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Plant Tissue Culture of *Catharanthus roseus* or Madagascar Periwinkle as a Continuous Source for the Increased Production of Alkaloids

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Abstract: ***Plants have been known for their medicinal properties across the world for a long time. In the field of medicine, medicinal plants have played a critical role. Catharanthus roseus is one such plant with enormous medical significance and potential. C.roesus, often known as Madagascar periwinkle, is a major drug-producing plant. More than 200 alkaloids are found in all parts of the plant, including the leaf, root, shoot, and stem, and are used to treat a variety of diseases. Vincristine and Vinblastine, two of the most important alkaloids, are produced from the leaves of C. roseus. Because the indole alkaloids vinblastine and vincristine are exceedingly expensive and are found in very low concentrations (0.0005 percent of dry weight) in leaves, half a tonne of dry leaves is required to obtain 1 kg of vinblastine, and 530 mg of leaves are utilised to make 1 kg of vinblastine. The medicinal efficacy of these bis-indole alkaloids (VBL and VCR) and the expensive expense of producing them in minute amounts have driven substantial efforts to enhance their levels using Plant-Tissue Culture and mutation induction. Cell suspension, hairy root, and callus cultures, shoot cultures, metabolic engineering, and regulation studies are all examples of in vitro approaches that can be used to improve the quality of the product.***

Keywords: *Catharanthus roseus*, Plant Tissue Culture, Enhanced Production, Alkaloids, Vincristine, Vinblastine

Key Messages: The results show that *in vitro* techniquesisan viable alternative for large-scale *Catharanthus roseus* multiplication and proved to be a continuous source for increased production of alkaloids.

1. Introduction

Secondary metabolites are natural compounds that plants create in huge quantities. These secondary metabolites are alkaloids, which have a wide range of medicinal characteristics and are commonly employed in traditional medicine and herbal medicines [5] F.E. Koehn & G. T. Carter, Nat. Rev. Drug Discover. 2005, 4, 206−220.Therefore, people are becoming increasingly interested in natural and organic treatments. Because these natural plant-based medications or goods are less expensive than those created through standard breeding procedures. Plants have a long history of being used as medicines to cure a variety of ailments. Traditional medicine and herbal remedies still rely heavily on the use of plants as medications[7] S.J. Murch, S. Krishna Raj & P.K. Saxena, Plant Cell Rep., 2000, 19, 698-704

Plant Tissue Culture

Plant Tissue Culture or *in-vitro* culture is defined as the "The culture of plant seeds, organ embryo, tissues, cells, and protoplast into suitable nutrient medium under sterile conditions. "The production of exact copies of plants that produce particularly good quality of plants, fruits, flowers, and antibiotics. It has the capability to produce mature plants. The production of multiples of plants in the absence of seeds. Micropropagation has been defined as "*in-vitro* regeneration of plants from organs, tissues, cells or protoplasts" (Beversdorf, 1990) and "the true-to-type propagation of a selected genotype using *in-vitro* culture techniques" (Debergh and Read, 1991). The regeneration of whole plants from plant cells that have been genetically modified [1] Beversdorf, W.D. (1990) In Progress in Pi. Cellular and Mol. Biol. eds. H.J.J. Nijkamp *et al.*, Kluwer Acad Publishers, Dordrech By the help of Plant Tissue Culture, we can get disease free plants and the desired product.[6] J. R. Dodds, L. W. Roberts (1995)

1.1 Environmental conditions for Plant Tissue Culture

1) Nutrient Media
2) Aseptic Conditions
3) Aeration of the tissue

1) Nutrient Media:

Media depends upon the type of plant tissue or cell used for culture. Generally, media consists of-

- Inorganic salts (both micro and macro elements)
- A carbon source (usually sucrose)
- Vitamins (e.g., nicotinic acid, thymine, pyridoxine)
- Amino acids(arginine)
- Growth regulators (e.g., auxins, cytokinin's, gibberellins)
- An optimum pH (5.7) is also very important

2) Aseptic Conditions

- Nutrient medium contains sugar which increases growth of microbes.

- These microbes compete with growing tissue and finally kill it.
- It is important to maintain Aseptic condition in Plant Tissue Culture.
- Sterilization is very important to kill the growth of microbes. [3] Debergh, P.C. and Read, P.E. (1991) Micro-propagation, in Micro-propagation Technology and Application eds. P.C. Debergh and R.H. Zimmerman, Kluwer Acad. Publishers, Dordrech

3) Aeration of the Tissue

- Proper aeration of the cultured tissue is also an important aspect of Plant Tissue Culture.
- It is achieved by occasionally stirring the medium by stirring or by automatic shaker

Plant Tissue Culture of *CATHARANTHUS roseus*

- This study or goal depicts the use of *Catharanthus roseus* plant tissue culture to create thousands of duplicates of c. roseus leaves for alkaloid extraction. Because the indole alkaloids Vinblastine and Vincristine are exceedingly expensive and are present in very low concentrations (0.0005 percent of dry weight) in leaves, half a tonne of dry leaves is required to obtain 1 kg of vinblastine, and 530 mg of leaves are required to create 1 kg of vinblastine.
- And, to create a big number *of Catharanthus* plants, we used the technique of Plant tissue culture, also known as micro propagation, which is the artificial process of propagating plants vegetatively through tissue culture under sterile conditions.
- Thousands of duplicates of plants can be made in a short amount of time this manner.
- After obtaining hundreds of copies of plants, leaves from *C. roseus* plants were harvested for alkaloids extraction.

2. Material and Methods

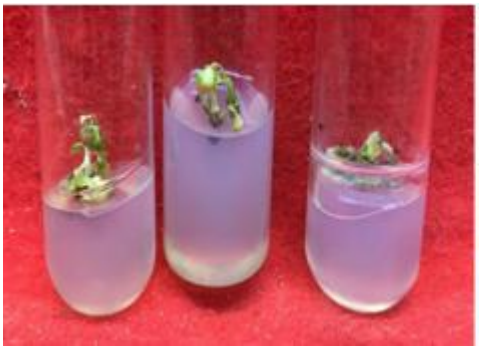
A) Plant Material

- Explants were taken from the garden grown plants of Vinca rosea.
- Young branches of healthy plants were cut and washed in running tap water.
- They were further washed in 1% detergent for 1-2 minutes and then washed in tap water for 6-8 times.
- Finally, 2 washing were done using sterilized distilled water.
- And then explants were taken for culture.

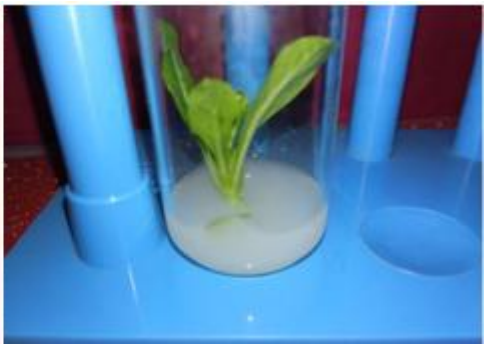
B) Medium and culture conditions:

- Morishige and Skoog's [MS] medium containing 0.8% agar and sucrose[30gm] were used during the experiment.
- The pH of the medium was adjusted to 5.8.
- Growth regulators were supplemented with various concentrations.
- Plant Growth Regulators used were: 2, 4-D, kinetin, IAA, BAP and NAA in different combinations. [4] Dew, R.A. (1991) Int. PI. Propanatot-s' Soc. Combined Proceedings

Abbreviations Used	Plant Growth Regulators
2,4-D	2,4-Dichlorophenoxyacetic acid
KIN	Kinetin
NAA	Naphthalene Acetic Acid
IAA	Indole-3-Acetic Acid



A



C

B

D

E

Figure in Reference to 2.1

A. Induction of shoots on MS basal Media supplemented with BAP (0.5 mg/l).
B. Shoots proliferation on MS- media +BAP 90.5 mg/l) + NAA (1.0 mg/l) + AC 3%.
C. Elongation of induced shoots on MS-medium + BAP (0.5 mg/l) + IAA (1.5 mg/l).
D. In vitro rooting on ½ MS + IBA (0.10 mg/l) after 4 weeks of culture.
E. A well-developed acclimatized plant in soil.

C) Induction and multiplication of Callus and Biomass:

- Induction of callus from leaf explants was initiated as per the standard protocol
- Different combinations of PGR regulators were used on growth and multiplication of biomass and callus.
- Three different combinations of PGR's used were (A) 0.1 mg/l of 2, 4-D + 0.5 mg/l of KIN + 1.0 mg/l of NAA (B) 0.5 mg/l of2,4-D + 1.0 mg/l of KIN + 2.0 mg/l of NAA and (C) 1.0mg/l of 2,4-D + 2.0 mg/l of KIN + 4.0 mg/l of NAA.)
- Callus and biomass obtained from both the medium were further used for alkaloid extraction

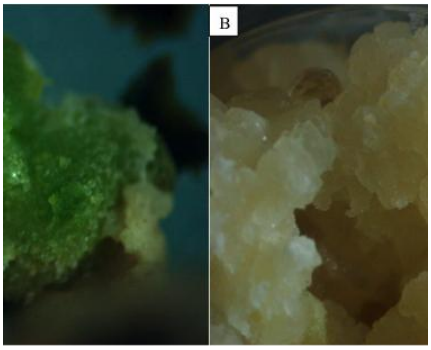


Figure in Reference to 2.1 C) Calluses growing on the medium

Results after Plant Tissue Culture

The findings of this study reveal that we can obtain thousands of copies of C. roseus plants using plant tissue culture or Micro propagation, and we can utilise as many leaves as we desire. The leaves were sub-cultured to produce callus, which was then used in the extraction process. In MS media with several PGR combinations, a good amount of green and white callus and biomass was formed. Vincristine and Vinblastine were extracted from these callus preparations using HPLC [8] Snith RH San Diego; Academic Press Incharge (1992) Plant Tissue Culture, Techniques and Experiments

Extraction of Alkaloids From Catharanthus Roseus Callus Obtained from Plant Tissue Culture Technique

[2] The idea of extracting and purifying alkaloids from Catharanthus roseus was taken from the study of "Screening of *Catharanthus roseus* secondary metabolites by High performance liquid Chromatography" by C. Tickhomiroff and M. Jolicouer, 2002

Vinblastine and Vincristine were extracted from callus extracts from various combinations using HPLC. After that, the biomass and callus were filtered and dried. The dried callus was combined with sulphuric acid in water, followed by four purification stages: partitioning with benzene, two chromatographic columns, and crystallisation in ethanol and sulphuric acid. After that, it was ready for Thin Layer Chromatography purification.

3.1 Methods

A) Thin Layer Chromatography

Thin Layer Chromatography is a technique used to separate compounds in a mixture.TLC can be used to analyze virtually any substance class, including pesticides, steroids, alkaloids, lipids, nucleotides, glycosides, carbohydrates, and fatty acids. The substance usually used is silica gel, aluminum oxide and cellulose.TLC has a very high sensitivity for alkaloid extraction.

Procedure

- On thin layer chromatography plates, the material will be applied.
- TLC will be used to identify alkaloids, and the chromogenic reaction with Ceric Ammonium Sulphate (CAS) will be used to chromatographically identify the alkaloids. UV light will be used to detect the alkaloids.
- Each alkaloid's mobility and characteristics will be compared to alkaloid standards, and their RF (retention factor), colours reaction with CAS, and UV will be used to detect them.
- The standards will be obtained from a different lab, and 0.0025 mg of each of the six standard alkaloids will be dissolved in 1ml ethanol and used for the alkaloids test.

B) Purification and Quantification of Vincristine and Vinblastine by HPLC

The homogeneity of the purified compounds was confirmed by HPLC analysis, which showed a single, symmetrical peak with RT 36.6 min (Figure 1a) and 34.9 min (Figure 1b) on C18 symmetry column of vinblastine and vincristine, respectively. Absorbance of the eluting compound showed high intensity at 220 nm and relatively low at 254 nm. The UV absorption analysis showed a peak representing absorption at 220 nm.

The absorbance maximum of standard vinblastine and vincristine was also obtained for comparison and is shown in Figure 1c and 1d respectively. The data of area peak vs. vinblastine and vincristine concentration, obtained in case of the standard sample was used to estimate the quantity of

fungal vinblastine and vincristine. The isolation of these vinca alkaloids from 1 liter culture filtrate yielded 56 mg and 44 mg of vinblastine and vincristine respectively

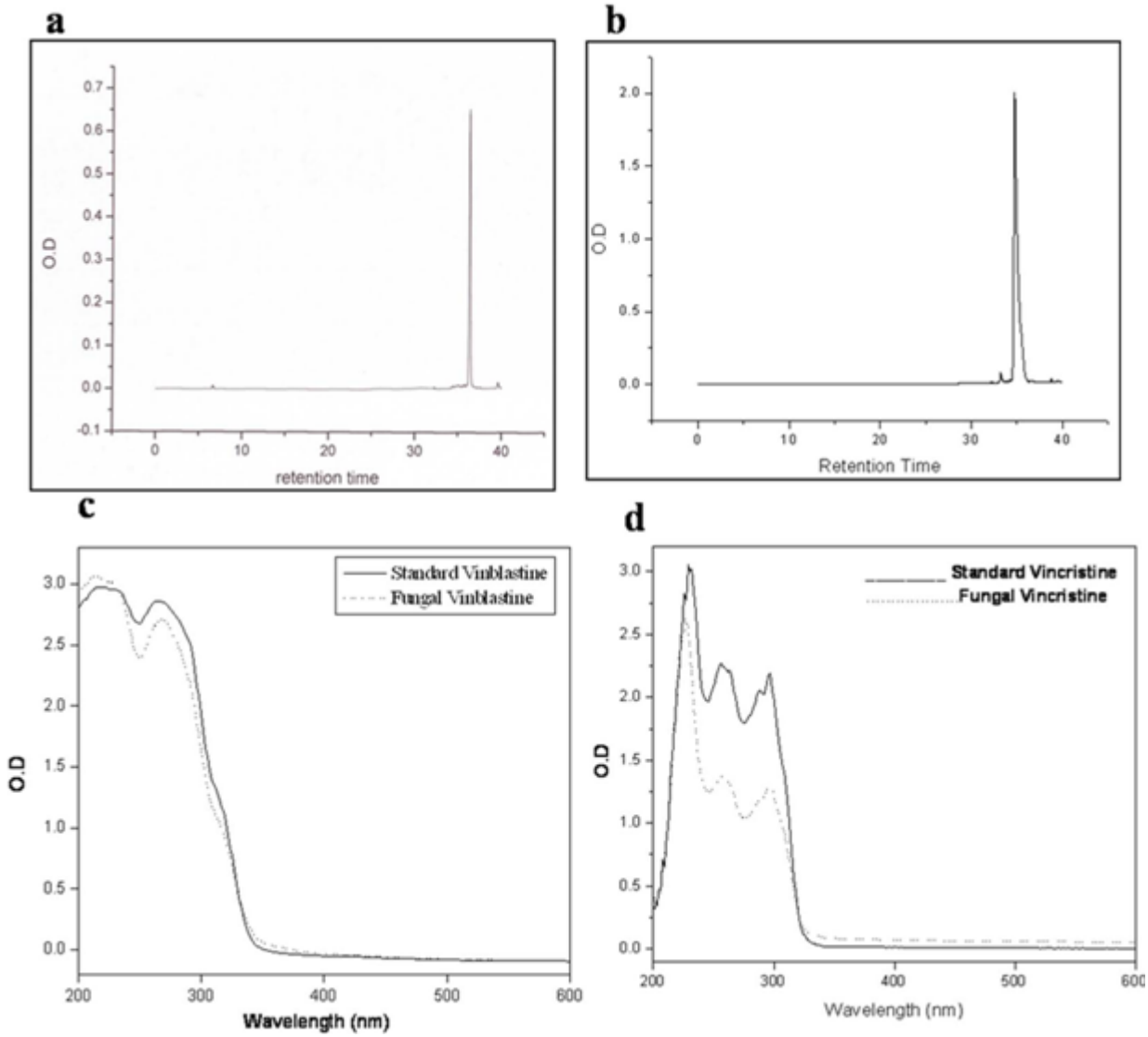


Figure in Reference to 3.1 B) HPLC and UV spectrum. (a) HPLC profile of pure fungal vinblastine with retention time of 36.6 min. (b) HPLC profile ofvincristine with retention time of 34.9 min. (c) UV absorption spectrum of standard vinblastine and vinblastine. (d) UV absorption spectrum ofstandard vincristine and vincristine

Results after HPLC

We were able to produce anti-cancer drugs vincristine and vinblastine in 56 mg/lit and 44 mg/lit, respectively, using plant tissue culture and micro propagation techniques, and we were able to isolate an increased amount of vincristine and vinblastine using Plant Growth regulators in different combinations.

S. No.	Concentrations of growth regulators(mg/lit)	Responsive callus (%)	Total Alkaloid Content(mg/l)
1.	0.1 mg/l of 2, 4-D + 0.5 mg/l of KIN	75%	63.56 ± 1.74
2.	0.5 mg/l of 2,4-D + 1.0 mg/l of KIN	67%	56.23 ± 3.06
3.	1.0mg/l of 2,4-D + 2.0 mg/l of KIN + 4.0 mg/l of NAA.)	58%	45.56 ± 2.18

Figure in Reference to 3.1 B) Effect of different combinations of Plant growth regulators on MS media and the content of Alkaloids produced.

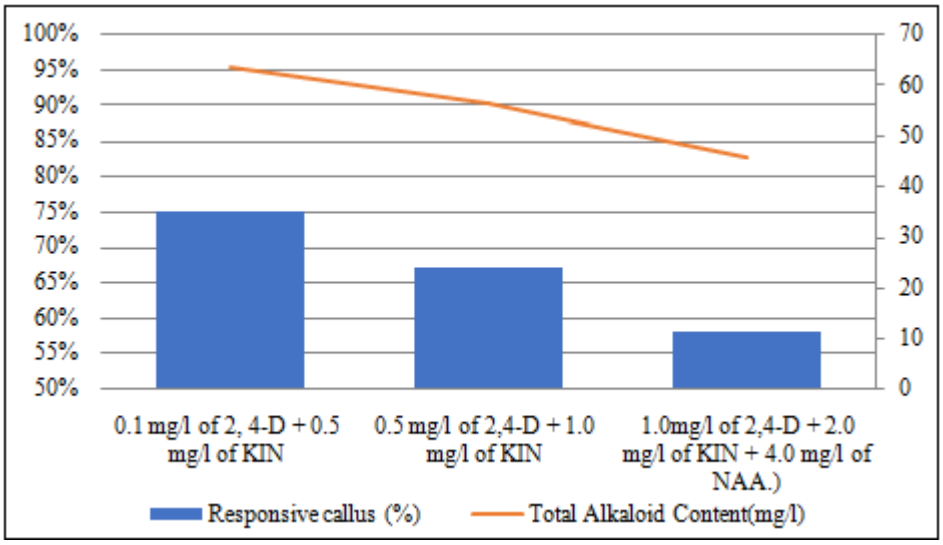


Figure 3.1: C) Graph showing response of callus by applying different combinations of plant growth regulators and amount of alkaloid produced

3. Conclusion

Increased amounts of vincristine and vinblastine were achieved after using Plant tissue culture technique and various combinations of Plant Growth regulators, according to this study.

This procedure was also cost effective because we grew the plants in our lab using an in vitro technique and harvested the leaves for further processing.

In the following study, we'll compare the quantity of alkaloids found in different C. roseus types, such as the white, pink, and purple kinds.

Author Contribution Statement

In this research work, the idea was conceived and designed by RS and SB. RS1 helped in conducting experiments. SB wrote the manuscript. Changes were suggested by RS. Therefore, all the authors approved the manuscripts.

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4. Statements and Declarations

- The authors have no competing interests to declare that are relevant to the content of this article.
- All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.
- The authors have no financial or proprietary interests in any material discussed in this article.
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