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Activities of phosphoenolpyruvate carboxylase and ribulose-1,5-bisphosphate carboxylase/oxygenase in leaves and fruit pericarp tissue of different coffee (*Coffea* sp.) genotypes

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Abstract

In order to study photosynthetic characteristics, phosphoenolpyruvate carboxylase (PEPC) and ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO) activities as well as soluble protein and chlorophyll contents were determined in leaf and fruit pericarp samples from diverse coffee genotypes (*Coffea arabica* cv. Colombia, Caturra, Caturra Erecta, San Pacho, Tipica, *C. stenophylla*, *C. eugenioides*, *C. congensis*, *C. canephora*, *C. canephora* cv. Arabusta, *C. arabica* cv. Caturra×*C. canephora* and Hibrido de Timor. We found a slightly higher PEPC activity in fruit pericarp than in leaves, while RuBPCO activity was much lower in pericarp than leaf tissue. Partial purification of PEPC and RuBPCO was carried out from leaves of *C. arabica* cv. Caturra and Michaelis-Menten kinetics for RuBPCO (K_m CO_2 = 5.34 μM), (K_m RuBP = 9.09 μM) and PEPC (K_m PEP = 19.5 μM) were determined. Leaf tissues of Colombia, Hibrido de Timor, and Caturra consistently showed higher content of protein [55.4-64.4 g kg^{-1}(f.m.)] than San Pacho, *C. stenophylla*, Tipica, Caturra Erecta, and Caturra×*C. canephora* [25.6-36.9 g kg^{-1}(f.m.)] and *C. canephora* cv. Arabusta, Borbon, *C. congensis*, *C. eugenioides*, and *C. canephora* [16.1-21.1 g kg^{-1}(f.m.)].

Additional key words: chlorophyll; enzyme activity; maize; Michaelis-Menten constants; total leaf proteins: *Zea mays*.

Introduction

Coffea arabica L. is the most important among the nearly 100 species of the *Coffea* genus and the only one cultivated in Colombia. The cultivar Colombia is widely spread in the country for its resistance to coffee rust (*Hemileia vastatrix* Berk *et* Br.) and among its parents is Híbrido de Timor, a natural hybrid from the crossing of *C. arabica* and *C. canephora* (Castillo and Moreno 1988). Changes made to the coffee cultivation methods during the last 20 years (from shaded to fully exposed to sunshine) require a better understanding of photosynthetic physiology in order to achieve enhancement of productivity. Nuñes *et al.* (1973) established that the photosynthetic behaviour of coffee plants was that of a typical C_3 one. However, Orozco and Cassalett (1974) observed anatomical features in leaf vascular bundle sheaths similar to those of C_4 plants (Laetsch 1974). These adaptations are not present in C_3 plants and have been confirmed by Vélez *et al.* (unpublished). Such characteristics may be associated with leaf photosynthetic activity, which is between 0.4 and 4.41 μmol(CO_2) m^{-2} s^{-1} (Nutman 1937, Nunes *et al.* 1968, Sondahl *et al.* 1976, Friend 1984).

Apple (*Malus* sp.) fruits have a system of refixing CO_2 through PEPC activity in an efficient manner, analogous to that exhibited by C_4/CAM forms, but with C_3 kinetics (Blanke and Lenz 1989). A similar form of PEPC has been found in rice (*Oryza sativa*) and other gramineous species (Imaizumi *et al.* 1990). Photosynthetic activity was found in fruits of soybean, pea, pepper, and barley (where 30 % of grain products come from pericarp photosynthetic activity), lemon and orange (Todd *et al.* 1961, Andrews *et al.* 1975, Steer and Pearson 1976, Atkins *et al.* 1977, Flinn *et al.* 1977, Bhatt and Srinivasa Rao 1993). Cannell (1985) found that photosynthetic activity of the coffee fruit could represent 20 to 30 % of the total photosynthesis of trees in full activity. However,

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Abbreviations: ATP – adenosine 5'-triphosphate; Chl – chlorophyll; DIECA – diethyldithiocarbamic acid; DTT – dithiothreitol; EDTA – ethylene diamine tetraacetic acid; MDH – malic dehydrogenase; NADH - nicotinamide adenine dinucleotide reduced; PEPC - phosphoenolpyruvate carboxylase; PVP-40 – polyvinylpyrrolidone; RuBPCO – ribulose-1,5-bisphosphate carboxylase/oxygenase;
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no detailed studies of photosynthetic characteristics of coffee fruit pericarp have been performed up to present. Great variation in the kinetic values of PEPC and RuBPCO in C_3 and C_4 plants, as determined in different tissues, is found in the literature.

As concerns RuBPCO kinetic constants in rice (*Oryza sativa*) leaves, K_m CO_2 is between 7.2 μM and 11.4 mM, K_m RuBP between 10 μM in cassava (*Manihot esculenta*) leaves and 75 μM in endosperm of germinating seeds of *Ricinus* sp. (Paulsen and Lane 1966, Benedict 1973, Osmond *et al.* 1975, Jensen and Bahr 1977, Jordan and Ogren 1981, Yeoh *et al.* 1981, Paul and Yeoh 1987, 1989, Makino *et al.* 1988, Woodrow and Berry 1988). For PEPC, K_m CO_2 values range from 27 μM in *Kalanchoe daigremontiana* to 0.3 mM in C_3 plants (Ting and Osmond 1973, Latzko and Kelly 1983, Amagasa 1984), while K_m PEP values fall between 27 μM in an isoenzymatic form of *K. daigremontiana* and 2.1 mM in maize protoplasts in darkness (Ting and Osmond 1973, Latzko and Kelly 1983, Tirumala Devi and Raghavendra 1992).

Materials and methods

Plants of *Coffea arabica* L. cv. Colombia, Caturra, Caturra Erecta, San Pacho, and Tipica, *C. stenophylla*, *C. eugenioides*, *C. congensis*, *C. canephora*, *C. canephora* cv. Arabusta, *C. arabica* cv. Caturra x *C. canephora*, Hibrido de Timor, and maize (*Zea mays* L.) ICA V305 were grown outdoors at the Centro Nacional de Investigaciones de Café – Cenicafé headquarters, Chinchiná (Colombia), at an altitude of 1425 m, average temperature of 20.3 °C, 2660 mm of yearly rainfall, and 83.5 % relative humidity, with an annual average sunshine of 1540 h (Cenicafé 1997). Leaf and fruit samples were obtained from the fourth node of branches of 2.5- to 3-year-old coffee plants from the collection of Cenicafé.

Leaf and fruit pericarp tissue extracts: 1 g of leaf or fruit pericarp tissue was ground in a chilled mortar with 4 cm^3 of modified Palmer (1986) extraction buffer: 0.05 M Tris-HCl, pH 8.0, 0.35 M sorbitol, 0.005 M EDTA, 0.005 M β-mercaptoethanol, and 3 % m/v PVP-40. The macerate was filtered through 4 layers of cheesecloth and an aliquot was taken for total Chl determination (Wintermanns and De Mots 1965). The remaining filtrate was centrifuged in an *Eppendorf 541-C* microcentrifuge at 4 °C and at 133 rps for 8 min. 1 cm^3 of supernatant was filtered through a *Biorad Econopac 10DG* column equilibrated with elution buffer [0.1 M Hepes-KOH, pH 7.5, 0.5 mM EDTA, 0.01 M magnesium acetate, 5 mM DIECA, 5 % (v/v) glycerol, 0.05 % *Triton X-100*, 0.02 M β-mercaptoethanol, 5 mM DTT, and 3 % (m/v) *PVP-40*] (Angelov *et al.* 1993). The eluate was used to determine the total soluble protein concentration (Bradford 1976).

PEPC and RuBPCO assays: PEPC activity was determined in a *Perkin-Elmer Lambda 3B* spectrophotometer with 1 cm^3 of 0.1 M Hepes-KOH, pH 8.0, 0.5 mM EDTA, 1 mM $MgCl_2$, 1 mM DTT, 0.01 M $NaHCO_3$, 0.2 mM NADH, and 5 units of pig heart MDH. Fifty mm^3 of the extract were incubated during 10 min at 35 °C and the reaction was started with 0.4 mM PEP (Angelov *et al.* 1993). RuBPCO activity was determined in 1 cm^3 of 0.1 M Hepes-KOH, pH 7.8, 0.5 mM EDTA, 0.01 M $MgCl_2$, 1 mM DTT, 5 mM phosphocreatine, 0.2 mM NADH, 0.01 M $NaHCO_3$, 1 mM ATP, 2 units of phosphoglycerate phosphokinase, 2 units of glyceraldehyde 3-phosphate dehydrogenase, and 1 unit of creatine phosphokinase, as modified from Usuda (1984). The enzyme (50 mm^3 of the extract) was incubated for 10 min at 25 °C and the reaction was started with 0.5 mM RuBP (Angelov *et al.* 1993). In both cases, enzyme activity was followed by NADH oxidation at 340 nm with an extinction coefficient of 6.22 mM (Segel 1976).

Michaelis-Menten kinetics for PEPC were determined using PEP as substrate, and for RuBPCO with CO_2 and RuBP.

Results and discussion

Soluble protein and Chl content in leaf tissues: Preliminary assays to determine soluble protein, Chl, and enzyme activity showed great variations mainly due to optic activity of polyphenols which interferred with that of NADH and Chl. Modification of Palmer (1986) extraction buffer solved this problem. Among the 13 genotypes three groups may be distinguished (Table 1). One group (Colombia, Híbrido de Timor, and Caturra) showed a high protein content [55.4-64.4 g kg^{-1}(f.m.)], another group (San Pacho, *C. stenophylla*, Típica, Caturra Erecta, and Caturra×*C. canephora*) showed a medium content [25.6-36.9 g kg^{-1}(f.m.)], and a third group showed a low protein content [16.1-21.1 g kg^{-1}(f.m.)]. In general, wild genotypes (*C. canephora*, *C. eugenioides*, *C. congensis*, and *C. canephora* cv. Arabusta), except for *C. stenophylla*, showed the lowest protein contents. Híbrido de Timor, a tetraploid interspecific hybrid resulting from spontaneous crossing of *C. arabica* and *C. canephora*

(Castillo and Moreno 1988), consistently showed a high leaf protein content. Leech *et al.* (1985) found that the degree of ploidy is closely related to concentration of RuBPCO in wheat. There was no correlation between the contents of protein and Chl, but the group with the highest protein content (Colombia, H. de Timor, and Caturra) also showed the lowest Chl/protein ratio. This could indicate a greater quantum efficiency of photosynthesis or a greater activity of photosynthetic carboxylation enzymes in these genotypes.

Table 1. Leaf protein content [g kg^{-1}(f.m.)], chlorophyll content [g kg^{-1}(f.m.)] and chlorophyll–protein ratio in twelve *Coffea* sp. genotypes. Means±SD of nine experiments.

Genotype	Leaf proteint	Chl ($a + b$)	Chl/protein
C. canephora	16.13 ± 6.25	1.59 ± 0.27	0.10
C. eugenioides	20.25 ± 4.10	1.77 ± 0.35	0.09
C. congensis	20.70 ± 3.87	2.60 ± 0.33	0.13
C. arabica cv. Borbón	21.16 ± 5.83	2.61 ± 0.04	0.12
C. canephora cv. Arabusta	21.16 ± 7.31	3.63 ± 0.35	0.17
C. arabica cv. Caturra×*C. canephora*	25.66 ± 2.35	3.97 ± 0.77	0.15
C. arabica cv. Caturra erecta	26.02 ± 7.00	2.86 ± 0.47	0.11
C. arabica cv. Típica	26.51 ± 5.43	2.39 ± 1.08	0.09
C. stenophylla	30.73 ± 11.4	2.33 ± 0.55	0.08
C. arabica cv. San Pacho	36.99 ± 2.04	4.00 ± 0.80	0.11
C. arabica cv. Caturra	55.44 ± 7.79	2.62 ± 0.42	0.05
Híbrido de Timor	63.14 ± 9.82	2.66 ± 0.30	0.04
C. arabica cv. Colombia	64.42 ± 9.54	2.33 ± 0.24	0.04

Enzyme activities in leaf and fruit pericarp extracts: Enzyme activities in leaf extracts (Table 2) indicated that the different coffee genotypes had a relatively high PEPC activity as compared to a typical C_3 plant [<17 nmol(NADH) kg^{-1}(f.m.) s^{-1}] (Nuñes *et al.* 1973), but lower than maize, the C_4 plant [143.2 µmol(NADH) kg^{-1}(f.m.) s^{-1}]. RuBPCO activity was greater in the three coffee genotypes than in maize, which indicates a typical C_3 biochemical behaviour in the coffee genotypes studied. Ting and Osmond (1973) have reported 320 µmol(NADH) kg^{-1}(f.m.) s^{-1} for PEPC of maize. The PEPC/RuBPCO ratio is representative of C_3 plants (<0.6) and lower than that of C_4 plants (1.7–5.0). Despite that PEPC activity in the three coffee genotypes was within the range of C_3, it was high. As proven for other crops (Meinzer *et al.* 1990), thermal stress may stimulate expression of C_4-type isoenzyme form of PEPC, which could be an explanation to the increased productivity of coffee fully exposed to sunlight.

In green fruit pericarp tissue from six genotypes of *C. arabica* (Colombia, Caturra, Borbón, and Tipica), Híbrido de Timor, and *C. canephora*, protein and Chl ($a+b$) contents were much lower than in leaf tissue of the same genotypes (Table 3). Apparently, genotypes with a higher Chl content trap more sunlight in the green fruit pericarp surface, but such efficiency also depends on fruit size, number of fruits per node, and plant architecture.

Híbrido de Timor had the highest PEPC activity [60 µmol(NADH) kg^{-1}(f.m.) s^{-1}] in green fruit pericarp, followed by Borbón and Caturra. On the other hand, Borbón had the highest RuBPCO activity [25.3 µmol(NADH) kg^{-1}(f.m.) s^{-1}]. In all cases, PEPC/RuBPCO ratio for green fruit pericarp tissue (1.85-17.49) was higher than that for leaf tissue and other C_3 plants (<0.6). This suggests that primary photosynthetic carboxylation in green fruit pericarp tissue is carried out mainly *via* an isoenzyme form of PEPC (probably C_4) more active than the form present in leaf tissue. Blanke and Lenz (1989) presented comparable activities of both enzymes in fruits and pods for other crops such as wheat, apple, pea,

Table 2. PEPC and RuBPCO activities [µmol s^{-1}] in leaves from two coffee (*Coffea arabica* L.) genotypes, Híbrido de Timor, and maize. Means± SDof three replicates.

Genotype	PEPC per kg^{-1}(f.m.)	kg^{-1}(Chl)	kg^{-1}(protein)	RuBPCO kg^{-1}(f.m.)	kg^{-1}(Chl)	kg^{-1}(protein)	PEPC/RuBPCO
Caturra	53.7±10.0	15.1±3.3	1.5±0.3	90.8±9.2	30.9±2.3	3.0±0.0	0.48
H. de Timor	57.7±11.7	17.4±3.3	1.6±0.7	98.5±10..0	32.9±3.5	3.2±0.0	0.53
Colombia	47.7±0.8	13,5±2.5	1.7±0.1	84.5±1.3	22.9±0.2	2.8±0.2	o.56
Maize	143.2±4.5	40.7±5.0	16.0±0.2	65.0±4.8	14.4±2.5	2.4±0.6	2.82

tomato, and citrus flowers. Fridlyand and Kaler (1986) suggest that high photosynthetic carboxylation activities found in C_3 plants can be explained by an unknown CO_2-concentrating mechanism. In coffee, Meinzer *et al.* (1990) found that efficiency in water utilization by plants under water stress could be explained by CO_2 fixation *via* PEPC. According to Cannell (1985), green coffee fruits supply 20-30 % of plant assimilates, contributing to the source–sink balance of the whole plant.

Table 3. Contents of chlorophyll, Chl (*a*+*b*) content and total proteins [g kg^{-1}(f.m.)], activities of PEPC and RuBPCO [µmol s^{-1}] in fruit pericarp samples from five coffee (*Coffea arabica* L.) genotypes and *Coffea canephora*. Means ± SD of three replicates.

Genotype	Chl (*a*+*b*)	Protein	PEPC per kg^{-1}(f.m.)	PEPC per kg^{-1}(Chl)	PEPC per kg^{-1}(protein)	RuBPCO per kg^{-1}(f.m.)	RuBPCO per kg^{-1}(Chl)	RuBPCO per kg^{-1}(protein)	PEPC/ RuBPCO
Colombia	0.12	1.17	22.5±3.3	193..8±29.0	19.2±0.3	6.9±0.7	5.9.0±7.0	5.9±0.7	3.26
Caturra	0.11	5.08	43.2±7.3	385.3±65.5	8.5±1.4	7.5±1.0	67.2±10.0	1.5±0.2	5.73
H. de Timor	0.11	0.70	59.7±7.0	552.3±66.2	84.8±10.0	3.4±0.0	31.5±4..0	4.9±0.5	17.49
Borbón	0.08	0.51	47.2±7.0	561.5±83.2	93.3±13.8	25.4±4.2	301..8±4.2	50.2±8.5	1.85
Tipica	0.06	0.80	58.8±6.3	600.5±113.0	73.5±7.8	4.8±0.5	85.7±10.2	6.0±0.7	12.25
C. canephora	0.08	3.42	50.7±6.5	603.2±77.3	14.8±1.8	6.9±1.0	81.5±12.2	2.0±0.2	7.35

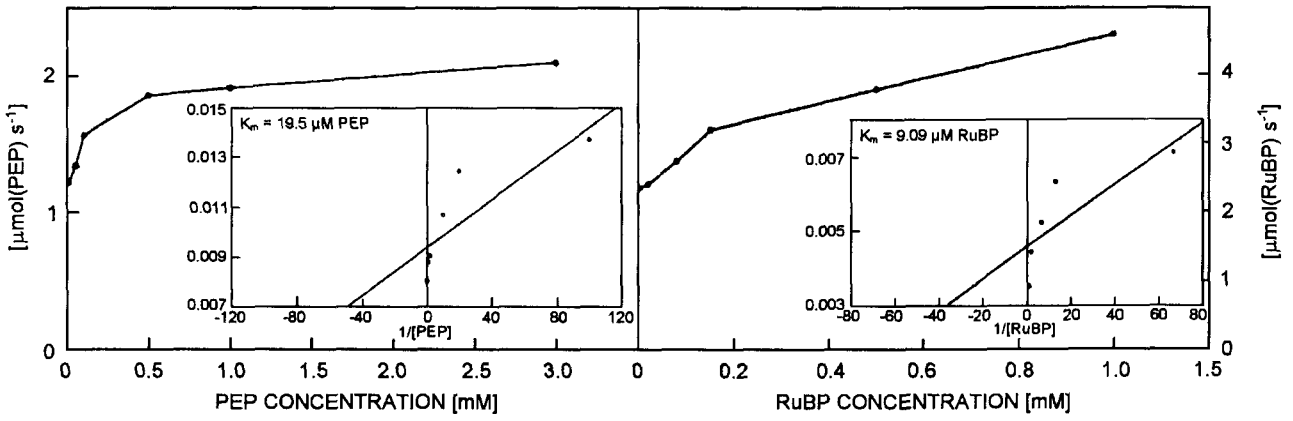

Fig. 1. PEPC (*left*) and RuBPCO (*right*) Michaelis-Menten kinetics and Lineweaver-Burk representations with PEP (*left*) or RuBP (*right*) as substrates in *Coffea arabica* L. cv. Caturra.

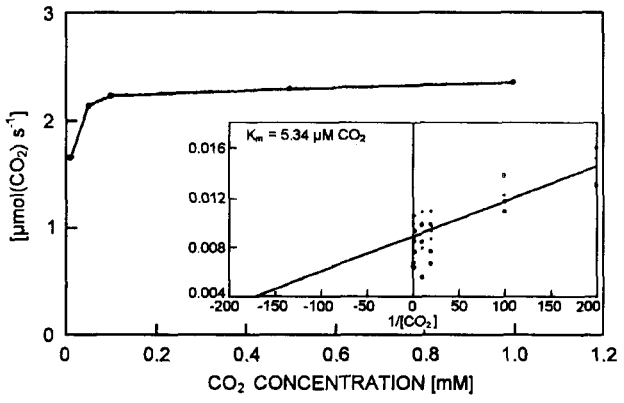

Fig. 2. RuBPCO Michaelis-Menten kinetics and Lineweaver-Burk representations with CO_2 as substrate in *Coffea arabica* L. cv. Caturra.

PEPC and RuBPCO enzyme kinetics in leaf extracts of *C. arabica* cv. Caturra: A typical Michaelis-Menten kinetics was found for PEPC using PEP as substrate (Fig. 1, *left*), with a maximum velocity of ~2 µmol(PEP) s^{-1}. K_m of PEP was 19.5 µM, a value within the range of C_3 plants PEPC, but in the lower limit, which suggests great affinity of the enzyme for the substrate in Caturra leaves. For RuBPCO, K_m of RuBP was 9.09 µM (Fig. 1, *right*), a value similar to those reported for the enzyme in other C_3 plants (Paul and Yeoh 1987). K_m for CO_2 5.34 µM (Fig. 2) was around the K_m reported for other crops. The K_m values have been used in *Manihot esculenta* Crantz to establish phyllogenetic differences within groups of genotypes (Paul and Yeoh 1987, 1989).

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