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BRIEF COMMUNICATION

Utilization of photosynthetically fixed $^{14}CO_2$ into alkaloids in relation to primary metabolites in developing leaves of *Catharanthus roseus*

N.K. SRIVASTAVA*, A. MISRA, A.K. SRIVASTAVA, and S. SHARMA

Central Institute of Medicinal and Aromatic Plants, P.O. CIMAP, Kukrail Picnic Spot Road, Lucknow-226 015, India

Abstract

Partitioning of current photosynthates towards primary metabolites and its simultaneous incorporation in leaf alkaloids was investigated in developing leaves of medicinally important *Catharanthus roseus.* Of the total $^{14}CO_2$ assimilated, the leaves at positions 1–6 fixed 8, 22, 25, 19, 13, and 8 %, respectively, and stem 3 %. Leaf fresh mass, chlorophyll content, and CO_2 exchange rate increased up to the third leaf. The total alkaloid content was highest in young actively growing leaves, which declined with age. Total ^{14}C fixed and its content in ethanol soluble fraction increased up to the third leaf and then declined. The ^{14}C content in primary metabolites such as sugars and organic acids was also highest in the 3rd leaf. The utilization of ^{14}C assimilates into alkaloids was maximum in youngest leaf which declined with leaf age. Hence the capacity to synthesize alkaloids was highest in young growing leaves and metabolites from photosynthetic pathway were most efficiently utilized and incorporated into alkaloid biosynthetic pathway by young growing leaves.

Additional key words: amino acids; chlorophyll; leaf area and dry mass; leaf ontogeny; net photosynthetic rate; organic acids; sugars; terpenoids; total alkaloids.

Catharanthus roseus (Apocynaceae) is an important source of alkaloids used as pharmaceutical agents. The alkaloid accumulation occurs in different plant parts and some are organ specific (viz. leaves, stem, roots, and flowers). Histochemical analysis using alkaloid specific reagents showed the presence of alkaloids in lactifers and specialized parenchyma cells. Studies using cell suspension culture, seedlings, and whole plants have revealed that alkaloid accumulation is dependent on several intrinsic and extrinsic factors (Verpoorte *et al*. 1997, Misra and Kumar 2000). These factors include irradiation ("white light" and UV-radiation) (Hirata *et al*. 1993), application of plant growth regulators (Smith *et al*. 1987), effect of wounding (Frischknecht *et al*. 1987, Naaranlahti *et al*. 1991), nutrient application (Maheshwari *et al*. 1991), water stress (Saenz *et al*. 1993), and biotic and abiotic elicitors such as jasmonic acid, salicylic acid, and Ca/nutrients (Verpoorte *et al*. 1997 and references therein). Total alkaloid content is also dependent on leaf age, where with increasing leaf age (from younger to older leaves) contents of catharanthine and vindoline decrease (Westekemper *et al*. 1980, Dues-Neumann *et al*. 1987).

The relation between leaf carbon assimilation capacity, content of primary metabolites, and their utilization into alkaloid accumulation have not been investigated yet. This is particularly important because various steps of alkaloid biosynthesis occur in different cell compartments/plant parts/organs and the availability of precursors through primary photosynthetic metabolites will significantly influence alkaloid accumulation. Through the process of C-assimilation, basic metabolites are made available which *via* different pathways act as precursors for different steps of biosynthesis and subsequent accumulation. We studied the ontogenic changes in distribution of photosynthetically fixed $^{14}CO_2$ into total alkaloids in relation to primary metabolites in developing *Catharanthus* leaves.

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*Fax: 91-0522-2342666, e-mail: nk.srivastava@cimap.res.in, neelsrivastava2003@yahoo.co.uk
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Plants raised from seedlings (cv. Dhawal having white flowers) were maintained in ceramic pots filled with soil in a glasshouse at ambient temperature (30–35 °C) and under irradiance of 800–1 000 μmol m^{-2} s^{-1} (sunlight). Four-months-old plants were used for study. Shoots with six leaf pairs were selected. The leaf pairs were numbered from top to the base of shoot, and the upper most leaf pair represented the youngest leaf. The area of the leaf pair was measured by an automatic area meter *Li-3000* (*LiCoR*, USA). Leaf fresh mass was determined immediately and dry mass was determined after oven drying at 80 °C until a constant mass.

A known mass of leaf tissue was ground with pestle and mortar, encased in ice in "dim light" till a fine paste was obtained, and extracted with 80 % acetone. Chlorophyll (Chl) absorbance was recorded on a *Spectronic 21D* spectrophotometer (*Milton Roy and Co*., New York, USA) and Chl concentration calculated according to Arnon (1949). Net photosynthetic rate (P_N) was measured in a closed system *LI-6000* (*LiCOR*, USA; cf. Srivastava and Luthra 1991a). Total alkaloids from the leaves were determined as described by Goswami *et al*. (1996).

For $^{14}CO_2$ incorporation studies 12 shoots having 6 leaf pairs each were cut under water and placed in vials with cut ends dipped in half strength Hoagland solution. The vials were then kept in a sealed plexiglas chamber around a central vial containing $Na^{14}CO_3$ solution (1.85 MBq, 1.78 TBq mol^{-1}) obtained from the isotope division of Bhabha Atomic Research Centre, Trombay, India. $^{14}CO_2$ was generated by injecting 2 M H_2SO_4 into the sodium carbonate solution through a PVC tube and uniformly distributed using a small electric fan. The twigs were exposed to $^{14}CO_2$ for 1 h. Afterwards saturated solution of KOH was run into the central vial and left for 15 min to absorb excess $^{14}CO_2$. The chamber was then opened and twigs assimilated labelled CO_2 for 6 h. Natural sunlight was between 800–1 000 μmol m^{-2} s^{-1} during the time of exposure. After the 6 h of exposure, label was determined in alkaloids and in primary metabolic fractions in leaves 1–6, stem top (between leaves 1–3), and stem base (between leaves 4–6). A known mass of separated leaves was processed for determination of ^{14}C incorporation into alkaloids whereas remaining parts were immediately fixed by immersing in boiling ethanol. The radioactivity in alkaloid fraction in leaves was determined in a scintillation counter (*Wallac 1409*, USA) using Bray's scintillation fluid (Dixit and Srivastava 2000a,b). Known mass of leaves and stem was extracted in boiling 80 % ethanol. The ethanol-soluble material was separated into neutral (sugar + sugar phosphate), acidic (organic acids), and basic (amino acids) fractions by *Amberlite* ion-exchange column (Srivastava and Luthra 1994). The total terpenoid fraction was extracted in chloroform and the label in this fraction was determined in PPO-POPOP-toluene cocktail. Ethanol-insoluble material was further hydrolyzed by enzyme diastase in 0.05 M acetate buffer (pH 5.2) at 50 °C (Dixit and Srivastava 2000a). Total ^{14}C accumulated was calculated as the sum of radioactivity incorporated in ethanol-soluble (including that in terpenoids) and -insoluble fraction and expressed per fresh mass. The radioactivity in eluates after ion-exchange separation was measured using Bray's scintillation fluid in liquid scintillation counter (*Wallac 1409*, USA) (Srivastava and Luthra 1994). The leaf position from apex to the base of the shoot represented a gradient in the tissue age and change in the physiological capacity of the leaf (Table 1). The leaf area increased from 1st to 5th leaf and its fresh mass up to third leaf whereas its dry mass increased up to 2nd leaf after which it remained steady till leaf 5. Chl content and P_N increased to 3rd leaf after which decline was observed. Thus based on dry mass (with similar values in 2nd and 3rd leaves) and Chl content, P_N, and fresh mass the physiological capacity of catharanthus leaves increased till 3rd leaf suggesting that the 3rd leaf was physiologically most active. However in contrast, the total alkaloid content of the first leaf was maximum which declined with increasing leaf age (Table 1).

In many plants, where secondary metabolites are of economic value and pharmaceutical use, similar results have been reported. In turmeric (*Curcuma longa* L.) leaf area, its fresh and dry masses, and P_N increase up to third leaf (Dixit and Srivastava 2000a), in *Mentha piperita* the 3rd leaf has maximum P_N, dry mass, and Chl *a/b* ratio (Srivastava and Luthra 1991a). Inter-specific variations in mint species for photosynthetic efficiency in relation to essential oil capacity have also been reported (Srivastava and Luthra 1991b). The content of terpenoid accumulation in leaves is dependent on developmental stage in cymbopogon (Luthra *et al*. 1991). In developing mint leaves the incorporation of $^{14}CO_2$ into metabolites such as sugars was highest followed by organic acids, amino acids, and essential oil at all stages of leaf development. The incorporation into sugars and amino acids declines as the leaf matures whereas incorporation in essential oil and organic acids increases with leaf expansion and then decreases (Srivastava and Luthra 1991a). Time dependent changes in incorporation pattern into primary and secondary metabolites were also reported in mint (Srivastava and Luthra 1991b). In turmeric youngest developing leaves assimilated maximum $^{14}CO_2$ into metabolites and essential oil. Of the total carbon assimilated, leaves 1–4 fixed 31, 23, 21, and 9 %, roots 4 %, rhizome 6 %, oil 0.01 %, and curcumin 4.6 % of rhizome fresh mass (Dixit and Srivastava 2000a).

The C-assimilation capacity of catharanthus leaves and the simultaneous incorporation of current photosynthate into alkaloids were studied by $^{14}CO_2$ feeding. The investigation of current photosynthate was reflection of efficiency of utilization of photosynthetic metabolites into biosynthetic pathways. The maximum $^{14}CO_2$ fixation increased from leaf one and was highest in leaf three and thereafter the fixation declined in leaf 4 to 6. When total ^{14}C incorporation was analyzed into major fraction, the

Table 1. Changes in leaf growth parameters, chlorophyll (Chl) content, net photosynthetic rate (P_N), total content of alkaloids, and partitioning of $^{14}CO_2$ photoassimilates in primary metabolic fractions and into alkaloids [kBq kg^{-1}(FM)] in leaves at different position of catharanthus.

	Leaf position from top						C.D. at 5 or 1 %	
	1	2	3	4	5	6		
Leaf area [cm^2 of leaf pair]	13.19	23.87	25.13	25.05	28.58	24.31	0.56	0.79
Leaf fresh mass [g of leaf pair]	0.42	0.55	0.60	0.56	0.57	0.51	0.04	0.07
Leaf dry mass [g of leaf pair]	0.07	0.09	0.08	0.08	0.07	0.06	0.01	0.01
Chl (*a*+*b*) [g kg^{-1}(FM)]	0.83	0.96	1.09	0.78	0.58	0.42	0.07	0.11
P_N [μg(CO_2) m^{-2} s^{-1}]	33.1	53.9	69.4	63.1	40.3	38.1	4.4	6.1
Total alkaloids [% DM]	0.89	0.69	0.50	0.57	0.57	0.25	0.12	0.16
Terpenoids	737	544	507	229	31	17	22	31
Ethanol-soluble	13498	36298	41849	32557	21786	13848	43	61
Ethanol-insoluble	268	939	843	542	204	199	9	12
Total	14504	37781	43200	33328	22021	14065	59	83
Sugars	39	84	154	50	33	31	6	9
Amino acids	23	33	31	38	25	24	4	6
Organic acids	28	22	56	15	24	28	2	3
Total alkaloids	2553	1876	1560	823	567	567	30	42

label in ethanol soluble fraction increased from leaf 1 to 3, and then declined to leaf 6. This trend was similar to total ^{14}C fixation. However, the ^{14}C content in the ethanol insoluble fraction increased from leaf 1 to leaf 2 and then declined to leaf 6. The ethanol soluble fraction was further analyzed into sugars, amino acids, and organic acids. The current ^{14}C contents in sugars and organic acids increased from leaf 1 to leaf 3 and in amino acids from leaf 1 to leaf 4 and then declined. At the same time, total ^{14}C partitioning into terpenoid fraction, through which alkaloid intermediates such as geraniol and secologanin are derived, was maximum in the 1st leaf and then declined. Incidentally the utilization of current photosynthate in total leaf alkaloid fraction was also highest in the 1st leaf and showed downward trend starting with leaf 2 (Table 1).

In order to check the transport of currently assimilated photosynthates through phloem tissue, the amounts were analyzed in top and basal regions of stem. The top portion had higher contents in ethanol-soluble and -insoluble fraction, total alkaloids, and sugars whereas the basal stem portion had higher contents of terpenoids, amino acids, and organic acids (Table 2).

Partitioning of leaf-assimilated $^{14}CO_2$ into roots and rhizome is also time dependent. In turmeric, the roots as well as rhizome receive maximum photosynthates from leaves after 24 h of feeding, with higher contents of sugars and organic acids than amino acids (Dixit and

Table 2. Partitioning of leaf assimilated $^{14}CO_2$ into primary metabolic fractions [kBq kg^{-1}(FM)] in stem top (between leaves 1–3 from apex) and stem base (between leaves 4–6) of catharanthus plant. T – terpenoids, ES – ethanol-soluble, EIS – ethanol-insoluble, Total – sum of T+ES+EIS, S – sugars, AA – amino acids, OA – organic acids.

Stem portion	Metabolic fractions						
	T	ES	EIS	Total	S	AA	OA
Top	67	5119	92	5279	44	22	12
Base	70	447	71	589	19	33	63
T-values	1.7	856.1	11.3	757.4	30.6	5.5	37.7

Srivastava 2000b). In addition there is substrate specificity in utilization of precursors towards secondary metabolic pathways as shown by feeding of labelled metabolites such as ^{14}C-acetate, ^{14}C-saccharose, and $^{14}CO_2$ which are differentially utilized and incorporated into terpenoid metabolism end products in cymbopogon (Luthra *et al*. 1993, Srivastava *et al*. 1998).

A positive correlation was found between leaf dry mass and Chl content (r = 0.829), ^{14}C-content in ethanol soluble (r = 0.868) and -insoluble fraction (r = 0.938). ^{14}C-content was positively correlated with ^{14}C-content in total alkaloids (r = 0. 980).

The youngest growing catharanthus leaves showed maximum utilization and incorporation of current photosynthate into alkaloid fraction. This was accompanied by concomitant pattern of increase in terpenoid fraction. However, which of the primary metabolites amongst sugars, amino acids, and/or organic acids are preferentially utilized for alkaloid biosynthesis cannot be specified. Similar to the trend of current photosynthate

utilization, the youngest leaf had maximum alkaloid content which declined with leaf position and age. There are earlier reports that in *C. roseus* plants the content of monomers catharanthine and vindoline in young leaves is high (Westkemper *et al*. 1980).

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