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Application of triacontanol modulates plant growth and physiological activities of *Catharanthus roseus* (L.)

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Abstract
Catharanthus roseus (L.) G. Don is well known medicinally important plant. Alkaloids derived from this plant are used in the treatment of cancer. Triacontanol (TRIA) proved as a potent plant growth regulator in agriculture. We have investigated the role of TRIA in enhancing the growth and physiological attributes of *C. roseus*. Therefore, an experiment was conducted to evaluate effect of TRIA on plant growth and physiological activities at 120 and 150 days after planting in earthen pots. Four concentrations of TRIA viz. 10^{-0} (Control) 10^{-7}, 10^{-6} and 10^{-5} M were applied through leaf spraying. Foliar application of TRIA at a concentration of 10^{-6} M caused a significant increase in plant growth and physiological parameters as compared to the control (10^{-0} M).

Keywords: *Catharanthus roseus*, vinblastine, vincristine, vindoline alkaloids, TRIA

1. Introduction
Sadabahar [*Catharanthus roseus* (L.)] belongs to the family Apocynaceae, is a medicinally important producing anticancer alkaloids and also commonly used in the treatment of childhood leukaemia and Hodgkins' disease [1]. The alkaloid contents are present in very low concentration. Hence the efforts have been made to increase the productivity [2]. Triacontanol (TRIA) has been reported as a pivotal plant growth regulator (PGR) when applied on number of crop plants [3, 4, 5]. It is proved that TRIA can improve growth and physiological processes in medicinal plants [6, 7]. Keeping the marvelous medicinal importance in mind, the present study was conducted to investigate the promotive effects of leaf-applied TRIA on the growth and physiological performance of *C. roseus*.

Materials and methods
Plant materials and growth conditions
In this experiment, plants cultured in pots (25 cm diameter × 25 cm height) under natural environmental conditions. Each pot contained 5 kg of homogenous mixture of soil and farmyard manure (4:1) with following characteristics; 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 arranged in randomized blocks and factorial design. Five replicates for each treatment (Each replicate with three plants), thus each treatment was consist of a total of 15 pots each containing a single healthy plant. The pots were sufficiently watered regularly to avoid water deficit.

Triacontanol Treatments
Treatments of TRIA were applied through foliar application at 15 days interval when plants were having 2-3 true leaves. A total of five sprays of TRIA for each treatment (10^{-0}, 10^{-7}, 10^{-6} and 10^{-5} M) were given to the plants using a hand sprayer. The control plants were sprayed with double distilled water (10^{-0}) only. Plants were analyzed for growth and physiological parameters at 120 and 150 DAP (days after plantation).

Growth attributes
The growth attributes viz. number of leaves per plant, average leaf-area and fresh and dry weights of plants were determined at 120 and 150 days after planting (DAP). Five plants from each treatment were uprooted carefully followed by recording the number of leaves and fresh weight per plant. Plants were washed and then dried in an oven at 80 °C for 24 h prior measuring the plant dry weight. Only 10% of the randomly selected leaves of each sample (consisting of five plants) were used to determine the leaf area using graph paper sheet [8]. The average area per leaf was multiplied by the total number of leaves to estimate the total leaf area per plant.

Net photosynthetic rate and stomatal conductance
Plants were sampled for all physiological parameters at 120 and 150 DAP. Net photosynthetic rate and stomatal conductance were evaluated in intact leaves, randomly selected among youngest fully expanded leaves.

Measurements were taken on sunny days around 1100 h, using an infra red gas analyzer (Li-Cor 6400 Portable Photosynthesis System, Lincoln, NE, USA).

Total contents of chlorophyll and carotenoids

Total content of chlorophyll and carotenoids in leaves was estimated according to Lichtenthaler and Buschmann[9]. Fresh tissue from the interveinal leaf area was grinded with 100% acetone using mortar-pestle. The optical density (OD) of the pigment solution was recorded at 662, 645 and 470 nm to determine chlorophyll *a*, chlorophyll *b* and total carotenoids content, respectively, using a spectrophotometer (model UV-1700, Shimadzu, Tokyo, Japan). Total chlorophyll content was assessed by the sum of chlorophyll *a* and *b* contents. The content of each photosynthetic pigment was expressed as mg g^{-1} leaf FW.

Activity of nitrate reductase (NR)

Activity of nitrate reductase (E.C. 1.6.6.1) was estimated in youngest fully developed leaves by using the method developed by Jaworski [10]. A sample with 200 mg of fresh chopped leaves, was transferred to plastic vials, containing 2.5 mL phosphate buffer (pH 7.5), 0.5 mL potassium nitrate solution and 2.5 mL of 5% isopropanol. The vials, with the reaction mixture were incubated for two hours at 30 ˚C. After incubation, 1% sulphanilamide and 0.02% N-(1-naphthyl) ethylenediamine dihydrochloride (NED-HCL) were added. The test tubes were kept for 20 minutes at room temperature for maximum color development. The OD of the solution was recorded at 540 nm. Activity of NR was expressed as n mol g^{-1} FW h^{-1}.

Activity of carbonic anhydrase (CA)

The activity of carbonic anhydrase (E.C. 4.2.1.1) was measured in fresh leaves selected randomly, using the method described by Dwivedi and Randhawa [11]. Two hundred mg of chopped leaf-pieces were transferred to Petri plates. The leaf pieces were dipped in 10 mL of 0.2 M cystein hydrochloride solution for 20 minutes at 4 °C. The solution adhering to leaf pieces was removed with the help of a blotting paper, followed by immediate transferring to a test tube containing 4 mL of phosphate buffer (pH 6.8). Four 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 enzyme activity was expressed as mol CO_2 kg^{-1} leaf FW s^{-1}.

Activity of tryptophan decarboxylase (TDC)

The activity of tryptophan decarboxylase (E.C. 4.1.1.28) was carried out according to the method of Islas *et al*. [12]. Frozen material (1g) were pulverized in a cold mortar to fine powder and homogenized with 1.25 mL of 0.1 M HEPES (pH 7.5), containing 3 mm, dithiotreitol, 5 mm EDTA and 200 mg of polyvinylpolypyrrolidone. The extract was filtered through four layers of cheesecloth and then it was centrifuged at 18000×g for 30 min. The resulting supernatant was used as source of enzyme. The protein content of the enzyme extract was determined as described by Peterson [13], using albumin as standard. The activity of tryptophan decarboxylase (TDC) enzyme was expressed as nmol min^{-1} mg^{-1} protein.

Statistical analysis

According to simple randomized design, the data obtained from research were analyzed statistically using SPSS-17 statistical software (SPSS Inc., Chicago, IL, USA). Duncan's Multiple Range Test (DMRT) at $p<0.05$ was used to compare the means.

Results

Growth attributes

The foliar application of TRIA significantly increased the growth of *C. roseus* at both the growth stages. There was a progressive increase in values of growth parameters with increasing TRIA concentration up to 10^{-6} M. At TRIA 10^{-5} M, the values declined significantly but gave higher value compared to the control (Table 1). The maximum enhancement of growth attributes was attained at 150 DAP with 10^{-6} M TRIA. Application of TRIA at 10^{-6} M increased the number of leaves by 20.0%, leaf-area by 10.8%, plant fresh weight by 39.9% and plant dry weight by 43.0%, in comparison to the control. Plants, analyzed at 150 DAP, exhibited more growth than that at 120 DAP. As for stage, 150 DAP proved superior over 120 DAP for all growth attributes,. Plants of 150 DAP exhibited 21.3, 14.9, 12.8 and 13.9% higher values for number of leaves, average leaf area, plant fresh and dry weights, respectively over 120 DAP. Effect of TRIA × Stage interaction (10^{-6} M TRIA-150 DAP) was also significant and gave higher values regarding number of leaves per plant, average leaf area, fresh weight per plant and dry weight compared to 10^{-0} M TRIA-120 DAP, the poorest interaction (Table 1).

Table 1: Effect of four foliar concentrations of triacontanol (TRIA) on number of leaves and average leaf area, fresh and dry weights per plant of *Catharanthus roseus* L. 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)	Number of leaves per plant	Average leaf area (cm^2)	Fresh weight per plant (g)	Dry weight per plant (g)
	TRIA10^0(M)	205.0	10.60	66.70	14.65
120	TRIA 10^7(M)	224.0	11.23	75.23	16.80
	TRIA 10^6(M)	238.0	11.67	91.38	20.54
	TRIA 10^5(M)	230.0	11.40	84.25	18.90
150	TRIA 10^0(M)	245.0	12.15	71.83	16.25
	TRIA 10^7(M)	262.6	12.74	86.82	18.92
	TRIA 10^6(M)	302.0	13.53	102.4	23.66
	TRIA 10^5(M)	278.0	13.20	97.10	21.90
Means of Treatments	TRIA 10^0(M)	225.0^d	11.37^d	69.26^d	15.45^d
	TRIA 10^7(M)	243.3^c	11.98^c	81.02^c	17.86^c
	TRIA 10^6(M)	270.0 ^{a}	12.60 ^{a}	96.89 ^{a}	22.10 ^{a}
	TRIA 10^{-5}(M)	254.0 ^{b}	12.30^b	90.67^b	20.40^b

Means of Stages	120 DAP	224.2[b]	11.23[b]	79.39[b]	17.72[b]
	150 DAP	271.9[a]	12.91[a]	89.54[a]	20.18[a]
LSD at 5%	T	4.1	0.15	4.32	1.20
	S	5.5	0.17	5.05	1.17
	T × S	9.6	0.32	9.36	2.30

T: Treatments; S: Stages

Physiological and biochemical attributes

As compared to the control, TRIA significantly enhanced net photosynthetic rate (P_N), stomatal conductance (Gs), chlorophyll and carotenoid contents and the highest values of all variables were found in plants treated with TRIA at 10^{-6} M (Table 2). The data revealed that the effect of (i) treatments as well as that of (ii) T×S interaction was significant for the above parameters studied. Plants sampled at 150 DAP proved better than 120 DAP; it improved the value with regard to P_N (31.3%) and Gs (23.3%) owing to application of TRIA (Table 2). As per interaction effects, 10^{-6} M TRIA-150 DAP, exhibited the highest P_N (61.5%) and Gs (42.7%), over the poorest interaction, 10^{-0} M TRIA-120 DAP.

TRIA significantly increased the photosynthetic parameters in the treated plants (Table 2). Of the four TRIA concentrations, 10^{-6} M resulted in the greatest increase in the photosynthetic parameters. As compared to the control, application of TRIA at 10^{-6} M enhanced the total chlorophyll content by 21.8% exceeding the control. As compared to control, TRIA increased total carotenoids content by 7.89%. Plants of 150 DAP surpassed 120 DAP by 18.3 and 4.12% regarding total chlorophyll and carotenoids content, respectively. Interaction 10^{-6} M TRIA-150 DAP registered the best results over the 10^{-0} M TRIA-120 DAP, which gave the lowest values of content of total chlorophyll and carotenoids. Application of TRIA at 10^{-6} M proved the best and considerably enhanced the activities NR, CA and TDC over their respective control (Table 2). The interaction of 10^{-6} M TRIA-150 DAP also produced more activities of NR, CA and TDC than the 10^{-0} M TRIA-120 DAP, which showed the lowest value (Table 2).

Table 2: Effect of four foliar concentrations of triacontanol (TRIA) on total chlorophyll and total carotenoids contents, activities of nitrate reductase, carbonic anhydrase and tryptophan decarboxylase, net photosynthetic rate and stomatal conductance of *Catharanthus roseus* L. 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)	Total chlorophyll content (mg g^{-1} FW)	Total carotenoids content (mg g^{-1} FW)	Nitrate reductase activity [nmol NO_2^- g^{-1} (FW) h^{-1}]	Carbonic anhydrase activity [mol (CO_2) kg^{-1} (FW) s^{-1}]	Tryptophan decarboxylase activity (nmol min^{-1} mg^{-1} protein)	Net photosynthetic rate [μ mol (CO_2) m^{-2} s^{-1}]	Stomatal conductance [mmol (CO_2) m^{-2} s^{-1}]
120	TRIA 10^{-0}(M)	1.030	0.187	218.6	5.32	20.5	12.45	0.510
	TRIA 10^{-7}(M)	1.120	0.194	230.2	5.63	22.0	14.00	0.540
	TRIA 10^{-6}(M)	1.246	0.200	251.4	6.06	24.2	15.01	0.581
	TRIA 10^{-5}(M)	1.186	0.196	238.0	5.90	22.6	14.90	0.565
150	TRIA 10^{-0}(M)	1.220	0.194	223.4	5.40	22.0	16.26	0.620
	TRIA 10^{-7}(M)	1.316	0.199	245.5	5.80	23.6	17.65	0.650
	TRIA 10^{-6}(M)	1.494	0.210	262.7	6.31	26.9	20.11	0.728
	TRIA 10^{-5}(M)	1.390	0.204	254.0	5.96	24.7	19.98	0.712
Means of Treatments	TRIA 10^{-0}(M)	1.125[d]	0.190[d]	221.0[d]	5.36[d]	21.3[d]	14.35[c]	0.565[c]
	TRIA 10^{-7}(M)	1.218[c]	0.196[c]	237.8[c]	5.72[c]	22.8[c]	15.82[b]	0.595[b]
	TRIA 10^{-6}(M)	1.370[a]	0.205[a]	257.1[a]	6.18[a]	25.5[a]	17.56[a]	0.654[a]
	TRIA 10^{-5}(M	1.288[b]	0.200[b]	246.0[b]	5.93[b]	23.6[b]	17.44[ab]	0.638[ab]
Means of Stages	120 DAP	1.145[b]	0.194[b]	234.5[b]	5.73[a]	22.3[b]	14.09[b]	0.549[b]
	150 DAP	1.355[a]	0.202[a]	246.4[a]	5.87[a]	24.3[a]	18.50[a]	0.677[a]
LSD at 5%	T	0.031	0.002	7.40	0.18	0.21	0.53	0.024
	S	0.034	0.003	7.24	0.22	0.20	0.80	0.035
	T × S	0.065	0.005	14.6	0.40	0.41	1.33	0.059

T: Treatments; S: Stages

Discussion

TRIA enhanced plant height and dry matter accumulation in various plants due its growth-promoting effect. Improvement in number of leaves and leaf area index might contribute to the enhanced values of dry weight of TRIA treated plants in the present study (Table 1). The PGR-treated plants possessed comparatively a greater leaf-area index, which could presumably be due to stimulation of cell division and cell enlargement as stated regarding the positive effect of TRIA on plants [14, 15]. The growth promoting effect of TRIA has previously been well established to increase productivity of medicinal plants [3, 4]. In fact, TRIA has proved to be an excellent promoter of growth and productivity of crops as explored by several workers [14, 15, 16, 17, 18]. Previous studies have shown that TRIA, applied either to the root medium or to the leaves as a foliar spray, enhanced the growth as well as yield of vegetable and cereal crops [19, 20]. Foliage of the plants sprayed with the PGRs employed elevated the content of both chlorophyll and carotenoids in comparison to control plants (Table 1).

In addition, the enhancement in the chlorophyll content might have resulted in increased photosynthetic rate. Earlier studies have revealed an increase in the rate of both CO_2 fixation and photosynthesis in different plants as a result of TRIA application [5]. Further, in this study, increased photosynthesis was parallel with the elevated levels of leaf chlorophyll and carotenoids contents (Table 2). Activity of

CA was positively affected by the TRIA. The activity of the enzyme increased to the maximum extent at 120 DAPS. Present study revealed that the leaves treated with TRIA improved the CA activity considerably at both sampling stages. The enhancement of CA activity due to TRIA application might also be ascribed to the *de novo* synthesis of CA, which might involve the genes associated with its transcription and translation in the cell [21]. TRIA-treated leaves of *C. roseus* also possessed a greater activity of NR as compared to control plants. It is reported that the level of NR is highly affected by the environmental factors such as light and temperature and PGRs [22, 23]. Application of TRIA also enhanced the TDC activity positively at both stages (Table 2). It is documented that TDC enzyme played a role in enhancing the production of terpenoid indole alkaloids (TIA) biosynthesis. Naeem *et al*. [24, 25] reported the positive effect of other PGRs on TDC activity in *C. roseus*. The positive role of TRIA in increasing growth, yield and quality as well as physiological processes in various medicinal plants including *Artemisia annua* L. [26], *Coriandrum sativum* L. [27], *Cymbopogon flexuosus* Steud, Watts. [28], *Mentha arvensis* L. (Srivastava and Sharma 1991; Naeem *et al*. 2010), *Papaver somniferum* L. [29] and *Withania somnifera* L. [30] has earlier been reported.

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. Ries and Houtz [3] suggested 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 essential oil (EO) content in lavender, spearmint, Japanese mint, and coriander as a result of application of epibrassinolide (EBL), ethrel, gibberellic acid and TRIA has been reported by various researchers [29, 31, 32, 33]. 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 [15, 17] leading to the improved yield and its attributes in this study. 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 [34, 35, 6, 14, 16, 17]. *C. roseus* exhibited a better performance at 150 DAP as compared to 120 DAP. Variation in two growth stages recorded due to more number of leaves and biomass production at 150 DAP.

Conclusions

Application of TRIA applied as spray at a concentration of 10^{-6} M is highly recommended, as it causes a significant improvement in the overall growth and physiological attributes of *C. roseus.*

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