Pak. J. Bot., 54(6): 2167-2172, 2022. . DOI: http://dx.doi.org/10.30848/PJB2022-6(7)

ESTABLISHMENT OF AN EFFICIENT CELL SUSPENSION CULTURE SYSTEM FOR *LONICERA JAPONICA* THUNB.

NING WANG, HAO CHEN, QINGHAI GAO AND JINGJING QIAN*

Anhui Science and Technology University, No.9, Donghua Road, Fengyang Country, Anhui Province, 233100, China
**Corresponding author's email: Qianjj@ahstu.edu.cn*

Abstract

Lonicera japonica Thunb (*L. japonica* T.) has a high medicinal value. Establishing a cell suspension system lays the research foundation for large-scale extraction of the medicinal products of *L. japonica T.*. Using stem segments from *L. japonica T.* tissue culture seedling as material, the effects of various hormone combinations on the growth of *L. japonica T. cells* were studied, the effects of initial inoculum, pH and sucrose concentration of culture medium on cell suspension culture were studied, and the optimal culture time of cell suspension culture was screened. This experiment established an efficient cell suspension culture system from the *L. japonica* T., stem segment to provide a foundation for large-scale extraction. The following were the observed results: (1) Different hormone combinations had varying effects on the growth of L. japonica. 1.5 mg/L Indol-3-Butyric acid (IBA) had the best effect on plantlet rooting in japonica cells, while 1.0 mg/L 6-Benzylaminopurine (6-BA) was suitable for bud differentiation, and 0.2 mg/L 6-BA + 0.8 mg/L 1-Naphthaleneacetic acid (NAA) had the best effect on callus dedifferentiation. (2) For the optimal cell suspension culture system, the best initial inoculum weight was 50 g/L, with a pH of 5.8 and a sucrose concentration of 3%. (3) The cell growth curves were determined using the optimal cell suspension system described above. Cell growth peaked at 18 days of suspension culture, with the fresh weight of cells being 676.0±12.66 g/L; 13.52 times higher than the initial inoculation amount.

Key words: *Lonicera japonica* Thunb; Tissue culture; Cell suspension.

Introduction

Lonicera japonica Thunb. (*L. japonica* T.) is a herbaceous plant of the lonicera family (Li, *et al.*, 2020). It is a traditional Chinese medicine with a long history (Bong *et al.*, 2021). The main extracts are chlorogenic acid, flavonoids (Wang *et al.*, 2016), and polyphenolic chemicals, which have antioxidant (Jiao *et al.*, 2018), anti-inflammatory (Park *et al.*, 2012), and cancer treatment (Park *et al.*, 2018) effects. Currently, the effective substance of *L. japonica* T. needs to be extracted from the flower, which is time-consuming and laborious (Jurgoński *et al.*, 2013).

Biotechnological methods for the production of plant cells, tissues, and organs through *In vitro* culture have been regarded as an alternative system for the large-scale production of bioactive compounds, and suspension culture of plant cells is a new technology for the mass production of a variety of secondary metabolites in a relatively short time (Haida *et al.*, 2019). Various studies, at home and abroad, have used this technology for the large-scale production of Chinese herbal medicines and other plants; and achieved an improvement of the content of target components in the plant cell culture (Sánchez-Ramos *et al.*, 2019). However, establishing a suspension culture system for *L. japonica* T., had not been previously observed.

In this research, the stem segment of the *L. japonica* T. tissue culture system was improved, the cells were induced, and the cell suspension culture system was established. This will provide the material basis for producing secondary metabolites by mass culture of *L. japonica* T. cells, the study of synthesis pathway of *L. japonica* T. metabolites, and provides a basis for the simple and rapid extraction of compounds.

Materials and Methods

The experimental materials for this research came from the Horticultural Laboratory of Anhui Science and Technology University, along with the stem segments of the test-tube seedlings with robust growth.

Establishing a callus culture framework for *L. japonica* T.

Induction: The shoot segments (approximately 1 cm in length) from young *L. japonica* T. plants were chosen, and the leaves and axillary buds were discarded. The shoot segments were cultured in the medium for 35 days.

Hormone application: The primary medium was the Murashige and Skoog (MS) medium, which contained 3% sucrose and 0.55% agar and had a pH of 6.2. Different concentrations of Indol-3-Butyric acid (IBA), 6-Benzylaminopurine (6-BA), and 1-Naphthaleneacetic acid (NAA) were added (as shown in Table 1). The medium was then subjected to sterilization at 121°C for 20 min. The explants were transplanted in a germ-free environment. A total of 33 treatments with 15 replicates per group were performed. The plantlets were cultured for 35 days, and the data were recorded according to the following formulas:

$$\text{Induction rate of adventitious buds (\%)} = \frac{\text{No. of budding (n)}}{\text{No. of inoculations (n)}} \times 100$$

$$\text{Mean No. of proliferation (n)} = \frac{\text{total No. of proliferation (n)}}{\text{No. of inoculations (n)}}$$

$$\text{Rate of rooting (\%)} = \frac{\text{No. of Roots (n)}}{\text{No. of inoculations (n)}} \times 100$$

$$\text{Mean root length (cm)} = \frac{\text{Total root length (cm)}}{\text{Total No. of roots (n)}}$$

$$\text{Callus formation frequency} = \frac{\text{No. of induced callus}}{\text{No. of inoculations}} \times 100$$

$$\text{Mean weight of induced callus} = \frac{\text{Weight of total induced callus}}{\text{No. of inoculations}}$$

Table 1. The ratio of different concentrations of hormones.

NO.	IBA ($mg \cdot L^{-1}$)	6-BA ($mg \cdot L^{-1}$)	NAA ($mg \cdot L^{-1}$)
A1	0.5	0	0
A2	1.0	0	0
A3	1.5	0	0
A4	2.0	0	0
A5	0	0.5	0
A6	0	1.0	0
A7	0	1.5	0
A8	0	2.0	0
A9	0	0.2	0.2
A10	0	0.2	0.4
A11	0	0.2	0.6
A12	0	0.2	0.8
A13	0	0.2	1.0
A14	0	0.4	0.2
A15	0	0.4	0.4
A16	0	0.4	0.6
A17	0	0.4	0.8
A18	0	0.4	1.0
A19	0	0.6	0.2
A20	0	0.6	0.4
A21	0	0.6	0.6
A22	0	0.6	0.8
A23	0	0.6	1.0
A24	0	0.8	0.2
A25	0	0.8	0.4
A26	0	0.8	0.6
A27	0	0.8	0.8
A28	0	0.8	1.0
A29	0	1.0	0.2
A30	0	1.0	0.4
A31	0	1.0	0.6
A32	0	1.0	0.8
A33	0	1.0	1.0

Establishing a suspension culture system for *L. japonica* T. cells

Primary culture: The best-performing cells were transplanted into MS liquid medium with the previously mentioned hormone mixture, supplemented with 3% sucrose, and the pH adjusted to 5.8. The cells were then cultured in a shaker at 110 rpm, and at a temperature of 22±2℃.

Effects of inoculation size on the growth of *L. japonica* T. suspension cells: Cells with uniform growth, viscous, light green, and well-dispersed conditions were collected. The collected cells were then transplanted into 50 ml MS liquid medium in batches of 1.0 g, 1.5 g, 2.0 g, 2.5 g, and 3.0 g to achieve final concentrations of 20 g/L, 30 g/L, 40 g/L, 50 g/L, and 60 g/L, respectively. All cells were subsequently cultured in a shaker at 110 rpm, and at a temperature of 22±2℃. Each inoculation unit represented a treatment. Three replicates were made for each treatment. The cells were cultured for 21days. From the ninth day, the fresh weight of cells were counted every three days for five total times.

Effects of pH on the growth of suspension cells of *L. japonica* T.: To render the final proportion of cells to 50 mg/L, the cells with uniform growth, viscous, light green, and well-distributed conditions were inoculated into MS liquid medium with various pH values (5.6, 5.8, 6.0, and 6.2). The cells were then cultured in a shaker at 110 rpm, and at a temperature of 22±2℃. Each pH unit represented a treatment. Three replicates were made for each treatment. After 21 days, the cells were collected and weighted. The yield of cells was calculated as follows: Yield of cells = total weight of cells-inoculated weight.

Effects of sucrose on the growth of *L. japonica* T. suspension cells: Cells with uniform growth, viscous, light green, and well-dispersed conditions were inoculated into 50 mL MS liquid medium (pH 5.8) to make the final proportion of cells to 50 mg/L. Then 0.5 g, 1 g, 1.5 g and 2 g of sucrose was added into the medium to make the final concentration of sucrose as 1%, 2%, 3% and 4%, respectively. The cells were then cultured in a shaker at 110 rpm, and at a temperature of 22±2℃. A medication was assigned to each sucrose concentration. Each treatment was replicated three times, and the cells were extracted and weighed after 21 days.

Suspension cells of *L. japonica* T. with a dynamic growth curve: Using the ideal conditions tested in sections 1.3.2, 1.3.3, and 1.3.4, cells with uniform growth, viscous, light green, and well-dispersed conditions were inoculated into MS liquid medium and then cultured in a shaker for 21 days at 110 rpm, and at a temperature of 22±2℃. The fresh weight of cells was measured every three days for seven total times from the beginning of the cultivation. Each sampling represented a treatment, and each treatment had three replicates. The dynamic growth curve of cells was drawn according to the results.

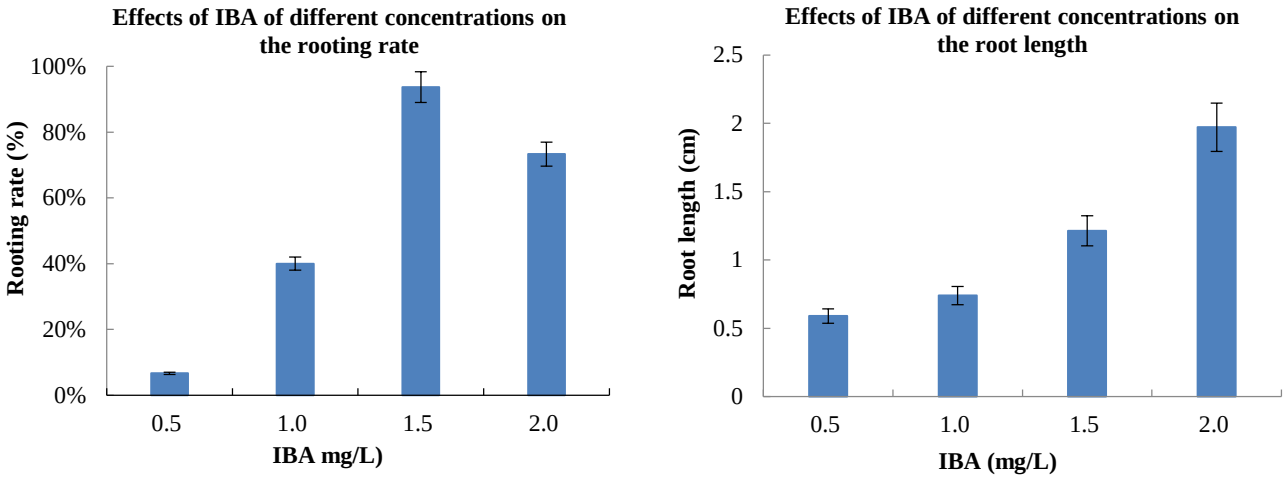

Fig. 1. Effects of IBA of different concentrations on the rooting rate and length of tissue culture of *L. japonica* T.

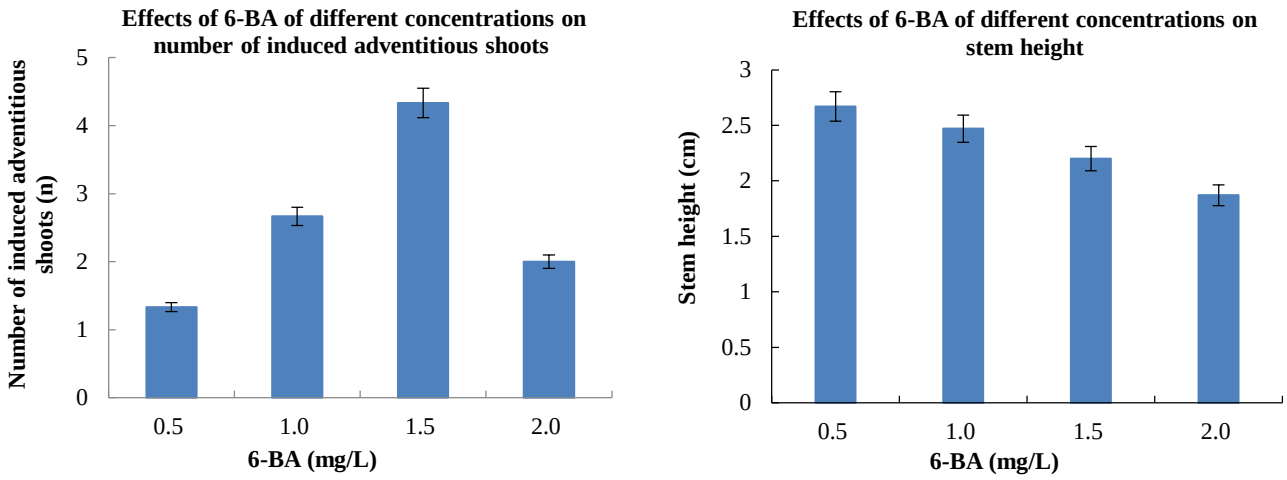

Fig. 2. Effects of 6-BA of different concentrations on number of induced adventitious shoots and stem height.

Results

Effects of hormones on the growth of explants of *L. japonica* T.: IBA has the ability to trigger the rooting of *L. japonica* T. explants, but its effectiveness is dependent on the amount of IBA used (Fig. 1). The root length was related to the concentration of IBA in the medium in a positive way (Fig. 1A). The mean root length reached 1.97±0.19 cm under 2.0 mg/L IBA induction. However, a higher concentration of IBA (2.0 mg/L) inhibited the rooting of plantlets, which could be seen for the rate of rooting result where it was only 73.33%±4.0%, which was 0.78-fold of that under the lower concentration of IBA (1.5 mg/L). Furthermore, the roots were not strong under a higher concentration of IBA treatment.

The addition of 6-BA to the MS medium was used to induce adventitious buds. After 35 days of cultivation, the growth of adventitious buds decreased as the concentration of 6-BA increased (Fig. 2A). The best growth occurred under 0.5 mg/L 6-BA induction, up to 2.67±0.12cm; the mean length of adventitious buds was up to 2.50±0.10cm under 1.0 mg/L 6-BA induction. In a specific range, 6-BA had a positive effect on the induction rate of adventitious buds. When the concentration of 6-BA was between 0.5 and 1.0 mg/L, the induction of adventitious buds increased, reaching a peak of 93.30%±0.31% at 1.0 mg/L. The induction rate of adventitious buds steadily decreased when the concentration of 6-BA was 1.0-2.0 mg/L.

The combinations of A9, A13, A15, A17, A29, and A33 had the best induction of callus, up to 100%. The combination of A12 had the most significant contribution to the proliferation of callus, up to 23.30±0.06g after 35 days of cultivation, and the cells were well dispersed, representing the suitable condition for suspension culture. Therefore, this combination (MS + 6-BA 0.2 mg/L + NAA 0.8 mg/L) is considered the optimal proportion for callus induction and proliferation (Fig. 3).

Scale of the initial inoculation: When the scale of inoculation was less than 30 g/L, the cell growth rate was slower, and the cell fresh weight peaked at 21 days. When the scale of inoculation was 40-50 g/L, the cell growth rate was higher, and the cell fresh weight peaked at 18 days. The most significant growth reached 675.94±12.07g/L under the inoculation size of 50 g/L, which was 39.05, 6.13, 37.94, and 62.21 g/L higher than other concentrations, respectively. When the scale of inoculation reached 60 g/L, the growth of cells slowed due to fewer nutrients and faster senescence, and their fresh weight was lower than that of the 50 g/L inoculation size. The results show that the initial inoculation size of 50 g/L was optimal for the suspension culture of *L. japonica* T. cells.

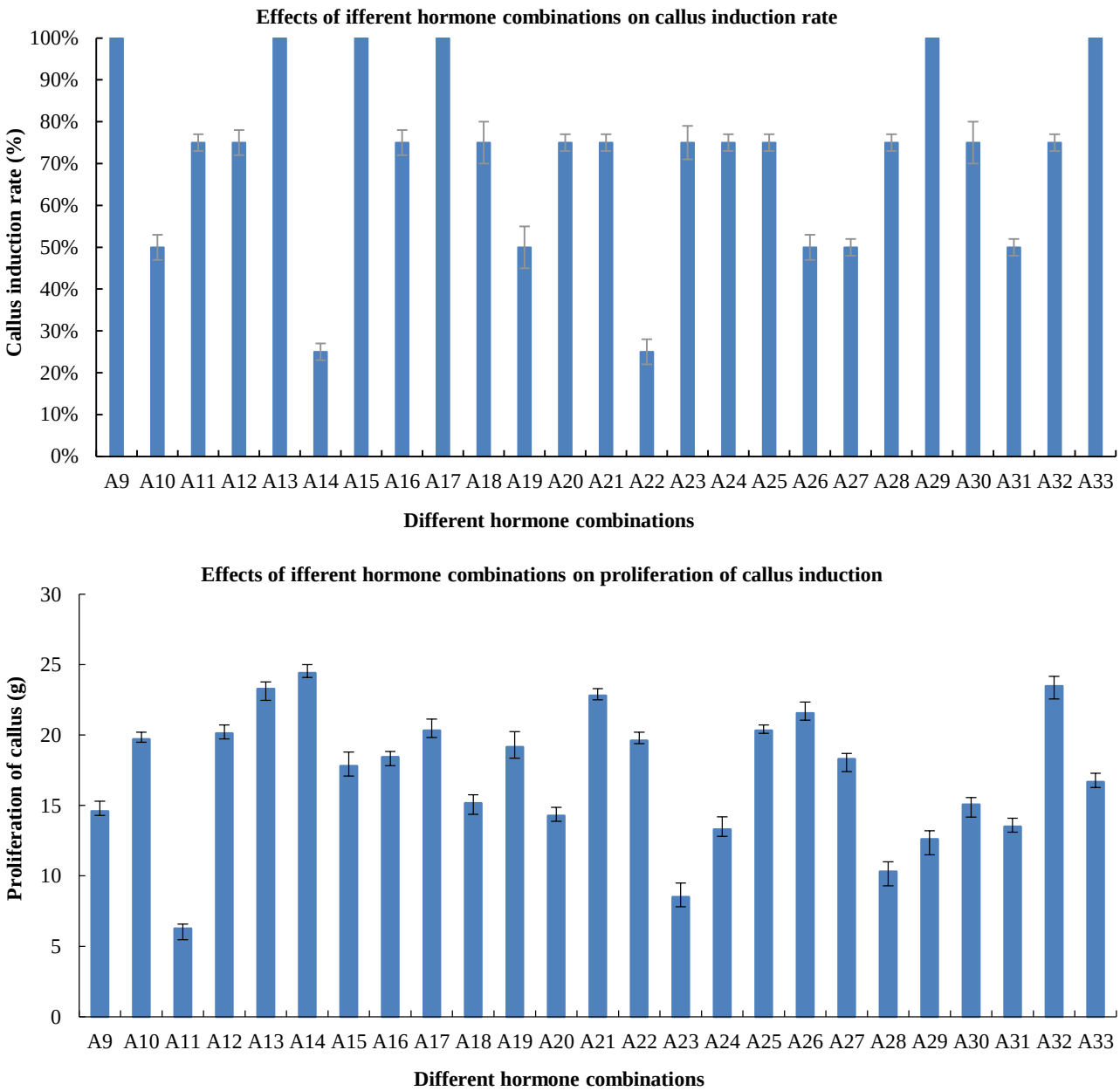

Fig. 3. Effects of different hormone combinations on callus induction rate and proliferation of callus induction.

pH: When the pH was raised from 5.6 to 5.8, the fresh weight of suspension cells increased from 546.05±18.36 g/L to 655.84±20.84 g/L, as shown in Fig. 4B. While the pH value was greater than 5.8, the fresh weight of the cells decreased significantly. When the pH was 6.2, the fresh weight of the cells was just 462.20±14.43 g/L. Furthermore, as shown in Fig. 5, pH variations may influence cell colour, as the cells changed from beige to yellow and formed a mass-like shape. As a result, the optimum pH for *L. japonica* T. suspension cells is 5.8.

Sucrose concentration: As shown in Fig. 4C, under lower sucrose concentration (1%), the growth of cells was slower; the fresh weight was only 334.21±22.68 g/L. With the increase of sucrose concentration, the growth of cells became faster and reached a peak at 3% sucrose concentration. However, when over 3%, the growth of the cells was inhibited. The fresh weight of cells was 534.18±21.35 g/L at 4% sucrose, only 81.65% of that of the 3% sucrose concentration.

The growth curve of suspension cells under optimal conditions: In a range of 24 days of culture, the growth curve of suspension cells displayed an 'S' pattern (Fig. 6). In detail, 0-6 days represented a lag phase since the cells grew slowly and gained less weight. The fresh weight was only 36.24±0.83g/L, which was 20.1% of the inoculation scale. From 6-15 days, the cell growth reached the logarithmic growth phase. During this time, the growth of cells was faster. At 15 days, the fresh weight was up to 619.60±21.36 g/L. Subsequently, the growth of cells remained higher speed until 18 days, which represented a stable phase. At 18 days, the fresh weight had reached 676.16±12.66 g/L. The decline period lasted from 18-24 days, during which the fresh weight of the cells steadily decreased. As a result, the best culture time for suspension cells was 18 days.

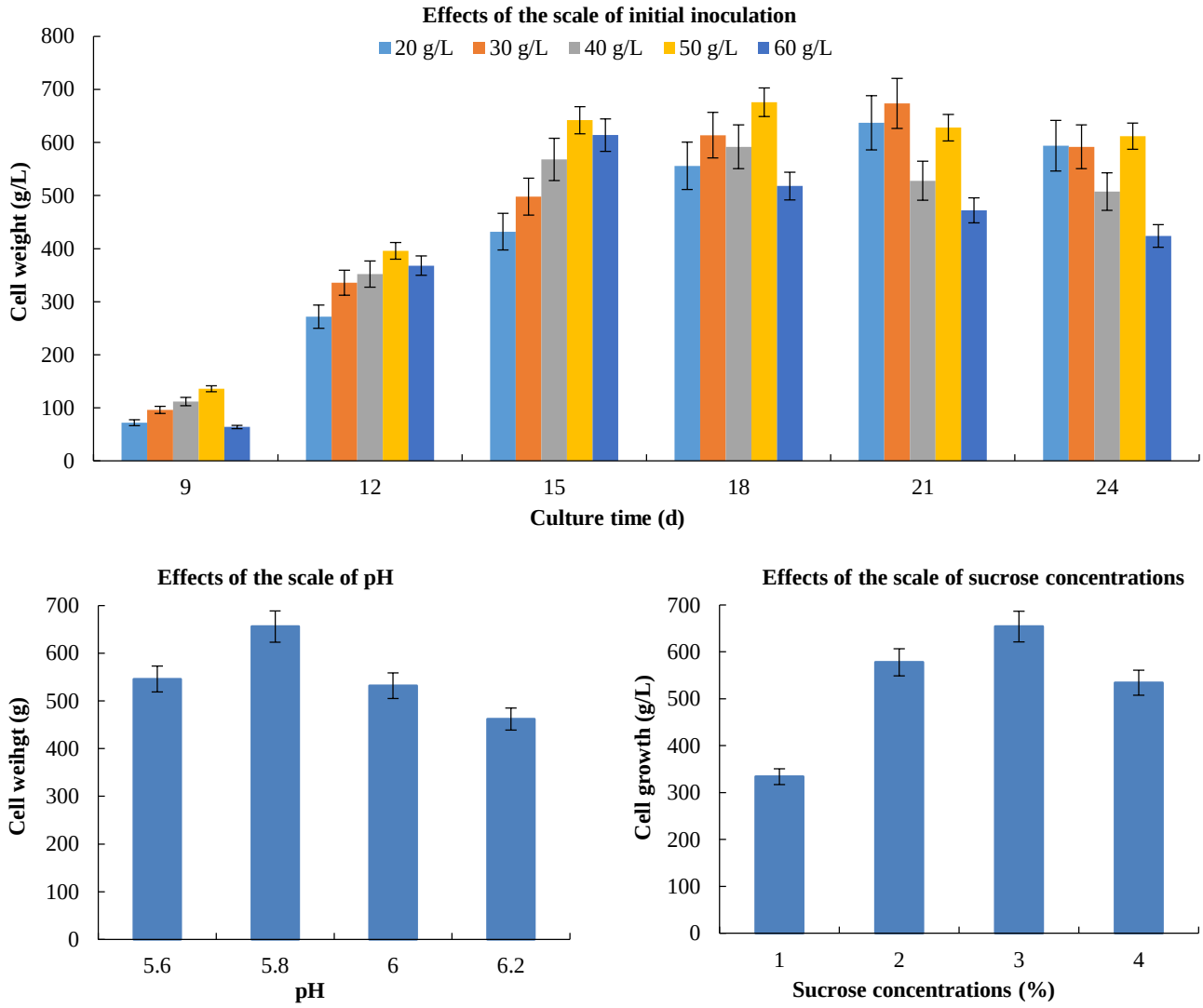

Fig. 4. Effects of the scale of initial inoculation (A), pH (B), and sucrose concentrations (C) on the cell growth of *L. japonica* T. suspension cells.

Fig. 5 Effects of different pH value on the color of *L. japonica* T. suspension cells.

Discussion

Cell suspension culture is a technique in which free plant cells or small cell clusters are cultured and grown in a liquid culture medium. A good suspension system must first meet the requirements of loose callus, good uniformity, and a fast proliferation rate. The growth state of plant callus and the type of structure formed can usually be regulated by the type of hormone and its concentration, especially the concentration and ratio of auxin and cytokinin (Ahmad *et al.*, 2021). The key factors affecting the establishment of plant cell suspension system are the composition of the medium (Sivanandhan *et al.*, 2013), the initial inoculum amount (Villegas-Quiceño *et al.*, 2018) and cultivation days, the composition of the medium includes hormone content (Khandy *et al.*, 2017), sucrose content (Deepthi *et al.*, 2017), and pH value (Naik *et al.*, 2020). This research

discovered the optimal conditions for rooting, adventitious bud, and callus induction by plant hormone, optimal inoculation scale, pH value, sucrose concentration, and incubation time for *L. japonica* T. cell suspension culture. Additionally, the essential system for the rapid propagation and cell suspension culture of *L. japonica* T. ex vivo was established.

In the final analysis, the optimal medium for adventitious buds induction was MS + 1.0 mg/L 6-BA; the optimal medium for root growth was MS + 1.5 mg/L IBA; the best conditions for suitable callus are MS + 0.2 mg/L 6-BA + 0.8 mg/L NAA; the best conditions for cell growth are MS + 0.6 mg/L 6-BA + 0.5 mg/L NAA + 0.2 mg/L 2,4-D; and the best initial inoculation scale is 60 g/L, with pH 5.8, 3% sucrose, and 18 days of cultivation.

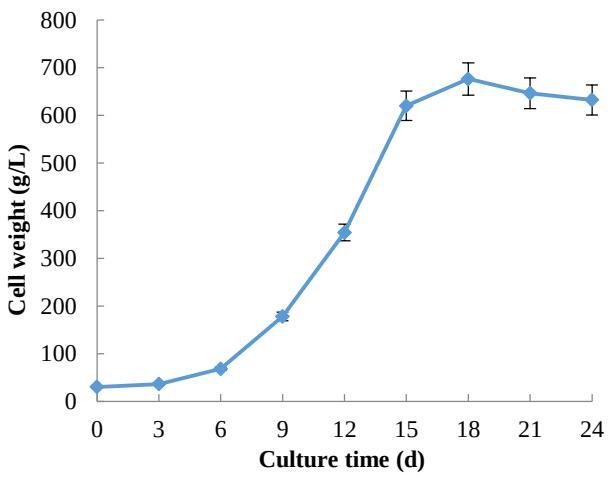

Fig. 6. Growth curve of L. japonica T. suspension cells.

Acknowledgement

This work was supported by the Industry-university Research (grant number ZY1008); person foundation of ASTU (grant number SKYJ201702); Key research and development plan of Anhui Province Funding (grant number: 201904a06020015); Key research and development plan of Anhui Province Funding (grant number: 202004a07020031).

References

Ahmad, A., Q. Muhammad Tahir, A. Shoukat, M.M. Aslam, M. Tariq, M. Hakiman and F.A. Joyia. 2021. The effects of genotypes and media composition on callogenesis, regeneration and cell suspension culture of chamomile (*Matricaria chamomilla* L.). *PeerJ*., 250: 885-898.

Bong, F.J., N.J.Y. Chear, S. Ramanathan, M.K. Nethia, S. Sreeramanan and L.C. Bee. 2021. The development of callus and cell suspension cultures of sabah snake grass (*Clinacanthus nutans*) for the production of flavonoids and phenolics. *Biocat. & Agri. Biotech.*, https://doi.org/10.1016/j.bcab.2021.101977.

Deepthi, S. and K. Satheeshkumar. 2017. Cell line selection combined with jasmonic acid elicitation enhance camptothecin production in cell suspension cultures of *Ophiorrhiza mungos* L. *Appl. Microbiol. Biotechnol*., 101(2): 545-558.

Haida Z, Syahida A, Syed MA, Maziah M and Hakiman M. 2019. Factors affecting cell biomass and flavonoid production of *Ficus deltoidea* var. kunstleri in cell suspension culture system. *Scientific Rep. Rts.*, 9: 1-8.

Jiao M, Zhao M, Lin L and Wang Y. 2018. *Lonicera japonica* Thunb. extract improves the quality of cold-stored porcine patty through inhibition of lipid and myofibrillar protein oxidation. *Int J of Food Sci Tech*., 53(4): 986-993.

Jurgoński, A., J. Juśkiewicz and Z. Zduńczyk. 2013. An anthocyanin-rich extract from kamchatka honeysuckle increases enzymatic activity within the gut and ameliorates abnormal lipid and glucose metabolism in rats. *Nutrition*, 29: 898-902.

Khandy, M.T., D.V. Kochkin, S.V. Tomilova, B.A. Galishev, E.S. Sukhanova, A.G. Klyushin, I.M. Ivanov and A.M. Nosov. 2017. Obtaining and study of callus and suspension plant cell cultures of *Tribulus terrestris* L., a producer of steroidal glycosides. *Appl Biochem Microbiol*, 53(8): 800-806.

Li, R.J., X.P. Kuang, W.J. Wang and W.X. Li. 2020. Comparison of chemical constitution and bioactivity among different parts of *Lonicera japonica* Thunb. *J Sci Food Agric.*, 100(2): 614-622.

Naik, P.M. and J. Al-Khayri. 2020. Influence of culture parameters on phenolics, flavonoids and antioxidant activity in cell culture extracts of date palm (*Phoenix dactylifera* L.). *Erwerbs – Obstbau*, 62(2): 181-188.

Park, C., W.S. Lee, M.H. Han, K.S. Song, S.H. Hong, A. Nagappan, G.Y. Kim, G.S. Kim, J.M. Jung, C.H. Ryu, S.C. Shin, S.C. Hong and Y.H. Choi. 2018. *Lonicera japonica* Thunb. Induces caspase-dependent apoptosis through death receptors and suppression of AKT in U937 human leukemic cells. *Phytotherapy Research*, 32(3): 504-513.

Park, K., S.R. Kang, H.S. Park, D.H. Lee, A. Nagappan, J.A Kim, S.C. Shin, E.H. Kim, W.S. Lee, H.J. Chung, S.J. An and G.S. Kim. 2012. Regulation of Proinflammatory Mediators via NF-κB and p38 MAPK-Dependent Mechanisms in RAW 264.7 Macrophages by Polyphenol Components Isolated from Korea *Lonicera japonica* Thunb. *Evidence-Based Complementary and Alternative Medicine*, doi:10.1155/2012/828521.

Sánchez-Ramos, M., L. Alvarez, A. Romero-Estrada, A. Bernabé-Antonio, S. Marquina-Bahena and F. Cruz-Sosa. 2020. Establishment of a cell suspension culture of ageratina pichinchensis (Kunth) for the improved production of anti-inflammatory compounds. *Plants*, 9(10): 1398.

Sivanandhan, G., G. Kapil Dev, M. Jeyaraj, M. Rajesh, M. Muthuselvam, N. Selvaraj, M. Manickavasagam and A. Ganapathi. 2013. A promising approach on biomass accumulation and withanolides production in cell suspension culture of *Withania somnifera* (L.) dunal. *Protoplasma*, 250(4): 885-98.

Villegas-Quiceño, A.P., J. Arias-Echeverri, D. Aragón-Mena, S. Ochoa-Cáceres and M.E. Arias-Zabala. 2018. Multi-objective optimization in biotechnological processes: application to plant cell suspension cultures of *Thevetia peruviana*. *Revista Facultad de Ingeniería Universidad de Antioquia*, (87): 35-40.

Wang, L., J. Qiu, J. Hu and J. Li. 2016. Research progress on chemical constituents of *Lonicerae japonicae* flos. *Bio. Med. Research International*, 2016(3): 1-18.

(Received for publication 10 August 2021)