Exploring photosynthesis for power generation

A thesis submitted towards partial fulfillment of
BS-MS Dual Degree Programme
by

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Certificate

This is to certify that this dissertation entitled “Exploring photosynthesis for power generation” towards the partial fulfilment of the BS-MS Dual Degree programme at the Indian Institute of Science Education and Research, Pune represents original work carried out by Bhumika Sumesh Upadhyay at IISER Pune under the supervision of Dr. Sagar Pandit, Assistant Professor, IISER Pune during the academic year 2018-19.

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Declaration

I, Bhumika Sumesh Upadhyay, hereby declare that the matter embodied in the report entitled “Exploring photosynthesis for power generation” are the results of the work carried out by me at IISER Pune under the supervision of Dr. Sagar Pandit and the same has not been submitted elsewhere for any other degree.

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Abstract

Biophotovoltaics is a novel approach to generate electricity which utilizes plant's ability to harvest light energy. Under this concept, the photosynthetic electrons from plants are collected to produce electric current. So far, the research in this field has mainly focused on increasing the efficiency of the electrode to capture more photosynthetic electrons. However, not much work has been done towards optimizing the plant's photosynthetic machinery in itself. In this project, we have explored the effects of naturally occurring herbivory on plant's photosynthetic machinery as a means to enhance the biophotovoltaic current. Using jasmonic acid (JA) treatment we simulated the effects of herbivory in plants and found that JA treatment enhances the net current output from the biological material like thylakoid. We have also studied the sustainability of the photosynthetic system in the biophotovoltaic cell by testing the photoactive response life of the biological materials. Taken together, our work paves way for incorporating further biological research in the field of biophotovoltaics for power generation.

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1. Introduction

In any developing economy, one of the most fundamental requirements is a sustainable energy source. Our society is aware of the importance of using renewable energy sources over the non-renewable ones. In this regard, people have tried to explore natural resources such as wind, sunlight, and water, to be used as a renewable energy source.

However, there are few challenges in such schemes such as:

- High capital investment
- Prolonged installation time
- High maintenance cost
- Weather dependency

Hence, in addition to the so far mentioned energy sources, biological systems have also been used as a pool of energy from which one could draw electrons and generate electric current.

1.1 Biological systems in Energy production

One of the primary sources of energy is the sun. Solar energy is harvested artificially with the help of solar panels made up of semiconductor materials and is converted into electrical energy to be further used by humans. Similarly, in nature plants harvest solar energy and convert it into chemical energy, by the process of photosynthesis. Hence, since past years there have been various schemes to incorporate this light harvesting property of the plants in electrical energy production. Use of plants and biological systems over solar panels is advantageous as they grow and self-repair whereas solar panels tend to lose efficiency over time (Sherwani and Usmani, 2010). There are different ways of incorporating biological systems in energy production.

Biofuels are one such way of using biological systems as an energy source. Biofuel is the fuel obtained from the stored plant biomass and animal waste. It mainly includes fuelwood, charcoal, biodiesel, biogas and pyrolysis oil. It is produced by

unconventional ways like agriculture and not by conventional means like geological processes which produce fossil fuel. Though biofuel is a renewable source of energy, its production isn't a popular approach as it involves growing and harvesting crops which can be processed only for the production of biofuel (Cheng and Timilsina, 2011). The whole process in itself is quite arduous. It has also been argued to cause extensive diversion of resources like labor, machinery, fertilizer, etc. away from food crops, which would inevitably lead to food scarcity.

The microbial fuel cell is another approach which uses the plant's ability to harvest solar energy to produce organic matter. Plants produce reducing equivalents like acetate. The heterotrophic microorganisms then catalyze the oxidation of such organic molecules released by the plant. This oxidation reaction leads to the release of electrons which are captured by the electrodes of the fuel cell (McCormick et al., 2015). However, usage of a microbial fuel cell is limited as it requires a constant supply of organic substrate for the microorganisms.

Biophotovoltaics is an upcoming alternative approach where the artificial system draws electrons directly from photosynthetic machinery of green plants. However, bio-photovoltaics is different from photosynthetic microbial fuel cells and other light-harvesting bio-electrochemical systems as it does not require any reducing elements produced by plants or any specific microbe. Instead, the plant's thylakoid and photosynthetic protein complexes are directly employed as a source of electrons to draw electricity (McCormick et al., 2015).
Therefore, this approach overcomes limitations as mentioned earlier of other biological system-based energy sources.

1.2 Biophotovoltaics and recent research

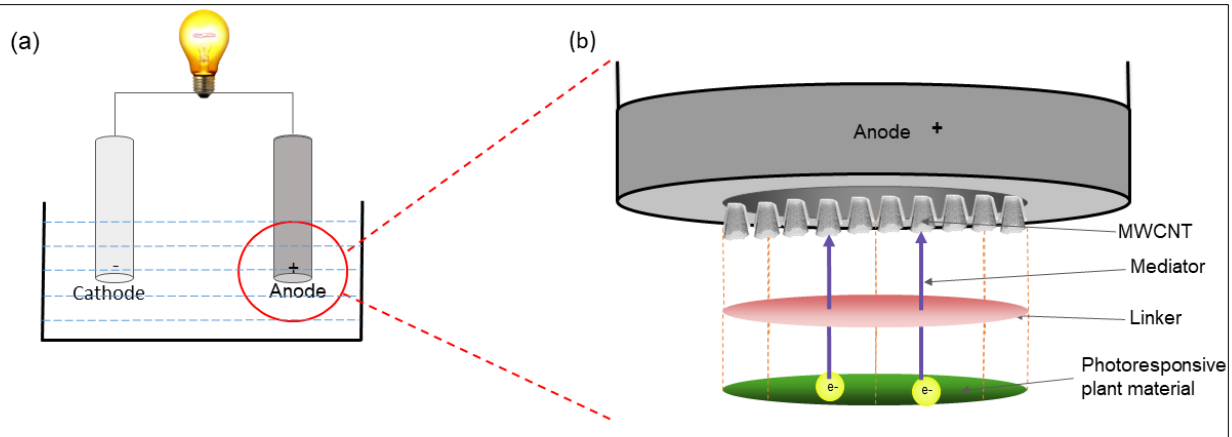


Figure 1: Biophotovoltaic cell component

(a) Biophotovoltaics cell incorporates a biological material modified anode and a cathode with mediator molecule in the electrolyte to facilitate electron carriage

(b) Biophotovoltaics cell anode component; Anode is modified with Multiwalled carbon nanotube (MWCNT) to which photoresponsive biological material is immobilized using a liker molecule.

The term Biophotovoltaics was coined in 2015 (McCormick et al., 2015). 'Bio' here stands for biological material like plant and 'photovoltaics' stands for a material that generates electricity by conversion of light energy by photovoltaic means.

A typical biophotovoltaic cell comprises a cathode and an anode [figure:1(a)]. The anode here is modified with the photoresponsive biological material which generally encompasses plant photosynthetic components. The plant material is attached to the electrode with the help of a biocompatible conductive linker molecule [figure:1(b)]. A mediator molecule facilitates the transfer of the photosynthetic electrons to the electrode. When the light is incident on the plant material, the electrons generated in the process of photosynthesis, are then captured and directed towards the anode.

So far, the primary research towards advancements in this field has focused upon creating better electrode or better linker molecule or finding a better mediator to facilitate the detection of photosynthetic electrons more efficiently by the anode. Although studies like these have provided the information of the system's maximum potential for power generation, it has neglected a few important concepts like the low

sustainability, low half-life and no self-repair of the plant material (thylakoid membrane or protein complex) used in vitro. Also, the major research in this field has been towards artificially upgrading the electrode for enhanced current production but has mostly neglected exploring the biological photosynthetic machinery for better performance of these systems.

A study by Calkins' group in 2013 was a significant milestone in the field of biophotovoltaics. In their research, they devised a photosynthetic electrochemical cell which generated a maximum power density of 5.3 $\mu W/cm^2$. This cell was prepared using thylakoid modified anode and laccase modified cathode and flashing light on thylakoid generated electrons which were captured by the anode. A linker molecule- 1-Pyrenebutanoic acid succinimidyl ester (PBSE) was used to connect thylakoid to the electrode. To increase the electron capturing efficiency of the electrode, it was modified with multiwalled carbon nanotube (MWCNT) (Calkins et al., 2013). Along with this, a few other studies were undertaken with combination of different plant material like- Photosystem I (Mershin et al. 2012; Yehezkeli et al 2013, Gizzie et al., 2015), Photosystem II (Yehezkeli et al., 2012; Yehezkeli et al 2013), thylakoid (Lee et al., 2016; Ahmed et al., 2009) and Cyanobacteria (Anderson et al. 2015; Çevik et al., 2018) with different electrodes.

Further designs were tested to modify electrode to make it more biocompatible (Ibrahim et al., 2018), to prepare a porous translucent electrode (Wenzel et al., 2018) or even to modify it to eliminate the need to immobilize the thylakoid (Pinhassi et al., 2016). Approaches to improve the linker molecule (Çevik et al., 2018) and mediator (Kato et al., 2012) to obtain higher photovoltaic current were tested. Various attempts were made to increase the sustainability of the biophotovoltaic cell by switching to more stable analyte like chloroplast (Hasan et al., 2017) or cyanobacteria (Schuergers et al., 2017). To establish direct contact between the electrons generated during photosynthesis and electrode, a nanobionics technique to introduce single-walled carbon nanotube to penetrate inside the lipid bilayer of chloroplast and increase the current output was tested (Giraldo et al., 2014). A more recent approach in the research of biophotovoltaics was when the biophotovoltaics devices were modified both for light

harvesting and charge storage (Falk and Shleev, 2018). In addition to that, a design to prepare next generation biophotovoltaic cells by printing live cyanobacteria cells was also introduced (Sawa et al., 2017). In an attempt, to commercialize the idea of biophotovoltaics, key methods were suggested to reduce prices and increase efficiencies (Tanneru et al., 2019).

Though there have been attempts to incorporate genetically engineered cyanobacteria to increase net current output (Schuergers et al., 2017), there haven't been many studies which explicitly explore the process of photosynthesis and its machinery in biophotovoltaics.

1.3 Photosynthesis

Photosynthesis is the process by which the plants produce glucose by fixing carbon dioxide in the presence of water and sunlight. This whole process takes place inside the chloroplast organelle in the plant cell. The chloroplast is made up of two regions- membrane bound stacked compartments called thylakoid and the surrounding clear fluid called stroma.

The process of photosynthesis is divided into two steps:

1. Light reaction
2. Dark reaction or Calvin cycle

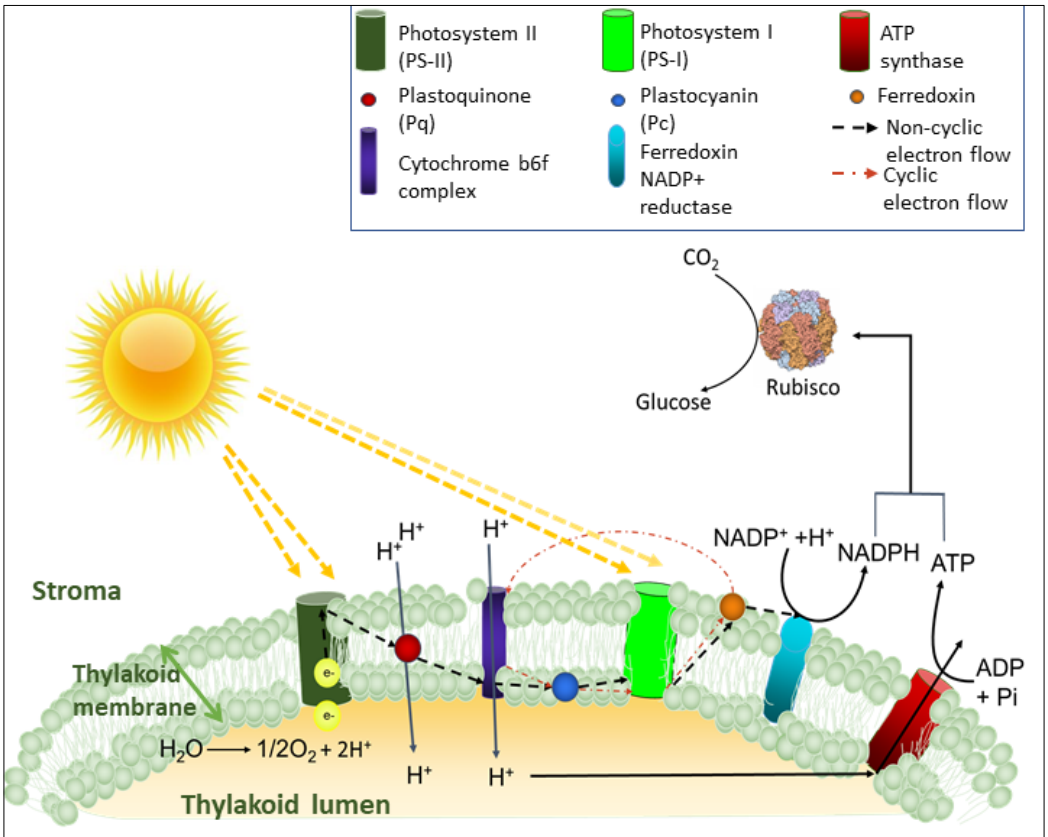


Figure 2: A schematic representation of the process of photosynthesis in chloroplast

Electrons excite when the light energy falls on the photosystem complexes in transmembrane region of the thylakoid. They then travel through the photosynthetic electron transport chain (PET) in transmembrane region of the thylakoid. End products are NADPH+ and ATP which are further utilized by Rubisco in the stroma to fix carbon dioxide.

Light reaction is the first reaction in the process of photosynthesis. It takes place in the thylakoid region of the chloroplast. At the end of the light reaction, Adenosine triphosphate (ATP) and reduced Nicotinamide adenine dinucleotide phosphate ($NADPH^+$) are produced, which are required in the dark reaction of photosynthesis.

It starts in the Photosystem II (PS-II) protein complex of thylakoid transmembrane region. The photosystem complex contains chlorophyll and other accessory pigment molecules. These molecules absorb photons when the light is incident and excites electrons to a higher energy state. These electrons are then taken up by the reaction center chlorophyll molecule located in PS-II and channelize the electrons to Plastoquinone in the transmembrane region of thylakoid. These energized electrons then transfer through the photosynthetic electron transport (PET), facilitating the pumping of protons from the stroma into the thylakoid lumen, creating a proton concentration gradient across the thylakoid membrane (Krause and Weis, 1991).
The electrons lost by PS-II are replenished by the electrons coming from the photolysis of water. The photolysis of water results in the formation of an oxygen molecule, proton, and electrons.

Photon gradient across the thylakoid membrane finally results in the formation of ATP by a transmembrane protein- ATP synthase. ATP synthase produces ATP by the phosphorylation of Adenosine diphosphate (ADP).
Phosphorylation can further be categorized into two types, based on the nature of the flow of electrons [figure 2].

1. Noncyclic Photophosphorylation

The photoexcited electrons take a linear path and further result in the formation of both ATP and NADPH.

$NADP^+ + H^+ + e^- \longrightarrow NADPH+$

$ADP + P_i + e^- \longrightarrow ATP$

The electrons in noncyclic photophosphorylation are fully utilized at the end of light reaction in the abovementioned chemical conversion

2. Cyclic Photophosphorylation

The photoexcited electrons take a cyclic path around PS-I and further result in the formation of ATP. Here, the electrons do not get utilized in the preparation of $NADPH^+$ but return to the plastoquinone pool.

An increase in the net cyclic photophosphorylation results in the net electron flux around the thylakoid.

The second reaction following the light reaction in the process of photosynthesis is the dark reaction. It takes place in the stroma region of the chloroplast. It is in this reaction that the fixing of carbon dioxide and the formation of glucose take place. The most important enzyme in this process is Rubisco. It catalyzes the dark reaction utilizing both ATP and $NADPH^+$. Another enzyme which is essential in the dark reaction is Rubisco activase (RCA). It is the enzyme which promotes the activation of Rubisco by removing inhibitory sugar phosphates from its active sites (Portis, 2003). So, in its absence, the activity of Rubisco is compromised.

There are various ways which affect the process of photosynthesis by upregulating or downregulating the components of the photosynthetic machinery. One such way is by herbivory on plants.

1.4 Effect of Herbivory on photosynthesis

Plants are the organisms which invest energy in growing stem, leaves, fruits and other body parts. Herbivory is the act by which the herbivore animals feed on the plant parts. So, to cope up with herbivory, plants have evolved different methods to limit the effects of herbivory. One of them is through chemical means.

When a plant experiences herbivory, it responds by upregulating jasmonic acid in its cells. Jasmonic acid is a phytohormone which activates the expression of protease inhibitors and other anti-herbivore metabolites. This serves as a defense of plant towards herbivory.

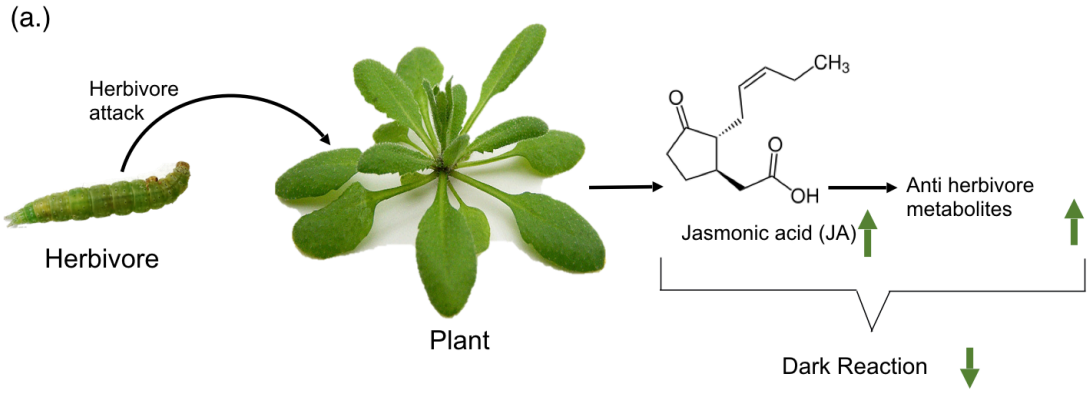


Figure 3: Schematic representation of the effects of herbivory in plants

(a) Herbivory induced Jasmonic acid upregulation decreases photosynthesis in plants

(b) Jasmonic acid mediated effects on photosynthetic machinery

Out of the several effects of the jasmonic acid surge in the cells, one is downregulation of dark reaction (Halitschke et al., 2003; Mitra and Baldwin, 2014) [figure 3(a)]. This effect is seen as a result of downregulation of Rubisco enzyme.

In a few separate studies on the effects of downregulation of the Rubisco, it was also observed that the cyclic electron flow increases in Rubisco (Alric et al., 2010) and Rubisco activase (RCA) mutants (Jin et al., 2008) as compared to the wild-type plants.
As a result of this, the level of NADPH+ rises and the process of cyclic photophosphorylation gets upregulated [figure 3(b)].

Another effect seen as a result of the increase in Jasmonic acid is the reduction in the expression levels of a transmembrane Ferredoxin NADP-reductase (FNR) (Guo et al., 2017) [figure 3(b)].
Which catalyze the following reaction:
[2 reduced ferredoxin + $NADP^+$ + H^+ $\rightleftharpoons$ 2 oxidized ferredoxin + $NADPH^+$]

This decrease in the amount of FNR results in a high amount of reduced ferredoxin in the plant cell.
As a result, PGRL1-PGR5 (Proton gradient regulation 1 and 5) complex then switches the transfer of electrons from reduced ferredoxin to cytochrome b_6f complex to maintain the distribution of electrons in the photosynthetic system. This also increases the cyclic electron flow (DalCorso et al., 2008).

So, it could be implied that upon JA treatment as the activity of Rubisco and FNR is downregulated and as a result, the process of cyclic photophosphorylation gets upregulated. This upregulation of cyclic photophosphorylation leads to an increase in cyclic electron flow in thylakoid and further increase the electron flux around thylakoid.

1.5 Ways to biologically increase the biophotovoltaic current

Perturbing the process of photosynthesis could lead to alteration in the photosynthetic electron flow and the biophotovoltaics current. A potential approach of increasing biophotovoltaics current production is by the knockdown of photosynthesis related genes or by using a chemical inhibitor of the photosynthetic machinery. If the chlorophyll molecule and reaction center at PS-II is not disturbed, the excitation of electrons will mimic the wild-type by regularly producing photocurrent. However, by reducing the functionality of the photosynthetic specific electron carrier proteins, their electron carrying capacity would be reduced. As a result, the number of excited electrons would exceed the carrying capacity of the electron carrier proteins and could lead to excess electron accumulation around the thylakoid. This further could enhance the total current of the bio-photovoltaic source.

A study by Pisciotta et al. reported that the transfer of electrons by Plastoquinone to Cytochrome-b6 complex was blocked by the addition of 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (DBMIB) to thylakoid, thus increasing the biophotovoltaic current output (Pisciotta et al., 2011). Following this, Bradley et al. hypothesized that if there is high light stress at the water-splitting complex, the number of electrons generated will be high. However, the downstream proteins would not be able to capture the electrons at such a high rate. This would then lead to an increase in the net electron pool accumulation (Bradley et al., 2012).

The chemical treatments and generating photosynthetic mutants serve the purpose of understanding the role of an individual component of the photosynthetic machinery, but this approach can’t be extrapolated under natural conditions.

One of the most prominent ways to increase the electron flux around thylakoid without opting for a synthetic biology approach could be by mimicking an ecological process. One such process is that of herbivory in plants. This could be done by treating the plant with jasmonic acid. As the results of this treatment are naturally found in the

ecosystem, it could be one of the adequate alternatives to increase the current output from the biophotovoltaics device.

This project addresses the problem of low current output and photoresponsive life in previous biophotovoltaics systems by trying to create a biophotovoltaic anode with biological materials. The primary goal was to prepare the biophotovoltaic anode with high photoresponsive life while also mimicking the effects of herbivory in the increment of photosynthetic electron flux.

1.6 Experimental outline

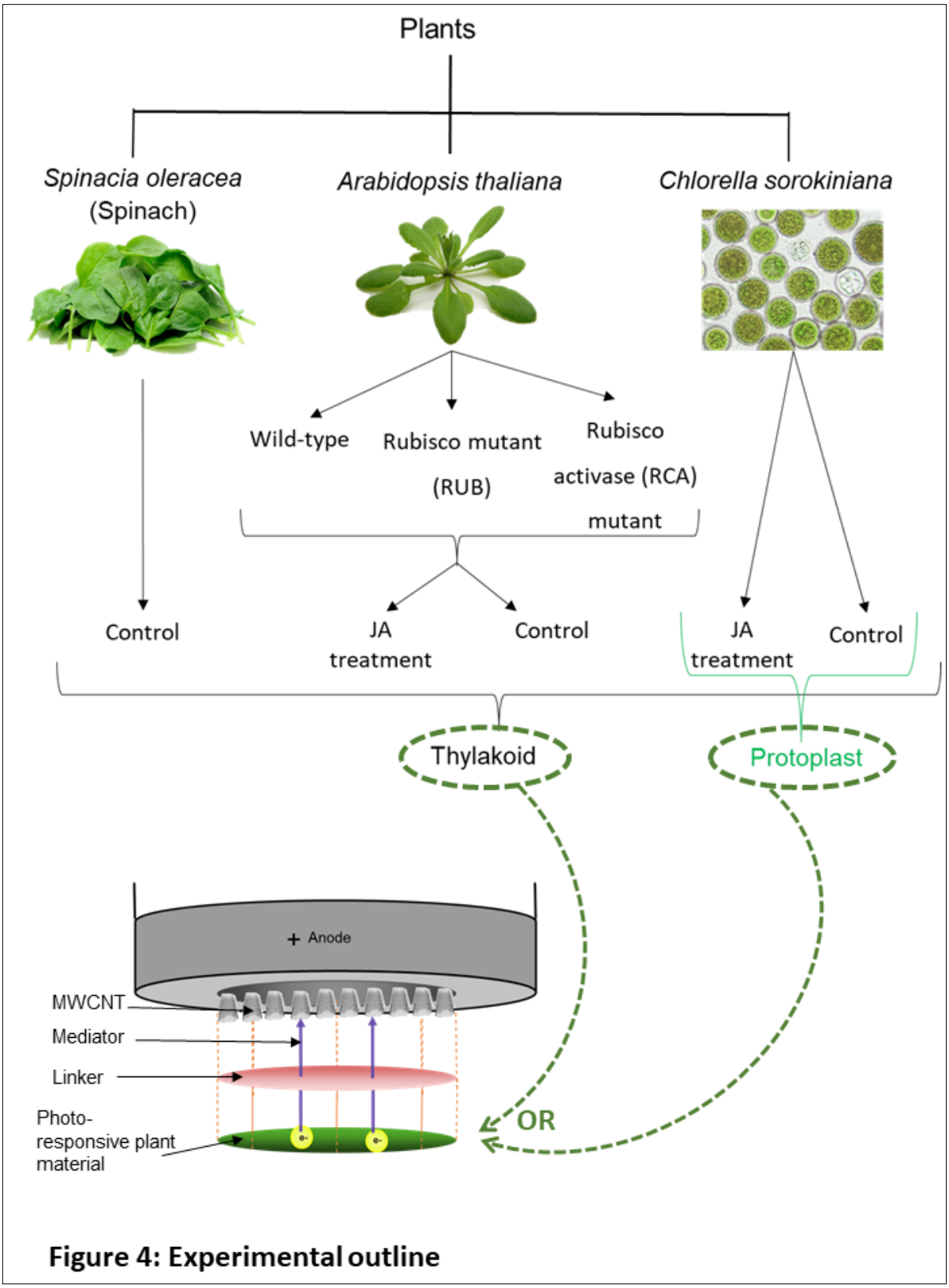


Figure 4: Experimental outline

There are three parts of the project:

(i) Optimization of the biophotovoltaic anode

(ii) Testing the effect of JA on biophotocurrent

(iii) Increasing the sustainability of the biophotovoltaic anode

(i) Optimization of the biophotovoltaic anode

The standardization of electrode for electrochemical measurements was done by increasing the electron capturing efficiency of an electrode by coating it with multiwalled carbon nanotube (MWCNT). 1-Pyrenebutanoic acid succinimidyl ester (PBSE) was used as the conductive linker molecule which attached the thylakoid to MWCNT coated electrode.

Spinacia oleracea (Spinach) was used in this process to obtain thylakoid. Spinach is known to have a high content of chloroplast and hence thylakoid. Due to its abundance and easy availability throughout the year, it has been used in many bio-photovoltaics studies (Calkins et al., 2013). Spinach is used here to obtain PS II enriched membrane as per the method by Berthold, Babcock and Yocum.

(ii) Testing the effect of JA on bio-photocurrent

The testing of JA treatment on bio-photocurrent was done by using the thylakoid obtained from *Arabidopsis thaliana.*

A. thaliana is the model organism for the study of genetic manipulation in plants. Thylakoid used here was harvested from three types:

(a.) Wild type

(b.) Knockdown plant lines of Rubisco small subunit 2B (RUB)

(c.) Knockout plant lines of Rubisco activase (RCA)

The mutants will provide information about the changes in biophotovoltaic current with regards to changes in photosynthetic electron flow.

Leaves of these plants are sprayed with jasmonic acid to mimic the effect of herbivory and observe changes in biophotovoltaic current.

(iii) Increasing the sustainability of the biophotovoltaic anode

To test for increasing the sustainability of the biophotovoltaic anode, photosynthetic parts from *Chlorella sorokiniana* were used.

These are monoplastidic algae. Most of their cell volume is occupied by the chloroplast (Carfagna et al., 2013). Unlike higher plants, each cell is a unique individual and capable of surviving. Both, the extracted thylakoid and the protoplast of these cells were used to check the photovoltaic current.

These algal species were also treated with jasmonic acid, and further used to obtain protoplast and thylakoid to check photovoltaic current.

Materials and Methods

2.1 Chemicals

BG 11 Broth and Murashige and Skoog (MS) medium for culture and growth were purchased from HiMedia Laboratories Pvt. Ltd.. Tricine, Dialysis Membrane - 50 and 2-(N-morpholino) ethanesulfonic acid (MES) buffer were also bought from HiMedia Laboratories Pvt. Ltd.. Diuron was bought from Sigma-Aldrich Corporation.
Multiwalled carbon nanotube (MWCNT) was purchased from Aldrich with 95+% purity.

2.2 Plant Growth and Treatment

Spinach plants were planted in the IISER-Pune greenhouse at room temperature. This allowed the fresh leaf tissue collection, minimizing senescence.
Arabidopsis thaliana plants were grown on MS media plates and then were transferred to soil. The plants were kept in growth chamber with 70% humidity, 24^0C temperature, 130 µmol/m^2 luminescence and in 10 hours light and 14 hours dark exposure.
Plants were sprayed with 0.625 µmol JA (dissolved in 30% (v/v) ethanol–water and the leaf tissue were kept for 60 minutes before thylakoid extraction. Control plants were sprayed with 30% (v/v) ethanol-water.
Chlorella sorokiniana was obtained from National Chemical Laboratory (Pune) and was cultured in BG11 media with light intensity of 2000 Lux, at temperature 23-25^0C with occasional shaking manually.

2.3 Jasmonic acid (JA) Treatment

Arabidopsis thaliana plants were sprayed with 0.625 µmol JA (dissolved in 30% (v/v) ethanol–water and the leaf tissue were kept for 60 minutes before thylakoid extraction (Mitra and Baldwin, 2014).

Jasmonic acid dissolved in ethanol at concentration of 10^{-5} M was added to the *C. sorokiniana* culture. The final ethanol concentration in the culture media didn't exceed 1% (v/v) (Czerpak et al. 2006).

2.4 Plant material extraction/preparation

Berthold, Babcock and Yocum (BBY) method to extract thylakoid

Photosystem enriched thylakoid was extracted from spinach. For this process, approximately 150g of leaf lamella was used. The extraction was carried out in cold room under dark according to Carpentier et al. Ultracentrifugation was performed at different steps to pool down PS II rich thylakoid membrane. The obtained thylakoid was stored in amber color tubes at -80^{0}C (Carpentier and R. ed. 2004).

Thylakoid membrane

Extraction of thylakoids from leaves of *A. thaliana* and cells of *C. sorokiniana* was performed according to Chen et al. *A. thaliana* leaf mass ranged from 4 to 10g whereas, the pellet cell mass of *C. sorokiniana* ranged from 0.8 to 1.5g. All the experiments were performed in cold room under low light condition. The obtained thylakoid membrane was stored in amber color tubes at -80^{0}C (Chen et al., 2016) .

Protoplast

For preparing the protoplasts, lysozyme was added to the *C. sorokiniana* culture at a concentration of 5 mg/L and was kept at 37 °C for 10 h (Gerken et al., 2013). This resulted in increased permeability of the electrically non conducting cell wall of *C. sorokiniana* cells and hence increased current output.

2.5 Chlorophyll loading

The absorbance of extracted thylakoids and prepared protoplasts at the wavelength of 645nm and 663nm were measured using microplate photometers.

The concentrations of chlorophyll a, chlorophyll b and total chlorophyll were calculated using the following equation:

Total Chlorophyll: 20.2(A645) + 8.02(A663)

Chlorophyll a: 12.7(A663) – 2.69(A645)

Chlorophyll b: 22.9(A645) – 4.68(A663)

(Rajlakshmi and Banu, 2015)

Accordingly, the thylakoids and protoplasts were loaded on the electrode, considering the total chlorophyll.

2.6 Anode Coating

Working electrode was modified with multiwalled carbon nanotube (MWCNT), before coating thylakoid. To coat MWCNT (size ranging from 0.5-200 μm) on the electrode, it was suspended in solvent [figure:6(b)]. Then the MWCNT suspension was kept for sonication to obtain homogeneous dispersion for 5-6 hours. The suspension was coated onto the working electrode by drop casting and then was dried at 70 ^{0}C for 2 to 3 hours.

Further, 10 μmol 1-Pyrenebutanoic acid succinimidyl ester (PBSE) was also drop casted on the MWCNT coated electrode and was kept in ice bath for 10 minutes. The coating on electrode was further followed by drop casting plant material, and was kept for incubation in ice bath (Calkins, et al. 2013).

2.7 Scanning Electron Microscopy (SEM)

The plant material (thylakoid/protoplast) was drop casted on multi walled carbon nanotube (MWCNT) coated electrode. The plant material-MWCNT and MWCNT coated electrodes were then kept at 60°C for three hours and then overnight in the desiccator before obtaining SEM images (Ryu et al., 2018).

The SEM imaging was performed by the Biology Department, Central Microscopy Facility at IISER Pune.

2.8 Photoelectrochemical activity analysis

Half-cell reactivity of the analyte (here: plant material) was investigated using three electrode system. The chronoamperometric readings were taken using platinum wire counter electrode and a silver-silver chloride (Ag/AgCl) reference electrode.

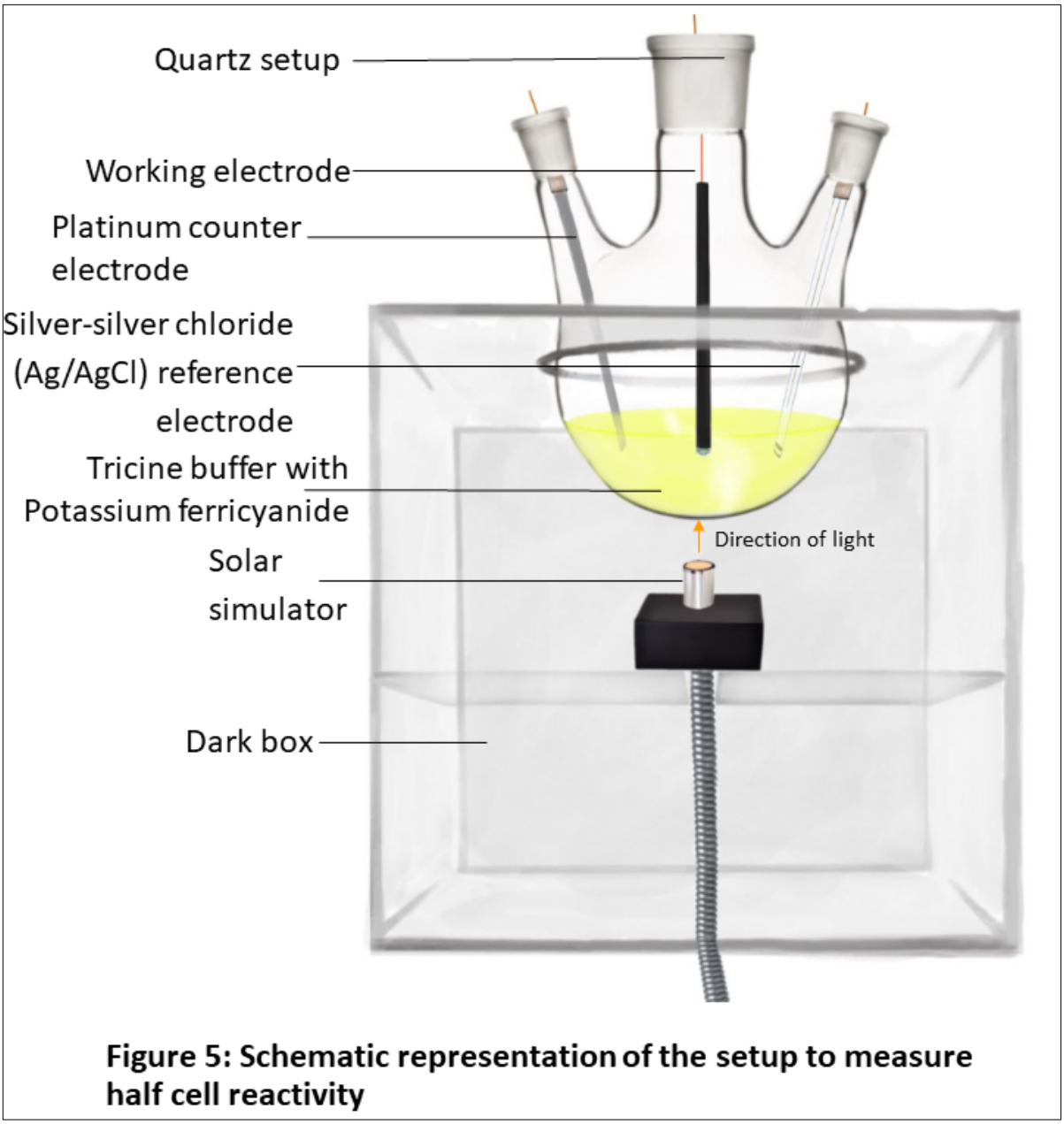


Figure 5: Schematic representation of the setup to measure half cell reactivity

Testing was done using CH760E. (CH Instruments) electrochemical workstation in the dark at room temperature. Pet photoemission tech 150 WSS-EM solar simulator

was used as the light source to incident light of intensity 100 mW cm^2. 0.1 M Tricine buffer with 7.8 pH was used as the electrolyte. The redox mediator was 1.5 mM $[Fe(CN)_6]^{-3}$.

2.9 Statistical analysis

The statistical analysis was done using student t-test and one-way ANOVA and the statistical significance ($P \leq 0.05$) was determined by Fisher's least significant difference *post hoc* test using the software- Graphpad Prism.

Results and Discussion

3.1 Optimization of Biophotovoltaic anode

The first objective of the project was to establish the concept of photovoltaics in the context of the photosynthetic biological material. To achieve that, it was important to test the photovoltaic activity of the biological material using electrochemical tests.
The first step to measure the electrochemical activity and biophotovoltaic response of the analyte (here: photosynthetic biological material), is to prepare a photo-responsive analyte coated anode. This anode would serve as the basis for biophotovoltaic activity and hence, the concept of harnessing electrons from the photosynthetic machinery.

The components of a photovoltaic anode are as follows:

- The primary working electrode
- Multiwalled Carbon nanotube (MWCNT)
- A linker molecule- to attach the analyte to MWCNT
- Photoresponsive analyte- plant's photosynthetic material

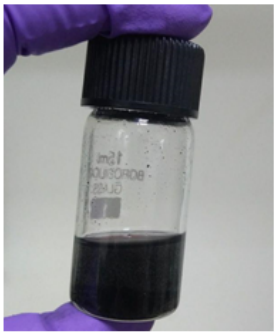
To prepare a biophotovoltic anode, the electrode is coated with MWCNT and linker molecule and is further modified with a photo-responsive analyte.
Figure1(b) shows the direction of the flow of excited electrons from the photovoltaic analyte to the electrode.

Optimization of component 1: MWCNT even coating

(a)

Solvent	Specificities	PVDF(mM)	Result
DMF	10mM DMF	-	high phase separation seen
DMF	Absolute DMF	-	phase separation seen
DMF +PVDF	Absolute DMF	10	phase separation seen
DMF +PVDF	Absolute DMF	50	phase separation seen
DMF +PVDF	Absolute DMF	100	phase separation seen
Ethanol +Nafion	With activated carbon	-	high phase separation seen
	High Nafion, high MWCNT	-	homogeneous dispersion

(b)



(c)

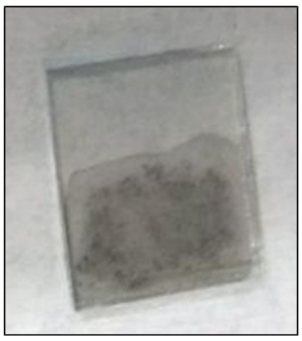


Figure 6: MWCNT modified anode preparation

(a) MWCNT suspension optimization [DMF:Dimethylformamide, PVDF: Polyvinylidene fluoride] the highlighted preparation was further used for modifying electrode

(b) MWCNT dispersed in Nafion containing ethanol

(c) MWCNT even coating on anode (iv) MWCNT provide a directional channel for electrons to reach the electrode

The primary electrode was modified by coating it with MWCNT. Uniform coating of MWCNT helps in replicability and also improve the electron capturing efficiency of the electrode.

To uniformly coat the electrode, MWCNT has to be evenly dispersed in a solvent, with least phase separation. Optimization of MWCNT dispersion was carried out by dispersing different concentration of MWCNT in various solvents [figure 6 (a)].

Optimization of component 2: Finding the primary working electrode

Optimization of the working electrode was achieved by separately testing two electrodes as the working electrode for higher current output.

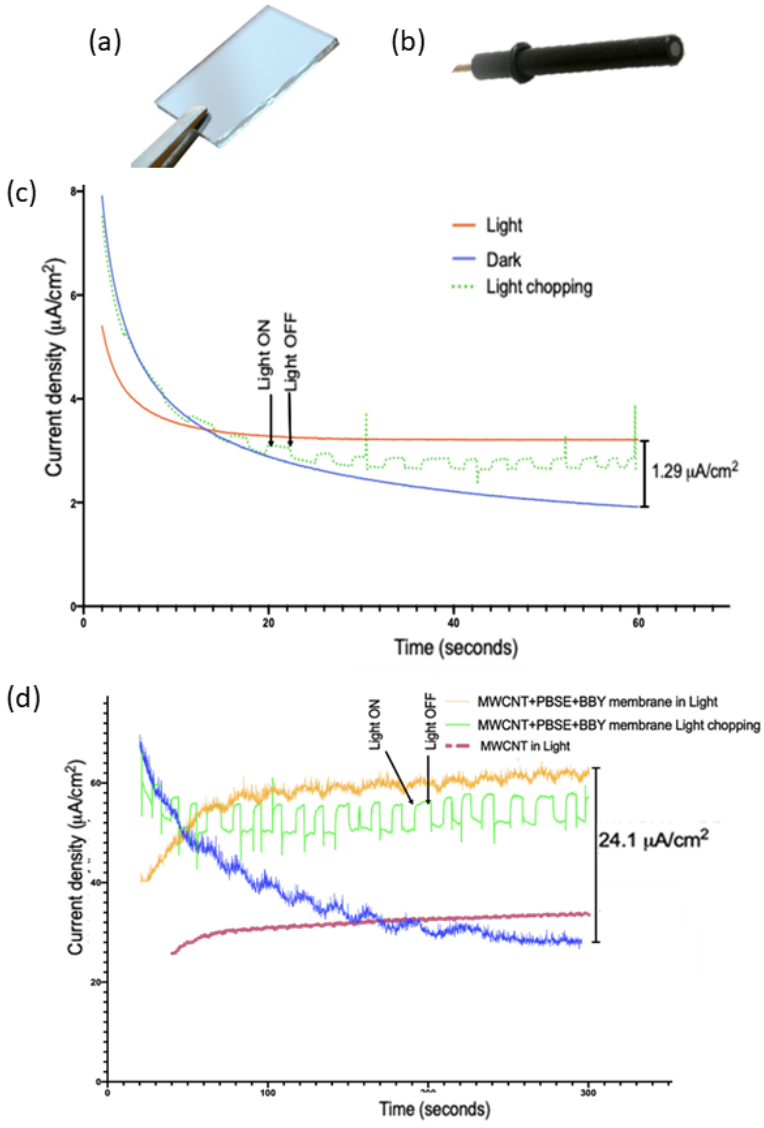


Figure 7: Optimization of working electrode
(a) Fluorine doped titanium oxide (FTO) glass sheet;
(b) Glassy carbon electrode; Chronoamperometric readings of
(c) FTO electrode and (d) Glassy carbon electrode

1. Fluorine-doped titanium oxide (FTO) glass electrode:

FTO glass was taken as the working electrode due to its transparency. This facilitated no blockage of light during analyte illumination. The surface area of the electrode available for analyte coating was 1.00 cm^2.

2. Glassy carbon (GC) electrode

It has a smaller surface area (0.07 cm^2) which facilitates homogeneous analyte coating.

The selection of electrode was made on the bases of maximum current obtained, i.e., the difference between the dark and light current. The value of obtained maximum current density for glassy carbon electrode was about 19.45 folds higher than the FTO glass electrode [figure 7]. Hence, the glassy carbon electrode was considered for further experiments.

Optimization of component 3: Thylakoid linking to the electrode

It was necessary for the photoresponsive components in thylakoid to be in close vicinity of the electrode to facilitate easier carriage of electrons and hence improving biophotovoltaic responsiveness of the electrode.

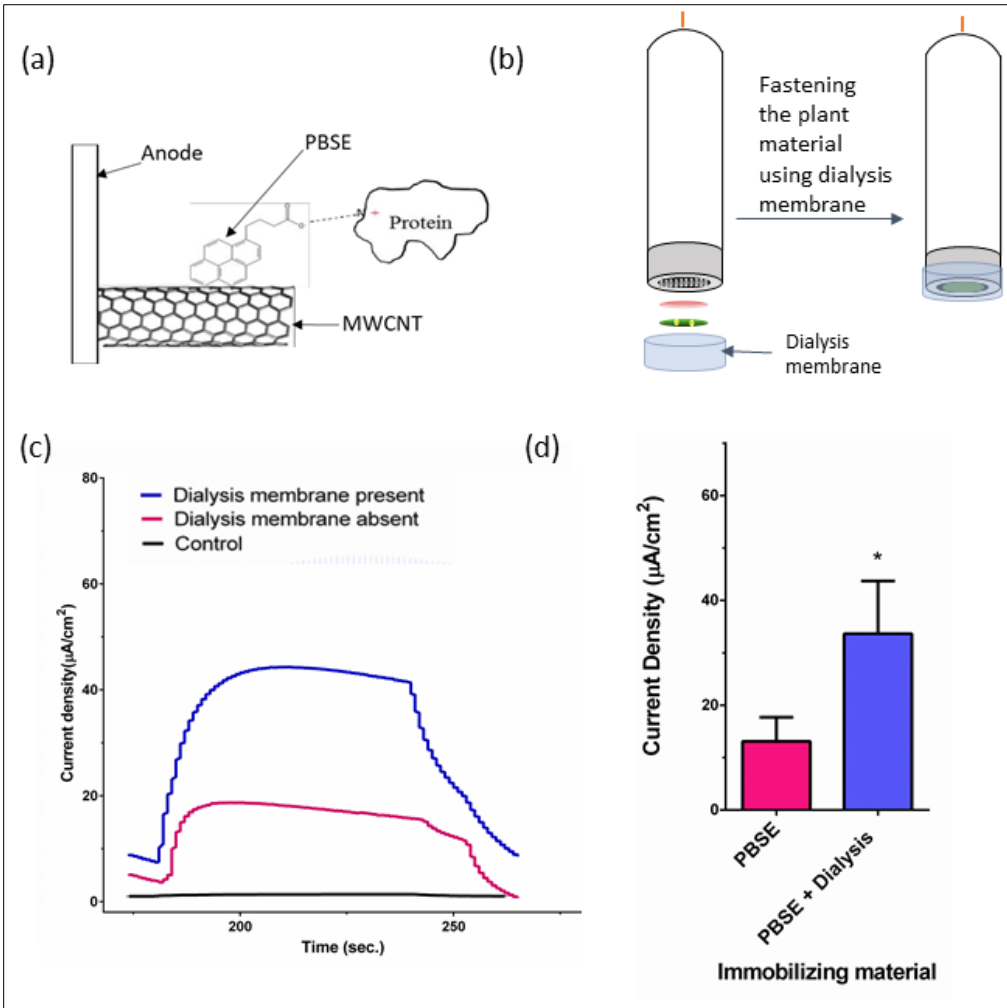


Figure 8: Linking thylakoid to electrode

(a) PBSE connects thylakoid proteins to MWCNT

(b) Dialysis membrane covering the thylakoid modified electrode

(c) Chronoamperometric data for testing current density obtained from dialysis membrane enclosed electrode

(d) Student t-test based quantification of current density obtained from dialysis membrane supported MWCNT-thylakoid modified electrode [$P<0.05$, $n=5$ for dialysis membrane supported and $n=3$ for dialysis membrane covered electrode]

PBSE was used as the linker molecule. It was used to connect thylakoid protein to MWCNT [figure 8(a)].

However, PBSE used in this project could not keep the thylakoid membrane with a high concentration of phospholipid attached to the MWCNT. Hence, the thylakoid membrane could only be loosely bound to the electrode. As a result, on immersing the electrode inside the buffer, a sufficiently higher proportion of the thylakoid membrane and its transmembrane proteins not stay attached to the electrode but dissolved in the buffer. This resulted in the loss of significant amount of transmembrane proteins in the buffer.

As a result, there was a need to provide extra support to the membrane to stay attached to the anode. Dialysis membrane was used to overcome this shortcoming. Considering the size of the protein molecules in the thylakoid molecule, the dialysis membrane was used to cover the electrode after it has been modified with thylakoid, wrapping the thylakoid inside it, keeping it close to the electrode [figure 8(b)]. This helped in the retention of the thylakoid membrane and its proteins in the vicinity of the electrode.

It was observed that the current density increased 2.39-fold after using a dialysis membrane to cover the coated layer of thylakoid.

Optimization of component 4: Mediator

Optimization of the mediator molecule was carried out by using two electron carriers:

1. Potassium Ferrocyanide: $Fe(CN)_6^{4-}$
2. Potassium Ferricyanide: $Fe(CN)_6^{3-}$

$Fe(CN)_6^{3-}$ exhibits the least photoactivity among the popular mediators so is the ideal candidate to decipher the photoelectric activity of the analyte (Calkins et al., 2013). It was found that the current density obtained from a system when potassium ferricyanide was used as the mediator molecule was significantly higher in comparison when potassium ferrocyanide was used.

Since potassium ferricyanide could be reduced more easily than ferrocyanide, it has a better electron carrying capacity and hence is the better mediator among the two.

3.2 Scanning electron microscopy (SEM) imaging of thylakoid biofilm on MWCNT electrodes

It was observed that the sole coating of the electrode with the MWCNT resulted in some photovoltaic activity, i.e., exhibited some photocurrent on illumination. However, when the MWCNT electrode is further coated with thylakoid, it results in total coverage of MWCNT and attenuates penetration of light to MWCNT.

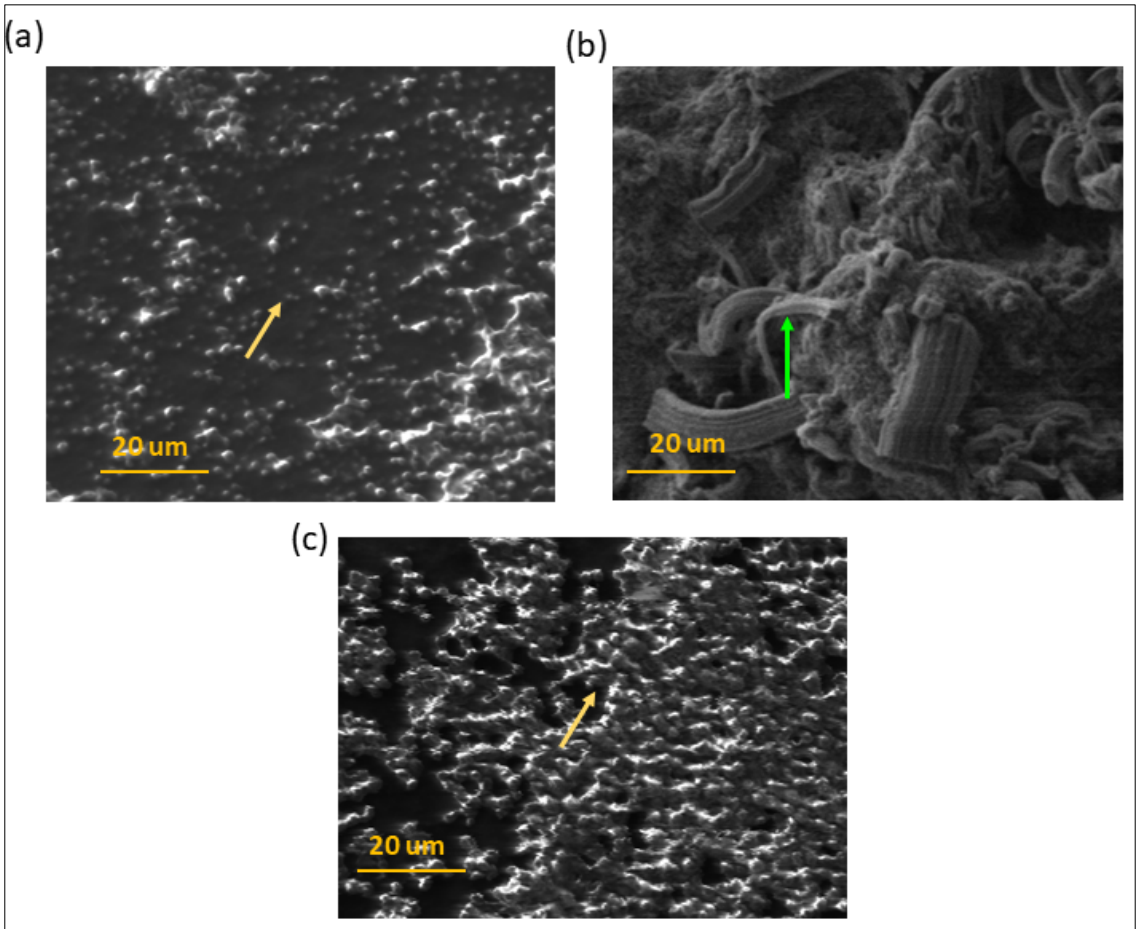


Figure 9: Scanning electron microscopy of the thylakoid-MWCNT electrode

(a) SEM image of thylakoid modified electrode

(b) SEM image of MWCNT modified electrode

(c) SEM image of MWCNT-thylakoid modified electrode

[yellow arrow shows thylakoid, green arrow shows MWCNT]

Hence, it could be inferred that no photochemical activity is obtained due to MWCNT with thylakoid coating on it. The extend of coating and its consistency was investigated using SEM imaging.

Hence, the current obtained after flashing light on thylakoid coated MWCNT electrode was due to the photovoltaic activity of thylakoid and not due to MWCNT.

The SEM images showed the distribution of thylakoid coated on the electrode [figure 9(a)]. The SEM images of thylakoid-MWCNT electrode show that the thylakoid covers the MWCNT coating on the electrode [figure 9(c)].

3.3 Herbicide 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) treated thylakoid coated electrode displayed significantly reduced photovoltaic activity

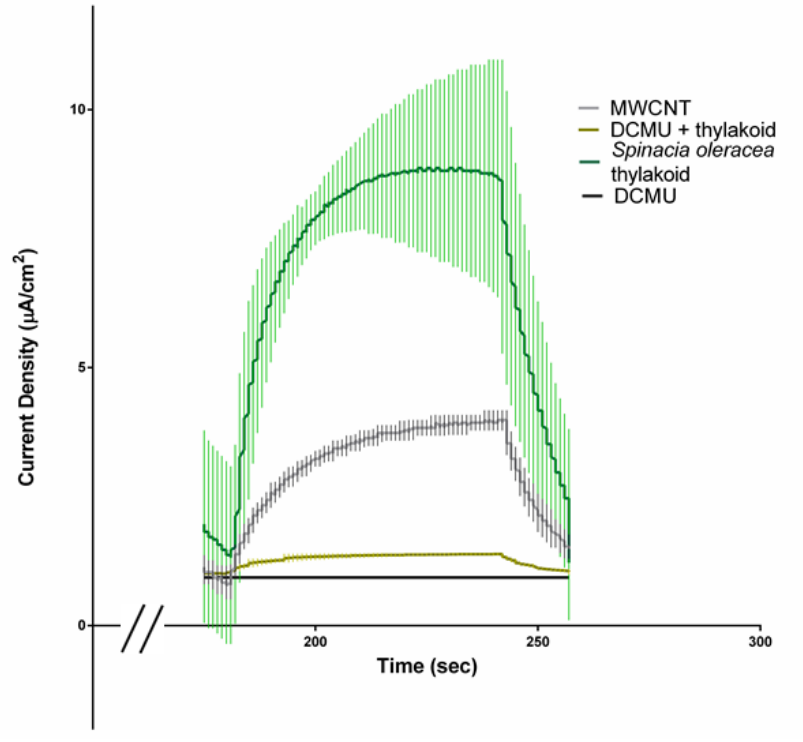


Figure 10 : Chronoamperometric test to check photo-responsiveness of: MWCNT modified electrode; DCMU treated thylakoid-MWCNT modified electrode; healthy thylakoid-MWCNT modified electrode and DCMU modified electrode

Photosystem complex rich thylakoids were extracted from the leaves of the spinach plant. They were further coated on MWCNT electrode with chlorophyll concentration between 8.6-9.7 μg/cm^2.

The electrode exhibited significant photovoltaic activity. The thylakoids used here emit electrons as a response to incident light and increases current density.

This photoresponsive thylakoid-MWCNT electrode was considered a positive control.

To prepare the electrode to be used up as the negative control, the thylakoid used for it was rendered non-photoresponsive. One of the ways was by treating it with herbicide.

To decipher the changes in photovoltaic activity in herbicide-treated plants, they were treated with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). DCMU captures the electrons that are emitted as a result of photolysis of water, resulting in negligible electron transport across the PET chain.

On average, the current density of 0.38 μA/cm^2 was observed in the DCMU treated thylakoid-MWCNT electrode. This current density was significantly less than using healthy thylakoid-MWCNT thylakoid.

After the standardization of thylakoid-MWCNT electrode was achieved, changes in biophotovoltaic current due to perturbations in activity of photosynthetic components was studied. To do so, herbivory was considered due to its changes on the photosynthetic electron flow. To decipher the differences in biophotovoltaic current due to herbivory in plants, the plants were treated with jasmonic acid (JA). Here, the plants under consideration were *A. thaliana*.

3.4 Jasmonic acid treatment increases the current density in *A. thaliana* wild-type plant

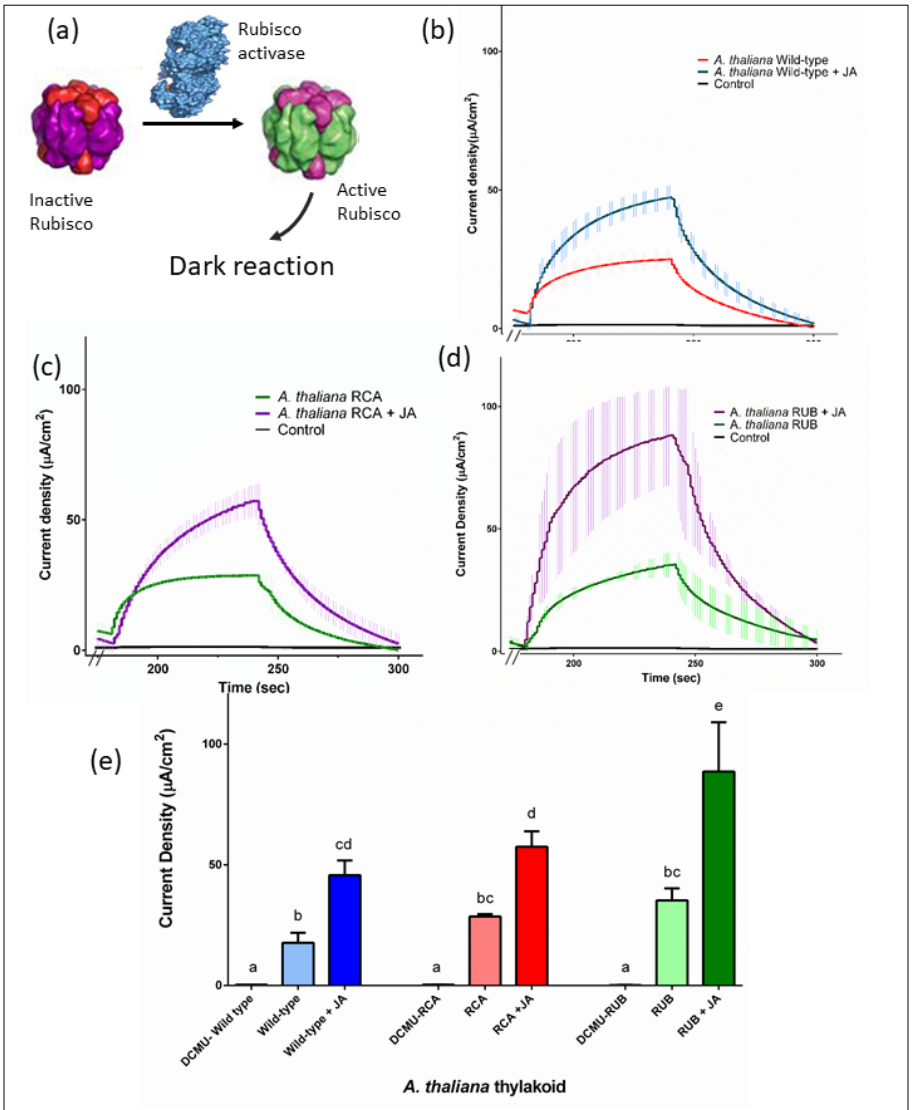


Figure 11: Effect of JA treatment on current density

(a) Function of rubisco; amperometric data (i-t curve) for glassy carbon electrode modified with (b) thylakoid of JA treated and untreated wild-type *A. thaliana*, (c) thylakoid of JA treated and untreated RCA mutant of *A. thaliana* (d) thylakoid of JA treated and untreated Rubisco mutant of *A. thaliana;* (e) One-Way ANOVA Post Hoc Tests based quantification of Current density (µA/cm2) for A. thaliana wild type, RCA mutant and RUB mutant [(mean±SE) $F_{8,18}$ =45.67, P<0.0001,n=3]

Wild type *A. thaliana* were treated with jasmonic acid. Further thylakoid was extracted from both JA treated and control. These thylakoids were coated separately on

MWCNT electrodes with chlorophyll concentration between 8.85-8.92 μg/cm^2 and 8.09-9.5 μg/cm^2 respectively.

It was observed that the average current density obtained from thylakoid after jasmonate treatment was significantly higher (approximately 2.58-fold) compared to the thylakoid obtained from the control [figure 11(b)].

It could be implied that this difference in current density between the JA treated and control is because JA changes the activity of the photosynthetic machinery and hence, the photosynthetic electron flow. One of the possibilities for this surge in current density could be due to the reduced activity of Rubisco (Halitschke et al., 2003) (Mitra and Baldwin, 2014) which further leads to an increase in cyclic photophosphorylation (Alric et al., 2010). This increase in cyclic electron flow results in excess electron flux in the vicinity of the thylakoid. These are then captured and are perceived as an increase in photovoltaic current.

To further investigate if the increase in current is exclusively due to downregulation of Rubisco activity, Rubisco mutants (RUB) and Rubisco activase mutants (RCA) were treated with jasmonic acid, and their biophotovoltaic activity was studied.

3.5 Jasmonic acid treatment increases the current density in Rubisco activase (RCA) and Rubisco (RUB) mutants

Knockdown plant lines of Rubisco small subunit 2B (RUB) and knockout plant lines of Rubisco activase (RCA) of *A. thaliana* were used individually for testing the effect of JA on photovoltaic current.

RCA and RUB mutant plant line were treated with jasmonic acid. Further thylakoids were extracted from both JA treated and control RCA mutant plant lines. The thylakoid was then coated on MWCNT electrode keeping chlorophyll concentration between 8.845-9.302 μg/cm^2 for JA treated RCA and 8.89-9.35 μg/cm^2 for JA control RCA. Likewise, thylakoids were also extracted from both JA treated and control RUB

plants. They were then coated on MWCNT electrode with chlorophyll concentration between 8.86-9.88 μg/cm^2 for JA treated RUB and 8.1-9.78 μg/cm^2 for JA control RUB.

It was observed that the current density obtained from the thylakoids of both the mutants after jasmonate treatment showed a statistically significant increase as compared to control mutants. The average increase in current density obtained from thylakoids of JA treated RCA was approximately 1.9-fold higher compared to the control [figure 11(c)], and that seen in jasmonate treated RUB was about 2.67 times as compared to the control RUB plants [figure 11(b)]. A high standard deviation in current density was observed in Rubisco mutants.

Statistically, there was no significant change found between current density obtained from thylakoids of JA treated wild type and thylakoids of RUB or RCA mutant [figure 11]. In addition to that, no statistically significant current density change was observed from thylakoids of JA treated wild type, and JA treated RCA mutants. However, there was a significant difference between the current density obtained from JA treated wild type and JA treated RUB.

Previous studies have found that on downregulating the activity of Rubisco (Alric et al., 2010) or RCA (Jin et al., 2008), there is an increase in the cyclic photophosphorylation. Hence, it could be hypothesized that the increase in current, seen here is the result of the increase in cyclic electron flow in the thylakoids under the electrochemical study.

In the case when the RUB and RCA plants were treated with jasmonic acid, there was a significant increase in current density as compared to the control.

This points out to the possibility that apart from the effect of downregulation of Rubisco activity, other factors are also contributing synergistically towards increasing the current density. One of which could be the downregulation of FNR (Guo et al., 2017). This result in further upregulation of the cyclic electron flow around the thylakoids and increase in current under electrochemical studies.

The current density obtained from thylakoids extracted from JA treated RCA mutants is not significantly higher when compared to the JA treated wild-type plants. This indicates that the effect of RCA knockout, on the downregulation of the activity of Rubisco is already included among the other impacts of JA surge in the cell and the effect is enhanced on JA treatment.

3.6 Current density produced by *C. sorokiniana* protoplast and thylakoid increased upon jasmonic acid treatment

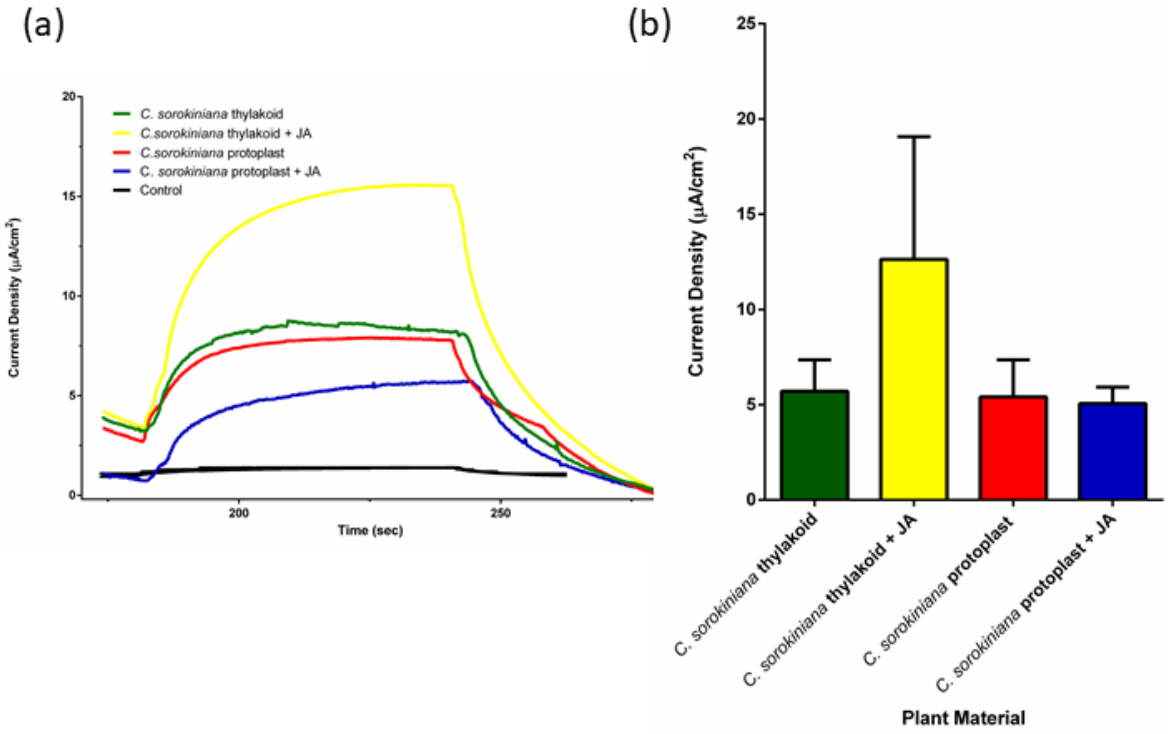


Figure 12: Photocurrent comparison between protoplast and thyalkoid

(a) Amperometric data (i-t curve) for JA treated and untreated protoplast and thylakoid of C. sorokiniana

(b) (b) One-Way ANOVA Post Hoc Tests based quantification of Current density (µA/cm2) for JA treated and control C.sorokiniana thylakoid and protoplast [(mean±SE) $F_{3,8}$ =3.238, P=0.3376 ,n=3]

Protoplasts were prepared from JA treated and control *C. sorokiniana*. Further thylakoids were also extracted from both JA treated and control *C. sorokiniana* cells. Both protoplasts and thylakoids obtained from control cells were coated on MWCNT electrode with chlorophyll concentration between 9.34-9.92 µg/cm^2 and 7.24-8.65 µg/ cm^2 respectively. Also, protoplasts and thylakoids obtained from both JA treated *C.*

sorokiniana were coated on MWCNT electrode with chlorophyll concentration between 9.04-9.42 µg/cm^2 and 9.02-9.61 µg/cm^2 respectively.

As a major part of the total cell volume of *C. sorokiniana* consist of the chloroplast, it could be interpreted that there should not be any substantive change in the current density obtained from both thylakoids and protoplasts.

The difference between the current density of control thylakoids and protoplasts was not statistically significant. The average current density obtained from the JA treated thylakoids was approximately 2.21-fold higher compared to the control [figure: 12(b)]. However, due to the high standard deviation in current density from JA treated thylakoids, a statistically significant difference between current density from JA treated and control was not seen. Also, JA treatment to protoplasts did not show a statistically significant change. The current obtained from thylakoid was drastically reduced when obtained from *C. sorokiniana* as compared to the current from *A. thaliana.*

In the case when protoplast was used as an electron source for chronoamperometric testing, the entire cell wall-less algal cell with all its organelles was utilized. Hence, the physiological activities of all the organelles along with chloroplast affect the photovoltaic current. One of the reasons for no significant effect of JA treatment on the current density from the protoplast could be due to the changes in cellular respiration and another protease activity. One of the possibilities for less photovoltaic current from thylakoid of *C. sorokiniana* could be less amount of PSII compared to PS I. PS II is the source of the photo-excited electrons. It has been reported that in the leaves of *A. thaliana*, PSII/PSI ranged 1.01-20 whereas, in algae PSII/PSI was found to be 0.35-0.46 (Nakamura et al., 1976; Wientjes et al. 2017).

3.7 Intact *C. sorokiniana* protoplast produce biophotovoltaic current for longer duration than the isolated thylakoid

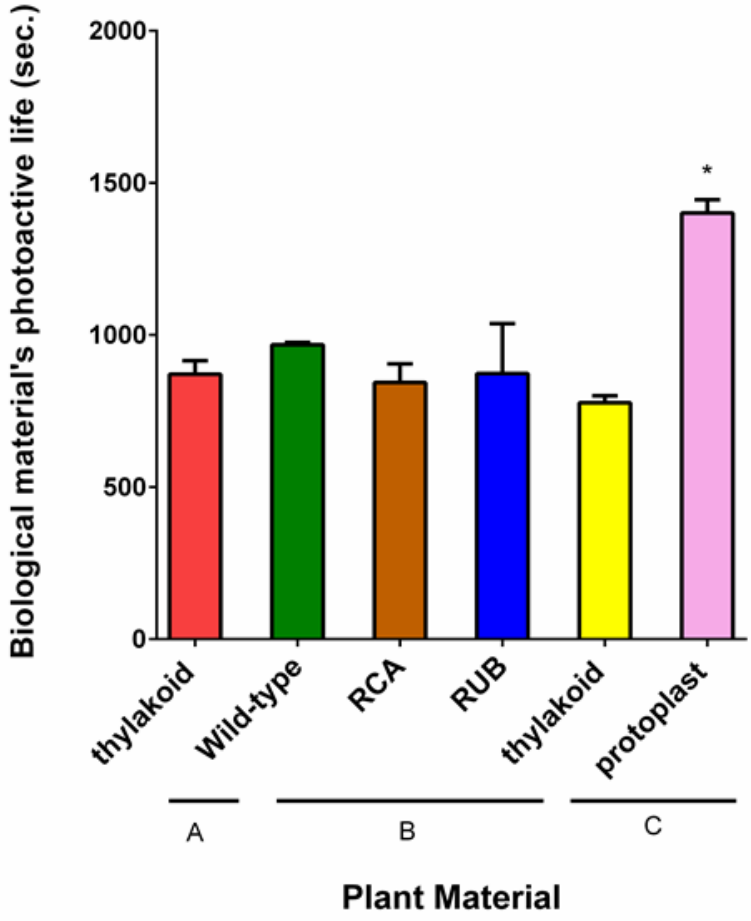


Figure 13: Quantification of biological material photoactive life

One-Way ANOVA Post Hoc Tests based quantification of biological material photoactive life of for *A. thaliana* wildtype, RUB, RCA, *C. sorokinana* thylakoid and protoplast [(mean±SE) $F_{5,12}$ =26.20, P=0.001,n=3]

Here, A: *S. oleracea*; B: *A. thaliana*; C: *C. sorokiniana*

The current density was observed for an extended period of 1800 seconds for thylakoids and protoplasts to compare the activity between the two.

It was observed that the decrease in current density in chronoamperometry reading in the case of thylakoids was significantly greater than that for protoplasts [figure:13].

Thylakoids and chloroplasts are found to be living in symbiosis inside the plant cell. Hence, the isolated chloroplasts and thylakoids don't remain viable for longer

period. This also results in the decrease of their photoactivity over time. The protoplast is a cell without the cell wall. Hence, it could remain isolated for long and have a longer photoactive life than thylakoid. This makes the protoplast to be more sustainable electron source for biophotovoltaic devices as compared to thylakoid.

Conclusion

In this project, it was found that the herbivory-induced cyclic photophosphorylation can be used for enriching the photosynthetic electron flow in a biophotovoltaic setup and the same was successfully tested. A significant increase of 2.58 folds in the steady-state current density from *A. thaliana* was observed upon the jasmonic acid treatment. This finding has high implication in the field of biophotovoltaics. Maximum current density up to 80 μA/cm2 was observed here which is higher than previously reported 68 μA/cm2 using the identical setup. Further, photoactivity for a relative longer period was observed in the case of *C. sorokiniana*. This signifies that the use of protoplast in the biophotovoltaics system can be a more durable approach for practical purposes. A protoplast containing a higher density of PSII in thylakoid will be desired for maintaining a higher density for this duration.

One of the potential future goals following this project could be to prepare the complete photovoltaic cell for power generation. A further futuristic approach could be that of utilizing the entire plant while also ensuring to keep it alive.

By the findings in this project, we could conclude that incorporating the intricacies of biological processes and machinery in the field of semiconductors photovoltaics; we could enhance the efficiency of biophotovoltaic devices for power generation.

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