Orchid Micropropagation Using Temporary Immersion System

Subjects: Plant Sciences

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Temporary immersion system (TIS) overcomes the limitations of SS and liquid culture systems by temporarily submerging the plants in the liquid medium for shorter periods, followed by exposing them directly to the gaseous environment by draining the liquid medium. The shorter immersion time and more prolonged gas exposure lower the detrimental effects of hyperhydricity and asphyxia on plants, providing optimal environmental conditions for efficient nutrient absorption under the least liquid contact. Greater gas exposure improves oxygen transport to cultured cells minimizing oxygen limitation and lowering the asphyxia effect on the plant tissues in TIS. Furthermore, enhancing headspace with carbon dioxide (CO_2) and culture agitation by hydrodynamic forces without mechanical devices in TIS allows normal development and increases plant tissue multiplication with regular photosynthetic activities and minor shear stress.

Keywords: orchids ; micropropagation ; explants ; semi-solid media ; temporary immersion system

1. Important Factors Influencing Temporary Immersion System (TIS)

The culture medium provides nutrients required by the plants to grow and develop. Using a suitable medium in the TIS system is essential for successful plant propagation. Though MS medium is employed most frequently in TIS, the need for an appropriate culture medium depends on the nutrient requirement of plant species during its developmental period [1]. The volume of the culture medium is also crucial in influencing plant multiplication rate, leaf and root formation, and growth. The shoot multiplication rate was enhanced from 8.3 shoots (in 30 days) to 23.9 shoots (30 days) when the medium volume in the TIS bioreactor was increased from 5.0 to 50.0 mL per explant in *Saccharum* spp. [2]. Escalona et al. [3] also established the optimum medium volume for maximum shoot proliferation of pineapple at a higher volume of 200 mL per explant. Roels et al. [4] demonstrated the escalated shoot multiplication rate (11.9 to 13.8), shoot length (4 to 5 cm), leaf number (3.1 to 3.7), and root number (2.8 to 3.2) when the medium volume was raised from 10 to 30 mL in TIS. Uma et al. [5] observed the highest shoot production of bananas (24 shoots) in a 250 mL volume of the medium as compared to shoots generated (20 shoots) in other volumes (100 mL and 500 mL) tested. However, a larger culture volume than its optimum level was not favorable for plant growth as extracellular chemicals excreted from the cultures were diluted in a higher volume [1]. Container size of the TIS may also influence the plant growth as bigger vessels can accumulate larger medium volumes avoiding culture overcrowding and early shortage of nutrients. Monette [6] demonstrated longer shoot development in grapevine in bigger containers of square wide-mouth Mason jars (910 mL) compared to smaller Erlenmeyer flasks (125 mL). Krueger et al. [7] also revealed the advantage of larger culture vessels (7 L) over smaller baby food jars (140 mL) in imparting a positive influence on shoot elongation and multiplication by providing larger head space and lowering culture congestion.

TIS provides better culture aeration cultures than continuous and partial immersion liquid culture systems [8]. Exposure of the plants to gas is essential to avoid unwanted asphyxia in the culture [9]. Plants usually require different oxygen and carbon dioxide concentrations for proper development [10]. Bioreactors fitted with pressure and flow regulators can provide proper gas exposure to the cultures to enhance multiplication and growth. Increasing oxygen and carbon dioxide to a certain level is warranted as they are the crucial components of photosynthesis affecting plant growth and metabolism [10]. The oxygen transfer rate depends on its mass transfer coefficient, which is easily influenced by agitation and air flow rate and the design of the bioreactor [11]. The growth of seedless watermelons was enhanced when the oxygen level was raised by 40% inside the TIS [12]. The plants in the TIS bioreactor are temporarily submerged in the liquid medium for a short duration, during which they receive nutrients from the medium. When exposed to the gas after short immersion, the plants acquire oxygen and carbon dioxide, which are also essential for their development [11]. Optimizing the immersion time and frequency is vital as lengthening the immersion time will increase the hyperhydricity effect, and more prolonged gas exposure will enhance tissue drying and moisture loss, deterring normal development and proliferation [10]. The

immersion time and frequency in TIS varied considerably with species and micropropagation process involved. Potato tuberization was effective when the plants were submerged for a longer duration (1 h) every 6 h, while somatic embryogenesis was accelerated with a brief immersion time of 1 min every 12 h in *C. arabica* [13][14]. The shoot multiplication rate of *Coffea* microcuttings also changed with different immersion times with plant submersion durations of 1, 5, and 15 min every 6 h producing multiplication rates of 3.5, 5.4, and 8.4, respectively [15]. Immersion frequency also influenced culture growth in TIS as immersion of 4 h six times per day produced maximum shoots (17.33) in *Dianthus caryophyllus* but with the highest rate of hyperhydricity [16]. The immersion of explants for 4 h four times daily produced the most desirable shoot growth (14.33 new shoots) without any hyperhydricity effect. Bello-Bello et al. [17] found the highest shoot multiplication rate (10.78 shoots per explant) of *Hylocereus undatus* in 2 min immersion time at 4 h intervals, while the least shoots (5.46 shoots per explant) were recorded in immersion frequency of every 16 h. Furthermore, the most extended shoots were formed at an immersion frequency of every 4 h, and the short shoots were noticed in other immersion frequencies (every 8, 12, and 16 h).

Explant inoculation density is one of the key factors that influence the growth of culture in the TIS. The optimum density of the explant should be determined as its increase in a culture vessel with constant medium volume may lead to poor aeration and congestion, affecting plant growth [17]. Pérez-Alonso et al. [18] showed potato microtuber formation improved from 168 to 234 when inoculum density from 60 explants per TIS was increased to 90 explants. However, there was an enhancement of the total fresh weight of microtubers per TIS (164.7 g) with less inoculum density of 60 explants compared to fresh weight (47 g) obtained with a higher density of 90 explants [18]. García-Ramírez et al. [19] also demonstrated the effect of inoculum density on the physiological development and morphology of *Bambusa vulgaris* shoots by taking 6, 12, and 18 explants per TIS. The inoculum density of 12 explants was more favorable for shoot growth as shoot and leaf number, shoot length, and chlorophyll content increased. Nevertheless, higher inoculum density was not suitable for culture growth with the accumulation of less total chlorophyll, lower dry mass, and water content of the shoots. Posada-Pérez et al. [20] employed inoculation densities of 100, 200, and 300 somatic embryos per TIS of papaya to determine the optimum density for best plant growth. The maximum response for callus, leaf and root formation, and root length were witnessed with the inoculation of 100 somatic embryos per TIS. Uma et al. [5] used varied inoculum densities (3, 6, and 12 explants) per TIS to ascertain the optimum inoculum density for the best shooting response in bananas. The TIS with explant densities of 3 and 6 generated higher shoots (24 shoots/explants) than those with inoculum densities of 12 explants.

2. Benefits and Drawbacks of TIS

The benefits of using TIS over a conventional SS system are enormous in terms of lowering the production and labor cost by preventing frequent subculturing using higher culture liquid volume and containers, enhancing plant multiplication rate by accelerating nutrient absorption through uniform intermittent contact with liquid medium and plant tissues, normal morphological plant development by reducing hyperhydricity and asphyxia effects, and the possibility of successful large scaleup with full automation of culture systems [5][11][12]. Lorenzo et al. [2] reported 46% reduction in the production cost of shoot multiplication of *Saccharum* spp. using TIS compared to the conventional SS culture system. Pineapple generation using TIS produced a 100-fold increase in shoot generation and lowered the production cost by 20% compared with standard liquid medium. Bello-Bello et al. [17] established in vitro protocols for scaling-up *Pitahaya* propagation using TIS, which produced a multiplication twice that of semi-solid and partial immersion media. Ducos et al. [21] also succeeded in scaling up culture production by generating 2.5 million pregerminated embryos of *Coffea canephora* annually in a 40 m^2 size culture room using 100 TIS units of 10 L volume each. Ptak et al. [22] demonstrated the positive influence of metatopolin and benzyladenine on plant development with two times more production of morphologically normal plants from SEs in TIS than SS medium without any hyperhydricity. TIS also helps reduce medium browning due to oxidation, plant contamination through air vents, and lower agitation stress on plant tissues due to the absence of mechanical devices [23][24]. Though TIS is mainly utilized for plant micropropagation, it also offers an alternative to SS and liquid culture for increasing biomass yield and secondary metabolite production at a low cost. Pavlov and Bley [25] found high biomass accumulation (18.8 mg g^{-1}) and betalains yield (9.6 mg g^{-1}) when hairy roots of *Beta vulgaris* were cultured in TIS at immersion frequency of 15 min immersion every 60 min interval. Kokotkiewicz et al. [26] reported the TIS to be more effective in phenolic secondary metabolite accumulation (xanthones and benzophenone derivatives) in the cell culture of *Cyclopia genistoides* than liquid culture systems providing a possible cheap alternative source of the phytochemicals from plants. Kunakhonnuruk et al. [27] found the production of biomass per clump of *Drosera communis* (3.40 g fresh weight and 0.36 g dry weight) in TIS thrice and 1.8 times greater than SS and continuous immersion systems, respectively. Furthermore, the maximum plumbagin yield per replication (17.31 µg/replication) was attained in cultures grown in TIS compared to those in SS and continuous culture systems. TIS has also been applied for in vitro production of foreign proteins in plant cells and tissues. Michoux et al. [28] obtained high expression of a modified form of the green fluorescent

protein (GFP^+) and a vaccine antigen, fragment C of tetanus toxin (TetC), in transgenic cells cultured in TIS with thidiazuron for inducing proper shoot initiation. The yield of GFP^+ (660 mg L^{-1} of bioreactor) and TetC (95 mg L^{-1}) were much higher than protein expression witnessed in transformed cells in suspension cultures.

Despite having the many advantages of TIS, it also includes one of the main limitations of difficulty in scaling up to the commercial scale. The volume of containers involved in the TIS can be increased to 10–20 L, which is still less for plant propagation at the commercial level. However, the utilization of larger vessels might hamper the performance of TIS as the use of 10 L jars did not produce normal embryo development of *C. canephora* due to uneven light distribution [21]. The increased plant biomass in bigger culture containers disrupted light penetration and resisted nutrient and oxygen transfer [21][29]. Utilization of larger culture vessels may not necessarily be an effective approach to scale up the process for plant propagation. One way of overcoming this shortcoming is by using several smaller-size containers (1–5 L) that may be subjected to simultaneous operation under preset culture parameters in an automated fashion that will ensure plant propagation at a commercial scale. The TIS bioreactors should be designed to provide a particular microenvironment conducive to the growth and development of complex differentiated plant tissue and organs [30]. Many TIS with varying designs are developed to meet the specific culture requirements for large-scale plant multiplication. Some popular TIS available in the markets are Twin-Flask, Ebb-and-Flow, RITA, Thermo-photobioreactor, BioMINT, SETIS, and PLANTIMA [31]. However, these commercially available TIS are associated with several drawbacks, such as complex automation, unsuitable for forced ventilation and CO_2 enrichment, low headspace, humidity in the growth chamber, no nutrient medium renewal, occupation of more space in the growth chamber, tilting platforms requirements, difficulty in biomass harvesting, complex automation and construction, and high cost and energy requirement [31]. Attempts have been made to refine and improve the TIS bioreactors circulating in the markets so that the existing shortcomings are eliminated and more effective, cheap, simple, and easy to store and handle compact bioreactor designs with autoclavable and reusable plastic elements with options for multiple uses are readily available.

3. Application of TIS for In Vitro Orchid Propagation

The first instance of the application of TIS for orchid culture was evident when Tisserat and Vandercook [32] established an automated plant culture system (APCS), which did away with the necessity of the frequent manual subculturing of the culture to freshly prepared medium after every 4 to 8 weeks in either agar solidified or liquid medium. The shoot tips of *Potinera* orchid hybrid were subjected to different immersion frequencies to determine the optimum immersion frequency for the best plant growth response. Orchid tips subjected to less immersion frequency (4 and 1 immersion cycles/day) showed a lower survival rate when compared to those with a higher immersion frequency of 12 or 24 immersion cycles/day. When grown in an automated culture system, the orchid tips generated higher tissue mass than those in the agar-gelled medium. The orchid tissue production employing the APCS was four times higher in nine months than in the tissue mass generated in the same period on agar solidified medium.

Young et al. [33] used TIS and a continuous immersion system (CIS) to multiply PLBs of *Phalaenopsis* orchid from PLB explants obtained from in vitro grown leaf segments. TIS with the charcoal filter attached produced maximum PLBs (about 18,000) in eight weeks of incubation from 20 g of inoculum on 2 L Hyponex medium. The different aeration rates at 0.5 or 2.0 volumes of air per volume of medium min^{-1} (vvm) did not impact the PLB multiplication as they generated a similar amount of biomass. Liu et al. [34] used air-driven periodic immersion (API) bioreactors to culture PLBs obtained from lateral buds of the flower stalks of *Doritaenopsis.* Comparison of PLB growth on solid, liquid, and API showed the highest PLB proliferation and growth in the API system. PLBs growth increased by 4–6 fold when immersion time was set for 5 min at 4 h intervals. The increased level of PLBs formation in the API system may be due to the combined nature of both solid and liquid cultures. Yang et al. [35] also examined the feasibility of producing PLBs in bioreactors using shoot tips derived from in vitro plantlets of *Oncidium* ‘Sweet Sugar’. To initiate bioreactor cultures, 30g fresh weight (FW) PLB pieces were used, with PLBs being submerged in the medium throughout the culture period in the CIS while an immersion period of 1 h after every hour was applied for the TIS. The two bioreactor systems exhibited different growth rates of PLBs with a maximum growth ratio (10.9) witnessed in the CIS. Superior PLB proliferation may be due to the increased rate of nutrient uptake by the cultures because of their constant contact with the medium in the continuous immersion system. The earlier studies showed TIS appropriate for shoot multiplication [36][37], while the CIS is suitable for culture of storing organs such as adventitious roots [38][39], bulblets [40], and microtubes [41]. Ekmekçigil et al. [42] applied a thin cell layer and RITA temporary immersion bioreactor to mass propagate PLBs and shoots of *Cattleya forbesii* at the commercial level. The highest PLB production (PLBs per RITA—2237 PLBs and per explant—111.9 PLBs) was recorded when 20 tTCL-PLB explants were inoculated in 250 mL of medium with an immersion frequency of 1 min/4 h. Similar inoculum density in lower medium volume (150 mL) at the same immersion frequency generated the maximum shoot formation (shoots per RITA—3998 shoots and shoots per explant—199.9 shoots). Fritsche et al. [43] also found TIS favorable for PLB

multiplication in *Cattleya tigrina* with MS medium incorporated with 30 g L^{-1} sucrose and Morel vitamins. Fresh weight increment rate (FWI) of PLB formation was significantly improved in TIS with a 2-fold increase in PLB proliferation (77.3 g) in comparison to that of PLB formation (35.3 g) in a continuous immersion system on the gelled medium.

Hempfling and Preil [44] used in vitro grown shoots of *Phalaenopsis* cv. Jaunina as inoculum for adventitious shoot multiplication and rooting in TIS. The shoot formation was maximum at 25.4 after 12 weeks of culture with an immersion frequency of eight immersion per day and immersion time of 10 min each. An increase in the time interval of medium recharge to four weeks significantly reduced the shoot multiplication rate to 14.5. The rooting response was tested by exposing shoots of 4–7 cm from TIS cultures to TDZ-free medium incorporated with 0.5 and 1.0 mg L^{-1} IAA or NAA. Maximum shoots were rooted (93.8%) with the production of the highest root number (3.7 roots per shoot) in medium supplemented with 1.0 mg L^{-1} IAA after subjecting to six immersions per day with an immersion time of 10 min each. Pisowotzki et al. [45] investigated the effect of PVPP on the in vitro shoot development of *Phalaenopsis*-hybrids grown in TIS. They observed that the phenolic compounds extracted from liquid and plant tissues exhibited the same peak pattern after HPLC separation, though the concentration of these compounds varied between different tissues, with their concentration higher in shoots than leaves. There was a reduction in biomass production and shoot proliferation rate when the phenolic compound was withdrawn from the culture medium, indicating its positive influence on culture growth. Biomass generation and shoot multiplication were higher in TIS system than in the conventional SS. Ramos-Castellá et al. [46] investigated the efficiency of TIS (RITA) for shoot multiplication of *Vanilla planifolia*. The highest shoot multiplication rate (14.27 shoots per explant) was best observed in TIS, applying an immersion frequency of 2 min every 4 h. The most appropriate medium volume for shoot multiplication was determined at 25 mL per explant, delivering the highest multiplication rate of 17.54 ± 1.14 shoots per explant. The TIS and partial immersion system did not produce a significant change in shoot length (12.67 ± 1.2), but solid medium generated the shortest shoots (10.47 ± 1.01 mm). The shoots were successfully rooted in TIS when transferred to ½ MS medium enriched with 0.44 NAA at an immersion frequency of 2 min every 4 h. Ramírez-Mosqueda and Iglesias-Andreu [47] employed three different TIS, viz., Recipient for Automated Temporary Immersion (RITA), Temporary Immersion Bioreactors (BIT), and Gravity Immersion Bioreactors (BIG) for establishing efficient in vitro protocols for *Vanilla planifolia* micropropagation. Shoot multiplication was the highest (18.06 shoots/explant) in BIT systems compared to shoot formation observed with RITA (12.77 shoots/explant) and BIG (6.83 shoots/explant). However, the maximum shoot length was witnessed in BIG (1.69 cm) and RITA (1.64 cm) compared to the BIT system. The shoots were rooted effectively in the three TIS with the highest number of roots noticed in BIT (4.27 roots/explant), followed by BIG (2.76 roots/explant) and RITA (1.90 roots/explant). Ramirez-Mosqueda and Bello-Bello [48] established commercially applicable in vitro propagation protocols for *Vanilla planifolia* by employing SETIS bioreactor. This bioreactor differs from RITA, BIT, and BIG systems in having horizontally placed larger-capacity vessels (4000 mL) with more headspace for plant development, enabling easy scaleup to commercial scale. Plant height and leaf number were recorded highest in TIS at 4.24 cm and 3.72 per explant, respectively. TIS also reported elevated percentages of closed stomata and a stomatal index indicating a high functionality of stomata, favoring a low transpiration rate. The survival rate of the in vitro propagated plants after acclimatization was also high (98%) for TIS.

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