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RESEARCH ARTICLE

Enhancing the Production of Anticancer Leaf-Alkaloids of *Catharanthus Roseus* (L.) G.Don, Using Methyl Jasmonate and Irradiated Sodium Alginate in Pot Experiments

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Abstract

Catharanthus roseus (L.) G. Don yields anticancer alkaloids, *viz.* vinblastine and vincristine, in its leaves. Gamma irradiated sodium alginate (ISA) encourages the plant growth as well as production of alkaloids and other active constituents in different medicinal and aromatic plants (MAPs). Methyl jasmonate (MeJA) is a potent elicitor of secondary metabolites in plants, especially under adverse environmental conditions. In view of these considerations, three pot-experiments were conducted in the net house according to simple randomized design in order to reveal the effect of foliar application of ISA and MeJA (potent elicitors) and of their combinations on growth, physiological attributes and content and yield of total as well as anticancer alkaloids of *Catharanthus roseus*. Foliar spray of ISA and MeJA, applied alone as well as in combination, resulted in significant improvement in most of the parameters studied. Combined application of ISA and MeJA exerted most favourable effect on the parameters studied, enhancing the total leaf-alkaloids yield by 71.48 and 57.32%, leaf-vincristine yield by 44.53 and 37.82% and, leaf-vinblastine yield by 33.93 and 26.24% at 6 and 9 months after sowing, respectively, in comparison to the control.

Keywords: *Catharanthus Roseus;* Vincristine; Vinblastine; Irradiated Sodium Alginate; Methyl Jasmonate; Alkaloid Yield

Introduction

Periwinkle [*Catharanthus roseus* (L.) G. Don] belongs to Apocynaceae family. Medicinal value of this plant resides in its several medicinally significant alkaloids. Two of its highly important leaf-alkaloids, viz. vinblastine and vincristine, are of chemotherapeutic value, which can inhibit the growth of cancer cells by hindering the formation of mitotic apparatus during cell division [1]. *Catharanthus roseus* (L.) G. Don contains several valuable alkaloids, which are distributed in all parts of the plant. The commercially important anticancer-alkaloids are mainly present in the leaves. Gamma-irradiated polysaccharides, viz. sodium alginate, carrageenan and chitosan, are known to encourage various biological and physiological activities of plants including seed germination, growth of shoot and root, production of flower, induction of phytoalexin, stimulation of antimicrobial activity, amelioration of heavy metal stress and plant growth in general [2-6]. Like other plant growth encouraging substances, gamma irradiated sodium alginate (ISA) has been reported to enhance overall plant growth [7-9]. In fact, foliar application of ISA is known to enhance the total alkaloid production and active constituents in different MAPs such as *Papaver somniferum, Foeniculum vulgare, Mentha arvensis, Eucalyptus citridora, Artemisia annua, Cymbopogon flexuosus and Trigonella foenum-graecum* [4,5,9,10-13]. Exogenous application of methyl jasmonate (MeJA) is new to the list of plant hormones and has been reported as potent elicitor of secondary metabolite production in many plant species [14]. The role of MeJA in activating the expression of genes [15] with regard to accumulation of phytoalexins and other secondary metabolites has been confirmed in various plant systems [16]. Taking together the importance of ISA as growth supporting substance for plants and that of MeJA as elicitor of defense responses and alkaloid production in *Catharanthus roseus*, it was hypothesized if foliar spray of ISA and/or methyl jasmonate could enhance the growth, other physiological activities and production of alkaloids in *Catharanthus roseus*, specifically that of vincristine and vinblastine. Three pot experiments were conducted in this regard.

Materials and Methods

Plant materials and growth conditions

Healthy seeds of *Catharanthus roseus* were procured from Indian Agricultural Research Institute (IARI), New Delhi. The seeds were surface sterilized with 70% ethanol with frequent shaking and then were washed with deionized water. Seeds were sown in earthen pots (25 cm diameter × 25 cm height), each pot carrying 5 kg of homogenous mixture of sterilized soil and farmyard manure. The soil used in the experiments carried the following characteristics: texture sandy loam, pH (1:2) 7.5, E.C. (1:2) 0.46 dSm^{-1}, available N, P and K 102.0, 7.8 and 145.6 mg kg^{-1} of soil, respectively. A basal dose of N, P and K (15:25:25 kg ha^{-1}, respectively) was applied in the form of urea, single superphosphate and muriate of potash, respectively, at the time of planting. The pot experiments were conducted in the natural conditions of net-house at the Botany Department, Aligarh Muslim University (A.M.U.), Aligarh (27˚ 52' N latitude, 78˚ 51' E longitude, and 187.45 m altitude). The average temperature of November, December and January, the coldest months, is about 19.4 °C, 12.9 °C and 11.2 °C, respectively, with the average winter temperature of about 14.5±4 °C. The summer season extends from April to June and the average temperature of May and June is about 34.5 °C and 45.5 °C, respectively. The monsoon extends from the end of June to the middle of October with a temperature range between 26 °C to 30 °C. The average annual rainfall is about 847.3 mm. The relative humidity (RH) of the winter season ranges from56% to 77% with an average RH of 66.5%. In the summer, it ranges from 37% to 49% with an average RH of 43%. In the monsoon season, the relative humidity ranges from 63% to 73%, with an average RH of 68%. The meteorological data were recorded during the investigation period at the Meteorological Observatory, Department of Physics, A.M.U, Aligarh.

Pot Experiments

Three pot-experiments were conducted during the winter season of 2012 and 2103 on *Catharanthus roseus* (L.) According to simple randomized design in order to reveal the individual effect of irradiated sodium alginate (ISA), methyl jasmonate (MeJA) and of their combination on growth, yield and alkaloids production. Enhancement of content and yield of total leaf alkaloids, specifically of those of vincristine and vinblastine, was the precise aim of this project. The treatments-scheme regarding the experiments is given in Table 1. Each treatment was replicated five times and each replicate contained one plant (one plant in each pot). Foliar spray of ISA and that of MeJA was applied when the plants were two months old. Totally, six sprays of aqueous solutions of ISA and that of MeJA were carried out at 10 days interval, using a hand sprayer. In experiments 1 and 2, growth and chemical analysis of the crop was carried out at 6 months (180 days) after sowing (MAS), while in Experiment 3 the crop analysis was made at 6 and 9 MAS in terms of the shoot and leaf parameters, physiological parameters, leaf-nutrient contents, and content and yield of total alkaloids and those of anticancer alkaloids (vincristine and vinblastine) in the leaves.

S. No.	ISA treatments (Experiment 1)	MeJA treatments (Experiment 2)	ISA + MeJA treatments (Experiment 3)
1.	CONTROL	CONTROL	CONTROL
2.	UISA (20 mg L^{-1})	MeJA 10 mg L^{-1}	UISA (40 mg L^{-1})
3.	ISA 20 mg L^{-1}	MeJA 20 mg L^{-1}	ISA 40 mg L^{-1} + MeJA 20 mg L^{-1}
4.	ISA 40 mg L^{-1}	MeJA 30 mg L^{-1}	ISA 40 mg L^{-1} + MeJA 30 mg L^{-1}
5.	ISA 80 mg L^{-1}	MeJA 40 mg L^{-1}	ISA 80 mg L^{-1} + MeJA 20 mg L^{-1}
6.	ISA 160 mg L^{-1}		ISA 80 mg L^{-1} + MeJA 30 mg L^{-1}
7.			ISA 160 mg L^{-1} + MeJA 20 mg L^{-1}
8.			ISA 160 mg L^{-1} + MeJA 30 mg L^{-1}

CONTROL: deionised water; UISA: unirradiated sodium alginate; ISA: irradiated sodium alginate; MeJA: methyl jasmonate. All the experiments were conducted using earthen pots in the natural conditions of net house

Table 1: Treatment-schemes related to foliar spray of ISA (Experiment 1), MeJA (Experiment 2) and ISA + MeJA (Experiment 3) on *Catharanthus roseus*

Preparation of aqueous solution of ISA and MeJA

The natural polysaccharide, namely sodium alginate, was purchased from Sigma Aldrich (USA). The solid material of sodium alginate was sealed in a glass tube with atmospheric air. The sample of sodium alginate was irradiated by gamma-rays using Co-60 as a source at 520 Kilo Gray (kGy) at the dose rate of 2.4 kGy h^{-1} in Gamma Radiation Chamber, BARC, Mumbai (India). Spray-solution of MeJA was prepared by diluting the compound using ethanol (95% purity; Sigma-Aldrich Corp., USA). As per treatment-scheme, solutions of various concentrations of the ISA and MeJA were sprayed on plants at scheduled rates and dates.

Determination of shoot and leaf parameters

At the scheduled sampling date(s), plants from each treatment-pot were harvested and their roots were washed carefully with tap water to eliminate the soil and other foreign particles. The water, adhering to roots, was removed with blotting paper and the fresh mass of whole shoot and then that of the detached leaves was recorded; shoot length was also recorded simultaneously. Fresh leaves were employed to measure physiological parameters, thereafter. The leaf-area was assessed with the help of graph-paper method using a millimetre graph-paper [17]. Leaf area was estimated by the following equation:

Leaf area (cm^2) = x/y

Where x is the weight (g) of the area covered by the leaf outline on a millimetre graph paper, and y is the weight of one cm^2 of the same graph paper. The average leaf-area was determined by measuring the area of 3 leaves (upper, middle and lower) with regard to each plant of the sample (consisting of five plants). Leaf-yield was recorded by weighing all plant-leaves using an electronic balance. Samples of the leafless shoots and plucked leaves were dried at 80 °C for 24 h using a hot-air oven; after that, the dry mass of shoots and leaves, was measured, separately. The dried leaves were turned to fine-powder using pestle-mortar, followed by their chemical and HPLC analysis.

Determination of total leaf-chlorophyll content

Total content of chlorophyll in the fresh leaves was estimated using the method of [18]. The fresh tissue from interveinal leaf area was grinded with sufficient amount of 80% acetone using mortar-pestle. The optical density (OD) of the solution was recorded at 662 and 645 nm for chlorophyll a and chlorophyll b, respectively, using a spectrophotometer (Shimadzu UV-1700, Tokyo, Japan). Total chlorophyll content was estimated adding up the values of chlorophyll a and chlorophyll b. Total chlorophyll content was expressed as mg g^{-1} FW.

Determination of nitrate reductase (NR) activity

Activity of nitrate reductase (E.C.1.6.6.1) was determined in fresh leaves by the intact-tissue assay method based on reduction of nitrate to nitrite [19]. Chopped leaf-pieces (250 mg) were incubated for 2 h at 30 °C in a 5.5-mL reaction mixture, which contained 2.5 mL of 0.1 M phosphate buffer, 0.5 mL of 0.2 M potassium nitrate, and 2.5 mL of 5% isopropanol. Subsequently, the nitrite formed was determined at 540 nm after azo-coupling with sulphanilamide and naphthylene diamine dihydrochloride, using the spectrophotometer. The NR activity was expressed as nmol of nitrite formed per h per g of leaf fresh-weight (nmol NO_2 h^{-1} g^{-1} FW).

Determination of carbonic anhydrase (CA) activity

Activity of carbonic anhydrase (E.C. 4.2.1.1) was measured in fresh leaves, using the method described by [20]. Fresh leaf pieces (200 mg) were transferred to Petri plates. The leaf pieces were dipped in 10 mL of 0.2 M cysteine hydrochloride solution for 20 minutes at 4 °C, followed by transferring the filtrate-content of each Petri plate to 10 mL test tube. To each test tube, 4 mL of 0.2 M sodium bicarbonate solution and 0.2 mL of 0.022% bromothymol blue were added. The reaction mixture was titrated against 0.05 N HCl using methyl red as indicator. The CA activity was expressed as mol CO_2 kg^{-1} leaf FW s^{-1}.

Determination of leaf-nutrient contents

Leaf -N, -P and -K contents were determined on dry-weight basis. Total nitrogen content in leaves was determined using the dry leaf-powder by standard Kjeldahl method [21]. The leaf powder was digested in sulphuric acid using salicylic acid and sodium thiosulphate. Ammonia of the digest was distilled into boric acid solution using 40% NaOH solution and mixed indicator (bromocresol green plus methyl red indicator). The ammonia absorbed in boric acid was titrated against the standard solution of sulphuric acid to determine total nitrogen in the leaf-sample. In order to determine phosphorus and potassium contents, the leaf-powder was digested in di-acid (nitric acid + perchloric acid). Total phosphorus content in the leaf tissue was determined by vandate-molybdate method recording the absorbance at 420 nm of the yellow-colour complex (vando-molybdo-phosphoric heteropoly complex) formed due to reaction of extracted plant orthophosphates with molybdate and vandate, using the spectrophotometer [22]. Phosphorus content in the aliquot was estimated with the help of standard curve prepared by using potassium dihydrogen orthophosphate (KH_2PO_4). Potassium content in the nitric-perchloric acid digest of the leaf-sample was estimated using a flame photometer (Corning Limited, Nalstead Essex, England) equipped with K filter.

Estimation of total leaf alkaloids content

Total content of leaf-alkaloids was estimated according to [23]. The leaves and roots were dried in a hot-air oven at 80 °C for 24 hours. The samples were powdered and passed through a 72 mesh sieve. Five hundred mg powder of leaves as well as of roots was transferred into a 100 mL round-bottom reflux flask separately, followed by adding a known volume of ethyl alcohol. Then, the mixture was refluxed for 6 hours. Thereafter, the content was filtered, followed by adding 50 mL of dilute HCl. The mixture was shaken for 15-20 minutes. The upper diethyl ether layer was discarded and the lower water layer was decanted into a beaker; the content was made slightly basic by adding ammonia solution. The decanted content was again transferred into a separating funnel with 50 mL of diethyl ether; it was decanted again. To this decant, anhydrous sodium carbonate was added. The mixture was again decanted in a pre-weighed dry porcelain dish and then the content was evaporated till dryness, followed by weighing the dried content.

Total alkaloid content (%) was calculated using following formula:

$$\text{Total alkaloid content } (\%) = \frac{WA - WE}{WR} \times 100$$

Where,

WE =Weight of empty porcelain dish (g)
WA=Weight of porcelain dish after evaporation (g)
WR= Weight of the dried powder (g)

Estimation of vincristine and vinblastine contents

Extraction of samples and the chromatographic condition of high-performance liquid chromatography (HPLC) instrument were achieved through the method of [24]. Freshly harvested leaves were oven dried at 60 °C for 48 hours and then powdered, using pestle-mortar. A volume (30 mL) of 90% ethanol was added to 5 g of leaf powder; the content was left over night and then filtered. The residue was re-extracted with 90% ethanol (3 × 30 mL) at room temperature (27 °C); the pooled alcoholic extract was filtered and concentrated *in vacuo* at 40 °C. The dried residue was re-dissolved in ethanol (10 mL), diluted with water (10 mL) and then acidified with 3% hydrochloric acid (10 mL). This was then extracted with hexane (3 × 30 mL), the hexane extract was discarded and the aqueous portion of the content was cooled to 10 °C; it was basified with ammonium hydroxide to pH 8.5 and then was extracted with chloroform (3 × 30 mL). The combined chloroform extract was washed with water, evaporated to dryness and re-dissolved in 1 mL of chloroform. After that, it was passed through a silica Sep-Pak cartridge (Waters Corporation, Milford, Massachusetts, USA), pre-saturated with chloroform and then washed successively with 5 mL each of chloroform and chloroform-methanol mixture (9:1, v/v) and dried over anhydrous sodium sulphate before being evaporated to dryness. The residue obtained was dried to constant weight in order to determine the contents of vincristine and vinblastine alkaloids. An aliquot (10 mg) of the crude alkaloid was dissolved in 1 mL of HPLC grade methanol; 10 µL of it was subjected to HPLC analysis.

HPLC analysis

Chromatographic separations were carried out using HPLC (LC-20AD, Shimadzu, Science Inc., Kyoto, Japan). Solvents were filtered by using a Millipore Filter System (Merck Life Science Pvt. Ltd., Bengaluru, India) and the analysis was performed on C18 reversed-phase column, 10 mm (30 cm × 3.9 mm I.D.). A constant flow rate of 0.6 mL/min was used during analysis. The composition of mobile phase was optimized by using acetonitrile: 0.1 M phosphate buffer: glacial acetic acid (38:62:0.3); pH 4.14, flow rate 0.6 mL/min, column temperature 26 °C, and detector wave length 254 nm. For standard, stock solutions of vincristine and vinblastine were prepared dissolving 1 mg of each of the alkaloids in 1 mL of methanol. The solutions were subjected to HPLC and the retention time (Rt) for vincristine and vinblastine were noticed.

Statistical analysis

Each pot was treated as one replicate and all the treatments were repeated five times. The data were analyzed statistically using SPSS-17 statistical software (SPSS Inc., Chicago, IL, USA). Means were statistically compared using Fisher's Least Significant Difference (LSD) at $p<0.05$.

Results

In general, both the control (water-spray treatment) and UISA treatment (unirradiated sodium alginate applied at 20 mg L^{-1}) gave the lowest values and proved statistically similar in effect. On the other hand, the application of ISA improved the parameters studied significantly in comparison to the control (water-spray treatment) as well as to the UISA treatment. For most of the parameters studied, ISA applied at 80 mg L^{-1}, proved the best treatment or sometimes it was at par with 160 mg L^{-1} of ISA, resulting in the highest values (Tables 2a, 3a and 4a). Application of 20 mg L^{-1} of MeJA, equalled by 10 mg L^{-1} of MeJA, was optimum treatment, while 40 mg L^{-1} of MeJA proved deleterious for growth parameters, physiological parameters and leaf-nutrient contents in most cases (Tables 2b, 3b and 4b). On the other hand, MeJA application at 30 mg L^{-1}, followed by that at 40 mg L^{-1}, proved the best foliar spray treatment for alkaloid parameters (Tables 2b, 3b and 4b). Interestingly, application of ISA with methyl jasmonate (MeJA) improved the parameters studied significantly in comparison to the control (water-spray treatment) and UISA treatment. For most of the parameters studied, combined application of ISA at 80 mg L^{-1} + MeJA at 20 mg L^{-1} resulted in the highest values (Tables 2,3 and 4).

Shoot and leaf parameters

ISA, applied at 80 mg L^{-1}, gave maximum values for shoot and leaf parameters (shoot length, shoot fresh mass, shoot dry mass, average leaf area, leaf fresh mass and leaf dry mass). However, 80 mg L^{-1} of ISA was at par with 160 mg L^{-1} of ISA for shoot and leaf parameters and resulted in the enhancement of shoot length by 33.33%, shoot fresh mass by 20.95% and shoot dry mass by 26.31% in comparison to control plants sprayed with DDW. As per Table 2a, application of MeJA gave the highest values of shoot parameters at 20 mg L^{-1}; however, the values were statistically equal with those given by 10 mg L^{-1} of MeJA. Thereafter, the values decreased significantly at 30 and 40 mg L^{-1} of MeJA. Treatment 20 mg L^{-1} of MeJA enhanced the shoot length by 10.20%, shoot fresh mass by 12.86% and shoot dry mass by 8.23% in comparison to control. Compared with the control, treatment 20 mg L^{-1} of MeJA enhanced the average leaf area, leaf fresh mass and leaf dry mass by 7.22, 17.55 and 28.57%, respectively; however, the effect of 20 and 30 mg L^{-1} of MeJA was statistically at par about leaf dry-mass. Treatment 40 mg L^{-1} of MeJA proved invariably deleterious regarding the leaf parameters (Table 2b). Combined application of ISA 80 mg L^{-1} + MeJA 20 mg L-1 gave maximum values for shoot and leaf

parameters enhancing the shoot length by 20.57 and 30.32%, shoot fresh mass by 19.42 and 25.11%, shoot dry mass by 28.56 and 22.41% (Table 2), average leaf area by 18.64 and 11.70%, leaf area index by 21.83 and 8.53% and leaf number by 22.82 and 33.03%, at 6 and 9 MAS, respectively (Table 2.1).

Treatments	Shoot parameters					
	Shoot length (cm)		Shoot fresh mass (g)		Shoot dry mass (g)	
	6MAS	9MAS	6MAS	9MAS	6MAS	9MAS
CONTROL	48.60^{d}	68.60^{e}	65.02^{d}	88.40^{f}	14.25^{b}	23.42^{d}
UISA (40 mg L^{-1})	49.10^{d}	70.40^{e}	65.08^{d}	89.20^{f}	14.28^{b}	23.65^{d}
ISA 40 mg L^{-1} + MeJA 20 mg L^{-1}	55.80^{b}	78.50^{c}	74.68b^{c}	98.60^{d}	16.67^{ab}	25.26^{c}
ISA 40 mg L^{-1} + MeJA 30 mg L^{-1}	54.20^{c}	74.80^{d}	73.23^{c}	95.70^{e}	16.28^{ab}	24.85^{c}
ISA 80 mg L^{-1} + MeJA 20 mg L^{-1}	58.60^{a}	89.40^{a}	77.65^{a}	110.60^{a}	18.32^{a}	28.67^{a}
ISA 80 mg L^{-1} + MeJA 30 mg L^{-1}	56.20^{b}	85.20^{b}	76.42^{ab}	106.20^{b}	18.09^{a}	28.16^{ab}
ISA 160 mg L^{-1} + MeJA 20 mg L^{-1}	54.90^{bc}	82.40^{b}	75.86b^{c}	102.70^{c}	17.76^{a}	27.54^{b}
ISA 160 mg L^{-1} + MeJA 30 mg L^{-1}	53.40^{c}	76.80^{cd}	73.80^{c}	99.20^{d}	17.28^{a}	25.86^{c}
LSD (p<0.05)	1 .44	3.25	1.90	2.87	2.09	1.09

CONTROL: deionised water; UISA: unirradiated sodium alginate; ISA: irradiated sodium alginate; MeJA: methyl jasmonate; MAS: months after sowing; values followed by the same letter in a column are not significantly different according to Fisher's Least Significant Difference (LSD) at p<0.05

Table 2: Effect of different concentrations of ISA and MeJA combinations on shoot parameters of *Catharanthus roseus* at 6 and 9 months after sowing

Treatments	Leaf parameters					
	Leaf number		Leaf area index		Average leaf area (cm²)	
	6MAS	9MAS	6MAS	9MAS	6MAS	9MAS
CONTROL	184^{g}	224^{d}	9.25^{d}	9.72^{c}	10.89^{e}	8.54^{f}
UISA (40 mg L^{-1})	192^{f}	230^{d}	9.28^{d}	9.78^{c}	10.95^{e}	8.60^{f}
ISA 40 mg L^{-1} + MeJA 20 mg L^{-1}	202de	256^{c}	10.12^{c}	10.14^{b}	11.93^{c}	8.98^{de}
ISA 40 mg L^{-1} + MeJA 30 mg L^{-1}	198^{ef}	242^{d}	9.97^{c}	10.00^{b}	11.39^{d}	8.82^{e}
ISA 80 mg L^{-1} + MeJA 20 mg L^{-1}	226^{a}	298^{a}	11.27^{a}	10.68^{a}	12.92^{a}	9.54^{a}
ISA 80 mg L^{-1} + MeJA 30 mg L^{-1}	218^{b}	284^{b}	11.07^{ab}	10.55^{a}	12.84^{a}	9.36^{ab}
ISA 160 mg L^{-1} + MeJA 20 mg L^{-1}	212^{bc}	275^{b}	10.73^{b}	10.42^{ab}	12.64^{b}	9.22^{bc}
ISA 160 mg L^{-1} + MeJA 30 mg L^{-1}	208^{cd}	264^{c}	10.21^{c}	10.28^{ab}	12.06^{c}	9.10^{cd}
LSD (p<0.05)	7.28	9.27	0.51	0.43	0.17	0.19

CONTROL: deionised water; UISA: unirradiated sodium alginate; ISA: irradiated sodium alginate; MeJA: methyl jasmonate; values followed by the same letter in a column are not significantly different according to Fisher's Least Significant Difference (LSD) at p<0.05

Table 2.1: Effect of different concentrations of ISA and MeJA combinations on leaf parameters of *Catharanthus roseus* at 6 and 9 months after sowing

	Shoot parameters			Leaf parameters		
Treatments	Shoot length (cm)	Shoot fresh mass (g)	Shoot dry mass (g)	Average leaf area (cm²)	Leaf fresh mass (g)	Leaf dry mass (g)
CONTROL	45.0^{b}	42.0^{b}	9.5^{bc}	10.00^{e}	12.02^{d}	3.84^{c}
UISA (20 mg L^{-1})	47.0^{b}	42.0^{b}	9.7^{b}	10.50^{d}	12.34^{c}	3.82^{c}
ISA 20 mg L^{-1}	48.0^{b}	44.0^{b}	9.9^{b}	11.50^{c}	12.51^{c}	4.14^{b}
ISA 40 mg L^{-1}	50.0^{ab}	45.0^{b}	10.2^{b}	11.90^{b}	13.26^{b}	4.22^{b}
ISA 80 mg L^{-1}	60.0^{a}	52.3^{a}	12.0^{a}	12.50^{a}	14.42^{a}	4.82^{a}
ISA 160 mg L^{-1}	55.0^{a}	50.8^{a}	11.5^{a}	12.40^{a}	14.01^{b}	4.22^{b}
LSD (p<0.05)	5.102	3.170	0.512	0.334	0.299	0.129

CONTROL: deionized water; UISA: unirradiated sodium alginate; ISA: irradiated sodium alginate; values followed by the same letter in a column are not significantly different according to Fisher's Least Significant Difference (LSD) at p<0.05

Table 2a: Effect of irradiated ISA on shoot and leaf parameters of *Catharanthus roseus*

	Shoot parameters			Leaf parameters		
Treatments	Shoot length (cm)	Shoot fresh mass (g)	Shoot dry mass (g)	Average leaf area (cm^2)	Leaf fresh mass (g)	Leaf dry mass (g)
CONTROL	48.0^{ab}	38.1^{b}	8.5^{a}	8.30^{ab}	12.13^{c}	3.64^{c}
10 mg L^{-1}	50.6^{a}	41.9^{a}	8.8^{a}	8.50^{a}	12.50^{b}	4.50^{b}
20 mg L^{-1}	52.9^{a}	43.0^{a}	9.2^{a}	8.90^{a}	14.26^{a}	4.68^{a}
30 mg L^{-1}	43.7^{c}	37.7^{b}	7.8^{b}	7.80^{c}	12.23^{c}	4.60^{a}
40 mg L^{-1}	41.4^{c}	36.0^{b}	7.3^{b}	7.70^{c}	11.52^{d}	3.59^{c}
LSD (p<0.05)	3.682	3.301	0.595	0.310	0.157	0.084

CONTROL: deionised water; MeJA: methyl jasmonate; values followed by the same letter in a column are not significantly different according to Fisher's Least Significant Difference (LSD) at p<0.05

Table 2b: Effect of MeJA on shoot and leaf parameters of *Catharanthus roseus*

Physiological parameters

Physiological parameters (NR activity, CA activity and chlorophyll content) were best affected by 80 mg L^{-1} of ISA resulting in an increase of 22.72% in comparison to the control; however, the values obtained with 160 mg L^{-1} of ISA were statistically equal regarding CA activity and chlorophyll content. In comparison to the control, 80 mg L^{-1} of ISA enhanced these values by 20 and 20.83%, respectively (Table 3a). Of the MeJA treatments, 20 mg L^{-1} of MeJA proved optimum treatment for all physiological parameters, with 10 mg L^{-1} giving statistically at par values. MeJA applied at 30 and 40 mg L^{-1} always proved significantly detrimental (Table 3b). Physiological parameters were best affected by ISA 80 mg L^{-1} + MeJA 20 mg L^{-1} (Table 3). Hence, ISA 80 mg L^{-1} + MeJA 20 mg L^{-1} proved optimum combination of treatments for all physiological parameters resulting in the increase in chlorophyll content by 12.80 and 11.52%, CA activity by 11.05 and 8.29% and NR activity by 15.42 and 10.66% at 6 and 9 MAS, respectively (Table 3).

Treatments	Physiological parameters					
	NR activity [nmol NO_2- g^{-1} (FW) h^{-1}]		CA activity [mol (CO_2) kg^{-1} (FW) s^{-1}]		Total chlorophyll content (mg g^{-1} FW)	
	6MAS	9 MAS	6MAS	9 MAS	6MAS	9 MAS
CONTROL	201.6^{d}	191.20^{e}	4.34^{f}	4.22^{e}	1.312^{e}	1.275^{e}
UISA (40 mg L^{-1})	205.2^{d}	191.62^{e}	4.36^{f}	4.25^{e}	1.315^{e}	1.280^{e}
ISA 40 mg L^{-1} + MeJA 20 mg L^{-1}	223.7^{b}	198.56^{d}	4.64^{d}	4.38^{cd}	1.423^{bc}	1.382^{bc}
ISA 40 mg L^{-1} + MeJA 30 mg L^{-1}	218.3^{c}	195.24^{d}	4.52^{e}	4.33^{d}	1.410^{cd}	1.320^{d}
ISA 80 mg L^{-1} + MeJA 20 mg L^{-1}	232.7^{a}	211.60^{a}	4.82^{a}	4.57^{a}	1.484^{a}	1.422^{a}
ISA 80 mg L^{-1} + MeJA 30 mg L^{-1}	227.1^{b}	208.17^{ab}	4.76^{ab}	4.52^{ab}	1.467^{ab}	1.402^{ab}
ISA 160 mg L^{-1} + MeJA 20 mg L^{-1}	223.7^{b}	205.64^{bc}	4.72^{bc}	4.48^{b}	1.397^{cd}	1.378^{bc}
ISA 160 mg L^{-1} + MeJA 30 mg L^{-1}	218.3^{c}	202.85^{c}	4.68^{cd}	4.41^{c}	1.369^{d}	1.346^{cd}
LSD (p<0.05)	4.62	4.19	0.070	0.057	0.050	0.040

CONTROL: deionised water; UISA: unirradiated sodium alginate; ISA: irradiated sodium alginate; MeJA: methyl jasmonate; FW: fresh weight; NR: nitrate reductase; CA: carbonic anhydrase; MAS: months after sowing values followed by the same letter in a column are not significantly different according to Fisher's Least Significant Difference (LSD) at p<0.05

Table 3: Effect of different concentrations of ISA and MeJA combinations on physiological parameters of *Catharanthus roseus* L. at 6 and 9 months after sowing

	Physiological parameters			Leaf-nutrients		
Treatments	NR activity [nmol NO_2- g^{-1} (FW) h^{-1}]	CA activity [mol (CO_2) kg^{-1} (FW) s^{-1}]	Total chlorophyll content (mg g^{-1} FW)	Leaf-N content (%)	Leaf-P content (%)	Leaf-K content (%)
CONTROL	220^{d}	4.5^{c}	1.20^{b}	3.20^{b}	0.30	3.30^{b}
UISA (20 mg L^{-1})	222^{d}	4.6^{bc}	1.21^{b}	3.23^{b}	0.30	3.31^{b}
ISA 20 mg L^{-1}	241^{bc}	4.9^{b}	1.30^{b}	3.32^{ab}	0.31	3.42^{b}
ISA 40 mg L^{-1}	248^{b}	5.1^{ab}	1.31^{ab}	3.39^{ab}	0.32	3.44^{ab}
ISA 80 mg L^{-1}	270^{a}	5.4^{a}	1.45^{a}	3.52^{a}	0.33	3.59^{a}
ISA 160 mg L^{-1}	253^{b}	5.2^{a}	1.39^{a}	3.45^{a}	0.33	3.53^{a}
LSD(p<0.05)	9.872	0.324	0.134	0.211	NS	0.145

CONTROL: deionised water; UISA: unirradiated sodium alginate; ISA: irradiated sodium alginate; NR: nitrate reductase; CA: carbonic anhydrase; FW: fresh weight; NS: non-significant; values followed by the same letter in a column are not significantly different according to Fisher's Least Significant Difference (LSD) at p<0.05

Table 3a: Effect of ISA on physiological parameters and leaf-nutrient contents of *Catharanthus roseus*

Treatments	Physiological parameters			Leaf-nutrients		
	NR activity [nmol NO_2- g^{-1} (FW) h^{-1}]	CA activity [mol (CO_2) kg^{-1} (FW) s^{-1}]	Total chlorophyll content (mg g^{-1} FW)	Leaf-N content (%)	Leaf-P content (%)	Leaf-K content (%)
CONTROL	225^{b}	5.0^{b}	1.20^{a}	2.70^{ab}	0.22^{ab}	3.10^{c}
10 mg L^{-1}	246^{a}	5.2^{ab}	1.26^{a}	3.63^{a}	0.26^{a}	3.96^{ab}
20 mg L^{-1}	253^{a}	5.4^{a}	1.31^{a}	2.90^{a}	0.27^{a}	4.50^{a}
30 mg L^{-1}	198^{c}	4.4^{c}	1.14^{ab}	2.16^{b}	0.22^{ab}	3.80^{b}
40 mg L^{-1}	182^{d}	4.3^{c}	1.08^{ab}	2.40^{b}	0.22^{ab}	3.00^{c}
LSD (p<0.05)	7.557	0.349	0.155	0.911	0.042	0.654

CONTROL: deionised water; UISA: unirradiated sodium alginate; NR: nitrate reductase; CA: carbonic anhydrase; FW: fresh weight; values followed by the same letter in a column are not significantly different according to Fisher's Least Significant Difference (LSD) at p<0.05

Table 3b: Effect of methyl MeJA on physiological parameters and leaf-nutrient contents of *Catharanthus roseus*

Leaf-nutrient contents

Foliar application of ISA at 80 mg L^{-1} gave the highest values for leaf-nutrient (leaf-N and -K) contents, with 160 mg L^{-1} proving statistically equal to 80 mg L^{-1} (Table 3a). 80 mg L^{-1} increased the content of leaf-N by 10% and leaf-K by 8.87%.With regard to the effect of MeJA application, 20 mg L^{-1}, equalled by 10 mg L^{-1}, gave the highest leaf nutrient contents enhancing leaf N, P and K by 7.40, 22.72 and 45.16% respectively; while, 30 and 40 mg L^{-1} of MeJA resulted into significantly lower values (Table 3b). Combined application of ISA 80 mg L^{-1} + MeJA 20 mg L^{-1}, mostly followed by ISA 80 mg L^{-1} + MeJA 30 mg L^{-1}, gave the highest values with regard to leaf-nutrient contents, surpassing the control (water-spray treatment) by 26.79 and16.71% regarding leaf-N content, by 25.92 and 20.88% regarding leaf-P content and by 7.46 and 6.94% regarding leaf-K content at 6 and 9 MAS, respectively (Table 3.1).

Treatments	Leaf-nutrients					
	Leaf-N content (%)		Leaf-P content (%)		Leaf-K content (%)	
	6 MAS	9 MAS	6 MAS	9 MAS	6 MAS	9 MAS
CONTROL	2.945^{d}	2.214^{d}	0.324^{ab}	0.249^{b}	3.482^{c}	3.024^{c}
UISA (40 mg L^{-1})	2.952^{d}	2.220^{d}	0.326^{ab}	0.255^{b}	3.490^{c}	3.030^{c}
ISA 40 mg L^{-1} + MeJA 20 mg L^{-1}	3.483^{c}	2.417^{b}	0.384^{a}	0.268^{a}	3.522^{c}	3.176^{ab}
ISA 40 mg L^{-1} + MeJA 30 mg L^{-1}	3.407^{c}	2.356^{c}	0.368^{a}	0.262^{ab}	3.613^{b}	3.118^{d}
ISA 80 mg L^{-1} + MeJA 20 mg L^{-1}	3.734^{a}	2.584^{a}	0.408^{a}	0.301^{a}	3.742^{a}	3.234^{a}
ISA 80 mg L^{-1} + MeJA 30 mg L^{-1}	3.667^{a}	2.515^{ab}	0.397^{a}	0.291^{a}	3.692^{a}	3.202^{a}
ISA 160 mg L^{-1} + MeJA 20 mg L^{-1}	3.562^{b}	2.458^{b}	0.386^{a}	0.283^{a}	3.596^{b}	3.194^{a}
ISA 160 mg L^{-1} + MeJA 30 mg L^{-1}	3.461^{c}	2.392^{bc}	0.376^{a}	0.276^{a}	3.531^{c}	3.136^{b}
LSD (p<0.05)	0.06	0.07	0.05	0.03	0.06	0.04

CONTROL: deionised water; UISA: unirradiated sodium alginate; ISA: irradiated sodium alginate; MeJA: methyl jasmonate; MAS: months after sowing; values followed by the same letter in a column are not significantly different according to Fisher's Least Significant Difference (LSD) at p<0.05

Table 3.1: Effect of different concentrations of ISA and MeJA combinations on leaf-nutrient contents of *Catharanthus roseus* L. at 6 and 9 months after sowing

Leaf-alkaloids content

Treatments	Contents of leaf-alkaloids					
	Total alkaloid (mg g^{-1})		Vincristine (mg kg^{-1})		Vinblastine(mg kg^{-1})	
	6 MAS	9 MAS	6 MAS	9 MAS	6 MAS	9 MAS
CONTROL	6.0^{f}	5.8^{f}	42	39	205	201
UISA (40 mg L^{-1})	6.0^{f}	5.8^{f}	43	39	205	201
ISA 40 mg L^{-1} + MeJA 20 mg L^{-1}	6.4^{e}	6.2^{e}	43	39	206	202
ISA 40 mg L^{-1} + MeJA 30 mg L^{-1}	6.7^{d}	6.6^{d}	44	41	206	202
ISA 80 mg L^{-1} + MeJA 20 mg L^{-1}	7.2^{c}	7.0^{bc}	44	41	207	203
ISA 80 mg L^{-1} + MeJA 30 mg L^{-1}	7.8^{a}	7.3^{a}	46	43	208	203
ISA 160 mg L^{-1} + MeJA 20 mg L^{-1}	7.5^{b}	7.2^{ab}	45	42	207	205
ISA 160 mg L^{-1} + MeJA 30 mg L^{-1}	7.3^{bc}	6.8^{cd}	45	42	206	204
LSD (p<0.05)	0.24	0.21	NS	NS	NS	NS

CONTROL: deionised water; UISA: unirradiated sodium alginate; ISA: irradiated sodium alginate; MeJA: methyl jasmonate; NS: non-significant; MAS: months after sowing values followed by the same letter in a column are not significantly different according to Fisher's Least Significant Difference (LSD) at p<0.05

Table 4: Effect of different concentrations of ISA and MeJA combinations on leaf-alkaloid contents of *Catharanthus roseus* L. at 6 and 9 months after sowing

Treatments	Contents of leaf-alkaloids			Yield of leaf-alkaloids		
	Total alkaloid (mg g^{-1})	Vincristine (mg kg^{-1})	Vinblastine (mg kg^{-1})	Total alkaloid (mg $plant^{-1}$)	Vincristine (µg $plant^{-1}$)	Vinblastine (µg $plant^{-1}$)
CONTROL	7.0^{c}	49	211	26.88^{d}	188^{e}	810^{e}
UISA (20 mg L^{-1})	7.0^{c}	49	211	26.74^{d}	187^{e}	806^{e}
ISA 20 mg L^{-1}	8.0^{ab}	49	212	33.12^{c}	203^{d}	877^{d}
ISA 40 mg L^{-1}	8.1^{ab}	50	212	34.18^{c}	211^{c}	894^{c}
ISA 80 mg L^{-1}	8.7^{a}	51	213	41.93^{a}	245^{a}	1026^{a}
ISA 160 mg L^{-1}	8.4^{a}	51	213	35.45^{b}	215^{b}	899^{b}
LSD ($p<0.05$)	0.43	NS	NS	1.170	3.767	4.115

CONTROL: deionised water; UISA: unirradiated sodium alginate; ISA: irradiated sodium alginate; NS: non-significant; values followed by the same letter in a column are not significantly different according to Fisher's Least Significant Difference (LSD) at $p<0.05$

Table 4a: Effect of irradiated ISA on leaf-alkaloid content and leaf-alkaloid yield per plant of *Catharanthus roseus*

Treatments	Contents of leaf-alkaloids			Yield of leaf-alkaloids		
	Total alkaloid (mg g^{-1})	Vincristine (mg kg^{-1})	Vinblastine (mg kg^{-1})	Total alkaloid (mg $plant^{-1}$)	Vincristine (µg $plant^{-1}$)	Vinblastine (µg $plant^{-1}$)
CONTROL	6.8^{e}	49^{d}	209^{d}	24.75^{e}	178^{e}	761^{e}
MeJA 10 mg L^{-1}	7.4^{d}	54^{c}	215^{c}	33.30^{c}	243^{d}	967^{c}
MeJA 20 mg L^{-1}	8.0^{c}	65^{b}	230^{b}	37.44^{b}	304^{b}	1076^{b}
MeJA 30 mg L^{-1}	8.9^{a}	75^{a}	239^{a}	40.94^{a}	345^{a}	1099^{a}
MeJA 40 mg L^{-1}	8.7^{b}	73^{a}	238^{a}	31.23^{d}	262^{c}	854^{d}
LSD ($p<0.05$)	0.034	2.467	5.473	1.924	8.608	11.123

CONTROL: deionised water; MeJA: methyl jasmonate; values followed by the same letter in a column are not significantly different according to Fisher's Least Significant Difference (LSD) at $p<0.05$

Table 4b: Effect of MeJA on leaf-alkaloid content and leaf-alkaloid yield per plant of *Catharanthus roseus*

80 mg L^{-1} of ISA was the best treatment, while 160 mg L^{-1} of ISA gave the second best value regarding total leaf alkaloids content (Table 4a). 80 mg L^{-1} of ISA enhanced the leaf alkaloids content by 24.28%, while leaf-contents of vincristine and vinblastine were not significantly enhanced. Application of MeJA at 30 mg L^{-1} resulted in the highest increase in the content of total leaf alkaloids (30.88%), while 40 mg L^{-1} of MeJA was the second best treatment in this regard (Table 4b). Combined application of ISA 80 mg L^{-1} + MeJA 30 mg L^{-1} resulted in maximum value regarding total leaf alkaloids content, surpassing the control by 30 and 26% at 6 and 9 MAS, respectively (Table 4).

Leaf-alkaloids content

Yield of total leaf-alkaloids attained the highest value as a result of 80 mg L^{-1} of ISA, while the second best value was attained at 160 mg L^{-1} (Tables 4a). Of the MeJA treatments, 30 mg L^{-1}, followed by 20 mg L^{-1}, gave the highest value regarding leaf-alkaloids yield; while, 40 mg L^{-1} of MeJA gave the lowest value (Table 4b). Combined treatment ISA 80 mg L^{-1} + MeJA 30 mg L^{-1}, followed by ISA 160 mg L^{-1} + MeJA 20 mg L^{-1}, resulted in the highest yield of total leaf-alkaloids, exceeding the control by 30 and 25.86% at 6 and 9 MAS, respectively (Table 4.1)

Treatments	Yield of leaf-alkaloids					
	Total alkaloid (mg $plant^{-1}$)		Vincristine (µg $plant^{-1}$)		Vinblastine (µg $plant^{-1}$)	
	6 MAS	9 MAS	6 MAS	9 MAS	6 MAS	9 MAS
CONTROL	24.2^{f}	23.2^{e}	169.3^{e}	156.0^{e}	826.2^{h}	804.0^{g}
UISA (40 mg L^{-1})	24.8^{f}	24.4^{e}	177.6^{e}	163.8^{e}	846.7^{g}	844.2^{f}
ISA 40 mg L^{-1} + MeJA 20 mg L^{-1}	27.8^{e}	26.0^{d}	187.9^{d}	164.2^{e}	900.4^{f}	850.4^{f}
ISA 40 mg L^{-1} + MeJA 30 mg L^{-1}	30.7^{d}	28.9^{c}	201.5^{c}	179.6^{d}	943.5^{e}	884.8^{e}
ISA 80 mg L^{-1} + MeJA 20 mg L^{-1}	33.8^{c}	32.2^{b}	206.8^{c}	188.6^{c}	972.9^{d}	933.8^{d}
ISA 80 mg L^{-1} + MeJA 30 mg L^{-1}	41.5^{a}	36.5^{a}	244.7^{a}	215.0^{a}	1106.6^{a}	1015.0^{a}
ISA 160 mg L^{-1} + MeJA 20 mg L^{-1}	38.4^{b}	35.3^{a}	230.4^{b}	205.8^{b}	1059.8^{b}	1004.5^{b}
ISA 160 mg L^{-1} + MeJA 30 mg L^{-1}	36.7^{b}	32.9^{b}	226.3^{b}	201.6^{b}	1036.2^{c}	979.2^{c}
LSD ($p<0.05$)	1.85	1.43	5.33	4.20	7.50	7.20

CONTROL: deionised water; UISA: unirradiated sodium alginate; ISA: irradiated sodium alginate; MeJA: methyl jasmonate; MAS: months after sowing; values followed by the same letter in a column are not significantly different according to Fisher's Least Significant Difference (LSD) at $p<0.05$

Table 4.1: Effect of different concentrations of ISA and MeJA combinations on leaf-alkaloid yield of *Catharanthus roseus* L. at 6 and 9 months after sowing

Leaf-vincristine and -vinblastine content

There was no significant effect of ISA on leaf-content of vincristine and vinblastine (Table 4a). According to Table 4b, application of MeJA at 30 mg L^{-1} enhanced the leaf-content of vincristine and vinblastine by 53.06 and 14.35%, respectively. However, 30 and 40 mg L^{-1} of MeJA gave statistically equal values both for vincristine and vinblastine content. On the other hand, the effect of ISA + MeJA treatment on leaf-content of vincristine and vinblastine was not significant (Table 4).

Leaf-vincristine and -vinblastine yield

The highest yield of leaf-vincristine and leaf-vinblastine was attained with 80 mg L^{-1} of ISA, while the second best value was attained at 160 mg L^{-1} of ISA (Table 4a). Leaf analysis revealed that in comparison to the control, 80 mg L^{-1} of ISA resulted in 30.31% and 26.66% increase in the yield of leaf-vincristine and leaf-vinblastine, respectively (Table 4a). On the other hand, 30 mg L^{-1} of MeJA, followed by 20 mg L^{-1} of MeJA, gave the highest yield both of vincristine and vinblastine. 30 mg L^{-1} of MeJA resulted in 93.82% and 44.41% increase in leaf-vincristine and leaf-vinblastine yield, respectively (Table 4b). The highest yields of vincristine and vinblastine were attained with ISA 80 mg L^{-1} + MeJA 30 mg L^{-1}. This treatment enhanced the leaf-vincristine yield by 44.53 and 37.82% and leaf-vinblastine yield by 33.93 and 26.24% at 6 and 9 MAS, respectively (Table 4.1).

Discussion

The present study showed significant improvement in the values of shoot and leaf characteristics, physiological parameters, leaf-nutrient contents and the content and yield of alkaloids as a result of application of ISA usually at 80 mg L^{-1} (Tables 2a, 3a and 4a). In most cases, MeJA gave maximum values at 20 mg L^{-1} for shoot and leaf attributes (Table 2b). Regarding shoot parameters, 20 and 10 mg L^{-1} of MeJA gave equal values. Treatment 20 mg L^{-1} of MeJA, equalled by 10 mg L^{-1} of MeJA, resulted in the highest values, while 40 mg L^{-1} gave the lowest values for physiological parameters and leaf-nutrient contents (Table 3b). Generally, foliar spray of ISA applied with MeJA (ISA 80 mg L^{-1} + MeJA 30 mg L^{-1}), showed significant improvement in the values of all the parameters studied, including the content and yield of total as well as anticancer alkaloids, viz. vincristine and vinblastine, in this study, employing *Catharanthus roseus* (L.) G. Don as the test plant (Tables 2,3 and 4).

Plant growth and development is known to be governed by several exogenous and endogenous factors, including the growth regulators [25]. In the present investigation, the foliar application of ISA along with that of MeJA, enhanced the leaf-area (Table 2.1), which obviously provided increased opportunity for light harvesting, leading to the accumulation of enhanced plant dry matter, compared to the water-spray control. It has already been reported that natural polysaccharides such as sodium alginate, carrageenan and chitosan, in their irradiated form, proved effective in promotion of germination and shoot growth and in exerting the positive effect on physiological parameters as well as plant nutrient contents [3-5,7,26-33]. In this respect, the irradiated natural polysaccharides might resemble the endogenous growth elicitors that might function as signal molecules to trigger the synthesis of different enzymes and activate various plant responses, exploiting the gene expression, as argued by [34]. Increase in alkaloid contents because of combined application of ISA and MeJA might be due to the anticipated improvement in secondary metabolism of *Catharanthus roseus*. Considering the above results, it may be speculated in this study that ISA generally proved as elicitor of overall growth and development (Tables 2a, 3a and 4a), while MeJA elicited the secondary plant metabolism (Table 4b). Expectedly, the yield of the anticancer alkaloids (vincristine and vinblastine) was significantly increased because the ISA treatments significantly increased the dry mass of the leaves (Table 2a).

While the values of most other parameters were decreased when 30 and 40 mg L^{-1} of MeJA were applied because of the growth-decreasing effect of higher doses of MeJa, application of MeJA (30 and 40 mg L^{-1}) increased both content and yield of total alkaloids as well as those of the anticancer alkaloids (Table 4b) [35]. In fact, Jasmonic acid (JA) and its derivatives such as methyl jasmonate (MeJA: a methylated derivative of JA) are elicitors that act as the intracellular signal compounds in the elicitation of plant defence responses [36]. Exogenous application of MeJA has been shown to be a potent elicitor of the secondary metabolites biosynthesis in the case of many medicinal plant species [37-41]. The role of jasmonate (jasmonic acid and its methyl derivative, MeJA) in activating the expression of genes in the accumulation of phytoalexins and other secondary metabolites has already been confirmed in various plant systems including *Catharanthus roseus* (L.) G.Don [16,42,43].

Expectedly, combined application of ISA and MeJA (ISA 80 mg L^{-1} + MeJA 30 mg L^{-1}) proved significantly effective in increasing the yield of total alkaloids as well as those of the anticancer alkaloids in the leaves (Tables 4 and 4.1) due to growth-encouraging effect of ISA and MeJA-induced secondary-metabolism. Thus, the present study revealed that such a combined application of the two elicitors (ISA and MeJA) might increase the production of the extraordinarily expensive anticancer alkaloids (vincristine and vinblastine) significantly. Hence, the present technique might be employed for reducing the cost of cancer chemotherapy.

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