Development of Automatic Dual Sequence Control Temporary Immersion Bioreactor Systems for Micropropagation *Coelogyne pandurata* Lindl.

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Abstract. *Coelogyne pandurata* Lindl. is an endemic orchid species of Indonesia from East Kalimantan and Papua. It is locally known as the "black orchid" due to the distinctive black coloration on its labellum with intricate green and hairy lines. Unfortunately, the population of this orchid has significantly declined in its natural habitat, rendering it a rare endangered species. Temporary immersion bioreactor systems have been proven to be efficient for endemic plant micropropagation with performance enhancements and innovations. This study aims to develop an optimized bioreactor design and innovate the automatic control performance of temporary immersion bioreactor systems based on previous research. The control system developed consists of three modules, namely Graphical User Interface (GUI) module, Sequence Control (SC) module, and Hardware Interface (HI) module. The GUI module receives information regarding time and duration of immersion and gas exchange, then the information is synchronized by the SC module, which plays the role of starting and stopping the processes, while HI module executes the order of the automatic control system in the immersion and gas exchange process. The developed bioreactor design and control system offer convenience, require less labor, and ensure precise control over the optimum conditions for black orchid micropropagation.

Keywords: Automatic Software Development, *Coelogyne pandurata* Lindl., Micropropagation, Temporary Immersion Systems

1 Introduction

Indonesia is one of the countries in the world with the largest tropical forests, which host a diverse range of flora, including many species of the Orchidaceae family. This family comprises about 800 genera and more than 25,000 species in total, with an estimated 5,000 of them found in Indonesia, some of which are classified as endemic to the region [8]. *Coelogyne pandurata* Lindl. is an endemic orchid species of Indonesia from East Kalimantan and Papua. It is locally known as black orchid because of the distinctive black coloration on its labellum with green and hairy lines [5]. The black orchid is an epiphytic sympodial orchid. Its pseudostems are either located closely or at a distance, each bearing two oblong and folded leaves. The flowers are arranged in clusters, with each cluster containing approximately 5-14 buds. The sepals and petals are lancet-shaped, pointed, and typically green. As an orchid, the seeds inside the fruit are microscopic in size due to the lack of endosperm [3]. Nowadays, the population of this orchid has greatly decreased in nature and is classified as a rare, endangered orchid [13]. The development of an efficient bioreactor system for micropropagation can contribute to the ex-situ conservation and propagation of plants, especially rare endemic orchid species in Indonesia.

Fig. 1. Morphology of the Black Orchid. A. A Plant with flower; B. Plant at the base of pseudobulbs; C. Close up a flower; D. Black labellum; E. Mature seeds; F. Fruit. Bars: 5 cm in A and B; 1 cm in C, D, and F; 0,5 mm in E [3].

In nature, black orchids thrive optimally at altitudes ranging from 1,000-15,000 meters above sea level, with humidity levels between 60-85%, CO_2 gas content at 500-1.200 ppm, light intensity at 40%-50%, and pH range of 6.4- 6.6 [7, 9]. Meanwhile, when cultivated *in vitro*, it is typically incubated at 21-23ºC temperature and exposed to a TL lamp emitting around 1000 lux of light [10, 11].

Micropropagation using temporary immersion systems (TIS) is an alternative to reduce production costs, increase biological yield and quality, as well as reducing the culture time [4]. A TIS involves and combines the advantages of cultivating explants that are temporarily exposed to nutrient liquid culture and air in automated bioreactors systems for large-scale production plantlets under controlled conditions. The optimal system operation varies for each species due to many factors and variations in cultivation conditions. The bioreactor system makes it easy to monitor and control physical parameters including pH of media, temperature, and gas environment that supports plantlet growth. Furthermore, subculturing is unnecessary because the bioreactor system and the liquid medium allows for medium exchange. To date, many TIS variations have been developed, including the Temporary Immersion Bioreactor (TIB®), Temporary Immersion (RITA®), Ebb-and-Flow bioreactor, SETIS™, and Monobloc Advance TIS (MATIS®) [1].

Currently, technology developments are advancing rapidly. This can be seen from the widespread use of computers to facilitate the creation of automatic tools, harnessing technological advancements [12]. In the field of electronics control, we frequently encounter chips capable of storing and executing programmed manners, in which an electrical component known as microcontroller is employed to automate the operation of devices [14].

Recent technological advancements have unlocked innovations in designing tools with a system capable of automatically monitoring and controlling *in vitro* culture ecological conditions. The concept behind this tool is that it can perform momentary soaking as needed and adjust environmental variables, such as temperature, humidity, light intensity, pH, and CO_2 gas which can change rapidly based on external factors. This automated design, control, and monitoring can be used in the cultivation industry to ensure optimal plant growth. The creation of this tool involves an LCD monitor, which displays parameters within the culture room.

2 Materials and Discussion

2.1 Plant Materials and Culture Condition

Coelogyne pandurata Lindl. aged 8 months was used as plant material. Plantlets were obtained from the Biotechnology Laboratory at the Faculty of Biology, Gadjah Mada University. The liquid medium used was MS, supplemented with various combinations of BAP and 2,4-D hormones. Organic materials extracted from Ambon banana peels were also added as organic biocides. All cultures were maintained at a temperature range of 21-25°C, with 60-85% humidity, a medium pH range of 6.4-6.6, a CO_2 gas content range of 500-1.200 ppm, and a light intensity around 1000 lux using white LED lamps.

2.2 Design Modifications Bioreactors for Temporary Immersion Bioreactor Systems

The Temporary Immersion Bioreactor (TIB) system maximizes the gas exchange and nutrient absorption by alternating between plantlets immersion in the liquid medium and dry periods [2, 6]. Plantlets in the *in vitro* culture can absorb nutrients through both their roots and leaves. In the TIB, when plantlets are immersed in the liquid medium, their entire epidermis is exposed to it, thus maximizing the nutrient absorption [2, 6]. Therefore, the plantlets grown in the TIB system are expected to exhibit superior growth and reproduction rates compared to the plantlets grown in the conventional solid or liquid medium culture. Moreover, the TIB system enables the removal of any volatile compounds, such as ethylene, by promoting ventilation in the culture vessel through forced aeration.

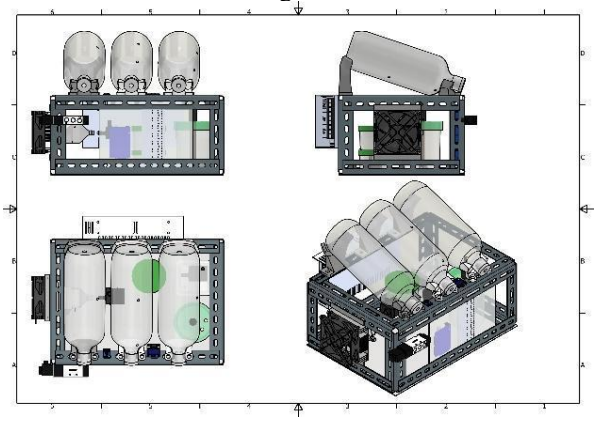

Fig. 2. 3D Design Hardware of Temporary Immersion Bioreactor Systems.

The modified temporary immersion bioreactor design in this paper consists of two chambers, the lower one for nutrient medium storage and the upper one for the culture room. The culture room shape is flexible to change depending on the needs. However, the culture room design demonstrated in this paper consists of three transparent glass bottles, each with 1 L volume and covered with a plastic port cap. The lower and upper containers are connected by silicone hose, and a 0.22 µm millipore filter is attached to each area where medium and gas exchange enters and exits to maintain the sterile condition (Fig. 2). The current optimal system is configured with a CO_2 gas injection volume of 0.1 vvm (aeration volume/medium volume/minute), and the timer is set to immerse the plantlet in the liquid medium for 1 minute every 20 minutes.

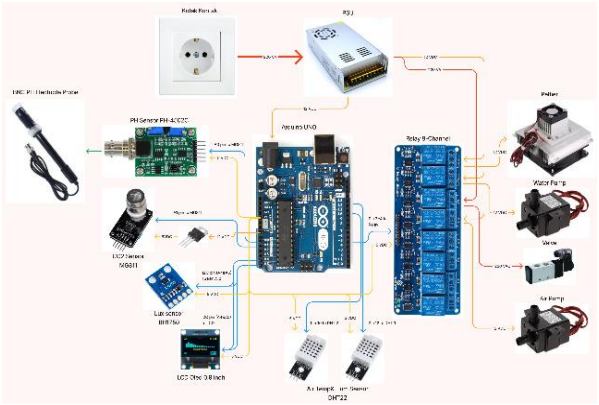

Fig. 3. Hardware of Temporary Immersion Bioreactor Systems.

The hardware components used in the temporary immersion bioreactor systems include a water pump for circulating the culture medium, an air pump for pushing the CO_2 gas and some fresh air into the system, a peltier device as the airflow cooler, a relay for connecting Arduino Uno to actuator systems, a 5 port pneumatic valve for adjusting airflow, a PSU for voltage supply, and sensor packages to monitor the live conditions inside the culture system. Several sensor systems installed in Arduino Uno include pH medium, light intensity, CO_2 gas, temperature, and humidity sensors (Fig. 3).

The installation of various sensors is crucial for monitoring and optimizing the TIB culture condition. Specifically, the temperature and humidity sensor (*DHT22*) is used to assess the environmental condition of the culture room. Additionally, a CO_2 sensor (*MG811*) was integrated to monitor CO_2 levels and gas exchange in the culture room. The effect of CO_2 supplementation was known to enhance the plantlets survivability during acclimatization phase. This improvement can be beneficial for micropropagation of commercial crops. A pH sensor (*PH4502C*) was also installed to assess the quality of the culture medium, as the provision of poor-quality culture medium adversely affects plantlet growth and development. Lastly, a light intensity sensor (*BH1750*) was added to measure the amount of light exposure in the culture room.

2.3 Automatic Software Development

The automatic controls in this system are regulated by signals generated from programmable digital timers. Synchronous schedules for both digital timers are manually assigned and must be created precisely to control the schedule. A well-scheduled assignment allows the temporary immersion bioreactor system to operate as expected. Failure to do so will result in unexpected output or even be able to damage the plantlet and equipment, for example if both valves are activated simultaneously, the compressed air could result in overpressure. Moreover, the excessive period of immersion could also cause hyperhydration and thus reduce productivity. Scheduling digital timers is a very complex task, which is especially important when multiple TIBs are activated simultaneously. A bad digital timer programming due to human error can occur and cause serious damage to plantlets and cultivation equipment.

Computer software for automatic dual sequence temporary immersion control on Arduino principle was developed. Operator can set the immersion period and forced ventilation of each TIB by uploading the settings on the Arduino uno with the times shown on the front panel of the GUI. The duration of soaking and ventilation (cleaning) can be determined by changing the numerical values on the GUI (image not shown). The SC module starts the immersion or forced ventilation process according to the specified value. The TIB status is monitored by the HI module, which sends signals to the targeted digital output devices. To control TIBs simultaneously, SC modules are arranged in series. Many global variables are specifically created for each TIB and are used to serve the parallel operation of GUI modules, SC modules, and HI modules.

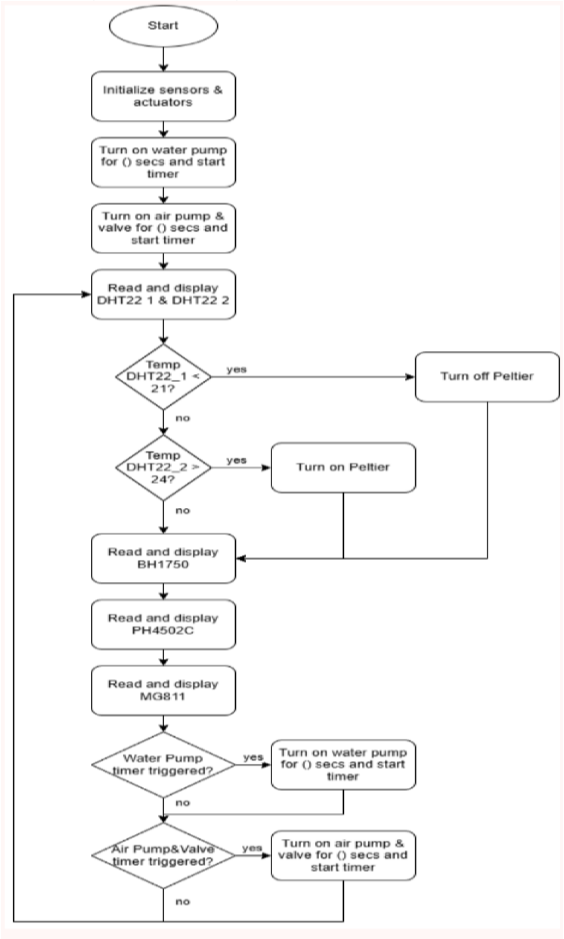

Fig. 4. Flow Chart of Temporary Immersion Bioreactor Systems.

The operational system flow starts from initializing sensors and actuators with multiple sequence control, then both pumps turn on for a few seconds and start the timer schedule. Media fluid transfer and gas exchange are controlled by Arduino Uno. The liquid medium culture is pumped into the culture bottle and remained there for twenty minutes, after which it flows back into the nutrient culture storage for reuse (Fig. 4).

Tool testing begins with the calibration process for each component used and testing is carried out on the entire series of microcontroller components that have been installed. Testing of DHT12 was successfully

carried out, this sensor can control changes in temperature and humidity provided in TIB culture. The MG811 sensor succeeded in reading changes in CO2 levels in TIB culture. The PH4502C sensor succeeded in reading changes in the pH value of the solution injected in TIB culture. The BH1750 sensor succeeded in reading changes of light intensity when different watts of LED lights are used. Testing the 128x6 resolution LCD monitor confirms that it displays reading in accordance with the created system. The sensor reading for temperature, humidity, light intensity, pH value, and CO2 levels were successfully carried out as was the testing of the entire series of microcontroller components was successfully carried out according to the provided flow chart in (Fig. 4).

It was observed that using the developed computer software allows the operator to modify the immersion conditions with less effort and more precision when compared with using conventional digital timers. The programmed control system was able to control the sequence of multiple TIBs as given. However, it was observed that the liquid medium transfer rate between the storage tank and the culturing tank reduced when liquid medium hose was obstructed with small plantlets or when insufficient air pressure was supplied to TIB. Therefore, the filling and draining process times needed to be extended to complete the liquid medium transfer. Manual adjustment of the liquid medium transfer rate was required. Further study on the application of fluid flow and pressure measurements in TIBs together with appropriate computer algorithms is needed to improve capability of software to detect and solve faults in liquid medium transfer. The fault detection function of the software may prevent the damage of plantlets due to hyperhydricity caused by unintentional extended immersion duration. In this study, we observed black orchids grow well in the right duration of immersion because it was controlled by an arduino microcontroller. We also observed that the growth rate of plants on controlled TIB was faster when compared to plants on solid media (Fig. 5).

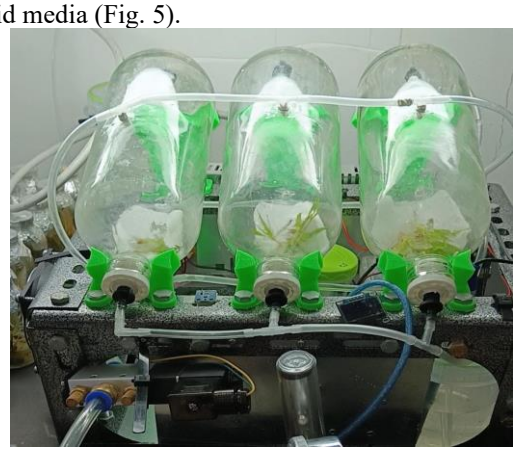

Fig. 5. Application Micropropagation *C. pandurata* in Temporary Immersion Bioreactor Systems.

2.4 Arduino Control System Algorithms

The output of these tools will be forwarded to the microcontroller as an entry point to be processed by the Arduino Uno Microcontroller system's brain. Arduino Uno Microcontroller receives data voltage and duration flow to compare it with the preceding value and makes a decision based on that input. Based on this input the decision is made by the Arduino Uno microcontroller. The main program module to support this project has been embedded in Arduino Uno microcontroller and is presented below.

```
//OLED - Display
#include <Adafruit_GFX.h>
#include <Adafruit_SSD1306.h>
#define SCREEN_WIDTH 128// OLED display width, in pixels
#define SCREEN_HEIGHT 64// OLED display height, in pixels
Adafruit_SSD1306 display(SCREEN_WIDTH, SCREEN_HEIGHT, &Wire, -1);

//DHT22 - Temperature & Humidity
#include "DHT.h"
#define DHTPIN_1 8     // DHT PIN 8
#define DHTPIN_2 9     // DHT PIN 9
#define DHTTYPE DHT22    // DHT 22  (AM2302), AM2321
DHT dht1(DHTPIN_1, DHTTYPE);
DHT dht2(DHTPIN_2, DHTTYPE);
float h1, t1, h2, t2 = 0;

//BH1750 - Lux
#include <BH1750.h>
BH1750 lightMeter

//PH-4502C - PH
#define PHPIN A0
#define PH_SAMPLE 5
#define ADC_RESOLUTION 1024.0

//MG811 - CO2
#define READ_SAMPLE_INTERVAL (50)
//define how many samples you are going to take in normal operation
#define  READ_SAMPLE_TIMES (10)
//define the time interval between each samples in
#define  DC_GAIN (8.5)
//define the DC gain of amplifier
#define  ZERO_POINT_VOLTAGE (0.171)
//define the output of the sensor in volts when the concentration of CO2 is 400 PPM
#define   REACTION_VOLTAGE   (0.030)
//define the voltage drop of the sensor when move the sensor from air into 1000 ppm
#define MG_PIN (A1)
//define which analog input channel you are going to use
```

3 Conclusion

The Arduino program designed in this study is capable of controlling immersion bioreactor systems while working independently and integratedly. The program developed has been used in micropropagation of black orchids at the root and leaves elongation stages. TIB system controlled by the Arduino program was able to

achieve a survival rate of 98%. Modification to immersion conditions for each temporary immersion system could be simply made on LCD screen or arduino software. The upper chamber design for *in vitro* culture can be flexibly adjusted as needed without the need to modify the control system. Additionally, the features of fluid transfer disturbance detection systems due to inhibition of small plantlets and insufficient air pressure should be developed to minimize risk of hyperhydricity explants.

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Authors Contribution A.D.R., L.A.U., and S.A.A.C. wrote the manuscript, E.S. supervised all the process. All authors listed have made direct and intellectual contributions to the work, and approved it for publication.

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