In-vivo Dehydration Sensing in Transgenic Tobacco Plants Using an Integrated Electrochemical Chip

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Abstract
In this paper, we demonstrate in-vivo plant based dehydration sensing using a bio-electrochemical sensor. In-vivo sensing reports on the plant's status as provided by its gene expression, responding to stress. Plant based sensing provides precise, real-time information acquired from the plants themselves; it complements the information provided by ex-vivo sensors sampling the plant's surroundings, e.g. in the soil, or sensors measuring the plant's electrical conductivity. In this paper, we present a method in which the plant's dehydration levels are monitored in real time using in-vivo techniques using the plant as the sensor. In this method, the expression of the β-D-glucuronidase enzyme, expressed under drought conditions, is monitored using its reaction with a substrate, which produces an electrochemically active product. The product is oxidized on the working electrode of a three-electrode electrochemical chip mounted on the leaves of Nicotiana tabacum plants. Electrochemical sensing showed earlier detection compared to other methods, e.g. visual inspection and conductivity measurements.

Keywords: drought plants, in-vivo plant sensing, biosensors, electrochemical sensing

I. INTRODUCTION

Global food security has become a very important issue in recent years. Due to the increase in the global population, the demand for food will continue to increase. As per current projections, by 2050, additional 70% food produce would be required to meet the needs [1]. Currently, over a billion people around the world are suffering from malnutrition owing to lack of food, and over two billion are consuming food deficient in vitamins and other nutrients [2]. Modern sensing technologies, integrated with local and global cloud based logistics systems can be used for optimizing food production, storage, processing, and consumption using the information gathered in all stages of the food supply chain. Moreover, the information gathered from such precision agriculture systems would serve as a guide for the following seasons. The key requirements from agricultural sensors are precise and reliable data output, economic viability, and ease of use for farmers [3]. Sensors which are currently available for agricultural use are mainly based on soil testing technologies, which are used to monitor the water content, soil nutrient levels, etc. This indirect sensing may not provide a reliable estimate of water or nutrients actually used by plants. Continuous monitoring of plant hydration status can assist in providing better automated irrigation. The majority of common methods used for estimation of plant hydration are based either on evapotranspiration models or on soil moisture sensors. A plant-based approach estimates the plant's hydration status directly from the plant. It offers earlier detection and is expected to be closer to the actual status of the plant compared to other indirect measurements [4], [5].

There have been a number of attempts at obtaining hydration information directly from plants. These use a wide range of sensing techniques ranging from bioluminescence sensing to ex-vivo electrochemical measurement [6]. Afzal *et al*. investigated plant water levels based on leaf thickness and leaf electrical capacitance in tomato plants [7]. However, as leaf thickness changes over time with plant growth, and as leaf capacitance can depend on factors other than hydration, this sensing methodology does not guarantee reliable results. A terahertz range time-domain spectrometer was used for analyzing water dynamics in *Arabidopsis* plants [8]. As terahertz range electromagnetic waves are highly attenuated due to water, this works as an extremely sensitive non-contact method for water level estimation in plants as well as other materials [9]. A large number of dielectric materials such as dehydrated living tissue are typically transparent in the terahertz range, which further improves the ability of this technique to be used for estimating hydration level in leaves. This technique can provide reliable information on water levels, however, it is expensive and impractical for on-field use.

Integrated electrochemical biosensors for monitoring gene expression under stress have already been developed. Using these sensors, real-time monitoring of β-glucuronidase (GUS) expression in transgenic tobacco plants has been demonstrated, using its activity as a biomarker for functional sensing. As a proof of concept, biosensing of GUS enzyme under constitutive expression in transgenic tobacco plants and Msk8 tomato cells, as well as in heat shock inducible BY2 tobacco cells has previously been demonstrated [10].

In this paper, we demonstrate real-time in-vivo detection of GUS enzyme driven by an *Arabidopsis* RD29 promoter [11] in *Nicotiana tabacum* plants using a simple three-electrode chip attached to a leaf and a portable potentiostat. We studied the dehydration of tobacco plants using in-vivo measurements and compared them with control plants, which were watered regularly.

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II. Experimental

A. Plants for drought sensing

In order to investigate the effect of dehydration levels on the expression of GUS, we studied plants stably transformed with the GUS enzyme driven by an *Arabidopsis* RD29 promoter that induces its expression under dehydration stress.

B. Fabrication of electrochemical chip

The electrochemical chip used here is similar to the one used in previous studies [10]. The three electrode chip was fabricated using photolithography followed by metal deposition with sputtering of Ti (15 nm)/Au (200 nm) on SiO_2 (500 nm)/Si wafers. Silver was deposited on the gold by electroplating followed by anodization of Ag using 0.1 M HCl and platinum electrode to get a stable Ag/AgCl pseudo reference electrode. Before each experiment, the stability of the pseudo reference electrode of the chip was validated by measuring the open circuit potential between the reference electrode of the sensor and a commercial Ag/AgCl (3.5M KCl) reference electrode, with different concentrations of KCl solutions (0.1 M-3.5 M KCl). Further validation of the chip was performed using cyclic voltammetry of 10 mM $K_3[Fe(CN)_6]$ in 0.1M KCl.

C. Biosensing of drought plants

The expression of the β-glucuronidase (GUS) enzyme in the plants was investigated using X-Gluc (5-bromo-4-chloro-3-indolylglucuronide) and a bio-electrochemical sensor. We also measured the soil moisture content (Lutron Professional Soil Moisture Meter (PMS-714)) and monitored the temperature of the leaves using infrared imaging (FLIR T660 thermal camera). The drought response was analyzed using 3 batches of the plants, each consisting of two plants, one watered regularly (control) and the other one maintained under drought (without watering). To prevent the rhythmic opening and closing of the stomata from affecting the measurement, the experiments were always started at the same time of day (10.00-11.00).

Small leaf discs from two *N. tabacum* plants, one maintained under drought conditions and one watered regularly, were immersed in X-Gluc solution and incubated at 37 °C for 24 hours. X-Gluc, a substrate for the GUS enzyme, stains the leaf disks with a blue colour on being cleaved by the GUS enzyme. The X-Gluc assay can only provide a qualitative analysis of the enzyme expression and cannot provide quantitative analysis or kinetics of the enzyme expression. To investigate the kinetics of the enzyme expression, which are expected to be directly proportional to the real-time dehydration levels of the plant, we used an in-vivo bio-electrochemical method. In order to do this, 4-nitrophenyl β-D glucuronide (PNPG), a substrate for the GUS enzyme, was injected into the leaf. Previous investigations have shown that PNPG produces 4-nitrophenol on reaction with the GUS enzyme [10]. This product can be oxidized at a potential of 0.4 V vs Ag/AgCl pseudo reference electrode to obtain electroactive species. Hence, chronoamperometric studies were performed at 0.4 V with respect to an Ag/AgCl reference using a portable Palmsens® potentiostat (Palm Instruments BV). In each experiment, 200 µl of 10mM PNPG in 0.1M PBS buffer, (pH 5.8) (which contains 0.1M PB and 0.1M Cl^-) or 0.1 M PBS (pH 5.8), were injected into the abaxial side of a leaf. The electrode was clipped onto the leaf and chronoamperometry was performed immediately.

III. Results and Discussion

Images of the drought plant with respect to the number of days under drought are shown in Fig. 1 and 2 for batches 1 and 2 respectively. For batches 1, 2, and 3, soil moisture content varied from 18% to 0%, 16% to 0%, and 24% to 11% in the span of 15, 30, and 25 days respectively (Fig. 3). The variation in soil moisture content for each plant depends on how tight the soil is packed. Hence, the soil moisture content cannot be determined accurately.

X-Gluc positive staining was observed from 3rd/4th day (light blue colour) onwards, which indicates the expression of the GUS enzyme (Fig. 3). However, drought levels cannot be quantified by this method.

Thermal imaging results (Figs. 1 and 2) showed that the temperature of the leaf increases when the plant is under drought. The leaf temperatures observed for batches 1 and 2 were in the span of 22-25 °C. The system used here has a precision of ±1 °C. Hence, such a setup cannot be used to accurately detect drought conditions.

Cyclic Voltammetry (CV) measurements were performed on the plants maintained under drought and control conditions, using a standard electrochemical chip. The results are shown in Fig. 4. We also present the CV results of an electrolyte with the product of the enzyme/substrate reaction (4-nitro phenol). The figure clearly indicates the plant sensor's potential for detection of drought induced stress.

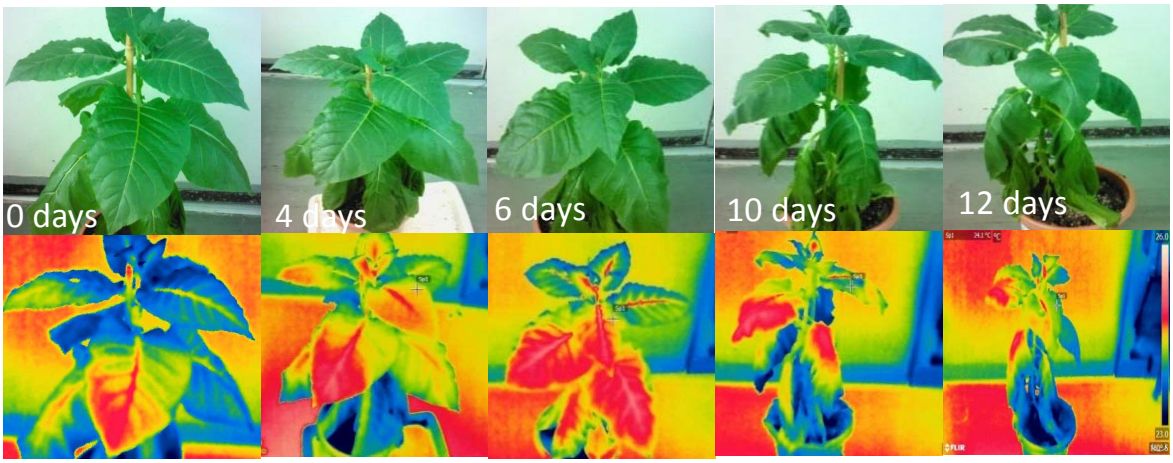

Fig. 1: Images of the plants under drought conditions (from batch 1) with respect to number of days along with infrared images

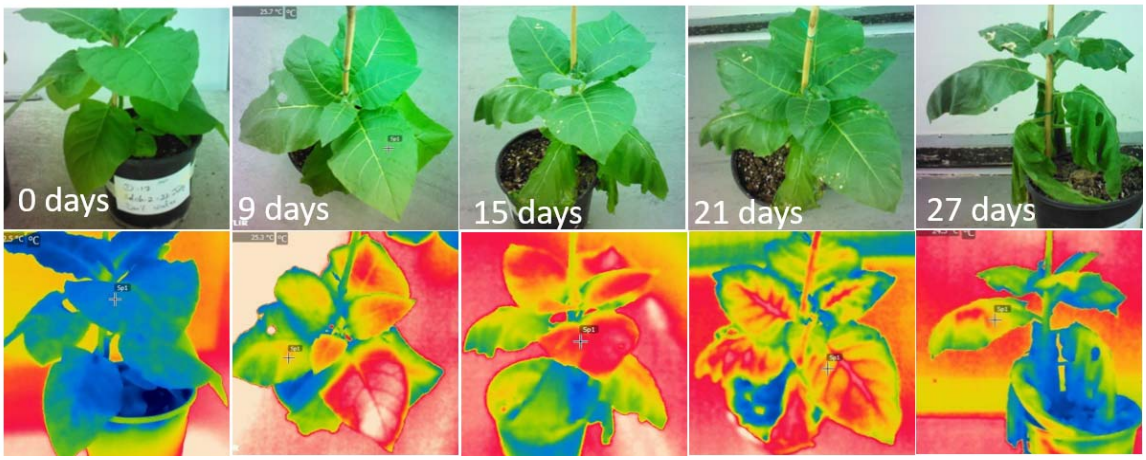

Fig. 2: Images of the plants under drought conditions (from batch 2) with respect to number of days along with infrared images

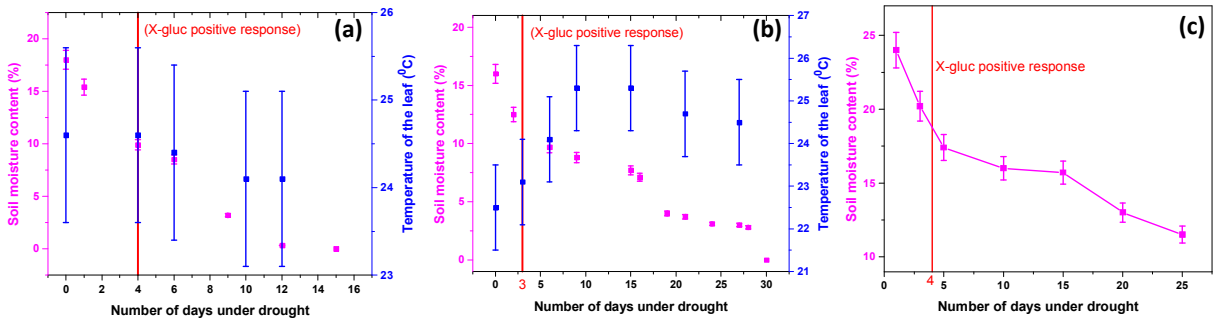

Fig. 3: Soil moisture content and leaf temperature for (a) batch 1, (b) batch 2, and (c) batch 3 drought plants

On performing chronoamperometry after injecting PNPG into the plants' leaves, a Faradaic current, generated due to the oxidation of the enzyme-substrate electroactive product, was observed. The current was significantly higher in the drought plant with the substrate, as compared to three control experiments (Fig. 5). Similar results were observed with the X-Gluc staining experiment, with blue coloured stains in samples from the plant under drought conditions and no colour change in samples from watered plants.

Due to the variability in biological factors such as stomata size and distribution, and the relative location of the electrodes with respect to the stomata, the magnitude of the observed current varies from sample to sample. Hence, it is necessary to normalize the current in order to compare between the results. In some cases, the transient current may reach very high values immediately after the beginning of the experiment, while in other samples there may be a significant delay. We assume that this transient delay does not represent any additional information, and exists only due to geometrical and structural effect in the electrodes and the leaves. Therefore, we calculated the effective current value at t=0 by linear extrapolation (using the current after the transient current decayed) and normalized the current vs. time using this extrapolated value. As the baseline for each experiment depends on various biological factors which vary

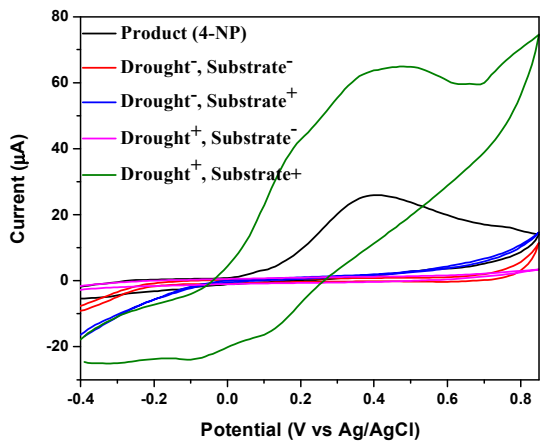

Fig. 4: Cyclic voltammetric measurements on drought and control plants with and without substrate, and product (10mM 4-NP). (Scan rate: 50 mV/sec)

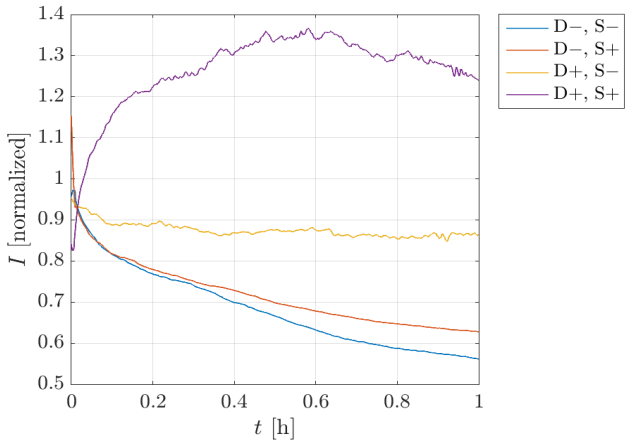

Fig. 5: Chronoamperometric measurements on drought plant with controls at 0.4V. D^-:0 days of drought plant; D^+: plant under drought; S^+: 10mM PNPG in PBS; S^-: PBS.

from plant to plant, this normalization process was performed independently for each current measurement.

There is a significant difference in electrochemical sensing of drought plants compared to watered plants. The magnitude of signal indicates the drought levels in the plant. Drought plants in three batches showed different magnitudes of signal on the same number of days under drought. This might be due to the difference in the conductance of each leaf. Fig. 6 shows the chronoamperometric results of batches 1 to 3. As a representation, the absolute values of the current was given in Fig.6c without normalization. The results indicated the magnitude of the Faradaic current did not always increase with the number of days under drought. On the first day under drought, there is no increase in current observed, whereas from the fifth day onwards, an increase in current is observed. This increase is consistent with the assumption that the product is generated following

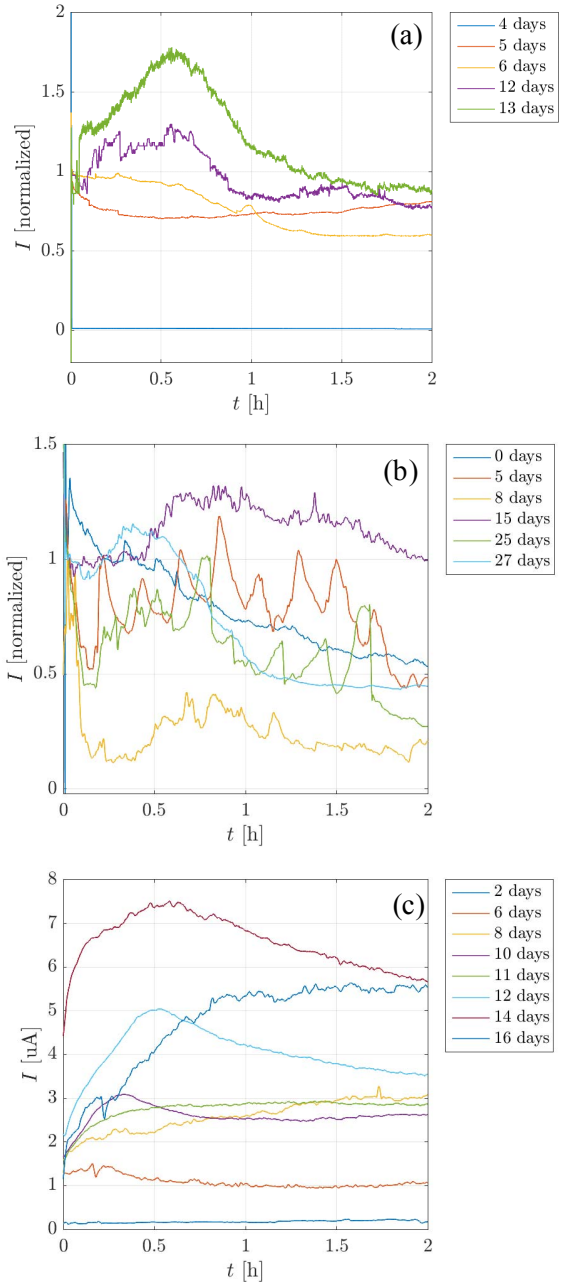

Fig. 6: Chronoamperometric measurements with plants under drought of (a) batch 1, (b) batch 2 and (c) batch 3 at 0.4V.

Michaelis-Menten kinetics and is transported to the electrodes by diffusion inside the leaf and from the stomata to the electrodes. For longer times without water, as the drought stress continues, we expect a larger quantity of product to be generated, and the electrochemical signal to increase. However, the increase in signal is not linear with time under drought. This has been observed for all plants under drought in batches 1 to 3 (Fig. 6)

We surmise the following reasons for this behaviour:

(i) The electrochemical detection depends on the enzyme-substrate product diffusing to the working electrode of the sensor through the stomata. Stomatal opening is a major factor in plant productivity and stress management [12]. Opening and closing of the stomata of leaves must be optimized because when they are open it carries out the gas exchange. However, at the same time, it results in loss of water due to evaporation. As the water levels are less in the drought plants, abscisic acid (ABA) will be released, [13] which causes the cell plasmolysed and results in the closing of the stomatal pores. Based on the percentage of the stomatal aperture opening, the product will be released through the stomata. Therefore, although we expect to have more product inside the leaf, the amount reaching the electrode may decrease due to the closure of the stomata.

(ii) For ideal operation of the electrochemical sensor, the Ag/AgCl pseudoreference electrode should be surrounded by an electrolyte with chloride ions. Under drought conditions, the amount of electrolyte on the pseudoreference electrode is very limited.

(iii) In the case of plants under drought, as the leaves are already dry, the injected substrate rapidly dries and the reaction with the enzyme is slowed down or even stopped. Therefore, the sensor cannot function as intended and the results are far than what is expected under ideal conditions (see for example reference [10]).

CONCLUSION

In-vivo sensing of the water levels in the plant was demonstrated by an electrochemical method using the plant itself as a sensor. Compared to ex-vivo sensing methods such as soil moisture content, thermal imaging and X-Gluc staining, plant based electrochemical sensing is faster. Hence it is more sensitive compared to ex-vivo techniques. However, the electrochemical signal deteriorates with increased time under drought. Under extreme drought conditions, the stomata are closed which reduces the electroactive analyte transport from the leaf. Also, dehydrated leaves prevent ideal operation of the sensor due to the lack of a chloride ion rich solution around the reference electrode. Further study is required to improve the signal; for example, by sampling the signal from the leaf using a penetrating electrode and/or introducing a wetting mechanism using a microfluidic apparatus to wet the working, auxiliary and reference electrodes.

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