Effect of light and methyl jasmonate on the accumulation of anticancer compounds in cell suspension cultures of *Catharanthus roseus*

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Background and objectives
Catharanthus roseus (Apocynaceae) is a medicinal plant that contains unique compounds used in cancer treatment. This investigation deals with enhancing the production of anticancer compounds (ajmalicine, vinblastine, and vincristine) in cell suspension cultures through elicitation by methyl jasmonate and light and dark treatments.
Materials and methods
For callus induction, leaf segments were cultured on solidified Murashige and Skoog (MS) medium with different 2,4-D and kin supplementations. Actively growing leaf was used for initiation of cell suspension culture by transferring 1 g of tissues in 100 ml Erlenmeyer flasks containing 20 ml liquid MS medium supplemented with 1.0 mg/l 2.4-D+1.0 mg/l kin. Different concentrations of MeJA (100, 200 and 300 μM) were added to cell suspension culture. Cell tissues were harvested at 2 and 4 days after elicitation. For light and dark elicitation, cell suspension culture was performed in 250 ml conical flasks containing 50 ml of liquid medium and inoculated with 1 g fresh calli, subjected to 16 h photoperiod and complete darkness; then callus tissues were collected at 2 and 4 days after elicitation. Estimation of ajmalicine, vinblastine, and vincristine was carried out using high-performance liquid chromatography in the elicited cultures compared with untreated calli.
Results and conclusion
Callus of *C. roseus* was produced from young leaves on MS medium with 1.0 mg/l of each 2,4-D and kin that recorded high callus initiation frequency (%). In cell suspension culture, viability of cells increased gradually with time until it reached their maximum at day 20 of culture, then declined until 30 day of culture. Adding methyl jasmonate (100 μM) showed higher level of ajmalicine after 2 days of culture and increased 19-fold than the control. The vinblastine content was decreased at 200 μM methyl jasmonate when cultures were treated for 2 days. With regard to vincristine accumulation in cell suspension, it was observed that there was no difference in the accumulation of vincristine. For light and dark exposure, it could be observed that cultures exposed to light condition gave the best results of ajmalicine and vincristine than cultures exposed to dark conditions, while the vinblastine content was better under dark at 2 or 4 days. In conclusion, the results suggest that methyl jasmonate efficiently enhances both of ajmalicine and vinblastine especially after 2 days, while for vincristine, there was no improvement. Regarding dark and light conditions, the yield of ajmalicine and vincristine was higher in light conditions, in contrast to vinblastine which is higher in dark than in light conditions in *C. roseus* suspension cultures. The results could be very effective for large-scale production for pharmaceutical industry.

Keywords:
callus and suspension cultures, *Catharanthus*, elicitation, high-performance liquid chromatography, light, methyl jasmonate

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Introduction

Catharanthus roseus, family Apocynaceae, is a renowned medicinal plant and is a major source of alkaloids, namely, vincristine and vinblastine, which own great importance in cancer treatment. These alkaloids interfere with the division of cancer cells [1]. Among these alkaloids, ajmalicine is found in the root and has been reported to have wide use in the treatment of circulatory diseases, especially in regulating normal cerebral blood circulation [2].

Plant tissue and cell culture-based technologies are promising options to increase indole alkaloids in various cultures of periwinkle by stress factors, bio-regulators, light irradiation, plant growth regulators, methyl jasmonate, and synthetic precursors. Detailed descriptions of results on the different elicitors are available in the recently published book chapter focusing on the biotechnology of periwinkle [3].

Currently, elicitation is considered the most influential technique frequently used for the production of secondary metabolites. Elicitor compounds are biotic or abiotic molecules that stimulate plant defense to protect the cell and whole plant, inducing signal transduction cascade and finally change the expression level of different regulatory transcription factors/genes, including secondary metabolic pathway genes [4].

Light was shown to have significant influences on vindoline and serpentine biosynthesis and stimulates peroxidase activity in *C. roseus* callus cultures [5]. Previous investigations of light also reported that under continuous light exposure, the total indole alkaloids in *C. roseus* suspension culture was boosted to about 2.8-fold compared with the control [5]. The biosynthesis of vindoline in *C. roseus* C20hi cell suspensions was improved by methyl jasmonate and light incorporation, while catharanthine production was not improved by either light or MJ elicitation [6]. On the contrary, in another research it was found that, in the callus culture of *C. roseus*, MeJa treatment displayed an unsteady decrease in vindoline accumulation compared with untreated calli [7]; gene expression analysis of the elicited calli cultures failed to enhance biosynthesis.

The current work aims to investigate the effect of MeJa and light on the accumulation of ajmalicine, vinblastine, and vincristine in *C. roseus* suspension cultures. Determination of these referred compounds was performed using the high-performance liquid chromatography (HPLC) analysis.

Material and methods

Seed sterilization and germination

Seeds of *C. roseus* were obtained from Medicinal Plants Research Institute, Ministry of Agriculture, Egypt. Seeds were immersed in 70% ethanol for 2–3 min, then rinsed three times in sterile distilled water. The seeds were then sterilized for 30 min in 20% commercial Clorox (5% NaOCl) containing 0.5% Tween 20. After rinsing three times with sterile distilled water, seeds were cultured on Murashige and Skoog (MS) medium [8] containing 3% (w/v) sucrose and solidified with 0.7% (w/v) agar. The culture medium was adjusted to pH 5.8 before autoclaving. The seeds were incubated in a culture room at 26±2°C and were kept under 16 h photoperiod of fluorescent 100 μmol/m^2S cool white light tubes and 8 h darkness. Leaf segments were separated and excised from 4-week-old seedlings as explants for further experiments.

Establishment of *Catharanthus roseus* tissue culture systems

Callus induction

Leaf segments were cultured on solidified MS medium with different supplementation: (a) 1.0 mg/l dichlorophenoxyacetic acid (2,4-D)+1.0 mg/l kinetin (kin), (b) 1.0 mg/l 2,4-D+0.5 mg/l kin, (c) 1.0 mg/l naphthalene acetic acid (NAA)+1.0 mg/l benzyl aminopurine (BAP) and (d) 1.0 mg/l NAA+0.5 mg/l BAP. Cultures were kept in the culture room under temperature of 26±1°C and light conditions of 16 h photoperiod and 100 μmol/m^2s^1. Initiated callus was observed after 4 weeks of cultivation and then the callus initiation frequency (%) was calculated based on the following equation: callus initiation frequency %= [(number of initiated calli)/(number of inoculated explants)]×100. The nature of callus tissues were visually estimated.

Cell suspension culture

Actively growing callii was used for the initiation of cell suspension cultures by transferring 1 g of tissues into 100 ml Erlenmeyer flasks containing 20 ml liquid MS medium supplemented with 1.0 mg/l 2.4-D+1.0 mg/l kin and sucrose (30 g/l). The flasks were placed on a rotary shaker (110 rpm) and incubated under temperature of 26±1°C in both dark and light conditions of 16 h photoperiod and 100 μmol/m^2s^1. Fresh weights, dry weights, and viability% were estimated every 3 days for 30 days. Healthy and fast growing suspension were used for elicitation experiments.

Elicitation of cell suspension culture with MeJA

MeJA solutions were prepared by diluting the MeJA stock (Aldrich, 95% purity). MeJA solutions were prepared in an aqueous solution of ethanol 50% (v/v) and were filter sterilized through a 0.45 μm Millipore filter (Minisart, Sartorius, Germany). Different concentrations of MeJA (100, 200 and 300 μM) were added to the cell suspension culture. Each 250 ml conical flask containing 50 ml of liquid medium and inoculated with 1.0 g fresh calli was

transferred into an orbital shaker at 110 rpm and incubated at 26±1°C and 16 h photoperiod. The cell biomass of cell suspension culture was harvested at 2 and 4 days after culture initiation, centrifuged at 9500 rpm for 20 min, supernatants were removed, and pellets (calli) were freeze dried for HPLC analysis.

Elicitation of cell suspension culture with dark and light exposure

Cell suspension culture was performed in 250 ml conical flasks containing 50 ml of liquid medium and inoculated with 1 g fresh calli, then transferred into an orbital shaker at 110 rpm and kept at 26±1°C in complete darkness and 16 h photoperiod conditions. Cell suspension cultures tissues were collected at 2 and 4 days after elicitation, centrifuged at 9500 rpm for 20 min, supernatants were removed, and pellets (calli) were freeze dried for HPLC analysis.

High-performance liquid chromatography analysis

Sample preparation

About 0.5 g of freeze-dried cell suspension cultures (calli) of each samples was transferred into a 10 ml centrifuge tube and then soaked with 5 ml extracting solution [2% formic acidic water : methanol (50 : 50 v/v)] for 2 h. The mixture was sonicated for 30 min and centrifuged for 10 min at 4000 rpm. The supernatant was transferred into another tube filtered with a 0.2 µm PTFE syringe filter and injected in the HPLC [9].

Instrument conditions

Agilent 1260 infinity HPLC series (Agilent, USA), equipped with a quaternary pump, the column used: kinetex 5 µm EVO C18 100 mm×4.6 mm (Phenomenex, USA), operated at 30°C. The separation is achieved using a ternary linear isocratic elution with (a) HPLC grade water 2% formic acid (v/v) and (b) methanol. The injected volume was 20 µl. Detection: WWD detector set at 254.

Results and discussion

Callus initiation

Data in Table 1 show the effect of different concentrations of various growth regulators on the percentage of callus initiation of leaf explants excised from *C. roseus* seedlings. From the recorded data, it could be observed that MS medium with 1.0 mg/l 2,4-D and 1.0 mg/l kin supplementation was the best medium for inducted calli, producing 72.7%. The other tested media gave moderate results or weak callus production ranging between 8 and 40% callus initiations. However, when the leaf explants were cultured in MS+1.0 mg/l 2,4-D+1.0 mg/l kin the callus seems to have a good growth characterized by the presence of new cells on the surface of the callus with light green and creamy callus tissues (Fig. 1a). The other culture looks more enlarged with the growth of young white callus (Fig. 1b,c). When a combination of MS+1.0 mg/l NAA and 0.5 mg/l BAP was used, leaf explants showed the lowest callus tissue formation with the appearance of some shoots (Fig. 1d).

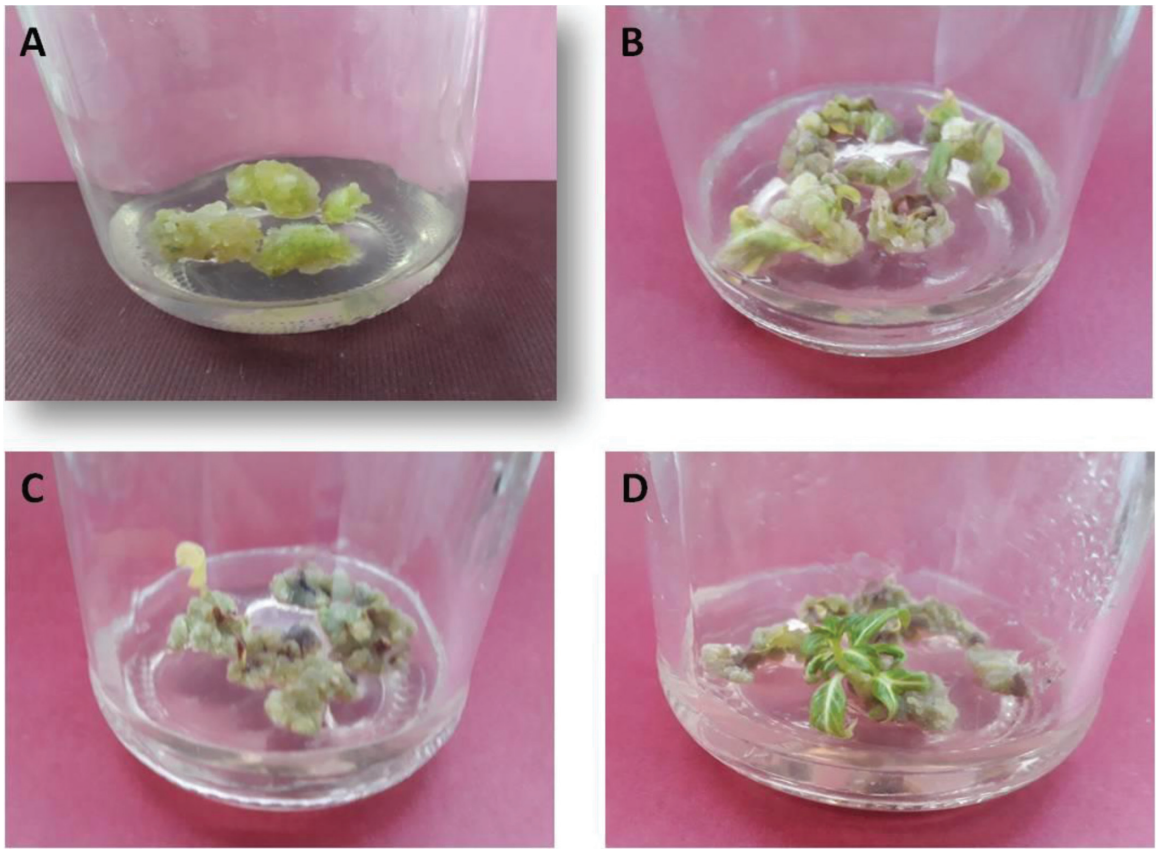
Table 1 Effect of Murashige and Skoog medium containing different growth regulators on callus induction and morphology

Growth regulator combinations	Callus induction (%)	Nature of callus tissue
1.0 mg/l 2,4-D+1.0 mg/l kin	72.7	Light green, creamy
1.0 mg/l 2,4-D+0.5 mg/l kin	40.0	White, creamy, compact
1.0 mg/l NAA+1.0 mg/l BA	12.8	White, compact
1.0 mg/l NAA+0.5 mg/l BA	8.0	Friable with shoots

NAA, naphthalene acetic acid.

In the previous studies, various researchers have tried *in vitro* culture of *C. roseus*; the responses were observed based on different growth regulator combinations and levels. 2,4-D was better to improve callus biomass and total alkaloid content. Besides, it was found that a combination of auxins with cytokinins is more effective for callus growth and alkaloid content enhancement. Given our results, MS medium with 1.0 mg/l 2,4-D+1.0 mg/l kin supplementation promoted a good amount of callus tissue reproduction [10]. Green callus also can be used for cell suspension culture and later for secondary metabolite production at an industrial level. In this context, it was observed that the highest callus frequency (100%) was obtained at 0.2 mg/l BA with 1.25 mg/l NAA combination from leaves of *C. roseus* [11]. Another study reported that the highest callus fresh weight obtained was with 1.0 mg/l 2,4-D +1.0 mg/l BA combination from the leaf of *C. roseus* [12]. It was also observed that the combination of 0.5 mg/l NAA and 1.0 mg/l BAP was the optimal for callus growth of *C. roseus* from hypocotyl explants [13]. Another work reported that the BAP at 2.0 mg/l with NAA at 1.0 mg/l combination was preferable for callus initiation from cotyledons of *C. roseus* [14]. Callus cultures from leaf explants of *C. roseus* were also obtained using 2 mg/l 2,4-D and 0.2 mg/l kinetin [15]. Recently, callus cultures from leaf explants of *C. roseus* were obtained with 1.5 mg/l BAP and 1.5 mg/l 2,4-D supplementation [16]. These reports

Figure 1



Callus induction of *Catharanthus roseus* from leaf explants cultivated on MS medium with different growth regulators after 4 weeks of cultivation, where (a) 1.0 mg/l 2,4-D+1.0 mg/l kin, (b) 1.0 mg/l 2,4-D+0.5 mg/l kin, (c) 1.0 mg/l NAA+1.0 mg/l BAP, and (d) 1.0 mg/l NAA+0.5 mg/l BAP. BAP, benzyl aminopurine; MS, Murashige and Skoog; NAA, naphthalene acetic acid.

strongly support the present study suggesting that 2,4-D and kinetin can support better growth and development of *in vitro* cultured leaf explants.

Cell suspension

Suspension culture was successfully developed using a leaf-derived callus, which was transferred to liquid MS medium supplemented with 1.0 mg/l each of 2,4-D and kin and incubated in a growth chamber on a rotary shaker with shaking (110 rpm) for 21 days with 16 h photoperiod (Fig. 2). Fresh weights and dry weights were estimated every 3 days. Data in Fig. 3 show that the fresh weight reached a maximum at the 20-day of culture, and then declined at 25 and 30 days of culture. Data in Fig. 4 show the viability (%) of cell suspension culture of *C. roseus* grown on an MS-liquid medium with 1.0 mg/l 2,4-D+1.0 mg/l kin supplementation. It could be observed that the viability of cells increased gradually with time until it reached their maximum at day 20 of culture, then declined until 30 day of culture, and was the lowest viability percentage.

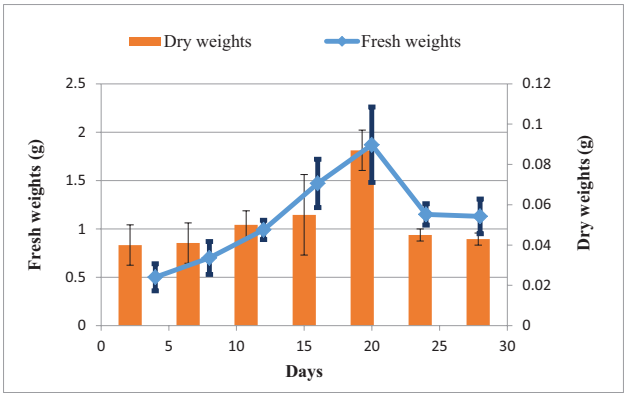
It was found that MS medium with 1 μM 2,4-D and 1 μM kinetin supplementation was beneficial to support the growth of callus cultures of *C. roseus* and the maximum callus biomass was obtained after 7 weeks of culture [17]. In our case also we have observed that kin and 2,4-D were effective in producing green calli, which might be suitable for suspension culture growth and alkaloid production.

Off-white callus with a friable texture was produced from young shoot tips using 1.5 mg/l 2,4-D and 0.5 mg/l kin supplementation and also it was observed that it was the optimal combination when this was used in the liquid medium, wherein it starts to disintegrate easily producing a friable cell suspension [18]. Suspension culture from *C. roseus* was established when they induced callus tissues from leaf explants; cell aggregation initiated when callus tissues were placed in a liquid that contained 2.0 mg/l NAA and 0.2 mg/l kinetin supplementation [19]. Also suspension culture from *C. roseus* was established when callus tissues were induced from shoot tip explants of *in vivo* grown plants and cultured on MS medium with 1.5 mg/l 2,4-D; the friable callus was transferred to an MS-liquid medium with 1.5 mg/l 2,4-D and 0.5 mg/l kinetin supplementation [20]. In our case, it was observed

Figure 2

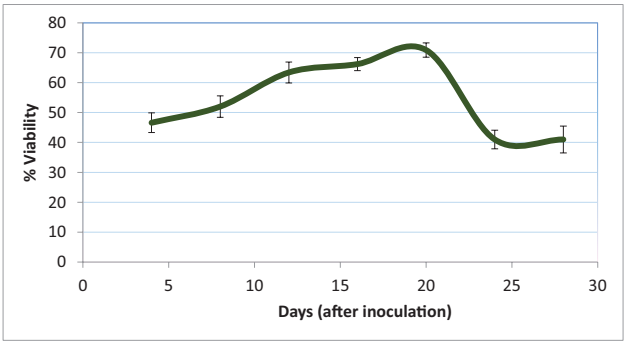
Cell suspension culture of *Catharanthus roseus* from leaf explant-derived callus after 1 month subculture on MS-liquid medium containing 1.0 mg/l 2,4-D+1.0 mg/l kin. MS, Murashige and Skoog.

Figure 3



Fresh and dry weights of cell suspension culture of *Catharanthus roseus* on MS-liquid medium supplemented with 1.0 mg/l 2,4-D +1.0 mg/l kin. MS, Murashige and Skoog.

Figure 4



Viability (%) of cell suspension culture of *Catharanthus roseus* on MS-liquid medium supplemented with 1.0 mg/l 2,4-D+1.0 mg/l kin. MS, Murashige and Skoog.

that the existence of 2,4-D and kinetin in the culture medium was necessary for the initiation of callus tissue, and this finding is in agreement with most studies.

Effect of methyl jasmonate on ajmalicine, vinblastine, and vincristine content

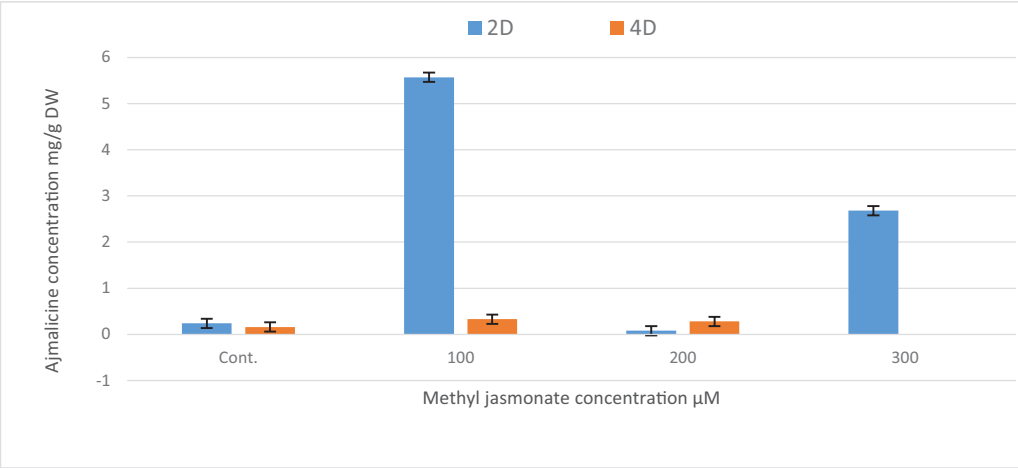
Ajmalicine content

Suspension culture that was subjected to different concentrations of methyl jasmonate was determined using HPLC for ajmalicine content. Data in Fig. 5 show the content of ajmalicine in calli collected from suspension cultures and treated with different concentrations of methyl jasmonate after 2 and 4 days of culture. It could be observed that methyl jasmonate (100 µM) treatment gave the highest content (5.6 mg/g dw) of ajmalicine after 2 days. In comparison with control, the ajmalicine content after 2 days of culture was increased 19-fold than the control. However, control accumulated the lowest ajmalicine content than other treatments (Fig. 5). There was a decrease in ajmalicine content in cell culture with methyl jasmonate treatments of 200–300 µM compared with 100 µM treatment.

Vinblastine content

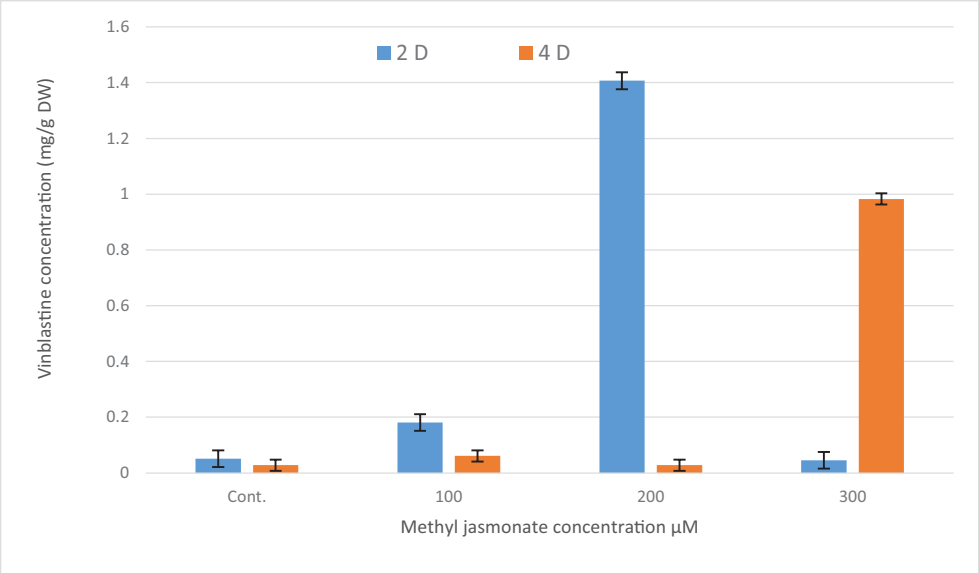
Data in Fig. 6 show the content of vinblastine in calli collected from suspension cultures treated with

Figure 5



Ajmalicine content (mg/g dw) in cell suspension cultures treated with different methyl jasmonate concentrations and control after 2 and 4 days of treatment.

Figure 6



Vinblastine content (mg/g dw) in suspension cultures treated with different methyl jasmonate concentrations and control after 2 and 4 days of treatment.

different concentrations of methyl jasmonate after 2 and 4 days of treatment. The highest vinblastine content was detected at 200 µM methyl jasmonate when cultures were treated for 2 days. It was a more than 140-fold increase than that in control. An increase in vinblastine content was observed at 300 µM methyl jasmonate after 4 days of elicitation. However, it could be observed that the content of vinblastine on callus without methyl jasmonate treatment (control) is the lowest one.

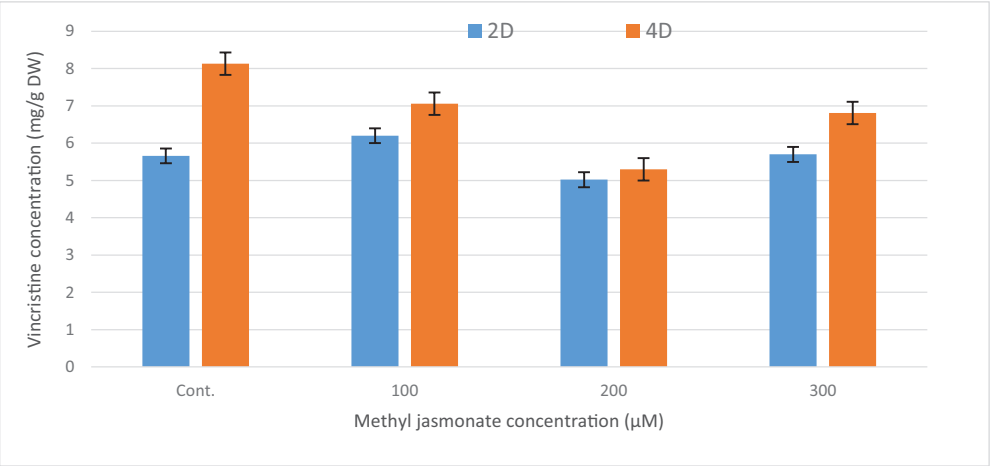
Vincristine content

Data in Fig. 7 show the content of vincristine in calli collected from suspension cultures treated with different concentrations of methyl jasmonate after 2 and 4 days of culture.

Results showed the effect of methyl jasmonate treatments on enhancing vincristine accumulation in cell suspension; it was observed that there was no difference in the accumulation of vincristine. A slight increase in vincristine content was recorded at 100 µM methyl jasmonate after 2 days of elicitation than other treatments, while the highest content of vincristine was recorded for control at 4 days.

Many studies have been previously attempted to obtain suspension cultures with a high production of indole

Figure 7



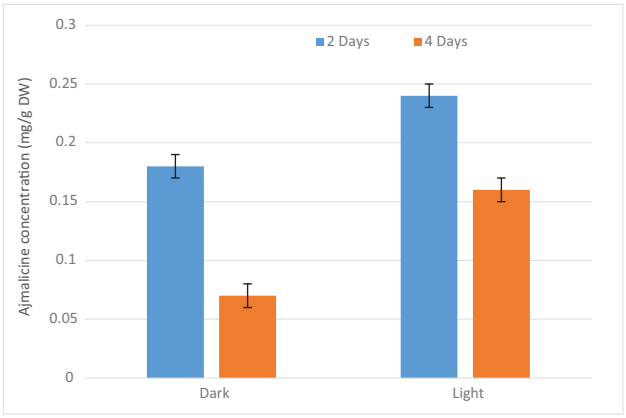
Vincristine content (mg/g dw) in suspension cultures treated with different methyl jasmonate concentrations and control after 2 and 4 days of culture.

alkaloids. *In vitro* culture of *C. roseus* was performed for the production of indole alkaloids and found that callus and cell suspension culture resulted in ajmalicine and catharanthine production from the cell culture [21]. Several studies have investigated the effects of light and methyl jasmonate on the accumulation of indol alkaloids. Different concentrations of MeJa (10, 100, and 250 µM) were added to a hairy root culture. The increasing concentration of MeJa increased the total alkaloid accumulation with a peak on the second day after elicitation. The different alkaloids showed different accumulation kinetics, but the accumulation of catharanthine was in parallel with the increasing MeJa concentration [22]. On the other hand, cyclodextrins were used combined with MeJa, and incorporated with short-term exposure to ultraviolet (UV). They found that the extracellular ajmalicine production was enhanced in cell suspension of *C. roseus* [23]. Also, it was observed by another study that the supplementation of methyl jasmonate into the culture medium significantly promoted the synthesis of tabersonine, catharanthine, and serpentine in *Glycyrrhiza uralensis* [24]. Our results are in line with most authors who found that elicitation of *C. roseus* culture with different elicitors significantly increased the accumulation of indol alkaloids. In the present results, methyl jasmonate had slight elicitation effect on vincristine yield, while it had strong elicitation activity concerning ajmalicine and vinblastine contents after 2 days of treatment at a concentration of 100 and 200 µM, respectively.

Effect of light and dark exposure on ajmalicine, vinblastine, and vincristine content

Data in Fig. 8 show the content of ajmalicine in calli collected from suspension cultures exposed to light and dark treatments after 2 and 4 days of culture. It could be observed that cultures exposed to light gave the best results than cultures exposed to dark conditions. Concerning vinblastine content, data presented in Fig. 9 show that dark condition gave the best levels of vinblastine than light condition especially after 2 days. However, vincristine content was better under light at 2 or 4 days (Fig. 10).

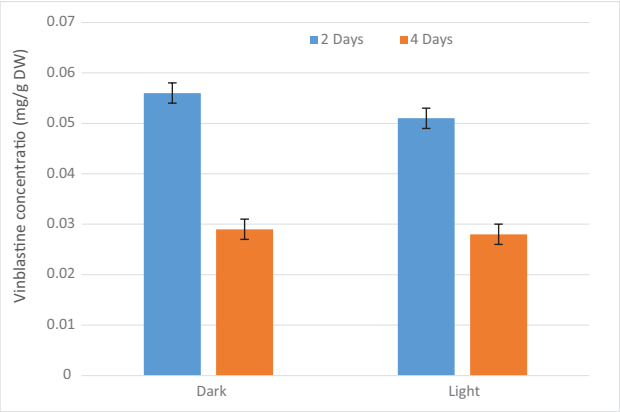
Figure 8



Ajmalicine content (mg/g dw) in suspension cultures exposed to light and dark treatments after 2 and 4 days of culture.

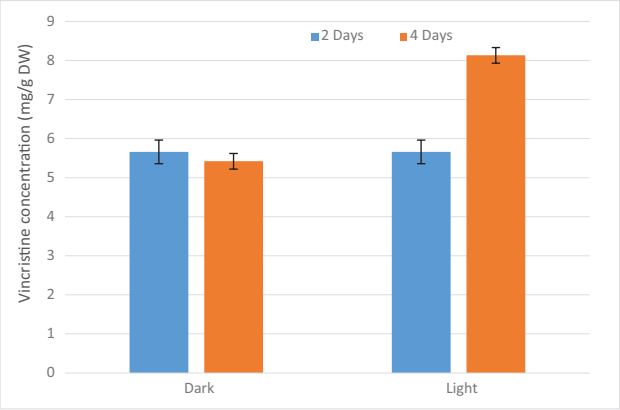
According to most author's investigations, the UV-B treatment proved to be effective in cell suspension cultures of *C. roseus*. The stationary phase of cultures was more responsive to a 5 min UV-B irradiation than late exponential phase of cultures, and it was observed that catharanthine and vindoline increased three-fold and 12-fold, respectively [25]. In this context, accumulation of vindoline was boosted by using MeJa with light combination in *C. roseus* cell

Figure 9



Vinblastine content (mg/g dw) in suspension cultures exposed to light and dark treatments after 2 and 4 days of culture.

Figure 10



Vincristine content (mg/g dw) in suspension cultures exposed to light and dark treatments after 2 and 4 days of culture.

suspensions, while catharanthine biosynthsis was improved by neither light nor MeJa elicitation [6].

Conclusion

Leaf explants of *C. roseus* cultured on MS medium supplemented with 1.0 mg/l 2,4-D and 1.0 mg/l kinetin was found to be the most effective for the initiation of callus tissue and suspension cultures. Methyl jasmonate treatment of *C. roseus* suspension cultures affects the ajmalicine content, where the highest content (5.6 mg/g dw) of ajmalicine content was observed with methyl jasmonate (100 µM) treatment. The highest vinblastine content was detected at 200 µM methyl jasmonate, which is the highest content in all treatments, when cultures were treated for 2 days. Also, there were no significant differences in the accumulation of vincristine between the different treatments of methyl jasmonate. On the other hand, light and dark treatments affect the content of ajmalicine. Cultures exposed to light treatment gave the best values of ajmalicine and vincristine, while vinblastine content was better under dark conditions.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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