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# Influence of Zn on Allocation of Leaf-assimilated $^{14}CO_2$ into Primary Metabolites in Relation to Production of Essential Oil and Curcumin in Turmeric (*Curcuma Longa* L.)

*N.K. Srivastava, S. Sharma and A. Misra*

Central Institute of Medicinal and Aromatic Plants, P.O. CIMAP, Lucknow-226015, India

**Abstract:** Role of Zn in C-assimilation and biosynthetic utilization of assimilates in production of essential oil and curcumin were studied in Turmeric (*Curcuma longa*). Changes in leaf growth parameters, $^{14}CO_2$ assimilation capacity and the distribution into primary metabolites and biosynthetic utilization into production of secondary metabolites-essential oil (in leaves) and curcumin (in rhizomes) were investigated by growing Turmeric plants in hydroponics solution culture at Zn supply- nil and 0.05 g $m^{-3}$. The total $^{14}C$ was highest in youngest leaves of +Zn plants leaves, which declined with leaf age. Zn starvation resulted in significant decline in assimilation capacity of $^{14}CO_2$ by all leaves from position 1 to 4. A significant decline in distribution in ethanol soluble, ethanol insoluble fraction and in metabolites pool of -sugars, amino acids and organic acids were detected in leaf, roots and rhizomes. Transport of total $^{14}C$ assimilates from leaves to rhizome was higher than that transported to the roots. In roots there was higher $^{14}C$ content in sugars whereas in amino acids and organic acids content was low. Biosynthetic utilization of current $^{14}C$ assimilate was highest by youngest leaf into essential oil and this biosynthetic capacity declined with leaf position. Similar decline in leaf's biosynthetic capacity was observed at -Zn. A significant decrease in transport of assimilates and biosynthetic utilization for production of curcumin (in rhizome) was also detected at -Zn. There were qualitative and quantitative changes in leaf growth parameters such as its area, fresh and dry biomass, chlorophyll (chl) content, $CO_2$ exchange rate, transpiration rate and stomatal conductance at all leaf position at -Zn the maximum effect being on young growing leaves. Zn deficiency significantly decreased leaf C assimilation capacity and distribution into sugars, amino acids and organic acids to roots and rhizome. The biosynthetic capacity to utilize these metabolites for production of oil and curcumin also declined.

**Key words:** Amino acids · leaf position · organic acids · primary and secondary metabolites · turmeric · Zn

## INTRODUCTION

Zinc (Zn) is an essential micronutrient and as component of carbonic anhydrase and other enzymes as dehydrogenases, transphosphorylases, RNA and DNA polymerases significantly influences pathways of carbohydrate and protein metabolism [1, 2]. Many of the intermediary metabolites of carbohydrate pathway provide carbon sources as sugars and derivatives as organic acids utilized as metabolites for essential oil production and amino acids of protein pathway as Phenyl ammonia lyase for cinnamate production and Trytophan utilized for alkaloid production etc. Thus there is a strong association between primary metabolic pathways and production/accumulation of secondary metabolites. Understanding of correlation between carbon assimilation and accumulation of secondary metabolites is an important area of functional plant biology, as many secondary metabolites are of great economic importance and pharmaceutical value [3]. Fundamental processes such as C-assimilation pathway and accumulation of secondary metabolic compounds are closely integrated and regulated by several intrinsic and extrinsic factors [4]. This aspect has been investigated in mints [5, 6], lemongrass [7], rose [8] and citronella [9].

Turmeric (*Curcuma longa* L. Syn. *C. domestica* Valen. Fam. Zingiberaceae) is cultivated for its rhizome, which is widely used as condiment, dyestuff and flavour, pharmaceutical and in cosmetic industry (as well as in religious and auspicious occasions) [10] thus has an

**Corresponding Author:** Dr. N.K. Srivastava, Central Institute of Medicinal and Aromatic Plants, P.O. CIMAP, Lucknow-226015, India (CIMAP Communication No. 2002-41J)

industrially important economic value. This crop is also grown extensively in India and also in Sri Lanka, Bangladesh, Pakistan, Thailand, China and Taiwan. Besides curcumin, dried rhizome powder also provides oleoresins and fatty oils. Recently Curcumin and its components demethoxycurcumin and bis-demethoxycurcumin has been recognized for its anticancer (antiangiogenic agent) activity [11, 12]. Rhizome development and yield in turmeric are reported to be dependent on several factors such as cultivation practices [13], genotype [14] and nutrient availability [15]. Despite the economic and medicinal value of this crop, little information is available on how physiological factors influence/regulate accumulation of oil and curcumin in turmeric. We have previously investigated and reported - availability of micronutrient for optimum yield and influence on production of oil and curcumin such as B [16] and Mn [17] including its effect on C-assimilation. Time dependent partitioning of $^{14}CO_2$ from leaves to rhizome and roots and biosynthetic utilization of current assimilate into oil and Curcumin product accumulation; C-assimilation and distribution of $^{14}CO_2$ assimilated metabolites by developing turmeric leaves [18, 19] and genotypic variability for C-assimilation [20].

Zn availability is now recognized as one of the most widespread micronutrient deficiencies affecting crop production in many countries [21, 22]. Keeping in view the role of Zn in regulating carbohydrate metabolism and simultaneous utilization of intermediary metabolites from these pathways it was considered essential to trace out role of Zn in $^{14}CO_2$ assimilated metabolites partitioning and biosynthetic utilization for production of essential oil by leaf and curcumin production by rhizome. This will establish relation ship between leaf's assimilation capacity and rhizome development that act as sink. The studies and findings highlights only the effect of presence and absence of Zn on interrelationship between C-metabolism and production of oil and curcumin and not on determining the critical level required for growth and development. In the present investigation we report affect of lack of Zn on leaf C-assimilation and partitioning in primary metabolic pool (sugars, organic acids and amino acids), their distribution to rhizomes and roots and the biosynthetic utilization of these metabolites in production of essential oil (in leaf) and curcumin (in rhizome). In addition data are supplemented with changes in leaf growth parameters such as $CO_2$ exchange rate, leaf area and its fresh and dry biomass.

## MATERIAL AND METHODS

**Plant culture conditions:** Uniform mother rhizomes of turmeric (*Curcuma longa* L.) were sprouted in ceramic pots (10,000 $cm^3$ capacity) filled with silica sand previously cleaned by hot acid digestion [23]. The sprouted rhizomes were then transplanted in an amber coloured glass pots (2500 $cm^3$ capacity) containing -Zn Hoagland nutrient solution purified by dithizone procedure to remove impurities of Zn [24]. Hoagland and Arnon's [25] nutrient solution was used except that Fe was supplied as Fe EDTA (5.6 g $m^{-3}$). Zn was supplied as $ZnSO_4$ in treated plants at 0.05 g $m^{-1}$ (+Zn) standardized previously for optimal plant growth whereas it was omitted in deficient plants (-Zn). Pots of treated and deficient plants were maintained in a glasshouse at ambient temperature of 30-35°C and photosynthetic active radiation (PAR) between 800-1000 μmol $m^{-2}$ $s^{-1}$. Routine practices for maintaining plants in hydroponics solution culture were followed. Tracer-feeding studies were performed at the emergence of new rhizome (about 4 month old plants) using $^{14}CO_2$ and growth parameters also measured.

**Leaf growth data:** The leaves were numbered from apex to the base of the shoot, with uppermost leaf representing the youngest leaf. Area of leaves was measured by an automatic area meter (Li-3000, LiCOR, Lincoln, USA). Leaf fresh mass was measured immediately and dry matter was determined by oven drying at 80 °C until a constant mass.

**Chlorophyll and gas exchange parameters:** A known mass of leaf tissue was ground and extracted with 80% acetone. Chl absorbance was recorded on a Spectronic 21D Spectrophotometer (Milton Roy and Co, New York, USA) and calculated according to method of Arnon [26]. The net photosynthetic rate ($P_N$), initial transpiration rate (E) and stomatal conductance (gs) were measured with a computerized Portable Photosynthesis System (Li-6000, LiCOR, Lincoln, USA) as described by Srivastava and Luthra [5].

**Extraction of essential oil:** Essential oil from leaves at different position was isolated by steam distillation technique using a mini Clevenger apparatus [27]. The isolated essential oil was extracted by diethyl ether [18].

**Determination of curcumin content in rhizome:** Curcumin content was assayed by the method of

American Spice Trade Association [28] spectrophotometrically in ethanol extract of fresh rhizome. A standard curve at different concentration was prepared with pure curcumin (Hi-Media, India) and absorbance recorded on a Spectronic 21D Spectrophotometer (430 nm) (Milton Roy and Co. USA) [29] and content estimated as described by Dixit and Srivastava [18].

**Tracer studies:** Tracer studies were performed by feeding of $^{14}CO_2$ to intact -Zn and +Zn plants. Pots with plants maintained in solution culture were placed in a sealed Plexiglas chamber (20000 $cm^3$ capacity) around a central vial containing $Na_2{}^{14}CO_3$ (1.85 MBq, 1.78 TBq $mol^{-1}$) obtained from the isotope division of Bhabha Atomic Research Centre, Trombay, India. $^{14}CO_2$ was freshly generated by injecting 2 M $H_2SO_4$ into the carbonate solution through a PVC tube and uniformly distributed using a small electric fan. The plants were allowed to assimilate $^{14}CO_2$ for 6 h at PAR of 800-1000 μmol $m^{-2}$ $s^{-1}$ standardised as the optimum time for maximal $^{14}CO_2$ incorporation into oil and curcumin [18]. The chamber was then opened for the remaining incorporation period of 24 h.

In order to determine the simultaneous distribution of leaf photo assimilates into primary metabolic pool and the biosynthetic utilization of current assimilates into oil (in leaves) and curcumin (in rhizome) exposed plants were immediately separated into leaves, rhizome and roots. Each separated part was divided into two portions. A known weight of the leaf and rhizome tissue was used to determine $^{14}C$ incorporation into oil and curcumin whereas remaining parts were immediately immersed in boiling ethanol to determine content in primary metabolites. To determine incorporation of $^{14}CO_2$ assimilated photosynthate into essential oil, a known mass of leaves was subjected to steam distillation as explained in Extraction of essential oil. The oil was extracted in ether and the label in ether-extracted aliquots was determined in a scintillation counter (Wallac 1409, USA) using PPO-POPOP-toluene cocktail. For determining utilization of current assimilates into biosynthesis of curcumin, a known mass of rhizome was ground, extracted in ethanol and processed as explained in Determination of Curcumin content in rhizome. The label in curcumin was measured in a liquid scintillation counter using Bray's scintillation fluid.

For determination of $^{14}C$ assimilate distribution into metabolic pool of sugars, amino acids and organic acids a known mass of tracer-fed leaves, rhizome and roots of +Zn and -Zn plants already fixed in boiling 80% ethanol were separated into ethanol soluble (ES) and ethanol insoluble (EIS) fractions. ES fraction was concentrated, depigmented and the resultant aqueous fraction was further separated by amberlite ion-exchange column chromatography into neutral fraction consisting of total sugars, acidic fraction consisting of total organic acids and basic fraction consisting of total amino acids [30]. The remaining EIS fraction was further hydrolysed by enzyme Diastase in 0.05 M acetate buffer (pH 5.2) at 50°C. The counts in ES fraction hydrolysed EIS fraction and in eluates after ion-exchange separation was measured using Bray's scintillation fluid in a liquid scintillation counter (Wallac 1409, USA).

Total $^{14}C$ accumulated was calculated as the sum of label incorporated in ES and EIS fractions and expressed on fresh mass basis (Bq/g.fr.wt.) [30].

**Statistical analysis:** Variations in the treatment were statistically analysed for significance by paired t-test. The results presented are means from three separate extractions.

## RESULTS AND DISCUSSION

Investigations were carried at stage when fresh rhizome started to develop. Growth parameters as leaf area, its fresh and dry biomass irrespective of Zn treatment continue to increase up to 3rd leaf indicating expansion. However in -Zn leaf area at position 2 and 3 were significantly lower with values at 168.62, 181.59 $cm^2$/leaf compared to 219.4 and 244.8 $cm^2$/leaf in +Zn, the fresh biomass at 4.69, 5.55 g/leaf than 6.50 and 7.00 g/leaf in +Zn and leaf dry weight at 0.68, 0.82 g/leaf in -Zn compared to 1.19 and 1.35 g/leaf in +Zn, respectively (Table 1).

There was simultaneous decline in leaf photochemical capacity as indicated by lower values of the chl. Content (a+b) in -Zn that was significantly low at 1st and 4th leaf at 0.64, 1.21 mg/g.fr.wt compared to 0.94, 1.52 mg/g.fr.wt. in +Zn. The leaf gas exchange parameters as $CO_2$ exchange rate (Pn), internal transpiration rate (E) and internal $CO_2$ concentration in -Zn were uniformly significantly lower than +Zn plants (Table 1). Thus there were qualitative and quantitative changes in leaf pigment and gas exchange parameters.

C-assimilation efficiency was determined by feeding of $^{14}CO_2$ as this is the most natural metabolic precursor to understand variability in C-metabolism. Analysis of assimilate into metabolic fractions in individual leaves

Table 1: Influence of Zn on changes in leaf area and biomass, net photosynthetic rate ($P_N$), stomatal conductance (gs) and transpiration rate (E) in developing leaves of turmeric

| | | Leaf position from apex | | | |
|---|---|---|---|---|---|
| Parameters | Zn | 1 | 2 | 3 | 4 |
| Leaf area | +Zn | 155.60 | 219.40 | 244.80 | 171.20 |
| [Cm2/leaf] | -Zn | 137.97$^{NS}$ | 168.62$^{*}$ | 181.59$^{**}$ | 122.40$^{NS}$ |
| Leaf fresh mass | +Zn | 4.80 | 6.50 | 7.00 | 3.20 |
| [g/leaf] | -Zn | 3.98$^{NS}$ | 4.69$^{**}$ | 5.55$^{*}$ | 2.65$^{NS}$ |
| Leaf dry weight | +Zn | 0.72 | 1.19 | 1.35 | 0.59 |
| [g/leaf] | -Zn | 0.56$^{NS}$ | 0.68$^{**}$ | 0.82$^{**}$ | 0.50$^{NS}$ |
| Chl (a+b) | +Zn | 0.94 | 1.02 | 1.28 | 1.52 |
| [mg $g^{-1}$FW] | -Zn | 0.64* | 0.97$^{NS}$ | 1.13$^{NS}$ | 1.21$^{*}$ |
| $P_N$ | +Zn | 3.53 | 4.10 | 4.77 | 4.08 |
| [µmol$CO_2$$m^{-2}$$s^{-1}$] | -Zn | 1.58$^{**}$ | 1.91$^{**}$ | 2.08$^{**}$ | 1.85$^{**}$ |
| E | +Zn | 0.27 | 0.29 | 0.31 | 0.28 |
| [mol $m^{-2}$$s^{-1}$] | -Zn | 0.16$^{**}$ | 0.17$^{**}$ | 0.18$^{**}$ | 0.17$^{**}$ |
| gs | +Zn | 0.31 | 0.36 | 0.40 | 0.38 |
| [mol $m^{-2}$$s^{-1}$] | -Zn | 0.18$^{**}$ | 0.19$^{**}$ | 0.20$^{**}$ | 0.19$^{**}$ |

*/** - Mean values significant at 5/1 % level of significance by paired t-test

Table 2: Effect of Zn on Incorporation pattern and distribution of leaf assimilated $^{14}CO_2$ into primary metabolic fraction in developing leaves, rhizomes and roots of turmeric

| | | Leaf position from apex | | | | | |
|---|---|---|---|---|---|---|---|
| Fractions | Zn Supply | 1 | 2 | 3 | 4 | Rhizome | Root |
| Total $^{14}C$ | +Zn | 7.32 | 5.79 | 4.71 | 3.02 | 1.62 | 1.17 |
| Incorporated | -Zn | 4.56$^{*}$ | 4.21$^{*}$ | 3.98$^{NS}$ | 3.41$^{NS}$ | 0.96$^{**}$ | 1.12$^{NS}$ |
| ES | +Zn | 6.70 | 5.36 | 4.38 | 2.85 | 1.47 | 1.06 |
| Fraction | -Zn | 3.76$^{*}$ | 3.66$^{**}$ | 3.25$^{NS}$ | 3.10$^{NS}$ | 0.81$^{**}$ | 1.01$^{NS}$ |
| EIS | +Zn | 0.61 | 0.43 | 0.33 | 0.18 | 0.13 | 0.09 |
| Fraction | -Zn | 0.78$^{*}$ | 0.61$^{NS}$ | 0.71$^{NS}$ | 0.29$^{NS}$ | 0.13$^{NS}$ | 0.12$^{NS}$ |
| Sugar + | +Zn | 4.00 | 3.05 | 2.70 | 1.37 | 1.24 | 0.41 |
| Sugar phosphate | -Zn | 2.90$^{**}$ | 2.63$^{*}$ | 2.22$^{**}$ | 1.95$^{**}$ | 0.63$^{*}$ | 0.66* |
| Amino acids | +Zn | 0.73 | 0.53 | 0.34 | 0.21 | 0.05 | 0.18 |
| | -Zn | 0.29** | 0.26$^{**}$ | 0.16$^{**}$ | 0.15$^{**}$ | 0.03$^{*}$ | 0.13$^{NS}$ |
| Organic acids | +Zn | 1.29 | 0.71 | 0.66 | 0.41 | 0.13 | 0.31 |
| | -Zn | 0.58$^{**}$ | 0.58$^{**}$ | 0.49$^{**}$ | 0.48$^{**}$ | 0.08$^{**}$ | 0.16* |

ES = Ethanol soluble fraction, EIS= Ethanol insoluble fraction

*/** - Mean values significant at 5/1 % level of significance by paired t-test. (All values in KBq $g^{-1}$FW)

reveal that in +Zn total $^{14}C$ content was highest in youngest leaf (7.32 KBq/g.fr.wt.) which declined with leaf position from 2 to 4 (5.79, 4.71 and 3.02 KBq/g.fr.wt). In -Zn the trend of $^{14}C$ content in leaves was similar to that of +Zn but the values was significantly lower in 1st and 2nd leaves at 4.56 and 4.21 KBq/g.fr.wt. ES fraction which represents mobile metabolic pool significantly declined in 1st and 2nd leaf of -Zn at 3.76 and 3.66 KBq/g.fr.wt compared to 6.7 and 5.36 KBq/g.fr.wt in +Zn leaves while in other leaves values were non significant. Further analysis of ES fraction into components by column chromatography revealed the content to be highest in sugars followed by organic acids and then in amino acids. Total sugar content was significantly lower at all leaf position 1-4 in -Zn at 2.90, 2.63, 2.22 and 1.95 KBq/g.fr.wt than in +Zn at 4.00, 3.05, 2.70 and 1.37 KBq/g.fr.wt. and also in total organic acid content in -Zn at 0.58, 0.58, 0.49 and 0.48 KBq/g.fr.wt as in +Zn at 1.29, 0.71, 0.66 and 0.41 KBq/g.fr.wt, respectively. Amino acid content was significantly lower at leaf position 1 and 2 in -Zn at 0.29 and 0.26 KBq/g.fr.wt than 0.73 and 0.53 KBq/g.fr.wt in +Zn. Thus distribution of $^{14}C$ in ES fraction and in sugars, amino acids and organic acids were lower in young growing leaves of -Zn in contrast to $^{14}C$ content in EIS fraction which was high in -Zn leaves (Table 2).

Upon analysis of transport of leaf assimilated $^{14}C$ to rhizome the total content was 41% lower in -Zn at 0.96 KBq/g.fr.wt than 1.62 KBq/g.fr.wt in +Zn. Amongst the two major fraction the significant decrease was in -Zn mobile ES fraction at 0.81 KBq/g.fr.wt than in +Zn at

Table 3: Influence of Zn on incorporation of $^{14}C$ assimilates into essential oil in leaf and curcumin in rhizome of turmeric.

| | | Leaf position from top | | | | |
|---|---|---|---|---|---|---|
| Fraction | Zn Supply | 1 | 2 | 3 | 4 | Rhizome |
| $^{14}C$ in essential Oil [Bq $g^{-1}$ FW] | +Zn | 33.32 | 5.16 | 3.56 | 3.02 | |
| | -Zn | 0.59* | 1.27* | 1.34* | 1.46* | |
| $^{14}C$ in Curcumin [Bq $g^{-1}$FW] | +Zn | - | - | - | - | 6.40 |
| | -Zn | - | - | - | - | 1.26** |

*/** - Mean values significant at 5/1 % level of significance by pair t-test

1.42 KBq/g.fr.wt. In contrast no significant difference was observed in EIS fraction in -Zn and +Zn. At the same time label content in sugars, amino acids and organic acids were significantly low in -Zn (Table 2).

When roots were analysed for distribution of these metabolites there was no significant difference in label content in total, ES, EIS and amino acid fraction. However in -Zn sugar content was significantly higher at 0.66 KBq/g.fr.wt than 0.41 KBq/g.fr.wt in +Zn while organic acids were lower at 0.16 and 0.31 KBq/g.fr.wt, respectively (Table 2).

The biosynthetic capacity to utilize current $^{14}C$ metabolites for oil production was highest in youngest leaf of +Zn, which continuously declined from leaf 1 to 4 from 33.32 to 3.02 Bq/g.fr.wt. Lack of Zn resulted in decline in utilization of metabolites in biosynthesis of oil. This effect was evident by low label content which were significantly uniformly low in leaves 1 to 4 at 0.59, 1.27, 1.34 and 1.46 Bq/g.fr.wt (Table 3). When calculated as % decrease in utilization, the values were 98, 75, 62 and 51%, respectively in leaves 1 to 4, respectively. At the same time utilization of current $^{14}C$ into biosynthesis of total curcumin by rhizome declined by approximately 40% from 6.40 to 1.26 Bq/g.fr.wt (Table 3).

Large amounts of photosynthate accumulated by leaves are required for root biomass development and related metabolic processes, which in annual crops could be 30% of the total photosynthate produced [31]. Thus rhizome and root development and biosynthetic production of Curcumin will depend on the translocation of assimilated metabolites from the leaves. Upon comparing the relative distribution of assimilates label between rhizome and roots, rhizome receive higher assimilates than roots.

Very little work on biosynthetic aspects of curcumin production has been reported. Curcumin belongs to diarylheptanoid group of natural products. Studies on Curcumin biosynthesis, including precursor-product relationship or assimilate utilization are very few in literature. Incorporation of 14C-phenylalanine into aromatic ring of capsaicin in capsicum [32] and gingerol in ginger has been reported by Denniff and Whiting [33]. The biosynthesis of curcumin would possibly appear to be related to the union of 2 cinnamate moieties with a central methylene ring supplied by malonate followed by subsequent hydroxylation and serial methylation to form final product. The proposed biosynthetic scheme was investigated by studying incorporation of various labeled precursors on 3-4 months old plants when rhizomes were being formed and active curcumin biosynthesis would be expected [34, 35]. Incorporation studies using 1- and 3-14C phenylalanine, 1- and 2-14C acetate and 14C malonate and 3H- cinnamic acid alone and together with 1-14C phenylalanine showed good incorporation at specific position in curcumin. These studies were consistent with the phenylalanine cinamate pathway [34, 35]. The present study highlights that current $^{14}C$ metabolites transported from leaves as sugars, amino acids and organic acids are utilized for biosynthesis of Curcumin in rhizome and lack of Zn significantly lowered biosynthetic utilization for oil and curcumin biosynthesis. Which of these metabolites are preferentially utilized, however cannot be specified. The possible mechanism could be either due to low transport of metabolites from leaf to rhizome, alteration in the biosynthetic steps or its effect on curcumin storage tissues (in rhizome). These intermediary steps need more investigation.

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