Boosting Anticancer Alakloids Vincristine, Vinblastine, and Vindoline in Cathranthus roseus Callus Culture Using Polyeeltheneglycol

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Doi: 10.21608/ajsr.2025.422217

Receiving the search 6 – 8 - 2024
Acceptance of publication 29 – 8 - 2024

Zainab Abdulwahid & Abdulminam Hussien Ali & Abdullah Hamad Lafta (2025). Boosting Anticancer Alakloids Vincristine, Vinblastine, and Vindoline in *Cathranthus roseus* Callus Culture Using Polyeeltheneglycol. ***The Arab Journal of Scientific Research***, AIESA, Egypt, 9(10), 123-140.

https://ajsr.journals.ekb.eg

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Abstract:

Periwinkle *Cathranthus roseus* is an ornamental plant that produces over 200 alkaloids, including vincristine, vinblastine, and vindoline, which are used to treat various types of cancer. However, the low yield of TIAs from periwinkle and the high cost of extraction has led researchers to explore alternative methods for their production. The study aims to investigate the influence of polyethylene glycol (PEG) as an elicitor to enhance alkaloid production in periwinkle callus cultures while examining the efficacy and safety of using PEG. Callus was induced and abiotic elicitation experiments were conducted to study callus growth enhancement. The dry weight of the callus was calculated, and vincristine and vinblastine were extracted and analyzed using high-performance liquid chromatography. The results showed that low to moderate concentrations of PEG4000 can enhance callus growth, while higher concentrations can reduce it. The addition of PEG4000 also increased the production of important alkaloids, including vindoline, vincristine, and vinblastine, by up to 2.65-fold. The findings suggest that PEG4000 can be used to optimize the production of alkaloids in C. roseus callus cultures.

Keywords: Periwinkle, *Catharanthus roseus*, polyethylene glycol (PEG), vincristine,vinblastine, vindoline

Introduction

Plants produce a wide range of secondary metabolites with diverse applications, including pharmaceuticals, dyes, and insecticides (24). Among these, periwinkle (*Cathranthus roseus*) stands out as an essential source of more than 130 different indole terpenoid alkaloids (TIAs), which have pharmaceutical uses (20, 26). Vincristine and vinblastine are bisindole alkaloids that are widely antineoplastic drugs and cancer chemotherapies (4, 23). vindoline, is also of great significance in the pharmacological function. In their research, Eltayeb *et al*., (6) demonstrated that the leaf extract of *C roseus* has the ability to inhibit the invasive properties of two types of breast cancer cells by regulating the activity of matrix metalloproteinases. Similarly, Goboza *et al*., (8) found that vindoline can promote insulin action, leading to a reduction in blood sugar levels in rats. However, the natural yield of these alkaloids in periwinkle is meager, making it necessary to find alternative methods for their production.

Callus culture is an unconventional technology that can be used to produce secondary metabolites and other natural products (18,12). By providing a continuous and reliable source of natural products in the long term, this technology is becoming increasingly popular among researchers. One of the key advantages of callus culture is the ability to use various elicitation factors to trigger and increase the yield of cell secondary metabolites (16, 21). Elicitors are compounds that stimulate the production of secondary metabolites in plants, and among the abiotic elicitors, polyethylene glycol (PEG) has been shown to be effective in enhancing the production of alkaloids in various plants, including periwinkle (13, 3, 1). However, some studies have raised concerns about the safety and effectiveness of using PEG as an elicitor (17, 10, 5).

ISSN: 2537-0367 **eISSN : 2537-0375**

The main aim of this study is to investigate the influence of PEG on biomass production and the accumulation of dimeric alkaloids, including vincristine, vinblastine, and vindoline, in callus cultures of periwinkle. Specifically, the study aims to examine the efficacy and safety of using PEG as an elicitor to enhance alkaloid production in periwinkle callus cultures. The hypothesis is that PEG will increase the yield of alkaloids in periwinkle callus cultures without causing any adverse effects on cell growth or viability. The present study will aid in the creation of a productive and long-lasting technique for manufacturing TIAs from periwinkle, which may be utilized for cancer treatment.

Materials and Methods

Plant Materials

Catharanthus roseus (L.) Don was selected as the plant species for this study, and pinkish-purple blooms were obtained from a reputable local nursery. The plants used in the experiment were identified and certified by the Department of Biology, College of Science, University of Basrah. Young and healthy leaves were carefully collected from the plants and washed with liquid dish soap and tap water for 15 minutes each to remove dust and dirt. To sterilize the leaves, 0.1% mercuric chloride solution containing 2-3 drops of Tween 20 was used for 4-5 minutes. To eliminate any remaining HgCl2 residue, the explants were washed five times with autoclaved distilled water. The leaves were then cut into small pieces measuring 0.4-0.5 cm and kept in wet sterile Petri dishes until inoculated into the culture vessels. All sterilization procedures were conducted under aseptic conditions to ensure the purity of the samples.

Callus induction

ISSN: 2537-0367 eISSN : 2537-0375

Sterilized leaf explants (0.4-0.5 cm) were inoculated into 250 ml jars containing 50 ml of MS Murashige and Skoog (19) basal medium supplemented with myo-inositol (100 mg L^{-1}), NaH2Po4 $2H_2O$ (150 mg L^{-1}), and 5% sucrose, as well as a combination of phytohormones, including naphthalene acetic acid (NAA) at 5 mg L^{-1}, benzyl aminopurine (BA) at 2 mg L^{-1}, and kinetin (KN) at 2 mg L^{-1}. The pH of the medium was adjusted to 5.8 using 0.1 M NaOH or 0.1 M HCl, followed by the addition of 7 g L^{-1} agar. The medium was then autoclaved at 121 °C for 20 minutes. All cultures were incubated in the dark at 27 °C in a plant growth room for two months. All chemicals utilized in the tissue culture experiment were ordered from Duchefa Company Holland.

Callus Treatments

Abiotic elicitation experiments were conducted using the callus culture obtained in step 1. To study the enhancement of callus growth, vincristine, vinblastine, and vandoline, one gram of callus was inoculated into a medium containing different levels of polyethylene glycol (PEG). Cultures that were not treated with abiotic elicitors (0 g) were used as controls.

Callus Fresh and Dry Weight

After a two-month treatment with different abiotic elicitors, calli were harvested and carefully rinsed with distilled water to remove any residual medium. The calli were then placed on filter paper to blot away excess water before being weighed to determine their fresh weight using a calibrated balance. To determine the dry weight of the calli, fresh callus samples were placed on filter paper and left to air-dry for 72 hours at 25°C. The dry weight of each callus was then calculated using an electronic balance.

Extraction of Vincristine and Vinblastine from Callus

ISSN: 2537-0367 **eISSN : 2537-0375**

To prepare the samples for analysis, 5 g of dried callus was ground into a fine powder and extracted three times with 90% ethanol for 12 hours at room temperature. The resulting alcohol extract was then passed through a Millipore filter and concentrated to 10 mL using a rotary evaporator. For further extraction, a 10 mL water extract was acidified with 3% HCl (10 mL) and then washed three times with 30 mL hexane. The aqueous fraction was then basified to pH 8.5 with ammonia and extracted three times with 30 mL of chloroform. The resulting chloroform extracts were washed with water, dried over sodium sulfate, and vacuum-concentrated to obtain the final sample. Finally, 10 mL of methanol was used to dissolve the sample (9).

Determination of Vincristine and Vinblastine by HPLC

The callus extracts were subjected to high-performance liquid chromatography (HPLC) analysis following the method described by Gupta *et al*. (9). The analysis was performed using an HPLC system (SYKAMN, Germany) equipped with a C18-ODS column and detector. The mobile phase used was 1.2 mL/min isocratic acetonitrile-0.1 M phosphate buffer containing 0.5% glacial acetic acid (30:70). The system was injected with diluted standards to analyze vincristine, vinblastine, and vendolaine. The results were recorded and analyzed using appropriate software.

Data recording and statistical analysis

A series of three in vitro experiments were conducted to investigate the response of calli to abiotic stress. Five vessels were used for each treatment. Data on callus induction and proliferation were collected after two months under both stress-free and stressful conditions. The experimental design followed a completely randomized design (C.R.D.) and the Genestats 0.7 program was utilized to determine the least significant difference (LSD) between the means of the treatments. The correlation between stress factors and the production of vincristine,

ISSN: 2537-0367 eISSN : 2537-0375

vinblastine, and vindoline was analyzed at a significance level of 0.05 using SPSS 24.

Results and Discussion

Influence of PEG on Callus Biomass and Alkaloid Production

The results of the experiment are presented in Table 1, which shows the callus weight for each PEG treatment. The callus fresh weight was highest in the 0.5 g PEG treatment with a mean of 4.44±0.4 g, while the lowest fresh weight was observed in the 2.0 g PEG treatment with a mean of 4.00±0.5 g. The mean callus fresh weight for all treatments was 4.20±0.6 g. The callus dry weight followed a decreasing trend with increasing PEG levels. The mean dry weight was 0.23±0.08 g, with the lowest dry weight observed in the 2.0 g PEG treatment (0.22±0.06 g). The LSD values for callus fresh and dry weight were 0.42 and 0.05, respectively. The data suggest that 0.5 g PEG treatment can enhance callus growth, whereas higher levels of PEG can reduce callus growth.

Table 1 Impact of various different concentrations of PEG_{4000} on the fresh and dry weight of *Catharanthus roseus* L. callus.
[*]Values followed by the different letters within the same group indicate statistically significant difference

PEG gm L^{-1}	Callus Wight (gm)	
	Fresh Weight+S E	Dry Weight +SE
Control	4.34±0.5[a]	0.33±0.04[a]
0.5	4.44±"0.4[a]	0.29±0.09[ab]
1	4.11±1.09[a]	0.24±0.1[b]
1.5	4.09±0.5[a]	0.23±0.07[ab]
2.0	4.00±0.5[a]	0.22±0.06[ab]
Mean	4.20± 0.6	0. 23± 0.08
LSD	0.42	0.05

SE= Standard error

ISSN: 2537-0367 eISSN : 2537-0375

The results of our study are consistent with previous findings reported by Sarmadi *et al*., (22), who observed a decrease in fresh and dry weights of *Taxus baccata* L. cultures with increasing levels of PEG. The decline in fresh and dry weight levels can be attributed to the reduction in water content, which causes a decrease in cell turgor pressure and oxidative stress levels, resulting in brown calli. However, low to moderate concentrations of PEG was found to reduce the accumulation and oxidation of phenolic compounds in calli, which was attributed to the absorption of phenolic compounds and a reduction in the activity of polyphenol oxidase. Mahmood *et al*., (14) also confirmed the inhibitory effect of PEG on callus growth and development of wheat, where growth and survival of callus were significantly reduced when transferred to a medium fortified with high levels of PEG. AL-Taha (2) reported similar results, where a reduction in callus growth was observed due to the decrease in cell turgor pressure caused by the reduction in water content.

Our HPLC analysis (Table 2, Fig. 1) revealed significant differences in the production of alkaloids in callus culture due to the addition of PEG. The highest
amount of vindoline, vincristine, and vinblastine was obtained on a medium containing 2 g PEG (15.90, 29.36, and 41.49 µg ml-1), followed by 1.5 g PEG (14.79, 28.22, and 40.19 µg ml-1), respectively. On the other hand, the lowest amount of vindoline, vincristine, and vinblastine was recorded in callus grown on a medium free of PEG and a medium containing 0.5 g PEG. Interestingly, the production of these alkaloids increased 2.65, 2.61, and 2.5-fold, respectively, with the addition of PEG. These findings suggest that PEG can be used to enhance the production of important alkaloids in *C. roseus* callus cultures.

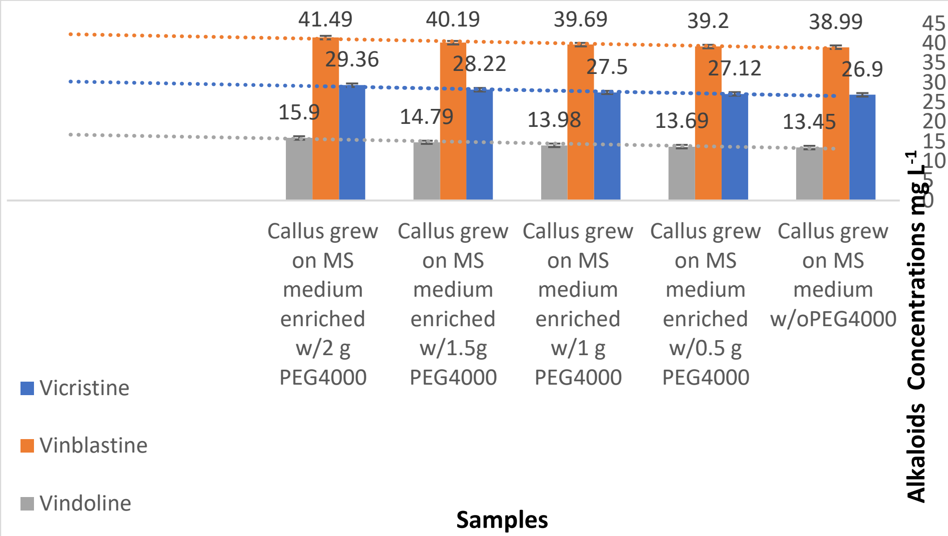
ISSN: 2537-0367 **eISSN : 2537-0375**

Table 2 Effect of various concentrations of PEG_{4000} on dimeric alkaloids content of *Catharanthus roseus callus*.

Samples	Vincristine			Vinblastine			Vindoline		
	RT/Min.	Area	Con. µg ml^{-1}	RT/ Min.	Area	Con. µg ml^{-1}	RT/ Min.	Area	Con. µg ml^{-1}
Callus grew on MS medium w/o PEG_{4000}	5.96	11253.98	26.90	7.93	15248.99	38.99	4.07	5521.08	13.45
Callus grew on MS medium enriched w/ 0.5 g PEG_{4000}	5.94	8642.58	27.12	7.96	9652.14	39.20	4.03	3256.58	13.69
Callus grew on MS medium enriched w/ 1 g PEG_{4000}	5.99	8965.28	27.50	7.93	10240.22	39.69	4.08	3521.49	13.98
Callus grew on MS medium enriched w/ 1.5 g PEG_{4000}	5.96	9242.58	28.22	7.92	11253.08	40.19	4.05	3652.18	14.79
Callus grew on MS medium enriched w/ 2 g PEG_{4000}	5.90	9521.28	29.36	7.92	12465.80	41.49	4.03	3854.08	15.90

w= with w/o= without, RT=Retention time, Con.= concentrations

ISSN: 2537-0367 eISSN : 2537-0375



w= with   w/o= without

Figure 1 The predictive curve for analyzing alkaloids from callus treated with different concentrations of PEG shows an increase in alkaloid production with an increase in the concentration of PEG's osmotic effect.

PEG is one of the most commonly used materials in water stress experiments and for creating drying conditions similar to the natural environment. It is widely used in various fields, including biochemical and industrial applications, due to its non-toxicity (15). Polyethylene glycol (PEG) has been widely used in *in vitro* plant culture to simulate water stress conditions, which can result in increased production of secondary metabolites (1). Taha *et al*. (24) found that adding a combination of PEG and chitosan to the growth medium of *C. roseus* increased the production of several alkaloids, including vincristine and vinblastine. Amirjani, (3) confirmed the stimulatory effect of PEG on the production of vincristine and vinblastine in *C. roseus* treated with 12% PEG for 72. Iskandar and Iriawati (10), demonstrated that exposing plants to drought

ISSN: 2537-0367 eISSN : 2537-0375

using PEG_{4000}, up to a concentration of 12% (w/v), did not have a significant impact on the production of vinblastine. While high vincristine concentration was obtained on a medium containing 0% of PEG. According to the study findings, there is a distinct pattern in the concentration of alkaloids that appears to be linked to the level of drought stress. This is likely due to the fact that the administered PEG concentration was not sufficiently high to reduce alkaloid biosynthesis while staying within the cells' tolerance limits.

Plant growth, morphology, and metabolic processes are all impacted by drought and salt stresses. To cope with these stresses, plants undergo adaptations that involve altering their metabolic processes. This includes producing and storing primary and specialized metabolites that aid in drought and salt resistance (27). Arabidopsis plants respond to drought stress by accumulating glucosinolates (11). In *C. roseus* plants, both drought and salt stress lead to an increase in the accumulation of terpenoid indole alkaloids (TIAs), such as ajmalicine, catharanthine, vindoline, vinblastine, and vincristine (12).

Theoretically, an increase in the concentration of PEG administered to the culture would result in a corresponding increase in the level of drought stress experienced by the plant cells. This, in turn, would trigger the plant cells to produce higher levels of abscisic acid as an initial response to drought stress (7). Abscisic acid (ABA) is a critical hormone in plant responses to drought stress. It regulates various physiological processes such as stomatal closure, which helps the plant to conserve water. The production of alkaloids is also regulated by ABA, and previous studies have shown that an increase in ABA levels can lead to promoting catharanthine production in *C. roseus* suspension cells (28).

Therefore, it is plausible to assume that the increase in abscisic acid levels triggered by PEG-induced drought stress

ISSN: 2537-0367 **eISSN : 2537-0375**

may have contributed to the observed increase in alkaloid production in the present study. However, further research is needed to confirm this hypothesis and to elucidate the underlying mechanisms involved.

It should also be noted that the effects of PEG-induced drought stress on alkaloid production may vary depending on the plant species and the type of alkaloids produced. The correlation analysis provides information on the stress factor of polyethylene glycol (PEG) and the TIAs compounds' production. The correlation between the compound vincristine and other alkaloids compounds was a strong inverse correlation (+), with R value of (0.975), followed by the compound vindoline with a value of (0.971), and then the vinblastine with a value of (0.966) Plate 1. The significant increase in the quantity of secondary compounds under the influence of the osmotic stress factor is attributed to the fact that growing cells under stress conditions can produce more polyamines, which are key components in the production of many secondary metabolites (25). Additionally, PEG increases the negative osmotic pressure of the cell, thereby increasing the exchange of solutes in the cell and consequently the production of secondary compounds. Therefore, caution should be exercised when extrapolating the findings of this study to other plant species or alkaloids.

In conclusion, the results of the present study suggest that PEG_{4000} in low concentrations with co-stress factor (sucrose 5%) induced drought stress can enhance alkaloid production in plants, possibly by increasing abscisic acid levels. This finding has potential implications for the pharmaceutical industry, as alkaloids are an important source of drugs and drug precursors. Further research is needed to explore the potential of using drought stress as a tool to enhance alkaloid production in plants.

ISSN: 2537-0367 **eISSN : 2537-0375**

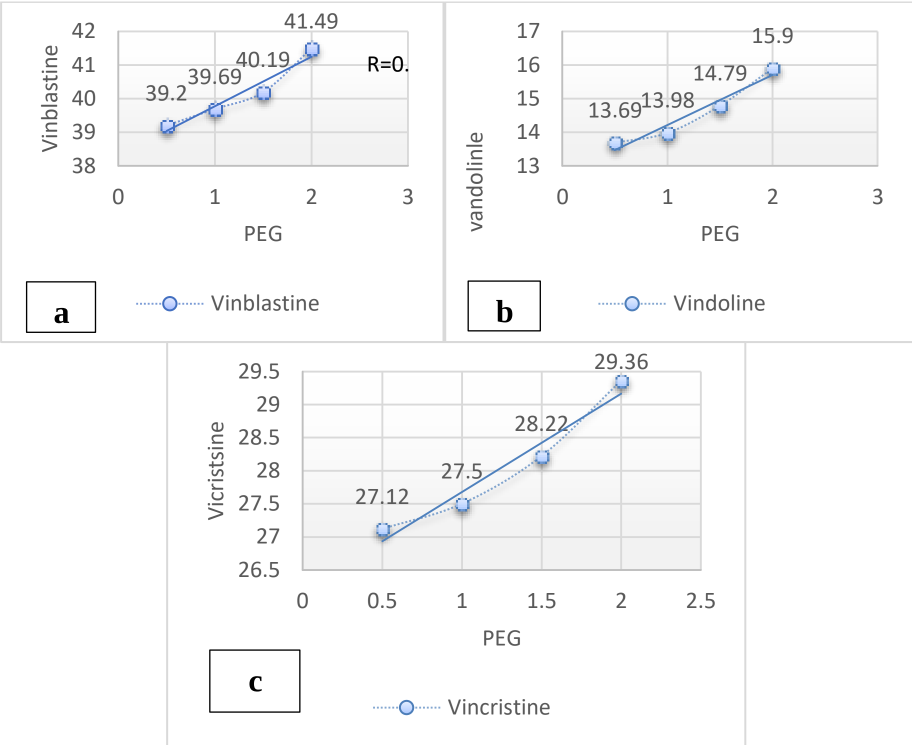


Plate 1 The curve shows the strength and direction of the correlation between the two variables TIA and PEG(Vinblastine (a), vindoline (b) and vincristine(c). The high correlation coefficients in this case indicate a strong positive correlation between PEG concentration and the concentrations of vinblastine, vindoline, and vincristine.

ISSN: 2537-0367 eISSN : 2537-0375

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ISSN: 2537-0367 **eISSN : 2537-0375**