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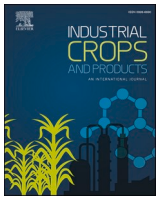

Kinetic analysis and modelling of therapeutically important dodeca-2E,4E,8Z,10E, Z-tetraenoic acid isobutylamides production from adventitious root cultures of *Spilanthes paniculata* Wall. ex DC.

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ABSTRACT

Spilanthes paniculata Wall. ex DC., toothache plant, is known for anaesthetic, anti-inflammatory, analgesic and antimalarial properties due to presence of N-alkylamides as primary therapeutic component. Current study focuses on production and kinetic modelling of dodeca-2E,4E,8Z,10E,Z-tetraenoic acid isobutylamides (DTAI) (an isomeric mixture) from in vitro adventitious root cultures induced from leaf-disc explants of *Spilanthes* on semi-solid MS medium fortified with auxins at varied concentrations. Highest root induction (86.66 %) was observed on medium supplemented with 2.5 μM Indole-3-acetic acid (IAA). These root cultures were transferred to suspension medium where MS salt strength, carbon source and inoculum density were optimized for root proliferation. Maximum fresh weight (85.33 ± 1.52 g/l) and dry weight (8.60 ± 0.15 g/l) of root biomass were obtained on ½MS (Only macronutrients were reduced to half strength) + 2.5 μM IAA. The DTAI was identified and quantified using HPLC, confirmed through mass spectrometry and highest productivity of 697.35 ± 25.03 μg/l was obtained after 18th day of inoculation. Growth kinetics of root biomass and DTAI production were studied using different kinetic models. Logistic model revealed maximum specific growth rate (μ_{max}) and root biomass (X_{max}) of 0.26 day^{-1} and 8.23 g/l (dry weight), respectively. Luedeking-Piret model calculated the growth associated constant (α) and non-growth associated constant (β), signifying DTAI production as growth associated phenomenon. Moreover, modified Gompertz model also applied to calculate the maximum DTAI productivity and rate of DTAI production, with a lag phase of 3.59 days. This study provides an optimized growth parameters for constant and quality production of DTAI without seasonal and geographical restrictions.

1. Introduction

N-alkylamides (NAAs) are a prominent group of naturally occurring secondary metabolites with different decarboxylated amino acids and unsaturated fatty acids attached through peptide linkage (Silveira et al., 2018). More than 300 N-alkylamides have been identified from more than 25 plant families, including Aristolochiaceae, Asteraceae, Brassicaceae, Convolvulaceae, Euphorbiaceae, Menispermaceae, Piperaceae, Poaceae, Rutaceae, and Solanaceae (Méndez-Bravo et al., 2011). These metabolites are popularly known to possess anti-viral (Cech et al., 2010) antimalarial (Rajendran et al., 2017), anthelmintic (Singh et al., 2014), anaesthetic (Barbas et al., 2016), insecticidal (Haw and Keng, 2003) (Sharma et al., 2012), anti-inflammatory (Chakraborty et al., 2004) and immune-modulating (Savadi et al., 2010) properties. The broad functional spectrum of these metabolites has led to an enormous increase in biomedical importance in the last two decades. Besides these traditional pharmacological properties, plants containing NAAs are used in food as spices (for their aromatic and tingling sensations) and body care products (wrinkle smoothening) (Borse et al., 2022; Debnath et al., 2024).

The demand for N-alkylamides is gradually increasing due to their higher market potential in medicinal, food and body care products. The dodeca-2E,4E,8Z,10E,Z-tetraenoic acid isobutylamides (DTAI) is one of the main bioactive components responsible for many therapeutic applications including immunostimulatory, anti-inflammatory and analgesic effects (Cai et al., 2024; Goey et al., 2012). The compound was earlier reported from the roots of *Echinacea* species, a wild population found in North America only. Overharvesting of wild *Echinacea* plants

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and lack of alternative resources have been a significant concern to the herbal industry (Bauer and Remiger, 1989; Kindscher et al., 2008). *Spilanthes paniculata* is a valuable natural source of these N-alkylamides, commonly distributed in different tropical and subtropical parts of the world. Recently, it has been declared threatened and included in the Red data book due to its overexploitation in the past few years (Bhat et al., 2016). As a result, the plant material from the wild resources has become inadequate due to the continuous expansion of market demand. Chemical synthesis of plant secondary metabolites is difficult due to their chiral and complex nature (Wu et al., 2021). Therefore, in vitro tissue culture methods have been an excellent alternative to produce N-alkylamides and reduce the burden on natural flora. Recent biotechnological research advances have shown that adventitious root culture is an attractive alternative to whole plant cultivation for producing biomass and secondary metabolites (Baque et al., 2012).

Mathematical modelling of plant biomass and metabolite production from in vitro liquid cultures could be advantageous for understanding the effects of various process parameters to better control any specific system (Chattopadhyay et al., 2002). The kinetic modelling of a biotechnological process also reduces the time consumed during optimization studies. Although kinetic modelling has been widely used in bioprocess applications, such techniques in plant cell culture applications must increase to reduce the already discussed experimental and development costs and ensure the economic feasibility of tissue culture processes and products (Pan et al., 2020).

Despite the extensive use of *S. paniculata* in traditional medicines, only a few in vitro cell, tissue and organ culture reports are available. The potential for producing bioactive NAAs from in vitro cultivation has not been explored yet. As far as we know, this is the first report on the adventitious root culture of *S. paniculata* for producing bioactive N-alkylamide. Our initial study observed the tendency of adventitious root formation from various explants of *S. paniculata,* and several NAAs were identified from the root biomass. Based on the initial findings, the current research focuses on developing a protocol for sustainable production of adventitious root biomass and NAAs under in vitro conditions. Three commonly used auxins, i.e. indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and Naphthalene acetic acid (NAA), were tested for root induction to find the suitable auxin and its optimized concentration. The induced roots were transferred to suspension medium for further proliferation. The effect of different carbon sources and macronutrient strengths on biomass and NAAs production were also investigated. Furthermore, the kinetics of root biomass growth and NAAs synthesis were studied to construct kinetic models. The logistic regression equation was used to build a kinetic model for adventitious root biomass growth, and NAAs production was modelled using Luedeking-Piret and modified Gompertz equations.

The study provides an alternative method to produce the representative therapeutic alkylamide dodeca-2E,4E,8Z,10E, Z-tetraenoic acid isobutylamides (DTAI) with anaesthetic, analgesic and anti-inflammatory activities through adventitious root cultivation in a liquid medium. These roots are differentiated plant organs, and their genetically & phytochemically stable nature and makes them a superior choice over the callus or hairy root cultures. Adventitious roots are considered a safer and scalable alternative for in vitro biomass production. The study can be utilized to overcome the non-availability of plant biomass due to seasonal and geographical restrictions in commercially producing the DTAI. The results of kinetic modelling studies can be utilized to overcome the problems associated with up-scaling, low metabolite yield and optimization of high-priced process conditions.

2. Materials and methods

2.1. Experimental materials

Spilanthes paniculata plants (Fig. 1A) were collected from the IIT Guwahati campus located in Guwahati, Assam, India and grown in greenhouse conditions maintained at 24–26 °C temperature, 70 %

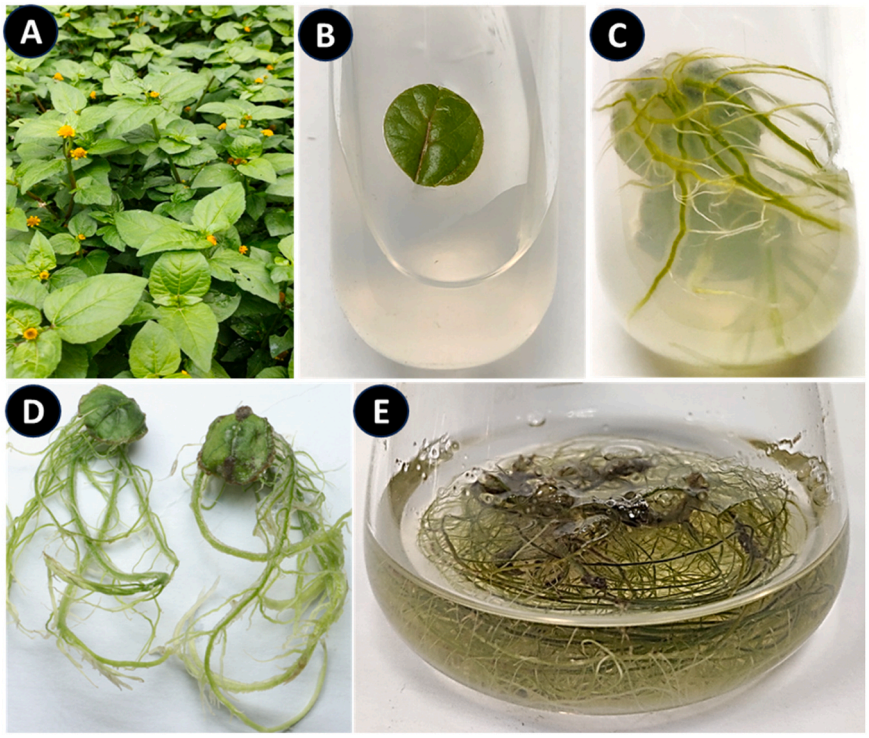


Fig. 1. Process of adventitious root induction from the leaf-disc explant of *S. paniculata* (A) Mother plant of *Spilanthes paniculata* (B) Leaf-disc inoculated as an explant on auxin supplemented MS medium (C) In vitro generated roots from the inoculated leaf-disc after 28 days of culture on semi solid medium (D) Adventitious roots to be inoculated in liquid medium of similar composition (E) Adventitious roots grown in shake flask after 21 days of inoculation.

humidity, and 16/8 h diffused light. Before starting the experiments, the plant was identified by a taxonomist and a herbarium bearing accession no. GUBH 79867 was submitted to Gauhati University. The greenhouse grown plants were used to initiate the in vitro nodal segment culture according to the protocol developed by Singh and Chaturvedi (2010). After three weeks of nodal segment culture, young leaves from in vitro raised shoots were taken as an explant for adventitious roots (ARs) induction.

2.2. Induction of adventitious roots in semi-solid medium

The leaf discs (10 mm dia.) of *S. paniculata* were prepared using a cork-borer and inoculated in test tubes (25 ×150 mm) on a slanted MS (Murashige and Skoog, 1962) medium supplemented with 2.5 μM auxins (IBA/IAA/NAA) as growth regulators, 3 % sucrose and 0.8 % agar (Fig. 1B). The auxin free medium was used as a control for the experiments.

The best root induction was observed on a medium supplemented with an auxin IAA (Fig. 1C). Therefore, further experiments were conducted using IAA supplemented MS medium. Different concentrations of Indole-3-acetic acid (IAA) (0.5, 1.0, 2.5, 5.0 μM) have been tested for maximum adventitious root induction. All medium pH was adjusted to 5.8 ± 0.2 with 1 N NaOH/HCl and autoclaved at 121 °C and 15 psi pressure for 20 minutes before inoculating the explant. After inoculation, cultures were incubated in an aseptically maintained room at 25 ± 2 °C under 16/8 h photoperiodic conditions. The effect of various factors was determined by calculating the number and length of the induced root.

2.3. Liquid medium culture of adventitious roots

To establish the suspension cultures, 28 days old adventitious roots from semi-solid medium were transferred to 250 ml shake flasks containing 100 ml of liquid MS medium supplemented with 3 % sucrose and 2.5 μM IAA (Fig. 1,D-E). Before inoculating, the roots were thoroughly washed with autoclaved distilled water under sterile conditions to remove the traces of agar. All the experiments were performed in an incubator shaker (Scigenics India) under 16/8 h photoperiodic conditions at a constant agitation speed (120 rpm) and a temperature of 25 ± 2 °C.

2.4. Effect of MS major salts and carbon source on adventitious roots proliferation in suspension medium

Different strengths of macronutrients were tested for further proliferation of root biomass and metabolites in suspension medium. After inducing in a semi-solid medium, roots were transferred to shake flasks (250 ml) containing 100 ml of MS suspension medium with different major salt strengths (0.25xMS, 0.5xMS, 0.75xMS, 1xMS, 1.5xMS & 2.0xMS) supplemented with 2.5 μM IAA and 3 % sucrose. After inoculation, other culture conditions were maintained in the same way as above. Root growth and metabolite production were analyzed after 18 days of inoculation, and the experiment was conducted in triplicates to check the reproducibility of treatments.

The carbon source has been recognized as a crucial factor responsible for adventitious root proliferation by providing energy to the growing tissues and acting as a signalling molecule. In another experiment set, different carbon sources, like glucose, fructose, maltose, sucrose, and mannitol were supplemented to 0.5x MS + 2.5 μM IAA medium at a 3 % concentration. Each flask (250 ml) containing 100 ml of suspension medium was inoculated with 1.0 g (FW) of root biomass as an inoculum and maintained under 16/8 h light/dark photoperiodic conditions at a constant agitation speed (120 rpm) and 25 ± 2 °C temperature. The experiment was performed in triplicates and designed in a completely randomized block fashion. The root biomass growth was recorded by measuring the fresh weight and dry weight of the biomass harvested from the shake flasks after 18 days of culture. The N-alkylamide quantification was also performed through HPLC analysis, and metabolite productivity was calculated using equation (1) (Wang et al., 2024).

Metabolite productivity (μg/L) = Dry weight (g/L) × Metabolite content (μg/g DW) …… (1)

2.5. Optimization of Inoculum density

The inoculum size in root suspension culture is key to maximizing the biomass and metabolite production. Different inoculum densities like 3, 6, 9, 12 and 15 g/l (FW) of root biomass were inoculated in flasks containing an optimized suspension medium with ½ MS (macronutrients reduced to half) + 2.5 μM IAA + 3 % sucrose composition. The experiment was performed in triplicates, and inoculated flasks were kept under a 16/8 h photoperiod, 120 rpm agitation speed and 25 ± 2 °C temperature. The biomass growth was monitored and recorded after 18 days of culture in terms of fresh and dry weight. Roots were harvested from each experiment, washed thoroughly with sterile distilled water and gently pressed on a blotting sheet to remove excess water. These roots were weighed for fresh weight determination, and the growth index for each inoculum density was calculated using Eq. (2) to determine the optimum inoculum size value for maximum root biomass production.

$$\text{Growth Index} = \frac{\textit{Final DW}(g/l) - \textit{Initial DW}(g/l)}{\textit{Initial DW}(g/l)} \quad (2)$$

2.6. Bioactive metabolite analysis

2.6.1. Root extract and sample preparation

In vitro grown root biomass was dried and ground in mortar & pestle for extract preparation. One gram of powdered material is soaked in 20 ml each of water, methanol, ethyl acetate and hexane (analytical grade, Merck, India) separately in a 50 ml shake flask and kept on shaker (120 rpm) for the next 12 hours. The mixture was centrifuged at 5000 rpm for 10 minutes, and the supernatant was collected and filtered using filter paper (Whatman no.1). Collected supernatant was dried using rotary evaporator (Buchi 200) and re-dissolved in HPLC grade methanol. The collected samples were filtered through 0.22 μM pore size syringe filters (Axiva, India) and stored in a refrigerator at −20 °C temperature for further use.

2.6.2. Preparation of reference standard

Reference standard of the isomeric mixture, dodeca-2E,4E,8Z,10E,Z tetraenoic acid-isobutylamide (DTAI) was purchased from Phytolab GmbH & Co. KG (Germany), which was pre-dissolved in acetonitrile at a specific dilution. Different concentrations ranging from 0.5 mg/ml to 0.03125 mg/ml were prepared and filtered through 0.22 μm syringe filters (Axiva) before analysis. The standard was run thrice during HPLC analysis for each concentration to check the repeatability and precision of the results. The linearity was checked by running the six different concentrations, and a calibration curve (Fig. S2) was plotted between the peak area (y-axis) and concentration (x-axis). The standard equation obtained from the curve was used to quantify the DTAI content in unknown samples and reported in μg/g DW.

2.6.3. High-performance liquid chromatography

Detection and quantification of DTAI was performed using the HPLC analysis protocol established by Singh and Chaturvedi (2012), slightly modifying the column temperature to 60 ºC. The HPLC system (UFLC, Shimadzu, Japan) equipped with a quaternary pump, up to 100 μl injection limit autosampler unit and BDS Hypersil RP-18 column (Thermo, USA) with 0.5 μm pore size and 4.6 mm× 250 mm dimensions. The mobile phase used for N-alkylamides detection was a mixture of 90 % acetonitrile and 10 % milli Q water with a flow rate of 0.5 ml/min. The UV detector detected the eluted peaks from the column at 230 nm wavelength. The compound peaks were collected and further analyzed

through electrospray ionization mass spectrometry.

2.6.4. Mass spectrometric analysis

Mass spectrometric analysis of the compound was performed on an Agilent quadrupole mass analyzer (UHPLC–QTOF-MS, Agilent Technologies, USA). All the peaks obtained from the HPLC were collected, concentrated, and further re-dissolved in methanol. The presence of dodeca-2E,4E,8Z, 10E, Z tetraenoic acid-isobutylamide was confirmed by comparing the mass spectra of collected peak fraction and commercially available reference standard. MassHunter Qualitative Analysis software (B.08.00 version, Agilent Technologies) was used for chromatogram processing.

2.7. Biomass and metabolites growth kinetics studies

To study various kinetic parameters of biomass and metabolite production, 0.9 g (FW) of adventitious roots were inoculated into 250 ml shake flask containing 100 ml of ½ MS (macronutrients reduced to half) suspension medium supplemented with 2.5 μM IAA and 3 % sucrose. The medium pH was adjusted to 5.8 before autoclaving, and inoculated cultures were kept under 16/8 h photoperiod, 120 rpm agitation speed, and 25 ± 2 °C temperature. During 24-day culture period, roots were harvested at 3-day intervals, and FW, DW, and DTAI content were analyzed. The DTAI productivity of each sample was also calculated according to equation (1).

The specific growth rate (μ) for each sample harvested at 3-day intervals was calculated from the slope of the graph between $\ln(X_t/X_0)$ vs. time 't'. X_0 and X_t are initial and final root biomass concentrations at different time intervals (days). The specific growth rate of root biomass (μ) depends on several factors such as pH, temperature, agitation (rpm), nutrient concentration, photoperiod and biomass production. Most of these factors were optimized and kept constant during culture period therefore, only substrate and biomass concentrations matter (Reiniati et al., 2017). Further, the unstructured models have been constructed to understand the adventitious root biomass growth and N-alkylamide production. The data obtained from dry weights of harvested root biomass and total metabolite productivity were utilized to calculate different kinetic parameters. The logistic regression equation was used to construct a kinetic model for biomass growth with time. According to Wang et al., (2024) & (Sivarathnakumar et al., 2019) the logistic equation was integrated to obtain the function of root biomass growth kinetics.

$$\frac{dX}{dt} = \mu_{max} X\left(1 - \frac{X}{X_{max}}\right) \tag{3}$$

Where - $\frac{dX}{dt}$ represents the rate of change of root biomass (X) with time (t), μ_{max} is the maximum specific growth rate of the root biomass, X_{max} is the environment's carrying capacity, representing the maximum root biomass concentration that the environment can sustain indefinitely without deteriorating.

On integrating Eq. (3),

$$X\ (t) = \frac{X_0 * \exp\mu_{max} t}{\left(1 - \left(\frac{X_0}{X_{max}}\right) * \ (1 - \exp\mu_{max} t)\right)} \tag{4}$$

The kinetic model for N-alkylamide production was constructed using the Leudeking-Piret (L-P) equation, which was developed to model the lactic acid production by *L. delbrueckii* (Zhang et al., 2018).

According to the Leudeking-Piret equation -

$$\frac{dP}{dt} = \alpha \frac{dX}{dt} + \beta X \tag{5}$$

Where: P is the metabolite productivity (μg/l), $\frac{dP}{dt}$ is the rate of change in productivity, α (g/g) and β (g/g/day) are empirical parameters representing the growth-associated and non-growth-associated components of product formation, respectively.

The logistic equation was substituted into the Eq. (5),

$$\frac{dP}{dt} = \alpha\mu_{max} X\left(1 - \frac{X}{X_{max}}\right) + \beta X \tag{6}$$

On integrating the Eq. (6),

$$P_t = P_0 + \alpha X_0 \left(\frac{\exp\mu_{max} t}{1 - \left(\frac{X_0}{X_{max}}\right) * \ (1 - \exp\mu_{max} t)} - \ 1 \right) + \beta \frac{X_{max}}{\mu_{max}} \ln\left[1 - \left(\frac{X_0}{X_{max}}\right) * (1 - \exp\mu_{max} t)\right] \tag{7}$$

Where: P_t is the metabolite productivity at time t (μg/l), P_0 is the initial productivity (μg/l), t is the culture period (days), X_0 and X_{max} are the initial and final root biomass DW (g/L) concentrations, respectively, and μ_{max} is the maximum specific growth rate (d^{-1})

The Modified Gompertz model is also one of the most widely used kinetic models for product formation. This model provides information on crucial parameters like lag time, maximum rate of product formation, and a potential maximum product concentration (Dodić et al., 2012). The modified Gompertz equations were derived from the Gompertz equation, which is expressed as follows -

$$Y = a.\exp\{-\exp(b - ct)\} \tag{8}$$

Where: Y is the metabolite produced (μg/l), 'a' is the Maximum metabolite concentration (μg/l), t is the culture period (day), b (no unit), and c (day^{-1}) are constants.

The Gompertz equation was modified to obtain the modified Gompertz equation to simulate the metabolite production, which is expressed as follows (Lo et al., 2010) -

$$P \ = Pm * \exp\{-\exp[(\frac{r_{p,m} * \exp(1)}{P_m}) * \ (tL–t) + \ 1]\} \tag{9}$$

Where: P is the product concentration (μg/l), P_m is the maximum product concentration (μg/l), $r_{p,m}$ is the maximum rate of product formation (μg/l.day), and t_L is the lag phase period (hr).

The experimental data was fitted into the equations, and kinetic parameters were calculated using commercially available Origin Pro 2024 software. The goodness of the fitting was estimated by obtaining the statistical indicator (R^2) value directly from the software.

2.8. Statistical analysis

Each experiment was performed in triplicate to ensure the reproducibility of the results. The results analysis and graphical representation were performed using Origin Pro 2024 (OriginLab Corporation) software. The mean ± standard deviation of the triplicate values is presented as the result. Tukey test was conducted to determine the statistical significance of different treatments, with $p < 0.05$ considered statistically significant.

3. Results and discussion

3.1. Adventitious root induction

The adventitious roots were initiated after 7–10 days of culture in all media except on basal medium (medium without auxin). Direct root induction (without callusing), with the highest induction efficiency of 86.66 %, was observed in the medium supplemented with 2.5 μM IAA (Fig. 3). The roots originated from the midrib portions of the leaf-disc along with the secondary and tertiary roots on this medium. A less

efficient and callus mediated root induction was observed on IBA and NAA supplemented media. Therefore, they were excluded from further experiments to maintain genetic and phytochemical stability over successive generations.

For IAA treated explants, adventitious root proliferation increases with an increase in IAA concentration and reaches the maximum root number (8.6 ± 0.25) and root length (10.53 ± 0.42 cm) at 2.5 μM IAA. However, the root number (Fig. 2A) and length (Fig. 2B) decrease on further increasing the IAA concentration. A similar pattern was observed in explants inoculated at various IBA concentrations. In IBA supplemented media, the highest number of roots and root lengths were 8.2 ± 0.24 and 7.9 ± 0.31 cm, respectively, and roots proliferated via callusing of explant. A significantly less adventitious root induction and higher callusing from explants were observed in NAA supplemented medium. The number of roots and root length in NAA incubated explants were 3.2 ± 0.09 and 4.1 ± 0.16 cm, respectively. Comparatively, the number and length of roots were higher for each treatment in the IAA group than in IBA and NAA. The MS medium supplemented with 2.5 μM IAA was found to be the most suitable for root proliferation. Therefore, MS + 2.5 μM IAA medium was utilized as the best root inducing medium for further experiments.

Adventitious roots are post-embryonic roots arising from any plant part except the primary root system. Apart from the auxins, certain endogenous and exogenous factors (Ca^{2+}, sugars, cGMP, MAPKs) also play a significant role in root formation (Li et al., 2009) These roots can be induced from the explant via direct or callus-mediated mode of root formation. In the current study, the rooting from the leaf-disc of *S. paniculata* originated through both direct and indirect methods (callus mediated). Direct rooting was observed in IAA supplemented medium, whereas the callus mediated rooting was obtained in IBA and NAA supplemented media. This study also supports the previous finding of adventitious root induction from various explants of *Orthosiphon stamineus,* highlighting that IAA is more potent for root induction and growth (Pick et al., 2009). IAA has been used alone (*Mondia whitei*, *Orthosiphon stamineus*) or in combination with IBA (*Withania somnifora, Artemisia vulgaris*) for root induction in various plants (Baskaran et al., 2016; Praveen and Murthy, 2010; Sujatha and Ranjitha Kumari, 2012).

3.2. Effect of medium salt strength on adventitious root biomass and DTAI accumulation

The medium salt strength significantly influences the adventitious root proliferation and N-alkylamide production in *S. paniculata*. In this experiment, the major salt strength in the MS medium was adjusted to 0.25xMS, 0.5xMS, 1xMS, 1.5xMS & 2.0xMS. The data collected in terms of fresh weight (FW) and dry weight (DW) are mentioned in Table 1. It was observed that response was better in media containing reduced salt strengths (0.5xMS) compared to the other treatments. However, further reducing the salt strength below 0.25x reduces the root biomass and metabolite productivity due to the lack of nutrients. The increased salt concentration also reduces biomass generation, probably due to the change in water potential caused by high salt concentrations. The highest FW (85.33 ± 1.52 g/l), DW (8.60 ± 0.15 g/l) and N-alkylamide accumulation (81.56 ± 1.44 μg/g DW) were observed ½MS + 2.5 μM IAA medium.

The salt strength of macronutrients is directly responsible for the medium's osmotic potential and ionic concentration. These parameters ultimately affect the cell growth, cell size, ion channel movement across the cell membrane and metabolism (Wang et al., 2024). Therefore, several studies have been conducted to check the effect of salt strength on biomass growth and metabolite production. According to (Lee and Paek, 2012), the adventitious root growth of *Eleutherococcus koreanum* Nakai was better at low salt strength (0.25, 0.5, and 0.75 MS) as compared to high salt strength (1, 1.5 and 2 MS). In another study, 0.25x MS medium was suitable for the root biomass and anthraquinone production in adventitious roots of *Morinda citrifolia* (Baque et al., 2010). The result of this experiment suggests that 0.5x MS medium was more suitable for adventitious root and DTAI production in *S. paniculata* than other salt strengths.

3.3. Effect of carbon source on adventitious root biomass generation

In vitro cultures are not entirely autotrophic, and a carbohydrate must be supplemented in the medium to serve as an energy source and maintain the medium's osmotic balance (Yaseen et al., 2013). The optimization of carbon sources is also necessary to maximize biomass and metabolite production in the adventitious root culture of *S. paniculata.* This study investigates the effect of different carbon

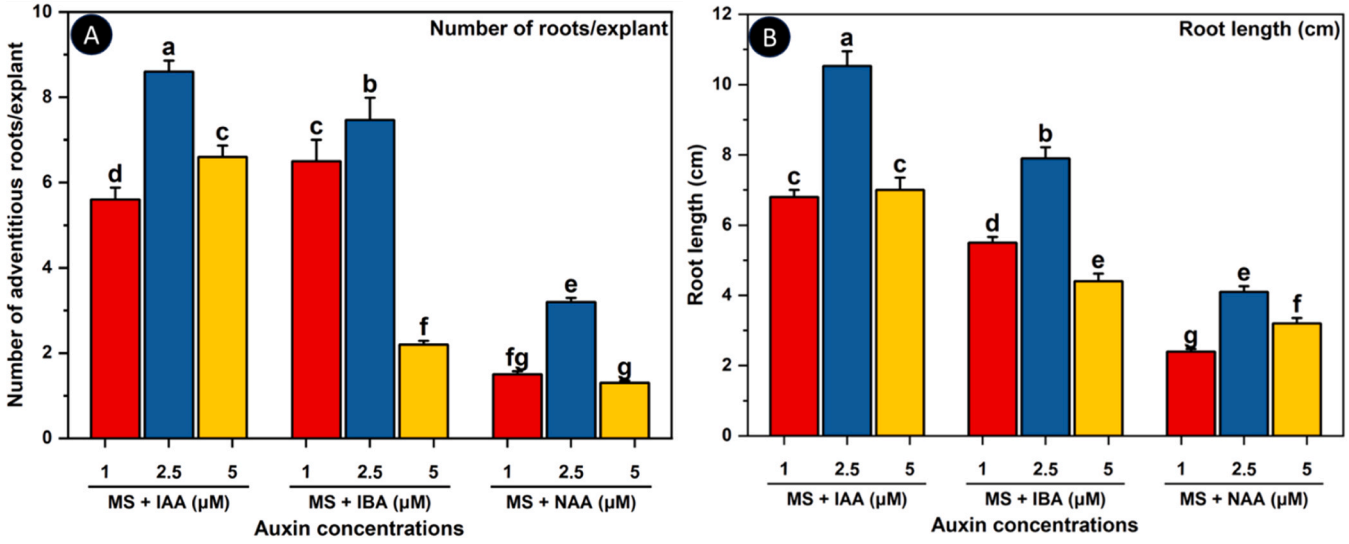


Fig. 2. Effect of different concentrations of IAA, IBA and NAA on the number and length of adventitious roots of *Spilanthes paniculata* (A) Number of adventitious roots after 28 days of inoculating the leaf-disc (B) Adventitious root length, Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA), and Naphthalene acetic acid (NAA). The data is the representation of the mean ± standard deviation of triplicate values obtained after the experiment. The letters mentioned at the top of each column indicate the significant difference between the treatments analyzed through the Tukey test at a significance level < 0.05.

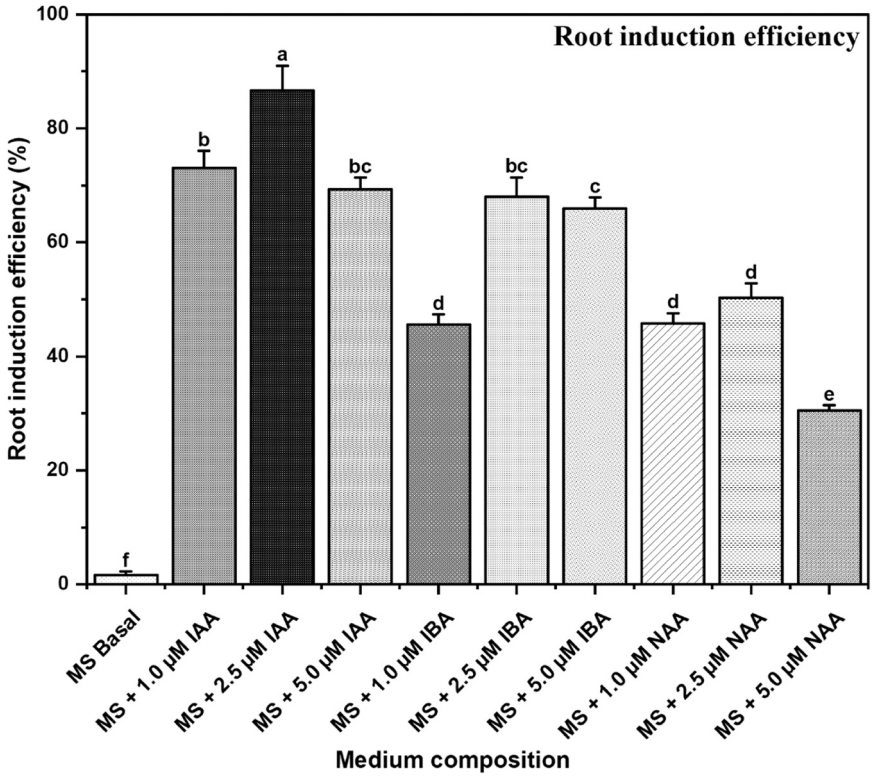


Fig. 3. Root induction efficiency of leaf-disc explants of *S paniculata* inoculated on semi-solid medium with different concentrations of IAA, IBA and NAA. MS basal medium was taken as control. The data is the representation of the mean ± standard deviation of triplicate values obtained after experiment. The letters mentioned on each column indicate the significant difference between the treatments analyzed through the Tukey test at a significance level < 0.05.

Table 1
Effect of MS Medium salt strength on adventitious root biomass proliferation and DTAI accumulation in *S. paniculata* after 18 days of culture.

Medium Composition	Fresh weight (g/l)	Dry weight (g/l)	Total DTAI content (µg/g DW)	Total DTAI content (µg/L)
MS Basal	30.66 ± 4.04 d	3.04 ± 0.40 e	29.10 ± 3.83 d	89.54 ± 23.71 f
0.5x MS	55.66 ± 3.78c	5.95 ± 0.40c	52.41 ± 2.85c	313.06 ± 38.87 d
0.25x MS + 2.5 µM IAA	73.66 ± 3.51 b	7.14 ± 0.34 b	70.11 ± 3.61 b	501.57 ± 49.54c
0.5x MS + 2.5 µM IAA	85.33 ± 1.52 a	8.60 ± 0.15 a	81.56 ± 1.44 a	697.35 ± 25.03 a
MS + 2.5 µM IAA	77.66 ± 2.30 ab	8.13 ± 0.24 a	73.72 ± 2.19 ab	599.87 ± 35.34 b
1.5x MS + 2.5 µM IAA	46.66 ± 2.51c	4.7 ± 0.25 d	44.29 ± 2.38c	208.59 ± 22.33 e
2x MS + 2.5 µM IAA	26.33 ± 4.50 d	2.59 ± 0.44 e	26.81 ± 2.40 de	69.97 ± 17.41 f
2.5x MS + 2.5 µM IAA	21.33 ± 4.041 d	2.15 ± 0.40 e	20.25 ± 3.83 e	44.76 ± 16.20 f

MS - Murashige and Skoog, IAA – indoleacetic acid. IBA – indole-3-butyric acid. NAA – 1-Naphthalene acetic acid. The data represents the mean ± standard deviation of triplicate values obtained after the experiment. The letters mentioned on the top of each column indicate the significant difference between the treatments analyzed through the Tukey test at a significance level < 0.05.

sources (glucose, fructose, maltose, sucrose, and mannitol) on the root biomass production of *S. paniculata.* Sucrose was found to be an ideal energy source for highest biomass accumulation yielded 83.6 ± 3.8 g/l fresh weight (FW) and 10.43 ± 0.63 g/l dry weight (DW) of root biomass followed by fructose (56.24 ± 0.77 g/l FW, 7.42 ± 0.35 g/l DW), maltose (42.4 ± 2.05 g/l FW, 5.20 ± 0.33 g/l DW), and glucose (39.01 ± 2.63 g/l FW, 5.24 ± 0.43 g/l DW). The least biomass production was observed in the medium supplemented with mannitol, which yielded 13.71 ± 1.45 g/l FW and 1.71 ± 0.16 g/l DW (Fig. 6 A). The difference in biomass production could be due to several factors facilitated by the carbon source. Molecular signalling, osmotic balance and energy supply are crucial factors depending on the concentration and complexity of the supplied carbon source. However, further studies are required to prove that these factors are involved in biomass and metabolite production in a plant cell. Like our findings, Murthy and Praveen (2013) also reported sucrose as the best carbon source for biomass and secondary metabolite accumulation in adventitious roots of *Withania somnifera* (L.) Dunal.

3.4. Inoculum density optimization

The optimum inoculum density is crucial to maximize the biomass and metabolite production during suspension cultures. In this experiment, inoculum sizes of 3–15 g/l (FW) were inoculated in shake flasks containing an optimized suspension medium. After 18 days of culture, root biomass was harvested to calculate the growth index for each inoculum size using the Eq. (2). The results showed that growth index increased with inoculum size, reached a maximum of 7.93 at 9.0 g/l, and decreased on further increasing the inoculum size (Fig. 6B). The highest harvested biomass was 80.37 g/l (FW) and 10.18 g/l (DW) at the inoculum size of 9.0 g/l. The decrease in growth index beyond 9.0 g/l inoculum size is probably due to the lack of available nutrients for the inoculated biomass for an 18-day culture period. Therefore, an optimum inoculum size of 9.0 gm/l was used for further experiments.

3.5. DTAI analysis through HPLC and mass spectrometry

HPLC and Mass spectrometry were performed to identify and quantify the DTAI content of the adventitious root samples of *S paniculata*. A range of mobile phase ratios like 70:30, 80:20, 85:15, 90:10 &

100:0 (acetonitrile:water) were used to get the satisfactory compound separation, and 90:10 (acetonitrile:water) was found to be an appropriate solvent ratio. One prominent peak at the retention time of 6.96 min. was obtained in the HPLC chromatogram at a UV wavelength of 230 nm and flow rate of 0.5 ml/min. HPLC analysis of commercially available reference standard also shows the compound peak at a similar retention time (6.96 min.), confirming DTAI's availability in the root samples (Fig. 10A-B). The obtained HPLC peak was collected, concentrated, and further analyzed through mass spectrometry (UHPLC-QTOF-HRMS) in a positive ion mode. The characteristic fragmentation pattern of the DTAI compound was obtained from mass spectrometry analysis. The highest peak of the spectrum at *m/z* 248.2028 was identified as the protonated ($M+H^+$) DTAI molecule. A small peak at the *m/z* value of 495.3961 was obtained due to the protonation of the dimerized DTAI molecule ($2\,M+H^+$). A characteristic *m/z* value of 167.1306 was also detected due to the fragmentation between the 6th and 7th carbon in the aliphatic chain of the molecule as shown in Fig. 10C (Goey et al., 2012). The metabolite was quantified using the standard curve equation mentioned in section 2.7.3.

3.6. Kinetics of adventitious root growth in S. paniculata

The growth pattern of adventitious roots was investigated by monitoring the changes in fresh weight (FW) and dry weight (DW) throughout the experiment. The growth curve typically followed a 'S-shaped (sigmoid) pattern, divided into an early lag phase, a log phase, a short stationary phase, and decline in biomass growth. No significant change in biomass was observed during the first 4–5 days of culture. From 6th day onwards, biomass began increasing significantly, reaching a peak on 18th day of culture and decreased (Fig. 4A). The specific growth rate for each 3-day interval was also calculated to understand the root biomass growth curve. The slope of the curve between natural log of X_t/X_0 and culture period (days) provided the specific growth rate (Fig. 4B). The lowest specific growth rate (0.0998 /day) was observed between 0 and 3 days and slightly increased from 3 to 6 days of culture, demonstrating an initial lag phase of biomass growth. From 7th day onwards, the specific growth rate increased rapidly and reached a maximum of 0.2456/day between 9 and 12 days of culture, indicating the exponential growth phase. From 12–15 days and 15–18 days, the specific growth rate was reduced, showing that biomass growth decreased. Subsequently, from day 18 – 21, the specific growth decreases rapidly and becomes negative (-0.0149/day), representing the senescence phase of biomass growth in the root culture of *S. paniculata*. The results show that maximum biomass production will occur after 18 days of culture, but the highest metabolic activity will occur between 9 and 12 days of culture. The best root biomass for further subculturing can be obtained after 12 days of culture.

The sigmoidal growth pattern of *S. paniculata* adventitious root biomass was in line with the Logistic regression equation for population growth. Therefore, dry weight data obtained from growth kinetics studies were used to develop the kinetic model by fitting it into the logistic equation. Different parameters such as initial biomass (X_0), maximum biomass (X_m) and maximum specific growth rate (μ_m) were estimated by putting the values into the OriginPro (2024b) software. A fitting curve was generated with an R^2 value of 0.9971 and a final equation was derived after substituting the values of different estimated parameters in Eq. (4) (Fig. 7A-C). The fitted curve and regression analysis show that the constructed model could precisely predict the adventitious root biomass growth of *S. paniculata*.

3.7. Kinetics of metabolite production

The DTAI content from adventitious root culture of *S. paniculata* was also measured at every 3-day intervals during a 24-day culture duration to elucidate the characteristics of bioactive metabolite production. Fig. 5A shows that DTAI content was much less immediately after inoculation and increased slowly for the first 9 days of culture. A dramatic increase in the content was observed from 9th to 15th day, followed by a gradual rise to the peak on 18th day of culture. Similarly, the DTAI productivity was also very low immediately after inoculation, but unlike the content, productivity increased rapidly from 7th day and reached a maximum on 18th day of culture, then decreased (Fig. 5B). The maximum DTAI productivity obtained during this culture period was 697.2 µg/l after 18th day of inoculation.

The L-P equation effectively explains the relationship between biomass growth and metabolite synthesis. The DTAI productivity data was fitted into the L-P equation to model the metabolite production through the Origin Pro 2024 program. The values of the different parameters (P_0, α, β) were calculated and substituted into Eq. (7) to derive a new equation with an R^2 value of 0.984 (Fig. 8A-C). The regression analysis and fitted curve showed statistical significance, indicating that the non-structural kinetic model constructed for product formation can

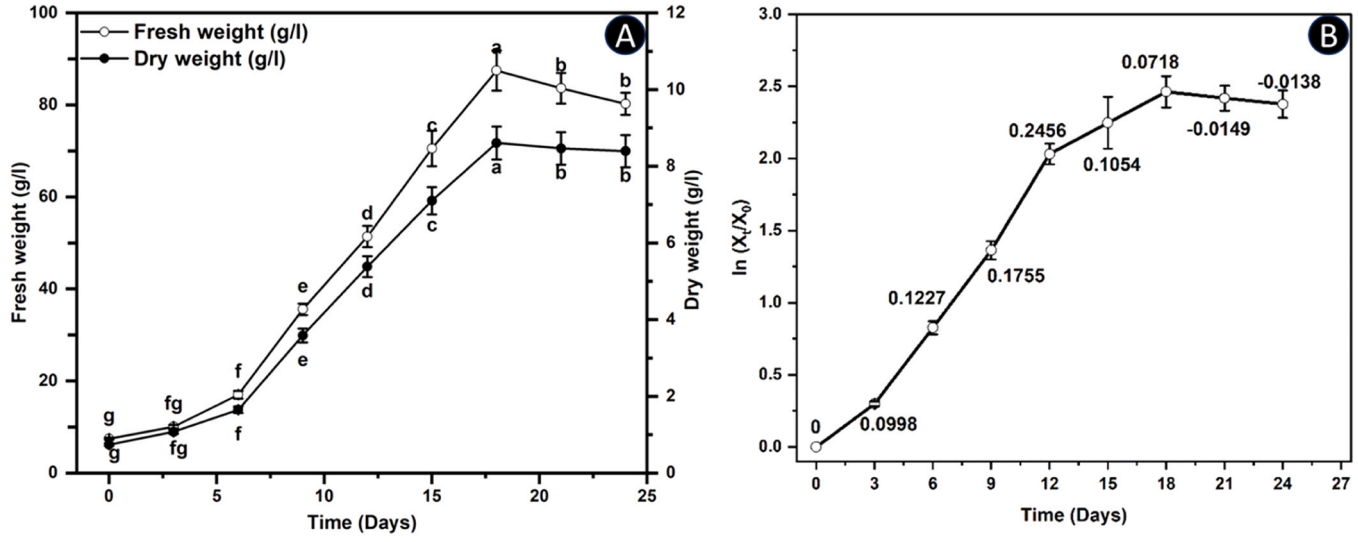


Fig. 4. Change in biomass production during adventitious root suspension culture of *S. paniculata* (A) Fresh weight and dry weight harvested at three days intervals (B) A graph between $\ln(X_t/X_0)$ of dry weight and time (days), the digits mentioned over points is the specific growth rate of root biomass between those two points. The data represents the mean ± standard deviation of triplicate values obtained after the experiment. The letters on each point indicate the significant difference between the treatments analyzed through the Tukey test at a significance level < 0.05.

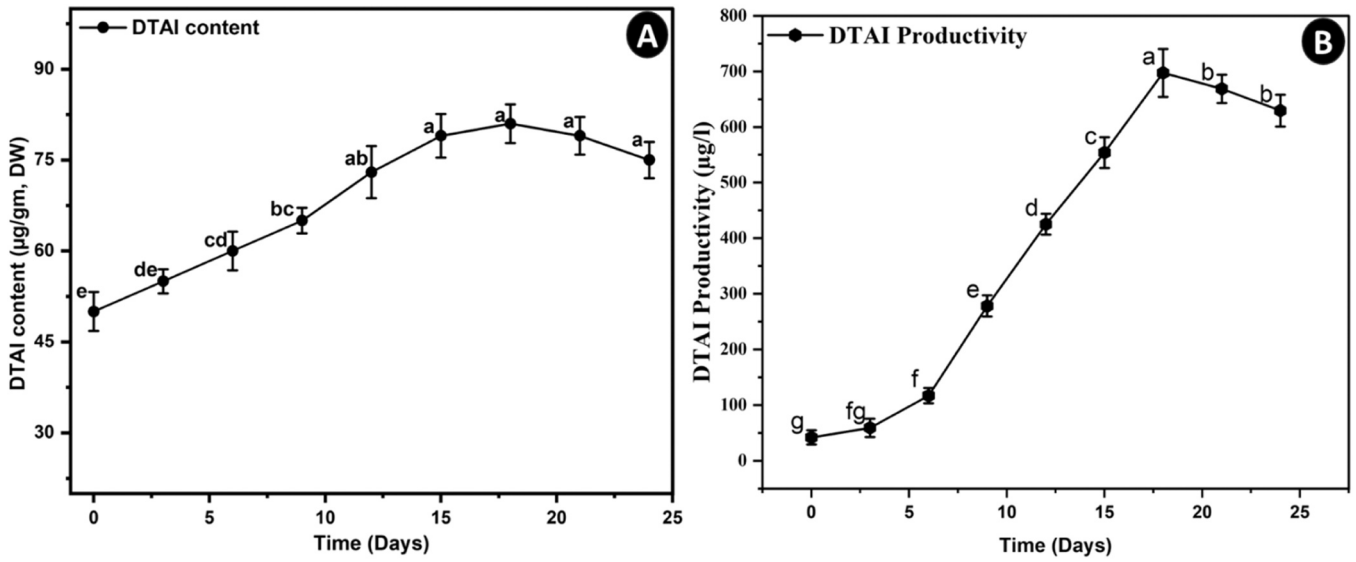


Fig. 5. Changes in DTAI profile with culture time during adventitious root suspension culture of *S. paniculata*. (A) Total DTAI content (B) Total DTAI productivity. The data represents the mean ± standard deviation of triplicate values obtained after the experiment. The letters on each point indicate the significant difference between the treatments analyzed through the Tukey test at a significance level < 0.05.

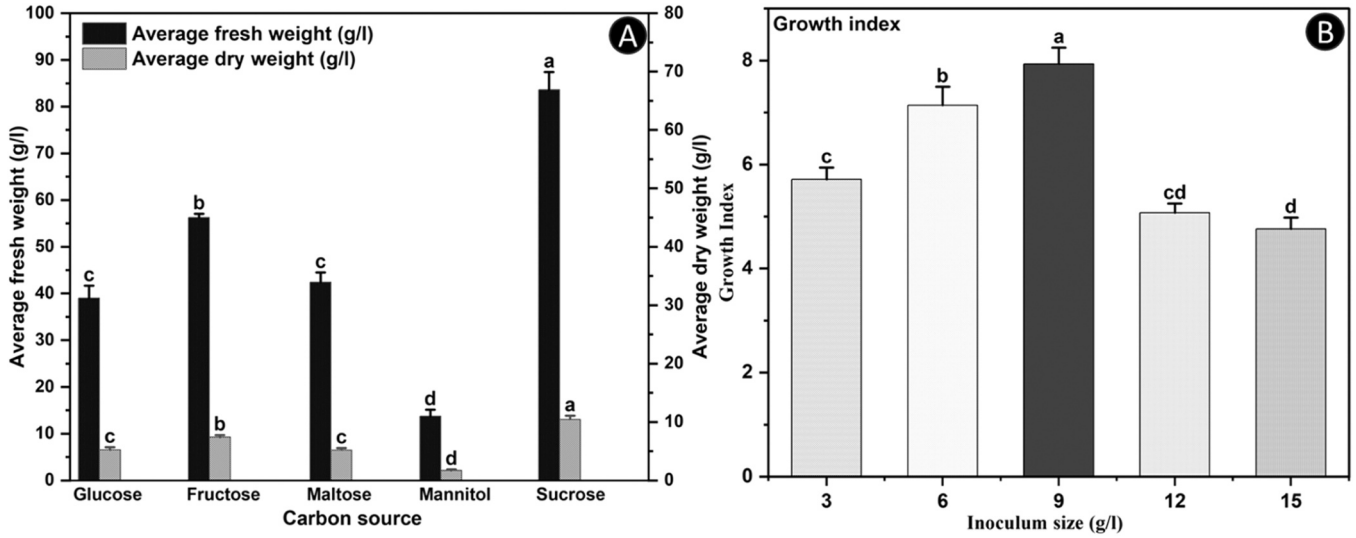


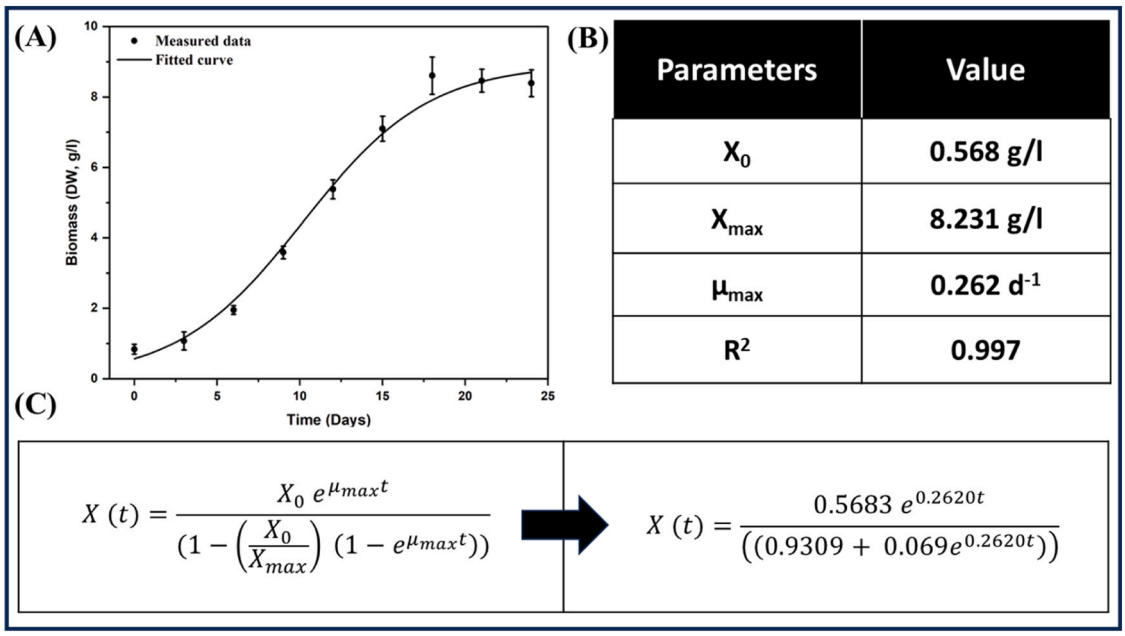
Fig. 6. (A) The effect of carbon sources on adventitious root proliferation of *S. paniculata* in suspension medium. (B) Optimization of root inoculum size in a shake flask (250 ml) containing suspension medium (100 ml). The data represents the mean ± standard deviation of triplicate values obtained after the experiment. The letters at the top of each column indicate the significant difference between the treatments analyzed through the Tukey test at a significance level < 0.05.

predict DTAI production. Our study shows that the value of α (87.524) is non-zero, whereas β (0.022) is approximately zero. This implies that the production of DTAI from the adventitious root of *S. paniculata* falls under the growth-associated metabolite production category.

The Leudeking-Piret (L-P) regression equation does not reveal any information about the initial lag in the product formation (t_L), maximum product concentration (P_m), and maximum rate of product formation ($r_{p,m}$). The modified Gompertz model can calculate these values and provide more information on product formation kinetics. The experimental data of DTAI productivity was fitted into the Eq. (9) through originPro 2024b software. A fitted curve with a good R^2 value of 0.9831 was obtained, and different parameters P_m, $r_{p,m}$ and t_L were calculated and substituted in the Eq. (9) to derive a new equation (Fig. 9A-C). The regression analysis and the statistical significance of the constructed model show that it can also predict the DTAI production well during adventitious root culture of *S. paniculata*.

The validation of the Logistic model for adventitious root biomass generation and Leudeking-Piret & modified Gompertz model for DTAI production was done by comparing the experimental data with predicted values (data obtained from derived equations in Figs. 7(C), 8(C) and 9(C)). The data shows a linear relationship between experimental and predicted values in all three models applied (Fig. S1). Since most of the values lie in the 95 % confidence band, it concludes that applied models can precisely predict the biomass and DTAI production from the adventitious roots of *S. paniculata* under in vitro conditions. The regression analysis data of all three models are obtained during modelling and compiled, as mentioned in Table S1.

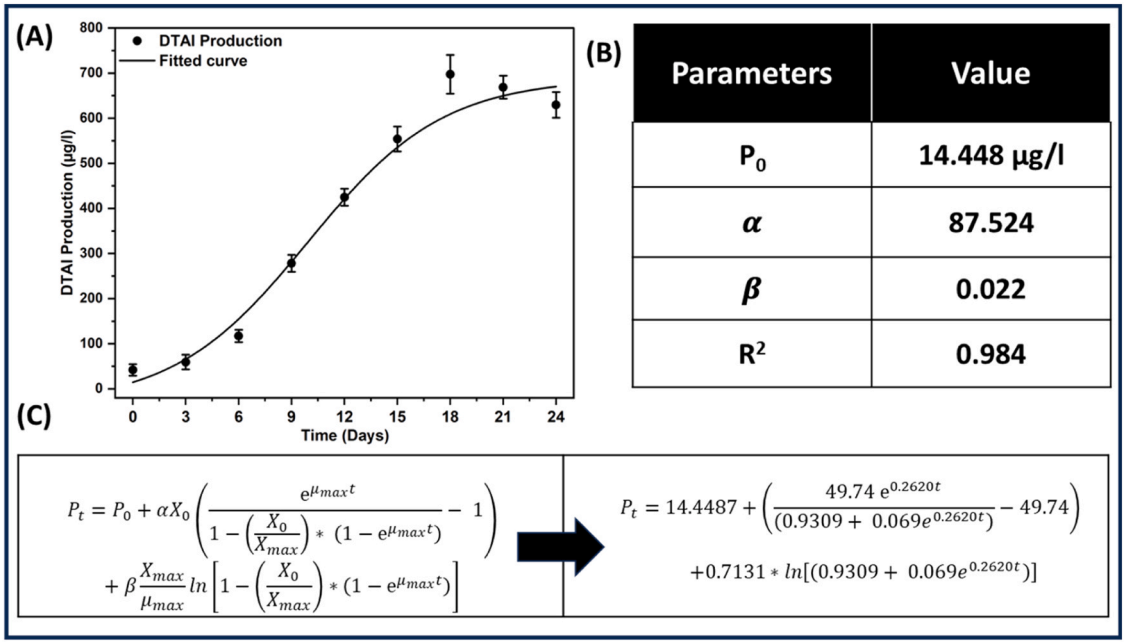
The growing interest in plant cell, tissue and organ culture for producing the structurally complex metabolite under in vitro conditions is challenging. The demand for numerous experiments to optimize the experimental conditions and parameters is costly, laborious, and time-consuming. Therefore, the obstacles of upscaling, low yields, and over-



Parameters	Value
X_0	0.568 g/l
X_{max}	8.231 g/l
μ_{max}	0.262 d^{-1}
R^2	0.997

$$X(t) = \frac{X_0\, e^{\mu_{max} t}}{\left(1 - \left(\frac{X_0}{X_{max}}\right)\left(1 - e^{\mu_{max} t}\right)\right)} \Rightarrow X(t) = \frac{0.5683\, e^{0.2620t}}{\left((0.9309 + 0.069 e^{0.2620t})\right)}$$

Fig. 7. Kinetic modelling (Logistic equation) of leaf-disc generated adventitious root biomass in *S. paniculata* (A) Fitted curve of biomass growth with time (B) Growth parameters and their values (C) Fitted equation with substituted values, X - Adventitious root dry weight (g/L). t - culture period. X_0 - initial adventitious root dry weight (g/L). X_{max} - maximum adventitious root dry weight (g/L), μ_{max} - maximum specific growth rate (per day). R^2 - correlation coefficients.



Parameters	Value
P_0	14.448 µg/l
α	87.524
β	0.022
R^2	0.984

$$P_t = P_0 + \alpha X_0 \left(\frac{e^{\mu_{max} t}}{1 - \left(\frac{X_0}{X_{max}}\right) * \left(1 - e^{\mu_{max} t}\right)} - 1\right) + \beta \frac{X_{max}}{\mu_{max}} ln\left[1 - \left(\frac{X_0}{X_{max}}\right) * \left(1 - e^{\mu_{max} t}\right)\right]$$

$$\Rightarrow P_t = 14.4487 + \left(\frac{49.74\, e^{0.2620t}}{(0.9309 + 0.069 e^{0.2620t})} - 49.74\right) + 0.7131 * ln[(0.9309 + 0.069 e^{0.2620t})]$$

Fig. 8. Kinetic modelling (Leudeking-Piret model) of DTAI production from adventitious roots of *S. paniculata* (A) Fitted curve of DTAI productivity with time (B) Productivity parameters and their values (C) Fitted equation with substituted values, X - Adventitious root dry weight (g/L). t - culture period. X_0 - initial adventitious root dry weight (g/L). X_{max} - maximum adventitious root dry weight (g/L), μ_{max} – maximum specific growth rate (per day), P_0 - Initial DTAI productivity (µg/L), P_t = productivity of DTAI at time t, α - growth associated constant, β - Non-growth associated constant. R^2 - correlation coefficients.

priced process parameters increase the demand for mathematical models (Diger et al., 2015). Many mathematical models focusing on product development have evolved in the last two decades. The Logistic model is commonly used to predict plant and microbial biomass growth. However, Luedeking-Piret & modified Gompertz equations are frequently used to construct the product formation kinetic models in plant cell/organ suspension culture. Our study finds that both the models applied for DTAI production are equally consistent with experimental data having good R^2 value and can be used to predict the product profile.

4. Conclusions

Adventitious roots from leaf discs of *S. paniculata* were induced on different auxin supplemented media, but the maximum number of roots and root length were observed on MS medium supplemented with 2.5 µM IAA. During suspension culture, the best root biomass growth was obtained when macronutrient concentrations in MS medium were

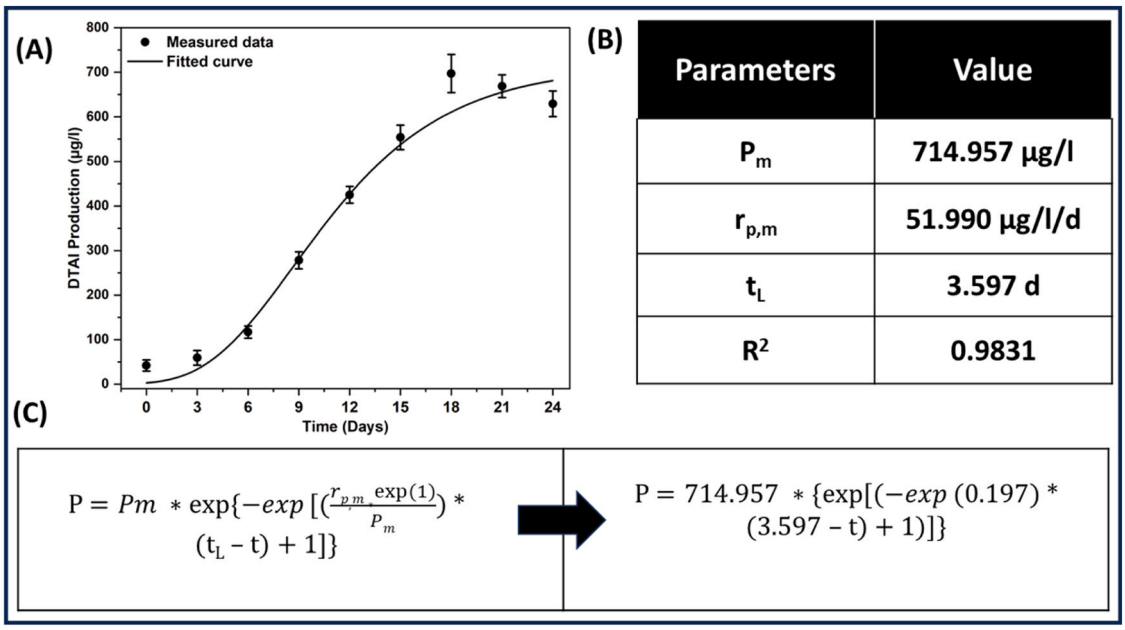


Fig. 9. Kinetic modelling (Modified Gompertz equation) of DTAI production from adventitious roots of *S. paniculata* (A) DTAI production parameters and their values (B) Fitted curve of DTAI production with time (C) Fitted equation with substituted values, P – DTAI production (µg/L). t - culture period. P_m – maximum product concentration (µg/L). $r_{p,m}$ – maximum rate of DTAI production (µg/L/d), t_L – lag phase time. R^2 - correlation coefficients.

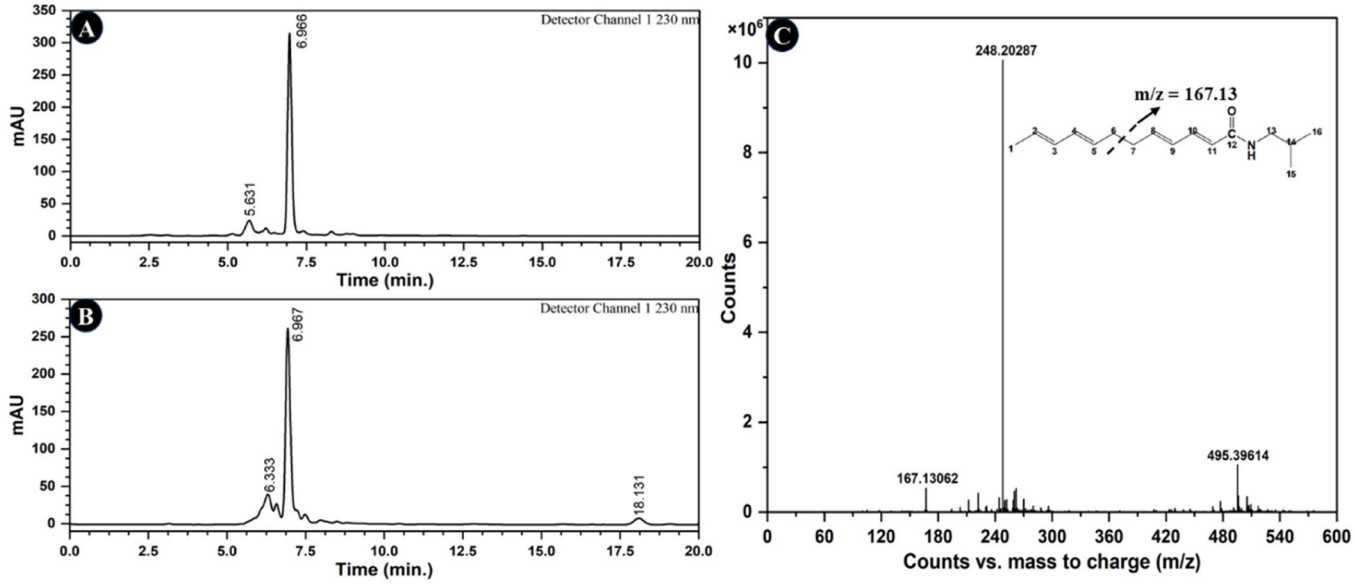


Fig. 10. HPLC and mass spectrometry analysis of dodeca-2E,4E,8Z,10E, Z-tetraenoic acid isobutylamides (DTAI) (A) HPLC analysis of commercially available reference standard showing the presence at retention time of 6.96 min. (B) In vitro generated *S. paniculata* adventitious root extract sample also showing the presence of DTAI at a similar retention time. (C) Mass spectrometric analysis HPLC purified DTAI sample of adventitious roots *S. paniculata.*

reduced to half. Different carbon sources have also been tested in suspension medium. However, sucrose was found to be the best for providing energy and maintaining the osmotic balance of the growth medium. The maximum fresh weight biomass (85.33 ± 1.52 g/l), dry weight biomass (8.60 ± 0.15) and DTAI productivity (697.35 ± 25.03 µg/l) was achieved on ½ MS medium supplemented with 2.5 µM IAA and 3 % sucrose. HPLC and mass spectrometry analysis were performed to identify and quantify the DTAI content in the root biomass generated from the optimized suspension medium. The growth kinetic studies showed that root biomass growth and DTAI productivity were maximum after 18th day of culture. A clear peak at *m/z* value of 248.2028 shows the presence of a protonated molecular ion ($M+H^+$). The kinetic models for biomass and metabolite production were also studied to check maximum biomass and metabolite productivities. The logistic equation fitted the biomass production data well, and the X_0, X_m and μ_m were calculated. DTAI production data was modelled using two different models (Luedeking-Piret and modified Gompertz model), and it was fitted slightly better with the Luedeking-Piret regression equation (regarding R^2 values). It is generally true that a model with more parameters has a higher R^2 value (Phukoetphim et al., 2017). The findings of this research can provide a reference for scaling up the process of consistent therapeutic metabolite production through adventitious roots of *S. paniculata,* irrespective of geographical and seasonal variations.

The present report is the first extensive study on in vitro adventitious

root biomass generation of *Spilanthes paniculata,* their chemical profiling, and kinetic modelling of biomass and a therapeutic compound, dodeca-2E,4E,8Z,10E, Z-tetraenoic acid isobutylamides (DTAI) production. It is the novel process of DTAI production and kinetic analysis report on *S. paniculata* under in vitro conditions. It is also one of the rare attempts to model the adventitious root biomass and DTAI production using logistic, Ludeking-Piret and modified Gompertz equations. The resulting kinetic parameters can be used as a theoretical basis to scale-up the production of adventitious root biomass of *S. paniculata* for large-scale DTAI formation.

CRediT authorship contribution statement

Chaturvedi Rakhi: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Formal analysis, Conceptualization. **Pachauri Krishna Kant:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Krishna Kant Pachauri and Rakhi Chaturvedi has patent METHOD AND SYSTEM FOR PRODUCTION OF N-ALKYLAMIDES FROM SPILANTHES SPECIES pending to IIT Guwahati. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.indcrop.2025.120997.

Data availability

Data will be made available on request.

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