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Boron deficiency induced changes in translocation of $^{14}CO_2$-photosynthate into primary metabolites in relation to essential oil and curcumin accumulation in turmeric (*Curcuma longa* L.)

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Abstract

Changes in leaf growth, net photosynthetic rate (P_N), incorporation pattern of photosynthetically fixed $^{14}CO_2$ in leaves 1-4 from top, roots, and rhizome, and in essential oil and curcumin contents were studied in turmeric plants grown in nutrient solution at boron (B) concentrations of 0 and 0.5 g m^{-3}. B deficiency resulted in decrease in leaf area, fresh and dry mass, chlorophyll (Chl) content, and P_N and total $^{14}CO_2$ incorporated at all leaf positions, the maximum effect being in young growing leaves. The incorporation of $^{14}CO_2$ declined with leaf position being maximal in the youngest leaf. B deficiency resulted in reduced accumulation of sugars, amino acids, and organic acids at all leaf positions. Translocation of the metabolites towards rhizome and roots decreased. In rhizome, the amount of amino acids increased but content of organic acids did not show any change, whereas in roots there was decrease in contents of these metabolites as a result of B deficiency. Photoassimilate partitioning to essential oil in leaf and to curcumin in rhizome decreased. Although the curcumin content of rhizome increased due to B deficiency, the overall rhizome yield and curcumin yield decreased. The influence of B deficiency on leaf area, fresh and dry masses, CO_2 exchange rate, oil content, and rhizome and curcumin yields can be ascribed to reduced photosynthate formation and translocation.

Additional key words: amino acids; leaf position; net photosynthetic rate; organic acids; rhizome; root; secondary metabolites; stomatal conductance; sugars; transpiration rate.

Introduction

Boron (B) is an essential micronutrient required for healthy plant growth and development. A number of metabolic pathways, primary (such as mobility of molecules through phloem, structural components of cell wall, membrane stability, maintenance of reproductive growth) and secondary (terpenoids) are affected by B (Marschner 1986). Till recently there was no indication that B is an enzyme component, however, purification and identification of the first boron-polyol transport molecules have provided evidence of its structural role in plants (Matoh *et al.* 1996, Blevins and Lukaszewski 1998). Most investigations on the role of B in metabolism tested what happens when it is withheld or re-supplied after deficiency. Among the secondary metabolic pathways, B influences accumulation of flavonoids (Carpena Artes *et al.* 1984), opium alkaloids (Srivastava *et al.* 1985), symbiotic nitrogen fixation (Yamagishi and Yamamoto 1994), accumulation of essential oils (Srivastava and Luthra 1992, 1993), and polyphenol biosynthesis (Watanabe *et al.* 1964).

Turmeric (*Curcuma longa* L. syn. *C. domestica* Valen, *Zingiberaceae*) is cultivated for its underground rhizome which is widely used as condiment, dye stuff, flavour, and in pharmaceutical and cosmetic industry (and also in religious and auspicious occasions) (Govindarajan 1980). The major constituents are essential oil, curcumin, and oleoresins stored in rhizome. Growth and development of leaves and rhizomes depend on cultivation practices (Randhawa and Mahey 1988) or genotype (Rao and Rao 1994). Despite the economic importance of the crop, little information is available on how micronutrients influence the physiological processes, regulating the secondary metabolite accumulation in turmeric. Growth of turmeric plant is influenced by deficiencies of

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Abbreviations: Chl – chlorophyll; E – transpiration rate; g_s – stomatal conductance; P_N – net photosynthetic rate; PPO – 2,5-diphenyloxazole; POPOP – 1,4-di-2-(5-phenyl oxazolyl)benzene.

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Fe and Zn (Rethinam *et al.* 1994, Dixit and Srivastava 2000a). A large proportion of photoassimilates accumulated by leaves are required for root growth and metabolism which in annual crops such as turmeric could be 30 % of the total photoassimilate produced (Marschner 1986). Thus rhizome development and the simultaneous accumulation of curcumin depend on the translocation of formed metabolites from the leaf. The amount of metabolites produced by leaves and the proportion of it that is translocated to the rhizome greatly influence size and yield, and also biosynthesis and accumulation of curcumin. Hence photosynthate partitioning to (sink) rhizome may be one of the factors controlling productivity apart from the biosynthetic capacity of rhizome. Since active precursors are produced in the leaves, the inherent photosynthetic capacity could be another regulatory factor. As B is involved in sugar transport, under B deficiency there might be alteration in the availability and content of translocated photosynthates that might be utilised in curcumin accumulation and rhizome development.

We report on assimilation and translocation of photosynthetically fixed $^{14}CO_2$ in leaf positions 1-4 from apex into essential oil and curcumin in relation to photosynthate contents in leaves, rhizome, and roots under B deficiency. In addition, we studied changes in net photosynthetic rate (P_N), Chl content, leaf area, fresh and dry matter accumulation in leaves, rhizome, and root, and changes in curcumin content.

Materials and methods

Plants: Uniform mother rhizomes of turmeric (*Curcuma longa* L.) were planted in ceramic pots (10 000 cm^3 capacity) filled with silica sand previously cleaned by hot acid digestion in steam for removal of B (Agarwala and Sharma 1961). The sprouted plants were transplanted in amber coloured glass pots (capacity of 2 500 cm^3) containing purified –B Hoagland nutrient solution. The macronutrient salt solutions were purified by alcohol re-crystallisation procedure to remove impurities of B (Hewitt 1966). The nutrient solution of Hoagland and Arnon (1938) was used except Fe, which was supplied as Fe-EDTA (5.6 mg kg^{-3}). B was supplied as boric acid (0.5 mg kg^{-3}) for control plants and omitted for deficient (–B) plants. Six pots each of control and deficient plants were maintained in a glasshouse at ambient temperature of 30-35 °C and photosynthetic active radiation (PAR) between 800-1 000 μmol m^{-2} s^{-1}. Routine practice for maintaining plants in solution culture was followed. With the onset of deficiency and the emergence of new rhizome (about 4-month-old plants) data were recorded.

Leaf growth parameters: The leaves were numbered from apex to the base of the shoot, the uppermost leaf being taken as the youngest one. Leaf area was measured by an automatic leaf area meter (*Li-3000 LiCOR*, Lincoln, USA). Leaf fresh mass was determined immediately and dry matter was determined after oven drying at 80 °C until a constant mass (48 h).

Chl content and gas exchange: 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) according to Arnon (1949). P_N, initial transpiration rate (E), and stomatal conductance (g_s) were measured with a portable photosynthesis system (*Li-6000, Licor*, Lincoln, USA) (Srivastava and Luthra 1991).

Essential oil from leaves was isolated by steam distillation technique using mini-clevenger apparatus (Clevenger 1928). The isolated essential oil was extracted by diethyl ether. Direct extraction by organic solvent and steam distillation process yielded the same results.

Determination of curcumin in rhizome: A known mass of fresh rhizome was ground in ethanol and further diluted in ethanol. A standard curve was prepared at different concentrations with pure curcumin and absorbance was recorded on a *Spectronic 21D* (*Milton Roy and Co.*, USA) spectrophotometer at 430 nm (American Spice Trade Association 1968, Prasad and Suresh 1997).

$^{14}CO_2$ incorporation: Pots with plants were placed in a sealed plexiglass chamber (20 000 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 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 optimum time for maximal $^{14}CO_2$ incorporation into oil and curcumin was 24 h (Dixit and Srivastava 2000b). The plants assimilated $^{14}CO_2$ for 6 h at irradiance of 800-1 000 μmol m^{-2} s^{-1}. At the end of this period, 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 for the remaining incorporation period of 24 h. Following the incorporation period, label content was studied in oil, curcumin, and metabolic fractions in leaves, rhizome, and roots. A part of the leaf and rhizome tissue was used to determine incorporation in oil and curcumin, whereas remaining parts were immediately immersed in boiling ethanol. To determine incorporation of $^{14}CO_2$-photosynthates into essential oil, a known mass of leaves was subjected to steam distillation as explained above. The radioactivity in ether aliquots was determined in a scintillation counter (*Wallac 1409* USA) using PPO-POPOP-toluene cocktail. For determining ^{14}C incorporation in curcumin, a known

Table 1. Changes in leaf parameters, net photosynthetic rate (P_N), stomatal conductance, (g_s), transpiration rate (E), and chlorophyll (Chl) content in leaves of different position of turmeric subjected to B deficiency. *,** mean values significant at 5 or 1 % level of significance by paired t-test.

		Leaf position from top			
		1	2	3	4
Leaf area [cm^2 $leaf^{-1}$]	+B	155.6	219.4	244.8	171.2
	–B	89.6	138.0**	149.3**	136.2*
Leaf fresh mass [g $leaf^{-1}$]	+B	4.85	6.54	7.02	3.25
	–B	3.37	4.02**	4.27**	4.03
Leaf dry mass [g $leaf^{-1}$]	+B	0.72	1.19	1.35	0.59
	–B	0.44	0.67**	0.82**	0.59
Chl (a+b) [g kg^{-1}(FM)]	+B	0.94	1.02	1.28	1.52
	–B	0.65*	0.80	1.14	1.38
P_N [μmol(CO_2) m^{-2} s^{-1}]	+B	3.53	4.10	4.77	4.08
	–B	1.72**	2.04**	2.46**	2.08**
E [mol m^{-2} s^{-1}]	+B	0.27	0.29	0.31	0.28
	–B	0.15**	0.17**	0.19**	0.16**
g_s [mol m^{-2} s^{-1}]	+B	0.31	0.36	0.40	0.38
	–B	0.17**	0.20**	0.20**	0.19**

mass of rhizome was ground and extracted in ethanol. The label in alcohol fraction was measured in a counter using Bray's scintillation fluid. A known mass of tracer-fed leaves, rhizome and roots of +B and –B plants was ex-tracted 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). Ethanol insoluble material was further hydrolysed by diastase in 0.05 M acetate buffer (pH 5.2) at 50 °C. Total ^{14}C accumulated was calculated as the sum of label incorporated in ethanol-soluble and -insoluble fractions and expressed on fresh mass basis. The radioactivity in ethanol soluble, hydrolysed ethanol-insoluble fraction, and in eluates after ion exchange separation was measured using Bray's scintillation fluid in liquid scintillation counter (*Wallac 1409* USA) (Srivastava and Luthra 1994).

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

Results and discussion

In +B and in –B plants studied at the same growth stage (4-month-old plants), leaves from top to bottom of shoot exhibited changes in physiological capacity. By the time fresh rhizomes started to grow, only four leaves remained on –B plants. Leaf area and fresh and dry masses increased till the 3rd leaf position and then declined in both +B and –B plants. In –B plants these values were significantly lower, the youngest leaf showing the maximum effect (Table 1). Chl content increased till the 4th leaf position, with –B plants having lower values. The decrease in Chl a content was significant, whereas the decrease in Chl b was not significant. The gas exchange parameters—P_N, initial E, and g_s—gradually increased and were maximal in the 3rd leaf. Due to B deficiency significant decrease in P_N, E, and g_s was observed at all leaf positions (Table 1). B deficiency thus reduced leaf area, chlorophyll (Chl) content, and P_N. This reduction in P_N should be reflected in contents of photosynthates and their translocation to rhizomes and roots, as well as in secondary metabolites.

The youngest leaf was characterised by maximum total $^{14}CO_2$ incorporation in both +B and –B plants (Table 2) which decreased as the leaf matured. In –B plants, total $^{14}CO_2$ incorporation was significantly lower than in control plants (Table 2). Similar trend of $^{14}CO_2$ fixation into ethanol soluble and insoluble fractions was also observed both in +B and –B plants. Ethanol soluble fraction was further analysed into sugars, amino acids, and organic acids. Contents of sugars and organic acids were significantly lower in all leaves of –B plants than +B plants. Significant reduction in amino acid content in the youngest –B leaf was observed, while in the 2nd-4th leaf the change was insignificant (Table 2).

Total ^{14}C content was higher in rhizome than in root. This difference was significantly lower in –B than +B plants (Table 2). The decrease was more significant in ethanol soluble fractions than in ethanol insoluble fractions. In rhizome and root the relative contents declined from sugars through organic acids to amino acids. The content of sugars in rhizome of –B plants decreased whereas the content of organic acids did not change and the content of amino acids was non-significantly greater. However, roots of –B plants contained less sugars, amino acids, and organic acids than roots of +B plants (Table 2).

Table 2. $^{14}CO_2$ fixation and partitioning into primary metabolic fractions [MBq kg^{-1}(FM)] in developing leaves, freshly developed rhizome, and roots of turmeric subjected to B deficiency. *,** mean values significant at 5 or 1 % level of significance by paired *t*-test.

Fraction		Leaf position from top				Rhizome	Root
		1	2	3	4		
Total ^{14}C incorporated	+B	7.32	5.79	4.71	3.02	1.62	1.17
	–B	2.97*	1.89**	1.82*	1.80*	0.94**	0.74**
Ethanol soluble fraction	+B	6.70	5.36	4.38	2.83	1.47	1.06
	–B	2.75*	1.75**	1.74*	1.72*	0.89**	0.68*
Ethanol insoluble fraction	+B	0.61	0.43	0.33	0.18	0.13	0.09
	–B	0.19**	0.13	0.08*	0.08*	0.03	0.06
Sugar+sugar phosphate	+B	4.00	3.05	2.70	1.37	1.20	0.41
	–B	1.39**	0.74**	0.73**	0.64**	0.56*	0.33
Amino acids	+B	0.73	0.53	0.34	0.21	0.05	0.18
	–B	0.56*	0.49	0.34	0.23	0.09	0.08
Organic acids	+B	1.29	0.71	0.66	0.41	0.13	0.31
	–B	0.51**	0.46**	0.38**	0.28**	0.13	0.14*

The youngest leaf had maximum content of ^{14}C-essential oil, which decreased to the 4th leaf. Assimilate content was significantly lower in –B than +B leaves (Table 3). There was significant less rhizome biomass in –B plants (Table 3). The relative curcumin content of rhizome increased under B stress (from 0.61 to 0.88 %), however, since there was significant decrease in rhizome biomass yield, the overall curcumin yield was low. The translocation and utilisation of $^{14}CO_2$ assimilated metabolites into curcumin was lower in rhizome of –B plants than in +B plants.

Table 3. Influence of B deficiency on ^{14}C-assimilate translocation into essential oil in leaves, rhizome yield, and curcumin content of turmeric. *,** mean values significant at 5 or 1 % level of significance by paired *t*-test.

	^{14}C in essential oil [kBq kg^{-1}(FM)] Leaf position from top				Rhizome yield [g plant^{-1}]	Curcumin [%]	Curcumin yield [g plant^{-1}]	^{14}C in curcumin [kBq kg^{-1}(FM)]
	1	2	3	4				
+B	33.3	12.5	3.5	3.0	166.1	0.61	1.014	6.40
–B	16.8	5.2**	1.5*	1.3*	58.7**	0.88**	0.518	5.81

B deficiency has more expressed effects on plant metabolism than deficiency of other micronutrients (Blevins and Lukaszewski 1998). The decrease in yield is connected with the reduction in Chl content and P_N. B deficiency results in the breakdown of chloroplast membrane that affects photochemical capacity and translocation of assimilates (Hudák and Herich 1976). Therefore the reduction in yield could be due to the loss of chloroplast membrane integrity, reduction in photosynthetic capacity, or low P_N accompanied by lower translocation of photosynthates in –B plants. In turmeric the deficiency in B resulted in both the reduction of total $^{14}CO_2$ fixed by leaves and the partitioning of assimilates towards the rhizome and roots.

Studies on biosynthesis, precursor-product relationship, or photosynthate partitioning in spice crops are scarce in the literature. Incorporation of ^{14}C-phenylalanine into capsaicin in capsicum (Bennett and Kirhy 1968) and gingerol in ginger (Deniff and Whiting 1976) has been reported. In a 6-d feeding trial, precursors such as ^{14}C-phenylalanine, ^{14}C-malonate, and ^{14}C-acetate were incorporated in different structural components of curcumin (Roughly and Whiting 1971, 1973). Our study confirmed that photosynthates are incorporated in curcumin, but did not specify if sugars, amino acids, or organic acids are preferentially utilised. Since there was higher content of curcumin under B stress, some steps of curcumin biosynthetic pathway might been selectively accelerated. In many essential oil bearing plants such as mints (Srivastava *et al.* 1990, Srivastava and Luthra 1991, 1993, 1994) or citronella (Singh and Luthra 1988) there is a positive relationship between primary and secondary metabolism that is dependent on nutritional status of plant (Srivastava and Luthra 1993). The development of rhizome and subsequent accumulation of curcumin depend on the translocation of photoassimilate coupled with biosynthesis of curcumin, but under B deficiency there is reduced translocation of photoassimilates to rhizome and roots which results in poor growth and low curcumin yield in turmeric, despite high relative curcumin content.

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