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Design and Construction of a Pneumatic Temporary Immersion Bioreactor System for the Multiplication of *Ananas comosus* var. Trujillana Red

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Abstract

The present work initially identified the design parameters of a temporary immersion bioreactor to later scale it to a complete system for the *in vitro* multiplication of *Ananas comosus* var. Trujillana Red. Thus, a low-cost pneumatic temporary immersion bioreactor system was designed and built with 24 tanks of 2 L each. The automation of the system was designed and implemented by means of a timer circuit whose design parameters were: duration of the propagation process, which depends on the multiplication period of the crop and is an open variable, which means that the operator decides when to turn off the system; the duration of each dive, which for reasons of complexity of the algorithm was standardized as one minute; immersion frequency, which was programmed for intervals of 1, 2, 3, 4, 5, 6, 7, 8 hours respectively and duration of aeration, which from a test run times of 0.20 were chosen, 30, 40, 50, 60, 70, and 80 seconds that correspond to the time of delivery of compressed air; additionally, the multiplication rate of *Ananas comosus* var. Trujillana Red in the immersion system which was 6.5 times per propagative unit inoculated in thirty days.

Keywords

Temporary Immersion, *Ananas comosus*, Bioreactors, Micropropagation

1. Introduction

Within tissue culture techniques, micropropagation is a technique developed for large-scale production of plants, which has been used successfully since the 60s

of the last century [1], with advantages such as, growth rates higher than in natural conditions, which allows to obtain a large number of plants in a short time, reducing the area necessary to maintain them, obtaining plants free of bacteria, fungi and viruses, the possibility of producing plants throughout the year [2].

However, this technique has certain limitations, it requires a large number of culture vessels, skilled labor, little possibility of automation, the propagation efficiency is limited and production costs are high [3]. There are commercial bioreactors that allow micropropagation of plants and have a great application in biotechnology, these reactors are sold abroad at a high cost, these prototypes are disposable.

A bioreactor is a device or system that supports a biologically active entity, in plant biotechnology it is a container whose main function is to provide a controlled environment that allows optimal conditions for micropropagation to be reached. Temporary immersion is a method that consists of intermittently moistening the tissue of a plant with a liquid medium for a short period of time, followed by drying by gravity, all of this occur inside a bioreactor [2] [3].

Bioreactors are a specialized technology, equipped and designed among others for the culture of plant tissues; recognizing the culture of cells, tissues and organs as an important tool for the clonal propagation of cultures of diverse commercial importance, the production of various secondary metabolites, the expression of complex proteins (molecular farming), conventional phytoremediation or through transformed roots [3].

A temporary immersion bioreactor is characterized by partially submerging the explants during iterative periods. Among its various applications are: cell cultures for the production of metabolites, somatic embryogenesis for the regeneration of plants, cultivation of prokaryotic microbes [4], production of phenolic compounds and flavonoids [3], production of the oxidative enzyme laccase that catalyzes the degradation of lignin [5]; also the production of ornamental species such as *Gypsophila paniculata* "rain". Thus, clonal multiplication based on plant tissue culture is preferred because it provides a commercial pathogen-free and genetically homogeneous production [6].

Using a temporary immersion bioreactor is the best alternative for solving the problems of *in vitro* culture in solid medium because it offers the advantages of being an important technology in considerably increasing the rate of biomass production in cultured explants. reduction of labor, offers low production costs, automation of the process [1], allows control of operating conditions and is sufficient to establish a practical system for the mass propagation of various species. All the advantages mentioned are due to the fact that the contact area of the culture medium with the explants is maximized, since these are immersed in the liquid medium.

At a technical and economic level, the use of bioreactors has advantages at the cost level, since the consumption of the substrate is efficient, it provides a greater contact area with the explant, reducing the cost per plant [4]. The design and construction of our own temporary immersion bioreactor imply a lower invest-

ment compared to commercial prototypes that are disposable and expensive; In addition, the highest growth speed is achieved in less time, therefore explants of the same size are obtained in less time [7].

Automation reduces the labor used in the process, which has an impact on operating costs, in addition, the control of the operating parameters allows micropropagation to be more efficient since optimal conditions are provided for the development of the explants [4]. Some process variables such as immersion time and frequency, photoperiod and CO_2 flux can also be controlled [8]. Finally, scaling is possible, this implies that working with a liquid medium allows a greater number of plants to be micropropagated in the same space, therefore increasing the operation volume will allow the production of more explants [2].

There is the problem of designing and building a temporary immersion bioreactor that is low cost and with equal or better efficiency than commercial prototypes. Acquiring them in the foreign market and their high price are reasons why this technology has not been implemented in developing countries.

In this regard, there are successful experiences in other countries in the construction of prototypes for this nature. In 2010, Monroy and Filgueira, at the Military University of Nueva Granada, built a prototype with two twin flasks for the micropropagation of *Dianthus caryophyllus* "carnation" using heat-resistant glass jars and perforated rubber stoppers into which glass tubes were inserted; air was added through a fish tank pump controlled by solenoid valves and a timer [8].

Using catalog materials, Acuña [7] in Mexico mentions the assembly of two twin bottles. In Chile, various projects have sought to introduce this technique in their universities and agricultural companies, in the particular case of *Vaccinium corymbosum* "cranberry" Arencibia *et al.* [9] mention the use of twin bottles designed for their study.

Gueguim *et al.* [4] built a prototype focused on the implementation of details by computer using a software to control the fermentation process of *Pleurotus pulmonius* "oyster mushroom". Juárez *et al.* [2] designed a pneumatic and a mechanical temporary immersion bioreactor for the propagation of *Aztekium hintonii*, an endangered cactus, this system consists of a single tank in both cases.

Cuba's experience in the use of temporary immersion systems led them to create the twin bottle system at the Bioplantas center, replacing the automated temporary immersion container (RITA) created at CIRAD in France, in this system it has been specially propagated bananas and pineapples [1] where they test the multiplication of the banana clone FHIA-18 (AAAB).

In Nigeria, under the patronage of the Federal Ministry of Science and Technology, the National Center for Genetic Resources and Biotechnology (NACGRAB) in 2012 built a complete system of twin flask temporary immersion bioreactors and test various agricultural species [10]. In 2014, in the same country, it employs an innovation in the temporary immersion bioreactors of twin flasks consisting of overlapping rectangular tanks of the SETIS brand of the

company BIOCHEMIE B.V [11].

In Peru, it has been propagated in bioreactors, pineapple of the Golden variety and potato in the La Molina National Agrarian University under international financing, despite this the system is imported and was not built in Peru, none of these systems are economically accessible enough and technically for its massive use so this work is the first in its construction in our country, as well as the first time that Trujillo red pineapple is introduced and multiplied *in vitro*.

Pineapple is the third most important tropical fruit in the world, after bananas and citrus fruits Prior to the discovery of the pineapple fruit by Christopher Columbus in 1493, the fruit was already a stable component of the crops and diet of the Native Americans of the tropical lowlands. The edible pineapple is classified taxonomically in: Order Bromeliales; Bromeliaceae family; Subfamily Bromelioideae; Genus: *Ananas*; Species: *comosus* [12].

Among the commercial pineapple varieties most in demand in Peru are the Criolla Selva, the Cayenna Lisa and the MD-2, whose production is mainly concentrated in the central jungle (Chanchamayo and Satipo provinces); However, important cultivated areas correspond to varieties such as Samba de Chanchamayo, Pucallpina, Trujillana Red and Motilona [13].

In Poroto, the Trujillana Red variety is the most commercialized for its resistance to transport and degree of acidity; in addition, varieties such as Española, Piña Blanca, Vanila, Hawaiana, Selvática, Ecuatoriana, and French Guiana are reported in the area, whose crops are spread over hamlets and annexes such as Platanar, Con Con, Miñate, Dos de Mayo, Shirán, Chille and Cambarra, among others [14].

Red pineapples have a rougher pulp (fibrous) and of somewhat inferior quality (high acidity) than white pineapples, especially in hot climates. Within this group, the "Trujillana Red" can be mentioned. The trujillana red pineapple planted in Poroto and surroundings in La Libertad (department of Peru), until 5 years ago, had a duration of 10 to 15 years of cultivation, but at present the duration of cultivation has been reduced to 4 to 5 years due to factors not yet defined, thus harming pineapple production [14].

Pineapple producers in Poroto and Salpo in La Libertad frequently source seeds from neighboring fields, having serious problems of uniformity in plantations and proliferation of pests and diseases. Therefore, it is important to clean the propagating material and multiply it by *in vitro* culture to avoid this constant contamination. This activity would also allow establishing seed fields or propagating units in order to avoid bringing plants that present serious health problems to the final field, in addition to selecting the seed by size and type to standardize each growing area [14]. In this way a uniformity of plants in the field is ensured and consequently good quality fruit at harvest time.

By using the micropropagation technique, by applying the SIT method, in pineapple cultivation; It is intended to show the impact that this has, with respect to the traditional methods of micropropagation; since a higher multiplication and acclimatization rate is obtained, as well as higher levels of survival in field

conditions [15].

The advantage of using the temporary immersion system, in the mass micropropagation in pineapple, is that the contact of the liquid medium, with the tissue of the explant, is made intermittently and not permanently; avoiding the vitrification of the explants, generating in a short time, a greater quantity of vegetative material, which maintains its quality. Using conventional propagation methods, they would be slow to produce and high production costs [16].

The objectives of the present work were to design and build a low cost pneumatic temporary immersion bioreactor system, design and implement the automation of the pneumatic temporary immersion bioreactor system and determine the multiplication rate of *Ananas comosus* var. Trujillana Red in the temporary immersion bioreactor system.

2. Material and Methods

For the sizing of the compressor, the friction losses in the pipes and accessories were calculated using the Darcy-Weisbach equation and the Colebrook-White formula automated in a spreadsheet [17] [18]. After sizing the compressor, a Werken brand oil-free air compressor with a capacity of 116 L/min was chosen at a maximum pressure of 0.8 MPa, which compressed the air and delivered it under pressure to feed the bioreactors.

The system transfers from 500 mL of culture medium in 10 seconds to 6 liters of culture medium when the 12 twin flasks (bioreactors) were used in 20 seconds at a compressor flow rate of 116 L per minute (**Figure 1**). This compressed air was distributed to the bioreactors through a network of half-inch pipes whose flow is regulated by stopcocks at the beginning and end of each level, whose terminals in each level are a total of eight and their diameter of 10 mm.

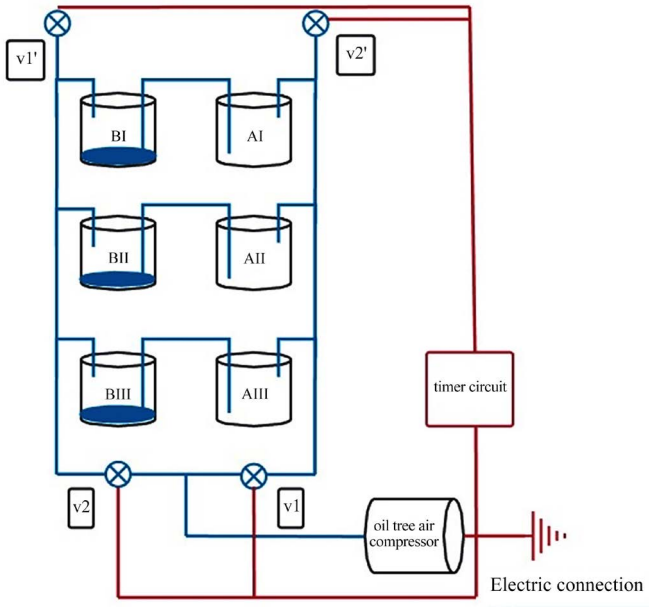

Figure 1. Electro-pneumatic diagram of the bioreactor system.

We opted for PTFE (polytetrafluoroethylene) membrane vent filters on a polypropylene support, autoclavable, Acro 50 brand from PALL corporation, ideal for gas filtration. The main property of this material is that it is practically inert, it does not react with other chemical substances except in very special situations. This is basically due to the protection of the fluorine atoms on the carbon chain. These filters were connected between the bioreactors and the aeration system outlets by means of a 10 mm wide autoclavable silicone hose (**Figure 1**).

The most suitable container should be made of clear, translucent and autoclavable plastic, preferably with a wide mouth [7]. There are various materials on the market such as Carboy, Polycarbonate and Boron-Silicate (glass). In our country these products are not common and are imported in the Tygon and Nalgene brands to name a few of them, following the parameters detailed by the aforementioned authors, the local market was searched for plastic containers whose manufacturing label promised heat resistance, These containers were subjected to an autoclave to evaluate their resistance, finding only one type of them that passed the test, of the PLASTIOLED brand of 2 L capacity, translucent (**Figure 1**). Although these containers support autoclaving, the lid did not have any hermetic connection for the entry and exit of the filtered air, these connections were added using 10 mm irrigation connectors, drilling two holes in the lid with the help of a drill with a 16 mm drill bit.

Based on what was mentioned by Gueguim *et al.* [4], about the control variables in automation, the following were taken, with their respective parameters:

1) Duration of the propagation process: it is the total time in hours or days that the bioreactor must operate while the plant multiplies. For this variable, it was considered to leave this period open, so the system will only stop repeating the frequency and duration of the dive due to unscheduled human action; that is, if it is disconnected from the electrical power source or is misconfigured to start another crop or frequency.

2) Immersion time: Indicates the period of time that the organism can be in contact with the culture medium. Regarding this variable, one minute was chosen as a fixed time for the duration of each dive. This is due to the complexity of executing an algorithm for the operation of the control circuit, which is why it was decided to choose a minimum time, in this regard suggests that the immersion times should be short, thanks to this the explants are covered with a film of liquid culture medium and in this way the desiccation of the same is avoided [1]. Resistance to gas diffusion is low and there is minimal breakdown of gas exchange between the tissues and the internal atmosphere of the culture flask.

3) Dive frequency: Indicates the occurrence of each dive expressed in minutes or hours. For the control system, the following hours were programmed as the space between each dive: 1, 2, 3, 4, 5, 6, 7 and 8 hours; from which it is understood that the highest frequency per day is approximately 24 times per day and the minimum three times.

4) Duration of aeration: Indicates the time in which dissolved oxygen is provided in the culture medium, in this time the air delivered by compression to the

system is sufficient to transfer the culture medium from one container to another, it was determined from of the operation of the complete bioreactor system in three repetitions, that the minimum average time to execute this translation process was ten seconds for the system of 12 twin bottles of 2 L capacity and 1/2 L of culture medium per system of twin jars (2 jars).

3. Results and Discussion

Pneumatic system

The materials, dimensions, components, pressure, volume, flow, speed, periodicity and frequency of immersion were analyzed for the correct pneumatic mechanism that guarantees immersion in the Bioreactors according to the requirements of the culture. The compressed air was distributed to the bioreactors through a network of half-inch pipes whose flow is regulated by stopcocks at the beginning and end of each level, whose terminals in each level are a total of eight and their diameter of 10 mm. The characteristics and calculations of friction losses in pipes and fittings using the Darcy-Weisbach equation and the Colebrook-White formula are shown in Table 1 and Table 2.

Control system

The timer circuit was designed to deliver compressed air based on this minimum time and aeration periods of: 10, 20, 30, 40, 50, 60, 70 and 80 seconds respectively were programmed. All the aforementioned parameters were controlled by a timer circuit manufactured to operate the compressor and four solenoid valves, two arranged at the inlets and two at the outlets of the aeration system pipes (Figure 1).

Electric connection: 220 volt wall, V1, V1', V2, V2': solenoid valves, A y B: tanks. Operating Principles of the Twin Bottle System: 1) Exposure period. The entire volume of the liquid medium is in the medium storage tank. The connections for both vessels are closed and the solenoid valves open to atmosphere; 2) Transfer of the liquid culture medium from the chamber with explants to the storage tank. The air line of the culture chamber opens, and the air line closes in the medium storage tank, the overpressure moves the medium from one tank to another; 3) Period of immersion. The propagules are immersed in the liquid medium. The medium storage tank is empty. Air lines for both containers are closed and solenoid valves open to atmosphere; 4) Draining the nutrient medium back to the growing medium tank. The culture chamber air line is open, while the medium storage tank air line is closed. The excess pressure moves into the storage tank Figure 2.

Once the operation of the bioreactor system had been determined, culture tests were carried out with the species known as "pineapple" in the mentioned variety (Figure 3). The introduced suckers were provided by the plant biotechnology laboratory of the Professional Academic School of Agronomy of the National University of Trujillo in a total of 30 suckers per bioreactor, for the efficiency test. For this, the culture medium proposed by Murashige and Skoog (1962) was used [19].

Table 1. Characteristics of the pipes and components for the design of the fluid conduction.

Components	Dimensions	Quantity	Unit of measurement
Whiskers (36 units)	Diameter	0.01	meters
	Length	7.2	meters
	Absolute roughness	0.0000015	meters
	Overall coefficient of minor losses	0.22	-
Whiskers holder (6 units)	Diameter	0.025	meters
	Length	7.2	meters
	Absolute roughness (PVC)	0.0000015	meters
	Overall coefficient of minor losses (cubit + valve)	0.99	-
Side carrier (4 units)	Diameter	0.025	meters
	Length	4	meters
	Absolute roughness (PVC)	0.0000015	meters
	Overall coefficient of minor losses (cubit + valve)	0.99	-
Matrix pipe	Diameter	0.012	meters
	Length	2	meters
	Absolute roughness	0.0000015	meters
	Overall coefficient of minor losses (T + cubit)	1.3	-
Filters (24 units)	Diameter	0.0125	meters
	Length	4.8	meters
	Absolute roughness	0.0000015	meters
	Overall coefficient of minor losses (Widening and membrane)	1.3	-
Tanks (24 units)	Diameter	0.12	meters
	Length	3.84	meters
	Absolute roughness	0.0000015	meters
	Overall coefficient of minor losses	0.22	-

Table 2. Total friction head losses in components.

COMPONENTE	hm (manometric height)	Pressure (Mpa)
Whiskers (36 units)	0.66	0.01
Whiskers holder (6 units)	9.82	0.1
Side carrier (4 units)	16.97	0.17
Matrix pipe	17.23	0.17
Filters x 24	7.83	0.08
Tanks	26.98	0.26
total losses	79.51	0.79*

*According to the calculations, a compressor had to be found that delivered more than 0.79 Mpa (The oil-free compressor had a capacity of 0.8 Mpa (81.58-gauge height).

Figure 2. Pneumatic Temporary Immersion bioreactor system assembled and in operation. (A) Matrix pipe (B) Valve (C) Side carrier (D) Whiskers holder.

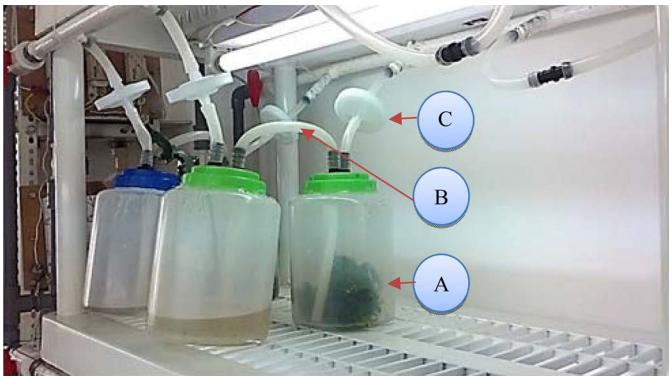

Figure 3. Bioreactors installed in the aeration and lighting system. (A) Tanks,)B) Whiskers, (C) Filters.

Said basic medium was supplemented with the MS vitamins, which were prepared in stock concentrated solutions and additionally with sucrose as a carbon source (30 g/L) and with the growth regulators naphthalene acetic acid (ANA) and benzyl amino purine (BAP) to a concentration in both cases of 2 $mg{\cdot}L^{-1}$.

For the introduction of the explants in the Bioreactors, a laminar flow cabinet was used, the parental material was sectioned by suckers, 30 suckers were introduced per previously autoclaved bioreactor and connected to the aeration system for incubation by temporary immersion (**Figure 3**).

Thirty days after the suckers were incubated, the multiplication rate (MR) expressed as a ratio of the average number of material obtained from suckers or propagative units per bioreactor (PUB) between the number of seeded parental material (SPM) was determined en the Equation (1).

$$MR = \frac{195(PUB)}{30(SPM)} = 6.5 \quad (1)$$

Under the conventional system of 200 mL glass containers, 2 to 3 buds are obtained for each sucker grown, from which the considerable increase in the multiplication rate in the bioreactor is contrasted (Figure 4).

The evolution of the biomass accumulation of pineapple *in vitro* seedlings, obtained in the bioreactor up to thirty days, was determined, weighing the average plant mass obtained every five days (Figure 5).

The use of liquid culture medium in the different stages of micropropagation, as in the case of bioreactors, favors the development of explants and, as reported for different plant species, there are significant increases in the proliferation rate [20], this effect was notorious in this work in which the multiplication rate per shoot in bioreactors (6.5 shoots per sown shoot) was almost three times that obtained in flasks (2 shoots per sown shoot).

The seedlings obtained were transplanted in fine blond peat, obtaining 100% rooting without the need to add rooting products, reaching the filling of the tray cone in 30 days (Figure 6).

Figure 4. Propagative units obtained in a bioreactor 30 days after sowing.

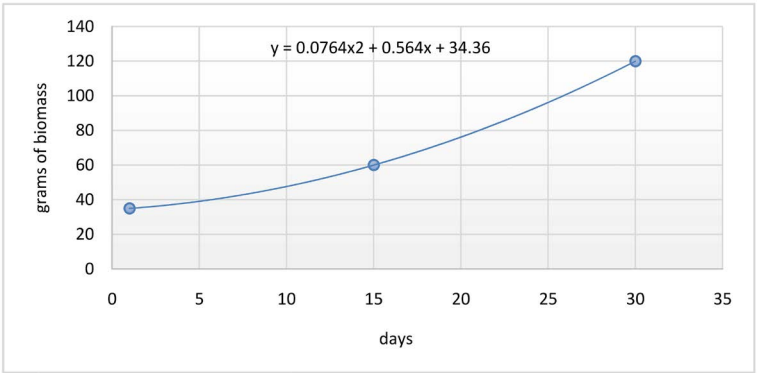

Figure 5. Evolution of the biomass accumulation of pineapple *in vitro* seedlings, obtained in the bioreactor up to thirty days.

Figure 6. Seedling behavior in substrate rooting. (A) Rooted seedling, (B) 1-tank clusters, (C) transplanted seedlings.

In the Automated Temporary Immersion System (bioreactors), Llanos [13] applied immersion frequencies of three minutes every three hours for a period of six to eight months, for Ángel and Gonzáles [16] in the multiplication phase, they determined that when using The Temporary Immersion System with a five-minute immersion time, every two hours, generated the highest number of shoots of Ananas like their Golden variety. Unlike the present work where he underwent immersion periods of one minute every eight hours.

Llanos [13] too, incubated the bioreactors at a temperature of 25˚C - 30˚C, with a photoperiod of 11 or 12 hours per day under white fluorescent light that together with the selected medium of BAP concentrations (2.1 mg·L^{-1}) - ANA (0.3 mg·L^{-1}) added to the medium of Murashige and Skoog [18], as it presented a higher multiplication index, it resulted in a multiplication rate of 11; while Newton *et al.*, supplemented the medium of Murashige and Skoog (1962) [18] with BAP (1 mg·L^{-1}) - ANA (0.25 mg·L^{-1}), in this work with constant light and a medium with the combination of concentrations BAP (2 mg·L^{-1}) - ANA (2 mg·L^{-1}) and in a period of 1 month a multiplication rate of 6.5 was obtained (**Figure 4**) It is essential to highlight that the propagative units (suckers) obtained had in average 2 cm long, with a longer incubation period they would have continued to develop, the differences may be due in addition to the incubation time to a higher content of ANA in this work.

The addition of regulators and their concentrations are very important, they managed to obtain around 5000 plants from a single explant with Murashige and skoog (MS) liquid medium added with water from coconut and transferred to BAP to form axillary shoots capable of rooting [19].

Regarding the volume of culture medium used in this case, it was 500 mL per 2 L container [20], in this regard demonstrated in a similar way with pineapple that a volume of medium of optimal culture for shoot proliferation, which was estimated to be 200 ml per explant for that species. In this case, larger volumes also led to a drop in the proliferation rate. At the end of the culture, the volume consumed per bioreactor was measured, which on average was 100 mL, which does not justify a higher volume of use for this design.

In temporary tissue culture immersion systems, it is evident that the immersion time is very important, since it regulates the absorption of nutrients and the expression of hyperhydricity [21]. The immersion times used for different jobs vary considerably; this is probably due to the wide variety of species, processes and temporary immersion micropropagation systems used. Long immersion times (1 h every 6 h) prove to be effective for potato tuberization, while short immersion times (1 min. every 12 h) stimulate the production of more somatic embryos in coffee and rubber [22].

The frequency of immersion in this trial was every 8 hours (3 times a day), other studies show that the effect of the frequency of immersion in the development of shoots can be explained by the availability of nutrients and this to at the same time explain the multiplication rate obtained. That using a higher frequency of immersion can present effects such as a decrease in oxygen concentration. The osmotic shock suffered by the explants during each immersion in the culture medium, possibly the more frequently the tissues suffer a level of stress that affects the response of the explant [23].

Aeration is one of the most influential factors in *in vitro* propagation through temporary immersion systems. In the first place, due to the entry of the air flow into the SIT containers, the mechanism of each immersion cycle is activated, which will eventually keep the explants in contact with the liquid culture medium [24], points out that the use of bioreactors with air lift shows an increase in the proliferation of meristematic buds and a reduction in tissue cuts, this being the desired result when propagating seedlings using SIT. The consulted authors do not refer to the delivered flow rates of pressurized air in the system, for this test a compressor flow rate of 116 L/min was used (**Figure 5**) which, when divided into each of the twelve connections for each bioreactor, is delivered 9.666 liters of air per minute at approximately 0.066 MPa of pressure since the losses in the pipe and accessories because they are very short are almost negligible.

4. Conclusions

A system of pneumatic temporary immersion bioreactors was designed and built for the multiplication of *Ananas comosus* var. Trujillana Red. The automation of the pneumatic temporary immersion bioreactor system was designed and implemented through a timer circuit that controlled the pneumatic mechanisms of the solenoid valves and air compressor, determining the immersion frequency, which was programmed for intervals of 1, 2, 3, 4, 5, 6, 7, 8 hours respectively and aeration duration of 0, 20, 30, 40, 50, 60, 70, and 80 seconds.

The multiplication rate of *Ananas comosus* var. Trujillana Red in a system of pneumatic temporary immersion bioreactors, was 6.5 seedlings for each incubated bud.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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