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# EFFECT OF PLANT GROWTH REGULATORS ON SUSPENSION CULTURE OF ADHATODA VASICA NEES

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## ABSTRACT

Suspension culture is one of common technique use in plant tissue culture for production of secondary metabolites. In the present study the callus was established using adhatoda vasica cells and then used to prepare suspension culture. two auxin and one cytokine used to manipulate the media and the effect was studied in terms of cell growth. Secondary metabolite production was analyzed by HPLC and found immobilized cells were showing higher biosynthetic capacity for vasicine

**KEYWORDS**: A. vasica, suspension culture, immobilization, cell growth, vasicine.

## INTRODUCTION

Plant-produced secondary compounds have been incorporated into a wide range of commercial and industrial applications, and fortuitously, in many cases, rigorously controlled plant *in vitro* cultures can generate the same valuable natural products. Plants and plant cell cultures have served as resources for flavors, aromas and fragrances, bio based fuels and plastics, enzymes, preservatives, cosmetics (cosmaceuticals), natural pigments, and bioactive compounds. Callus cultures are extremely important in plant biotechnology. Manipulation of the auxin to cytokinin ratio in the medium can lead to the development of shoots, roots, or somatic embryos from which whole plants can subsequently be produced.

Immobilization has been suggested as a strategy to improve the overall productivity of secondary metabolite in plant cell culture. Freely suspended plant cells mostly accumulate their secondary metabolites in the stationary phase of their growth cycle, at the point of time their growth stop. Entrapment of plant cells is one the means to create non-growth condition underwhich the production of secondary metabolites may be improved.[1, 2]

*Adhatoda vasica* is a well-known plant drug in Ayurvedic and Unani medicine. Adhatoda leaves have been used extensively in Ayurvedic Medicine primarily for respiratory disorders. Juice from the leaves and the decoction of the leaves and roots are helpful in asthma, bronchitis and chronic coughs and breathlessness.[3, 4, 5]

Used for bleeding due to idiopathic thrombocytopenic purpura, local bleeding due to peptic ulcer, piles, menorrhagia. Relief in pyorrhoea and for bleeding gums by locally application. Relieves or eases muscular spasms, cramps or convulsions.

Vasicine and vasicinone is major alkaloid responsible for pharmacological activities.

## MATERIAL AND METHOD

For the preparation of callus culture studies Murashige and Skoog medium (MS medium) and Gamborg $B_5$ were selected, as they are most commonly used for plant tissue culture studies and have almost all desired essential macro and micro nutrients which are essential for the growth of plant tissue. In the present study three plant growth regulators were selected in which one belongs to auxin class of plant growth regulator i.e. 2,4-Dichlorophenoxyacetic acid (2,4-D) and Indole 3 acetic acid (IAA) inducing cell elongation and cell division with all subsequent results for plant growth and development, another one is kinetin (kin.), a kind of cytokinin, that promotes cell division.

For propagation, nodal stem explants with auxiliary buds were used and for raising callus, Auxiliary leaves explants from Adhatoda vasica were collected and washed under running tap water to remove dirt traces. Explants were immersed in a detergent for 5 min, washed in water once, and then surface sterilized with 0.1%mercuric chloride for 4-5 minutes, and then explants were washed thrice with double distilled water. The surface sterilized leaves cut in to 1-2 $mm^2$ small pieces. The cut segments were then cultured individually on MS and $B_5$ medium containing different concentration of 2, 4-D, Kin. and IAA.

The callus was weighed, and homogenized with tissue homogenizer, under aseptic conditions. During homogenization small quantity of media was added to maintain the cell viability. After weighing the concentrated cell mass, selected plant tissue culture media was added in the cell suspension in this manner to get 1gm fresh weight of cells /ml.
The 2 ml of cell suspension (1gm/ml fresh weight) was then aseptically added to the 98 ml. of tissue culture media to make it 100 ml and which produce 2gm plant cell in 100 ml culture media. freely suspended cultured cells in Erlenmeyer flask (250 ml.) are placed in Remi shaker cum incubator at 90 rpm, 25 ± 2°C, and under direct light of 40W (08 hrs light period).

The production media was collected from cell culture flasks at every 5-day interval for the estimation of alkaloid, and several other studies were perform i.e. cell growth, cell viability etc.

**Cell number study in Suspension culture:** Cell number study is the most accurate method, which clearly indicates about the cell growth in the production medium. To quantify the cell number increment, 0.1 ml of cell suspension culture is pipetted out after proper shaking, to ensure the uniform distribution of cells in the culture medium, and was further diluted 10 times with distilled water, and triturate well to disrupt the large cell aggregates.

By means of haemocytometer the cells were counted by placing 1 drop of cell suspension on Newbrans chamber. The cells were counted and multiplied by dilution factor to calculate the cell number in 1ml.

**Fresh and Dry weight Study in Suspension culture:** For Fresh and dry weight analysis, 20.0 ml media was taken out after proper shaking to ensure uniform cell distribution, from each flask and pooled together to produce 100 ml medium, containing the suspended cells. The above collected medium (100 ml) was filtered through pre-weighed, wet Whatman No.41 filter paper, and weighed again after removing excess medium to calculate the fresh weight of cells. For dry weight analysis the above mentioned Whatman No.41 filter paper with wet cells was placed in oven at 90°C, and weighed until get a constant weight, to calculate the dry weight of cells.

**Table 1. Study of cell number in suspension culture**

| Days | Cell number ( x $10^5$/ $mm^3$) in MS Media Supplemented with | | | Cell number ( x $10^5$/ $mm^3$) in $B_5$ Media supplemented with | | |
|---|---|---|---|---|---|---|
| | 2 ppm 2,4-D 1 ppm Kin. 0.5 ppm IAA | 2 ppm 2,4-D 0.5 ppm Kin. 0.5 ppm IAA | 1.5 ppm 2,4-D 1.0 ppm Kin. 1.0 ppm IAA | 2 ppm 2,4-D 1 ppm Kin. 0.5 ppm IAA | 2 ppm 2,4-D 0.5 ppm Kin. 0.5 ppm IAA | 1.5 ppm 2,4-D 1.0 ppm Kin. 1.0 ppm IAA |
| 0 | 6.2±0.3 | 6.5±0.11 | 6.0±0.41 | 6.1±0.14 | 6.3±0.45 | 6.1±0.56 |
| 7 | 6.5±0.24 | 7.3±0.26 | 7.5±0.62 | 6.3±0.22 | 6.7±0.32 | 6.9±0.74 |
| 14 | 8.04±0.21 | 9.2±0.41 | 9.8±0.17 | 6.92±0.81 | 7.4±0.24 | 9.4±0.53 |
| 21 | 8.24±0.52 | 9.5±0.72 | 9.9±0.39 | 7.13±0.64 | 7.5±0.12 | 9.8±0.14 |

**Table: 2 Fresh weights Study in Suspension culture**

| Time in Days | Fresh weight (gm) / 10 ml of suspension culture in MS Media Supplemented with | | | Fresh weight (gm) / 10 ml of suspension culture in $B_5$ Media Supplemented with | | |
|---|---|---|---|---|---|---|
| | 2 ppm 2,4-D 1 ppm Kin. 0.5 ppm IAA | 2 ppm 2,4-D 0.5 ppm Kin. 0.5 ppm IAA | 1.5 ppm 2,4-D 1.0 ppm Kin. 1.0 ppm IAA | 2 ppm 2,4-D 1 ppm Kin. 0.5 ppm IAA | 2 ppm 2,4-D 0.5 ppm Kin. 0.5 ppm IAA | 1.5 ppm 2,4-D 1.0 ppm Kin. 1.0 ppm IAA |
| 0 | 0.45 | 0.43 | 0.45 | 0.44 | 0.43 | 0.42 |
| 7 | 0.81 | 0.93 | 0.88 | 0.65 | 0.69 | 0.67 |
| 14 | 1.68 | 1.80 | 1.52 | 1.23 | 1.42 | 1.47 |
| 21 | 1.80 | 1.97 | 1.78 | 1.31 | 1.60 | 1.57 |

**Table: 3 Dry weights Study in Suspension culture**

| Time in Days | Dry weight (mg) / 10 ml of suspension culture in MS Media Supplemented with (in mg) | | | Dry weight (mg) / 10 ml of suspension culture in $B_5$ Media Supplemented with (in mg) | | |
|---|---|---|---|---|---|---|
| | 2 ppm 2,4-D 1 ppm Kin. 0.5 ppm IAA | 2 ppm 2,4-D 0.5 ppm Kin. 0.5 ppm IAA | 1.5 ppm 2,4-D 1.0 ppm Kin. 1.0 ppm IAA | 2 ppm 2,4-D 1 ppm Kin. 0.5 ppm IAA | 2 ppm 2,4-D 0.5 ppm Kin. 0.5 ppm IAA | 1.5 ppm 2,4-D 1.0 ppm Kin. 1.0 ppm IAA |
| 0 | 22.3 | 21.4 | 22.7 | 20.2 | 21.3 | 21.4 |
| 7 | 29.4 | 30.1 | 27.2 | 24.5 | 26.2 | 23.3 |
| 14 | 58.8 | 61.4 | 59.4 | 55 .7 | 63.7 | 58.4 |
| 21 | 60.2 | 62.6 | 60.3 | 57.3 | 64.4 | 62.1 |

**Immobilization in calcium alginate**

The 1ml. of cell suspension corresponding to 1gm of fresh weight was added 10 ml of 2% sodium alginate (200 mg / 100 ml. water), and mix well to achieve the uniform distribution of cells throughout the sodium alginate solution.

This cell suspension was then poured drop-wise in the 3mM $CaCl_2$ solution, through sterile syringe and needle. This leads the formation of spherical beads of diameter between 2.5-3.0 mm.

Formed beads are then left in $CaCl_2$ solution for about 30 min for the stabilization of beads. The beads were then washed thoroughly with sterilized water to remove the traces of $CaCl_2$.

Washed beads were then dispersed in 50 ml production media for culturing in Erlenmeyer flask (250 ml. capacity).

**Study of cell number, Immobilized in calcium alginate**

For the estimation of cell number in the Calcium alginate beads it is necessary to dissolve the beads first to release the entrapped cells. Cell number was estimated by dissolving the randomly selected 10 beads in 10 ml. of Potassium phosphate buffer (pH 6.5). After ensuring the complete dissolution of beads, the cells were counted under the microscope with haemocytometer.

**Table 4: Cell number study immobilized in calcium alginate**

| S.N. | Time (in Days) | Cell number/ $mm^3$ of immobilized cells in MS Media Supplemented with | | |
|---|---|---|---|---|
| | | 2 ppm 2,4-D 1 ppm Kinetin 0.5 ppm IAA | 2 ppm 2,4-D 1 ppm Kinetin 0.5 ppm IAA | 1.5 ppm 2,4-D 1.0 ppm Kin. 1.0 ppm IAA |
| 1 | 0 | $3.3 \times 10^5$ | $3.61 \times 10^5$ | $3.42 \times 10^5$ |
| 2 | 7 | $3.41 \times 10^5$ | $3.75 \times 10^5$ | $3.42 \times 10^5$ |
| 3 | 14 | $3.48 \times 10^5$ | $3.76 \times 10^5$ | $3.46 \times 10^5$ |
| 4 | 21 | $3.52 \times 10^5$ | $3.80 \times 10^5$ | $3.50 \times 10^5$ |

**Fresh weight Analysis of Calcium Alginate Entrapped Cells:** For the Fresh and dry weight analysis, randomly selected 20 beads are taken out from the culture medium and dissolved in 25 ml of Potassium phosphate buffer (pH 6.5). After complete dissolution of beads, obtained cell suspension was centrifuged at 3000 rpm for 2 min.

**Table 5: Fresh weight analysis / 20 beads of cells Immobilized in calcium alginate in MS Media**

| S.N. | Number of days | Fresh weight / 20 beads of Immobilized cells in MS Media Supplemented with (in mg) | | |
|---|---|---|---|---|
| | | 2 ppm 2,4-D 1 ppm Kinetin 0.5 ppm IAA | 2 ppm 2,4-D 1 ppm Kinetin 0.5 ppm IAA | 1.5 ppm 2,4-D 1.0 ppm Kin. 1.0 ppm IAA |
| 1 | 0 | 51.5 | 52.1 | 56.2 |
| 2 | 7 | 53.7 | 54.3 | 58.7 |
| 3 | 14 | 57.2 | 60.7 | 62.3 |
| 4 | 21 | 60.2 | 62.1 | 61.5 |

**Quantitative estimation of Vasicine (By HPLC):** The Quantitative estimation of Vasicine, the major bioactive constituent in culture medium was done by High Performance Liquid Chromatography (HPLC) method given in Indian Herbal Pharmacopoeia, 1998 [200]. The dilutions of known concentration of vasicine (Provided by Laila Impex, Vijaywada) was

prepared by dissolving them in methanol in such a way to get the concentration range between 10-60 µg/ml.

**Sample preparation:** The fresh leaves of Adhatoda vasica were collected from the medicinal plant garden, Hi-tech College of Pharmaceutical Sciences, Bhubaneswar (Odisha), and dried in shed, and then powdered and passed through sieve No. 36 mesh size 75 Um. The powder was then reflux with 50 ml methanol for 2 hrs Filtered and the marc was subjected for extraction for another two cycles (1 hr each) with methanol. The filtrate was concentrated to 1ml, diluted with water to about 20 ml, The aqueous extract was acidified with 5 ml of 3% hydrochloric acid, Partitioned twice with 10 ml chloroform. Reject the chloroform fraction, and basify the remaining aqueous phase with 3% ammonia solution (5 ml), extract with chloroform (5 x 10 ml), concentrate the pooled chloroform fraction (1 ml), and dissolved in 100 ml methanol.[6]

**HPLC Standard curve for Vasicine**

**Instrument**: Shimedzu.

**Mobile phase**: Methanol: water (2:3).

**Flow rate**: 0.7 ml/min.

**Column**: Resolve C18 Spherical 5µ (3.9 mm X 15 cm).

**Detector**: SPD-M10 Avp.

**Wavelength**: 298 nm.

**Retention time**: 3.8 min.

## RESULTS AND DISCUSSION

**Table 6: Vasicine content in suspension culture and in immobilized cell culture.**

| Time in Days | Vasicine content (µg /ml / gm fresh weight) of *A.vasica* cells in suspension culture | | | | | |
|---|---|---|---|---|---|---|
| | MS medium supplemented with | | | $B_5$ medium supplemented with | | |
| | 2,4-D (1 ppm) + Kinetin (1.0 ppm) + IAA(0.5 ppm) | 2,4-D (1 ppm) + Kinetin (0.5 ppm) + IAA(0.5 ppm) | 2,4-D ( 1 ppm) + Kinetin (1.0 ppm) + IAA (1.0 ppm) | 2,4-D (1 ppm) + Kinetin (0.5 ppm) | 2,4-D (2 ppm) + Kinetin (0.5 ppm) | 2,4-D (1 ppm) + Kinetin (0.5 ppm) + IAA (0.5 ppm) |
| Initial day | 0.42 ± 0.02 | 0.42 ± 0.04 | 0.42 ± 0.07 | 0.42 ± 0.04 | 0.42 ± 0.05 | 0.42 ± 0.04 |
| 7 | 0.48 ± 0.11 | 0.54 ± 0.10 | 0.67 ± 0.04 | 0.51 ± 0.07 | 0.56 ± 0.07 | 0.52 ± 0.07 |
| 14 | 0.56 ± 0.02 | 0.77 ± 0.07 | 0.95 ± 0.12 | 0.61 ± 0.12 | 0.69 ± 0.11 | 0.76 ± 0.09 |
| 21 | 0.67 ± 0.08 | 1.40 ± 0.03 | 1.11 ± 0.09 | 1.26 ± 0.19 | 1.38 ± 0.08 | 0.98 ± 0.14 |

**Table 7: Vasicine Content in suspension culture of *A. vasica***

| Time in Days | Vasicine content (µg /ml / gm fresh weight) of *A.vasica* cells immobilized in Calcium alginate | | | | | |
|---|---|---|---|---|---|---|
| | MS medium supplemented with | | | $B_5$ medium supplemented with | | |
| | 2,4-D (1 ppm) + Kinetin (1.0 ppm) + IAA (0.5 ppm) | 2,4-D (1 ppm) + Kinetin (0.5 ppm) + IAA (0.5 ppm) | 2,4-D (1 ppm) + Kinetin (1.0 ppm) + IAA (1.0 ppm) | 2,4-D (1 ppm) + Kinetin (0.5 ppm) | 2,4-D (2 ppm) + Kinetin (0.5 ppm) | 2,4-D (1 ppm) + Kinetin (0.5 ppm) + IAA (0.5 ppm) |
| Initial day | 0.20 ± 0.05 | 0.17 ± 0.03 | 0.31 ± 0.05 | 0.19 ± 0.04 | 0.29 ± 0.05 | 0.31 ± 0.09 |
| 7 | 0.61 ± 0.07 | 0.48 ± 0.03 | 0.92 ± 0.07 | 0.58 ± 0.07 | 0.49 ± 0.07 | 0.90 ± 0.07 |
| 14 | 2.1 ± 0.0.7 | 1.59 ± 0.14 | 2.51 ± 0.21 | 2.47 ± 0.04 | 2.25 ± 0.04 | 2.7 ± 0.17 |
| 21 | 0.20 ± 0.09 | 0.17 ± 0.09 | 0.31 ± 0.14 | 0.19 ± 0.17 | 0.29 ± 0.22 | 0.31 ± 0.12 |

The above studies with various combinations of plant growth regulators are tabulated, and it was observed that the combination of 1.5 ppm 2,4-D, 1.0 ppm Kinetin and 1.0 ppm IAA has increased cell number significantly . This might be due to higher concentration of cytokinin which is responsible for cell division, and the higher the numbers in cell leads to higher the fresh and dry weight calculations. Calcium alginate immobilized cells shows higher vasicine biosynthetic capacity as compared to freely suspended cells, and this might be due to restricted growth which turns metabolic pathways to synthesize more secondary metabolites. MS media was more capable in vasicine synthesis as compared to $B_5$ media, this might be due to more organic and inorganic sources in MS medium, which provide necessary ions and energy for vasicine synthesis. The present study indicates that immobilization may be more useful technique for secondary metabolite synthesis.

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