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BRIEF COMMUNICATION

Influence of gibberellic acid on $^{14}CO_2$ metabolism, growth, and production of alkaloids in *Catharanthus roseus*

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Abstract

Changes in growth parameters, carbon assimilation efficiency, and utilization of $^{14}CO_2$ assimilate into alkaloids in plant parts were investigated at whole plant level by treatment of *Catharanthus roseus* with gibberellic acid (GA). Application of GA (1 000 g m^{-3}) resulted in changes in leaf morphology, increase in stem elongation, leaf and internode length, plant height, and decrease in biomass content. Phenotypic changes were accompanied by decrease in contents of chlorophylls and in photosynthetic capacity. GA application resulted in higher % of total alkaloids accumulated in leaf, stem, and root. GA treatment produced negative phenotypic response in total biomass production but positive response in content of total alkaloids in leaf, stem, and roots. ^{14}C assimilate partitioning revealed that ^{14}C distribution in leaf, stem, and root of treated plants was higher than in untreated and variations were observed in contents of metabolites as sugars, amino acids, and organic acids. Capacity to utilize current fixed ^{14}C derived assimilates for alkaloid production was high in leaves but low in roots of treated plants despite higher content of ^{14}C metabolites such as sugars, amino acids, and organic acids. In spite of higher availability of metabolites, their utilization into alkaloid production is low in GA-treated roots.

Additional key words: amino acids; chlorophyll; $^{14}CO_2$ photoassimilate partitioning; leaf; organic acids; root; stem; sugars.

Catharanthus roseus (L.) G. Don (Apocynaceae) derives its economic importance from highly valued leaf anti-cancer alkaloids vincristine and vinblastine and anti-hypertensive root alkaloid ajmalicine. Several endogenous and exogenous factors regulate alkaloid production. Among these, growth hormones are important endogenous regulators (Verpoorte *et al.* 1997).

While investigating the effect of growth hormones as elicitors of alkaloid production in *C. roseus* plants we observed continual effect of gibberellic acid (GA) on plant phenotype. Earlier studies have reported that GA application (at 50, 100, and 500 g m^{-3}) as foliar spray on transplanted cuttings increased plant height (Sadowska *et al.* 1984). Continuous availability (for one month) of GA at 5.8 and 11.6 μM resulted in elongation of shoots, lowering number of leaves with little effects on alkaloid content on seedlings grown on MS solid medium (El-Sayed and Verpoorte 2004). GA up to 1.2 μM induced changes in glutathione metabolism, which was associated with anthocyanin content in *C. roseus* cell cultures (Ohlsson and Berglund 2001). However, it is not clear what changes occur in the carbon metabolism of GA treated *C. roseus* plants and the subsequent distribution and utilization of assimilates for alkaloid production.

In the present study, we report the influence of GA on photosynthetic efficiency and alkaloid accumulation in *C. roseus*. C assimilation capacity was investigated by studying the incorporation of $^{14}CO_2$ and its partitioning into primary photosynthetic metabolites, viz. sugars, amino acids, and organic acids, into leaf, stem, and root and the subsequent utilization of current assimilates into production of total alkaloids. At the same time changes in plant growth parameters, CO_2 exchange rate, chlorophyll (Chl) content, and alkaloid content were also determined.

Plants raised from seeds (cv. Dhawal) obtained from the farm nursery of the Institute were maintained in soil

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pots in a glasshouse at ambient temperature of 30–35 °C and irradiance of 800–1 000 μmol m^{-2} s^{-1}, the latter measured by *Li-COR Li-188B* light meter (*Li-COR*, Lincoln, USA.). Plants in which flowering had just initiated (4-month-old) were foliar sprayed with GA (*Glaxo Fine Chemicals*, India) at concentration of 1 000 g m^{-3} (corresponding to 2.88 mM) by a hand held sprayer. *Tween 20* (0.1 %) was added as surfactant. Care was taken to wet both surfaces of the leaf to ensure maximum application. Equal numbers of plants were sprayed with *Tween 20* only. Observations were done three weeks after spray.

Plant height, leaf length of the 3rd leaf that is physiologically mature (Srivastava *et al.* 2004), and internode length (between second and third leaf) were measured. Carefully uprooted plants were separated into leaves, stem, and roots, and their fresh matter (FM) and dry matter (DM) after drying to constant value were recorded.

Chl amount was measured spectrophotometrically in 80 % acetone extract on a *Spectronic 21D* spectrophotometer (*Milton Roy & Co.*, New York, USA) and calculated according to Arnon (1949). Net photosynthetic rate (P_N) of 3rd leaf was measured in an open system by a computerized portable photosynthesis system *CI-310 PPS* (*CID Instruments*, USA).

Freshly harvested leaf, stem, and root samples of treated and untreated plants were oven dried at 60 °C for 48 h and powdered. A known amount of each plant material was extracted in 90 % ethanol (3 times), filtered, and concentrated to dryness. Dried residue was re-dissolved in ethanol, diluted with equal volume of water, and acidified with 3 % hydrochloric acid. The mixture was extracted with hexane (3 times), hexane fraction discarded, and aqueous extract cooled to 10 °C and basified with 3 % ammonium hydroxide to pH 8.5. This portion was further extracted with chloroform (3 times). The combined chloroform extract was washed with distilled water, evaporated to dryness, and weighed (Uniyal *et al*. 2001).

Exposure of plants to $^{14}CO_2$ was carried out 5 h after the beginning of the light period. Pots with GA treated and untreated plants were placed in an assimilation chamber around a central vial containing $Na_2{}^{14}CO_3$ solution (activity 1.85 MBq, specific activity 1 628 GBq mol^{-1}) obtained from the isotope division of Bhabha Atomic Research Centre, Mumbai, India. $^{14}CO_2$ was liberated by injecting 2 M H_2SO_4 into the carbonate solution through a PVC inlet tube and uniformly distributed within the chamber using a small electric fan. Plants were initially exposed to $^{14}CO_2$ for 1 h in natural sunlight of 800–1 000 μmol m^{-2} s^{-1} during the time of exposure. After 1 h, saturated solution of KOH run into the central vial to absorb remaining $^{14}CO_2$. The plants were removed from the chamber and allowed to assimilate $^{14}CO_2$ for next 6 h (Srivastava *et al.* 2004). After this exposure, plants were carefully uprooted from soil and separated into leaf, stem, and root. Each of the plant part was processed for determining the allocation of label into major primary photosynthetic metabolic fractions such as ethanol soluble (ES), ethanol insoluble (EIS), and chloroform soluble (CS). Simultaneously the biosynthetic capacity to utilize currently assimilated metabolites in total alkaloids was determined by quantifying the label into alkaloids in leaf, stem, and roots. The separated plant parts were divided in two portions: In a known mass of leaf, stem, and root incorporation of current assimilate in total alkaloids was determined using a liquid scintillation counter and PPO-POPOP-toluene cocktail (*Wallac 1409*, USA). Another portion of plant parts was fixed immediately in boiling ethanol to maintain metabolic status. The fixed material was ground in ethanol, filtered, filtrates were evaporated and diluted in a known volume of distilled water, this aqueous phase being termed as ethanol soluble fraction (ES). The unfiltered ground leaf tissue further hydrolyzed by enzyme diastase in 0.05 M acetate buffer (pH 5.2) at 50 ºC was termed as ethanol insoluble fraction (EIS). The aqueous phase was further extracted with equal volume of chloroform and this chloroform soluble fraction (CS) contained pigments and some of the terpenoid pathway derived metabolites (Srivastava *et al.* 2004). The ^{14}C label in fractions was determined as described above. The ES fraction was further separated into metabolites by passing through *Amberlite* ion exchange column chromatography into neutral (sugars), acidic (organic acids), and basic (amino acids) fractions. The ^{14}C content in eluates after column chromatography was measured in a liquid scintillation counter (Srivastava and Luthra 1994, Dixit and Srivastava 2000).

GA treatment produced marked phenotypic changes such as pale yellow leaves showing serrated white mid rib, long internodes, and elongated plants as compared to untreated plants (Fig. 1). A significant increase in plant height and increase in leaf length were observed (Table 1). Accompanying these morphological changes, leaf FM and DM declined significantly. Reduction in root

Fig. 1. Effect of GA application on visual morphological changes in leaf and internodes in flowering plants of *Catharanthus roseus*. *Left:* untreated plants, *right*: GA-treated plants.

Table 1. Changes in growth parameters, pigment content, net photosynthetic rate (P_N), plant biomass, distribution of total alkaloid in plant parts, metabolic profile of assimilated $^{14}CO_2$, and distribution of ^{14}C in primary metabolites of GA treated and untreated *Catharanthus* plants. Chl = chlorophyll; DM = dry matter, FM = fresh matter; P_N = net photosynthetic rate.

Material	Parameter	Untreated	GA-treated	CD 5 %	CD 1 %
	Plant height [cm]	42.33	69.66	15.45	25.56
	Length of 3rd leaf [cm]	3.60	5.60	1.49	2.46
	Length of 2nd internode of 3rd leaf [cm]	2.20	7.20	1.79	2.97
	Leaf FM [g plant^{-1}]	45.00	24.30	10.31	17.07
	Leaf DM [g plant^{-1}]	8.53	3.20	1.72	2.85
	Stem FM [g plant^{-1}]	11.66	12.33	4.14	6.85
	Stem DM [g plant^{-1}]	2.10	1.70	0.60	0.99
	Root FM [g plant^{-1}]	11.66	10.53	2.58	4.27
	Root DM [g plant^{-1}]	2.23	1.63	0.49	0.82
	Total plant FM [g plant^{-1}]	68.33	47.22	16.41	27.16
	Total plant DM [g plant^{-1}]	12.86	6.13	2.57	4.25
	Chl *a*+*b* [g kg^{-1}(FM)]	1.91	0.53	0.13	0.21
	Chl *a* [g kg^{-1}(FM)]	1.39	0.39	0.11	0.19
	Chl *b* [g kg^{-1}(FM)]	0.50	0.14	0.02	0.04
	P_N [μmol m^{-2} s^{-1}]	12.20	4.70	1.78	2.94
	Alkaloids in leaf [% DM]	0.73	1.13	0.11	0.18
	Alkaloids in stem [% DM]	0.25	0.34	0.03	0.06
	Alkaloids in roots [% DM]	0.68	1.96	0.19	0.32
	Total alkaloids [% DM]	1.67	3.44	0.29	0.49
Leaves	Ethanol soluble (ES) [kBq kg^{-1}(FM)]	101.60	46.00	10.80	17.88
	Ethanol insoluble (EIS) [kBq kg^{-1}(FM)]	613.60	804.60	257.69	426.40
	Chloroform soluble (CS) [kBq kg^{-1}(FM)]	25.30	17.00	5.15	8.53
	Total ^{14}C in fractions [kBq kg^{-1}(FM)]	740.60	867.60	257.54	426.16
	Sugar+sugar phosphates [kBq kg^{-1}(FM)]	3.60	3.10	1.40	2.30
	Amino acids [kBq kg^{-1}(FM)]	1.40	1.90	3.40	5.70
	Organic acids [kBq kg^{-1}(FM)]	2.70	2.20	1.00	1.70
	^{14}C in alkaloids [kBq kg^{-1}(FM)]	0.68	2.66	0.73	1.22
Stems	Ethanol soluble (ES) [kBq kg^{-1}(FM)]	84.00	27.30	7.58	12.55
	Ethanol insoluble (EIS) [kBq kg^{-1}(FM)]	555.60	1600.30	245.03	405.45
	Chloroform soluble (CS) [kBq kg^{-1}(FM)]	12.30	4.00	0.92	1.53
	Total ^{14}C in fractions [kBq kg^{-1}(FM)]	652.00	1631.60	243.80	403.41
	Sugar+sugar phosphates [kBq kg^{-1}(FM)]	3.50	2.70	0.77	1.28
	Amino acids [kBq kg^{-1}(FM)]	3.20	2.30	0.70	1.16
	Organic acids [kBq kg^{-1}(FM)]	3.50	2.90	0.56	1.16
	^{14}C in alkaloids [kBq kg^{-1}(FM)]	3.17	6.24	2.17	3.60
Roots	Ethanol soluble (ES) [kBq kg^{-1}(FM)]	2592.30	4311.00	170.67	282.41
	Ethanol insoluble (EIS) [kBq kg^{-1}(FM)]	1965.60	2381.00	575.69	952.59
	Chloroform soluble (CS) [kBq kg^{-1}(FM)]	78.60	197.30	10.60	17.54
	Total 14C in fractions [kBq kg^{-1}(FM)]	4636.60	6888.30	705.79	1167.85
	Sugar+sugar phosphates [kBq kg^{-1}(FM)]	3.00	3.80	1.30	2.15
	Amino acids [kBq kg^{-1}(FM)]	3.60	6.80	1.13	2.05
	Organic acids [kBq kg^{-1}(FM)]	1.60	7.70	0.89	1.47
	^{14}C in alkaloids [kBq kg^{-1}(FM)]	6.18	4.70	4.76	7.87

FM and its DM along with decrease in stem DM resulted in significant decrease in overall total plant DM (Table 1). Similar pattern of increase in shoot length and root growth have been reported in seedlings at GA applications of 5.8 and 11.6 μM after 4 weeks of treatment (El-Sayed and Verpoorte 2004). At a dose of 500 g m^{-3}, reductions in yield of shoot cutting of *C. roseus* have also been reported (Sadowska *et al*. 1984).

GA treatment significantly reduced total Chl and Chl *a* and *b* contents and P_N (Table 1). Thus, there was a concomitant decrease in Chl content and photosynthetic capacity. Total alkaloid contents in leaves, stems, and roots of GA-treated plants were significantly higher than in untreated plant parts (Table 1).

The major metabolic processes influenced in the hormone treated plants are the C-assimilation, distribution of metabolites into plant organs, and subsequent utilization by biosynthetic pathways (Verpoorte *et al*. 1997). ^{14}C

contents in ES and CS fractions from GA-treated leaves were significantly lower and in EIS higher than in untreated leaves. The decline in ^{14}C content in CS fraction further supports the decline in Chl content induced by GA. Since GA is biosynthesized from terpenoid pathway and localized in plastids, GA might exert feedback inhibition on terpenoid pathway (Lack and Evans 2001). Total ^{14}C content in ES+EIS+CS fractions in GA-treated leaves was higher than in untreated leaves. ^{14}C content in sugars and organic acids of treated leaves was lower but it was higher in amino acids as compared to untreated leaves. However, utilization of current assimilates for alkaloid production was significantly higher by leaves of treated plants than in the untreated ones (Table 1).

Since GA stimulates stem elongation, the content of assimilates should vary. Analysis of ^{14}C assimilates into metabolic fractions revealed that ^{14}C contents in ES and CS fractions were significantly lower whereas in EIS fraction significantly higher in treated than in untreated stems. As a result total ^{14}C content (sum of ES+EIS+CS) was higher in treated stems than in untreated ones. ^{14}C content in sugars, amino acids, and organic acids was again lower in treated variants than in the untreated one. Stems incorporate more assimilates into alkaloids in treated variant than in the untreated one (Table 1).

Source-sink relationship is significantly influenced by hormones including GA (Marschner 1986a,b), hence distribution of ^{14}C assimilates to roots was analyzed. ^{14}C contents in ES and CS fractions in treated roots were significantly higher than in untreated ones. Except in sugars, ^{14}C contents in amino acids and organic acids in treated roots were significantly higher than in untreated roots. Despite the higher contents in metabolites, capacity to utilize them for alkaloid production was low.

The higher accumulation of ^{14}C in metabolic fractions in treated plants does not represent higher C-fixation but is probably due to lack of its utilization in growth and developmental process. The biomass accumulation depends on photosynthetic efficiency that is reduced by GA treatment. The biomass production and alkaloid accumulation are entirely separate metabolic processes and hence ^{14}C distribution and biomass production are not interrelated. Distribution and accumulation of alkaloid content in plant parts in *Catharanthus* vary in roots, stems, and leaves (Misra and Kumar 2000). We observed similar results for distribution, irrespective of treatment. There is an imperative association between inter-organ assimilate transport, particularly shoot to root partitioning of metabolites, and biomass production (Walch-Liu *et al.* 2005). How distribution of these metabolites influences alkaloid production by providing organ specific precursor remains to be elucidated (Verpoorte *et al.* 1997, 1999). A number of sugars, amino acids, and organic acids may act as signals for alkaloid production and possibly biotransformations (Aerts *et al.* 1996).

GA treatment on whole plant basis thus resulted in negative morphological response in biomass production but positive response on alkaloid content. Effect is also on distribution of ^{14}C assimilates between plant parts and shoot-root transport and utilization for alkaloid production. GA enhances metabolic activity within pathways leading to accumulation of secondary metabolites, *e.g.* steroids (Ohlsson and Bjork 1988), anthocyanin (Weiss *et al.* 1992), and essential (terpenoid) oil production (Singh *et al.* 1999). Ontogenic studies in *Catharanthus* have revealed that young rather than old leaves efficiently utilize $^{14}CO_2$ assimilates for alkaloid production (Srivastava *et al.* 2004). Dose-dependent (11.6 μM GA) change in alkaloid content was reported in seedling stage (El-Sayeed and Verpoorte 2004) without influence on enzymes such as peroxidases. Which of the sugars, amino acids, or organic acids are preferentially utilized for alkaloid production cannot be specified and what is the change in the spectrum of alkaloids cannot be clearly defined. The present study indicates that GA treatment on whole plant produced negative phenotypic response in total biomass production with positive response in the content of total alkaloids in all plant parts. But on the whole, the total production of alkaloids was decreased.

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