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Full Length Research Paper

Partitioning of exogenously supplied ^{14}C substrates into primary metabolites and accumulation of total triterpenoids saponins in *CENTELLA ASIATICA*

N. K. Srivastava* and A. K. Srivastava

Central Institute of Medicinal and Aromatic Plants (Council of Scientific and Industrial Research, New Delhi), P. O. CIMAP, Kukrail Picnic Spot Road, Lucknow-226015, India.

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Comparative utilization of exogenously supplied carbon substrates as ^{14}C-saccharose and ^{14}C-acetate were analyzed for incorporation and accumulation into total triterpenoids saponins. Simultaneously, the metabolic status of the plant was determined by profiling assimilates into primary metabolic pool as sugars, amino acids and organic acids into shoots and roots of *CENTELLA ASIATICA*. ^{14}C-saccharose was preferentially utilized for accumulation of total triterpenoids into shoots- as determined by high ^{14}C content- compared to when ^{14}C-acetate was supplied. The content of ^{14}C label in metabolites in shoots as sugars, amino acids and organic acids was higher when saccharose was supplied as compared to acetate supply. Shoot to root partitioning of metabolites indicates higher ^{14}C content in sugars and organic acids in roots when ^{14}C-saccharose was supplied. The relative higher incorporation of ^{14}C-saccharose indicates the preferential utilization of metabolites from DOXP/terpenoid pathway that contribute to total triterpenoids accumulation.

Key words: ^{14}C assimilation, primary metabolites, total triterpenoids, *Centella asiatica*.

INTRODUCTION

Centella asiatica (L.) Urban is native to Asia and is commonly found in Sri Lanka, China, Indonesia, Malaysia, Australia, South and Central Africa, America and tropical regions of world. The medicinal value of plant is due to the presence of triterpenoids saponin-asiaticoside- in the leaves. The plant is an important ingredient of Ayurvedic pharmacopoeia. The plant has traditionally been used in treatments of a number of ailments but mainly in anxiety, mental disorders as memory enhancers and in skin diseases. Because of its multipurpose medicinal use, Centella based drugs and cosmetics are gaining popularity and emerging as an important medicinal plant in international market (Mathur et al., 2007). Chemical and pharmacological aspects have been reviewed (Srivastava et al., 1997a). Plant extracts have shown antibacterial (Srivastava et al., 1997b) and antifeedent activity (Srivastava et al., 1997c).

Despite the medicinal and economic importance of the plant, no basic information regarding relationship of primary carbon metabolism and accumulation of total triterpenoids saponin is available. The formation of triterpenoids in *Centella* occurs through the terpenoid biosynthetic pathway. Two routes are possible. One is through the operation of mevalonate pathway occurring in cytoplasm where acetate is the preferred metabolic precursor. The second route occurs through DOXP-pathway localized in chloroplast where sucrose is main metabolic precursor. Both these pathway may jointly contribute to the operation of terpenoid pathway leading to the accumulation of triterpenoids (Lichtenthaler, 2007). However, metabolites from which pathway are preferentially utilized is not clear. Feeding of labeled precursors is an important link between primary metabolism and secondary metabolite accumulation (Dixit and Srivastava 2000; Srivastava et al., 2004). To understand, which pathway contributes more, labeled

*Corresponding author. E-mail: nk.srivastava@cimap.res.in.

^{14}C-acetate and ^{14}C-sucrose were fed to intact plants. After uptake, the label incorporation was determined into total triterpenoids saponins in shoots and into primary metabolites to understand the shoot to root partitioning of metabolites with in the plants.

Seedlings of *C. asiatica* (L.) obtained from the farm nursery of CIMAP were initially raised in 10,000 cm^3 ceramic pots filled with acid washed clean silica sand (Agarwala and Sharma, 1961). After 2 week, seedlings were transplanted in a 1,000 cm^3 plastic container filled with acid washed silica sand. Nutrient solution of Hoagland and Arnon (1938) was used, except Fe, which was supplied as FeEDTA. These pots were maintained at ambient temperature (30 to 35°C) and irradiance (800 to 1000 µ mol m^{-2} s^{-1}) measured by a light meter (model LI-188B, LiCOR Inc., USA). Tracer studies were performed on 3 month-old plants. ^{14}C-Saccharose and ^{14}C-Acetate were obtained from the isotope division of Bhabha Atomic Research Centre, Mumbai, India. Label was given to intact rooted plants. A uniform dose of 0.05 mCi of saccharose and acetate were given by keeping the plant in a glass vial dipped in 10 ml of Hoagland and Arnon's complete nutrient solution and the plants allowed to uptake ^{14}C in sunlight. After uptake of different ^{14}C substrates plants were separated into shoot and root. Each plant part was processed for determining the partitioning of label into major primary photosynthetic metabolic fractions such as ethanol soluble (ES), ethanol insoluble (EIS) and chloroform soluble (CS) and into metabolic pools of sugars, amino acids and organic acids. Simultaneously the biosynthetic capacity to utilize currently assimilated metabolites into total saponins was determined by quantifying the ^{14}C label into total triterpenoids saponins in the shoot. Thus, the separated plant parts shoot and roots were divided in two portions:

1. A known weight of shoot tissue was processed for determining the incorporation of current ^{14}C assimilates in total triterpenoids saponins. Aerial shoots were oven dried at 50°C and powdered. A known weight of powdered material was defatted with n-hexane (3 x 20 ml). The hexane insoluble material was extracted with methanol (3 x 20 ml), filtered, concentrated, and final volume made up to known ml of methanol. This methanol extract was considered as total triterpenoids saponins (Verma et al., 1999). ^{14}C in total triterpenoids saponin (methanol extract) was counted using PPO-POPOP-toluene cocktail in a liquid scintillation counter (Wallac 1409, USA).

2. A known weight of shoot and root tissues were immediately fixed into boiling ethanol to maintain the tissue metabolic status. The fixed material was ground in ethanol, filtered, filtrates evaporated and diluted in a known volume in distill water, this aqueous fraction termed as ES fraction. The unfiltered ground leaf tissue further hydrolyzed by enzyme diastase in 0.05 M acetate buffer (pH 5.2) at 50°C was termed as EIS fraction. The aqueous ES fraction was further extracted with an equal volume of chloroform, this chloroform soluble fraction termed as (CS) fraction, which contained pigments, and terpenoid pathway derived metabolites (Srivastava et al., 2004). The ^{14}C label in ES and in EIS fractions was measured using Bray's scintillation fluid and in CS fraction using PPO-POPOP-toluene cocktail in a liquid scintillation counter (Wallac 1409, USA). The ES fraction was further separated into metabolites by column chromatography by passing through Amberlite ion exchange and separation into fractions consisting of neutral (sugar), acidic (organic acids) and basic (amino acids). The ^{14}C content in eluates after column chromatography was measured in a liquid scintillation counter (Wallac 1409, USA) using Bray's scintillation fluid. (Srivastava et al., 2004; Dixit and Srivastava, 2000; Srivastava and Luthra, 1994). The results presented are the mean values from three separate determinations and were statistically analyzed by the least significant different test.

When ^{14}C acetate was fed the ^{14}C label in total triterpenoids was 1987 Bq/g dry wt shoot whereas when ^{14}C saccharose was fed the ^{14}C counts in total triterpenoids was 3223 Bq/g dry wt shoot (Figure 1). The high ^{14}C content in total triterpenoids from saccharose indicated that compared to acetate, saccharose was preferred substrate for the accumulation by the terpenoid pathway. Thus, there was selectivity in utilization of substrate for total triterpenoids accumulation. The carbon assimilates were analyzed into major metabolic fractions in shoots and it was found that ^{14}C label from ^{14}C-saccharose into ES was 5479 Bq/ g shoot F. Wt., into EIS was 526 Bq/ g shoot F. Wt. and into CS was 272Bq/ g shoot F. Wt. were much higher than when ^{14}C acetate was supplied. The value when 14C-acetate was supplied was for ES 543 Bq/ g shoot F. Wt., for EIS 334 Bq/ g shoot F. Wt. and for CS 201 Bq/ g shoot F. Wt. Thus, the primary metabolic pool of ES, EIS and CS as indicated by ^{14}C label was higher when saccharose was supplied as compared to acetate.

Roots are important metabolic sinks and consume about 30% of leaf photo-assimilates (Marschner, 1986). The label in ES and EIS fractions was higher in roots but lower in CS fraction when ^{14}C-saccharose was supplied. Shoot to root partitioning of ES, EIS and CS fractions indicate that saccharose derived metabolites are more mobile than acetate derived metabolites.

ES fraction of the shoot was further analyzed into metabolic pool of sugar, amino acids and organic acids. The ^{14}C content into sugars, amino acids and organic acids were higher when ^{14}C-sucrose was external source as compared to when acetate was supplied (Table 1). Subsequent analysis of ES fraction of the root revealed higher ^{14}C content into metabolite sugar and organic acid when saccharose was supplied as compared to when acetate was supplied (Table 2).

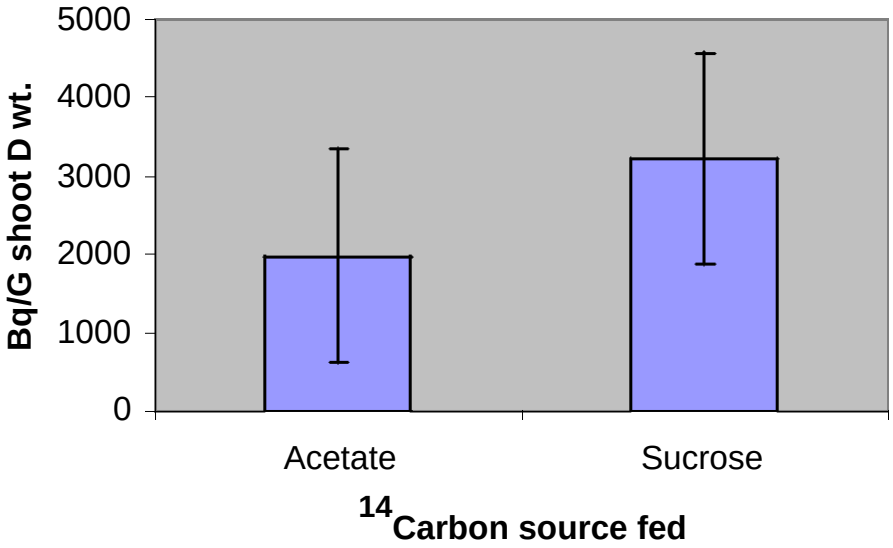

Figure 1. Changes in ^{14}C content in total triterpenoids saponin (methanolic extract) in shoot upon feeding of ^{14}C-Acetate and ^{14}C-Saccharose in *Centella*.

Table 1. Changes in ^{14}C content into primary metabolic pool in shoots of *Centella asiatica*. (All values in Bq/ g shoot F. Wt.).

Fraction	^{14}C-acetate	^{14}C-saccharose	SEM	SED
Sugars	173.0	335.0	54.0	76.0
Amino acids	322.0	429.0	79.0	112.0
Organic acids	512.0	631.0	202.0	286.0

Table 2. Changes in ^{14}C content into primary metabolic pool in roots of *Centella asiatica*. (All values in Bq/ g root F. Wt.).

Fraction	^{14}C-acetate	^{14}C-saccharose	SEM	SED
Sugars	222.0	1360.0	137.0	194.0
Amino acids	396.0	321.0	75.0	106.0
Organic acids	538.0	687.0	46.0	65.0

Interspecific variations have been reported with respect to utilization of exogenously supplied precursors ^{14}C-acetate, ^{14}C-glucose and ^{14}C-saccharose for essential oil synthesis (by terpenoid pathway) in *Cymbopogon* species. Acetate was most efficiently incorporated into essential oil in *Cymbopogon winterianus* and *Cymbopogon flexuosus* and glucose in *Cymbopogon martinii* (Luthra et al., 1993). Thus, formation of end product depends on the availability of the initial substrate to the terpenoid pathway. Not much work has been done on physiological aspects on the whole plant level as far as secondary metabolite accumulation is concerned. Efforts to enhance triterpene accumulation in callus cultures using precursors as farnesyl pyrophosphate, isopentyl pyrophosphate, leucine and squalene have been reported. Squalene treated callus produced highest biomass with higher total triterpenoids production (Mathur et al., 2007).

CONCLUSION

By feeding different ^{14}C substrates and analyzing distribution into primary metabolites into different plant parts and the biosynthetic capacity to accumulate total triterpenoids saponins production was monitored in *Centella*. At whole plant level, the capability to utilize different carbon sources depends on the metabolic status and availability/requirement of metabolic pool that together control biosynthetic potential to accumulate secondary metabolite. The relative higher incorporation of ^{14}C-saccharose indicates the preferential utilization of

metabolites from DOXP/terpenoid pathway that contribute to total triterpenoids accumulation as compared to ^{14}C-acetate indicating lower contribution of mevalonate/ terpenoid pathway derived metabolites.

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