

Improving biomass and dendrobine-type totalalkaloid (DTTAs) production of *Dendrobium nobile* through combining Temporary Immersion Bioreactor system (TIBS) with Endophyte MD33 elicitation

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Abstract

Dendrobium nobile Lindl. (*D.nobile*) is a perennial herb of the orchid family and is commonly used in traditional Chinese medicine. Due to the scarcity of *D.nobile* resources, the market is facing a shortage. To meet market demand, enhance cultivation efficiency, and reduce costs, the temporary immersion bioreactor (TIBS) has been employed for cultivation. The optimal cultivation conditions for *D.nobile* tissue culture seedlings have been determined through experimental research. These conditions include a liquid medium consisting of 1/2 MS, 30 g/L sucrose, 80 g/L potato, 0.5 mg/L NAA, 60 g/L banana, and a pH of 6.0. The optimal inoculation volume of TIBS per canister was 220 *D.nobile* seedlings, and the optimal submergence frequency of TIBS was set at 5 min/8 h, and the optimal submergence time was set at 6 min/6 h. In addition, it was observed that its endophytic fungus MD33, had a significant impact on the dendrobine-type total alkaloid (DTTAs) content and plant growth. This study demonstrates that utilizing TIBS for cultivation and co-cultivating with endophytic fungus significantly increased the biomass and bioactivity of *D.nobile* tissue culture seedlings.

Introduction

Dendrobium nobile Lindl. (*D.nobile*) is a perennial herbaceous plant that belongs to the orchid family and is primarily found in warm and humid regions. It typically grows on rocks or tree trunks in valleys at altitudes ranging from 500 to 1700 meters. This species holds significant ornamental value and occupies a distinct position in the global horticultural market (Zhang et al. 2021). Studies have demonstrated that *D.nobile* contains active components such as polysaccharides, alkaloids, and phenols. Among these, alkaloids serve various functions, including hypoglycemic, hypolipidemic, and anti-tumor properties (Long et al. 2023). Furthermore, research has shown that dendrobium possess the ability to inhibit the early replication of influenza viruses and combat influenza A H1N1 virus (Li et al. 2017). Due to its considerable medicinal and ornamental value, there is a high demand for *D.nobile* in the market. However, its natural reproductive capacity is relatively low, and the extensive excavation by humans has led to severe damage to the habitat and ecosystem of *D.nobile* (Zhang et al. 2023). To address these challenges and meet commercial demands, the plant tissue culture technique has been widely employed in the artificial cultivation of orchids. This technique enables faster propagation while ensuring the quality of the plants. The use of a liquid medium for orchid propagation, as compared to a semi-solid medium, allows for uniform nutrient uptake by the plant, thereby improving plant growth and reproduction (Xu et al. 2022).

The temporary immersion bioreactor system (TIBS) is a system that utilizes a liquid medium in intermittent contact with plant tissues to provide nutrients. Studies have reported successfully cultivating *Cattleya forbesii* Lindl. PLBs (Protocorm-like bodies) using a RITA® bioreactor based on the temporary immersion system, resulting in a significant increase in PLB yield and shoot regeneration (Münire et al. et al. 2019). However, the RITA bioreactor has a low capacity and is not suitable for large-scale commercial production. In contrast, the SETIS™ bioreactor model has greater light availability and a medium capacity of 4000 mL (Ahmad, 2020). Wagner A. Vendrame et al. (2023) used SETIS™ TIBS to culture *Brassavola*

nodosa (L.) Lindl., resulting in increased and improved plant growth propagation and rooting rate. The TIBS used in this study provides intelligent control or digitization, allowing for observation and experimental intervention conditions (Chen et al.2020). This significantly improves the efficiency of research and industrial utilization for the culture of *D.nobile* histocultures, enabling high-throughput long-term culture of the plant without the need for multiple transitions(Zhang et al.2018). This avoids germplasm degradation caused by too many inoculations in solid culture, and ensures the timely exchange of gases between the plant and the outside world. It provides the plant with sufficient carbon source and overcomes the limitations of traditional plant tissue culture technology(Zhang et al. 2022).

The quality of herbal medicines depends on the level of their secondary metabolite content, increasing the content of active plant components and cultivating high quality seedlings has become a key direction of today's research. The study of Szopa et al. (2019a) demonstrated that the use of bioreactors with a temporary immersion system significantly increased the accumulation of phenolic compounds and flavonoids in medicinal plants. In another study, Makowski et al. (2023) cultured *Pontechium maculatum* (Russian bugloss) using different culture methods and found that plants cultured with TIBS had the highest yield of phenolic derivatives among all methods, suggesting that it had a positive impact on the accumulation of secondary metabolites. This shows that TIBS can influence the production of plant secondary metabolites to some extent. Endophytic fungi colonise the interior of plants and not only metabolise the same or similar active products as the host plant, but also affect the synthesis, content and accumulation of secondary metabolites in medicinal plants (Li et al.2022). Ye et al. (2021) co-cultivated three kinds of endophytic fungi with *Houttuynia cordata* Thunb, and found that all three kinds of fungi had a positive effect on the host plant, which showed that endophytic fungi have practical value in the production of Chinese herbal medicine and can increase the accumulation of medicinal metabolites. Currently, few studies have been conducted on the use of endophytic fungi in co-culturing with plants in reactors, and in order to validate a possible strategy for expanding the production of secondary metabolites from medicinal plants in tissue culture, this paper co-cultivated *D.nobile* with the dendrobine-producing endophytic fungus MD33 using TIBS with a view to maximising the effects of both TIBS and the endophytic fungi, and the results obtained also contributed to a broad understanding of medicinal plant Biotechnology.

The main objectives of this study are as follow: Firstly, investigating the optimal conditions for cultivating *D.nobile* in TIBS. Secondly, comparing the effects of two different cultivation methods on *D.nobile* growth. and at last, the impact of adding endophytic fungi in TIBS on *D.nobile* growth and DTTAs accumulation was observed. This work provides a theoretical foundation for high-throughput plant cultivation in TIBS and co-cultivation experiments between endophytic fungi and plants, which has significant value for commercial production.

Materials and methods

Plant material preparation

Chishui City in Guizhou Province, China is the authentic production area of *D.nobile* and has obtained national geographical indication protection for orchids. The *D.nobile* seeds used in this experiment were collected from this region. The *D.nobile* pods were rinsed with running water for 30 minutes, shaken with 75% (v/v) ethanol for 30 minutes, rinsed with sterile water three times, and then shaken with 0.1% mercury chloride for 6 minutes, followed by rinsing seven times. The surface moisture was absorbed with sterile filter paper, and the sterile seeds were evenly spread on the germination medium (1/2 MS + 30 g/L sucrose + 30 g/L potato + 0.5 mg/L NAA + 5.5 g/L agar, pH = 6)(Zhang et al. 2022). The *D.nobile* seedlings obtained after 8 weeks of culture were transferred to solid culture bottles containing 200mL of nutrient medium (1/2 MS + 30 g/L sucrose + 80 g/L potato + 0.5 mg/L NAA + 5.5 g/L agar, pH = 6) and cultured in a growth chamber at a temperature of $24 \pm 1^{\circ}\text{C}$ and a light intensity of 2000 lux for 1 month before proceeding to the next step of the study (Zhang et al. 2018).

Effect of different bioreactor culture parameters

Experiments were conducted to culture *D.nobile* seedlings and observe the accumulation of dendrobine-type total alkaloid (DTTAs) using TIBS. The main factors, including intermittent immersion frequency (5 min/4 h, 5 min/6 h, and 5 min/8 h), immersion time (2 min/6 h, 4 min/6 h, and 6 min/6 h), sucrose concentration (20 g/L, 30 g/L, and 40 g/L), and inoculation amount (120 plants, 220 plants, and 320 plants) were tested. The *D.nobile* tissue culture seedlings obtained from 1 month culture in the nutrient medium were excised from their roots and then transferred to liquid medium (1/2MS + sucrose concentration set at different parameters + 80 g/L potato + 0.5 mg/L NAA + 60 g/L banana, pH = 6.0) according to the different parameter settings mentioned above and inoculated into TIBS according to the treatment parameters for the experiments. The experiment was replicated three times with three canisters of TIBS per treatment group. data were recorded 90 days after the start of the culture.

Cultivation of *D.nobile* seedlings in solid medium

D.nobile seedlings were removed from the nutrient medium cultured for 1 month and transferred into solid culture bottles containing 200 ml of *D.nobile* seedling propagation medium (1/2 MS + banana 60 g/L + potato 80 g/L + sucrose 30 g/L + NAA 0.5 mg/L + agar 6 g/L, pH = 6.0). Each bottle was inoculated with 10 seedlings and 15 bottles were inoculated in each treatment group and replicated three times. Data were recorded 90 days after the start of the culture.

Extraction of DTTAs from *D.nobile* tissue culture seedlings

Based on prior laboratory research, enhancements have been made to the conventional alkaloid extraction method (CHEN et al. 2019). Tissue-cultured seedlings of *D.nobile* were fetched for examination and dehydrated at a low temperature of 45°C . Subsequently, the desiccated sample was pulverized into a fine powder. A quantity of 0.3 g of the pulverized powder was taken and blended with 30 ml of 2% HCl. The sample was subjected to sonication for 10 minutes and then extracted over a duration of 12 hours on a shaker at 180 rpm and 28°C . The resulting mixture was filtered, and 10 ml of the resulting filtrate was obtained. The solution's pH was adjusted to 10 using ammonia, and subsequently combined with an

equal volume of dichloromethane. Then, 5 ml of the organic phase was mixed with 3 ml of buffer solution and 2 ml of bromocresol green solution. The mixture was then allowed to stand for 30 minutes. Afterward, 2.5 ml of the organic phase was extracted and combined with 0.5 ml of a 0.01 mol/L alkaline ethanol solution. The resulting mixture was agitated and allowed to settle for 5 minutes, with subsequent measurement of absorbance at 620 nm through an Ultraviolet-visible Spectrophotometer.

Co-cultivation of endophytic fungus MD33 from *D.nobile* with tissue culture seedlings

Trichoderma longibrachiatum MD33 is a fungus derived from *Dendrobium* stems with the ability to produce dendrobine (Sarsaiya et al. 2020a). Therefore, in this study, the endophytic fungus MD33 was co-cultivated with tissue-cultured seedlings of *D.nobile* in TIBS. Firstly, the MD33 fungal blocks were taken with a puncher and added to a 50 ml shake flask containing 20ml of potato dextrose broth (PDB) medium (Qian et al.2021). The shake flasks were placed on a shaker at 28°C and 120 rpm for 5 days, then the filtrate was collected and 20 ml of filtrate was added to the TIBS of the pre-cultured 3-month-old seedlings. The control group was added with 20 ml of PDB medium and three parallels were set for each treatment group. The immersion frequency was set at 5 minutes every 6 hours and the plants were removed after a total of 7, 14, 20 and 25 days of incubation, cleaned and measured for growth (plant height, stem diameter, root length). The plants were dried at low temperature and ground into a powder, which was then assayed as described above for the extraction of DTTAs.

Data collection and statistical analysis

Thirty plantlets were randomly selected from each TIBS container to measure their height and calculate the average value. Additionally, ten plantlets were randomly chosen from each TIBS container, and three roots were sampled from each of these plantlets to measure their length and determine the average value. Thirty seedlings were randomly selected from each TIBS container and stem diameter was measured from the top of the stem between the fourth and fifth leaves to calculate the average stem diameter.

The purpose of this study was to investigate the impact of various culture conditions on the botanical characteristics and DTTAs content of *D.nobile* cultured by TIBS. The data obtained from the experiments were analyzed using one-way ANOVA in SPSS 26 (Version 26.0 SPSS Inc., Chicago, IL, USA), with a significance level of $P < 0.05$ indicating statistically significant differences.

Results

Optimisation of culture parameters in different bioreactors

Effect of different immersion frequency on the growth of *D.nobile* tissue culture seedlings

The different frequency of immersion resulted in morphological differences in the seedlings, with the best growth of plant height and stem diameter at an interval of 8 h, which was significantly higher than the other two groups, as well as moderate root length, which has been reported to be too long for

transplanting (Table 1) (Zhang et al. 2020). The study found that the DTTAs content significantly increased with the extension of soaking time. The accumulation of DTTAs content in the 8-hour treatment group was 71.62% ($P < 0.05$) higher than that in the 4-hour treatment group (Fig. 1-A), reaching 999.97ug/ml. In summary, the optimal immersion frequency for culturing the tissue-cultured seedlings of *D.nobile* is 5 min/8 h in the TIBS.

Table 1
Effects of different immersion frequency cultures on the morphological characteristics of *D.nobile* seedlings

Treatment method	Treatment parameters	Plant height/cm	Stem diameter/cm	Root length/cm
Immersion frequency	5 min/4 h	1.34 ± 0.11 ^c	0.21 ± 0.15 ^b	1.05 ± 0.13 ^c
	5 min/6 h	1.86 ± 0.05 ^b	0.11 ± 0.09 ^c	1.95 ± 0.16 ^a
	5 min/8 h	2.45 ± 0.16 ^a	0.33 ± 0.08 ^a	1.39 ± 0.14 ^b
Different letters in each column represent means significantly different at $P < 0.05$.				

Effect of different immersion time on the growth of *D.nobile* tissue culture seedlings

When the immersion time was set to 2 minutes, the growth status of tissue-cultured *D.nobile* plants was moderate. Subsequently, when the immersion time was increased to 4 minutes, the plant's growth condition was poorer. However, when the immersion time was increased to 6 minutes, the plant's growth condition was optimal. Compared to the 2-minute group, the plant height increased by 45.59% ($P < 0.05$) and stem diameter increased by 23.07% ($P < 0.05$). In the 6-minute group compared to the 4-minute group, the plant height increased by 55.15% ($P < 0.05$) and stem diameter increased by 46.15% ($P < 0.05$) (Table 2). In addition, as shown in Fig. 1-B, the DTTAs content increased with the increase in immersion time. The DTTAs content accumulation in the 6-minute immersion group was 19.89% ($P < 0.05$) higher than that in the 2-minute group. In conclusion, setting the immersion time to 6 minutes is beneficial for plant growth and the accumulation of metabolites.

Table 2
Effects of different immersion time cultures on the morphological characteristics of *D.nobile* seedlings

Treatment method	Treatment parameters	Plant height/cm	Stem diameter/cm	Root length/cm
immersion time	2 min/6 h	1.98 ± 0.14 ^b	0.16 ± 0.06 ^a	1.53 ± 0.06 ^a
	4 min/6 h	1.36 ± 0.18 ^c	0.13 ± 0.05 ^c	1.03 ± 0.09 ^c
	6 min/6 h	2.11 ± 0.13 ^a	0.19 ± 0.05 ^b	1.88 ± 0.06 ^b
Different letters in each column represent means significantly different at P < 0.05.				

Effect of different Medium sucrose concentration on the growth of *D.nobile* tissue culture seedlings

The effect of different concentrations of sucrose on the growth of *D.nobile* seedlings is presented in Table 3. It was found that when sucrose concentration was 30 g/L, the height of the plant significantly increased to 1.90 cm, compared to the other two groups. At a lower sucrose concentration of 20 g/L, the stem diameter of the *D.nobile* seedlings was 0.16 cm, while at a sucrose concentration increased to 40 g/L, the optimal stem thickening growth of *D.nobile* was 0.20 cm. The seedlings with a sucrose concentration of 20 g/L had the lowest DTTAs content, while those with 30 g/L had the highest, representing a 51.92% (P < 0.05) increase compared to the 20 g/L treatment group (Fig. 1-C). Therefore, a sucrose concentration of 30 g/L was deemed most suitable for the cultivation of *D.nobile* tissue culture seedlings.

Table 3
Effects of different medium sucrose concentration cultures on the morphological characteristics of *D.nobile* seedlings

Treatment method	Treatment parameters	Plant height/cm	Stem diameter/cm	Root length/cm
Medium sucrose concentration	20	0.86 ± 0.03 ^c	0.16 ± 0.02 ^b	1.06 ± 0.05 ^c
	30	1.90 ± 0.13 ^b	0.19 ± 0.05 ^{ab}	1.49 ± 0.08 ^b
	40	1.31 ± 0.13 ^a	0.20 ± 0.07 ^a	1.28 ± 0.07 ^a
Different letters in each column represent means significantly different at P < 0.05.				

Effect of different inoculum concentration on the growth of *D.nobile* tissue culture seedlings

The utilisation efficiency of TIBS is closely related to the inoculum concentration. When the inoculum concentration was set at 220, the growth condition of the *D.nobile* tissue culture seedlings was the best, with an average plant height of 2.43 cm and an average stem diameter of 0.22 cm (Table 4). The

accumulation of DTTAs exhibited the highest increase of 16.9% ($P < 0.05$) in comparison to the 120 plant treatment group (Fig. 1-D). When the inoculum concentration was set to 120, the plants were smaller and had a more fragile growth state, while when the inoculum concentration was set to 320, the growth rate of the plants decreased and their growth state was not as good as the treatment group with an inoculum concentration of 220. Therefore, setting the inoculum concentration to 220 plants per TIBS container is more beneficial for tissue culture of the *D.nobile* plant.

Table 4

Effects of different inoculum concentration on the morphological characteristics of *D.nobile* seedlings

Treatment method	Treatment parameters	Plant height/cm	Stem diameter/cm	Root length/cm
inoculum concentration	120	1.07 ± 0.17^c	0.10 ± 0.11^c	0.98 ± 0.07^c
	220	2.43 ± 0.16^b	0.22 ± 0.09^b	1.68 ± 0.10^b
	320	1.64 ± 0.09^a	0.16 ± 0.04^a	1.26 ± 0.15^a
Different letters in each column represent means significantly different at $P < 0.05$.				

Table 5

Effects of TIBS and solid culture method on the growth status of seedlings of *D.nobile*

Treatment method	Plant height/cm	Root length/cm	Stem diameter/cm	Root-shoot ratio
Solid medium	1.74 ± 0.05	1.68 ± 0.04	1.28 ± 0.05	0.97
TIBS	$2.48 \pm 0.08^{**}$	$1.03 \pm 0.06^{***}$	$1.57 \pm 0.07^{**}$	0.42^{***}

Note: “**” means significant difference at $P = 0.01$ level; “***” means significant difference at $P \leq 0.001$ level.

Comparison of *D.nobile* seedlings in TIBS and solid culture:

In the comparison between tissue-cultured seedlings of *D.nobile* cultivated in solid and liquid intermittent immersion systems (Fig. 2), it was observed that the root system of seedlings grown in solid culture was well-developed, exhibiting a significantly higher root-shoot ratio compared to those grown in liquid intermittent immersion. The plants cultivated in the bioreactor exhibited a significantly increased plant height, with a 42.5% ($P < 0.01$) improvement over solid culture medium. The table also indicates shorter root length in plants grown in the bioreactor, indicating potentially ample nutrient supply provided by TIBS, whereas roots in solid culture require continuous growth to acquire nutrients, resulting in longer root length. Additionally, seedlings cultivated in the bioreactor can be directly transplanted without the need for seedling hardening, making them more suitable for transplantation. Most importantly, compared to solid culture, TIBS offers a larger capacity, resulting in significant improvements in fresh weight, dry

weight, and proliferation coefficient (Fig. 3), thereby promoting large-scale industrial cultivation of tissue-cultured seedlings of *D.nobile*.

The influence of endophytic fungus MD33 on the growth status and DTTAs content of *D.nobile* tissue culture seedlings

This study conducted co-cultivation of the endophytic fungus MD33 with *D.nobile* tissue culture seedlings in TIBS for 7, 14, 20, and 25 days to observe their growth. Figure 4-A shows that the plant height of the treatment group co-cultivated with MD33 was higher than the control group. After 14 days of co-cultivation, the plant height of the treatment group increased significantly by 29.23% ($P < 0.05$) compared to the control group. Figure 4-B shows that the stem diameter of all treatment groups was significantly higher than the control group. After 25 days of co-cultivation, the stem diameter of the treatment group increased by 89.96% ($P < 0.05$) compared to the control group, but the difference was not significant compared to around 20 days of co-cultivation. It was observed that root length of *D.nobile* tissue culture seedlings co-cultured with endophytic fungus MD33 was generally lower in the treatment group than in the control group (Fig. 4-C), with a significant difference of 25.92% ($P < 0.05$) at 14 days. In addition, the addition of endophytic fungus MD33 significantly affected the DTTAs content of *D.nobile* tissue-cultured seedlings (Fig. 5). The results showed that after 7 days of co-cultivation, there was a significant difference in DTTAs content between the treated group and the control group, with an increase of 38.83%. The difference in DTTAs content between co-cultivation for 20 days and co-cultivation for 14 days was not significant. However, after 25 days of co-cultivation, the DTTAs accumulation in *D.nobile* tissue-cultured seedlings increased significantly by 40.61% compared to the control group. These results indicate that co-cultivation with endophytic fungus MD33 can increase the DTTAs content of *D.nobile* tissue-cultured seedlings, with the most significant effect observed after 25 days of co-cultivation.

Discussion

In the field of plant biology, the study of cultivating plant seedlings with high levels of active compounds is of significant value in both fundamental knowledge and industrial applications. Recent advances in medical research have revealed the anti-tumour, hypoglycaemic and immune-enhancing properties of *D.nobile*. Given the high market demand and limited resources of this species, it is crucial to explore advanced cultivation techniques for high-throughput seedling production to meet market demands. This study aims to investigate the optimal conditions for TIBS cultivation and the impact of endophytic fungal stress on the biomass and DTTAs accumulation in *D.nobile* tissue culture seedlings. Additionally, the study also compares the effects of two different cultivation methods on the growth status of the plants.

Traditional cultivation techniques are expensive and have low productivity, making them unsuitable for commercial production. However, advanced cultivation techniques like TIBS can improve plant productivity and reduce labor and production costs. In a study conducted by M. Escalona Previous et al. (1999) using pineapple culture in solid, liquid, and TIBS, it was found that TIBS significantly increased biomass and proliferation rate. Similarly, Manuel Cabrera Jova et al. (2011) cultivated yam using the

TIBS, and the results demonstrated that the system could produce more plants without the need for adaptive cultivation. The author believes that by optimizing the sowing procedure, cultivation conditions, and frequency of medium exchange, it is possible to increase the biomass of the plants. In this study, TIBS was used to cultivate 200–300 *D.nobile* tissue culture seedlings at a time, achieving a high throughput output compared to traditional solid culture methods, and continuously optimising multiple parameters in the reactor. Among these parameters, the intermittent immersion frequency and immersion time were found to be crucial for plant cultivation using TIBS. Zhu et al. (2005) cultivated apple shoots using TIBS and discovered that setting the immersion frequency to 16 times a day significantly increased the reproduction rate. Zhang et al. (2018) optimized the cultivation conditions of *Bletilla striata* in TIBS and found that an intermittent immersion frequency of 3 minutes every 6 hours resulted in optimal growth. In this study, three optimized parameters were set, and the results showed that setting the parameters to 5 min/4 h resulted in stunted seedlings, while adjusting the interval to 5 min/8 h not only significantly improved plant growth status but increased the DTTAs content. It was observed that a lower immersion frequency yielded better seedling cultivation effects, which is in line with the findings of Zhang et al. (2018) and Ivanov et al. (2011). Zhang et al. (2018) proposed that frequent immersion of plants in liquid medium may not meet the respiratory needs of plant tissues and could lead to excessive dehydration and deformities in explants. Therefore, the immersion frequency set at 5 min/8 h yielded the best plant cultivation effect. Additionally, the study also demonstrated that immersion time had the greatest impact on plant height (Zhang et al. 2018). Gao et al. (2015) conducted a study on the cultivation of Chinese water chestnuts under different immersion times and found that the growth rate was highest at 15 minutes, with the growth rate decreasing as the immersion time increased. Agustine Christela Melviana et al. (2021) studied the effect of immersion time on the growth and shoot biomass conversion of *Stevia rebaudiana* Shoot and found that plants immersed for 30 minutes had higher proliferation rates and biomass compared to those immersed for 15 minutes. According to Gaussier et al. (2002), the ideal frequency for intermittent immersion varies depending on the type and species of explants. To study the effect of immersion time on the growth of *D.nobile* plantlets, this study set the immersion time to 2, 4, and 6 minutes every 6 hours. The results showed that both shorter immersion time (2 minutes) and longer immersion time (6 minutes) were beneficial for plant cultivation. According to the report by A. Ramos-Castellá et al. in 2014, similar observations were made when using TIBS for cultivating *Vanilla planifolia* Jacks. ex. Considering all factors, immersing for 6 min/ 6 h was determined to be the best parameter for cultivating *D.nobile* tissue culture seedlings.

Sucrose plays a crucial role in plant bodies as an important signaling molecule, facilitating the transmission of sugar signals and regulating plant growth and development processes (Smeekens et al., 2010). In a study conducted by Saleta Rico et al. (2022), it was found that adding 0.5% sucrose was beneficial for the growth of TIS cultured explants of shoots of *Cannabis sativa* L., but hindered the growth of traditional solid-cultured explants. Another study by Hu et al. (2014) examined the optimal sucrose concentration for TIBS cultured *D.nobile* seedlings and concluded that a concentration of 20 g/L was optimal. Results from this study showed that both excessive and insufficient sucrose concentrations in the culture medium were detrimental to growth status and DTTAs content when compared to a

concentration of 30 g/L. This suggests that low concentrations of sucrose lead to nutrient deficiencies and growth inhibition in plants, whereas high concentrations of sucrose hinder plant growth due to osmotic pressure and other factors. Furthermore, the size of the inoculum also plays a significant role in plant growth. Hu et al. (2014) demonstrated that insufficient inoculum size hinders efficient utilization of the culture medium, while excessive inoculum size leads to nutrient shortage, both of which are unfavorable for plant growth. In this experiment, when 120 plants were inoculated into TIBS, they exhibited slow growth and fragile states, which could be attributed to the crowded characteristic of *D.nobile* seedlings cultured in TIBS, where the high-throughput advantage of TIBS cannot be fully utilized when the number of seedlings is small (Zhang et al. 2020). This not only hampers growth but wastes jar capacity. When the inoculum size was increased to 320 plants, the limited growth space resulted in a decrease in growth trend. The study determined that the optimal inoculum size for *D.nobile* seedlings was 220 plants, which showed significant advantages in terms of plant growth status and DTTAs content.

Compared to traditional solid culture methods, the TIBS offers advantages such as low cost, high efficiency, and the elimination of multiple transfers. In their study, Na-Young Kim et al. (2020) compared the TIBS with conventional culture systems for cultivating virus-free apple seedlings. Their findings demonstrated that the TIBS enables rapid propagation of a large quantity of virus-free plants, making it suitable for large-scale cultivation. In order to assess the impacts of the TIBS and the traditional solid cultivation method on the cultivation of *D.nobile* seedlings, we observed an intriguing phenomenon. Specifically, the root length of *D.nobile* seedlings cultivated in solid culture bottles was found to be longer than that of seedlings cultivated in TIBS. In TIBS, a continuous and uniform nutrient supply was provided by regular immersion in liquid medium, which promoted more stable seedling growth. On the other hand, plants grown in solid culture bottles absorbed more nutrients, resulting in continuous growth and longer root length. Compared to traditional solid culture methods, when using the TIBS, there were significant increases in fresh weight, dry weight, and proliferation coefficient.

Endophytic fungi (EF) are a group of fungi that reside within and interact with host organisms, playing a crucial role in host growth, development, and the accumulation of secondary metabolites (Wang et al. 2019). Fabio C. Chaves et al. (2012) isolated an endophytic fungus, *Aspergillus oryzae* NRRL 35191, from coffee plants and observed that co-cultivation with cacao plants increased caffeine content and enhanced the plant's resistance to insects and pathogens. Wang et al. (2019) studied the effects of ginseng saponin and puerarin stimulants and symbiotic microorganisms on the synthesis of polysaccharides by the endophytic fungus *Chaetomium globosum* CGMCC 6882. The results showed that polysaccharide titers significantly increased when 16 µg/L of ginsenosides and puerarin were added, reaching as high as 4.55 g/L when co-cultivated with UV-irradiated *G.pentaphyllum*. The purpose of this study is to use TIBS as a cultivation system to provide conditions for continuous monitoring and experimental intervention. After adding endophytic fungi, the growth status and accumulation of secondary metabolites of *D.nobile* tissue culture seedlings were monitored. Li et al. (2009) successfully simulated the co-cultivation of *Taxus chinensis* var. *mairei* and the endophytic fungus (*Fusarium mairei*) in a 20-liter combined bioreactor and achieved an ideal production of paclitaxel. This study found that co-cultivation with endophytic fungus MD33 significantly increased the DTTAs content of *D.nobile* tissue

culture seedlings. These results also indicate that conducting co-cultivation experiments in TIBS is beneficial for exploring the interaction between microorganisms and plants, providing theoretical basis for large-scale commercial production.

Conclusion

In this study, the culture conditions for *D.nobile* seedlings in the TIBS were optimized. Additionally, the co-cultivation of the endophytic fungus MD33 with *D.nobile* seedlings in TIBS was investigated for the first time. The results demonstrated that the optimal conditions for cultivating *D.nobile* species in TIBS were as follows: an immersion time of 6 min/ 6 h, a submergence frequency of 5 min/8 h, a sucrose concentration in the medium of 30 g/L, and an inoculum size of 220 seedlings. These conditions resulted in a significant increase in biomass compared to both TIBS and the traditional solid culture method, as well as excellent growth status. Furthermore, the addition of the endophytic fungus MD33 to the TIBS and the co-cultivation with *D.nobile* seedlings led to improved growth status and a significant increase in DTTAs content. These findings provide valuable insights for the large-scale commercial production of *D.nobile* seedlings.

Declarations

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Author contributions

CYW conducted the experiments and wrote the manuscript. QX and YTF analysed the data and revised the article. JQ and CJS planned and designed the study.

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Data availability

The authors are unable or have chosen not to specify which data has been used. The data will be made available on request.

Conflict of interest the authors confirm that this article content has no conflict of interest.

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Figures

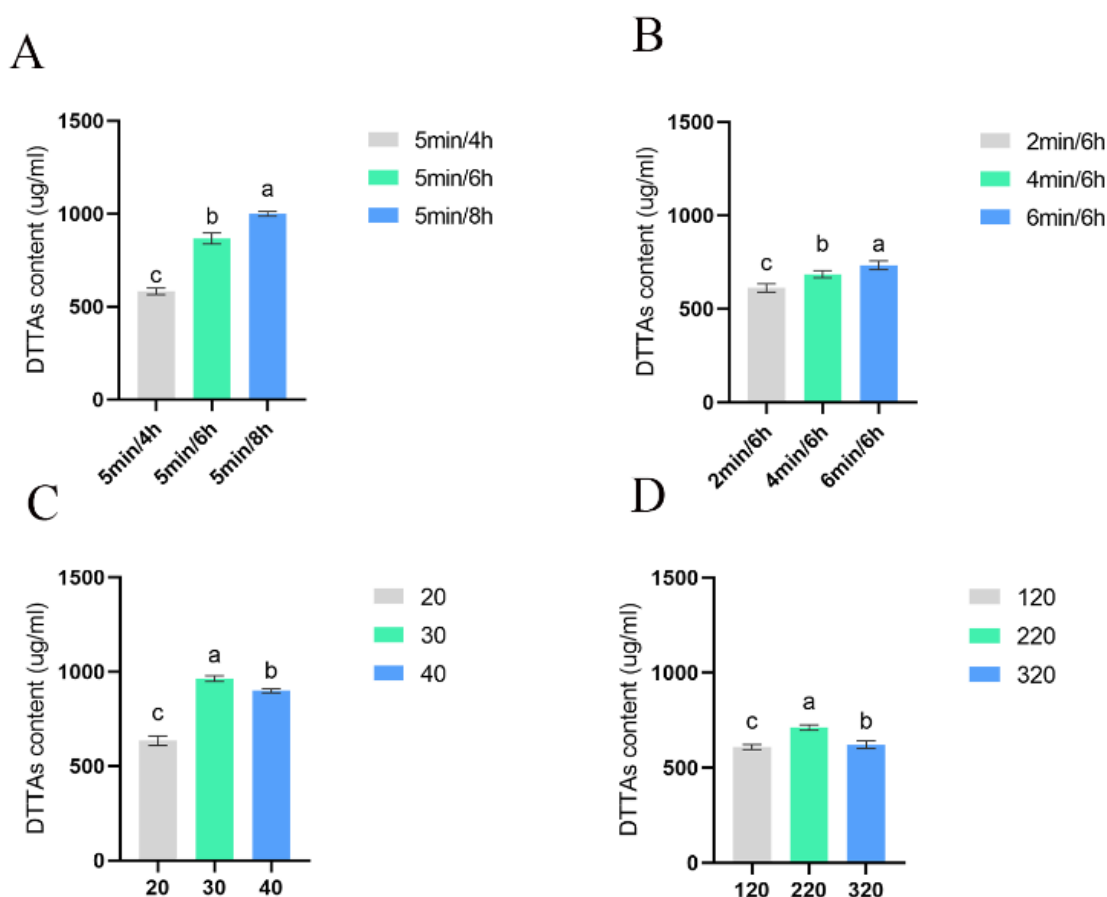


Figure 1

Effects of different treatment parameters on the total alkaloid content of *D. nobile* seedlings. A: different immersion frequency B: different immersion time C: different Medium sucrose concentration D: different inoculum concentration; Error bar represent mean $\pm$ SD, Different letters in each column represent means significantly different at $P < 0.05$.

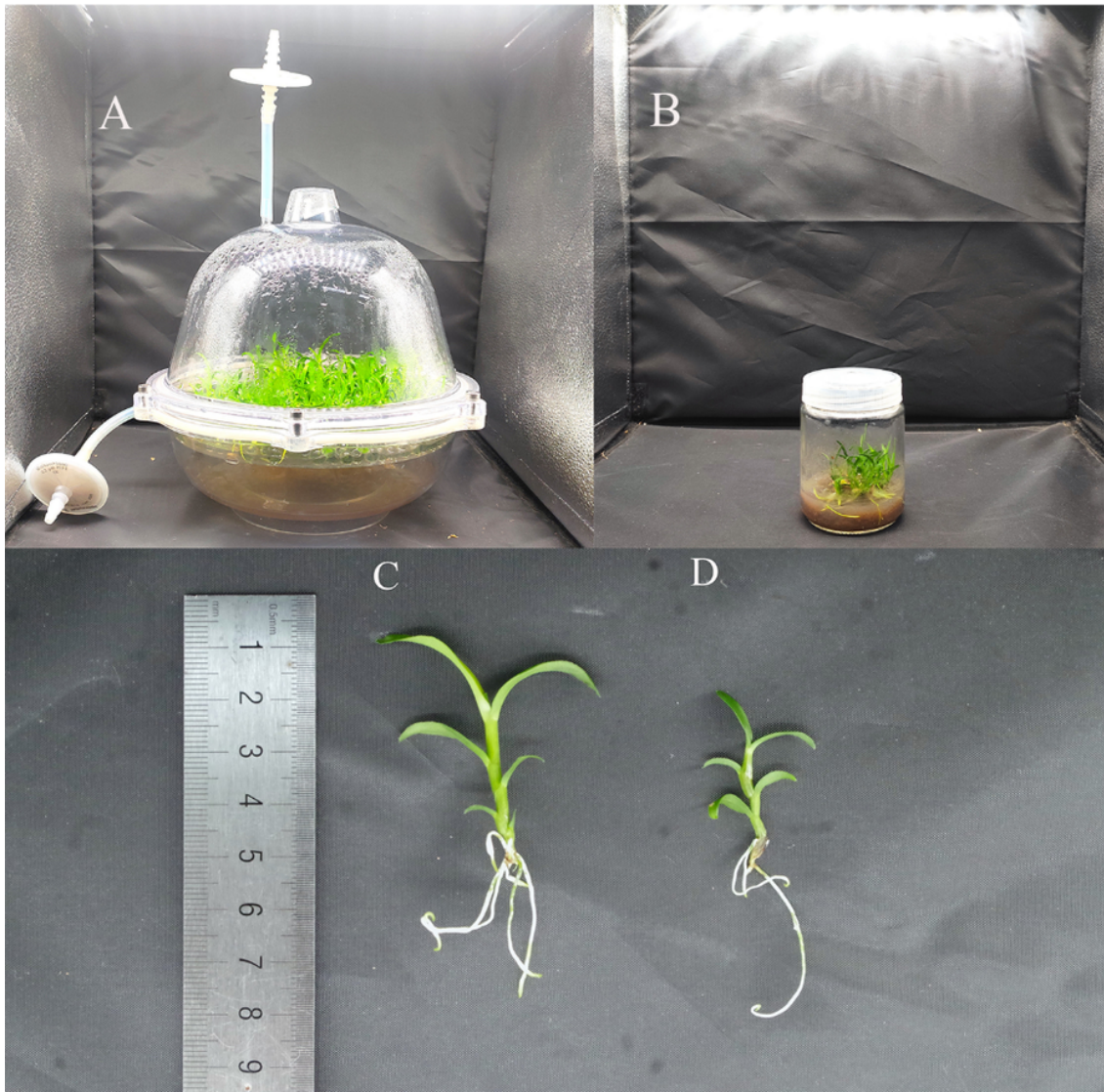


Figure 2

Effects of different culture methods on the growth status of *D.nobile* seedlings: TIBS culture (A), solid culture (B), morphology of TIBS system cultured seedlings (C), morphology of solid cultured seedlings (D)

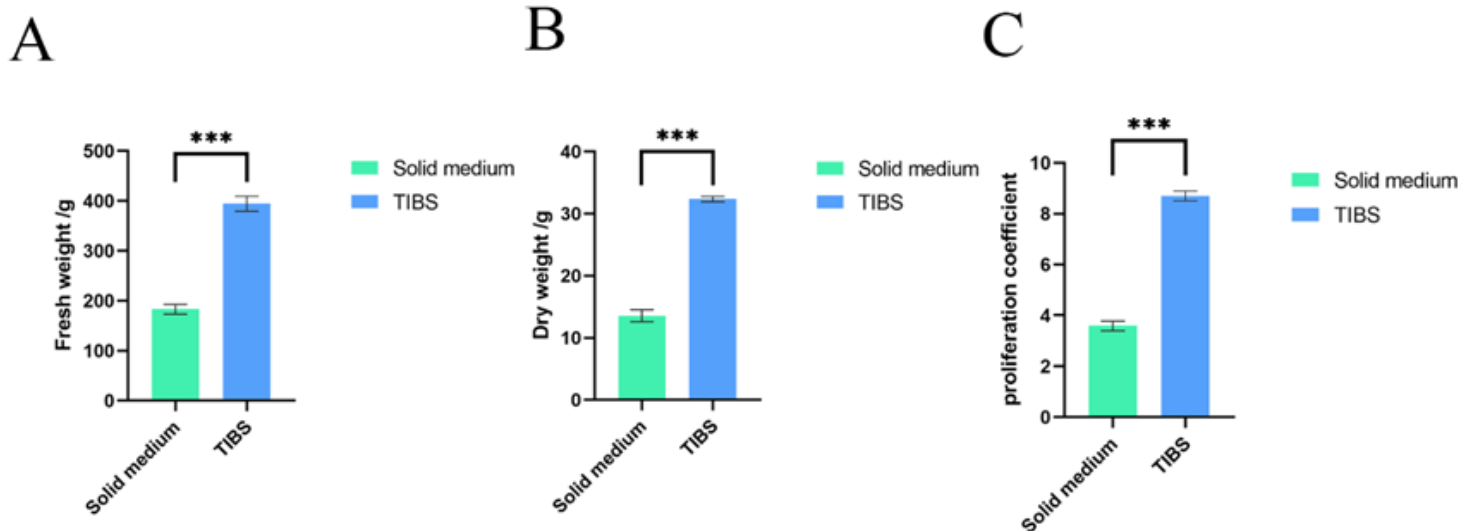


Figure 3

Effects of different culture methods on fresh weight(A), dry weight(B), and proliferation coefficients(C) of *D.nobile* seedlings; Error bar represent mean $\pm$ SD, * $p < 0.05$.

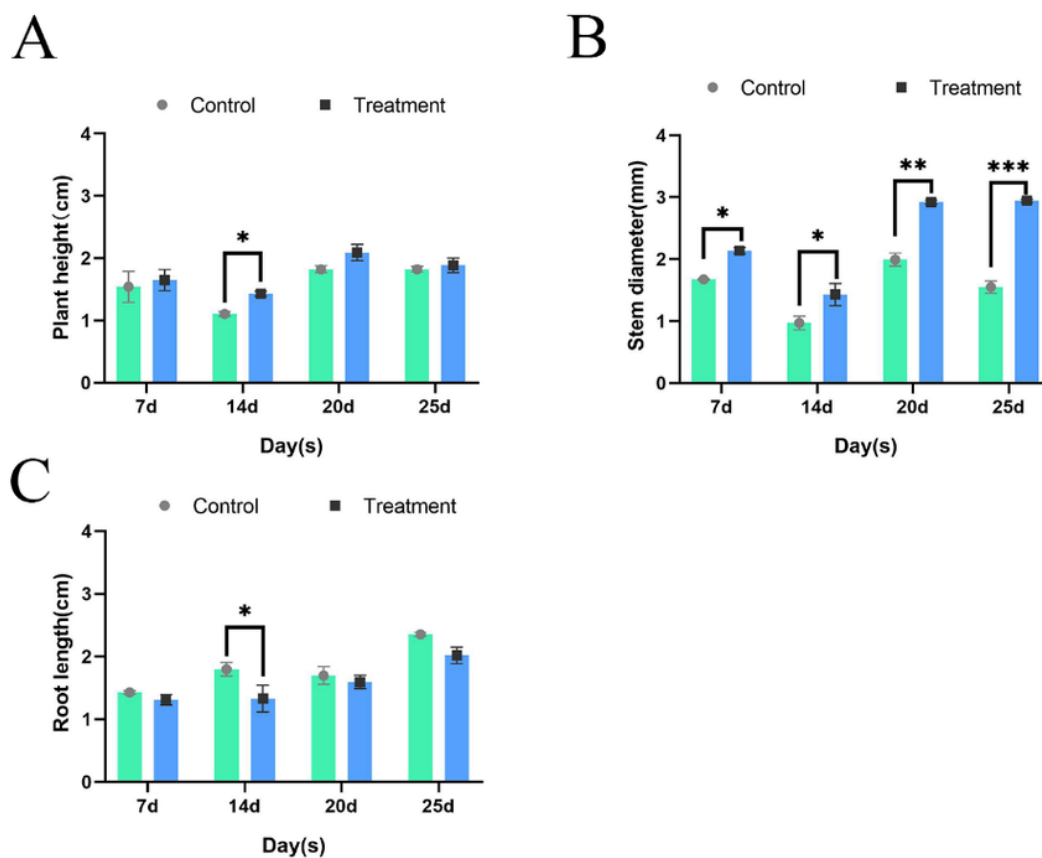


Figure 4

Effects of endophytic fungus MD33 on coculture growth state of *D. nobile* A: Plant height B: stem diameter C: Root length ; Error bar represent mean $\pm$ SD, *p < 0.05.

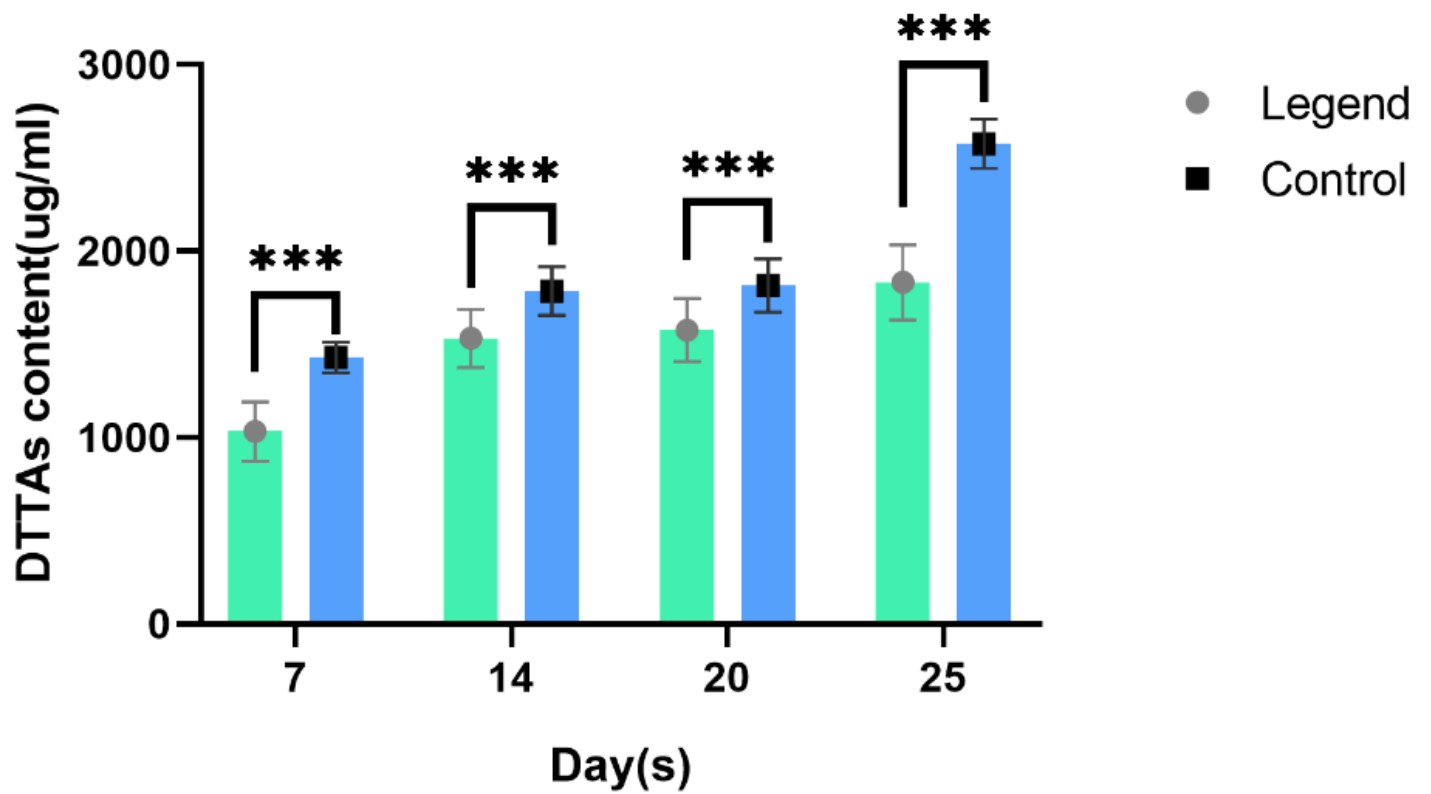


Figure 5

Effects of endophytic fungus MD33 on total alkaloids content after co-culture with *D.nobile* for different time periods ;Error bar represent mean $\pm$ SD, * $p < 0.05$.