METABOLIC FATE OF [^{14}C]GLUCOSE IN PERICARPS AND SEEDS OF *COFFEA ARABICA* AND *COFFEA CANEPHORA* FRUITS DURING DEVELOPMENT AND RIPENING

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To elucidate the overall metabolism of sugars in coffee fruits, in situ metabolism of exogenously supplied ^{14}C-glucose were investigated in segments of pericarps and seeds of two cultivars of *Coffea arabica* and *C. canephora* fruits obtained from different growth and ripening stages. The coffee fruits were categorized into five stages 1 to 5 which were corresponding to young, developing, mature (green), ripening (pink) and fully-ripened (red) fruits, respectively. The rates of uptake and metabolism of [U-^{14}C]glucose in fruits of the stages 1–3 was higher than in fruits of stages 4–5. Release of $^{14}CO_2$ which represented cellular respiration was high in both pericarp and seeds of *C. arabica* and *C. canephora* up to the stage 3, but it gradually reduced and the minimum was found in the fully ripened stage 5 fruits. The highest incorporation of radioactivity into the methanol-soluble metabolites was found in the stage 2 fruits. In pericarp, incorporation of radioactivity into the acidic component (mainly consisted of organic acids) was much higher than into basic component (mainly consisted free amino acids). In seeds, significant amounts of the radioactivity were found in the basic fractions especially at the stages 4 and 5. [U-^{14}C]glucose was converted to fructose and sucrose in both pericarp and seeds. Only small rate of sucrose synthesis from [^{14}C]glucose was detected 18 h after administration, however, its relative rate is slightly increased at stages 4-5 fruits. Incorporation of radioactivity into the methanol-insoluble metabolites (mainly consisted protein starch and cell wall constituents) was found in any stages of fruits. The maximum rates were found in pericarp and seeds of developing (stage 2) fruits of *C. arabica* and in those at early three stages 1–3 of *C. canephora*. Possible metabolic routes and relative participation of carbon in different metabolite biosynthesis in coffee fruits are discussed.

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Introduction

Coffee is a useful plant and many studies on its metabolites have been performed using coffee beans (seeds) from the view points of food chemistry and human health.[1-3] However, little is known on biosynthesis and metabolism of these metabolites in coffee plants. As a part of the series of our research to elucidate the primarily and secondary metabolism in coffee fruits, we have earlier investigated the biosynthesis of caffeine, trigonelline and chlorogenic acids in growing and ripening fruits of *Coffea arabica* and *Coffea canephora*.[4,5] In this subsequent study, we have examined carbohydrate metabolism in coffee fruits using *C. arabica*, cv. Mokka and cv. Catimor and *C. canephora* grown in the coffee field of the Hawaii Agriculture Research Center, Oahu Island.

In coffee plants, atmospheric CO_2 is fixed by photosynthesis mainly in leaves and a photosynthate, sucrose, appeared to be translocated to the sink including coffee fruits. In addition, limited amounts of CO_2 are also fixed at the surface of pericarps and translocated to seeds. Sucrose synthesized in leaves is loaded into phloem by the H^+-sucrose co-transporter. Loaded sucrose is translocated to fruit flesh through the phloem and is unloaded into the parenchyma tissue.[6] In the sink, sucrose is converted to monosaccharides and then metabolised. Geromel et al.[7,8] reported that invertase activity was high during early stage of coffee fruits and sucrose was hydrolysed to glucose and fructose. Therefore, in order to investigate the profile of sugar metabolism in pericarps and seeds during growth of coffee fruits, we have applied [U-^{14}C]glucose to the segments of pericarps and seeds and followed the metabolic fate. On the basis of the results obtained, overall sugar metabolism in coffee fruits has been discussed.

Experimental

Plant Material

Fruits of *Coffee arabica* cv. Mokka (MA2-7) and cv. Catimor (T5175-7-1), and of *Coffea canephora*, were obtained from the Experimental Station of Hawaii Agriculture Center, Kunia Station, Oahu Island, Hawaii. These coffee trees were cultivated at the same site at an altitude of ca. 150 m above sea level. Fruits were divided into five stages according to the growth and maturity of coffee fruits.

Radiochemicals and biochemicals

[U-^{14}C]Glucose (specific activity 9.1 GBq mmol^{-1}) was purchased from Moravek Biochemicals Inc, Brea, CA, USA. Standards of metabolites were purchased from Sigma-Aldrich, St. Louis, Mo, USA.

Administration and Analysis of ^{14}C-labelled Glucose

Administration and analysis were according to the methods described in our earlier papers[9-12] with slight modifications. Samples (~100 mg fresh weight) and 2.0 mL of 20 mM sodium phosphate buffer (pH 5.6) containing 1 mM [U-^{14}C]glucose and 0.5 % sodium ascorbate were placed in the main compartment of a 30-ml Erlenmeyer flask. The flask was fitted with a small glass tube that contained a piece of filter paper impregnated with 0.1 mL of 20% KOH in the centre well, to collect $^{14}CO_2$. Each reaction was started by adding a solution of the radioactive compound to the main compartment. The flasks were incubated for 18 h in an oscillating water bath at 27 ºC. After incubation, the plant materials were harvested using a stainless steel tea strainer, then washed with distilled water and frozen with liquid nitrogen. They were then stored at -80 ºC. Potassium bicarbonate that had been absorbed by the filter paper was allowed to diffuse into distilled water overnight, and aliquots of the resulting solution (usually 0.5 mL) were used for determination of the radioactivity with a liquid scintillation analyser.

The frozen plant materials were homogenized in 80 % methanol using a pestle and mortar. The homogenates were centrifuged at 12,000 x g for 10 min. The resulting supernatant was collected and the precipitate was re-suspended with 80 % methanol, and the supernatant was collected by centrifuging. The first and second methanol-soluble fractions were combined. After complete evaporation of the methanol, the methanol-soluble extracts were dissolved in distilled water. The methanol-soluble fraction were fractionated into sugars, amino acids and organic acids on two types of ion-exchange resins, Dowex 50W-X8 (H^+ form) and Dowex 1-X8 (Cl^- form).

The basic components including free amino acids and the acidic components including organic acids were eluted with 2 N NH_4OH from the Dowex 50W-X8 column and 5N HCO_2H from Dowex 1-X8 (Cl^- form), respectively. The neutral components including sugars were obtained in the effluent from the two columns.

Sugars were separated by TLC using butanol-acetic acid-water (4: 1:2 v/v) as the developing solvent. Development was repeated after drying to complete the separation.

Results and Discussion

Growth Stages of Coffee Fruits during Development and Ripening

In this paper, the five growth stages of coffee fruits from the sizes and maturation are defined. Fruits of stages 1 to 3 are green-coloured ones of small, medium and large sizes. They are roughly corresponded to the rapid expansion and pericarp growth stage, the endosperm formation stage and the dry matter accumulation stage as described by Cannell.[13] At stage 4, fruit skin colour was changed from green to pink and seeds were pre-ripened, and at stage 5, colour was changed to red and seeds are fully ripened. Fresh weights and sizes of fruits in stages 1 to 5 are shown in Table 1.

Metabolic Fate of [^{14}C]Glucose

In order to examine the metabolic fate of [U-^{14}C]glucose which is taken up by the segments of pericarps and seeds of coffee fruits, respiratory released $^{14}CO_2$ was collected with KOH in the centre well of the incubation flasks and intracellular ^{14}C-metabolites were extracted with 80 % methanol and the methanol-soluble and methanol-insoluble components were obtained.

Table 1. Growth stages and fresh weights of Coffee fruits.

Sample	Stage	Colour	Fresh weight (mg)
Coffea arabica cv. Mokka	1	Green	215± 3
	2	Green	822±16
	3	Green	915±14
	4	Pink	1037±40
	5	Red	1180± 9
Coffea arabica cv. Catimor	1	Green	236± 4
	2	Green	800±35
	3	Green	849±41
	4	Pink	1012±39
	5	Red	1288±11
Coffea canephora	1	Green	239±11
	2	Green	545± 3
	3	Green	622± 7
	4	Pink	778±73
	5	Red	1112±90

The compounds in the methanol-soluble components were fractionated by ion-exchange and thin-layer chromatography as illustrated in Figure 1.

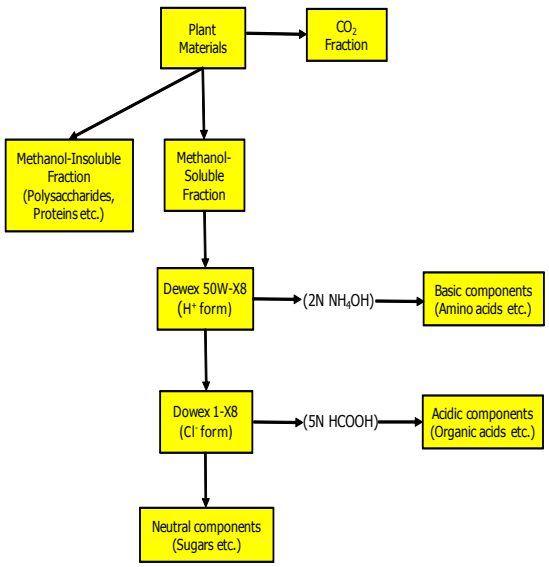

Figure 1. Flow sheet of the separation of ^{14}C-labelled metabolites

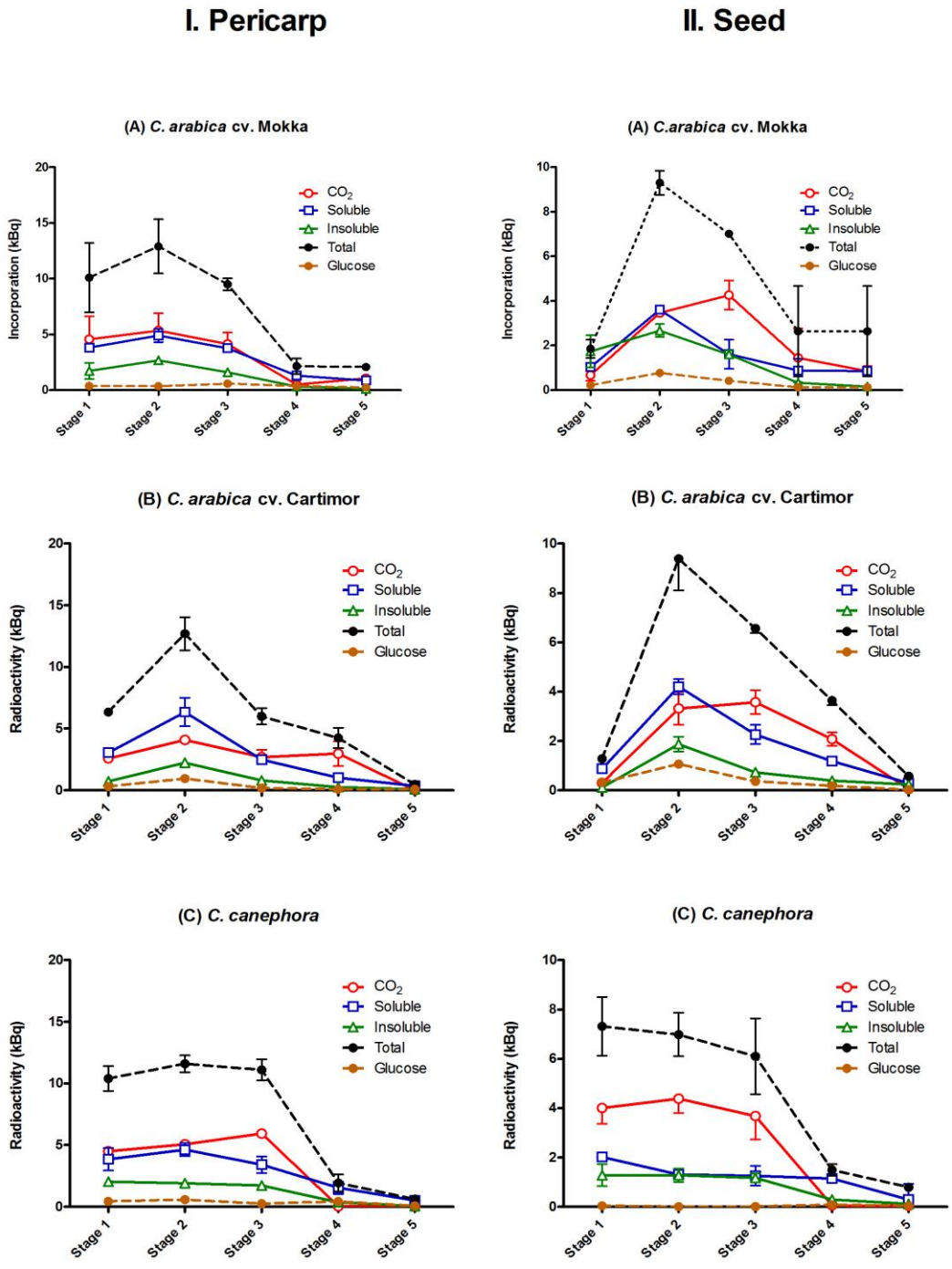

Figure 2. Changes in the incorporation of radioactivity from [U-^{14}C]glucose into CO_2, methanol-soluble and methanol-insoluble components.

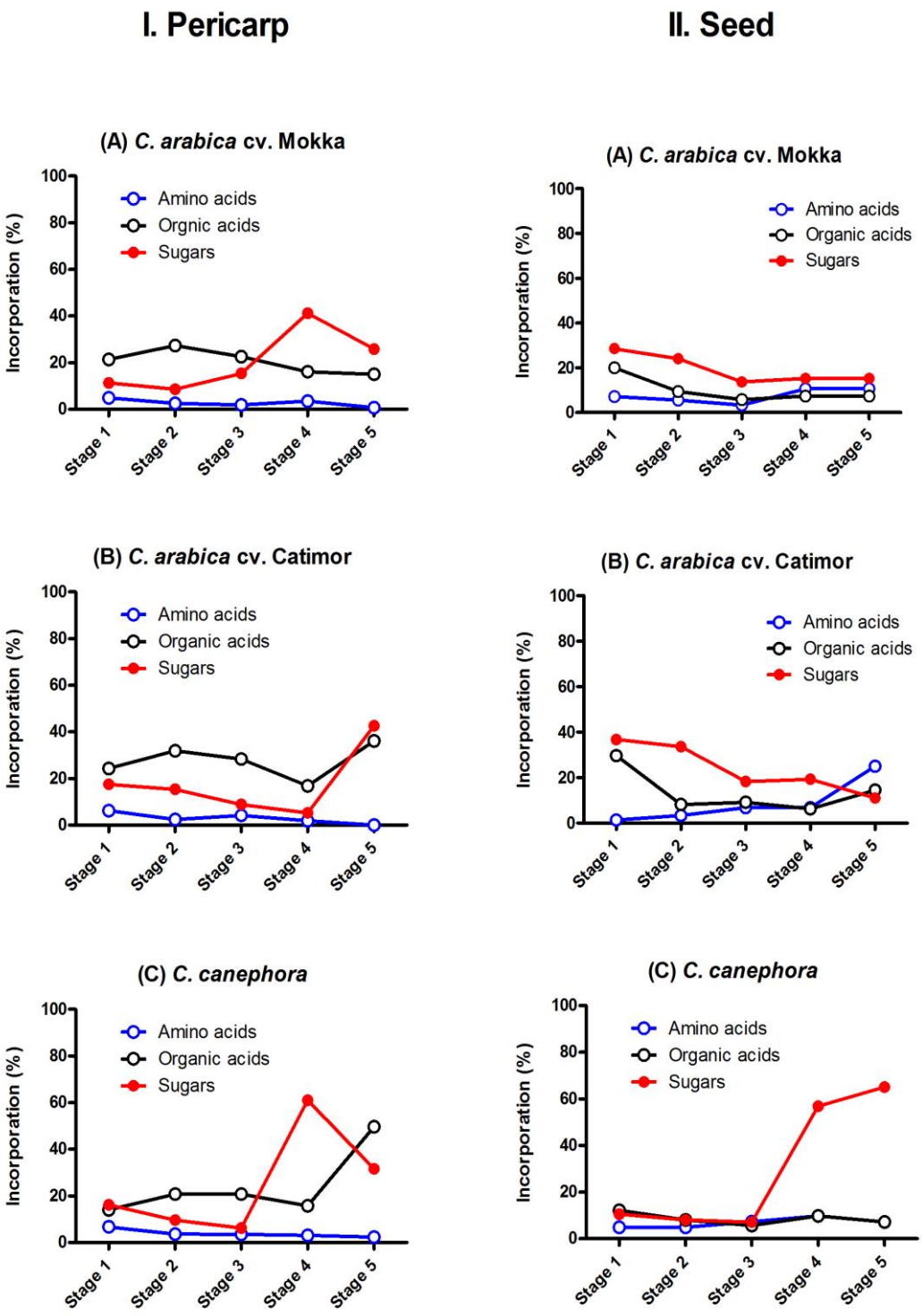

Figure 3. Distribution of the radioactivity from [U-^{14}C]glucose in the methanol soluble metabolites during growth. Incorporation into amino acids, organic acids and sugars is shown as % of total uptake shown in Figure 2.

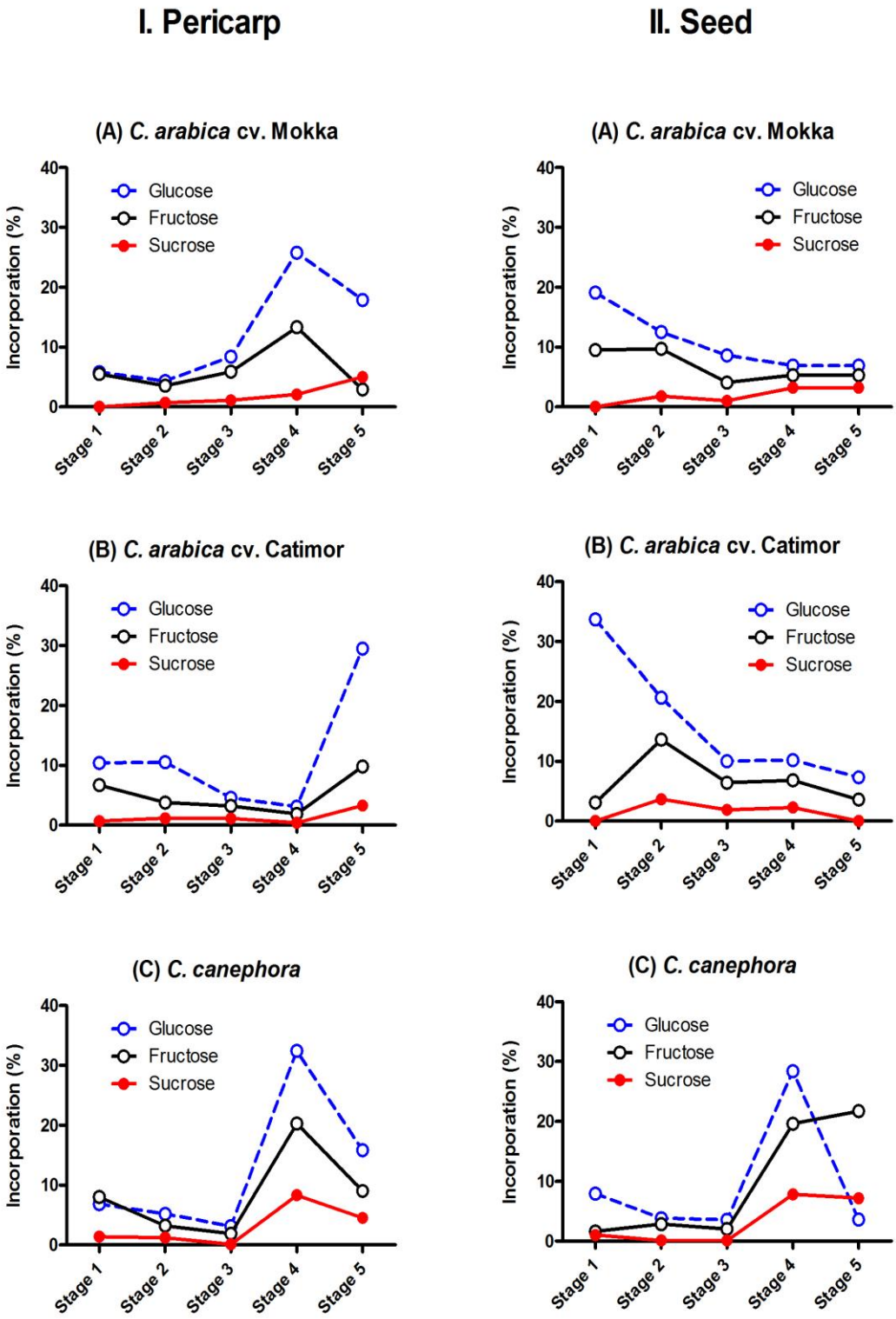

Figure 4. Incorporation of the radioactivity from [U-^{14}C]glucose into individual sugars during growth. Distribution of radioactivity in glucose, fructose and sucrose is shown as % of total uptake shown in Figure 2.

The total uptake of [U-^{14}C]glucose by the segments of pericarps and seeds of three coffee samples and incorporation of radioactivity into CO_2, methanol-soluble and methanol-insoluble fractions are shown in Figure 2. The radioactivity detected in glucose, some of which may be unmetabolized precursor, is also shown for reference. Since most of applied [U-^{14}C]glucose were metabolized and not retained in the tissues, uptake rates of [^{14}C]glucose seems to be directly related to the metabolic capacity of each tissue. In pericarps of *C. arabica* cv. Mokka, the rates of total uptake of [^{14}C]glucose during 18 h incubation in the fruits of stages 1–3 were 10–13 kBq/100 mg FW, but the rates reduced to ~2 kBq at the stages 4–5. Although slight difference was found (for example, higher value at stage 4 of *C. arabica* cv. Catimor), but the pattern of three coffee samples essentially resembled. Thus, in coffee pericarp, activity of glucose metabolism is active during development and is reduced on the ripening of the fruits.

The pattern of [^{14}C]glucose uptake in seeds was different between *C. arabica* and *C. canephora*. The rates of total uptake of [^{14}C]glucose in stage 1 seeds of *C. arabica* cv. Mokka and cv. Catimor were low (1.9 and 1.3 kBq/100 mg FW, respectively), and then increased at stage 2 (9.3 and 9.4 kBq, respectively) and after that they were gradually reduced. In contrast, the total uptake of [^{14}C]glucose was high in stages 1–3 of *C. canephora* seeds (6.1–7.3 kBq) and then decreased rapidly.

The incorporation of ^{14}C into CO_2, methanol-soluble and methanol-insoluble cellular components were examined. Most of CO_2 is released by the catabolic pathway of glucose, namely glycolysis and the tricarboxylic acid (TCA) cycle. In general, 35–60 % of glucose taken up by the tissues was catabolised in both pericarps and seeds. The respiratory glucose catabolism of exogenously supplied [^{14}C]glucose was markedly reduced in the ripening of fruits. Incorporation of ^{14}C into the methanol-soluble metabolites is usually higher than in methanol-insoluble metabolites. For example, in *C. arabica* cv. Mokka of green pericarps (Stages 1–3), 41–44 % of the total radioactivity was released as CO_2, 38–40 % of radioactivity was recovered as methanol-soluble fraction and the rest (17–21 %) was incorporated into methanol-insoluble fraction. No significant difference was found between in pericarp and in seeds, respectively 37–55 %, 33–56 % and 8–24 % of total radioactivity was found in CO_2, methanol-soluble and methanol-insoluble fractions of *C. arabica* cv. Mokka seeds of the stages 1–3.

Incorporation of radioactivity from [^{14}C]glucose into the methanol insoluble fraction usually occupied 10–20 % of total radioactivity. In this fraction mainly consisted of proteins and polysaccharides including starch and cell wall constitutes.[10] Special galactomannans and arabinogalactan polysaccharides of coffee seeds seem to be also included in this fraction.[14]

The methanol-soluble fraction was separated into three components using ion-exchange chromatography. Major components of the basic, acidic and neutral fractions of [^{14}C]glucose metabolism are amino acids, organic acids and sugars.[9] Therefore, data obtained in this study are shown as amino acids, organic acids and sugars and the values are presented as % of total radioactivity taken up by the samples (Figure 3). The radioactivity incorporated into amino acids was lower than that into organic acids, except for the later stages of seeds; the relative incorporation into amino acids (25 %) was higher than that into organic acids (15 %) in the stage 5 of *C. arabica* cv. Catimor seeds.

The neutral fraction was further analysed by TLC and found that most radioactivity was distributed in glucose, fructose and sucrose and the radioactivity located in other unidentified spots was extremely low (data not shown).

Changes in the distribution of radioactivity from [^{14}C]glucose into these three sugars are shown as % of total radioactivity taken up by the segments and shown in Figure 4. The radioactivity found in glucose may be mainly due to the unmetabolized glucose. The values were high in the later stages of pericarps in all coffee plants and the stage 1 of seeds of two cultivars of *C. arabica*. In general, radioactivity recovered in fructose was higher than in sucrose. Incorporation of radioactivity into sucrose from [^{14}C]glucose was detected both in pericarp and in seeds. The rates were slightly increased with the ripening of fruits. However, the activity of sucrose biosynthesis in pericarp and seeds of coffee fruits was restricted.

In situ metabolism of exogenously supplied [U-^{14}C]glucose suggests that possible metabolic pathways illustrated in Figure 5 are operative in pericarps and seeds of coffea plants. Glucose is converted to glucose-6-phosphate (G6P) and enters to the glycolytic pathway and then metabolised by the TCA cycle. In these processes, $^{14}CO_2$ is released at the steps of pyruvate dehydrogenase, isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase. The results of current studies indicate that active glycolysis and subsequent respiration occur during the development of fruits and its activity decreased with ripening the fruits. This is probably due to the fact that ATP is required for the development of coffee fruits. Organic acids, such as malate and citrate, are the members of the TCA cycle. Probably these two compounds are the major ^{14}C-metabolites during short (18 h) incubation as shown in other plant materials.[10] Considerable radioactivity from [U-^{14}C]glucose may be incorporated into amino acids via 2-oxoglutarate and then incorporated into proteins.[9] [U-^{14}C]glucose is utilized for the synthesis of sucrose and polysaccharides including cell wall constitutes and starch via UDP- and ADP-glucose.[14]

Coffee seeds accumulate sucrose (4–8 % dry weight), caffeine (1–2 %), chlorogenic acids (6–10 %), trigonelline (0.7–1 %) and polysaccharides (45–50 %).[15] However, little or no radioactivity from [U-^{14}C]glucose into these compounds was detected 18 h after administration. This may reflect that the distance of the pathway from glucose was long and biosynthetic velocity of these compounds is rather slow compared with the primary metabolites which turnover is fast. These secondary metabolites and storage compounds are gradually accumulated.

In conclusion, carbohydrate metabolism in young developing fruits is active and reducing sugars, such as glucose, are utilized for energy metabolism to produce ATP. Large portion of carbon-skeleton of sugars are released as CO_2 by respiratory metabolism, i.e., glycolysis and the TCA cycle, and the rest was utilized for the synthesis of organic acids, amino acids and sugars. These precursors are gradually converted to the storage compounds which are accumulated in coffee seeds.

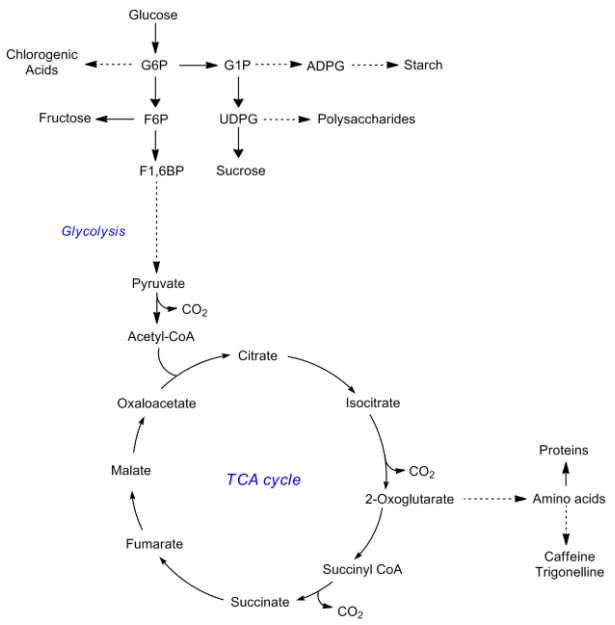

Figure 5. Possible metabolic routes of [^{14}C]glucose in pericarps and seeds of *Coffea arabica* and *C. canephora*. ADPG, ADP-glucose; F6P, fructose-6-phosphate; F1,6BP, fructose-1,6-bisphosphate; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; UDPG, UDP-glucose.

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