Egypt. J. Chem. Vol. **68**, No.2, pp. 211 -219 (2025)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Different Carbon Sources and Their Concentrations Affect Alkaloid Accumulation In Transformed Root Cultures Of *Catharanthus Roseus*

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Abstract

Catharanthus roseus is a perennial small herb that produces terpenoid indole alkaloids (TIAs) of high medicinal importance. Carbon source is an absolute requirement for growth, development, and secondary metabolite production in plant tissue culture systems. TIAs are found in limited quantities within the plant, and their commercial demand is rapidly increasing. This study aims to evaluate the effects of different types and concentrations of carbon sources on alkaloid accumulation in hairy root cultures of *C. roseus*. Leaf explants were infected with *Agrobacterium rhizogenes*, strain ATTCC 15834, and several parameters were investigated for the maximum efficiency of the transformation protocol. In addition, the effects of sucrose, glucose and fructose on the accumulation of vincristine, vinblastine, ajmalicine and catharanthine in hairy root cultures were analyzed by HPLC. The results indicated 30-min period as the best time for inoculating the explants with *A. rhizogenes*. Maximum transformation efficiency was obtained with the supplementation of acetosyringone at a concentration of 100 µM into the co-cultivation media. Successful transformation was confirmed by polymerase chain reaction (PCR) with the RolA gene serving as an indicator of effective integration. While fructose at a concentration of 30 g/L significantly enhanced the levels of vinblastine, ajmalicine and catharanthine on hairy root cultures of *C. roseus*, sucrose at a concentration of 30 g/L was superior over other types of carbon sources for the maximum production of vincristine. These results offer insights into the optimal growth conditions, particularly the appropriate carbon source and provide a basis for further research aiming at enhancing commercial production of TIAs.

Keywords: Hairy root; *Agrobacterium rhizogenes*; carbon source; HPLC**;** Alkaloids.

1. Introduction

The use of medicinal plants dates back to the dawn of humanity. Ancient healers relied on these plants to treat a wide range of ailments. Amongst the plethora of medicinal plants identified, *Catharanthus roseus* has been widely utilized to heal many illnesses in numerous countries. This legendary splendid plant produces more than 130 different terpenoid indole alkaloids (TIAs), some of which demonstrate remarkable pharmacological properties **[1].** For example, vinblastine and vincristine are commercial TIAs used in anticancer chemotherapy nowadays. In addition, catharanthine and vindoline are precursors of these important two TIAs **[2].** *C. roseus* also produces ajmalicine and serpentine, which belong to TIAs and used as antihypertensive and antioxidant agents **[3].** However, these important alkaloids are present in extremely low concentrations in this plant. As such, there is a need for increasing the alkaloids level to meet the accelerated commercial demand.

Various *in vitro* systems such as plant cell and organ cultures, immobilized plant cultures, transformed cultures, bioreactor cultures are increasingly being employed for enhancing secondary metabolites production, often utilizing techniques like elicitation, precursor supply, media composition and genetic engineering **[4].** Plant tissue culture techniques allow for the mass production of disease-free plants (or specific tissues) in short durations, in small spaces with high level of clonal stability **[5-8].** This technology offers distinct advantages for inducing secondary metabolites, facilitating more reliable production and enabling faster, more efficient phytochemical isolation compared to traditional methods involving entire plant organs **[9, 10].** The induction of hairy root cultures, in particular, demonstrates a valuable tool for maximizing the production of target secondary metabolites in medicinal plants. This approach has drawn much attention in the last few decades because hairy root cultures develop faster than the adventitious roots or even conventional plant cultures and accumulate greater amounts of particular secondary metabolites than both adventitious roots and native-grown plant roots **[11].** The composition of the culture medium is one of the most important factors that applied for increasing the level of secondary metabolites in hairy root cultures. In particular, the carbon source in tissue culture media is a vital demand for growth, development, and metabolite

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Receive Date: 23 May 2024, Revise Date: 19 June 2024, Accept Date: 24 June 2024
DOI: 10.21608/ejchem.2024.292105.9759

production within the system **[12].** Different concentrations of carbon sources may considerably affect the performance of the culture, which is attributable to their effects on the energy supplied to the cell and the osmotic potential of the medium. The dysregulation of these physiological factors related to cell survival leads to remarkable modifications in cell expansion and division, as well as in the biosynthesis of secondary metabolites **[13].** Few attempts in hairy root cultures of *C. roseus* have been reported recently. For example, **Hanafy et al. [11]** determined the accumulation of some TIAs in the transgenic hairy roots. Furthermore, they evaluated the antimicrobial effects of these hairy root cultures. In addition, **Benyammi et al. [14]** investigated the relationship between biomass production and alkaloids accumulation of three selected hairy root lines and found that the evolution of accumulation of ajmalicine and catharanthine are positively correlated with the development of the biomass growth. **Rady et al. [15]** investigated the impact of two activators on two genes and reported that these activators work differently. They pointed out the importance of the experimental factors on the impact of these activators. The main objective of this investigation was to evaluate the impact of different types and concentrations of carbon sources on alkaloid accumulation in hairy root cultures of *Catharanthus roseus*.

2. Results

2.1. Induction of hairy root cultures

The effects of several parameters such as inoculation time and supplementation of acetosyringone on transformation efficiency of C. roseus by using A. rhizogenes strain ATCC 15834 were investigated. The results showed that different inoculation periods exhibited different hairy root induction frequencies (Figure 1). The highest frequency of hairy root induction (68%) was obtained with the 30 minutes treatment followed by the 20 minutes treatment (52%), whereas the lowest percentage of hairy root frequency (39%) was noticed in the 10 minutes treatment. It was observed that extending the inoculation period over 30 minutes caused browning and reduced survival of the explants. Control explants (0.0 min; no infection with A. rhizogenes strains ATCC 15834) failed to induce hairy root.

The results showed that irrespective of acetosyringone concentration, all explants induced considerable frequencies of hairy root at varying degrees (Figure 2). Among the different concentrations, 100 µM of acetosyringone proved to induce maximum efficiency of hairy root (88%). Two hundred µM of acetosyringone leads to decrease the efficiency slightly (83%). It was observed that the lowest frequency of hairy root induction (50%) obtained by 50 µM of acetosyringone was significantly higher than that of the control samples (13%; no addition of acetosyringone).

Hairy roots were initiated directly from the wound sites of explants after 10 days of culture on solid 1/2 MS medium. Subculture was conducted every two weeks on a fresh medium. Figure (3) represents stages of initiation of hairy root cultures on solid 1/2 MS medium.

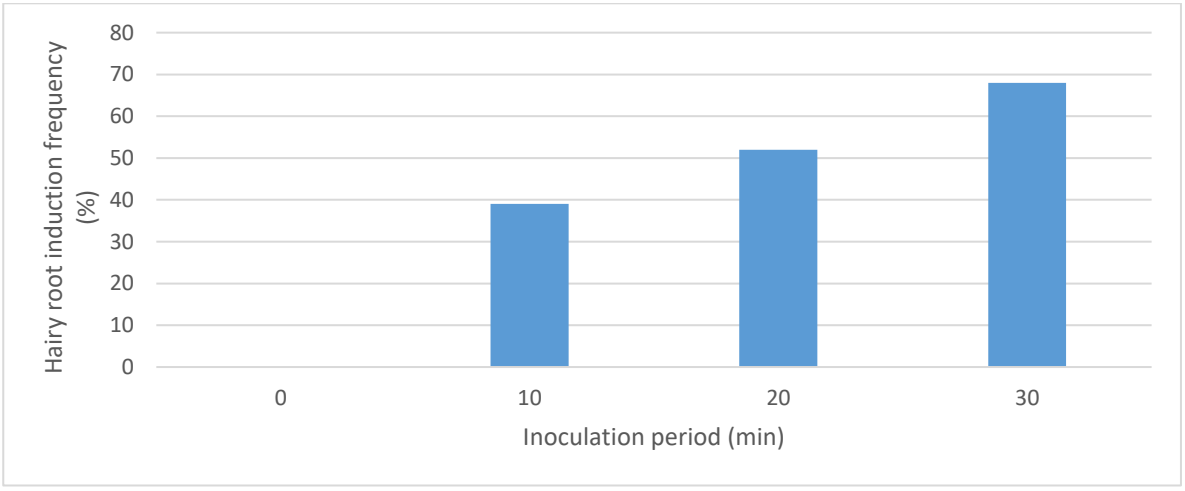

Figure 1. Effect of different inoculation periods on hairy root induction frequency of *Catharanthus roseus*.

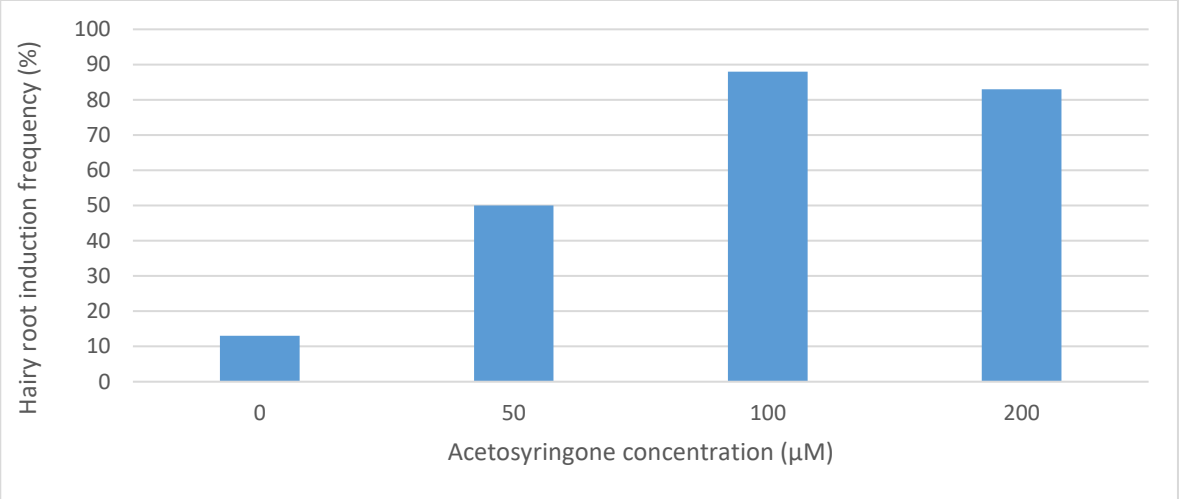

Figure 2. Effect of different concentrations of acetosyringone on hairy root induction frequency of *Catharanthus roseus*.

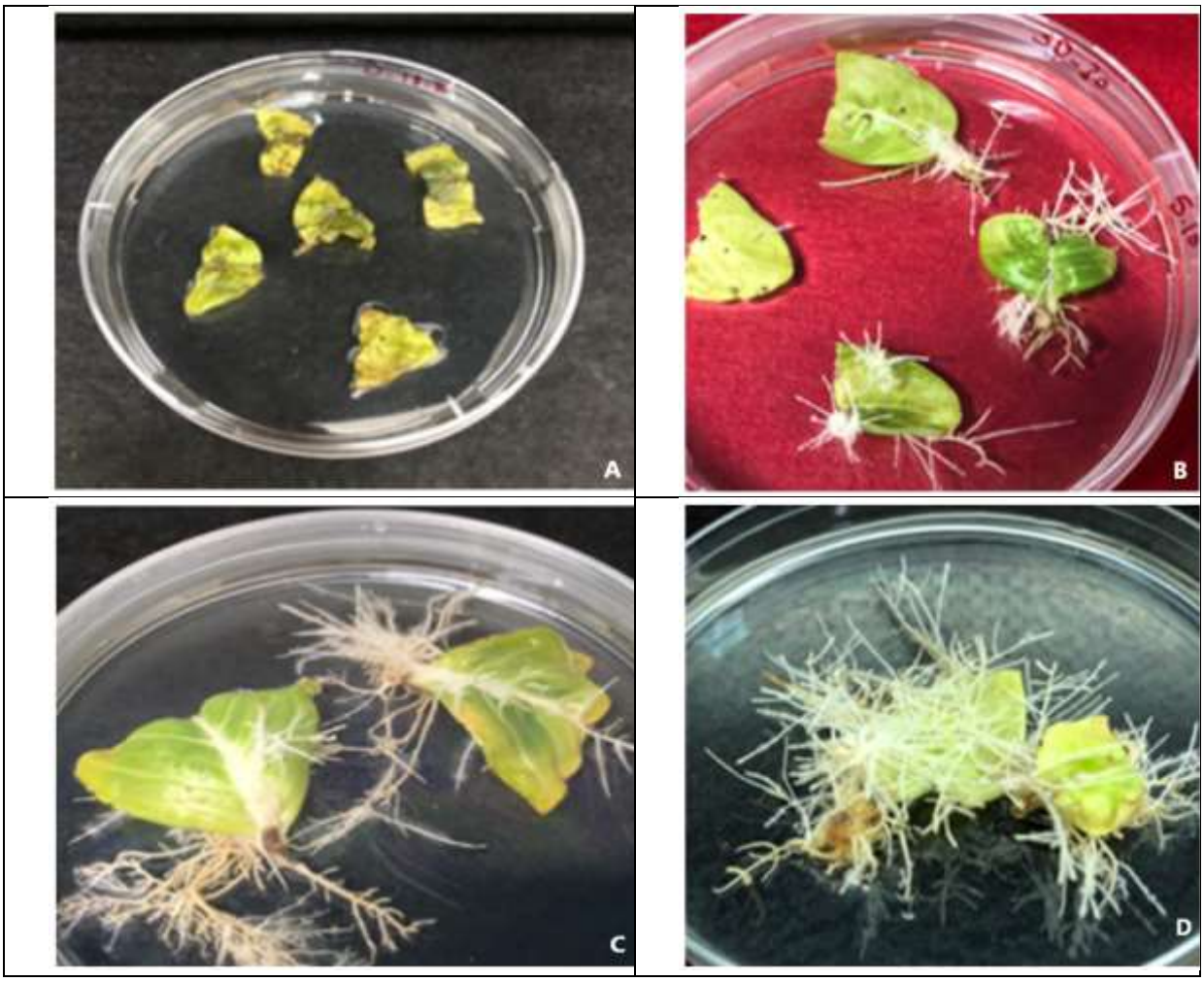

Figure 3. Induction of hairy root culture: (A) leaf explants after infection with *Agrobacterium* strains ATCC 15834; (B) hairy root culture after two weeks of infection; (C) hairy root culture after four weeks of infection; (D) hairy root culture after six weeks of infection.

2.2. Molecular confirmation of transgenic hairy roots

Agrobacterium rhizogenes has a large Ri-plasmid that contains rol genes A, B, C, and D. Infection of plant cells with Agrobacterium rhizogenes introduced the T-DNA into plant genomic DNA, encouraging the formation of hairy roots from the explants. Polymerase chain reaction (PCR) analysis of rolA gene and gel electrophoresis indicated that Catharanthus roseus hairy roots showed bands for the estimated PCR product (304 bp). Figure (4) indicated that the induced roots (in all tested samples; lane 3-7) were hairy roots originating from Catharanthus roseus explants. The PCR product (304 bp) was not observed in wild type roots (negative control; lane 2) whereas it was noticed in the Ri-plasmid of Agrobacterium rhizogenes (positive control; lane 1).

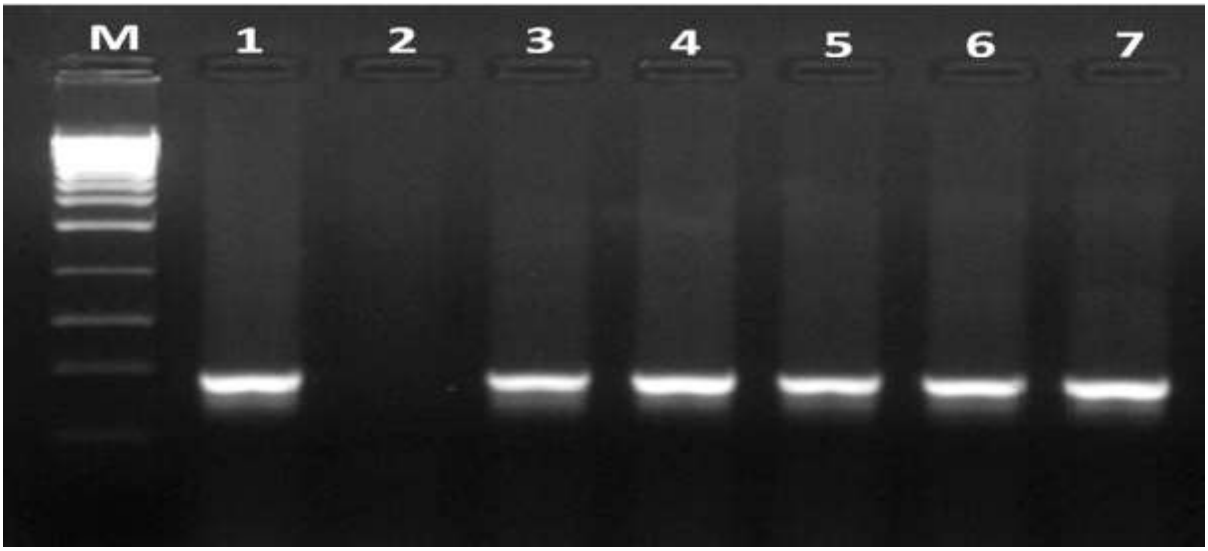

Figure 4. Represent PCR analysis of *rol*A gene in transformed roots by *Agrobacterium rhizogenes* ATCC 15834. Lane M, marker 1000 bp ladder, lane 1, positive control (plasmid DNA), lane 2, negative control (DNA roots of non-transformed roots), and lane 3–7, transformed roots. PCR product size is 304 bp.

2.3. Effect of different carbon sources on the accumulation of some terpenoid indole alkaloids (TIAs)

High performance liquid chromatography (HPLC) was used to analyze hairy root cultures for the production of some terpenoid indole alkaloids (TIAs) namely, vincristine, vinblastine, ajmalicine, and catharanthine after treatments with different types and concentrations of carbon sources. These alkaloids were determined using HPLC analysis by comparing peaks retention time to the standard. Figure (5) showed the content of vincristine in hairy root cultures treated with different types and concentrations of carbon sources. Vincristine contents ranged from 0.009 µg/g DW to 0.167 µg/g DW. In this regard, the highest level of vincristine was observed in the medium supplemented with 30 g/L sucrose (0.167 mg/g DW), followed by the medium supplemented with 30 g/L fructose (0.085 mg/g DW) whereas the medium supplemented with 60 g/l fructose recorded the lowest level of vincristine (0.009 µg/g DW).

Data in Figure (6) showed the content of vinblastine in hairy root cultures treated with different types and concentrations of carbon sources. The contents of vinblastine ranged between 0.105 µg/g DW and 3.662 µg/g DW. Although the highest content of vinblastine was achieved in the media continuing 30 g/L fructose, the lowest level was obtained in the medium containing 60 g/L fructose.

The HPLC profile of ajmalicine isolated from the hairy root cultures is shown in Figure (7). The content of ajmalicine ranged from 0.068 µg/g DW to 1.039 mg/g DW. The results indicated that the optimum carbon source for the maximum production of ajmalicine is fructose at a concentration of 30 g/L (1.039 µg/g DW) followed by sucrose at a concentration of 30 g/L (0.906 µg/g DW) whereas 60 g/L sucrose exhibited the lowest level of ajmalicine (0.068 µg/g DW).

Figure (8) showed the effects of different types and concentrations of sucrose, glucose and fructose on the accumulation level of catharanthine in hairy root cultures of Catharanthus roseus. The level of catharanthine ranged between 0.034 µg/g DW and 1.148 µg/g DW. In this regard, the highest accumulation was recorded in the hairy root cultures which were grown on media supplemented with 30 g/L fructose followed by media containing 30 g/L sucrose (0.433 µg/g DW) whereas media containing 60 g/L fructose exhibited the lowest level of catharanthine (0.034 µg/g DW).

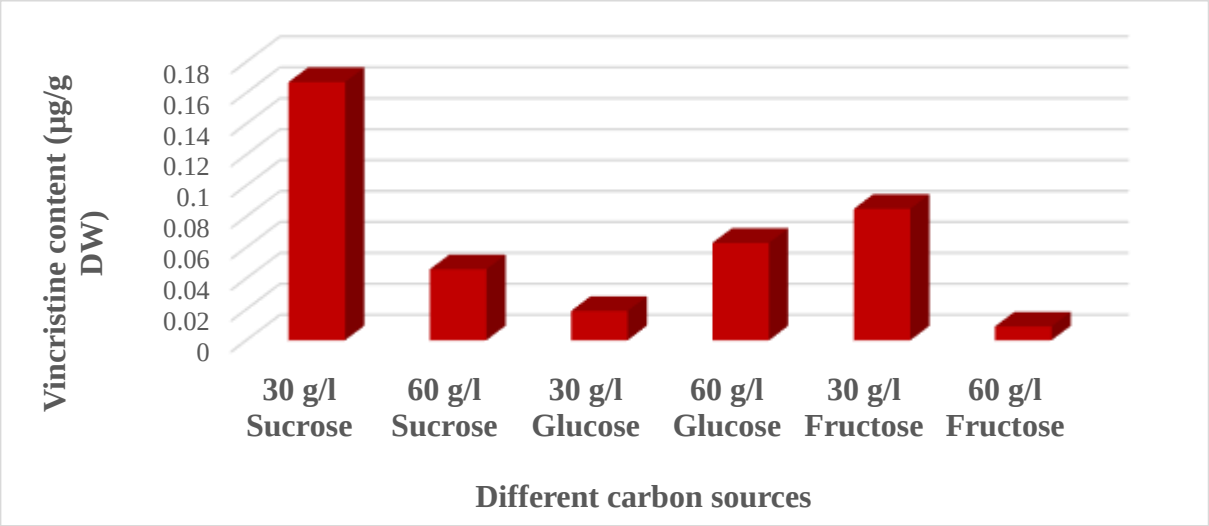

Figure 5. Effect of different carbon sources on the accumulation of vincristine in hairy root culture of *Catharanthus roseus*.

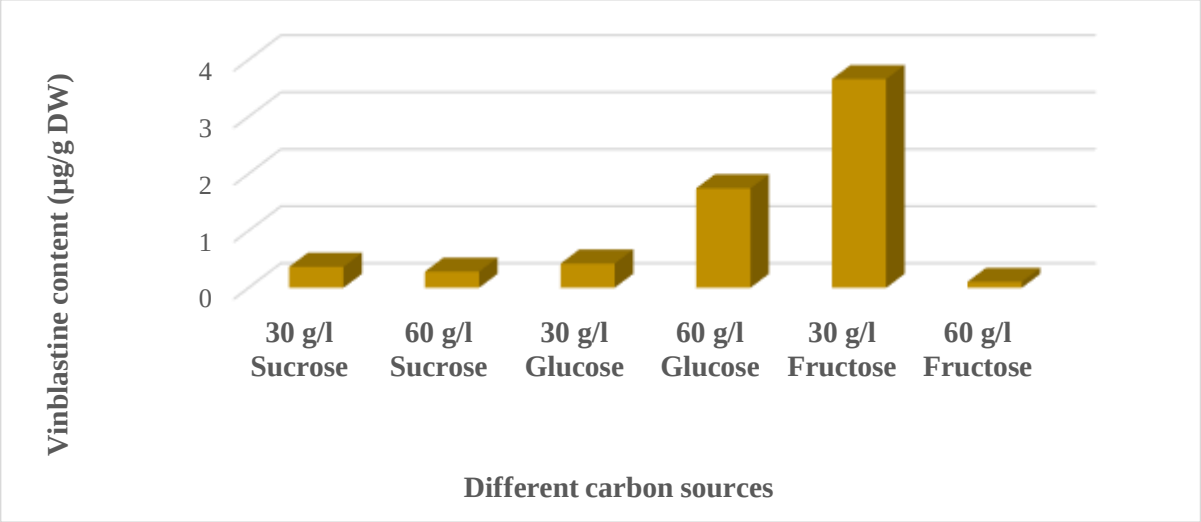

Figure 6. Effect of different carbon sources on the accumulation of vinblastine in hairy root culture of *Catharanthus roseus*.

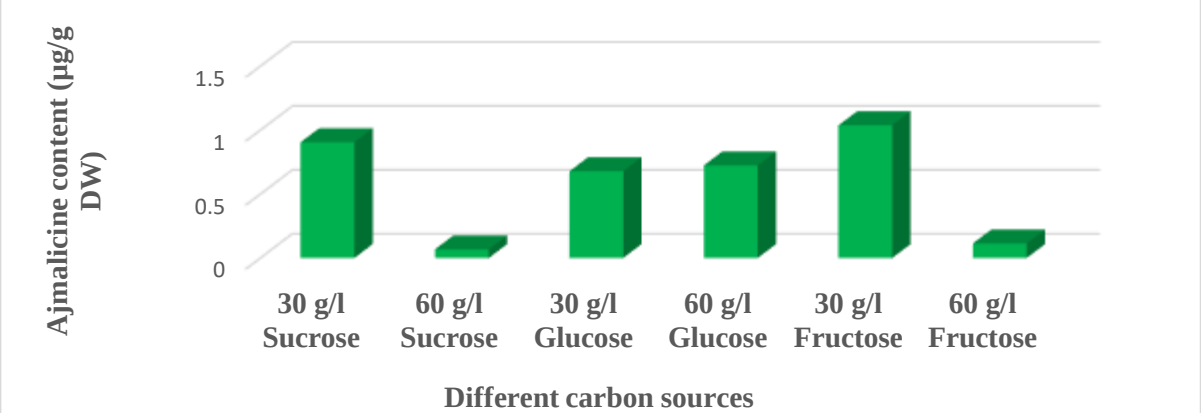

Figure 7. Effect of different carbon sources on the accumulation of ajmalicine in hairy root culture of *Catharanthus roseus*.

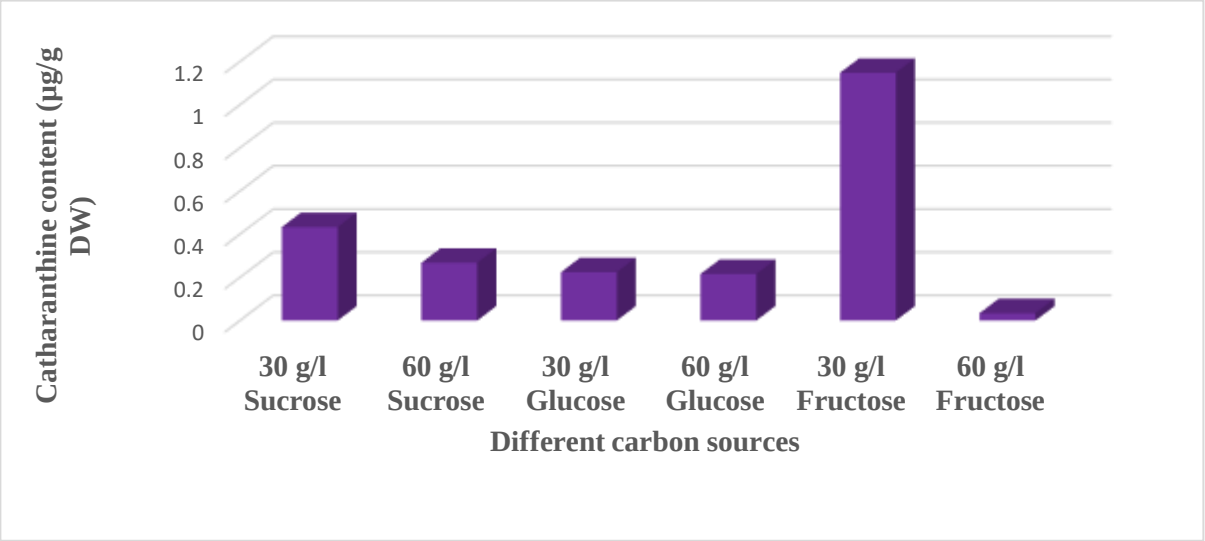

Figure 8. Effect of different carbon sources on the accumulation of catharanthine in hairy root culture of Catharanthus roseus.

3. Discussion

Nowadays, the screening of natural sources for novel biologically active metabolites is of utmost importance. In particular, secondary metabolites from plants have received remarkable attention owing to their pharmaceutical properties and various applications in many industries. Catharanthus roseus is an important medicinal plant because of the presence of remarkable bioactive compounds such as terpenoid indole alkaloids (TIAs). Therefore, selecting the most appropriate transformation methods and strains are essential factors for enhancing the accumulation of TIAs. In this study, Agrobacterium rhizogenes strain ATCC 15834 was selected and used to successfully develop hairy root cultures. **Gangopadhyay et al. [18]** examined the transformation efficiency of various A. rhizogenes strains for transgenic hairy roots induction in Plumbago indica. Among the tested strains, the ATCC 15834 strain was found to be the most efficient strain for maximum production of hairy roots compared with the other two bacterial strains. Similarly, **Setamam et al. [19]** confirmed the superiority of ATCC 153834 strain over other strains in Capsicum frutescens. The remarkable impact of Agrobacterium rhizogenes strain AR15834 on transformation efficiency of Hyscyamus muticus was confirmed **[20].**

Phenolic compounds such as acetosyringone are known for their ability to enhance transformation efficacy of numerous plant species **[21, 22].** In this work, the addition of acetosyringone, at a concentration of 100 µM, into the co-cultivation medium significantly enhanced the transformation efficiency (Figure 2). Upon increasing the concentration of acetosyringone to 200 µM, a slight decrease in the transformation efficiency from 88% to 83% was observed. In accordance with our results, supplementation of acetosyringone at a concentration of 100 µM was found to have remarkable effect on hairy root induction of Trachyspermum ammi explants infected with all used strains of agrobacterium rhizogenes **[23].** It has been previously reported in wheat and barley that the presence of acetosyringone improves the transformation efficacy; whereas, upon further increasing the concentration above 150 µM, an abrupt decrease was noticed **[24, 25].** On the contrary, some investigators reported that the natural production of phenolics in plants might be sufficient and the use of exogenous acetosyringone may not be necessary for bacterial induction **[26, 27].**

Polymerase chain reaction (PCR) is a well-established analytical method for efficiently confirming successful transformation. To investigate the genetic status of the hairy roots, PCR test, targeted towards rolA gene of Agrobacterium rhizogenes, was conducted. Amplification of a 304 bp rolA DNA fragment from hairy root lines demonstrated successful integration of T-DNA to Catharanthus roseus genome (Figure 4). As rolA gene is located on Ri plasmid of the Agrobacterium rhizogenes, its presence in plant-derived DNAs confirms the integration to C. roseus genome. Similarly, numerous reports applied PCR for confirming the success of their transformation protocols **[28-30].** In addition, many workers used specific primers for RolA gene for PCR analysis in different plant species including Hypericum perforatum **[31]** and Silene latifolia **[32]** and Catharanthus roseus **[33].**

In this study, supplementation with three carbon sources affected TIAs accumulation levels differently. Moreover, different concentrations of the same carbon sources remarkably impacted the performance of the culture [Figures 5-8]. The varied effects could be attributed to their effects on the energy available for the cells and the osmotic potential of the medium **[34].** Additionally, carbon sources may serve as signaling molecules and/or regulators of gene expression regulating plant growth and development and metabolite production **[35].** Our results also indicated fructose as the best carbon source for the accumulation of vinblastine, catharanthine and ajmalicine whereas sucrose contributed to the maximum level of only vincristine [Figures 5-8]. These results are consistent with previous studies of increased active compound accumulation in hairy roots supplemented with various sugars. **Park et al. [36]** reported that while Fructose was responsible for the greatest

increase in baicalin accumulation, sucrose proved to be the best sugar for the enhancement of baicalein production in hairy root cultures of Scutellaria baicalensis. In contrast, numerous studies indicated sucrose as the best carbon source for the accumulation of bioactive compounds such as friedelin and epifriedelanol in hairy roots of Cannabis sativa **[37],** rosmarinic in hairy roots of Agastache rugosa **[38]** and betacyanin and betaxanthin in Beta vulgaris hairy roots **[39].** In general, sucrose is commonly considered the best carbon source in plant tissue culture as it is present in the phloem sap of most plant species. Nevertheless, a number of studies have illustrated that certain plant species can grow on alternative sugar other than sucrose **[36].** Furthermore, specific plant tissues have the ability to utilize different sugars at the same time **[40]. Guo et al. [41]** illustrated that addition of galactose resulted in the maximum accumulation level of rosmarinic acid in hairy root cultures of Salvia castanea.

4. Experimental

4.1. Hairy root induction and cultures

Leaf explants were obtained from plants grown in vivo for 30-40 days. Leaf segments (one cm) were cultured on inoculation media [1/2 MS medium containing sucrose (3.0%)] contaminated with the adjusted density (OD600 nm = 0.6-0.8) of Agrobacterium Rhizogenes strain ATCC 15834 for various times (ten minutes, twenty minutes and thirty minutes) **[16].** Thereafter, the explants were blotted dry on sterile filter paper (Whatman No. 1) and placed in the dark at 25 ± 1°C on 1/2 MS solid medium [1/2 MS medium containing sucrose (3.0%)] supplemented with different concentrations of acetosyringone (50 µM, 100 µM and 200µM). After 48 hours of co cultivation, the explants were moved to the 1/2 MS solid medium containing sucrose (3.0%) supplemented with 500 mg/L cefotaxime to remove the agrobacterium, and placed in the dark at 25 ± 1°C. Transformed hairy root cultures were induced from the wound sites of explants after ten days of cultivation. Afterwards, the root initials were separated from the explants and sub-cultured on fresh 1/2 MS solid medium at 25 ± 1°C every 2 weeks.

4.2. PCR confirmation of transformation

Successful transformation was confirmed by polymerase chain reaction (PCR) using rolA specific primers as previously reported **[17].** Total DNA was extracted from transgenic hairy root lines using a DNeasy mini kit (Qiagene) according to the manufacturer's instructions. Negative control samples were obtained from non-transformed roots, while positive control samples were obtained from the Agrobacterium rhizogenes plasmid. Primers with a sequence of 5´-GTTGTCGGAATGGCCCAGAC-3´ and 5´-CGTAGGTCTGAATATTCCGGTC-3´ were applied for rolA gene amplification. The amplification cycle consisted of denaturation at 95°c for one minute, annealing at 55°c for one minute and extension at 72°c for one minute. After 30 cycle the final extension was at 72°c for five minutes. PCR products (304 bp) were detected using 1.5% agarose gel electrophoresis.

4.3. Effect of carbon sources on TIAs accumulation in hairy root cultures

To investigate the impacts of carbon sources on TIAs production, different concentrations (30 g/L and 60 g/L) of sucrose, glucose and fructose, individually, were added into 1/2 MS liquid medium. The initial culture density was 1.0 g fresh weight with 50 mL liquid media in 250 mL eyeliner flasks, shaken in a 110 rpm by rotary shaker at 25 °C in a fully dark condition.

4.4. High performance liquid chromatography (HPLC) analysis

Fresh samples were extracted using a mixture of 2% formic acidic water: methanol (50:50 v/v) for two hours. Submerging 0.5g of samples in 100ml of the mixture, then were performed for a period of 24 hours before heating at 60°C for three hours. All samples were filtered through a 0.45 µm filter paper before subjection to centrifugation at 10.000 rpm. The supernatant layer was evaporated and concentrated by vacuum distillation; the precipitates were re-dissolved in the same extraction mixture, and then were stored at 4°C till in the refrigerator further using for HPLC analysis. The alkaloid analysis (vinblastine, vincristine, catharanthine and ajmalicine) was performed on an Agilent HPLC system (1260 series) as described by Hanafy et al. **[11].**

5. Conclusion

As Catharanthus roseus produces important TIAs in extremely low concentrations, we successfully optimized a hairy root culture protocol for the maximum induction of these TIAs. Moreover, the effects of different carbon sources were investigated to enhance TIAs accumulation. Our results provide a basis for further research aiming at enhancing commercial production of TIAs.

Conflicts of interest

There are no conflicts of interest.

Acknowledgements

We would like to express our sincere gratitude to Academy of Scientific Research and Technology, Egypt, for providing financial support of the Project entitled: "Production of anticancer compounds from hairy root cultures of C. rosues using some abiotic, biotic elicitors and precursor feeding" under the program of the National Strategy for Genetic Engineering and Biotechnology. We are also grateful to the National Research Centre, Egypt for technical support and providing all required facilities.

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