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BRIEF COMMUNICATION

Utilization of exogenously supplied ^{14}C-saccharose into primary metabolites and alkaloid production in *Catharanthus roseus*

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Abstract

Partitioning of exogenously supplied U-^{14}C-saccharose into primary metabolic pool as sugars, amino acids, and organic acids was analyzed and simultaneous utilization for production of alkaloid by leaf, stem, and root in twigs and rooted plants of *Catharanthus roseus* grown in hydroponic culture medium was determined. Twigs revealed comparable distribution of total ^{14}C label in leaf and stem. Stems contained significantly higher ^{14}C label in sugar fraction and in alkaloids [47 kBq kg^{-1}(DM)] than leaf. In rooted plants, label in ^{14}C in metabolic fractions in root such as ethanol-soluble, ethanol-insoluble, and chloroform-soluble fractions and in components such as sugars, amino acids, and organic acids were significantly higher than in stems and leaves. This was related with significantly higher content of ^{14}C in alkaloids in stems and leaves. ^{14}C contents in sugars, amino acids, and organic acids increased from leaf to stem and roots. Roots are the major accumulators of metabolites accompanied by higher biosynthetic utilization for alkaloid accumulation.

Additional key words: amino acids; assimilate partitioning; leaf; organic acids; roots; stem; sugars.

Catharanthus roseus (L.) G. Don is economically important due to its highly valued anti-cancer leaf alkaloids vincristine and vinblastine and antihypertensive root alkaloids ajmalicine and serpentine. Because of medicinal value, high price, and low alkaloid content, the plant is extensively studied (Verpoorte *et al.* 1999, Van der Heijden *et al.* 2004). Since alkaloid production in *C. roseus* is under strict developmental and environmental control and transport of intermediates is compartmentalized, understanding the relationship between availability of primary metabolites and alkaloid accumulation is important (Verpoorte *et al.* 1997). Feeding of labelled sucrose, glucose, and fructose to hairy root cultures reveals preferential utilization of sucrose for biomass production and alkaloid content (Jung *et al.* 1992, Bhadra and Shanks 1997). There is no information, however, on metabolic utilization and distribution of sucrose and production of alkaloid at whole plant level.

Recent evidence indicates that triose phosphate pathway (DOXP pathway, operative in chloroplast) is also the major contributor of carbon for monoterpenoid stream of alkaloid production pathway (Contin *et al.* 1998). Saccharose is one of the primary precursors of mevalonate pathway. This sugar is also central primary metabolite easily transportable through various plant organs, where it is utilized according to metabolic requirements (Lewis *et al.* 2000). The objective of the present study was the utilization of saccharose-derived metabolites and utilization for alkaloid production at whole plant level in *C. roseus*. This was investigated by feeding of U-^{14}C-saccharose and analyzing its partitioning into major primary metabolic fractions and distribution into sugars, amino acids, and organic acids and the simultaneous utilization of current assimilates for total alkaloid production. At the same time, the transport of saccharose and distribution in leaf, stem, and root, and analysis of assimilates were performed in cut twigs and in rooted plants grown to flowering stage in hydroponics culture medium.

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Seeds of *C. roseus* (L.) G. Don (cv. Dhawal) obtained from the gene bank of CIMAP were initially raised in 10 000 cm^3 ceramic pots filled with acid-washed clean silica sand (Agarwala and Sharma 1961). After 4 weeks, seedlings were transplanted to 2 500 cm^3 amber coloured glass containers filled with nutrient solution. Nutrient solution of Hoagland and Arnon (1938) was used except Fe, which was supplied as Fe-EDTA. These pots were maintained at ambient temperature (30–35 °C) and irradiance (800–1 000 μmol m^{-2} s^{-1}) measured by a light meter (model *LI-188B*, *LiCOR*, USA). The nutrient medium was changed weekly and routine practice for maintaining plants in hydroponics culture was used.

Tracer studies were performed on 4-month-old plants. Feeding of U-^{14}C-saccharose was performed in the morning, about 5 h after the beginning of the light period, on cut twigs and rooted plants. Each twig (having six leaf pairs) was cut under water and placed in a glass vial containing 54 kBq of U-^{14}C-saccharose in 5 cm^3 of nutrient solution. The U-^{14}C-saccharose was obtained from isotope division of Bhabha Atomic Research Center, Mumbai, India (activity 3.7 MBq; specific activity 11.1 GBq $mmol^{-1}$). Similarly, rooted plants were given the same label as the twigs. Both treated materials were placed in natural sunlight that varied between 800–1 000 μmol m^{-2} s^{-1} during the time of feeding. After the initial uptake of the label, the vials were kept filled with 5 cm^3 of nutrient medium twice to ensure complete uptake of adhered label. After the uptake of label (6 h), twigs and the whole plants were separated into leaf, stem, and roots. In each plant part the distribution of label into major primary metabolic fractions such as ethanol-soluble (ES), ethanol-insoluble (EIS), and chloroform-soluble (CS) was determined and simultaneously the label incorporation in total alkaloids was quantified. The ^{14}C-fed leaf, stem, and root were divided in two portions: (*1*) In known mass of leaf, stem, and root tissues the incorporation of ^{14}C label in total alkaloids was determined by using PPO-POPOP-toluene cocktail in a liquid scintillation counter (*Wallac 1409*, USA). (*2*) Known masses of leaf, stem, and root tissues were immediately fixed into boiling ethanol so that the current metabolic status was maintained. The plant material was ground in ethanol, filtered, the filtrate evaporated and diluted in a known volume of water and this aqueous phase was ES. This ES fraction was further extracted with chloroform and termed as CS. The remaining unfiltered ground plant material was hydrolyzed by enzyme diastase in 0.05 M acetate buffer (pH 5.2) at 50 ºC and this fraction was termed as EIS (Srivastava *et al*. 2004). The content of label in ES and EIS fractions was determined in Bray's scintillation fluid and in CS fraction in PPO-POPOP-toluene cocktail in a liquid scintillation counter. The ES fraction was additionally separated into metabolic components consisting of neutral (sugars), acidic (organic acids), and basic (amino acids) components by *Amberlite* ion exchange column chromatography. The content of ^{14}C in eluates after column chromatography was determined in Bray's scintillation fluid in a liquid scintillation counter (Srivastava and Luthra 1994).

^{14}C fed leaf, stem, and root samples were oven dried at 60 °C for 48 h and powdered. A known mass of each plant material was extracted in 90 % ethanol, left overnight, and filtered 3 times. Dried residue was re-dissolved in ethanol, diluted with equal volume of water, and acidified with 3 % hydrochloric acid. This mixture was extracted with hexane that was then discarded. The aqueous extract was cooled to 10 °C and basified with 3 % ammonium hydroxide to pH 8.5. This portion was extracted with chloroform (3 times). The combined chloroform extract was washed with distilled water and evaporated to dryness. This dried extract represented total alkaloids (Uniyal *et al.* 2001).

Feeding of saccharose was performed at a growth stage when flowering had just initiated. At this stage, maximum demand for assimilates is expected and should be reflected in distribution of metabolites in leaf, stems, and roots. Analysis of ^{14}C into primary metabolic fractions in twigs revealed that label in ES fraction (which represents mobile and active metabolic pool) is marginally higher in leaves than in stems (Table 1). Similarly, label in the CS fraction (which represents pigment and terpenoid pathway derived metabolites) in leaves was higher than in stem (Table 1). However, ^{14}C content in EIS fraction was 2-fold higher in stem than in leaves. Thus the total ^{14}C content in stem was higher than in leaves (Table 1).

The ^{14}C content in sugars and amino acids in the ES fraction was higher in stems than leaves, significantly only in sugars (Table 1). In contrast, the label in organic acids was higher in leaves than in stems, but this difference was not significant. The incorporation of current assimilates for alkaloid production was significantly higher in stems than in leaves (Table 1).

Roots are important metabolic sinks and consume approximately 30 % of leaf photo-assimilates (Marschner 1986). The label in ES, EIS, and CS fractions was higher in roots than in leaves and stems (Table 1). Hence, total ^{14}C content was significantly highest in roots, followed by leaves and stems. Subsequent analysis of ES fraction revealed an increasing trend in labelling sugars, amino acids, and organic acids, with significantly high values in roots (Table 1). Utilization of metabolites for alkaloid production was significantly higher in roots and stems than in leaves.

Earlier studies on feeding different labelled C-sources to hairy root cultures of *Catharanthus* revealed better utilization of saccharose than glucose and fructose resulting for increase in biomass, while fructose enhanced the catharanthine content (Jung *et al.* 1992). In heterotrophic cultures the growth-limiting nutrient was nitrogen when saccharose was freely available (Bhadra and Shanks 1997).

Table 1. Distribution of assimilated U-^{14}C-saccharose into different metabolic fractions in leaves and stem, and in roots in twigs and in intact *Catharanthus* plants and incorporation into total alkaloids. All values [kBq kg^{-1}(FM)] except for alkaloids [kBq kg^{-1}(DM)].

Fraction	Leaf	Stem	CD 5 %	1 %	Leaf	Stem	Root	CD 5 %	1 %
Ethanol-soluble	3264.3	3086.3	1054.1	1744.2	480.6	406.6	2143.3*	150.1	222.2
Ethanol-insoluble	432.3	950.0	746.0	1234.8	105.3	174.3	996.0*	200.6	303.8
Chloroform-soluble	60.4	55.4	17.1	28.3	37.2	34.9	342.6*	78.5	118.9
Sugars	172.0	373.3*	186.6	308.8	38.2	41.5	573.6*	43.1	65.3
Amino acids	22.9	28.6	16.9	28.0	16.0	25.0	87.3*	23.8	36.1
Organic acids	76.6	61.5	87.9	145.3	24.1	32.2	133.3*	38.5	58.4
Total alkaloids	30.8	47.4*	10.0	16.5	19.0	58.4*	157.4*	39.2	59.4

We found that maximum ^{14}C-saccharose derived metabolites distribution and alkaloid production occurs in roots. Shoot to root partitioning of metabolites are important signals of overall growth and biomass production.

Ontogenic studies in *C. roseus* revealed that young leaves utilize $^{14}CO_2$ photo-assimilates more efficiently than old leaves. Phloem transport of assimilates showed that top portion of stem had higher ^{14}C content in ES, EIS, sugar and alkaloid fractions, while basal portion had higher content in CS, amino acid, and organic acid fractions (Srivastava *et al*. 2004). ^{14}C assimilation and transport of metabolites through phloem into rhizome lead to curcumin accumulation in turmeric (Dixit and Srivastava 2000a,b). Feeding of U-^{14}C-acetate, $^{14}CO_2$, and U-^{14}C-saccharose revealed variety in substrate utilization for essential oil (terpenoid) production in *Cymbopogon winterianus* (Srivastava *et al.* 1998).

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