The substrate utilization and concentration of ^{14}C photosynthates in citronella under Fe deficiency

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Abstract

Changes in the utilization pattern of primary substrate, viz. [U-^{14}C] acetate, $^{14}CO_2$ and [U-^{14}C] saccharose, and the contents of ^{14}C fixation products in photosynthetic metabolites (sugars, amino acids, and organic acids) were determined in Fe-deficient citronella in relation to the essential oil accumulation. There was an overall decrease in photosynthetic efficiency of the Fe-deficient plants as evidenced by lower levels of incorporation into the sugar fraction and essential oil after $^{14}CO_2$ had been supplied. When acetate and saccharose were fed to the Fe-deficient plants, despite a higher incorporation of label into sugars, amino acids, and organic acids, there was a lower incorporation of these metabolites into essential oils than in control plants. Thus, the availability of precursors and the translocation to a site of synthesis/accumulation, severely affected by Fe deficiency, is equally important for the essential oil biosynthesis in citronella.

Additional key words: acetate; amino acids; $^{14}CO_2$; *Cymbopogon winterianus*; oil; organic acids, primary and secondary metabolites; sugars; [U-^{14}C]; [U-^{14}C] saccharose incorporation

Introduction

Iron is involved in various photosynthetic processes, namely the chlorophyll biosynthesis (Miller *et al.* 1982, Morales *et al.* 1990), photosynthetic electron transport system (Terry 1980), formation of chloroplast ultrastructure (Nishio and Terry 1983), photosynthetic enzyme activity (Taylor *et al.* 1982, Arulanantham *et al.* 1990), and decrease in carotenoid contents (Abadia *et al.* 1991). Structure and function of the whole photosynthetic apparatus is affected (Marschner 1986, Abadía 1992).

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The essential oil biosynthesis and accumulation in aromatic plants, including *Cymbopogon* species, is strongly influenced by intrinsic (genotype, ontogeny) and extrinsic (environmental) factors (Lawrence 1986). Among these, nutrients are important (Barz and Koster 1981). The essential oil biosynthesis takes place in epidermal oil glands which are carbon heterotrophic (Croteau and Johnson 1984) and hence depend on the adjoining photosynthesising cells for a continuous supply of carbon precursors. Labelled compounds such as CO_2, saccharose, glucose, acetate, and mevalonate have been studied as substrates, however, CO_2 and saccharose are the best precursors of isoprenoid biosynthesis (Croteau *et al.* 1972, McGarvey and Croteau 1995). There are certain factors which govern selective translocation of the precursor to the site of synthesis and/or accumulation. The contents of saccharose metabolism enzymes, starch and starch mobilizing enzymes, enzymes of oxidative pathways, and leaf ontogeny influence the essential oil accumulation in *Cymbopogon* (Singh and Luthra 1987, Singh *et al.* 1990, 1991, Luthra *et al.* 1991). As Fe is involved in photosynthesis, and saccharose and CO_2 are the most likely sources of carbon utilized in terpenoid biosynthesis, the role of Fe in influencing the essential oil accumulation seems particularly important.

The essential oil biosynthesis in citronella is the integration of several metabolic pathways which requires linking of several steps such as continuous production of precursors, their transport and translocation to the active site of synthesis, and finally the oil accumulation. This sequence of steps depends upon normal functioning of associated metabolic pathways such as carbon fixation, respiration (providing energy), isoprenoid pathway, *etc.* Any disruption in normal metabolic pathways affects the sequence of steps of the oil biosynthesis. Thus, a plant may alter/adopt its metabolic pathways in response to a particular deficiency, *e.g.*, under Fe deficiency there might be an alteration in availability, utilization of precursors, as well as the content of photosynthates which may be utilized in the oil biosynthesis and accumulation.

In the present paper we describe some experiments on the substrate utilization, particularly of acetate, CO_2, and saccharose for the essential oil accumulation in relation to the contents of primary photosynthates. This may help in understanding the causes of limitation imposed on the photosynthetic carbon metabolism and essential oil accumulation due to an Fe deficiency.

Materials and methods

Plants: The slips of *Cymbopogon winterianus* Jowitt (*Poaeae*) were obtained from the farm nursery of Central Institute of Medicinal and Aromatic Plants, Lucknow, India. These slips were planted in ceramic pots filled with silica sand that had been previously cleaned by a hot acid digestion in steam for removal of Fe from the sand (Agarwala and Sharma 1961). The steam digestion process was repeated to remove even the traces of Fe impurity until its level was extremely low when measured by an atomic absorption spectrometer. The salts used in preparation of the nutrient solution were purified against Fe by dithizone solution (Hewitt 1966). Nutrient solution of

Hoagland and Arnon (1938) was used, except Fe which was supplied as FeEDTA (5.6 g m^{-3}) for control plants and completely omitted in Fe deficient ones (Srivastava and Luthra 1993). Six pots each of deficient and control plants were kept inside a glasshouse at ambient temperature (30-35 °C) and under irradiance (800-1000 µmol m^{-2} s^{-1}). With the onset of deficiency, photosynthetic and tracer studies were performed.

Chl and CO_2 exchange rate: A known mass of tissue of the third leaf was extracted with 80 % acetone, and Chl absorption was recorded on a *Pye Unicam* spectrophotometer (model *PU 8610*), according to Arnon (1949). The net photosynthetic rate (P_N) of the third leaf was measured in a closed system using a computerized portable photosynthesis system (model *LI 6000*, *LiCOR*, USA) (Srivastava and Luthra 1991).

Tracer studies were performed by feeding $^{14}CO_2$, [U-^{14}C] acetate, and [U-^{14}C] sucrose. For $^{14}CO_2$ incorporation studies, freshly excised leaves (12 pairs) were cut in the water and placed inside vials with cut ends dipped into half strength Hoagland solution. Afterwards, the vials were kept in a sealed plexiglass chamber (20 000 cm^3) around the central vial containing $Na_2{}^{14}CO_3$ solution (3.7 MBq; 2.77 MBq mol^{-1}) obtained from the isotope division of Bhabha Atomic Research Centre, Trombay, India. $^{14}CO_2$ was generated by injecting 4 M H_2SO_4 into the carbonate solution through a PVC tube, and uniform distribution was achieved with the help of a small electric fan. The leaves were permitted to assimilate $^{14}CO_2$ for 1 h at 800-1000 µmol m^{-2} s^{-1} (the harvesting of samples was done in the morning about 5 h after the beginning of the light period). At the end of 1 h, a 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 chase period of 24 h, after which the leaves were harvested.

For [U-^{14}C] acetate and [U-^{14}C] saccharose incorporation studies, freshly excised leaves were cut in water and placed in vials containing aqueous solution (1 cm^3) of labelled precursor obtained from Bhabha Atomic Research Centre, Trombay, India. [U-^{14}C] saccharose specific activity was 21 460 GBq mol^{-1}, [U-^{14}C] acetate specific activity 1277 GBq mol^{-1}. The vials were kept in the light [800-1000 µmol(photon) m^{-2} s^{-1}] and after the uptake of the labelled material they were filled with half strength Hoagland solution. Harvesting was done after 24 h.

Samples of $^{14}CO_2$ or [U-^{14}C] saccharose or [U-^{14}C] acetate feeding were divided after harvest in two groups before subsequent extraction and analysis:

(*1*) For determining the incorporation of label into essential oil, a known mass of tracer fed leaves was chopped and subjected to microscale steam distillation in mini-clevenger apparatus (Clevenger 1928). The oil was recovered by ether extraction and dehydrated with anhydrous sodium sulphate. The radioactivity in the ether aliquots was determined in a scintillation counter (*LKB Rack Beta 1215*) using PPO-POPOP-toluene cocktail.

(*2*) For determining the incorporation of label into primary photosynthates, a known mass of tracer fed leaves was extracted in boiling 80 % ethanol. The ethanol soluble material was separated into neutral (sugars and sugar phosphate), basic (amino acids), and acidic (organic acids) fractions using *Amberlite* ion exchange columns.

The ethanol soluble material was further enzymatically hydrolyzed by amyloglucosidase (*Sigma*) in 0.05 M acetate buffer (pH 5.2) at 50 °C. The total ^{14}C incorporation was calculated as the sum of the label incorporated in soluble and insoluble fractions, and expressed on the fresh matter basis. The radioactivity in hydrolyzed alcohol insoluble material and eluates after the ion exchange separation was measured using Bray's scintillation fluid in a scintillation counter (Srivastava and Luthra 1991).

Statistics: All measurements were taken in triplicate and the results are given as means + S.E. Variations in the treatments were statistically analysed for significance by paired *t*-test.

Results and discussion

With the onset of visual Fe deficiency symptoms, the photosynthetic efficiency was determined. The third leaf of Fe-deficient (-Fe) plants had a significantly lower CO_2 exchange rate [129 ± 15 mg(CO_2) m^{-2} s^{-1}] than control +Fe plants [726 ± 4 mg(CO_2) m^{-2} s^{-1}], and the total Chl content [0.420 ± 0.001 g kg^{-1}(f.m.) $leaf^{-1}$] was significantly lower than that of the control plants [1.020 ± 0.006 g kg^{-1}(f.m.) $leaf^{-1}$]. However, the Chl *a*/*b* ratio was significantly higher in Fe-deficient plants. Fe deficiency increases Chl *a*/*b* ratio which alters the leaf photochemical capacity either by preferential photodestruction of all Chl *b* or by an increase in Chl *a* containing reaction centres relative to Chl *b* (Terry and Abadía 1986). Thus, under Fe deficiency there was an overall reduction in the photosynthetic efficiency of the plants. This reduction in the photosynthetic capacity was reflected in the concentration of internal leaf photosynthates and their translocation into essential oil.

When $^{14}CO_2$ was fed, the total incorporation by -Fe plants was significantly lower than that of +Fe plants. When the total $^{14}CO_2$ fixed was separated into ethanol soluble (mobile) and ethanol insoluble (immobile) fractions, the +Fe plants had a higher incorporation in the soluble fraction, whereas the -Fe plants had a higher incorporation into the insoluble fraction. Among major photosynthates, the contents of sugars and sugar phosphates were significantly lower in the -Fe than in +Fe plants, while the organic acid pool was significantly higher in the -Fe than in +Fe plants. The amino acid pool (Table 1), however, was significantly not different between the two variants. The incorporation of photosynthates into essential oil was significantly higher in the +Fe than -Fe plants (Table 1). Thus the overall fixation of $^{14}CO_2$ into photosynthates and their subsequent transformation into essential oil was reduced in the -Fe plants. The transformation of photosynthates to essential oil depends on various factors. Changes in the essential oil content, CO_2 exchange rate, and distribution of photosynthates have been reported in developing peppermint leaves where the incorporation is maximum into sugars followed by organic acids, amino acids, and essential oil at all phases of leaf development. The incorporation into sugars and amino acids declines as the leaf matures, whereas that into essential oil and organic acids increases with the leaf expansion and then decreases (Srivastava and Luthra 1991). Similarly, when peppermint is subjected to Fe deficiency, the

photosynthetic efficiency decreases. Variation in the label incorporated into sugars, amino acids, and organic acids, and reduced transformation of photosynthates to essential oil in -Fe plants is accompanied by significant changes in partitioning of a photosynthate between leaf and stem (Srivastava and Luthra 1993).

Apart from the decrease in efficiency of photosynthetic fixation of $^{14}CO_2$, the uptake and subsequent metabolism of two main precursors, [U-^{14}C] acetate and [U-^{14}C] saccharose was also studied with respect to the photosynthate contents and then to the incorporation into oil. When [U-^{14}C] acetate was fed, the total incorporation by -Fe plants was significantly higher than the incorporation by +Fe plants. The -Fe plants had a significantly higher incorporation in ethanol soluble fraction than +Fe plants, whereas the contents of insoluble fraction was significantly not different between the two. The contents of sugar + sugar phosphates, amino acids, and organic acids in the -Fe plants were significantly higher than in +Fe plants. Among the three metabolites, the content of organic acid was maximum. Despite the higher concentration of ^{14}C metabolites in the -Fe plants than in +Fe ones, the incorporation of acetate into essential oil was significantly higher in the +Fe than in -Fe plants (Table 1).

When [U-^{14}C] saccharose was fed, the total incorporation by -Fe plants was significantly higher than by +Fe plants, with 90 % of incorporation remaining in the ethanol soluble fraction. However, the difference in the ethanol insoluble fraction was not significant. Among the three major metabolites in the -Fe plants, the maximum incorporation occurred in sugar + sugar phosphate fraction followed by organic acids and least in amino acid and these were significantly higher than the respective incorporation in the +Fe plants. The incorporation of ^{14}C saccharose into essential oil, however, was higher in the +Fe plants than in the -Fe ones (Table 1). The incorporation of higher amounts of label from acetate and saccharose into primary metabolites in the -Fe plants signified preferential utilization of precursors, *i.e.*, there was a difference in utilization of CO_2, acetate, and saccharose during Fe deficiency. However, despite the preferential substrate utilization of exogenously supplied precursors and higher content of ^{14}C metabolites, the incorporation into essential oil did not increase. This could be due to several factors regulating the flow of these metabolites towards essential oil biosynthesis. Although reviews are available on terpenoid metabolism (Goodwin 1979, Croteau 1987, Chappel 1995, McGarvey and Croteau 1995), very little is known about the control of transport of primary metabolites/precursors towards the oil biosynthesis. There are at least three semi-autonomous subcellular compartments that segregate the terpenoid biosynthesis. These may or may not be linked to the accumulation step. Monoterpenes are synthesized in plastids. But what factors control the translocation from the site of synthesis to the site of accumulation is not clear. Interspecific variations have been reported with respect to utilization of exogenously supplied precursors [2-^{14}C] acetate, [U-^{14}C] glucose, and [U-^{14}C] saccharose for the essential oil synthesis in *Cymbopogon* species: acetate was most efficiently incorporated into essential oil in *C. winterianus* and *C. flexuosus*, and glucose in *C. martinii* (Luthra *et al.* 1993).

Table 1. Changes in incorporation pattern of $^{14}CO_2$, [U-^{14}C] acetate, and [U-^{14}C] saccharose into primary photosynthates [s^{-1} g^{-1}(f.m.)] in leaves of citronella under Fe deficiency. */** Mean values significant at 5/1 % level of significance by pair *t*-test. NS = non-significant.

Fractions	[$^{14}CO_2$] Control	-Fe	[U-^{14}C] acetate Control	-Fe	[U-^{14}C] saccharose Control	-Fe
Total incorporation	9032±32	6889±24**	2388±32	6372±44**	4112±16	13907±34**
Ethanol soluble fraction	8777±46	6602±17**	2162±31	6134±22**	3909±11	13316±41**
Ethanol insoluble fraction	254±25	286±15^{NS}	226± 4	237±23^{NS}	204±26	290±19^{NS}
Sugar + sugar PO_4	1202± 5	565± 9**	58± 1	52± 2**	452± 9	786± 2**
Amino acids	74± 2	180± 1^{NS}	69± 1	109± 1**	51± 1	176± 4**
Organic acids	123± 2	151± 1**	188± 1	328± 5**	61± 2	241± 3**
Oil	10±>0	6±>0**	28±>0	10±>0**	33± 1	14±>0**

Thus the simultaneous reduction in growth and photosynthetic efficiency and fixation of $^{14}CO_2$ into essential oil indicate limited availability of a photosynthate for the oil biosynthesis in -Fe plants. Saccharose is the most efficiently utilized for the biosynthesis of essential oil followed by acetate and CO_2. Under Fe deficiency, there is a significant decrease in the incorporation of these precursors into essential oils. However, in *Mentha* under Fe deficiency, the incorporation of ^{14}C saccharose into essential oil is higher in the -Fe plants than in control ones (Srivastava and Luthra 1993). This may be due to a low content of saccharose in the -Fe plants. But the incorporation of saccharose into essential oil was low in the -Fe plants despite its higher content. A similar low incorporation of ^{14}C-acetate into essential oil in the -Fe plants was observed even though the incorporation into sugars was higher. Thus not only the concentration of sugars is responsible for a high essential oil biosynthesis and/or accumulation. Other factors responsible for efficient utilization of precursors for the oil biosynthesis are the transport of photosynthates or other precursors to the site of biosynthesis, energy status of adjoining photosynthesing cells, and their capacity to translocate the precursors to the oil biosynthesis in preference over their own requirement, the contents of relevant enzymes of the isoprenoid pathway, and factors that control the partitioning of available photosynthates.

Under Fe deficiency (except in the contents of ^{14}C sugars when $^{14}CO_2$ is fed to the -Fe plants), there is a higher accumulation of ^{14}C into sugars, amino acids, and organic acids in the -Fe plants. Nevertheless, despite the availability of photosynthates, the stoppage of precursors to oil biosynthesis may be due to the energy deficiency, or translocation factors, membrane permeability, or other control mechanisms which need to be investigated yet.

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