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Triacontanol Improves Production of Anticancer Alkaloids in *Catharanthus roseus* L.

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Abstract

Catharanthus roseus (L.) G. Don is a medicinal plant that bears indole alkaloids used in cancer chemotherapy. The anti-cancer alkaloids, *viz*. vinblastine and vincristine, are mainly present in leaves of *C. roseus*. As there is high demand and low yield of these alkaloids in *C. roseus,* alternative ways to improve alkaloid production are needed. Hence, it was hypothesized that Triacontanol (TRIA), a potent plant growth promoting substance for various medicinal and agricultural crops, would improve alkaloid production in *C. roseus*. A pot culture experiment was carried out and the effects of TRIA on production of total alkaloids including anticancer alkaloids were evaluated at 120 and 150 days after planting. Four concentrations of TRIA [10^{-0} (Control) 10^{-7}, 10^{-6} and 10^{-5} M] were tested through leaf spraying. TRIA at 10^{-6} M significantly increased yield attributes. As compared to the control (10^{-0} M), leaf-applied TRIA at 10^{-6} M improved the production (yield) of anti-cancerous alkaloids vinblastine (+71.6%) and vincristine (+73.1%) and caused the highest content and yield of vindoline.

Keywords: *Catharanthus roseus*, Triacontanol (TRIA), Vinblastine, Vincristine, Vindoline Alkaloids

1. Introduction

Catharanthus roseus (L.) G. Don (commonly known in India as 'Sadabahar' or 'Periwinkle', family Apocynaceae) is a medicinal plant that produces chemotherapeutically important alkaloids, *viz*. vinblastine and vincristine. These alkaloids are mainly present in leaves of *C. roseus* and are able to inhibit the growth of cancer cells, hindering the formation of mitotic apparatus during cell division[1]. Further, vinblastine has helped increase the chance of surviving childhood leukaemia, while vincristine is used to treat Hodgkins' disease[1]. However, the content of these useful indole alkaloids is very low *viz*. 1 g t^{-1} and 20 mg t^{-1}, respectively in *C. roseus* leaves. Efforts have been made to produce these alkaloids at large scale by cultures of plant cell suspensions and diverse tissues, such as hairy roots[2]. However, it was found that *C. roseus* cell cultures lacked the part of biosynthetic pathway of vindoline and, hence, cells failed to synthesize these valuable and complex compounds for commercial use[3]. Tryptophan de-Carboxylase (TDC) is the key enzyme, involved in the early stages of indole alkaloid biosynthesis[4], catalyzing the formation of tryptamine from tryptophan and, therefore, playing an important role in terpenoid indole alkaloids biosynthesis.

Triacontanol (TRIA), a long chain primary alcohol ($C_{30}H_{61}OH$), has been recognized as an important plant growth regulator for a number of crops[5,6,7]. TRIA

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has improved growth, yield, quality and physiological processes of several medicinal plants viz. *Artemisia annua*, *Coriandrum sativum, Cymbopogon flexuosus, Lavandula dentate, Mentha arvensis, Papaver somniferum,* etc. by various workers[8-11].

Due to the paramount medicinal importance of *C. roseus*, we hypothesized that foliar application of TRIA would enhance foliage yield and the overall production of anticancer (vinblastine and vincristine) alkaloids in *C. roseus* plants.

2. Materials and Methods

2.1 Plant Materials and Growth Conditions

A pot culture experiment was conducted in naturally illuminated environmental conditions of the net house at the Botany Department, Aligarh Muslim University, Aligarh, India. Healthy *C. roseus* seedlings of equal size were obtained from Woodbine Nursery, Civil Lines, Aligarh, India. The seedlings were then transplanted to earthen pots. Prior to transplantation, each pot (25 cm diameter × 25 cm height) was filled with 5 kg of homogenous mixture of soil and farmyard manure (4:1). Soil and chemical characteristics were: texture sandy loam, pH (1:2) 7.2, E.C. (1:2) 0.46 $dS\ m^{-1}$, available N, P and K 95.0, 9.2 and 144.5 mg kg^{-1} of soil, respectively. The experiment was factorial design and arranged in randomized blocks. Each treatment was replicated five times and each replicate had three plants. Thus, each treatment consisted of 15 pots, and each pot contained a single healthy plant. The pots were sufficiently watered as required and plants did not face water deficit.

2.2 Treatments

Foliar spray of different concentrations of TRIA was done at 15 days interval when plants were at stage of 2-3 true leaves. In total, five sprays of TRIA were done using a hand sprayer. The control plants were sprayed with double distilled water only. The applied treatments were: Control (deionised water, 10^{-0}), 10^{-7}, 10^{-6} and 10^{-5} M TRIA. Different aqueous concentrations of TRIA were finally prepared using double distilled water for the spray treatments on plants. Plants were sampled for yield and quality parameters carried out at 120 and 150 DAP. At sampling (120 and 150 DAP), five plants of each treatment were harvested and their roots were washed carefully with tap water to remove all adhering foreign particles. Water adhering to the roots was removed with blotting paper. Then, the plant fresh and dry weights were measured using an electronic balance.

2.3 Yield Attributes

Leaf-yield was recorded by weighing all plant leaves with an electronic balance, whereas herbage-yield was measured weighing the total plant biomass with exception of roots.

2.4 Total Alkaloid Content in Leaves and Roots

Total alkaloid content was estimated in leaves and roots. The detailed methodology for the estimation of total alkaloids content has already been described in our previous studies[12,13]. The leaves and roots were dried in a hot air oven at 80°C for twenty-four hours. Five hundred mg of the powdered sample was taken in a100 mL round bottom reflux flask. To it, a known volume of ethyl alcohol was added. Then, the mixture was refluxed for 6 hours. Thereafter, it was filtered, followed by adding 50 mL of dilute HCl, and then the filtrate was transferred to a separating funnel, to which 50 mL of diethyl ether was added. To the final decant, anhydrous sodium carbonate was added. Then, the mixture was decanted in a preweighed dry porcelain dish, followed by evaporating the content till dryness; lastly, the dried content was weighed. Total alkaloid content in leaves/roots was calculated as per the formula described in the previous studies[12].

2.5 Content of Vincristine, Vinblastine and Vindoline Alkaloids

Preparation of sample extraction and the chromatographic conditions were based on the method [14]. Freshly harvested leaves were oven dried at 80°C for 24 h and then grinded to fine powder. A volume (30 mL) of 90% ethanol was added to 5 g of leaf powder; it was left over night and then filtered. The residue was again extracted with 90% ethanol (3 × 30 mL) at room temperature (27°C), and 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 was cooled at 10°C. Afterwards, 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), pre-saturated with chloroform. Then, it 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 total alkaloid content. An aliquot (10 mg) of the crude alkaloid was dissolved in 1 mL of methanol, and 10 µL of it was subjected to HPLC analysis.

Chromatographic analysis was carried out using HPLC (model LC-10A, Shimadzu, Tokyo, Japan). Solvents were filtered by using a Millipore system and analysis was performed on a Waters µ Bondapak C_{18} reversed-phase column, 10 mm (30 cm x 3.9 mm I.D.). A constant flow rate of 0.6 mL min^{-1} was used during analysis. The composition of mobile phase was optimized by using acetonitrile, 0.1 M phosphate buffer and glacial acetic acid (38:62:0.3); pH 4.14, column temperature of 26°C, detector (UV-Vis detector) at 254 nm. For standard, stock solutions of vinblastine, vincristine and vindoline were prepared dissolving 1 mg of each in 1 mL of methanol. The solutions were analyzed and the retention time (Rt) for vinblastine (Rt-5 min), vincristine (Rt-4 min) and vindoline (Rt-10 min) were checked.

2.6 Statistical Analysis

The data were analyzed statistically using SPSS-17 statistical software (SPSS Inc., Chicago, IL, USA) according to simple randomized design. Means were compared using Duncan's Multiple Range Test (DMRT) at $p<0.05$.

3. Results

Compared to the other foliar concentrations, TRIA applied at 10^{-6} M concentrations proved to be the best. TRIA, applied at 10^{-6} M, improved yield and quality attributes of the crop significantly at both the growth stages. However, TRIA applied at 10^{-5} M did not further improve all the attributes but it significantly enhanced the above studied attributes in comparison to control. Except on the content of vinblastine and vincristine, the effect of TRIA treatments, growth stages and of TRIA × Stage

Table 1. Effect of four foliar concentrations of TRIA on leaf and herbage yields, content and yield of leaf and root alkaloids per plant of *C. roseus* L. at 120 and 150 DAP. Means within a column followed by the same letter(s) are not significantly different ($p\leq0.05$)

DAP	Treatments (M)	Leaf yield per plant (g)	Herbage yield per plant (g)	Leaf alkaloids content (%)	Leaf alkaloids yield (µg)	Root alkaloids content (%)	Root alkaloids yield (µg)
120	TRIA 10^{-0}(M)	53.75	62.35	0.740	85.0	1.56	46.0
	TRIA 10^{-7}(M)	59.10	68.63	0.770	90.0	1.62	57.6
	TRIA 10^{-6}(M)	67.72	80.74	0.821	106.1	1.72	72.2
	TRIA 10^{-5}(M)	64.70	75.40	0.810	104.1	1.69	70.0
150	TRIA 10^{-0}(M)	56.36	66.74	0.770	88.0	1.60	67.0
	TRIA 10^{-7}(M)	62.50	76.96	0.820	96.0	1.68	79.4
	TRIA 10^{-6}(M)	72.70	89.76	0.870	112.7	1.80	108.8
	TRIA 10^{-5}(M)	68.45	84.64	0.850	110.0	1.77	106.5
Means of Treatments	TRIA 10^{-0}(M)	55.05^{d}	64.54^{d}	0.755^{c}	86.5^{c}	1.58^{c}	56.5^{d}
	TRIA 10^{-7}(M)	60.80^{c}	72.79^{c}	0.795^{b}	93.0^{b}	1.65^{b}	68.5^{c}
	TRIA 10^{-6}(M)	70.21^{a}	85.25^{a}	0.845^{a}	109.4^{a}	1.76^{a}	90.5^{a}
	TRIA 10^{-5}(M)	66.57^{b}	80.02^{b}	0.830^{ab}	107.0^{ab}	1.73^{ab}	88.2^{b}
Means of Stages	120 DAP	61.32^{b}	71.78^{b}	0.785^{b}	96.3^{b}	1.65^{b}	61.4^{b}
	150 DAP	65.00^{a}	79.52^{a}	0.827^{a}	101.7^{a}	1.71^{a}	90.4^{a}
LSD at 5%	T	2.40	3.24	0.02	2.8	0.03	2.1
	S	2.48	3.12	0.02	2.9	0.03	1.6
	T × S	4.88	6.36	0.04	5.7	0.06	3.7

T: Treatments; S: Stages

Table 2. Effect of four foliar concentrations of TRIA on content and yield of vinblastine, vincristine and vindoline of *C. roseus* L. studied at 120 and 150 DAP. Means within a column followed by the same letter(s) are not significantly different ($p \leq 0.05$)

DAP	Treatments (M)	Vinblastine content (%)	Vinblastine yield (µg)	Vincristine content (%)	Vincristine yield (µg)	Vindoline content (%)	Vindoline yield (µg)
120	TRIA 10^{-0}(M)	0.016	14.0	0.0032	3.70	0.049	38.0
	TRIA 10^{-7}(M)	0.017	18.0	0.0032	5.33	0.054	60.0
	TRIA 10^{-6}(M)	0.017	23.9	0.0032	6.20	0.060	71.0
	TRIA 10^{-5}(M)	0.017	23.0	0.0032	6.00	0.056	69.0
150	TRIA 10^{-0}(M)	0.017	17.0	0.0037	4.10	0.067	72.0
	TRIA 10^{-7}(M)	0.018	24.0	0.0036	5.90	0.074	105.0
	TRIA 10^{-6}(M)	0.018	29.3	0.0037	7.30	0.080	137.0
	TRIA 10^{-5}(M)	0.017	28.6	0.0036	7.10	0.077	135.0
Means of Treatments	TRIA 10^{-0}(M)	0.016	15.5^{d}	0.0034	3.90^{c}	0.058^{c}	55.0^{c}
	TRIA 10^{-7}(M)	0.017	21.0^{c}	0.0034	5.61^{b}	0.064^{b}	82.5^{b}
	TRIA 10^{-6}(M)	0.017	26.6^{a}	0.0035	6.75^{a}	0.070^{a}	104.0^{a}
	TRIA 10^{-5}(M)	0.017	25.8^{b}	0.0034	6.55^{ab}	0.066^{ab}	102.0^{ab}
Means of Stages	120 DAP	0.016	19.7^{b}	0.0032	5.31^{b}	0.055^{b}	59.5^{b}
	150 DAP	0.017	24.7^{a}	0.0036	6.10^{a}	0.074^{a}	112.2^{a}
LSD at 5%	T	NS	0.30	NS	0.28	0.002	2.50
	S	NS	0.40	NS	0.51	0.002	2.60
	T × S	NS	0.70	NS	0.80	0.004	5.10

T: Treatments; S: Stages

interaction (T × S) was significant on all the parameters studied, (Tables 1-2).

3.1 Yield and Quality Attributes

TRIA, applied at 10^{-6} M concentration, excelled the control in leaf yield by 27.5% and in herbage yield by 32.1% (Table 1). Application of TRIA increased the content and yield of alkaloids when compared to the control. The spray of TRIA at the concentration of 10^{-6} M resulted in the maximum increase in the content and yield of leaf alkaloids. It exceeded the control by 11.9% in leaf alkaloids content and by 26.4% in leaf alkaloids yield (Table 1). Comparing the control, the foliar spray of 10^{-6} M TRIA also increased the content and yield of root alkaloids. It increased the root alkaloids content by 11.4% and the root alkaloids yield by 60.2% (Table 1). Plants of 150 DAP surpassed 120 DAP by 6.0 and 10.8% regarding leaf and herbage yield, respectively, (Table 1). Further, 150 DAP plants produced 5.35, 5.61, 3.64 and 47.2% higher values for content and yield of leaf and root alkaloids, respectively over 120 DAP plants, (Table 1). Noticeably, the effect of TRIA treatments, stages differences, and TRIA-stages interaction was found significant for leaf yield, herbage yield, content and yield of leaf and root alkaloids (Table 1). Interactions T×S (10^{-6} M TRIA-150 DAP), exhibited the highest content (17.6 and 15.4%) and yield (32.6 and 136.5%) of leaf and root alkaloids, respectively over 10^{-0} M TRIA-120 DAP, the poorest interaction (Table 1).

In the present study, there was observed no progressive increase in anticancer constituents (content of vinblastine and vincristine) as compared to the control, when TRIA was applied to the *C. roseus* crop (Table 2). The TRIA spray at 10^{-6} M considerably increased the yields of vinblastine and vincristine. It increased the vinblastine yield by 71.6% and vincristine yield by 73.1%, as compared to the control (Table 2). Besides, the application of TRIA at 10^{-6} M resulted in the maximum content and yield of vindoline. It increased the vindoline content by 20.7% and vindoline yield by 89.1% as compared to the control (Table 2).

Plants of 150 DAP produced higher yields of vinblastine, vincristine and vindoline over 120 DAP, the

former surpassing the latter by 25.4, 14.9% and 19.0%, respectively. TRIA effect, stages differences and TRIA-Stage interaction were found non-significant for content of vincristine and vinblastine (Table 2). However, interaction of 10^{-6} M TRIA-150 DAP for vinblastine and vincristine content was found significant and showed higher values for the above parameters by 109.3 and 97.3%, respectively over 10^{-0} M TRIA-120 DAP, the poorest interaction (Table 2). Treatment effect, stages differences and their interaction was also found significant for content and yield of vindoline alkaloid (Table 2). Interaction of 10^{-6} M TRIA-150 DAP, gave higher values for content (36.7%) and yield (89.5%) of vindoline, exceeding the interaction 10^{-0} M TRIA-120 DAP, which showed lowest value, (Table 2).

4. Discussion

The effect of TRIA has previously been well established in plant productivity[5,6]. TRIA significantly improved the yield attributes over their respective plants (Table 1). A significant increase in the above-mentioned yield parameters of the TRIA treated plants might possibly culminate in maximization of the leaf-yield and herbage yield of the plant in the present study. The positive role of TRIA in increasing growth, yield and quality as well as physiological processes in various medicinal plants including *Artemisia annua* L.[9], *Coriandrum sativum* L.[15,16], *Cymbopogon flexuosus* Steud, Watts.[10], *Mentha arvensis* L.[11,17], *Papaver somniferum* L.[18] and *Withania somnifera* L.[19] has earlier been reported. Study suggested[5] that TRIA, like other plant hormones might activate enzymes or alter a membrane, which could trigger a cascading effect resulting in increased metabolism and the enhanced accumulation of various critical intermediate compounds. The increase in the quality parameters like EO content in lavender, spearmint, Japanese mint, and coriander as a result of application of ethrel, gibberellic acid and TRIA has been reported by various researchers[11,17,20]. Presumably, as a result of application of TRIA, in turn, enhanced the rate of photosynthesis and translocation of photosynthates and other metabolites to the sinks[17,21] leading to the improved yield and its attributes in this study (Tables 1,2). Regarding TRIA, our results are in agreement with those of other researchers who reported enhancement in the seed-yield and yield attributes due to TRIA[8,21,22].

This study reports significant enhancement in the alkaloid production as result of TRIA application (Table 1). A positive role of growth hormones in regulation of indole alkaloids of periwinkle has been reported[23,24]. PGRs have, in fact, been reported to affect the tissue specific secondary metabolism and manipulate the alkaloid accumulation at particular sites[25]. Other PGRs also exerted a significant effect on alkaloid production and similar results were reported[25]. Distribution and accumulation of alkaloids in plant parts may vary in roots, stems, and leaves of *C. roseus*[26]. TRIA enhanced the yield of vincristine alkaloid as well (Table 2). Besides, Salicylic acid (SA) has been reported to increase the production of other alkaloids such as serpentine and tabersonine[27]. Similarly, SA could induce the accumulation of vindoline alkaloid in *Catharanthus* seedlings[27]. MS medium supplied with 2,4-D, KIN, and IAA enhanced the production of vincristine and vinblastine alkaloids during *in vitro* culture[28]. Reported[24] an SA-mediated accumulation of vincristine and vinblastine alkaloids under salinity stress in *C. roseus*. Noted[9] a significant enhancement in the artemisinin content of *Artemisia annua*. Found[8] a significant positive effect of cumulative application of TRIA and GA_3 on the yield of opium (*Papaver somniferum* L.) and its morphine content. Study examined[18] the effect of TRIA on alkaloid-biosynthesis as well as on the relationship between alkaloid production and physiological parameters in opium poppy.

Plants of *C. roseus* at 150 DAP exhibited a better performance than that at 120 DAP in respect to growth attributes, physiological and biochemical parameters, and yield and quality attributes. Variation in two growth stages occurred due to more number of leaves and biomass production as plants continued to grow irrespective to treatments. In the agro-climatic conditions of India, the above variations regarding several physiological and morphological traits in *C. roseus* have also been recorded in our previous study[12,29]. Interaction of 10^{-6} M TRIA-150 DAP, exhibited higher values for almost all parameters studied, exceeding the interaction 10^{-0} M TRIA-120 DAP, which is proved poorest interaction (Table 2). Interactions between them, that impact on crop performance and yield.

5. Conclusions

Triacontanol at 10^{-6} M improved significantly the production (in terms of yield) of vincristine, vinblastine and vindoline of *C. roseus*. Thus, application of TRIA as foliar spray could be used to enhance the anticancer alkaloids production in *C. roseus*.

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