

Advanced mass multiplication of pineapple (*Ananas comosus* L.) cv. MD2 JengkaPine commercial clones using the temporary immersion bioreactor system (RITA®)

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Abstract

The temporary immersion bioreactor system (TIB) is the most recent and advanced type of micropropagation technology for plant regeneration. This technology is preferable in pineapple plant material production because it offers efficient automated operation, high-quality products, and the best value for money. At the moment, conventional micropropagation manufacturing has limited mass production capabilities, while conventional propagation techniques are ineffective and limited. The efficacy of specific plant hormones in the culture media was examined to obtain the optimum response of multiplication and rooting of the culture. At the end of the study, it was observed that less gelling agent application with 3.0 mg/L BAP promoted optimum proliferation potential of clonal planting material at a mean of 5.5 shoots per replicate, compared to traditional in-jar culture supplemented with similar hormone composition, with only 1.63 (mean) shoots within 12 weeks. This study found that using less gelling agent, such as RITA®, a TIB brand, significantly improved the multiplication rate of JengkaPine commercial clones at a cheaper cost and in a shorter period of time. The rate of multiplication was about six times higher (5:3) than in jars using conventional micropropagation methods. *In vitro* rooting was observed as early as 1 week after transfer to MS media containing 1.0 mg/L indole acetic acid (IAA).

1. Introduction

Pineapple (*Ananas comosus* (L.) Merr.) is an economically important tropical fruit plant belonging to the Bromeliaceae family. The pineapple was first cultivated in America in 1492 by Christopher Columbus, who later took the specimens to Europe. Later, the fruit was introduced to the rest of the world by the Spanish and Portuguese through sea routes. Today, it is widely cultivated in the tropics and subtropics for its delicious flesh and the many benefits it provides (Rahman *et al.*, 2020). Pineapple was introduced to Malaya by the Portuguese in the 16th century. In 1888, the Malaysian pineapple industry began in Singapore, Johor, and Selangor with the cultivation of coconuts, areca nuts, rubber, and other fruit trees (Malaysian Pineapple Industry Board (MPIB), 2022). Since then, the crop has

been commercially grown and cultivated in all parts of the country. Nowadays, Malaysia is one of the largest pineapple producers in the world, along with other neighbouring countries such as Thailand, the Philippines, and others. The major pineapple cultivars grown in Malaysia include 'Moris', 'N36', 'Sarawak', 'Yankee', 'Josapine', 'Maspine' and recently 'MD2' (Lasekan and Hussein, 2018).

In recent years, the Malaysian pineapple variety MD2 has been at the top of the consumer popularity scale. Known in Malaysia by many trade names such as "Golden Ripe", "Super Sweet", "Rompine" or "Gold", MD2 pineapple is characterised by its active growth and development, thin and dark green skin, cylindrical shape, absence of typical pineapple burning sensation on the

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tongue and fragrant, super-sweet, golden flesh. MD2 also has a longer shelf life and higher vitamin C content than other pineapple varieties. MD2 pineapple is also not difficult to care for, as it does not require much watering, only good drainage (Harian Metro, 2021). Historically, MD2 pineapple was developed by the Pineapple Research Institute in Hawaii in 1961, and the first industrial planting of MD2 occurred in Costa Rica in the 1980s (Paniagua-Molina and Solís-Rivera, 2020). Nowadays, MD2 enjoys increasing popularity among agricultural entrepreneurs, as it not only satisfies the high demand of the local fresh fruit market but is also exported abroad. With the increasing demand and land use for planting MD2 pineapples to meet consumer demand, a problem arises. Classically, pineapple is propagated through scions or offshoots. Although scions are widely used in pineapple propagation, they have the disadvantage of low uniformity due to heterogeneous growth ability and the fact that scions can only be produced once per year. With existing propagation techniques, a single mother plant can usually produce 3 to 4 offshoots, which is usually insufficient for new planting and expansion of existing farms. On the other hand, the existing planting material can also spread diseases. There are also concerns about expensive and limited planting material, limited plantation capacity and high start-up capital to meet the increasing demand from local consumers and exporting countries. In conventional farming, the harvesting period for pineapples is 14-17 months, and they need to be replanted in time to ensure quality or fruit guarantee (Ayub, 2018).

To overcome this problem, an alternative breeding system is essential. The pineapple tree, which includes MD2, is a perennial plant that can only be propagated vegetatively, which means that there is limited ability to establish mass production of good planting material. One of the best alternative vegetative propagation methods is tissue culture or micropropagation technology. Tissue culture is a method of producing clonal planting material from explants such as plant cells, tissues, or organs on a special synthetic medium under controlled conditions. Tissue culture technology offers the advantage of mass propagation of genetically stable planting material at commercial levels within a relatively short period of time, regardless of season and limited space (Plant Cell Technology, 2020). Conventional micropropagation techniques would be the best alternative for mass production of genetically stable, disease-free seedlings for the MD2 pineapple cultivar, but they involve intensive manual handling and high production costs, especially if commercial production is desired. One of the recent advances in plant tissue culture is the temporary immersion bioreactor system (TIB). TIB is an automated system used in plant micropropagation that

uses a liquid medium instead of conventional closed containers and gelled medium with added sugars, which includes aeration of the culture. The increased uptake of nutrients via the liquid medium, along with the renewal of air inside TIB, allows better control of plant tissue contact with the culture medium and can improve the physiological state of explants (Vidal and Sánchez, 2019). Better aeration and circulation of the medium in culture can increase the speed of propagation of plantlets in large quantities, stabilise the growth of cultures, and reduce the space, energy, and labour requirements which are typically required for manual micropropagation. Selecting crops that can be grown year-round, such as pineapple, can also allow for minimal production flow costs and optimal use of equipment and resources (Su *et al.*, 2019; Valdiani *et al.*, 2019).

Therefore, the technique would be an excellent alternative propagation method for the mass production of clonal MD2 pineapple cultivar for the specific limited planting materials and limited production space issues. There is also a limited micropropagation protocol for the specific MD2 pineapple cultivar, and currently, there is none for the specific MD2 pineapple JengkaPine, a brand exclusive to UiTM Pahang branch. Micropropagation will be initiated and raised using selected explants, and its growth performance will be evaluated using the conventional micropropagation method and RITA®, a brand of TIB. Different types of plant growth regulators at different concentrations in singular or combinations will be investigated to demonstrate an efficient propagation system for JengkaPine and, at the same time, clearly define the advantages of tissue culture technology over manual propagation. The study aims to establish a method for the mass production of the stable clonal pineapple cultivar MD2 JengkaPine through the use of TIB while assessing the effectiveness of applying a specific plant hormone in the culture medium.

2. Materials and methods

2.1 Preparation of plant material and surface sterilisation method

Fresh suckers of pineapple cv. MD2 JengkaPine were obtained from the UiTM Pahang's farm as a source of plant starting material for *in vitro* germination. The time of the surface sterilisation protocol has been optimised based on the contamination rate of explants. Prior to explant preparation, the collected suckers of JengkaPine were washed under running tap water and immersed in Virkon's disinfectant and virucide treatment, including 3% hydrogen peroxide, for 5 min (Figure 1a). Later, the plant materials were left to dry by hanging at room temperature for several hours. Once dried, the outer leaves of the fungicide-treated plant

materials were removed (Figure 1b) and surface sterilised by soaking in 70% (v/v) ethanol for 5 min, followed by soaking in 90% sodium hypochlorite solution (Clorox®) added with a few drops of Tween 20 with gentle shaking for 15 min. This was followed by three rinsing procedures with sterile distilled water.

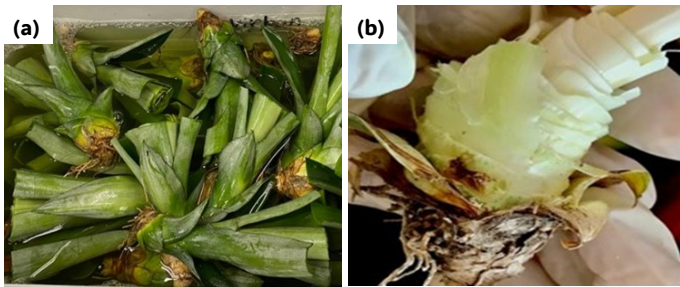


Figure 1. a) JengkaPine suckers are being submerged in Virkon's disinfectant and virucide treatment + 3% hydrogen peroxide solution. b) Removal of outer leaves prior to explant excision.

2.2 Culture media preparation for *in vitro* regeneration of explants in jars and temporary immersion bioreactor system (RITA®)

MS media was prepared with 4.41 g/L of Murashige and Skoog's (MS) salt with vitamins and 30.0 g/L of sucrose. The prepared solution was subsequently supplemented with 1.0–3.0 mg/L of benzylaminopurine (BAP) to aid in clonal shoot regeneration. MS solutions devoid of BAP served as controls. The MS media solutions were prepared in two conditions: semi-solid and liquid. Semi-solid culture media for explant initiation and propagation in jars (conventional plant tissue culture pathway) were made using 3.0 g/L gelrite to solidify, whereas liquid medium for culture establishment in a temporary immersion bioreactor system (RITA®) was made without gelrite. The pH of all media prepared was then adjusted to be within 5.6 to 5.8 and sterilised by autoclaving at 121°C (1 kg/cm²) for 20 min. Following autoclaving, the media solution was allowed to cool down, and approximately 50 mL of the media was directly poured into vessels for jar culture and approximately 250 mL for RITA®. Semi-solid media were left to set at room temperature.

2.3 Establishment of *in-jar* culture and maintenance

Explants used for culture were prepared by excising the surface-sterilised plant material of JengkaPine at an approximate size of 1 cm × 1 cm. The explants were then transferred and inoculated on the pre-prepared modified MS media. The cultures were then maintained in the incubation room at a temperature of 25±2°C and a 16/8 h photoperiod under continuous fluorescent light (1000 lux). Subcultures were carried out when required. The aseptically seedlings and proliferated clonal shoots from the procedure were used as a source of explants in the

subsequent procedures.

2.4 Establishment of a temporary immersion bioreactor (RITA®)

The temporary immersion bioreactor system (RITA®) used in this study was made with reference to the design of the Twin-Flask system (BIT®) by Escalona et al. (1999) and Mohamad Adam (2020). The system consisted of a container for plant culture connected to a liquid media vessel with a capacity of 1 L. The containers were joined together with a silicone tube (5 × 8 mm) that allows the liquid medium to move freely between them. Water motion for the liquid medium to flow and aerate the cultures at predetermined intervals was utilised by two air pumps (Kintons, AC 220V-240V, 50/60 Hz, 12W, max output: 4 × 10 L/min). The immersion time and frequency were scheduled accordingly, and each air pump had a timer to manage the onset. The use of air filters with a 0.2-micron particle size helped eliminate the possibility of culture contamination from airborne microbes. Autoclaving at 121°C (1 kg/cm²) for 20 min sterilised the culture vessels, medium reservoir, and silicone tubes.

2.5 Establishment of *in vitro* regeneration of *A. comosus* cv. MD2 JengkaPine in a temporary immersion bioreactor system (RITA®)

The clonal shoot clumps regenerated from the *in-jar* propagation were used as explants for *in vitro* propagation in the immersion bioreactor system (RITA®). The immersion frequencies were tested at three different levels, with 10 and 15 min of immersion time for each (Table 1). Each culture vessel was inserted with four clonal shoot clumps, and three complete sets of the RITA® system were utilised for each treatment, resulting in a total of twelve replicates for each treatment. The effectiveness of modified MS media in the RITA system to support morphological growth of clonal seedlings was observed and evaluated. The tested media that favour optimal culture growth were then used in the subsequent procedures. A culture of constantly immersed plantlets served as a control.

Table 1. Immersion frequency and duration of the temporary immersion bioreactor system (RITA®).

Time interval	Immersion duration (min)	
	Off	On
1	240	60
2	90	30
3	99	15

2.6 *In vitro* rooting of clonal shoots

The plantlets were cultivated in the temporary immersion bioreactor system (RITA®) for 3 weeks before being sub-cultured for 2 weeks in media with MS

composition for rooting induction. For the *in vitro* rooting media, MS media was prepared with 4.41 g/L of Murashige and Skoog's (MS) salt with vitamins supplemented with 1.0 mg/L indoleacetic acid (IAA).

2.7 Statistical analysis

All the data and variables of the study were laid out in a randomized complete block design (RCBD). Each treatment involved in *in vitro* propagation in jars was replicated at least ten times. The rate of germination (%), rate of germination response, number of clonal shoots regenerated, and morphological characteristics of each tested culture treatment are among the parameters measured to monitor optimal plantlet regeneration. Meanwhile, the growth rate and total number of clonal shoots developed were evaluated as propagation parameters in the temporary immersion bioreactor system (RITA®). SPSS 26 was used to do statistical analysis on the entire dataset. Statistical variance analysis was performed using a one-way ANOVA at $p \leq 0.05$ with the Tukey and Duncan tests. Each mean value was expressed as the mean $\pm$ standard error (SE).

3. Results and discussion

3.1 Establishment and proliferation of aseptic plantlets of *A. comosus* cv. MD2 JengkaPine in jar culture

Aseptic plantlets of JengkaPine were established from the excised explant cultured on MS medium supplemented with 2.0 mg/L BAP. The initiation of shoots was discovered as early as 4 weeks (Figure 2a), which afterwards proliferated significantly after transfer to fresh multiplication media containing 1.0–3.0 mg/L BAP. After 12 weeks (Figure 2b), the proliferation of clonal shoots was found to be optimal in cultures

inoculated with a higher concentration of BAP (3.0 mg/L), with a 90% rate of proliferation and a mean number of shoots of 1.63 ± 0.32 (Table 2). Subcultures improved the regeneration of new shoots in the following weeks (Figure 2c). The plantlets resulting from the optimal treatment were then propagated into masses and subsequently employed for automated mass multiplication in a temporary immersion bioreactor system (RITA®).

3.2 Establishment of clonal plantlets of *A. comosus* cv. MD2 JengkaPine in a temporary immersion bioreactor system (RITA®)

Aseptic plant materials regenerated from the best clonal shoot proliferation treatment in jar culture (MS + 3.0 mg/L BAP) were subjected to automated mass regeneration in a temporary immersion bioreactor system (RITA®). The RITA® system employed a similar composition of MS media for in-jar regeneration but in liquid form. A significant increase in the speed of clonal shoot regeneration, the rate of proliferation, and the mean number of shoots was observed after only 8 weeks of transfer in RITA®. The best mass multiplication treatment was also observed in a similar media composition as in jar culture, which was MS + 3.0 mg/L BAP (Figure 3a, Figure 3b) with a 100% rate and the mean number of clonal shoot regeneration at 5.50 ± 0.91 , followed by MS + 2.0 mg/L BAP immersion media with a mean number of clonal shoots at 5.13 ± 0.81 (Table 3). This finding is in line with the previous research reported by Sourze De Marchi *et al.* (2022), which showed that the RITA system helps pineapple crops grow. Better plant growth observed in twin flasks supports the idea that constant renewal of the internal atmosphere in TIB boosts pineapple growth. This process helps remove ethylene and raises respiratory rates, which improves cell division. The clonal shoots acquired from optimal immersion treatment were afterwards prepared for *in vitro* rooting (Figure 3c). Temporary immersion bioreactors are employed in the commercial cultivation of high-quality plant species (Florez *et al.*, 2016). These systems provide an optimised environment for plant propagation by periodically immersing plant tissues in a nutrient-rich liquid medium. This method enhances growth rates and overall plant health, making TIBs a valuable tool in the horticultural and agricultural industries. By enabling the efficient production of

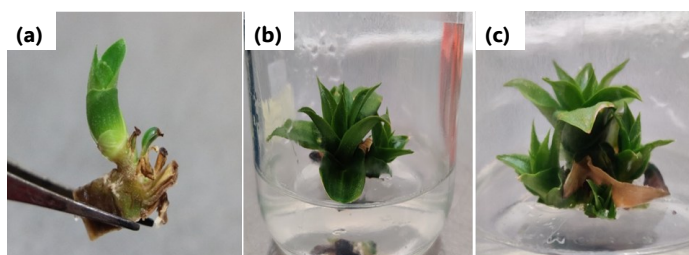


Figure 2. (a) Initiation of the shoot of *A. comosus* cv. MD2 JengkaPine on MS + 2.0 mg/L BAP after 4 weeks. (b) Clonal shoots multiplication on optimum proliferation media (MS + 3.0 mg/L BAP) after 12 weeks in jar culture. (c) Significant increase in clonal shoot regeneration following subcultures in the subsequent weeks.

Table 2. Clonal shoot proliferation treatment of *A. comosus* cv. JengkaPine after 12 weeks in jar culture

Treatment	Media composition (semi-solid)	Clonal shoot proliferation rate per treatment (%)	Mean number of shoots ($\pm$ SE)
T0	MS0 (Control)	25	0.25 ± 0.16
T1	MS + 1.0 mg/L BAP	60	0.63 ± 0.18
T2	MS + 2.0 mg/L BAP	50	0.63 ± 0.26
T3	MS + 3.0 mg/L BAP	90	1.63 ± 0.32

Table 3. Mass multiplication treatment of clonal shoots in RITA®.

Treatment	Media composition (semi-solid)	Clonal shoot proliferation rate per treatment (%)	Mean number of shoots ($\pm$ SE)
T1	MS0 (Control)	100	1.88 $\pm$ 0.30
T2	MS + 1.0 mg/L BAP	100	4.63 $\pm$ 0.73
T3	MS + 2.0 mg/L BAP	100	5.13 $\pm$ 0.81
T4	MS + 3.0 mg/L BAP	100	5.50 $\pm$ 0.91

superior plant varieties, TIBs contribute significantly to meeting the increasing demands for sustainable and high-yielding crops.

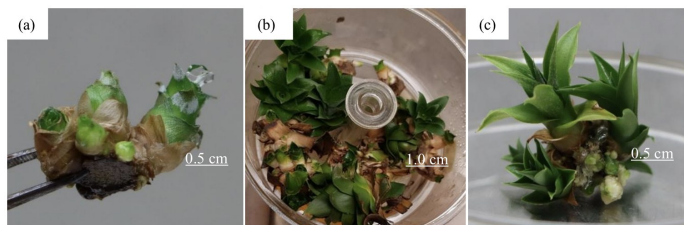


Figure 3. (a, b) Proliferated clonal shoots of *A. comosus* cv. MD2 (JengkaPine) for automated mass production in optimum proliferation treatment (MS + 3.0 mg/L BAP) in a temporary immersion bioreactor system (RITA®) after 8 weeks; (c) Clonal shoots ready for transfer to *in vitro* rooting media.

3.3 *In vitro* rooting of *A. comosus* cv MD2 JengkaPine

In vitro rooting of clonal shoots of *Ananas comosus* cv. MD2 JengkaPine acquired from earlier proliferation treatments was investigated using immersion and semi-solid media cultures containing MS salt and 1.0 mg/L indoleacetic acid (IAA). The study is particularly intended for clonal shoots without roots. At the end of the study, it was observed that both methods resulted in a positive response, with the initiation of new roots (Figure 4a) occurring as early as 1 week after culture. An abundance of roots could be seen by the second week (Figure 4b). When comparing the two methods of *in vitro* rooting, physical inspection revealed that the immersion method produced better results in terms of root texture, root length, and total number of roots. Although *in vitro* rooting on semi-solid rooting media in jars also promoted excellent rooting, the resulting roots have a slightly softer texture and are slightly shorter. As reported in Ayenew *et al.* (2013), this effect is due to better access for cultured cells to growth regulators, compared to the limited diffusion in solid media. These points show how the TIB system improves shoot multiplication and rooting, highlighting its advantages over traditional methods. Regardless, both approaches can be used for inducing rooting of the cultures, resulting in the production of healthy plantlets for further acclimatisation prior to field sowing. Rooted plantlets were later transferred to basal MS media for maintenance (Figure 4c).

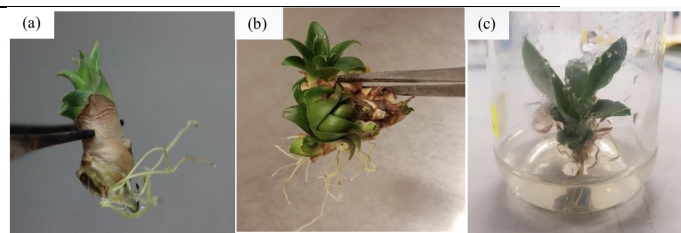


Figure 4. *In vitro* rooting of *A. comosus* cv. MD2 JengkaPine developed on (a) semi-solid agar medium containing MS + 1.0 IAA in jar culture and (b) immersion media of similar composition in a temporary immersion bioreactor system (RITA®) after 2 weeks. (c) Maintenance of rooted plantlets on basal MS media (MS0).

3.4 Comparison of clonal shoots proliferation performance of *A. comosus* cv MD2 JengkaPine in jar culture and temporary immersion bioreactor system (RITA®)

Based on the study's findings, it was discovered that cultivating *Ananas comosus* cv. MD2 JengkaPine plantlets in a temporary immersion bioreactor system (RITA®) encouraged better aseptic plantlet regeneration. Researchers have been increasingly using immersion culture to grow a variety of plants, including pineapple, as described by Ayenew *et al.* (2013), Amiladziffar (2018) and Reis *et al.* (2018). Among the particular reasons for the improved plant regeneration through the temporary immersion bioreactor system is the nature of the system, which is a liquid culture and is automatically operated. Liquid culture systems offer several advantages compared to other culture methods. One significant advantage is the capacity to maintain very uniform cultural settings, which leads to consistent results. Additionally, liquid media may be quickly manipulated or replaced, giving a greater degree of control over the environment in which the plant is growing. The use of liquid medium in *in vitro* culture has been thoroughly explored and is frequently recognised as an excellent technology for mass production due to its capacity to eliminate manual work and allow for easy modifications to medium composition (Berthouly and Etienne, 2005).

Micro-cutting and somatic growth in *in vitro* culture are two processes that are said to benefit significantly from the bioreactor setting. It improves plant development quality by enhancing nutrient contact and gas exchange, making it a cost-effective alternative to other expensive methods implied for research and mass

production in plant tissue culture (Teisson and Alvard, 1995). Different aspects of micropropagation have been found to benefit from temporary immersion. This includes shoot proliferation through the optimisation of nutrient medium and culture containers, as well as the enhancement of overall plant material quality. Moreover, plant material propagated using temporary immersion techniques often performs better during acclimatisation than plant material obtained using semi-solid or liquid media techniques (Etienne and Berthouly, 2002). Increased nutrition, improved gas exchanges, and agitation are all factors that contribute to the effectiveness of the system. The duration and frequency of immersion are other important elements that has a big effect on how well the system works (Teisson *et al.*, 1996).

4. Conclusion

The temporary immersion bioreactor system is an advanced micropropagation technology for plant regeneration, offering efficiency, high-quality products, and cost savings. It is ideal for pineapple mass production due to its efficiency and value for money. The goal of this study was to improve the tissue culture procedure for JengkaPine, an MD2 pineapple brand exclusive to the UiTM Pahang Branch. This was done by using a variety of plant hormone applications in the culture medium in RITA®, a brand of temporary immersion bioreactor system, at a lower cost by using less gelling agent. At the end of the research, it was found that the rate of multiplication of JengkaPine commercial clones increased significantly in a much shorter time than with the traditional micropropagation technique in jars using the same formulation of culture media.

Conflict of interest

The authors declare no conflict of interest.

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