Microtubers production by using Temporary Immersion System (TIS) bioreactor to potato varieties

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Abstract. As the national potatoes growth has become lower from year to year, it is now known that the problems came from the lack of certified potato seed varieties and the minimum access to sophisticated technology to make a good potato variety. The solution that can be made is to utilize microtubers as an efficient factor. The purpose of this research is to find the most effective method in micro tubers cultivation in vitro by comparing the conventional tissue culture method and the Temporary Immersion System (TIS) bioreactor method to four different potato varieties (Granola L., Dayang Sumbi, Atlantic Malang, Maglia). This research uses a split-plot design with a completely randomized design by using two factorial. The result of this study shows that the microtubers in the multiplication and production step using the TIS bioreactor method has a higher average compared to the conventional tissue culture method. As the various details, Dayang Sumbi has the highest parameter such as most sprouts, primer roots, diameter, wet weights, and fastest time growth. Granola L. excel in planlet height and most tubers. Atlantic Malang in the most multiplication and nodus. Meanwhile Maglia excel in most leaves.

1. Introduction
As the national potatoes growth has become lower from year to year. The low productivity of potatoes has also failed to fulfill the national potato consumption figures optimally. Several factors are considered to be the cause of the low productivity of national potatoes, including: The availability of certified and quality potato seed sources is minimal, which is only 10% of the total national need for potato seeds [1]. Handling and complicated seed distribution patterns, as well as overlapping regulations on seed systems, have hampered the dissemination and adoption of a large number of other high-yielding potato varieties [2]. Given the crucial importance of this problem, the use of micro tubers can be an alternative solution as a propagule in providing quality and more efficient potato seed sources [3].

There are several advantages given by using microtubers over in vitro propagated plants since they can be stored and transplanted directly into the field without an acclimatization stage [3]. Handling and shipping became easier, thus facilitating commercialization and international exchange of germplasm [3,4]. Some problems associated with microtuber production in conventional flasks are the low yield of tubers and the small tuber size that limits direct transplanting to field conditions.
Approaches have been made to improve tuber quality and the number of tubers per plant by changing the culture medium components such as high sucrose content and the addition of plant growth regulators; manipulation of culture conditions, temperature, light, and photoperiod [5]. But all these efforts are considered not giving the maximum results. To improve tuber quality and the number of microtubules produced per plant several semi-automated systems has been developed, based on temporary immersion as well as bioreactors. These semi-automated systems also allow the reduction of intensive manual handling and hence increase productivity and decrease the costs of production [6,7].

Another interesting thing is that the micro tubers produced through the TIS Bioreactor can produce several G0 tubers equivalent to the yield of plantlet base seeds with much better fresh weight and bigger diameter of G0 tubers [6]. Unfortunately, due to the lack of information regarding the potential of micro tubers as a quality seed source, proving the potential of micro tubers from various potato varieties become necessary. In addition, to determine the extent of the influence of the use of TIS Bioreactor culture, it is also important to optimize the production and cultivation of micro potato tubers.

2. Materials and methods

2.1. Plant material and culture media
Potato plantlets (*Solanum tuberosum* L. cvs. Atlantic Malang, Dayang Sumbi, Granola L. and Maglia) came from culture laboratories Plants and Vegetables Research Center of Indonesia (BALITSA) and multiplied on MS medium [8] with 30 g/l sucrose and solidified with 7 g/l agar. Shoots were subcultured every 21 days in glass pots (250 ml capacity) containing 30 ml of culture medium. For tuber induction and development, a similar medium but supplemented with 80 g/l sucrose and 5 mg/l Benzyl amino purine (BAP) was used. The pH of the medium was adjusted to 5.8 before autoclaving at 121°C for 20 min.

2.2. Description of the Temporary Immersion System (TIS)
Glass vessels (4,000 ml) with silicone caps were employed. A pair of vessels were connected by an autoclavable silicone tubing (ID=6 mm), one was used as a medium reservoir and another one as a culture vessel. A sterilizable filter (0.22 µm) was fitted to each flask for ventilation. The immersion frequency and duration were regulated using a programmable timer connected to three-way solenoid electrovalves (Figure 1). By opening one electrovalve or the other, the medium was flushed from the reservoir vessel to the culture vessel or in the opposite way. Each system, containing a 2,000 ml multiplication medium, was inoculated with 60 single nodal segments and incubated in illuminated conditions (24-h photoperiod), and temporarily immersed 2 minutes every 3 hours for shoot growth. After 21 days the whole medium was exchanged for 3,500 ml medium for tuber induction (80 g/l sucrose and 5 mg/l BAP) and plants were incubated in dark conditions for 6 weeks. Immersion times during the tuberization stage were 2 minutes every 6 hours.
2.3. **Cultures in semi-solid medium (control)**

As a control, we used the methodology of a conventional culture system for the production of potato microtubers. Seven single nodes were transplanted into glass pots (250 ml capacity) containing 30 ml of multiplication medium solidified with agar and incubated 20 ± 2°C under LED tubes (1,000–3,000 lux) with a 24-h photoperiod. Twenty-one days later, the plants will be added to liquid containing 20 ml medium for tuber induction and incubated in the continuous dark.

2.4. **Determination of plant and tuber size**

Twelve vessels of temporary immersion systems and 20 glass pots with semi-solid cultures as control were inoculated with each cultivar (Atlantic Malang, Dayang Sumbi, Granola L., and Maglia). The quality of the plants and tubers was determined by measuring the number of internodes, the speed of multiplication, the length of the plantlets (cm), the number of shoots. The parameters on the microtuberization stage are; the number of tubers per plant, fresh weight (g), and diameter (cm) of the tubers at the end of the tuberization stage (6 weeks). Microtubers were classified into three categories: less than 4 mm, from 4 to 6 mm, and larger than 6 mm in diameter. This research uses a split-plot design with a completely random design by using two factorial. Data were processed using a simple ANOVA and mean separation was done by Duncan’s multiple range test.

2.5. **Field studies**

As additional data, Direct planting of plantlet seeds resulting from shoot multiplication in TIS culture was also carried out. This is useful for knowing the percentage of viability and morphology of plantlet seeds under field conditions. The first step in the acclimatization process is that the plantlet material is immersed in a solution bactericide (Agrept) and fungicide (Anthracol) with a dose of each (0.5 g/500 l) for five minutes to prevent contamination of the plantlets as well as to clean the plantlets from the remnants of the culture media.

The clean plantlets were then cut ±3 cm long, then soaked again in sterile distilled water. Next, the cut ends of the plantlets were dipped in a 20% bio root gel solution to stimulate root formation, and then the micro cuttings were planted in acclimatization media. The acclimatization stage itself lasts up to 4 WAP, and during the process, humidity is continuously maintained by spraying the nutrient solution once a day. Infield experiments, the percentage of surviving plants was evaluated 7, 14, 21, and 28 days after planting. The morphological appearance of the seeds was also observed.
3. Results

3.1. Multiplication stage
Plants of 4 genotypes cultivated in TIS Bioreaktor showed vigorous growth. The stem length was about 2-fold greater than plants cultivated on solid media. The plants in temporary immersion also had more internodes, shoots, main roots, and leaves per plant. Respectively compared with 4 cultivars on conventional culture. These plants had larger leaves, thicker roots, and longer internodes comparing with control (Figure 2). No symptoms of hyperhydricity were observed during the culture period.

![TIS Bioreactor vs Conventional](image)

Figure 2. Morphological differences in TIS Bioreactor culture and conventional culture.

3.2. Tuber induction stage
Tubers were observed in the temporary immersion vessels 7 days after the culture under tuber induction conditions. Microtubers were induced at all plant nodes, indicating that microtubers in TIS Bioreaktor are formed faster than conventional culture. Of the 4 cultivars, only 3 cultivars (Atlantic Malang, Dayang Sumbi, Maglia) were successful but only occurred in one replication each (Table 1). Granola L. cultivar failed to grow tubers at all, due to contamination in all replicates. In TIS Bioreactor culture, contamination is indeed the main problem causing failure in the multiplication and microtuberization processes. Microbial contaminants that attack initially are latent in solid media but will appear suddenly in liquid media which is an ideal living environment for microbes in general [10].

Regarding the difference in the time of formation of microtubers in different potato varieties, Leclerc (1994) [11] has also stated that there is a correlation between the time of formation of microtubers and genetic differences in each variety [12]. The fastest cultivar to form tubers is cv. Dayang Sumbi with an average time of 5.6 days/plant. Meanwhile, cv. Atlantic Malang produces tubers with the longest average time of 20.58 days/plant (Table 1).

| Culture System Treatment | Average Time for Formation of Microtubers (±SD) |
|---------------------------|-----------------------------------------------|
|                           | Atlantic Malang | Dayang Sumbi | Granola L. | Maglia       |
| TIS Bioreactor            | 8.67±15.01     | 5.6±9.81     | 0          | 7±12.12      |
| Conventional              | 20.58±18.76    | 19.67±2.30   | 20.41±4.59 | 19.42±2.26   |

Table 1. The average time of microtuber formation of 4 potato varieties using the TIS Bioreactor culture system and conventional culture was 49 days.
Assimilation translocation competition that occurs between stolons also affects the sooner or later a tuber is formed [13]. Below, during the microtuberization process, the number of stolons growing on cv Atlantic Malang plantlets was very large, even when the plantlets were close to 70 days old, the stolons continued to grow accompanied by callus thickening (Figure 3).

**Figure 3.** The morphology of the rapid growth of stolons of the Malang Atlantic variety plantlet; In TIS Bioreactor culture (A) & In a conventional culture (B). Thick callus formation (C) & Late growth of tubers aged 70 days in conventional culture (D).

The induction of microtubers can be observed from the appearance of inflammation in the nodes, middle, and sub-apical region or the tip of the stolon [14]. In other words, induction of the tuberization process was not restricted to specific regions (nodes) of the plants [5,15] (Figure 4).

**Figure 4.** Formation of tubers in some parts of the plantlet; Tubers growing side by side with stolons (A); at the node (B); at the end of the stolons (C).

During the immersion culture, the liquid medium was in contact with all parts of the plant for a short time, so that tuber induction was more uniform between axillary shoots, resulting in more tuber formation. At the same time, nutrient uptake was greater and therefore, the number, tuber size, and weight were greater than semi-solid culture with a significant difference. Respectively compared for the fourth cultivars on conventional culture (Table 2; Table 3 & Table 4).
Table 2. The average number of microtubers of 4 potato varieties using the TIS Bioreactor culture system and conventional culture was 49 days.

| Culture System Treatment | Average for number of Microtubers (±SD) |
|--------------------------|----------------------------------------|
|                          | Atlantic Malang | Dayang Sumbi | Granola L. | Maglia    |
| TIS Bioreactor           | 0.79±0.45       | 1.20±0.69    | 0          | 1.14±0.66 |
| Conventional             | 0.41±0.38       | 1.16±0.14    | 1.58±0.14  | 1.08±0.38 |

The analysis of tuber according to number (Table 2) showed that the cv. Granola L. in conventional culture produced the highest number of microtubers with an average of 1.58 pieces. Meanwhile, the Atlantic Malang in conventional culture produced the least number of microtubers with an average value of 0.41 pieces.

Table 3. The average diameter of microtubers of 4 potato varieties using the TIS Bioreactor culture system and conventional culture was 49 days.

| Culture System Treatment | Average for Diameter of Microtubers (±SD) |
|--------------------------|----------------------------------------|
|                          | Atlantic Malang | Dayang Sumbi | Granola L. | Maglia    |
| TIS Bioreactor           | 4.53±2.61       | 7.53±4.34    | 0          | 6.97±4.02 |
| Conventional             | 1.66±1.44       | 6.42±1.61    | 4.52±0.38  | 4.89±1.20 |

The analysis of tuber according to size (Table 3) showed that the cv. Dayang Sumbi in the TIS Bioreactor produced the largest average diameter of microtubers which was 7.53 mm. Meanwhile, the Atlantic Malang in conventional culture produced the smallest microtubers with an average value of 1.66 mm. If observed based on the ideal tuber diameter size classification, the results showed that the TIS Bioreactor increased the number of microtubers larger than 6 mm; 26 tubers for Atlantic Malang, 38 tubers for Dayang Sumbi, and 28 tubers for Maglia (Table 4). Tubers of this size are the most desirable for commercial production because they can be planted directly into the field without an acclimatization stage and stored for longer periods without a detrimental loss of weight [5].

Table 4. Microtuber classification of the 4 potato varieties was different in TIS Bioreactor culture and conventional culture based on tuber diameter size.

| Diameter of Tubers | Atlantic Malang | Dayang Sumbi | Granola L. | Maglia |
|-------------------|-----------------|--------------|------------|--------|
|                   | TIS  | Conv | TIS  | Conv | TIS  | Conv | TIS  | Conv |
| <4mm              | 4    | 3    | 8    | 2    | -    | 17   | 16   | 4    |
| 4-6mm             | 13   | 1    | 19   | 2    | -    | 2    | 18   | 6    |
| >6mm              | 26   | 1    | 38   | 10   | -    | 0    | 28   | 3    |

Source: Jimenez et al., 1999 [5].

The larger diameter of the microtubers in the TIS Bioreactor culture makes the lenticels on the tuber skin more visible. This is one of the fundamental differences related to the morphological
appearance of microtubers produced from the two culture system treatments [14]. Lenticels have the function of a place for gas exchange between cells under the epidermis of the tuber to occur with the air around the plantlet's living environment (Figure 5).

**Figure 5.** Comparison of diameters of microtubers produced by the 4 potato varieties in TIS Bioreactor culture and conventional culture.

Based on observations related to the treatment of the culture system, it is known that the use of TIS Bioreactor culture can increase the average wet weight yield of microtubers more than tubers from conventional culture (Table 5).

**Table 5.** The average weight of microtubers of 4 potato varieties using the TIS Bioreactor culture system and conventional culture was 49 days.

| Culture System Treatment | Average for Weight of Microtubers (±SD) |
|--------------------------|----------------------------------------|
|                          | Atlantic Malang | Dayang Sumbi | Granola L. | Maglia      |
| TIS Bioreactor           | 151.12±87.24    | 247.72±143.02 | 0          | 244.48±141.15 |
| Conventional             | 21.83±21.38     | 117.33±44.12  | 46.50±10.99 | 95.83±49.07  |

The analysis of tuber according to number (Table 5) showed that the Dayang Sumbi in the TIS Bioreactor culture produced the highest weight of microtubers with an average value of 257.72 mg. Meanwhile, the Atlantic Malang in conventional culture produced the lowest average weight with a value of 21.83 mg. The following is a classification of microtubers based on the weight of tubers produced by the 4 potato varieties in both treatments of the culture system;

**Table 6.** Microtuber classification of the 4 potato varieties was different in TIS Bioreactor culture and conventional culture based on tuber weight.

| Weight of Tubers | Atlantic Malang | Dayang Sumbi | Granola L. | Maglia |
|------------------|-----------------|--------------|------------|--------|
|                  | TIS  | Conv | TIS  | Conv | TIS  | Conv | TIS  | Conv |
| < 100 mg         | 11   | 3    | 18   | 3    | 19   | 17   | 17   | 9     |
| 100-250 mg       | 14   | 1    | 23   | 9    | -    | 0    | 22   | 4     |
| > 250 mg         | 18   | 1    | 24   | 2    | -    | 0    | 23   | 0     |

Source: Aslan & Iqbal, 2010 [16].
In general, the microtubers produced from the TIS Bioreactor culture were dominated by tubers with a weight of 100-250 mg and > 250 mg. Meanwhile, in conventional culture, the micro tubers produced had an average weight of < 100 and 100-250 mg. Related to this, microtubers with a weight of > 250 mg, are considered to have a shorter dormancy period than microtubers with a weight of < 250 mg, so they are more recommended as seeds to produce better mini tubers (G0) [16]. A similar opinion was also expressed by Wattimena (1995)[4] in [17], which stated that to be a good propagule seed, microtubers must meet certain criteria, namely > 5 mm in diameter with fresh or weight of tubers > 100 mg/tuber.

3.3. Acclimatization stage

Based on the data obtained, the percentage of seed viability of the 4 potato varieties from both types of culture systems did not show a significant difference in yield. Plantlet seeds derived from the TIS Bioreactor culture system had a 91.5% lower viability percentage than plantlet seeds from a conventional culture with a percentage of 93% (Table 7). Meanwhile, plantlet seeds with the highest percentage of viability were Maglia and Dayang Sumbi varieties with values reaching 98% and 95%, respectively. While the plantlet seeds that have the lowest percentage of viability is the Granola L. variety with a value of 85%. and the second place is the Malang Atlantic variety with a value of 91% (Table 7).

Table 7. Percentage viability of seeds of 4 varieties of potato plants based on the treatment of the culture system.

| Treatment     | Varieties      | Weeks After Planting (WAP) % |
|---------------|----------------|-----------------------------|
|               |                | 1   | 2   | 3   | 4   |
| TIS Bioreactor| Atlantic Malang| 100 | 100 | 96  | 90  |
|               | Dayang Sumbi   | 100 | 100 | 96  | 94  |
|               | Granola L.     | 100 | 98  | 96  | 84  |
|               | Maglia         | 100 | 100 | 98  | 98  |
| Conventional  | Atlantic Malang| 100 | 98  | 96  | 92  |
|               | Dayang Sumbi   | 100 | 100 | 96  | 96  |
|               | Granola L.     | 100 | 98  | 96  | 86  |
|               | Maglia         | 100 | 100 | 98  | 98  |

In general, plantlet seeds from the TIS Bioreactor culture showed better morphological visuals, with stems that grew stronger and leave perfectly wide. Meanwhile, plantlet seeds from conventional culture have thinner stems with smaller leaves (Figure 6). These morphological differences have also been seen in the results of the previous shoot multiplication stage. In this case, the treatment of different culture systems in the in vitro phase also directly affected the shoot morphology of the 4 potato varieties in the acclimatization phase.
4. Discussions
Temporary immersion is a valuable option for potato microtuber production. The technique not only induces more tubers per plant than solid medium but also increases the size and weight of the tubers. This allows new opportunities for commercial laboratories dealing with potato seed production because these tubers can be stored and directly transplanted without an acclimatization stage. The temporary immersion system can also be used for shoot multiplication during the planting season when \textit{in vitro} plants can be immediately acclimatized and transplanted. Thus, several strategies are accessible by combining the induction and storage of microtubers with \textit{in vitro} plant production, according to the seasonal patterns of potato farming. Further improvements should be made to increase tuber size and the number of tubers per plant by manipulating the immersion programs, culture conditions, and medium exchange frequency.

5. Conclusions
In general, the use of TIS Bioreactor culture is more optimal in the process of shoot multiplication and micro tuber production. However, TIS Bioreactor cultures are more susceptible to contamination than conventional cultures. Meanwhile, based on several research parameters, the Dayang Sumbi variety is considered superior to other potato varieties. At the acclimatization stage, the difference in the culture system did not significantly affect the proportion of seed viability but rather affected the morphology of the seed.

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