

# Improving large-scale biomass and total alkaloid production of *Dendrobium nobile* Lindl. using a temporary immersion system and MeJA

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## Research

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# Abstract

## Background

*Dendrobium nobile* Lindl. is an important pharmacopeial plant with medicinal and ornamental value. This study sought to provide technical means for the large-scale artificial production of total alkaloids in *D. nobile*. Seedlings were cultured *in vitro* in a temporary immersion bioreactor system (TIBS). The four tested immersion frequencies (min/h; 5/2, 5/4, 5/6, and 5/8) influenced the production of biomass and total alkaloid content. In addition, to compare the effects of different concentrations of phytohormone methyl jasmonate (MeJA) and treatment time on biomass and total alkaloid accumulation, MeJA was added to the TIBS medium after 50 days. Finally, the production of total alkaloids in a semi-solid system (SSS), TIBS, and TIBS combined with the MeJA system (TIBS-MeJA) were compared.

## Results

The best immersion frequency was found to be 5/6 (5 min every 6 h) that ensured good levels of biomass and total alkaloid content in plantlets. 10  $\mu$ M MeJA and 20 days of culture were the most favorable for alkaloid accumulation in the seedlings cultured in TIBS. The maximum content and productivity of total alkaloid on use of TIBS-MeJA was 2.32- and 4.69- fold, respectively, higher in terms of content, and 2.04- and 10.27- fold, respectively, higher in terms of productivity than those on use of TIBS and SSS.

## Conclusions

Our results show that TIBS-MeJA is suitable for the large-scale production of total alkaloids in *in vitro* seedlings; therefore, they provide technical means for the large-scale artificial production of total alkaloids in *D. nobile*.

## Highlights

- Application of a temporary immersion bioreactor system (TIBS) for the mass propagation of *nobile*.
- Application of a TIBS-MeJA system for the artificial large-scale production of total alkaloid of *nobile*.
- Optimization of culture conditions for better induction of total alkaloid accumulation of *nobile*.
- Comparative analysis on functional efficiency of TIBS and semi-solid system (SSS) culture.
- The TIBS culturing significantly improves accumulation of total alkaloid and biomass of *nobile*.
- The MeJA inducing significantly improves accumulation of total alkaloid of *nobile* using TIBS tissue culture.

## Introduction

*Dendrobium*, which comprises approximately 1200–1500 species, is one of the largest genera in the family Orchidaceae. There are roughly 80 species within the genus *Dendrobium* in China[1]; of these, *Dendrobium nobile* Lindl is one of the most widespread species within the genus and is an important herb that has many medically important secondary metabolites, including alkaloids, flavonoids, and bioactive polysaccharides [2, 3]. *D. nobile* is described as “the strong body” in the ancient Chinese medical book, Compendium of Materia Medica. Over the past few decades, the species *Dendrobium communis* have been collected in large quantities, because of the high medicinal and ornamental value, resulting in the species becoming increasingly rare, and active preservation orders have been implemented in numerous counties, especially *D. nobile*. In order to solve the shortage of *D. nobile*, many researchers are leaning on tissue-culture instead of wild plants [4].

Alkaloids are the most common active compounds in *D. nobile* and are found in all parts of the plant. Modern pharmacology studies have shown that alkaloids can relieve pain and antipyretic effects, reduce the heart rate and blood pressure, slow respiration, and alleviate barbiturate poisoning [5, 6]. Furthermore, alkaloids have numerous therapeutic activities, including hypoglycemic, anti-cataract, anti-tumor, anti-cell withering, antioxidant, and anti-Alzheimer’s disease activities [7, 8]. However, *D. nobile* alkaloids are mainly obtained from three-year-old plants, which are not only expensive due to their long culture duration but also require a lot of space.

Plant tissue culture technology is a rapid and mass propagation method of medicinal plants, and also has the potential to increase the yield of secondary metabolites [9]. However, diverse culture systems influence the propagation and production efficiency of bioactive compounds [10–12]. At present, use of TIBS has a better performance in plant biomass and active substance accumulation, especially in officinal plants [13–16]. TIBS is a liquid culture method that allows the explants to contact the medium intermittently, thus renewing the atmosphere and supplying the nutrients to meet the growth of plants [17]. This semi-automatic micropropagation system is considered to be an effective method to reduce production costs and labor to a greater degree as compared with those of traditional culture methods, such as the semi-solid system (SSS) [18].

Many studies have confirmed that the phytohormone methyl jasmonate (MeJA) can enhance the biosynthesis of secondary metabolites in officinal plants [19–21]. MeJA, as an elicitor, plays a signal role in the biosynthesis of plant alkaloids and has been reported to promote the accumulation of metabolites in *Dendrobium* Plants [22–25]. In the present study, TIBS and MeJA were used to promote biomass and alkaloid accumulation in *D. nobile* seedlings.

## Results

### Effect of immersion frequency on plantlet biomass and total alkaloid during TIBS culture

The TIBS delivers an extremely aerobic environment for plant growth, as it provides forced ventilation through aeration. The immersion frequency is the most significant parameter for system productivity, which not only affects plant growth and micropropagation, but also affects bioactive compound accumulation [26]. In this study, four different immersion frequencies (5/2, 5/4, 5/6, and 5/8) were designed using TIBS culture, and a traditional semi-solid system (SSS) culture was used as a control. After 80 days of growth in the TIBS, there were significant differences in the morphology of seedlings under different immersion frequencies, especially 5/2, and the plantlets were dwarfed and crowded in the tank. The best plantlet morphology was found at an immersion frequency of 5/6 where the plants were taller and the roots showed superior morphology; the second immersion frequency was 5/8, followed by 5/4 (Fig. 1). Figure 2a shows the biomass of seedlings cultured in the reactor for 80 d under different immersion frequencies. The maximum values of fresh weight and dry weight, 349.23 g/L and 54.48 g/L, respectively, appeared in the immersion frequency of 5/6. For these parameters, the second best immersion frequency was 5/8, followed by 5/4 and 5/2. When the immersion frequency was fixed at 5/6, the fresh weight and dry weight of the seedlings in the TIBS tank increased with increasing culture time (Fig. 2b). Moreover, as can be seen in Fig. 2, the rate of increase in fresh weight increased after 40 days of culture, while that of the dry weight increased after 60 days of culture.

Different immersion frequencies and culture times directly affect the accumulation of bioactive compounds in plants, especially medicinal plants [27, 28]. Figure 3 shows the effects of immersion frequencies and culture time on the total alkaloid content and productivity of plantlets using TIBS culture. The highest total alkaloid content was found at an immersion frequency of 5/6 for all culture times. The total alkaloid productivity at the immersion frequency 5/8 was slightly higher than 5/6 after 20 days of cultivation, but the highest productivity was observed at an immersion frequency of 5/6 at other culture times. Moreover, the total alkaloid content and productivity obtained by TIBS culture were significantly higher than those obtained by the traditional semi-solid system culture at all culture times. Therefore, TIBS culture was more conducive to the accumulation of bioactive substances in *D. nobile* plantlets than SSS culture, which was similar to the results of Ashraf et al. (2013) [29]. As seen in Fig. 3, total alkaloid content and productivity were positively correlated with culture time, and the highest total alkaloid content (3.20 mg/g) and productivity (174.44 mg/L) appeared under the immersion frequency 5/6 after 80 days of cultivation.

## Effect of MeJA concentration during TIBS culture

MeJA treatment has significant effects on biomass accumulation during plant cell, tissue, and organ cultures [30–32]. In this research, different concentrations of MeJA were added to the liquid medium after the plantlets were cultured in TIBS for 50 days. After 30 days of induction culture, different concentrations of MeJA were shown to have significant effects on the growth and proliferation of *D. nobile* plantlets (Fig. 4). Treatment with MeJA resulted in the necrosis of some plantlets, especially at 20  $\mu$ M MeJA, where almost half of the plantlets were necrotic. Furthermore, from the perspective of individual plants, as the concentration of MeJA was increased, plantlets became shorter, with worse root growth, and a small number of plantlets became yellow. The control group without MeJA did not show

necrosis and exhibited the best growth, including of the roots. Figure 5 shows the fresh and dry weights of seedlings treated with different concentrations of MeJA for 30 days (Fig. 5a) and 10  $\mu\text{M}$  MeJA for different times (Fig. 5b) cultured using TIBS. With MeJA concentrations ranging from 0 to 20  $\mu\text{M}$  (interval of 5  $\mu\text{M}$ ), the fresh weight of plantlets decreased gradually, with the maximum value obtained at 10  $\mu\text{M}$  MeJA, as shown in Fig. 4. The fresh weight and dry weight of plantlets increased with the extension of culture time, but the growth rates were different in the experimental group treated with 10  $\mu\text{M}$  MeJA; the rate of increase in fresh weight was first low and then high, while the dry weight showed the opposite effect, which was similar to the results of the study by Bayraktar et al. (2016) [33].

MeJA treatment has remarkable effects on the accumulation of bioactive compounds in plant tissue culture [34, 35]. Bioactive compound accumulation in *D. nobile* plantlets cultured using TIBS was significantly affected by MeJA treatment. The total alkaloid content and productivity in the MeJA treatment groups were markedly lower than those in the control group after 10 days of TIBS culture. However, the accumulation rates of total alkaloid in plantlets were significantly higher in the treatment groups than in the control group. In particular, in the 10  $\mu\text{M}$  MeJA treatment group, the content and productivity were significantly higher than other treatment groups and control group after 20 and 30 days of TIBS culture, and the maximum content (7.41 mg/g DW) and productivity (316.59 mg/L DW) were observed after 20 days of culture (Fig. 6). Thus, we can surmise that MeJA enhances the synthesis of alkaloids in *in vitro* propagated seedlings, as has been shown in previous studies [36, 37].

## Comparison of SSS, TIBS and TIBS-MeJA

The biomass and total alkaloid content of *in vitro* seedlings grown under the three culture modes were compared in order to provide the most suitable scheme for the artificial production of alkaloids by *D. nobile*. Table 1 shows that the total alkaloid content, fresh weight, and dry weight of TIBS cultured seedlings were all significantly higher than those in the traditional semi-solid culture system. Specifically, TIBS cultured seedlings treated with MeJA (TIBS-MeJA) contained the maximum total alkaloid content, which was 2.32- and 4.69- fold higher than TIBS and SSS cultures without MeJA. However, the maximum fresh and dry weights were obtained in MeJA-free TIBS culture, because many plant tissue culture systems have diminished biomass after MeJA treatment [38]. Table 1 also shows that the dry weight of plantlets cultured using the TIBS-MeJA system was 0.88- and 2.19- fold higher than that of plants grown in TIBS and SSS, respectively; thus, the maximum total alkaloid productivity appeared at TIBS-MeJA and was 2.04- and 10.27- fold higher than that of the other two culture systems. Therefore, we propose that the combination of TIBS and MeJA is an ideal method for the artificial production of alkaloids from *D. nobile* plant tissue culture seedlings.

Table 1

Total alkaloid and biomass production of *D. nobile* after 80 days of culture in different systems

Culture system	Total alkaloid content (mg/g DW)	Fresh weight (g/L)	Dry weight (g/L)
SSS	1.58 ± 0.03c	185.02 ± 6.75c	21.80 ± 0.68c
TIBS	3.20 ± 0.05b	349.23 ± 11.63a	54.48 ± 3.69a
TIBS-MeJA	7.41 ± 0.17a	283.68 ± 6.76b	47.73 ± 0.85b

TIBS-MeJA means seedlings are cultured with MeJA using TIBS. Values in each column followed by different letters are significantly different at  $p \leq 0.05$  as of Post Hoc Multiple Comparisons Test.

## Discussion

TIBS is designed using liquid medium to intermittently contact with plant tissue to provide nutrition, which accords to the natural plant growth conditions. This system provides an advantageous growth environment for plantlets in liquid culture, including suitable nutrients and effective gas exchange, to ensure the healthy growth of seedlings [39, 40]. Immersion frequency is an important culture parameter of TIBS, which plays a decisive role in plant growth and accumulation of metabolic components. The results of this study show that a low immersion frequency (5/6 and 5/8) was beneficial to the accumulation of total alkaloids in plantlets, while a high immersion frequency (5/2 and 5/4) had the opposite effect (Fig. 3), which was similar to the findings of Ivanov et al. (2011) [41] and Malik et al. (2018) [42]. The reason for this may be that a low immersion frequency provided a drought environment, triggering plant stress and resulting in the accumulation of secondary metabolites.

It is known that MeJA has a high effect on plant growth and accumulation of active substances. However, the optimal concentration and treatment time of MeJA for maximum yield of active substances varied with the culture systems, e.g. 100  $\mu$ M MeJA showed a maximum level of saponin content in cell suspension culture of *Leucas aspera Spreng* over a period of 18 days [43], treatment with 150  $\mu$ M MeJA for 72 hours enhanced camptothecin production in tissue cultures of *Ophiorrhiza mungos* L. var. *angustifolia* (Thw.) Hook. f. [44], and induction of 100  $\mu$ M MeJA for 7 days promoted the production of valerian acid in valerian hairy root cultures of *Valeriana officinalis* [45]. Therefore, it is necessary to screen MeJA concentration and treatment time in order to obtain the maximum accumulation of bioactive compounds. In this study, we added different concentrations of MeJA to the TIBS liquid medium that had been cultured for 30 d. Results showed that the maximum total alkaloid content of plantlets was found at 10  $\mu$ M MeJA after culture for 20 more days.

Many medicinal plants, such as *Dendrobium* species, grow for a long time in nature, requiring growth periods of around three years; hence, the period of extraction of bioactive compounds from plants is very long. In order to quickly obtain medicinal plant compounds plant tissue culture technologies which can effectively shorten the growth cycle have been designed and adopted. For example, plant tissue culture

for sustainable valorization of secondary metabolites of *Bryophyllum* sp. [46], and *in vitro* shoot culture of *Rhododendron fortunei* used for the commercial production of raw materials for extracting bioactive phytochemicals [47]. Previous studies have reported that the use of bioreactor culture systems and MeJA induction could promote the accumulation of plant bioactive compounds [36, 37, 48, 49]. In the present study, we combined the bioreactor systems with MeJA to more efficiently produce alkaloids from *D. nobile* tissue culture seedlings. The results show that the TIBS-MeJA system was more conducive to the synthesis of alkaloids and could effectively shorten the production cycle, thus reducing production costs.

## Conclusions

TIBS can be applied for the large-scale biomass of *D. nobile*, and total alkaloid accumulation can be improved by selecting appropriate TIBS immersion frequencies. Moreover, treatment with MeJA has a high elicitation effect on bioactive compound accumulation in *in vitro*-cultured *D. nobile* seedlings, and about 10  $\mu\text{M}$  MeJA benefited the production of total alkaloids. Thus, we propose that TIBS and MeJA are necessary for the large-scale production of alkaloids. In this study, to obtain the highest total alkaloid content and productivity, 10  $\mu\text{M}$  MeJA was added to the TIBS liquid medium after plantlets were cultured for 50 days, and the culture was continued for 20 more days. Large-scale biomass and total alkaloid production of *D. nobile* were successfully accomplished using TIBS and MeJA.

## Materials And Methods

### Plant materials and preparation

*D. nobile* seeds were collected from Yunnan Province, China, and grown in a greenhouse at Nanjing Normal University. Mature capsules of *D. nobile* obtained by artificial pollination were surface sterilized using 75% alcohol and 10% hydrogen peroxide, then the sterile seeds were seeded on  $\frac{1}{2}$ MS (Murashige and Skoog, 1962) medium (pH 6.0), replenished with 25  $\text{g}\cdot\text{L}^{-1}$  sucrose, 80  $\text{g}\cdot\text{L}^{-1}$  CW (coconut water), 0.5  $\text{mg}\cdot\text{L}^{-1}$  NAA ( $\alpha$ -naphthaleneacetic acid), and 7.2  $\text{g}\cdot\text{L}^{-1}$  agar. Seeds were cultured in a photoperiod of 10 h light/14 h dark at  $25 \pm 1^\circ\text{C}$  for 30 days after 5 days of dark culture, and plantlets (stem length: 2–3 cm) from the seeds were used as plant material for further experiments.

#### TIBS and SSS culture of *D. nobile*

The temporary immersion bioreactor system (TIBS) was provided by Biofunction Co. Ltd. (Nanjing, China) with culture volume of 6.6 L, which includes controller, culture tank, Connecting tube, air filter (0.22  $\mu\text{m}$ ), etc. (Fig. 7). The liquid  $\frac{1}{2}$ MS medium (pH 6.0, 1 L) containing 25  $\text{g}\cdot\text{L}^{-1}$  sucrose, 0.5  $\text{mg}\cdot\text{L}^{-1}$  NAA, and 80  $\text{g}\cdot\text{L}^{-1}$  CW, and approximately 300 *D. nobile* plantlets were placed in each container for all treatments. Four immersion frequencies, 5/2, 5/4, 5/6, and 5/8, were compared for biomass and alkaloid content of *D. nobile* seedlings in TIBS. Here, the immersion frequencies indicate the time immersed in liquid culture and the interval; “5/2” for example, indicates that the plants were immersed in liquid medium for 5 min every 2 hours.

Semi-solid system (SSS): 300 explants were grown in 20 bottles (each bottle volume 0.5 L) of semi-solid medium averagely. SSS medium was added to  $7.2 \text{ g}\cdot\text{L}^{-1}$  agar as support. All cultures were maintained in 10 h photoperiod under cool white light (1800 lx) at  $25 \pm 1^\circ\text{C}$  for 80 days.

## MeJA elicitation experiments

MeJA (Sigma-Aldrich, 392707) was dissolved in ethanol to prepare a stock solution and filter-sterilized through a  $0.22 \mu\text{m}$  nylon filter. When the plantlets were cultured in TIBS for 50 days, MeJA was added through aseptic replacement of all liquid media, and the immersion frequency was 5/6. MeJA was used as the elicitor at final concentrations of 5, 10, 15 and  $20 \mu\text{M}$  ( $\mu\text{mol}\cdot\text{L}^{-1}$ ), and liquid medium without MeJA was used as control group. All TIBS cultures were maintained under cool white light (1800 lx) at  $25 \pm 1^\circ\text{C}$  for a 10 hr photoperiod with, and plantlets were harvested after 10, 20, and 30 days to determine fresh weight, dry weight, and alkaloid accumulation.

## Determination of biomass and total alkaloid content

The tissue culture seedlings of *D. nobile* were taken out from the culture containers and washed with tap water. The fresh weight (FW, g/L) was weighed after the water on the plant surface was absorbed with absorbent paper using analytical balance. The dry weight (DW, g/L) was weighed after plant dried in the oven at  $60^\circ\text{C}$  for 36–48 hr to absolute dryness. 0.5 g dried plant was grinded into powder using mortar, then added ammonia solution and let stand for 0.5 hr. Following this, the mixed liquids were poured into 50 mL flask and then added 25 mL chloroform for extraction. The chloroform in the flask was dried with a rotary evaporator after maintained in a water bath at  $70^\circ\text{C}$  for 2.5 hr, after which 5 mL of chloroform was poured into the flask to dissolve the dry residue and then 2 mL of chloroform extract was aspirated and added chloroform to 10 mL. Then, 5 mL of pH 4.5 potassium hydrogen phthalate buffer and 2 mL of 0.04% (w/v) bromocresol green solution were mixed and poured into the chloroform extract. Afterwards, shook the mixture violently for 3 min, then let it stand for 30 min, and then added 1 ml alkaline alcohol ( $0.01 \text{ mol}\cdot\text{L}^{-1}$  NaOH) to 5 ml lower fractions for analysis. The absorbance value was determined by spectrophotometer at 620nm, and then the total alkaloid content was calculated by a standard curve equation:  $y = 0.063x + 0.027$ , ( $R^2 = 0.996$ ) (Y and X are the absorbance and content of dendrobine) which was obtained with dendrobine as the reference standard. The content and productivity of total alkaloid were calculated by the following formula: content ( $\text{mg}\cdot\text{g}^{-1}$  DW) = dendrobine (mg)  $\times$  5/0.5 g and productivity ( $\text{mg}\cdot\text{L}^{-1}$ ) = DW ( $\text{g}\cdot\text{L}^{-1}$ )  $\times$  content ( $\text{mg}\cdot\text{g}^{-1}$  DW) [23, 38].

## Declarations

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### Authors' contributions

BHZ and XYD conceived and designed research. BHZ, ZTN and CL conducted experiments. QYX and WL contributed analytical tools. XJL and XYD analyzed data and wrote the manuscript. All authors read and approved the final manuscript.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request.

## Ethics approval and consent to participate

Not applicable.

## Consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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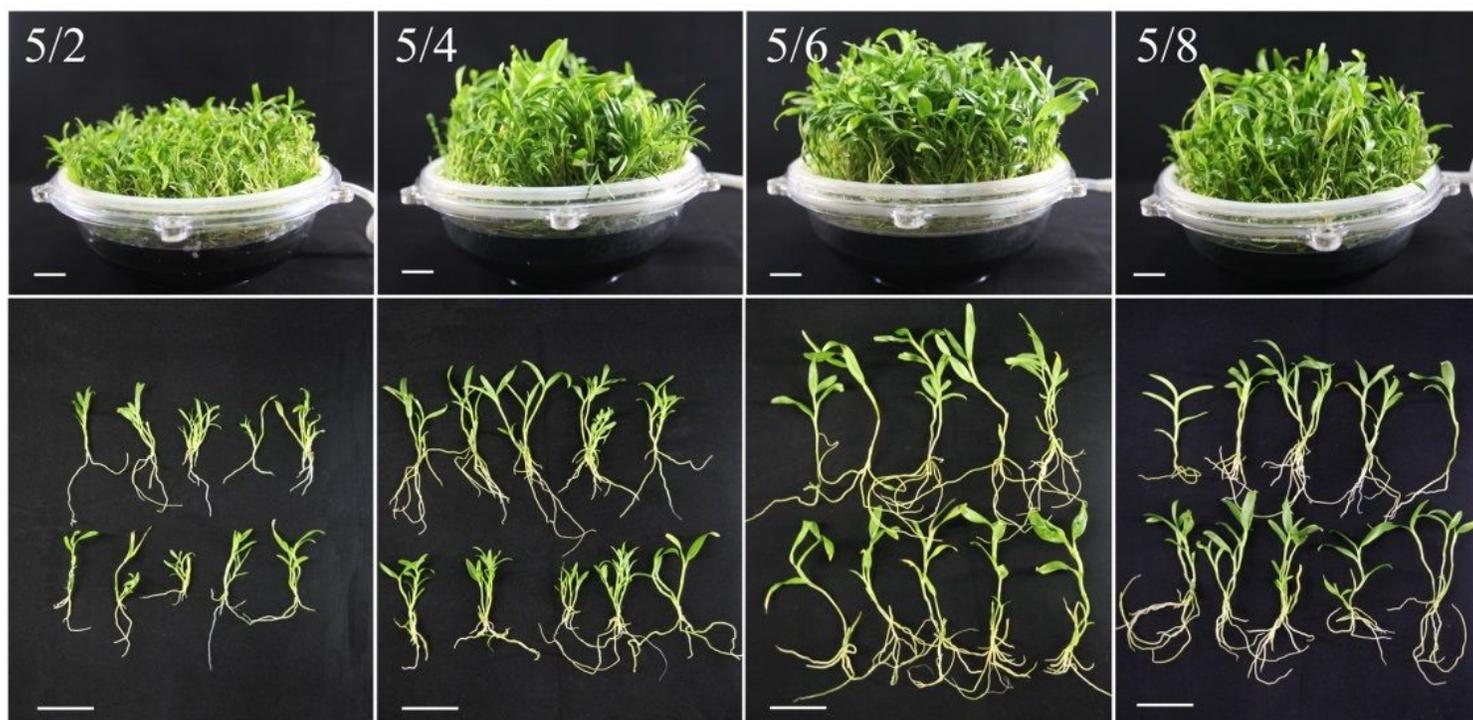
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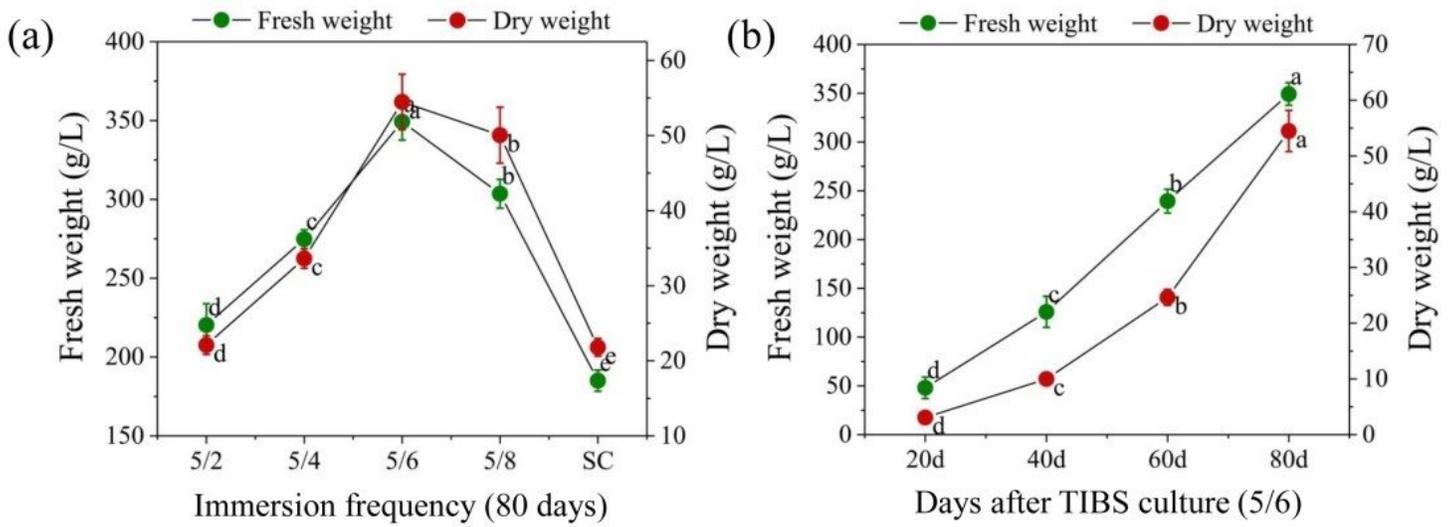
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## Figures



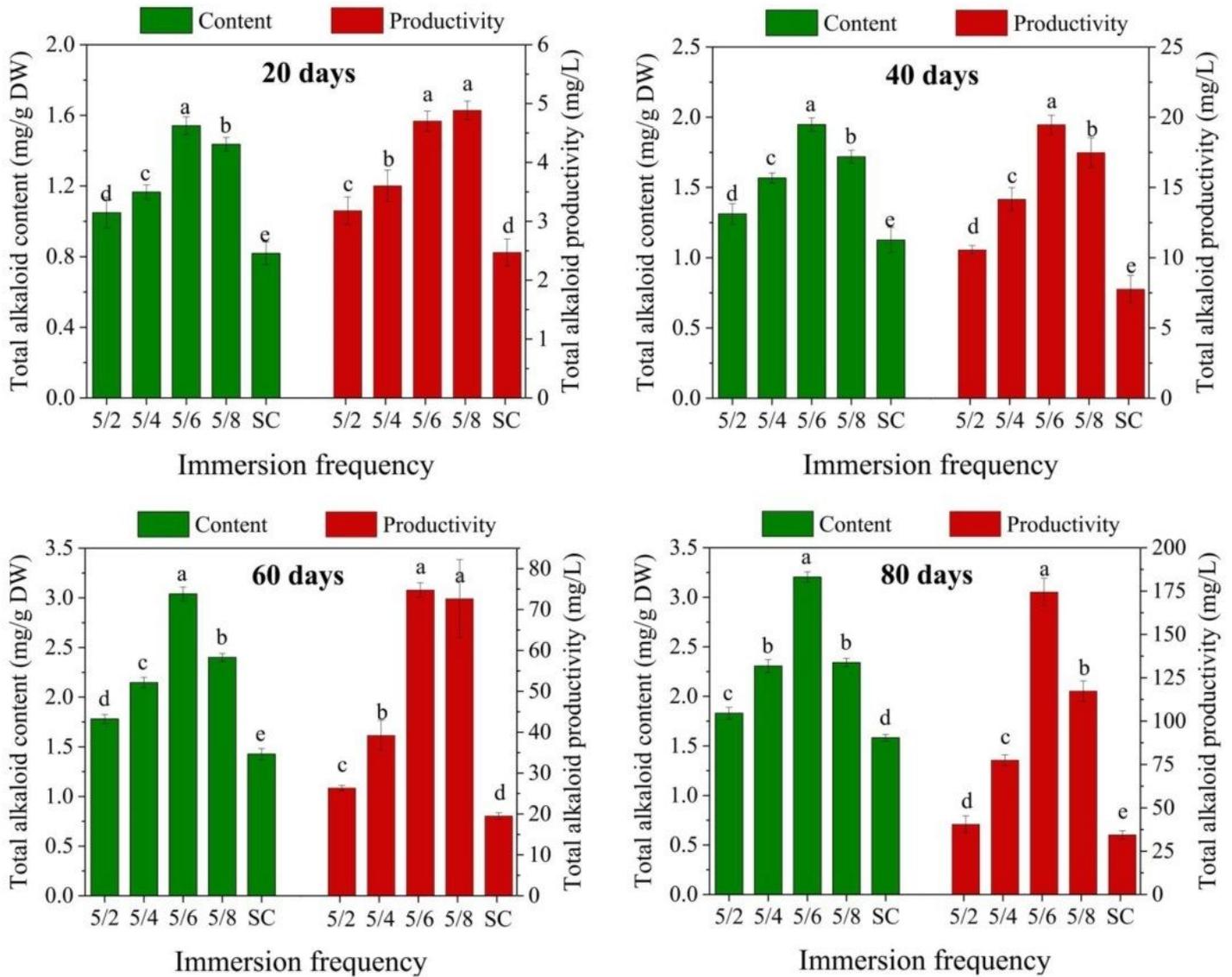
**Figure 1**

Effect of immersion frequency in TIBS on growth of *D. nobile* for 80 days. a/b means the immersion frequency was every b h for a min. Scale bar=2cm.



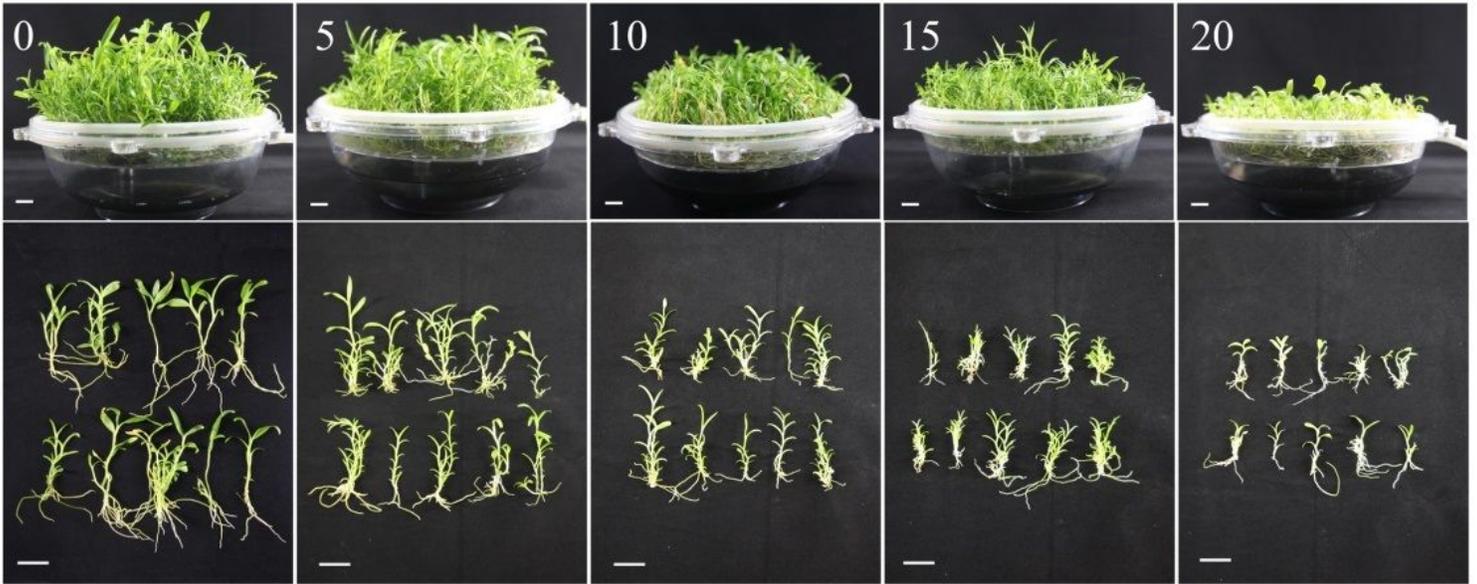
**Figure 2**

(a) Effect of immersion frequency on biomass accumulation of *D. nobile* after 80 days of TIBS culture.  
 (b) Effect of culture time on biomass accumulation of *D. nobile* at an immersion frequency of 5 min every 6 hr of TIBS culture. Data represents the mean  $\pm$  standard error of three replicates. Mean values followed by the same letters within a column are not significantly different according to Duncan's multiple range test at 5% level.



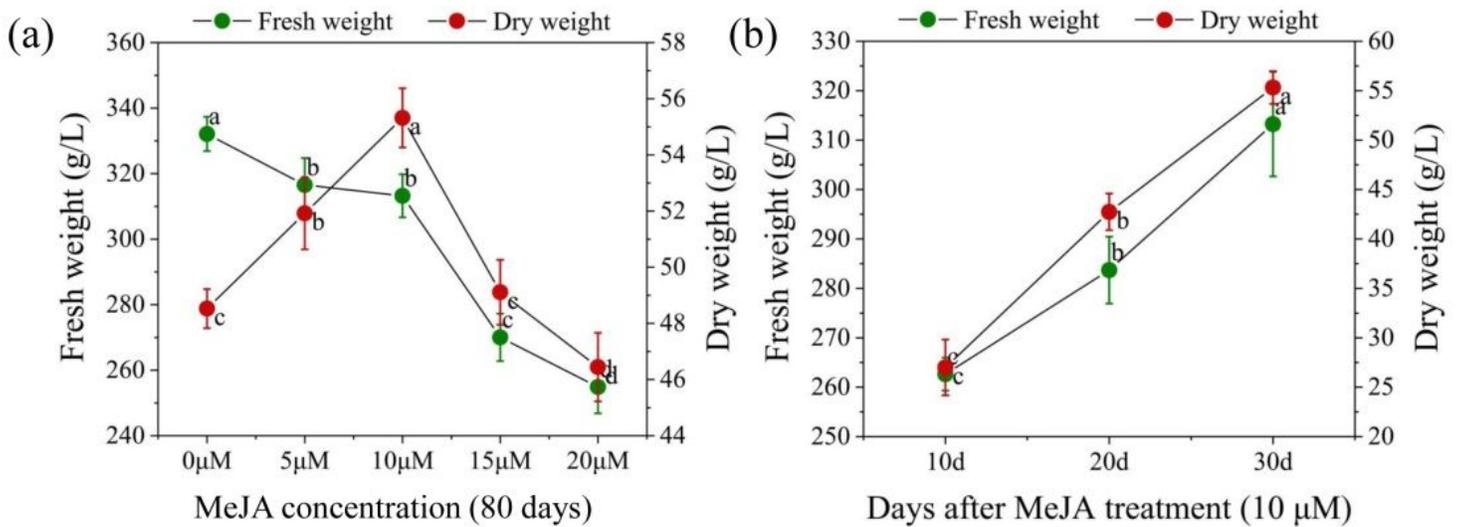
**Figure 3**

Effect of immersion frequency on total alkaloid accumulation of *D. nobile* after different culture times. SC means semi-solid system culture as a control. Data represents the mean  $\pm$  standard error of three replicates. Mean values followed by the same letters within a column are not significantly different according to Duncan's multiple range test at 5% level.



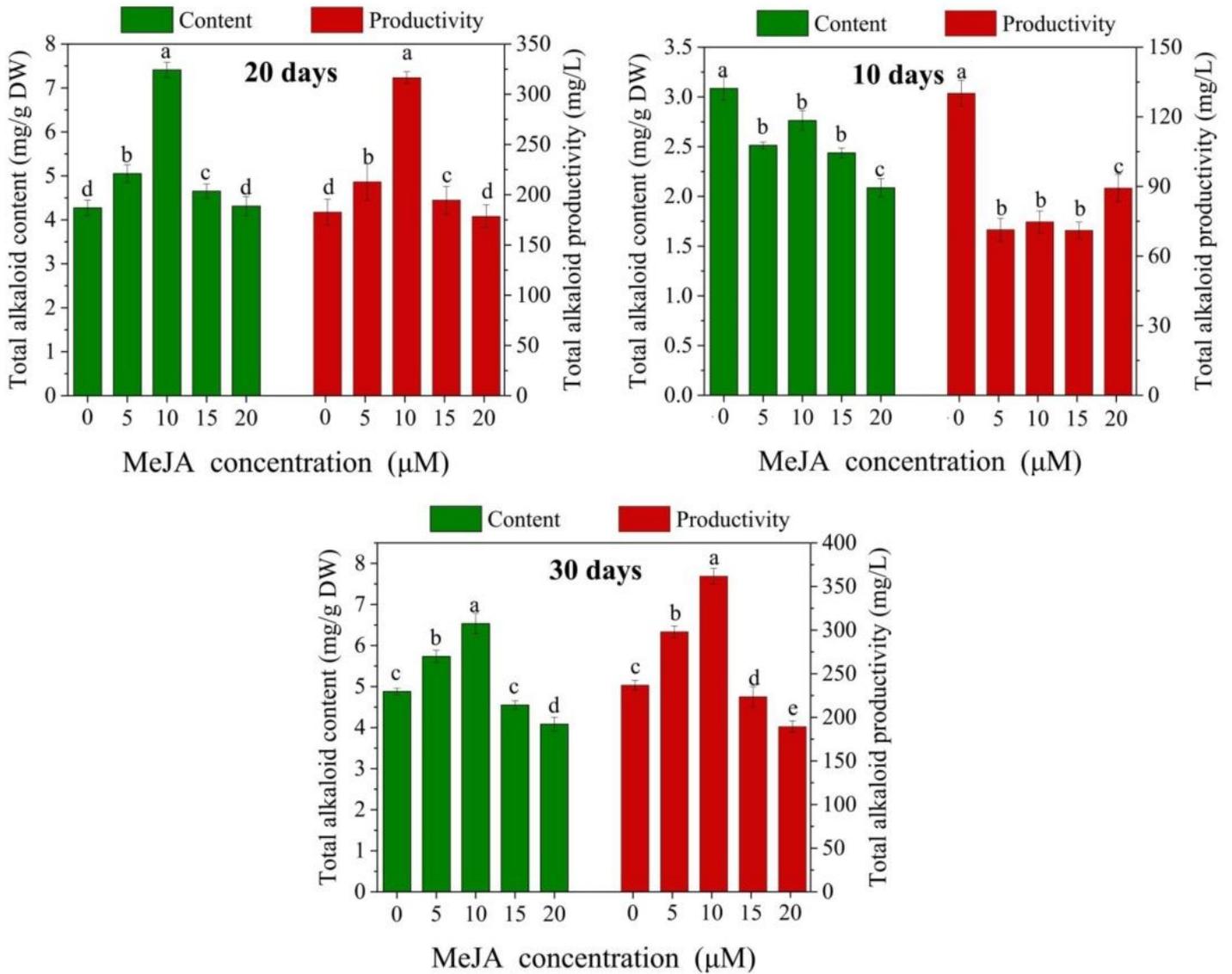
**Figure 4**

Effect of MeJA content in TIBS medium on growth of *D. nobile* for 80 days. Arabic numeral means the MeJA concentration ( $\mu\text{M}$ ). Scale bar  $\square$  2cm.



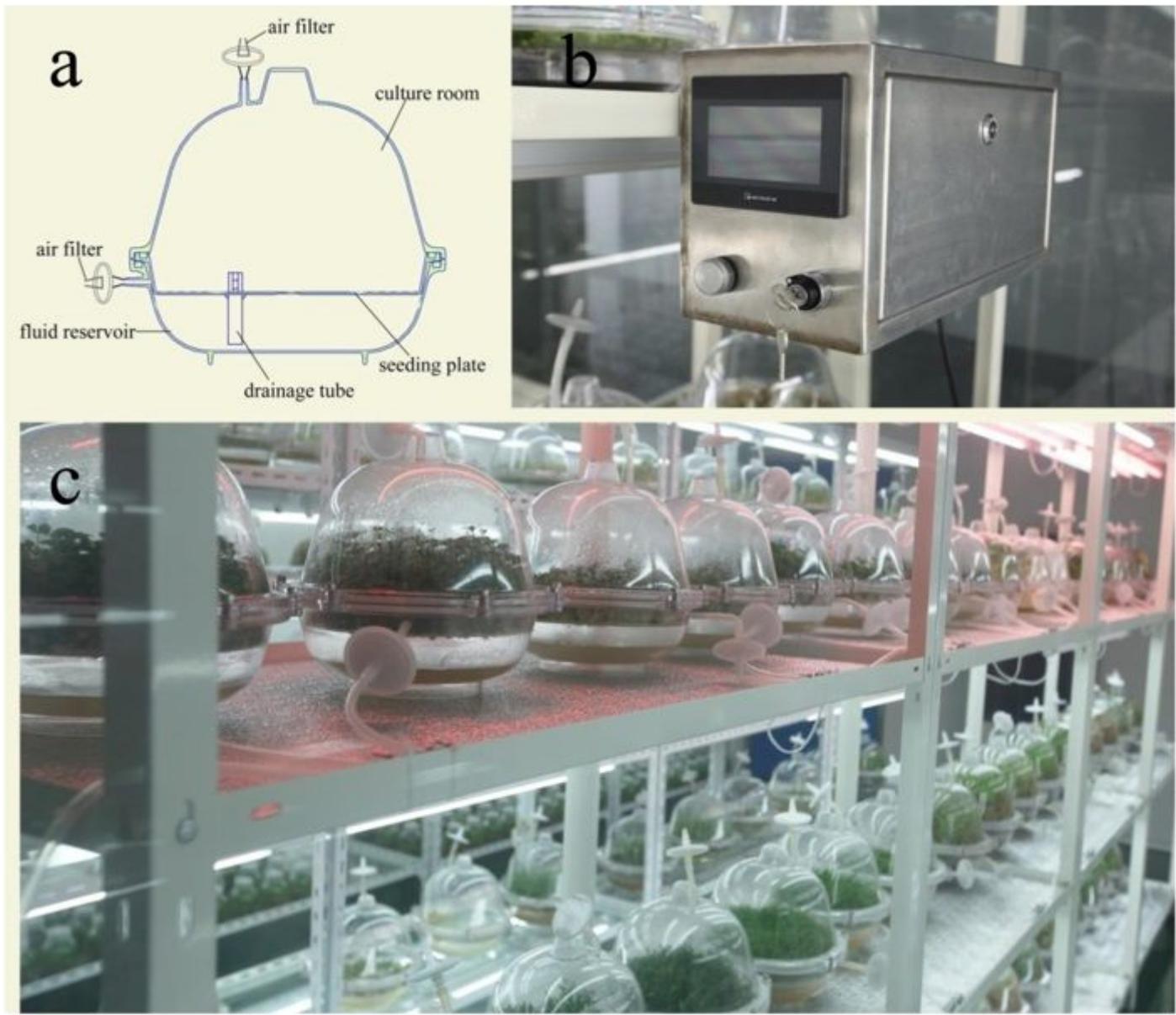
**Figure 5**

(a) Effect of MeJA concentration on biomass accumulation of *D. nobile* after 80 days using TIBS culture. (b) Effect of culture time on biomass accumulation of *D. nobile* at a MeJA concentration of 10  $\mu\text{M}$  using TIBS culture. Data represents the mean  $\pm$  standard error of three replicates. Mean values followed by the same letters within a column are not significantly different according to Duncan's multiple range test at 5% level.



**Figure 6**

Effect of MeJA concentration on total alkaloid accumulation of *D. nobile* after different culture times. Data represents the mean  $\pm$  standard error of three replicates. Mean values followed by the same letters within a column are not significantly different according to Duncan's multiple range test at 5% level.



**Figure 7**

(a) TIBS tank. (b) TIBS controller. (c) a typical culturing array

## Supplementary Files

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