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RESEARCH ARTICLE

Sucrose metabolism during fruit development in *Coffea racemosa*

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Abstract

Sucrose is one of the compounds in the raw coffee bean that has been identified as an important precursor of coffee flavour and aroma. In order to increase our knowledge of sucrose metabolism in coffee species, sucrose and reducing sugar content were investigated in the fast fruit-ripening coffee species *Coffea racemosa*. Fruits were harvested regularly from anthesis, until the point of complete fruit maturation and tissue development, followed by measurement of fruit tissue weight. Pericarp was the most abundant tissue, and always represented around 70–80% of fruit fresh weight. The perisperm present in young fruits was rapidly replaced by the endosperm at around 40 days after flowering (DAF). In the latter tissue, total and reducing sugars decreased during development. However, sucrose content was maintained at a relatively high level throughout fruit development, with a peak at 40 DAF that coincided with the highest level of sucrose synthase (SUS) activity detected in this tissue. For all endosperm developmental stages analysed, very low invertase activity was observed, suggesting a limited role for this enzyme in sucrose metabolism. Northern blot experiments using *SUS1* and *SUS2* cDNA sequences from *Coffea arabica* as probes were carried out to study the expression of SUS-encoding genes. The *SUS1* transcripts of *C. racemosa* overlapped with the peak of SUS activity in the endosperm, whereas *SUS2* transcripts accumulated mainly during the latest stages of endosperm development. Altogether, these results suggest that the SUS1 isoform of SUS is essential for sucrose accumulation in the fruits of *C. racemosa*.

Introduction

Coffea racemosa Lour. (Rubiaceae) originates from East Africa and is characterised by abundant branching, high leaf drop during the dry season and rapid fruit maturation (Guerreiro Filho, 1992). *C. racemosa* produces a pale coloured aromatic beverage that is low in caffeine (Silva, 1956; Mazzafera, 1990) and of medium quality (Chevalier, 1947). It is consumed locally in several countries in East Africa (e.g. Mozambique), but is not commercially exploited as a result of its low productivity. Like most *Coffea* species, *C. racemosa* is diploid ($2n = 2x = 22$) and allogamous, with several self-incompatibility alleles (Silva, 1956; Mônaco & Carvalho, 1972). This species has several other interesting agronomical characteristics, which have been transferred to commercial coffee varieties by traditional breeding programmes. Because it can be easily crossed with *Coffea arabica* ($2n = 4x = 44$ chromosomes),

the use of *C. racemosa* in coffee breeding programmes is of particular interest for the development of new coffee hybrids that are resistant to biotic and abiotic stresses while maintaining high beverage quality (Guerreiro Filho *et al.*, 1991). *C. racemosa* is highly resistant to the leaf miner (*Perileucoptera coffeella*) and to several *Meloidogyne* spp. and is weakly infected by leaf rust (*Hemileia vastatrix*) under field conditions (Guerreiro Filho, 1992, 2006). It also presents a tolerance to drought and high temperatures, which is mainly explained by its deep root system. At the genomic level, *C. racemosa* was also one of the three coffee species used for the Brazilian coffee genome initiative (Vieira *et al.*, 2006). More than 15 000 expressed sequence tags (ESTs) of *C. racemosa* were generated from fruit cDNA libraries and clustered into 919 contigs and 3107 singletons. Today, these ESTs are the only ones from this species sequenced in the world.

The complete development of *C. racemosa* fruits is achieved over a shorter period (60–110 days) than in *C. arabica* (180–250 days) and *Coffea canephora* (270–330 days) (Wormer, 1964; Berthaud & Charrier, 1988). Compared with *C. arabica* fruits, those of *C. racemosa* are relatively small (8–11 mm length, 4–6 mm width), black coloured at maturity and very aromatic (Chevalier, 1947). They also have a thick and aqueous mesocarp (pulp), a fine endocarp and a small, yellow-coloured endosperm (Carvalho, 1967). The composition of the *C. racemosa* endosperm (bean) has not been studied in depth (Lopes, 1974), but recent studies report a trigonelline content close to 1% of dry weight (DW), a level intermediate to that of *C. arabica* (1.13%) and *C. canephora* (0.82%) (Campa *et al.*, 2004). Caffeine content is relatively low, varying from 0.8% to 1% (Lopes, 1974; Mazzafera & Magalhães, 1991; Mazzafera *et al.*, 1991). In the search for genetic factors controlling beverage quality, particular attention has been given to the sucrose content in coffee endosperm, which is considered to be a key precursor of flavour and aroma (Guyot *et al.*, 1996; Casal *et al.*, 2000; Leroy *et al.*, 2006). In *C. racemosa*, the average sucrose content has been estimated at 6.44% DW, closer to that of *C. canephora* (6.10%), compared with that of *C. arabica* (9.32%) (Guyot *et al.*, 1988; Ky *et al.*, 2001; Campa *et al.*, 2004).

Sucrose and its cleavage products (glucose and fructose) are also central molecules controlling the development of sink organs (Sturm & Tang, 1999; Koch, 2004). Sucrose transport from source leaves into sink organs is controlled by 'sink strength', which is defined as the ability of these organs to attract sucrose (Ho, 1988). In this scheme, sucrose synthase (SUS: EC 2.4.1.13) is often the predominant sucrolytic activity, providing assimilated carbon (i.e. uridine diphosphate-glucose) for respiration, starch synthesis and cell wall synthesis (Wang *et al.*, 1993; Fu & Park, 1995; Weber *et al.*, 1997). However, an increase in SUS activity associated with sucrose accumulation has been reported in several fruits, such as peach, citrus fruits and muskmelon (Lingle & Dunlap, 1987; Moriguchi *et al.*, 1990; Hubbard *et al.*, 1991; Komatsu *et al.*, 2002). Initial studies of sugar metabolism, sucrose-metabolising enzymes and genes coding for these enzymes in coffee were recently published (Leroy *et al.*, 2005; Geromel *et al.*, 2006). They showed that SUS exists as two isoforms, encoded by the *CaSUS1* and *CaSUS2* genes, which are phylogenetically distinct and differentially expressed during endosperm development. In addition, the SUS2 isoform was identified as one of the main enzymes controlling sucrose accumulation in coffee beans because the expression of its corresponding gene overlapped with the peaks in SUS activity and sucrose accumulation observed at maturation.

The present study was conducted to increase our basic knowledge of sucrose metabolism during the development of fruits in coffee species. Concentrations of sucrose, total and reducing sugars, as well as the activities of sucrose-metabolising enzymes, were monitored in the endosperm throughout fruit development of the fast-ripening *C. racemosa*. Relationships between sucrose and reducing sugar contents, the activities of sucrose-metabolising enzymes and the expression of SUS genes are discussed and compared with the information already available for *C. arabica*.

Materials and methods

Plant material

Fruits were collected from a 15-year-old plant of *C. racemosa* maintained in the living coffee collection at IAPAR (Instituto Agronômico do Paraná, Londrina, PR, Brazil). Fruits were harvested every 2 weeks after the main flowering (27 October 2004) until full ripening (3 January 2005). The phenological stages were as follows: stage 1 [12 days after flowering (DAF)], small fruits with seed mainly formed of aqueous perisperm; stage 2 (26 DAF), aqueous endosperm tissue progressively replacing the perisperm; stage 3 (40 DAF), milky endosperm; stage 4 (54 DAF), hard white endosperm with the remaining perisperm forming a thin green pellicle surrounding the endosperm and stage 5 (68 DAF), ripening fruits with pericarps turning to purple. To ensure developmental synchrony of harvested fruits, cross-sections were made to visually inspect the tissues that were separated, and the tissues were immediately frozen in liquid nitrogen and stored at −80°C for further analysis.

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Fresh weight evaluations

Fresh weights (FWs) were evaluated from 120 (3 × 40) fruits at 12 DAF, from 60 (3 × 20) fruits at 26 DAF and from 30 fruits (3 × 10) for the subsequent harvests. Tissue (perisperm, endosperm and pericarp) development was followed by weighting separated tissues obtained from 30 (3 × 10) fruits.

Sugar determination and enzyme analysis

Fruit tissues were lyophilised, ground with a mortar and pestle and extracted with 80% ethanol (300 mg per 1 mL) in a Polytron homogeniser. Extraction proceeded for 30 min at 75°C in sealed tubes and the supernatant was obtained after centrifugation. The extraction was carried out three times with the same volume of ethanol, and the combined supernatants were used for the analysis of total soluble sugar, sucrose and reducing sugar (Geromel *et al.*, 2006). Acid invertase (AI) was assayed in the direction of sucrose cleavage and SUS was assayed in the direction of sucrose synthesis, as previously described (Geromel *et al.*, 2006). Results of sugar contents are expressed in mg g^{-1} of dry weight (DW). For enzymatic activities, protein content was determined with a ready-to-use Bradford (1976) reagent (Bio-Rad, Hercules, CA, USA) and is expressed in nkat mg^{-1} protein. In both cases, results are given as mean ± SD of three independent extractions. Extracts obtained for enzyme analysis were used in Western blot experiments. The proteins were separated by 10% (w/v) polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue to demonstrate equal loading of proteins (data not shown). Proteins were further transferred to polyvinylidene difluoride membranes using a Mini protean electrophoresis apparatus (Bio-Rad). The membranes were probed with a polyclonal antibody directed against SUS from *Pisum sativum* using the protocol described by Déjardin *et al.* (1997) and developed using an antirabbit secondary antibody conjugated with alkaline phosphatase.

Expression analysis

Total RNA was extracted from frozen fruits (−80°C), as previously described (Marraccini *et al.*, 2001). Total RNA (15 μg) was denatured in 12.55 M formamide, 2.2 M formaldehyde and 20 mM 3-(*N*-morpholino)-propanesulfonic acid (MOPS) buffer, pH 7.0 [also containing 5 mM Na acetate and 0.1 mM ethylenediamine tetraacetic acid (EDTA)] at 65°C for 5 min and fractionated on a 1.2% (w/v) agarose gel containing 2.2 m formaldehyde in MOPS buffer. After transfer to nylon membranes (Amersham Biosciences, São Paulo, SP, Brazil), filters were prehybridised and hybridised as described for the use of the Ultrahyb™ buffer (Ambion, USA). Probes used corresponded to partial sequences of *CaSUS1* (GenBank accession number AM087674) and *CaSUS2* (GenBank accession number AM087675) cDNAs from *C. arabica* coding, respectively, for isoforms 1 and 2 of SUS (Geromel *et al.*, 2006) were labelled by random priming with 50 μCi of [α-^{32}P]dCTP (Amersham Biosciences) according to Sambrook *et al.* (1989). After hybridisation, membranes were washed under stringent conditions [2 × 15 min in 2× sodium chloride-sodium citrate buffer (SSC), 0.1% sodium dodecyl sulfate (SDS) at 65°C and 2 × 15 min in 0.1× SSC, 0.1% SDS at 65°C) and exposed to Kodak X-Omat AR-5 film (Kodak, São Paulo, SP, Brazil) with an intensifying screen. Amounts of RNA samples loaded were controlled by loading equal abundances of 18S and 26S rRNAs on gels stained with ethidium bromide.

Results

Fruit development

Under our field conditions, fruits of *C. racemosa* completed their development in 68 DAF (Fig. 1). The fruit growth curve, as measured by FW, exhibited a biphasic course with a first peak at 12–26 DAF and a second between 40 and 54 DAF (Fig. 2A). During fruit development, the pericarp was the most abundant tissue, representing almost 70–80% of total fruit FW (Fig. 2B). The perisperm tissue, originating from the maternal nucleus tissue, disappeared rapidly between 12 and 40 DAF, concomitant with a rapid expansion of the endosperm that occurred between 26 and 40 DAF. At this time, the endosperm occupied the entire locule space and its size was equal to that of the mature stage.

Sugar content in developing fruits

Sugar (total soluble sugars, sucrose and reducing sugars) content was measured in each tissue during fruit development (Fig. 3A–C). After a decrease at 26 DAF, total soluble sugar content increased regularly in the pericarp during maturation, reaching 448 mg g^{-1} DW at maturity (68 DAF) (Fig. 3A). Sucrose content represented one third of the total sugars in the pericarp at 12 DAF and less than 20% at maturity (Fig. 3B). This is because of a sudden accumulation of reducing sugars that occurred during the 2 weeks preceding the harvest (Fig. 3C).

In the perisperm, total sugars accumulated up to 130 mg g^{-1} DW at 26 DAF and decreased as this tissue gradually disappeared. The levels of sucrose and reducing sugar content in this tissue followed this pattern, with sucrose content always representing around 40% of total sugars. The endosperm had a high content of

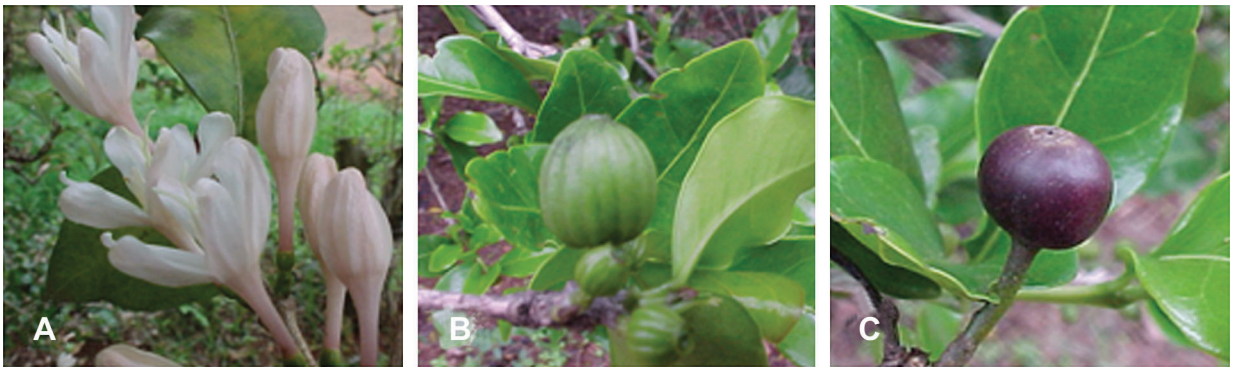

Figure 1 Development of *Coffea racemosa* fruits: flower at anthesis (A), green fruit at around 40 DAF (B) and fruit at maturity, around 68 DAF (C). Photographs were provided by Dr Oliveiro Guerreiro Filho, Instituto Agronômico de Campinas (IAC), Campinas, SP, Brazil.

soluble sugars (195 mg g^{-1} DW) at 26 DAF. Throughout its development, these soluble sugars decreased continuously. This decline was mainly related to reducing sugars, with only trace levels being detected at the mature stage (Fig. 3C). In contrast to observations in *C. arabica* and *C. canephora* (Rogers *et al.*, 1999), the decrease in reducing sugars was not accompanied by a concomitant increase in sucrose. Sucrose concentrations appeared to be relatively high during endosperm maturation, reaching a maximum value (90 mg g^{-1} DW) at 40 DAF and declining to 67.9 mg g^{-1} at the mature stage. At this time, sucrose represented approximately 100% of total soluble sugars.

Enzymatic activities in the endosperm during fruit development

Acid invertase and SUS enzyme activities were measured to determine if they might be correlated with variations in the amounts of soluble sugars. Protein extracts were prepared during endosperm maturation from 26 to 68 DAF (Fig. 4A). Low AI activity was observed in developing endosperm during all the phenological stages analysed. However, SUS activity showed a peak (3.02 nkat mg^{-1} protein) at 40 DAF that coincided with the rapid expansion of the endosperm. This activity declined steadily towards the end of maturation such that it was barely detectable in mature fruits.

A Western blot analysis of endosperm proteins was also carried out using antibodies against the major SUS isoform of pea teguments and corresponding to the protein product of the *PsSUS1* (AJ012080) gene (C. Rochat, personal communication). Under semidenaturing electrophoresis conditions, a SUS isoform (estimated molecular weight of 90.0 kDa) was detected in protein extracts with a peak at 40 DAF (stage 3); the isoform was detected in lesser amounts at 26 and 54 DAF (Fig. 4B). It is worth noting that the antibodies also reacted with another SUS isoform of lower molecular weight, which was revealed by a faint band at 26 and 54 DAF. However, no antibody cross-reaction was detected near maturation at 68 DAF.

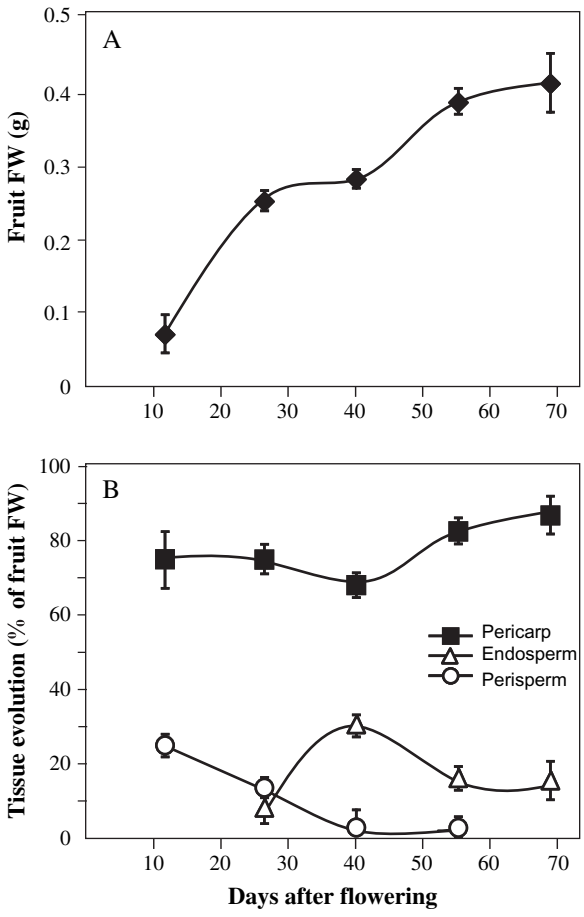

Figure 2 Weight of tissues during *Coffea racemosa* fruit ripening. (A) Fresh weights (FW) are given in grams for the entire cherry (◆). (B) Evolution of pericarp (■), perisperm (○) and endosperm (△) tissues, expressed as a percentage of cherry FW. Error bars indicate standard deviations (n = 3).

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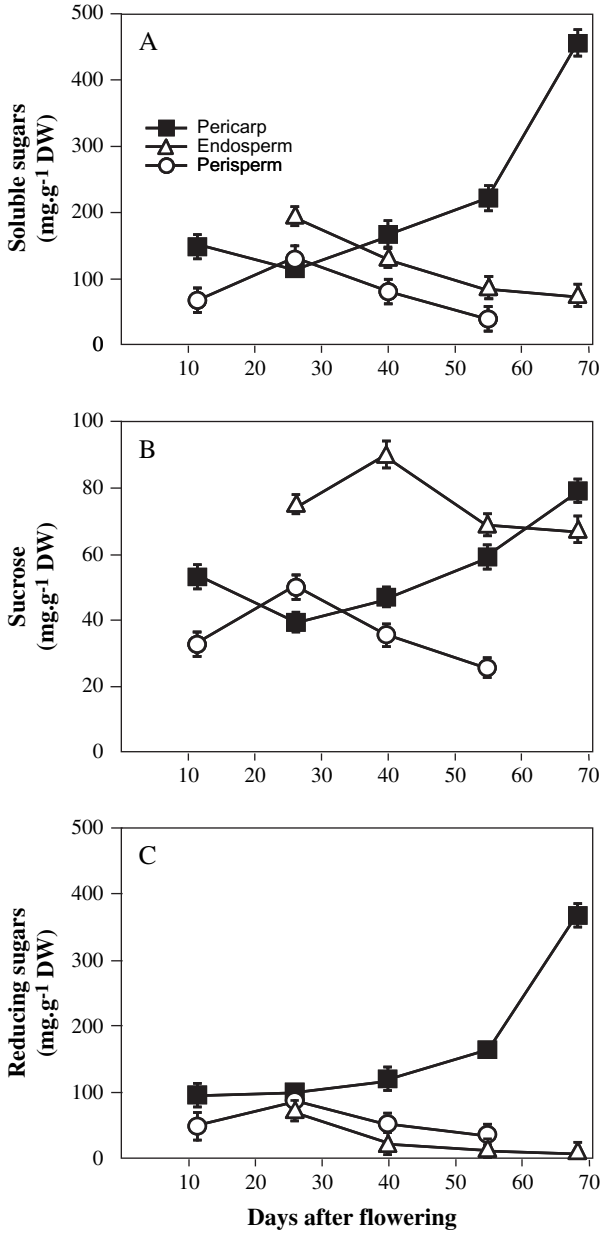

Figure 3 Total soluble sugar (A), sucrose (B) and reducing sugar (C) contents were measured in pericarp (■), perisperm (○) and endosperm (△) tissues separated from the fruits of *Coffea racemosa* during ripening. Values are given in mg g^{-1} of dry weight (DW). Error bars indicate standard deviations of repetitions (n = 3).

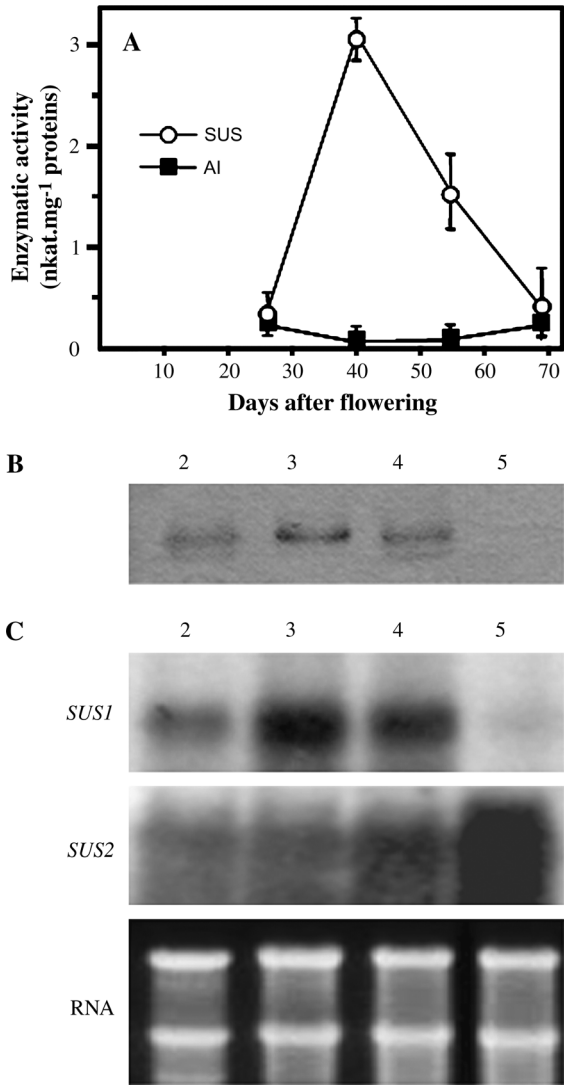

Figure 4 Sucrose synthase (SUS) and acid invertase (AI) activities in the endosperm of developing *Coffea racemosa* fruits. (A) Activities expressed in nkat mg^{-1} proteins. Error bars indicate standard deviations of repetitions (n = 3). (B) Western blot: proteins were extracted from developing endosperm (stage 2, 26 DAF; 3, 40 DAF; 4, 54 DAF and 5, 68 DAF) and probed with polyclonal antibodies raised against the abundant SUS isoform from *Pisum sativum*. (C) Expression of *SUS1* and *SUS2* genes of *Coffea racemosa* in developing endosperm. Total RNA (15 μg) was isolated from endosperm at regular stages (given before) of fruit development and hybridised independently with labelled probes corresponding to *SUS1* and *SUS2* cDNAs from *Coffea arabica*. Total RNA stained with ethidium bromide was used to monitor the equal loading of the samples.

Expression of SUS genes in the endosperm during fruit development

To analyse the expression of the SUS-encoding genes of *C. racemosa*, total RNA from endosperm harvested between 26 to 68 DAF was hybridised with *CaSUS1* and *CaSUS2* probes from *C. arabica* (Geromel *et al.*, 2006) (Fig. 4C). With *CaSUS1* probe, transcripts of approximately 2.9 kb were strongly detected in the endosperm between 26 and 54 DAF, with a peak at 40 DAF. With *CaSUS2* probe, transcripts of approximately 2.9 kb were weakly revealed at the earliest stages of endosperm development (26–40 DAF) but accumulated at a high

concentration at maturation (68 DAF). It is worth noting that at this time, the *C. racemosa* orthologous gene of *CaSUS1* was not expressed.

Discussion

Coffea racemosa fruits completed their development in 68 DAF. Despite this, the fruit growth curve exhibited a biphasic course that is commonly observed for other coffee species (Söndahl & Baumann, 2001). In *C. racemosa*, the pericarp constitutes the predominant tissue during fruit development. As in other coffee species, the perisperm is present in young fruits and is rapidly replaced by the growing endosperm (De Castro & Marraccini, 2006). The latest stages of fruit development are characterised by a significant increase in pericarp FW. In parallel, endosperm FW decreases drastically, characterising its intense dehydration during the latest stages of maturation (Eira *et al.*, 2006). These results show that the main difference in tissue development between the *C. racemosa* and *C. arabica* species concerns with the ratio of pericarp/endosperm FW, which reaches 5.4 in *C. racemosa* (this study) and 2.0 in *C. arabica* (Geromel *et al.*, 2006) at maturity.

Overall, sugar profiles in *C. racemosa* present some similarities with those observed in *C. arabica*, particularly in the pericarp, where sucrose and other sugars (total and reducing) accumulate late in development (Geromel *et al.*, 2006). This is also the case in the perisperm, where reducing sugars and sucrose present a transient accumulation early in the development. As previously reported in *C. arabica* and *C. canephora* (Rogers *et al.*, 1999), reducing sugar content decreased regularly throughout endosperm maturation of *C. racemosa*. However, sucrose levels appear relatively high and stable during endosperm development. This differs significantly from the situation observed in other coffee species, where sucrose contents were low in young endosperm and increased just before the harvest (Rogers *et al.*, 1999; Geromel *et al.*, 2006). In *C. racemosa*, sucrose content in mature beans was evaluated to be 6.8% DW, close to the value reported for this species (mean 6.44% with a range of 5.26–7.04% DW) by Campa *et al.* (2004). It is worth mentioning that lower contents of soluble and reducing sugars are observed in the pericarp exactly when sucrose has accumulated in the endosperm. This could characterise intensive sucrose exchange between these tissues. Vaast *et al.* (2006) estimated that pericarp photosynthesis may account for approximately 30% of the total carbon allocated in coffee fruits. The role of the pericarp in supplying photosynthates to developing coffee beans was also demonstrated by ^{14}C feeding experiments (Geromel *et al.*, 2006).

At an enzymatic level, our results show low AI activity in the developing endosperm of *C. racemosa*, thus suggesting that this enzyme plays a limited role in the developing coffee beans of *C. racemosa*. SUS activity was noteworthy in the endosperm, with a peak at 40 DAF that declined towards maturation. It is worth mentioning that maximum SUS activity coincided perfectly with the highest sucrose content, with the rapid endosperm expansion and with the peak of SUS isoform identified by Western blot. In fact, this experiment reveals the presence of two SUS isoforms of similar molecular weights at 26 and 54 DAF but not at 40 DAF. This could reflect the presence of two closely related SUS1 isoforms with similar epitopes leading to cross-immunoreaction with the antibodies, as reported for the SUS-SH1 and SUS1 isoforms of maize endosperm (Echt & Chourey, 1985; Chourey *et al.*, 1986). This could be tested using SUS isoform-specific antibodies (Duncan *et al.*, 2006). SUS isoforms detected by Western blot could also characterise the same protein but in different post-translational modification states, implicating ubiquitination or SUMOylation (small ubiquitine-like modifier), for example as these would increase the molecular weight by 8 to 10 kDa (Kurepa *et al.*, 2003).

In developing seeds, the sucrose-cleaving activity of SUS has been correlated with starch synthesis (Winter & Huber, 2000). As coffee endosperm has no apparent starch reserves (Rogers *et al.*, 1999; Bradbury, 2001), this is not a likely role for SUS in developing coffee fruits. Our results are consistent with the action of SUS in controlling sucrose accumulation in the fruits of *C. racemosa*. They also present some important differences with those previously reported in *C. arabica* (Geromel *et al.*, 2006). First, SUS activity is detected during endosperm expansion in *C. racemosa* but not in *C. arabica*. Second, the maximum SUS activity in *C. racemosa* was estimated to be 3.02 nkat mg^{-1} proteins at 40 DAF, considerably lower than the peak in SUS activity (18.7 nkat mg^{-1} proteins) observed at 234 DAF in *C. arabica*. It is tempting to speculate that the threshold differences in SUS activity observed between *C. racemosa* and *C. arabica* are responsible for the differences in sucrose level in the mature beans of these species (Campa *et al.*, 2004). A comparative study of SUS activity during fruit development in different coffee species with plants of the same age and cultivated under identical field conditions should clarify this point.

In *C. arabica*, two cDNAs (*CaSUS1* and *CaSUS2*) coding for phylogenetically distinct isoforms of SUS have been sequenced (Geromel *et al.*, 2006). Their corresponding genes presented different expression profiles during endosperm development, with *CaSUS1* being expressed in the earliest stages, while *CaSUS2* was exclusively expressed during maturation. The expression of SUS-encoding genes in the developing endosperm of *C. racemosa*

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was observed using *SUS1* and *SUS2* sequences as specific probes. Northern blots carried out under stringent conditions revealed transcripts hybridising with the two probes tested. This demonstrates that *C. racemosa* contains orthologous genes highly similar to those cloned and sequenced in *C. arabica*. A search of *C. racemosa* sequences homologous to *CaSUS1* and *CaSUS2* cDNAs was made within the Brazilian Coffee Genome Project (Vieira *et al.*, 2006). Twenty-two ESTs homologous (identity $\geq$97%) to *CaSUS1* cDNA were identified. After clusterisation, they form a partial contig of 1950 bp encoding a putative polypeptide of 334 amino acids that is 99% identical to the CaSUS1 protein (data not shown). However, *C. racemosa* ESTs related to the *CaSUS2* cDNA were not found. This situation resembles the one observed during the screening of the Brazilian Coffee Genome Project, where only 18 *CaSUS2*-homologous ESTs were encountered within a total of 130 792 ESTs from *C. arabica* (De Castro & Marraccini, 2006). This small number of ESTs is unexpected considering the high expression level of this gene observed at the mature stage both in *C. racemosa* (this study) and *C. arabica* (Geromel *et al.*, 2006). It could be explained by particular difficulties in reverse transcription of the corresponding mRNA or by an under-representation of ESTs specific of mature coffee fruits in the cDNA libraries of this project.

In *C. racemosa*, as in *C. arabica*, the *SUS1* gene is expressed during endosperm expansion and the *SUS2* gene is expressed at maturation. However, a profile comparison of gene expression and enzymatic activities in both species reveals some important differences. The first concerns *SUS1*, whose expression seems higher and more extended over endosperm development in *C. racemosa* than in *C. arabica*. In *C. racemosa*, *SUS1* expression overlaps perfectly with the peak in SUS activity, whereas no SUS activity accompanies the presence of *SUS2* transcripts. This is in contrast to the case observed in *C. arabica*, where the absence of SUS activity was found to coincide with *CaSUS1* expression, leading to the conclusion that CaSUS2 protein was the SUS isoform controlling sucrose accumulation ('source' function) in mature beans (Geromel *et al.*, 2006). Such discrepancies cannot be explained by methodological artefacts because strictly identical methods were used in both studies. Several lines of reasoning could explain the absence of SUS activity in the mature beans of *C. racemosa*. For example, it is possible that SUS2 transcripts are not translated, a situation that was observed for the *Sh* gene, coding for the SS1 isoenzyme of SUS in maize seedlings subjected to anaerobic stress (McElfresh & Chourey, 1988; Taliercio & Chourey, 1989; Guglielminetti *et al.*, 1996). This situation was also reported during tomato fruit development, where SUS mRNA and protein levels were not closely coupled (Wang *et al.*, 1994). Even after translation, the SUS2 protein could require some post-translational modifications to be activated. This has been reported in soybean, where sucrose-cleaving activity in root nodule organogenesis requires the binding of early nodulin (ENOD) 40 peptide A (Röhrig *et al.*, 2004). SUS protein has also been shown to be subject to reversible phosphorylation, controlled either by calcium-dependent protein kinase (CDPK) or plant sucrose non-fermenting (SNF)1-related (SnRK1) protein kinases (Huang & Huber, 2001; Halford *et al.*, 2003). Such post-translational modifications control SUS distribution between the cytosol, plasma membrane and actin cytoskeleton (Winter & Huber, 2000). They may also act on SUS protein turnover and limit SUS amounts in soluble extracts (Hardin *et al.*, 2003). *In vitro* translation experiments, as well as modified extraction procedures for proteins and immunolocalisation assays with SUS2-specific antibodies should clarify these points.

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