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AN IMPROVED, SIMPLE, INEXPENSIVE AND HIGHLY FLEXIBLE HYDROPONIC SETUP FOR ROOT MITOCHONDRIA ISOLATION FROM *ARABIDOPSIS* AND *NICOTIANA* PANTS

MUHAMMAD WAQAR HAMEED[1,3*] AND AZIZ-UD-DIN[2,3]

[1]*Dr. Panjwani Center for Molecular Medicine and Drug Research, University of Karachi, Karachi-75270, Pakistan*
[2]*Department of Genetics, Garden Campus, Hazara University, Dhodial Mansehra, Pakistan*
[3]*Max Planck Institute of Molecular Plant Physiology, Am Muhlenberg 1, 14476 Potsdam-Golm, Germany*
[*]*Corresponding author's e-mail: mwaqar13@gmail.com*

Abstract

Hydroponic setups are frequently developed and improved as they are convenient platforms for studying whole plant physiology. Mostly, the available systems produce small amounts of plant material and are therefore, unsuitable for studies requiring large quantities of plant material like isolation of mitochondria. To address this issue, we have modified a hydroponic setup that can sustain hundreds of *Arabidopsis* and *tobacco* plants until adult plants are established. The setup is very flexible and easy to construct. It is based on the use of recyclable and sterilizable plastic-net-pots and media containers, which are easily available from the local suppliers. The modified seed-pots and styrofoam sheets facilitate the transfer and harvesting of seedlings. We have used the Percoll based two-step density gradient centrifugation method for the isolation of root mitochondria from the hydroponically grown plants.

Key words: Hydroponic, *Arabidopsis*, *Nicotiana*, Roots, Mitochondria isolation.

Introduction

Arabidopsis and tobacco has been accepted as model plant species in many laboratories for multiple reasons. These include a brief life cycle, well-annotated genome, acquiescence to tissue culture and the ease at which they can be genetically transformed (Kaul *et al.*, 2000). To benefit from the huge molecular resources linked to these plant species, physiologists have adapted the available technologies to suit these physiological models. One such adaptation includes the development and modification of hydroponic systems, so as to provide a flexible experimental platform that would allow manipulation of both the shoot and root environments. The hydroponic systems generally serves as a convenient mean to study whole plant physiology in the laboratory owing to easy accessibility to all plant parts and the simplicity with which the nutrient profile of the growth medium can be manipulated (Alatorre-Cobos *et al.*, 2014). Although, phytagel or agar grown seedlings share similar advantages but here, the seedlings can only be maintained for around two weeks and therefore limited physiological experiments can be conducted using these systems (Ahn *et al.*, 2004; Conn *et al.*, 2013).

During the past decade multiple hydroponic systems have been developed independently in several laboratories reflecting their need and widespread use. Hydroponic systems described in the literature were basically designed to meet specific experimental needs and are therefore, unsuitable for various other experimental assays. The key limitation associated with these systems include (a) the use of a small media tanks for nutrients that abridged the chances of scalability (Arteca & Arteca, 2000; Robison *et al.*, 2006), (b) the prerequisite to sterilize component of the setup (Arteca & Arteca, 2000; Schlesier *et al.*, 2003) and the use of specific materials (like pre-fabricated seed holders) that increases the cost (Tocquin *et al.*, 2003), (c) the use of bigger sponge or rockwool plugs preventing access to the full root system (Gibeaut *et al.*, 1997; Robison *et al.*, 2006; Smeets *et al.*, 2008) and the requirement to move seedlings from one growth environment to the other (Hermans & Verbruggen, 2005). Although each of these systems is uniquely designed to suit its endpoint analysis, we sought to streamline the complete process to provide a universal and fully adaptable system for the growth of *Arabidopsis* and tobacco plants.

The most significant problem associated with the hydroponic systems that necessitates the use of aseptic conditions is the growth of algae in the hydroponic media, on agar-based or rockwool plugs, and/or on the shoots and roots of the seedlings (Alatorre-Cobos *et al.*, 2014; Conn *et al.*, 2013). Principally, algae nurtures due to the application of non-sterile phosphorous-rich medium and its exposure to light. This algae affects plant growth by hindering the nutrient up-take efficiency of seedlings, which in turn changes the global proteome and transcriptome composition of a plant (Boller & Felix, 2009; Huang *et al.*, 2011). This is why illumination of the hydroponic solution must be avoided if seedlings are to be used for any physiological investigations.

The major reason to design the current hydroponic system is to enhance the plant growing capacity of existing systems so as to cultivate enough *Arabidopsis* and tobacco plants that could be used for root mitochondria isolation. After considerable modifications, we present here a simple, cost-effective and highly flexible hydroponics system for the cultivation of *Arabidopsis* and tobacco plants, which addresses our considerations and streamlines the methodology allowing other researchers to adopt this procedure.

Materials and Methods

Hydroponic setup and media composition: To use as hydroponic media containers plastic boxes of 12″ width, 18″ length and 12″/18″ height were obtained. The base of a 2″ plastic-net-pot was removed with a pair of scissors and rockwool discs of ~2″ diameter and 0.5″ height fitted into these pots. To provide holdings for the net-pots and to cover the top of the media containers styrofoam sheets of 18″ length and 12″ width was taken and 40-holes were prepared in each using a power drill mounted with a 2″ hole-saw (Bosch, UK; Fig. 1b). The hydroponic media tanks can hold ~22L and ~36L of the nutrient solution depending upon their height (12″/18″). An aquarium pump (MA-300, ViaAqua Million Air, USA) was connected to the base of each media tank to aerate the nutrient solution. The growth media applied constitute upon 250µM $CaCl_2$, 1mM $MgSO_4$, 1mM NH_4NO_3, 0.1mM Fe-EDTA, 50µM KCl, 1mM KH_2PO_4, 10µM $MnSO_4$, 2µM $ZnSO_4$, 0.1mM H_3BO_3, 0.1µM Na_2MoO_4 and 1.5µM $CuSO_4$. A 10x stock was prepared and applied at the rate of 100 ml per 900 ml in water.

Plant material and growth conditions: *Arabidopsis thaliana* ecotype *Columbia* and *landsberg erecta*, *Nicotiana tabacum* var *Petit havana* and *Samsun* seeds were planted onto the surface of rockwool plugs soaked with the nutrient solution (Fig. 1). The plastic net-pots were fitted into a 24-cell plastic tray insert (Plant Pots Direct, UK) and placed into a climate chamber adjusted with 150 $\mu mols^{-1}m^{-2}$ of light intensity and 12/12h of light/dark cycle. Inside the chamber the relative humidity was maintained at 75% and the temperature was kept at 20/18°C for the day/night. After the germination of seeds, pots were shifted into bigger media tanks fitted with aquarium pumps (MA-300, ViaAqua Million Air, USA).

Root length and fresh weight: After ten weeks of germination seedlings were harvested in ice chilled water (Fig. 1-e) and root fresh weight and length was recorded. Root length was measured as a distance between the root-shoot junction and tip of the main root (Fig. 2). To calculate fresh weight, roots were placed in a weighing boat and placed onto a balance after removing extra water/media using a salad spinner (TOKIG, Germany; Fig. 3).

Mitochondria isolation: For the isolation of mitochondria, we have followed the two step-Percoll gradient centrifugation method. It was opted to remove contaminants (like plastids and peroxisomes) from the crude organelle pellets (Millar *et al.*, 2001). In short, *Arabidopsis* and tobacco roots were harvested in the buffer (0.4M Mannitol, 1mM EGTA, 0.1% BSA, 50mM Tricin, 20mM β-mercaptoethanol, 1% w/v Polyvinylpyrrolidone, NaOH pH 7.8) and homogenized using a Polytron blender (9500 min^{-1}). The homogenate was filtered through cheesecloth and nylon net. The filtrate was centrifuged twice and the resulting pellet was resuspended in wash media [0.4M Mannitol, 10mM MOPS (KOH), 1mM EGTA, 0.1% BSA, pH 7.8 with NaOH] and loaded on top of Percoll step gradient [40, 28, and 20% (v/v) Percoll]. After centrifugation, the enriched mitochondria were (Fig. 4) and diluted in 3 volumes of mannitol wash media and centrifuged at 11,500 rpm. The pellet was resuspended in sucrose wash media [0.3M Sucrose, 0.1% (w/v) BSA and 10mM TES-NaOH, pH 7.5] and loaded on top of a 28% Percoll gradient (Millar *et al.*, 2001). After centrifugation mitochondrial band near the top of the gradient was collected and concentrated by two successive centrifugations. The final pellet was suspended in mannitol wash media.

Isolation of nucleic acids: Genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method (Doyle & Doyle, 1990). Briefly, mitochondrial pellet was mixed with CTAB buffer (2% CTAB, 1.4M NaCl, 100mM Tris-HCl, 20mM EDTA, 100mM β-mercaptoethanol) and incubated at 65°C. To remove cell debris, samples were centrifuged and the supernatant was incubated with equal volumes of chloroform-isoamyl alcohol (24:1). The clear upper phase was collected after centrifugation. DNA was precipitated with 2.5 volumes of ethanol and pelleted by centrifugation at 15,000g. The pellet was suspended in DNase free water. For the isolation of RNA, 1-2ml of TriFast reagent (Erlangen, Germany) and 0.2ml chloroform was added to the mitochondrial pellet. The mixture was centrifuged and the upper aqueous phase was collected. RNA was precipitated with 0.7 volumes of isopropanol and pelleted by centrifugation. The final pellet was washed once with 70% ethanol and suspended in RNase free water.

Polymerase chain reaction (PCR): Gene specific primers were designed for Northern blot analysis, including mitochondrial *cox1-AT-NT* (5′-AGATACCCGTGCCTACTTCAC-3′, 5′-CGACCACGAAGAAACAACAAATCC-3′), plastidial *psbA-AT* (5′-CGGCCAAAATAACCGTGAGC-3′, 5′-GAAAGCCTATGGGGTCGCTT-3′), plastidial *psbA-NT* (5′-ATAGACTAGGCCAGGATCTTAT-3′, 5′-ATTTTACCATGACTGCAATTTTAGAG-3′), plastidial *16s-rRNA-AT-NT* (5′-CATGGATCCATCTCATGGAGAGTTCGATCCTG-3′, 5′-CATGGATCCTACGGCTACCTTGTTACGACTTC-3′) and mitochondrial *18s-rRNA-AT-NT* (5′-ACCCCAGTCGAAGACCCCACC-3′, 5′-CGCCCGAAGCATCGGACCAA-3′). PCR constitute upon 3-5ng of template DNA, 2mM dNTPs, 10pm of each primer, 25mM $MgCl_2$, 2.5 units of *Taq* polymerase and 5x PCR buffer. To amplify the DNA fragments, PCR cycler was programmed for denaturation at 94°C (30 sec), primer annealing at 52-60°C (30 sec) and strand elongation at 72°C (2 min). All steps were repeated 30 times and the final elongation was executed at 72°C for 5 min.

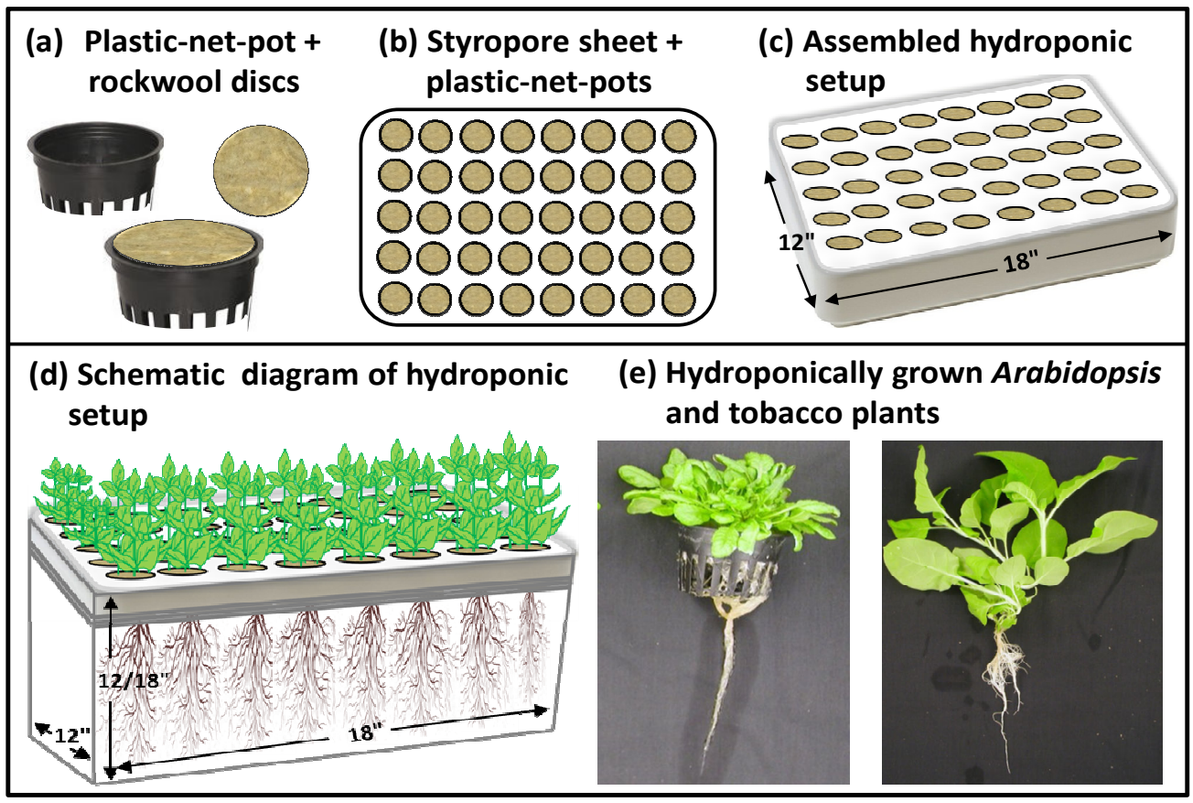

Fig. 1. Hydroponic setup and its assembly (a) Rockwool discs of 2" diameter were prepared and fitted into the plastic net-pot (2" diameter) with a removed base to facilitate root growth. (b) Forty hole of ~2" diameter were made in the styropore sheet (18″ length and 12″ wide) and plastic-net-pots plus rockwool plugs were fitted into them. (c) Stropore sheet was fitted on top of media tanks (18″ length, 12″ width, 12″/18″ height). Multiple seeds were sown on each rockwool disc and after germination extra seedlings were pulled-out to leave only 6-8 *Arabidopsis* and 2-3 tobacco seedlings per net-pot to maintain the required plant density. (d) Schematic diagram of hydroponic setup presenting seedling growth. (e) An example of *Arabidopsis* and tobacco plants harvested from the hydroponic cultures.

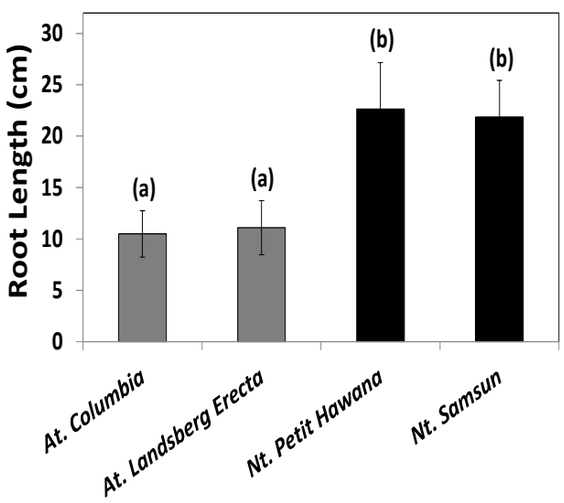

Fig. 2. Measurement of root length from *Arabidopsis thaliana* (At) ecotype *Columbia* and *landsberg erecta*, *Nicotiana tabacum* (Nt) var *Petit havana* and *Samsun*. After harvesting of seedlings, root length was determined and average root length was calculated from fourteen independent measurements. Different letters above the bars represent significant difference (one way ANOVA; $p<0.05$).

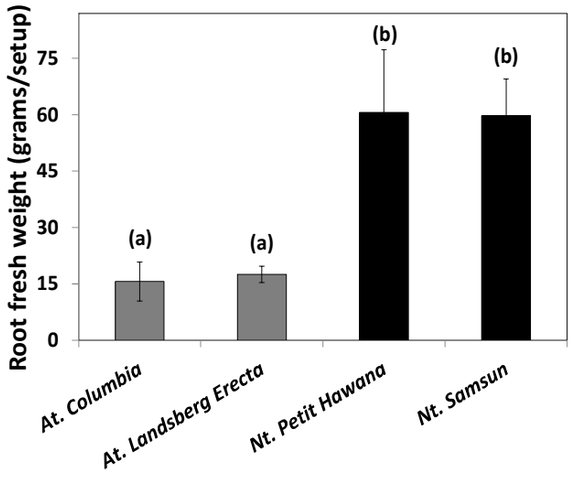

Fig. 3. Root fresh weight from *Arabidopsis thaliana* (At) ecotype *Columbia* and *landsberg erecta*, *Nicotiana tabacum* (Nt) var *Petit havana* and *Samsun* was calculated. For this roots were harvested after ten weeks of seed germination. After removing the extra medium (using a salad spinner), root fresh weigh was calculated in grams per setup. The bars represent standard deviation and letters indicated mean values that are significantly different (one way ANOVA; $p<0.05$).

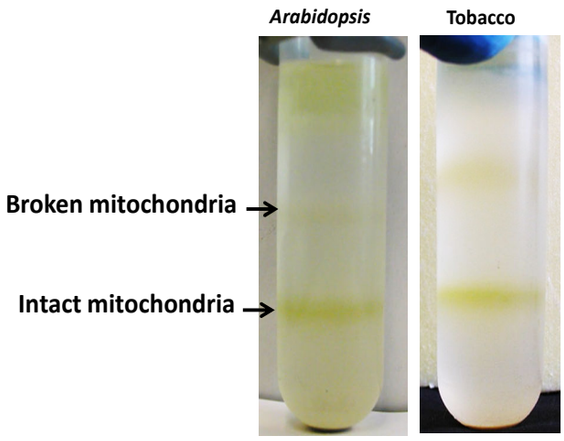

Fig. 4. Mitochondria isolated from *Arabidopsis thaliana* and *Nicotiana tabacum* roots. Intact mitochondria were aspirated and collected by successive centrifugation and washing steps.

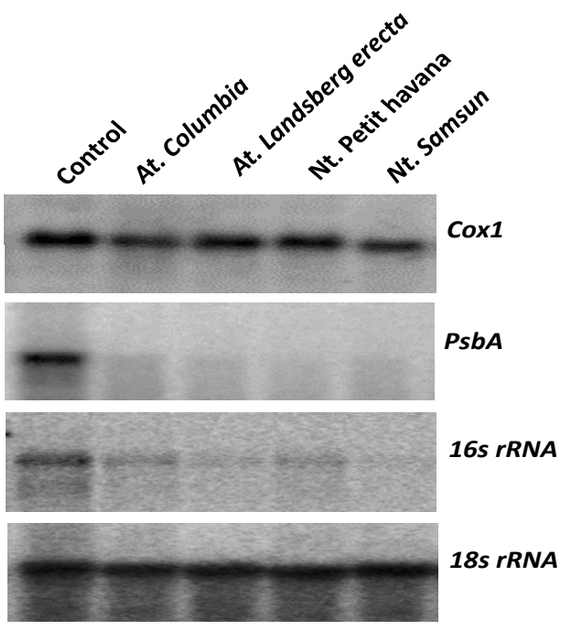

Fig. 5. Northern blot analysis on RNA isolated from root mitochondria. Radiolabelled probes for mitochondrial *Cox1,* plastidial *psbA* and*16s rRNA* were generated to detect the presence or absence of RNA species from these organelles. The probe against mitochondrial *18s rRNA* was used as a loading control. Control lane represents RNA isolated from tobacco leaves.

Northern blots: 5µg of RNA was resolved onto a formaldehyde agarose gel and directly transferred onto a Hybond nylon membrane (Amersham) by capillary blotting using a 20x SSC buffer (3M NaCl, 300mM tri-sodium citrate). Megaprime DNA Labeling System (GE Healthcare) was used to prepare the (α-32P)dCTP labelled probes. Before hybridization, the membranes were incubated at 65°C in the church buffer (7% SDS, 1% BSA, 1mM EDTA, 0.5M $NaHPO_4$). Probes were denatured (95ºC for 5min) and hybridized to the membranes at 65°C. The labeled blots were washed with wash buffer I (2x SSC, 0.1% SDS) and II (0.5x SSC, 0.1% SDS) at 65°C. Hybridization signals were detected using a Typhoon Trio scanner (GE Healthcare).

Results

Assembly of hydroponic system: We present here a modified hydroponic system for the long-term growth of *Arabidopsis* and tobacco plants. The assembly consists of plastic net-pots, rockwool discs, styropore sheets, media tanks, plastic tray inserts and plastic trays. The base of plastic-net-pots was removed with a pair of scissors to facilitate root growth. Rockwool discs of 0.5″ height and ~2″ diameter were sliced and fixed into the plastic-net-pots (Fig. 1-a). Each rockwool disc was carefully sliced to meet the predefined thickness, as thinner discs were found to lose their shape and fall into the media tank, while the thicker discs were avoided to minimize losses at the time of harvest. Forty of these plastic-net-pots were fitted into each styropore sheet (Fig. 1-b), which was in-turn fixed on top of a media tank (Fig. 1-c). Media tanks of different heights were used for *Arabidopsis* and tobacco because of the differences in the expected root length and seedling size of the two plant species.

All components of the hydroponic system were chosen opaque to avoid light from approaching the nutrient media. It was necessary to elude algae from growing into the hydroponic media. Algae normally grows upon exposure of non-sterile phosphorous-rich medium to light (Huttner, 2003). It utilizes all the nutrients, dies and decomposes leading to hypoxic media. The roots, therefore, do not receive adequate oxygen and becomes vulnerable to pathogen attack (Conn *et al.*, 2013). Hence, it is essential to prevent algae from growing into the nutrient media. In our system, we have seldom found algae growing on the surface of rockwool plugs. Therefore, to preclude algae growth, the pre-cultures were covered with plastic lids until the seedlings fully cover the rockwool surface. At later stages of seedling growth, we observed no algae growth on the rockwool plugs until harvesting.

The hydroponic cultures were first started as a pre-culture in a 24-cell plastic tray insert and after the roots have passed through the rockwool disc they were shifted to bigger media tanks (Fig. 1-c and 1-d). During pre-culturing, the volume of nutrient solution was kept constant to facilitate seed germination and seedling growth. The pre-cultures were not aerated at this stage because of the small volumes of media applied (Arteca & Arteca, 2000). However after transferring the cultures to bigger media containers the nutrient media was continuously aerated till harvesting. Aeration of the media was necessary to avoid any harmful effects on plant/root growth due to limitations in the availability of oxygen in the bigger media tanks.

Nutrients, media composition and refreshing time: The media composition used in the current investigation is based on Hoagland's solution (Gibeaut *et al.*, 1997), which has been shown repeatedly to support growth for a number of diverse plant species (Rocha *et al.*, 2010;

Tocquin *et al.*, 2003). It generally consists of both macro and micro nutrients. Since, micronutrients are needed in extremely minute amounts therefore a 10x stock was prepared and used at the rate of 100mL per 900mL of water. The media needs to be periodically refreshed to avoid strong changes in pH (acidification of media) which normally happens due to the depletion of some nutrients more rapidly than others. One of the factors that speed up media acidification is the number of plants grown per setup. The reported time period for media refreshing is after every 15 days with a plant density of 6-24 per setup (Huttner, 2003; Rocha *et al.*, 2010; Tocquin *et al.*, 2003). Since, we intended to grow >300 *Arabidopsis* and >100 tobacco plants per setup which is much more than the one reported in the literature therefore, the media refreshing time needs to be revised. To define an appropriate media refreshing time for our study, we have selected two times points *i.e.* after 7 and 15 days. Here, we observe no difference in plant growth and phenotype when the media was refreshed after every 7th day, whereas upon changing the media after every 15th day only tobacco plants became senescent and start to flower. In the light of these observations, we scheduled media refreshing after every 15th day for *Arabidopsis* and 7th day for tobacco. Following this schedule, we observed that both *Arabidopsis* and tobacco produced deep green leaves and white roots at the time of harvest (Fig. 1-e).

Number of plants per setup (plant density): Number of plants per hydroponic setup mainly depends upon the size of the experimental setup and the plant material needed to carry out the analysis. The capacity of available hydroponic setups is very low and can only support 6-24 plants, essentially due to the small amounts of tissue needed *i.e.* for single cell sampling, turgor measurement, electrophysiology, ionomic and molecular investigations (Conn *et al.*, 2011; Conn *et al.*, 2013; Dietrich *et al.*, 2012; Millar *et al.*, 2001; Tocquin *et al.*, 2003). Since, we would like to collect large amounts of root material for mitochondrial preparations, the existing hydroponic systems needs to be modified to support >300 *Arabidopsis* and >100 tobacco plants simultaneously. For this, we have used bigger pots and deeper media tanks (Fig. 1). By using this system we could not only grow hundreds of *Arabidopsis* and tobacco plants per setup but were also able to maintain them for longer periods of time. In this way, we were able to collect sufficient root mass for a mitochondrial preparation. However, due to the small size of seeds especially for *Arabidopsis*, it was very tedious to sow the exact number of seeds per pot (6-8 *Arabidopsis* and 2-3 tobacco) therefore, more seeds were planted and after germination extra seedlings were pulled-out to maintain the desired plant density per pot and ultimately per hydroponic setup.

Determination of root length and mass: To determine gain in root mass both root length and fresh weight was calculated at the time of harvest. Here, we found that the average root length for *Arabidopsis* var *Columbia* is ~10cm and that of *Landsberg erecta* is ~11cm, while, for that of *Nicotiana tabacum* var *Petit havana* and *Samsun* was found to be ~22cm (Fig. 2). Likewise, average root fresh weight was found ~15g for *Columbia* and ~17g for *Landsberg erecta* and ~60g for tobacco cultivars (Fig. 3). Typically around 100g of fresh tissue is required for a single mitochondrial preparation (Millar *et al.*, 2001; Ramirez-Aguilar *et al.*, 2011). Therefore, to collect 100g of root material we have to pool roots from 6-7 *Arabidopsis* and 2 tobacco setups.

Purity of root mitochondria: Mitochondria were isolated from *Arabidopsis* and tobacco roots following the published protocol (Millar *et al.*, 2001; Ramirez-Aguilar *et al.*, 2011) (Fig. 4). To check, the purity of isolated mitochondria Northern blotting experiments were conducted using gene specific probes for *Cox1* (mitochondrial), *PsbA* (plastidial), *16s rRNA* (plastidial) and *18s rRNA* (mitochondrial). Here the signals for mitochondrial *Cox1* probe could be easily detected from both *Arabidopsis* and tobacco root mitochondria while, plastidial *PsbA* and *16s rRNA* produced very faint signals (Fig. 5). This indicated that the isolated root mitochondria are highly pure and exhibit no plastid contaminations at the RNA levels.

Discussions

In the current study, we have modified a conventional hydroponic system to substitute the existing systems to cultivate *Arabidopsis* and tobacco plants. Primarily, it is comprised of plastic net-pots, rockwool discs, styropore sheets, plastic media tanks and aquarium pumps. This setup is very simple, employs inexpensive raw material and needs low maintenance. It not only permits an easy access to the root system but also allows monitoring of root growth at any stage of growth. Furthermore, the media can be easily manipulated and the number of plants per setup can be attuned according to experimental needs. This setup offers an easy swapping of plastic-net-pots and the styropore sheets between different media tanks making the up-grading possible to any scale.

Another advantage of our setup is the control over algae growth which was made possible by using opaque objects that prevented light from approaching the hydroponic media. Usually, the main cause of algae growth in the hydroponic cultures is the use of non-sterile phosphorus rich media and its exposure to light (Huttner, 2003). Algae affect plant growth by utilizing nutrients, by depleting dissolved oxygen and by changing the global transcriptome and proteome of a plant (Conn *et al.*, 2013; Huang *et al.*, 2011). Thus, it is extremely important to stop algae from growing into the hydroponic system. In our case, we rarely noticed algae growing on top of rockwool plugs. To diminish or minimize algae growth, we covered the pre-cultures with plastic lids until the seedlings were bigger enough to cover the rockwool surface. Furthermore, to prevent the media from becoming hypoxic, the media tanks were continuously aerated (Arteca & Arteca, 2000; Delhaize & Randall, 1995; Gibeaut *et al.*, 1997; Hirai *et al.*, 1995; Rodecap *et al.*, 1994).

The reported hydroponic systems could only support few plants (6-24) per setup (Conn *et al.*, 2011; Conn *et al.*, 2013; Dietrich *et al.*, 2012; Millar *et al.*, 2001; Tocquin *et al.*, 2003) and to manage the required plant density we have grown >300 *Arabidopsis* and >100 tobacco plants per setup (Fig. 1). The root material was harvested and mitochondria were isolated from them (Fig. 4). To improve the purity of mitochondria, we have used Percoll based two-step density gradient centrifugation method because Percoll minimizes the exposure of mitochondria to changes in osmolality and also improves purity by eliminating damaged mitochondrial membranes, plastids and peroxisomes (Millar *et al.*, 2001; Neuburger *et al.*, 1982; Struglics *et al.*, 1993). The isolated mitochondria were found highly pure since they exhibited no plastid contaminants as tested through Northern blots. Although, additional set of experiments are needed to prove the purity of mitochondria like transmission electron microscopy, flow cytometry and Western blots, if these mitochondria are to be used for physiological and/or biochemical studies (Lanza & Nair, 2010; Picard *et al.*, 2011). But as we only aimed to isolate mitochondria for transcriptome analysis therefore, only Northern blots were considered sufficient to show that the enriched mitochondria are free from plastidial RNA. In other words, although we haven't shown that the isolated mitochondria are free of damaged mitochondrial membranes, plastids and peroxisome but we have shown that they are free of plastidial RNA and can be used for transcriptome and proteome analysis.

Conclusions

In conclusion, our hydroponic device can be rapidly assembled to grow *Arabidopsis* and tobacco plants. Together with the common benefits of this system, like control over plant density, composition of growth medium and access to the root system, it also offers specific benefits like successful seed germination, smooth plant growth, high yield, flexibility and low maintenance. The use of plastic-net-pots, styropore sheets and plastic media containers also affluence eases up the system. It short, our hydroponic system employs common greenhouse material which can be bought easily from the market and can be reused several times without affecting the quality of the hydroponic setup.

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