitable for immobilization of microorganisms, Sigma-Aldrich) and 4.3g of MOPS were dissolved in 225 ml water. Then the suspension was stirred at 180 rpm and 95 ℃ until completely dissolved. The dissolved gases during the stirring process in the alginate solution were removed with a vacuum pump. Then, the alginate solution was sterilized at 121 ℃ for 30 min. A $CaCl_2$ solution was prepared by dissolving 2.49 g $CaCl_2$ and 4.7 g of MOPS in 225 ml DI water. The solution was autoclaved at 121 ℃ for 45 min.

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libraries for automating complex experiments to a NoSQL-based Cloud Database (DynamoDB).

Experimental design

The experiment was started on Dec. 9th and lasted for 50 days. A total of 48 tomato seeds were prepared, of which 24 were coated with alginate, and 24 seeds were directly attached to Miprobe without alginate. Tomato seeds were surface sterilized with 30% bleach. A total of 4 treatments include 1 control, were conducted on 24 alginate coated seeds, with 6 replicates in each treatment. Same treatments were conducted on seeds without alginate coating, with 6 replicates in each treatment (Table 1). A completely randomized design was adopted in this experiment (Salkind, 2010).

The seeds were embedded in root riot cubes (Hydrodynamics International Inc., USA) about 1cm deep. The tray was cultured in a dark room, and light was provided by two Giixer 1000W LED grow lights (NOVA SILK ROAD SARL, France) in full spectrum for 24 hours per day. The seedling cubes were put in starter trays and soaked in water, and sterile tap water was added every two days to keep the water level at 2 cm.

On day 18, 1 ml deionized water (control), Hoagland nutrient solution (210 ppm N, 31 ppm P, 235 ppm K, pH = 7), 0.5 g/L algae extract solution, and Hoagland nutrient solution containing 0.5 g/L algae extract (F+A) were applied to tomato seedlings as soil drench and this step was repeated every two days after day 18. The air temperature during the experiment was 20 ℃ and humidity was 27 %.

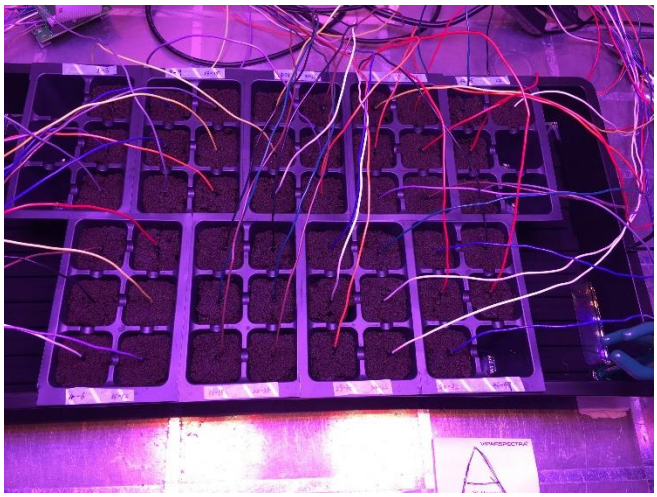

Fig. 3.1 Experimental Setup

Ash-free dry weight of tomato seedlings

The harvesting of tomato seedlings was conducted on day 50. The shoots and roots of tomato seedlings were dried separately in a 70 ℃ oven to constant weight.

Normalization of raw Miprobe signal and statistical analysis

The normalized data was aimed to improve visualization and obtained by subtracting the average of control probe signals from other probe signals. Statistical analyses were conducted using SPSS 18.0. The data was analyzed using a one-way analysis of variance (ANOVA), to test difference among the means, the level of significance was set at $p<0.05$.

Control (coated)	Control (uncoated)	Fertilizer (coated)	Fertilizer (uncoated)	Algae extract (coated)	Algae extract (uncoated)	F+A (coated)	F+A (uncoated)
N21	N33	N41	N11	N08	N18	N43	N34
N15	N42	N38	N27	N10	N07	N29	N02
N6	N20	N09	N44	N46	N14	N32	N19
N48	N37	N13	N30	N35	N25	N04	N40
N01	N23	N39	N45	N26	N03	N36	N16
N12	N28	N17	N24	N22	N05	N47	N31

Table 1 Randomization and Three Different Nutrient Treatments with Alginate- Coated and Uncoated Probes. F+A stands for fertilizer and algae extract.

3.3. Results and Discussion

Before the nutrient treatments, severe leaf chlorosis symptoms were observed (Fig. 3.2a). Specifically, the growth of new leaves was hindered, the old leaves turned yellow, and the growth of tomato seedlings was slow.

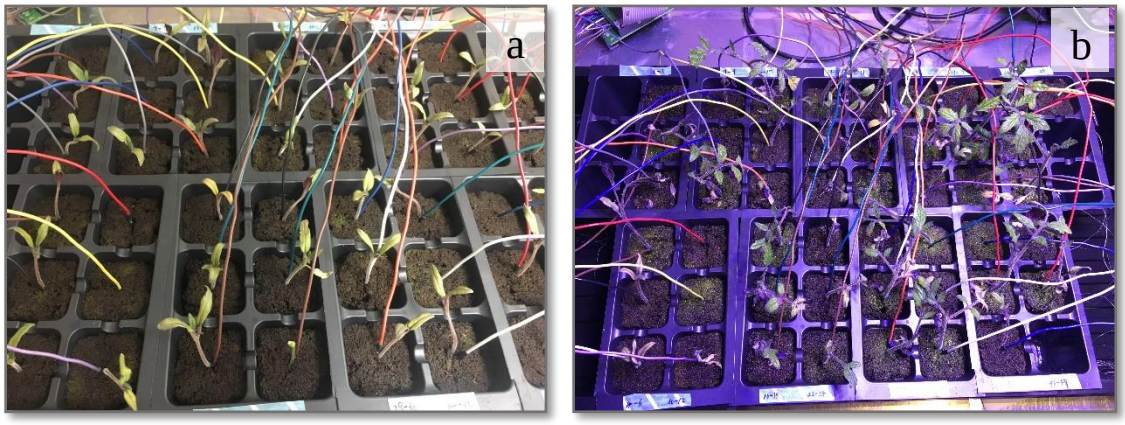

Fig. 3.2 The Growth Condition of Tomato Seedlings. **a** - Day 18, **b** –Day 46.

The uncoated probe signals are prone to have fluctuations, such as N25 and N05 in algae uncoated (Fig. 3.3b), N16 in fertilizer uncoated (Fig. 3.3d), and N45 in fertilizer and algae uncoated (Fig. 3.3f). The added nutrients might have triggered the fluctuations of N25, N45, and N16, because they were timed to coincide with the treatments. However, treatments applied at other times did not lead to similar fluctuation. The reason for the fluctuation remains unknown. It is possible that the biofilm on the uncoated probe's surface is more susceptible to environmental disturbances such as adding of nutrients. The fluctuation of N05 appeared more frequently than others, which might have been due to the damage of the probe.

Notably, all treatments triggered signals spikes on Day 20 when treatments were applied for the first time. Following treatments were applied every two days and also triggered similar signal spikes, however, with lower amplitude (Fig. 3.3 and Fig. 3.4). This may be because the addition of fertilizer for the first time stimulates more significant microorganisms and plant responses, and the following additions of fertilizer have less effect on microorganisms and plants. Additionally, the signal spikes triggered by the first treatments (day 18) on alginate coated probes have higher amplitude than that of the uncoated probes (Fig. 3.4). The amplitude difference may be that the alginate matrix is beneficial to the inoculation and growth of microorganisms and the microbe abundance is high on the coated probe surface. Hence, the microbial response is stronger. For the first treatment, there is a 13.5mv increase in algae extract-treated Miprobe signal and 19.33mv increase in both fertilizer-treated and fertilizer and algae extract-treated Miprobe signal (Fig. 3.4). Therefore, it can be concluded that chemical fertilizer is able to stimulate more significant Miprobe responses. Chemical fertilizer is the main reason for the probe signal

increase when fertilizer and algae extract were applied simultaneously. In addition, since the normalization has eliminated most environmental impacts, such as the replenishment of water, the spikes may also be affected by the nutrient itself.

The Miprobe signal after the first treatments (day 18) varies with different types of fertilization. Both coated and uncoated, algae extract-treated probe signal showed an upward trend after day 18 (Fig. 3.4 a, d). The alginate coated probe signal kept increasing before Day 7, while the uncoated probe signal became stable after Day 28. There is no significant difference in dry weight between the control (0.0267g ± 0.013SD) and the algae extract-treated seedlings (0.0233g ± 0.012SD). However, the normalized Miprobe potential still exhibit increases in both coated and uncoated probe signals after the treatment of algae extract. The bio-stimulating effect of algae extract might have caused the high level of Miprobe responses. The seedlings developed higher physiological activities to fight against the stress without contributing to biomass accumulation. Another possible explanation is that algae extract can be better decomposed and utilized by soil microorganisms as an organic slow-release fertilizer, thus tomato seedlings were at a disadvantage in the competition for nutrients. Hence tomato seedlings have fewer effects on the probe signal.

Fertilizer-treated, alginate coated probes exhibit a downward signal trend after day 18, while uncoated probes show an upward signal trend from day 18 to day 30 and a downward signal after day 30 (Fig. 3.4 b, e). Chemical fertilizer is efficient and easy to be absorbed by tomato seedlings. When treated with chemical fertilizers, the sudden increase of rhizosphere microbes and root activity led to signal spikes. However, the impact time of chemical fertilizer is relatively short, which causes the rapid decline of the signal. Another

possible explanation is that the fixed amount of fertilizer is not enough to support the plant's growth with time. The lack of replicates might be the reason for the different signal curves under the same treatment. Some probe signals behaved differently than others. For example, N38 and N41 in fertilizer treated-alginate coated showed a downward trend (Fig. 3.3c) after fertilization, which led to a decline in the signal in Fig. 3.4b, while other probe trends in fertilizer-coated are similar to those in the uncoated group. Algae proliferation (Fig. 3.2b) was found on the soil surface in the fertilizer treatment and may affect some probes' signal.

Fertilizer and algae extract-treated, alginate coated probe signal kept increase after the first treatment and became stable on about day 30. Then slightly decrease after day 40. The uncoated probe signal also kept increase after the first treatment; however, the signal started to decrease after day 30 (Fig. 3.4 c, f). There are fewer available replicates and more signal variance (N16) in uncoated probes, which might have caused the different signal curves under the same nutrient treatment. Besides, Algae proliferation (Fig. 3.2b) was also found on the soil surface in this treatment and may affect some probes' signal. Algae extract addition can provide additional carbon sources than solely fertilizer-treated probes, which may account for the high and persistent signal strength in fertilizer and algae extract-treated probe signals. Also, microbial activities can decrease the pH over time, particularly with nitrification, which could also affect the probe signal. There would be a significant lag between bacterial impacts, such as nitrification and plant growth hormone production, and physiological responses by plants, which may also account for the probe signal trend in algae extract-treated groups.

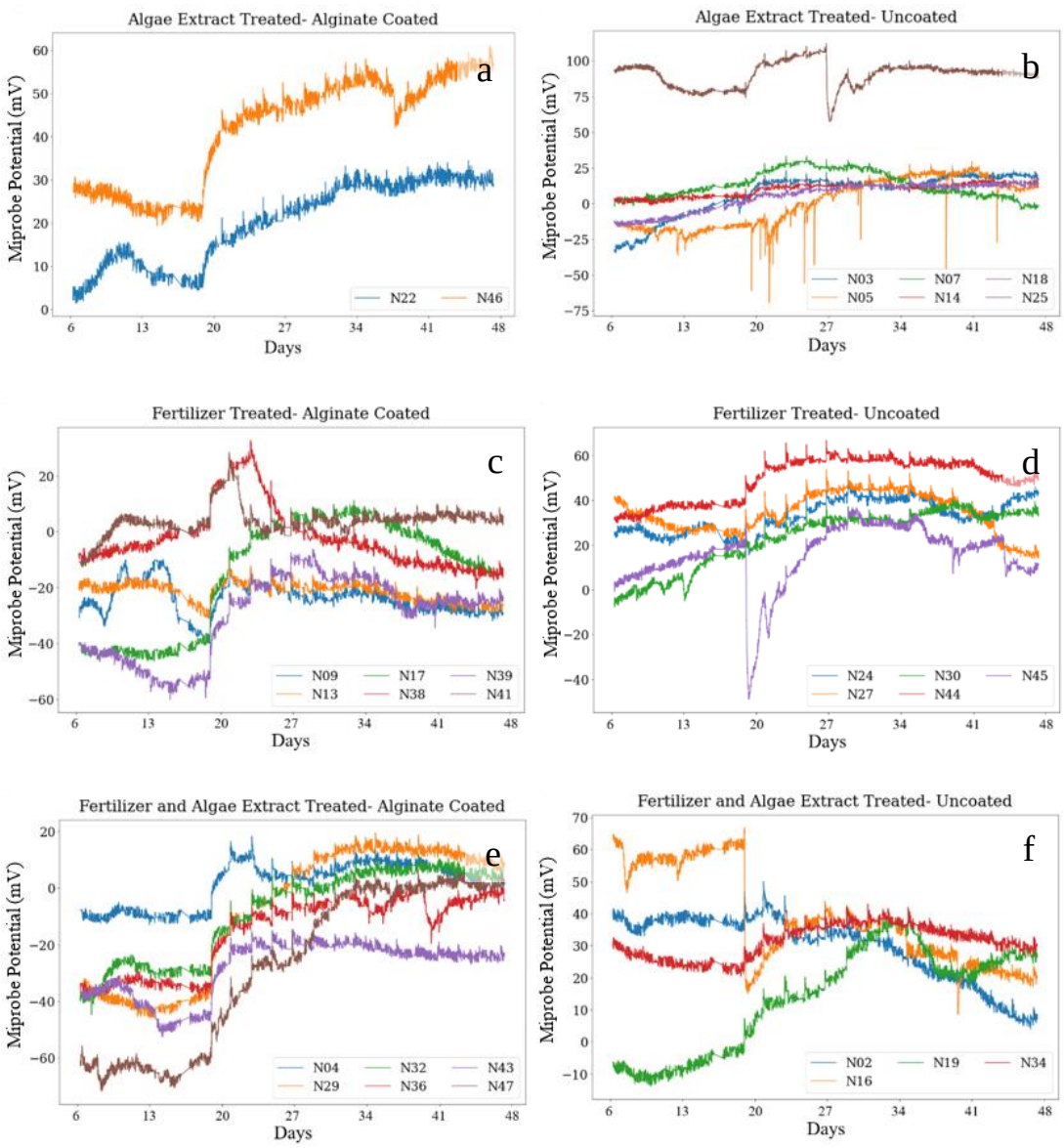

Fig. 3.3 - Normalized Miprobe Potential. Data was obtained by filtering out control signals (i.e., Fig. 3.3a is obtained by subtracting the average of alginate-coated control probe signals from alginate-coated algae extract- treated probe signals.) Non-germinating seeds are not included in data analysis. Therefore, for example, only 2 replicates are shown in Fig. 3.3a.

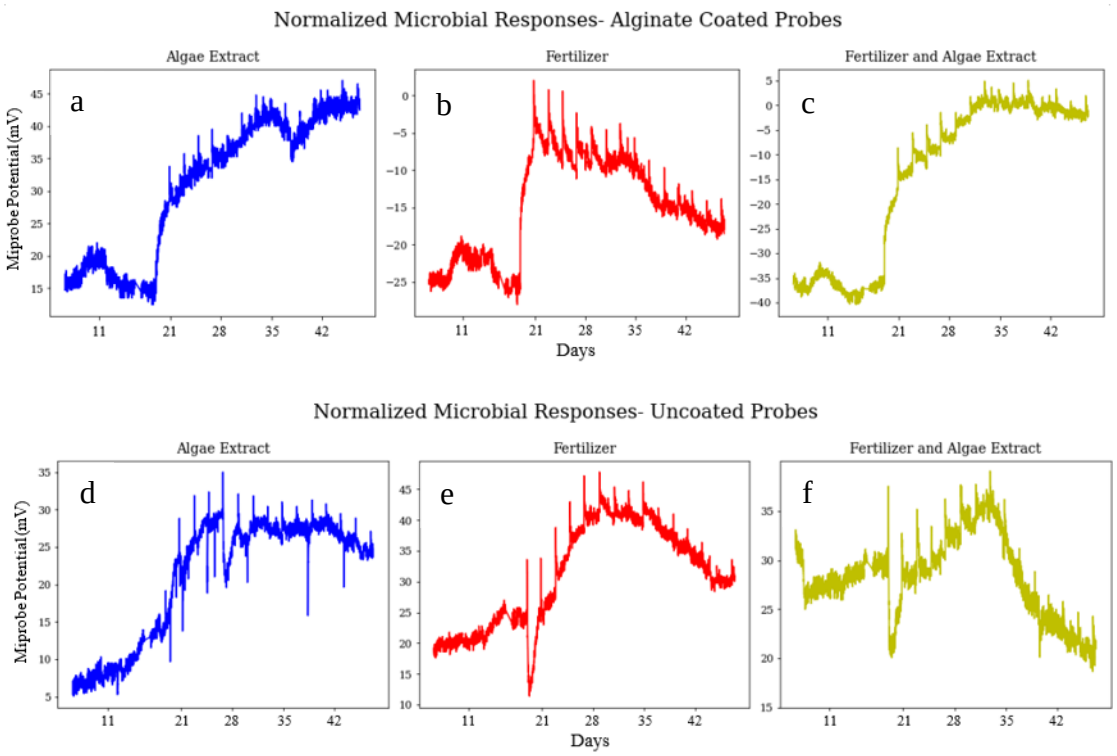

Fig. 3.4 - The average of Normalized Miprobe Potential. Overall growth conditions of seedlings under each treatment.

3.4. Conclusion

The uncoated probe signal is prone to have transmission failure after treatments. On the other hand, alginate coating can effectively avoid unexpected signal fluctuations, making the alginate coated probes more reliable. Coated probes are more sensitive to the treatments, although all the probes can accurately respond to treatments. The different signal curves triggered by different fertilizations demonstrated that Miprobe is also sensitive to different types of fertilizers.

The relationship between signal strength and biomass remains unknown because the algae extract-treated probe signal also exhibits higher signal strength than the control. High Miprobe response for algae extract-treated probes maybe because of the activities of

soil microorganisms. Additionally, the nutrient itself may also exert an impact on the Miprobe signal. It is known that the Miprobe signal is affected by multiple environmental factors; however, how many factors will exert impacts and to what degree still requires more investigation.

The individual plant will have more significant effects on the probe signal when the number of replicates is low. Therefore, it is necessary to consider each growth module as the experimental unit and individual plants as observation units. More replicates may be needed better to represent the overall growth condition under a particular treatment. The 24-hour photoperiod and root oxygen deficiency might have caused the poor growth, although it did not affect the analysis of the probe signal. In future experiments, a better growth condition is needed to test the function of Miprobes further.

CHAPTER 4

EVALUATION OF MIPROBE ON BIOMASS ESTIMATION

Abstract

The experiment was conducted under a 12/12 day-night cycle to study the raw signals, the relationship between biomass and the probe signal. It was found that the raw signal of the probe exhibited a day/night cycle. The probe signal presented an upward trend during the day, a downward trend during the night. Other results showed that the normalized biomass of tomato seedlings treated with fertilizer (11.78 mg ± 11.63 SD) was initially higher than that of tomato seedlings treated with fertilizer and algae extract (6.58 mg ± 11.83 SD) on day 21. Subsequently, the normalized biomass of seedlings treated with fertilizer and algae extract (44.15 mg ± 8.81 SD), became higher than that of tomato seedlings treated with fertilizer (34.70 mg ± 11.83 SD) on day 29. On the other hand, the fertilizer-treated probe signals were first higher from day 10 to day 25 and then became lower after day 25. Therefore, the intersections between the two treatments on the probe signal and normalized DW in the same period indicate that the biomass can be positively correlated to the probe signal. However, early fertilization did not contribute to the biomass accumulation, but the probe signal increased significantly, indicating that biomass was not the only factor affecting the probe signal. Other biological activities such as respiration, photosynthesis, and soil microbial activities should also be considered.

4.1. Introduction

Efficient plant phenotyping and site-specific crop management rely on rapid and accurate biomass and yield estimation. (Li et al., 2020). Above-ground biomass (AGB)

closely relates to crop nutrition status and yield, thus becoming an indicator of crop growth status. However, AGB is particularly troublesome, because it largely depends on subjective, destructive, inaccurate, and labor-intensive ground-based measurements (Reynolds et al., 2000; Walter et al., 2018). Remote sensing is an efficient technique for measuring growing season crop canopies and providing information on the spatial variability of crop AGB and yield. (Li et al., 2020). With the rising need for biomass and yield estimation for crop management and the next generation of phenotyping, many studies have investigated commercially available, non-destructive technologies such as UAV-based imaging (Li et al., 2020; Walter et al., 2018). Many imaging methods have been studied, such as RGB cameras, multispectral cameras, and LiDAR to facilitate non-destructively AGB measurements in field trials (Ehlert et al., 2009; Hosoi and Omasa 2009; Montes et al., 2011; Winterhalter et al., 2011; Bendig et al., 2014; Eitel et al., 2014; Amaral et al., 2015; Bendig et al., 2015; Li et al., 2015; Pittman et al., 2015; Schirrmann et al., 2016a; Schirrmann et al., 2016b).

In this chapter, raw Miprobe signal was investigated, and the ability of biomass estimation by Miprobes was evaluated and discussed. Fertilizer and algae extract were applied to tomato seedlings to achieve different biomass accumulation rates and further verify the results from the previous chapter.

4.2. Materials and Methods

Cultivation and harvesting of *Chlorella vulgaris*

The cultivation method is based on Garcia-Gonzalez & Sommerfeld, 2016. The microalga *Chlorella vulgaris* was cultivated at the Arizona State University, Arizona

Center for Algae Technology, and Innovation (AzCATI). Seven 4’ x 48’ production row panel photobioreactors with standard BG-11 culture medium were adopted (Stanier et., al 1971), bubbled with air mixed with 1% carbon dioxide for algae cultivation. The biomass was harvested on day 14 by centrifugation and then frozen until further processing. The frozen biomass was thawed at 4 ℃ for 24 hours, spread onto ten metal trays to a thickness of 1.5 cm, and then dried at -40 ℃ for about 48 hours with freeze-drying (Millrock Max Series). The biomass was collected and stored at 4 ℃ for further use (Garcia-Gonzalez & Sommerfeld, 2016).

Preparation of *Chlorella vulgaris* extract

The preparation method is based on Mutale-joan et al., 2020. 0.3g dry *Chlorella vulgaris* biomass was hydrolysed in 20 ml of 0.2M sulfuric acid. The mixtures were heated at 95 ℃ for 2 hours, stirring continuously, and mixed with sonic for 10 minutes every 30 minutes. The slurry was then sterilized at 121 ℃, cooled to room temperature, and centrifuged at 4000 rpm for 10 min at 4 ℃ (Mutale-joan et al., 2020). The supernatant was balanced to pH 7 with NaOH and stored at 4 ℃.

Preparation of alginate

3.3 g of sodium alginate (Alginic acid sodium salt from brown algae, BioReagent, suitable for immobilization of microorganisms, Sigma-Aldrich) and 4.3g of MOPS were dissolved in 225 ml water. Then the suspension was stirred at 180 rpm and 95 ℃ until it is completely dissolved. The dissolved gases during the stirring process in the alginate solution were removed with a vacuum pump. Then, the alginate solution was sterilized at

121 ℃ for 30 min. $CaCl_2$ solution was prepared by dissolving 2.49 g $CaCl_2$ and 4.7g of MOPS in 225 ml DI water. The solution was autoclaved at 121 ℃ for 45 min.

Alginate Coating of seeds

The seeds of tomato 'Golden Jubilee' (Marde Ross & Company) were surface sterilized with 30% bleach for 15min, then washed 3 times with sterile water. Coating of seeds was carried out under sterile conditions in a laminar flow hood. The alginate was added dropwise with the aid of a 10-ml sterile syringe on the tomato seeds and the probe surface. The coated seeds and probes were then boned together, soaked in 0.1 M $CaCl_2$ solution to form the alginate hydrogel. The excess Ca^{2+} ions were washed out with sterile tap water after 3 min of the formation of alginate hydrogel.

Miprobe lab instrumentation

MiProbe Gen2 Electrodes and Raspberry Pi 4 Compute Module were adopted in this experiment. The Miprobe Gen2 is able to work in soils and aqueous environments, and the graphite, titanium, carbon fiber–based sensing electrodes benefit the outdoor/long-term deployments and soil application of the probe. The Gen2 Miprobe uses ORP (oxygen reduction potential) as a reference. The configuration of Raspberry Pi 4 is as follows:

- eMMC-based OS storage
- Quad-Core 1.5ghz 64bit ARMv8 SoC
- Supports 2 Cameras, 2 LCD Displays + 2x HDMI Outputs
- MicroSD/USB Storage Options
- Compatible with all Raspberry Pi 3B/4 Peripherals

- Supports PCIe-based ML/AI Accelerators

MiProbe Lab Instrumentation utilizes a cross-platform (Linux/Mac/Windows) Python SDK for automatically logging data off the USB/Serial interface. This allows automation of data logging from each sensor connected to the host device (usually a Raspberry Pi 3B or 4 running Linux). The Python SDK provides a demo logging tool and libraries for automating complex experiments to a NoSQL-based Cloud Database (DynamoDB).

Experimental design

The experiment was started on Feb. 22nd, 2021 and lasted for 36 days. A total of 48 tomato seeds and probes were coated with alginate. Tomato seeds were surface sterilized with 30% bleach. 2 nutrient treatments were applied on day 8, with 16 replicates under each treatment. Each seedling was treated with either 1 ml deionized water (Control), or Hoagland solution (210 ppm N, 31 ppm P, 235 ppm K, pH = 7) (Fertilizer), or Hoagland solution containing 0.5 g/L algae extract (Fertilizer and Algae extract). Treatments were repeated every two days. A completely randomized design was adopted in this experiment (Salkind, 2010).

The seeds were embedded in root riot cubes (Hydrodynamics International Inc., USA) about 1cm deep. The tray was cultured in a dark room, and the light was provided by two Giixer 1000W LED grow lights (NOVA SILK ROAD SARL, France) in full spectrum with a 12/12 day/night cycle per day. The seedling cubes were put in starter trays

and soaked in water, and sterile tap water was added every two days to keep the water level at 2 cm. The temperature during the experiment was 20 ℃ and humidity was 25 %.

Fig. 4.1 Experimental Setup

Ash-free dry weight of tomato seedlings

The first harvesting of tomato seedlings was conducted on day 14. Subsequent harvestings were conducted every 7 days after day 14. The shoots and roots of tomato seedlings were dried separately in a 70 ℃ oven to constant weight.

Statistical analysis

Statistical analyses were conducted using SPSS 18.0. The data was analyzed using a one-way analysis of variance (ANOVA), to test difference among the means, the level of significance was set at $p<0.05$.

Normalization of raw Miprobe signal and dry weights

The normalized data was aimed to improve visualization and obtained by subtracting the average of control probe signals from other probe signals. Normalized dry weights were obtained by subtracting the DW of control from the DW of treatments.

	Control	Fertilizer	Fertilizer + Algae Extract
1st Harvest (Day 14)	N34	N45	N11
	N37	N08	N14
	N15	N31	N27
2nd Harvest (Day 21)	N30	N46	N28
	N01	N40	N42
	N02	N22	N39
	N16	N38	N19
3rd Harvest (Day 29)	N10	N12	N44
	N17	N23	N29
	N43	N32	N36
	N18	N03	N25
4th Harvest (Day 36)	N05	N24	N09
	N47	N04	N21
	N20	N41	N06
	N33	N07	N26
	N35	N48	N13

Table 2 Randomization, Harvest Dates and Different Treatments with Alginate-coated Probes.

4.3. Results and Discussion

It was observed that all tomato seeds had germinated on day 12. The probe signal showed day-night cycles after germination (Fig. 4.2). The growth light starts to work at 6 a.m. and is automatically turned off at 6 p.m. During the day, the probe signal presented a downward trend. The treatments caused the signal fluctuation at around 3 pm. During the night, the probe signals showed an upward trend (from 6 p.m. to 6 a.m.), and the signal began to decline again after 6 a.m.

It is worth noting that the minimum Miprobe potential that appeared at about 6 a.m. on day 12, day 13, day 14 gradually rising over time. However, the maximum Miprobe potential that appeared at about 6 p.m. on day 12 and day 13 stay the same. In other words, the amplitude of the raw signal is getting smaller with time. The similar maximum values

might have indicated that the photosynthetic activity stays the same on day 12 and day 13, while the rising minimum values might have indicated that the seedlings' activity, such as respiration at night, was getting stronger. As Fig. 4.2 shows, each treatment has a baseline. For example, the control started from 90mv on 03/06. The differences between the baselines may be because of each Miprobe and individual plants. As mentioned in Chapter 1, the Miprobe monitors the changes over time rather than the absolute value. Hence, the effect of the baseline of each treatment are eliminated from normalization.

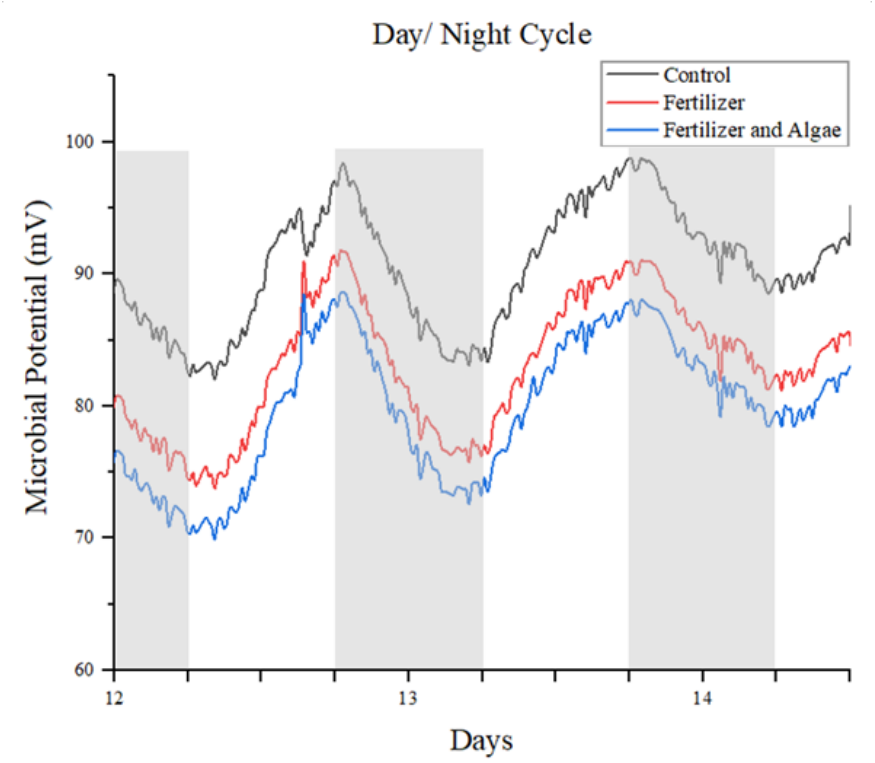

Fig. 4.2 Day Night Cycle. Grey areas represent night.

The normalized data showed that the probe signal stabilized fast after the tomato seeds were sowed (Fig. 4.3), which is different from the first week's probe signal in a sterile environment (Fig. 2.8). It can be concluded that the stabilization time is directly related to

the microbe abundance in the environment. The biofilm formation is slower in a sterile environment and faster in natural environments, such as natural soil.

Tomato seeds were sowed on day 0. During day 0 and day 8 when no treatments were applied, there is a slight decrease in both treatment's signals. The slight decrease in the first few days indicates that the probe may not work well initially. There were few signal variances when no treatments were added, and a few mV variances is always acceptable in Miprobe measurements. However, both treatments (fertilizer, fertilizer and algae extract) triggered signal spikes on day 8 when the first treatments were applied. Treatments were applied every two days, and each treatment triggered the same spikes. This phenomenon is the same as that found in previous experiments.

The harvest of tomato seedlings might have caused the signal fluctuation on day 29. A possible explanation for the sudden drop of fertilizer-treated probes signal is that some high-baseline plants were randomly harvested on day 29. Although it is still unknown why the probe signal will have a sudden drop after the harvest, based on the results from Chapter 3, the fewer replicates in the treatment, the more fluctuation on the average will occur, because individual plants will exert more impact on the average of normalized probe signals.

After the second treatment (day 10), the signal strength of the fertilizer-treated probes became higher than that of the fertilizer and algae extract-treated probes. However, the signal of fertilizer and algae extract-treated probes became higher after approximately day 26. On the other hand, the average of normalized DW of fertilizer-treated tomato seedlings on day 21 (2nd harvest) was 11.78 mg ± 11.63 SD, which is higher than that of fertilizer and algae extract-treated tomato seedlings (6.58 mg ± 11.83 SD). However, the

average of normalized DW of fertilizer and algae extract-treated seedlings became higher (44.15 mg ± 8.81 SD vs. 34.70 mg ± 11.83 SD) on day 29 (3rd harvest). The differences between DW are subtle and insignificant, and it may be because of the poor growth condition of tomato seedlings. A better growth environment, such as a bigger seedling tray or more nutrients, is worth consideration to minimize the SD. Also, it is necessary to consider the individual plant as an observational unit in future experiments. The intersection point was between day 21 to day 29, indicating that the fertilizer and algae extract-treated seedlings grow better during this period. Meanwhile, the probe signals intersected, and the signal strength of fertilizer and algae extract–treated probe became higher after the intersection point. Therefore, the Miprobe signal strength can be closely related to the biomass of tomato seedlings.

Notably, the early fertilizations did not contribute to biomass accumulation. Because in the first harvest (day 14), the normalized DW of the fertilizer-treated and fertilizer and algae extract-treated seedlings were 1.20 mg and 0.30 mg, which means the DW of control and the DW of two nutrient treatments were almost the same. However, the probe signals exhibited a dramatic increase in the first few days after the first fertilization. It is possible that the tomato seedlings were in nutrient saturation before day 14 (first harvest). The probe signal can be affected by both soil microorganisms and seedlings and it is clear that fertilization affects soil microbe activities. In the first few days after fertilization, soil microbes might have a more significant impact on the probe signal, while with the plant biomass increase, the plant might have a more significant impact on the probe signal. In summary, the biomass may positively correlate to the probe signal, but soil microbes and other biological activities need to be taken into consideration.

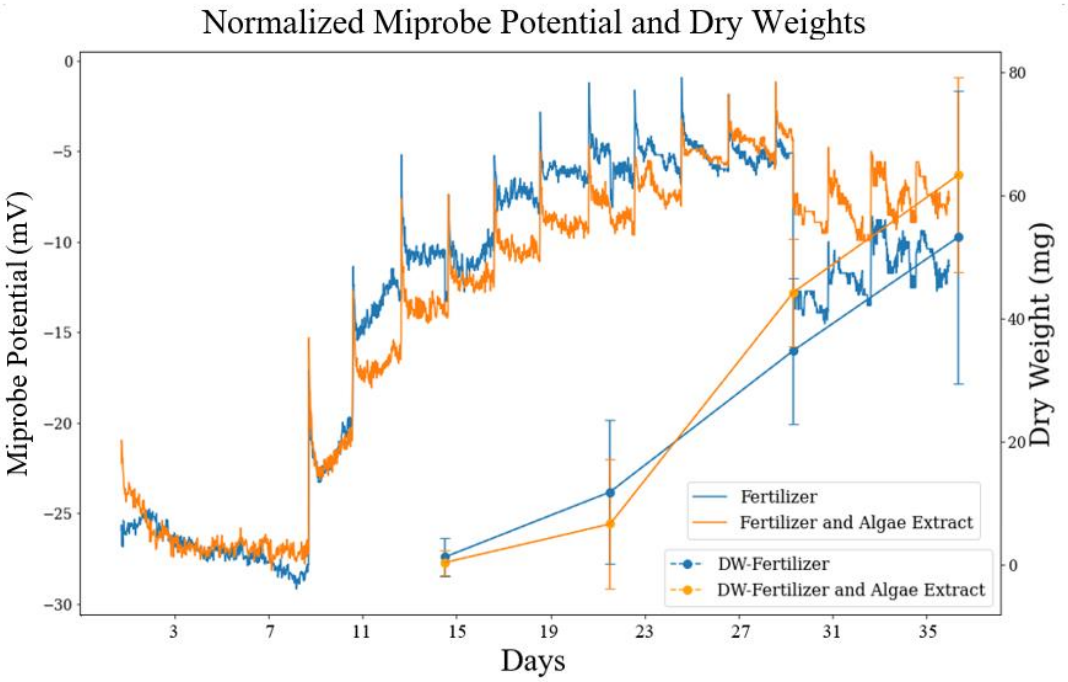

Fig. 4.3 Normalized Miprobe Potentials (solid line) and Dry Weights (dashed line). Data was obtained by subtracting control from treatments. Markers on dashed line represent 4 harvests. The harvests were conducted on day 14, day 21, day 29, and day 36.

4.4. Conclusion

Raw probe signal shows day/night cycles, indicating that the tomato seedlings can significantly affect the probe signal. The subtle changes in the amplitude of the Miprobe potential demonstrated that the Miprobe is capable of transmitting the plant's respiration activity.

Both treatments triggered the signal spikes, which once again confirmed that the Miprobe is sensitive to fertilization. The two treatments have different signal curves. At the beginning of the treatments, fertilizer-treated probes have higher signal strength, while the fertilizer and algae extract–treated probe took over afterward. It is also notable that the normalized dry weights of the two treatments also have an intersection in the middle of the experiment, indicating that the probe signal strength can be positively correlated to DW.

However, high signal strength trigger by early fertilization without contributing to the accumulation of biomass indicated that DW might not be the only reason for the probe signal increase. Multiple reasons such as soil microbial activities, root respirations may also account for the probe signal. It is essential to measure multiple plant growth parameters such as chlorophyll content, photosynthesis, and respiration rate, to study the relationship between plants and the probe signal.

CHAPTER 5

CONCLUSION

The present results show that the MiProbe signal is unstable in the first few days of application, but that the signal stabilizes after seed germination. Alginate coating did significantly reduce signal fluctuations and make the probe more sensitive to nutrient treatments. The raw signal also exhibited day/night cycles, indicating that plant activity does significantly impact the probe signal. There are subtle changes in raw signal with time, however, which is why normalization was required to improve visualization. Normalized data showed all MiProbes recorded signal spikes after the nutrient treatments and that the signals were affected by the addition of different types of fertilizers.

Many unknowns still require further investigation, such as the relationship between plant biomass and the probe signal. The intersections between the two treatments on the probe signal and normalized DW in the same period indicate that plant biomass can positively correlate to the probe signal. However, low biomass also triggered high signal strength in algae extract-treated probes. Moreover, early fertilizer addition also triggered immediate high signal strength without representing accumulated biomass. These findings suggest plant biomass does not contribute to probe signal alone. Other plant activities such as respiration, photosynthesis, and soil microbial activities should also be considered in future experiments.

For future experiments, it is important to repeat the above experiments and study the performance of Miprobe when no plants are present. A better fertilizer ratio and dosage, plus larger growth spaces are needed to adapt to the growth of plants. The effect of the depth of the probe inserted into the soil and the distance from the root on the probe signal

is still unclear and also needs further study. In addition to redox and nutrient, the effects of other environmental factors—such as the effects of temperature, humidity, and soil types—is also worthy of investigation. It is essential to measure as many biotic and abiotic parameters as possible, including no plants are present. It will help elucidate the relationship between Miprobe signal and soil microorganisms, plants, and the inorganic environment.

Moreover, the influences of plant biotic (pests) and abiotic (drought and salinity) stress on the probe signal are also worth investigating. This will permit better understanding of the relationship between plant physiological activities and probe signals. Long-term experiments are also needed to study how different crops affect the probe signal in different growth periods. Such an experiment will require multiple reference probes, for which precisely analyzing different treatments using different reference probes may be challenging.

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