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Transient Occurrence of Seed Germination Processes during Coffee Post-harvest Treatment

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• *Background and Aims* The chemical composition of green coffee and thus the final coffee quality are specifically determined by the mode of post-harvest treatment, i.e. the wet and dry processing. Recently, it was shown that metabolic processes, i.e. germination and, a slightly delayed stress-related metabolism are executed during the course of processing. The specific ambient conditions of either post-harvest treatment may influence differentially the extent and time course of these metabolic reactions; therefore, the incidence and intensity of germination processes in coffee seeds were analysed during processing.
• *Methods* Expression of the germination-specific isocitrate lyase was monitored using competitive RT-PCRs analyses. Resumption of cell cycle activity and cell division were determined by flow cytometry, as well as by the abundance of β-tubulin quantified by Western blot analyses.
• *Key Results* The extent and the time courses of germination processes in coffee seeds differed significantly between wet and dry processed beans. The highest germination activity occurred 2 d after the onset of wet processing, whereas the corresponding maximum in the course of dry processing appeared about 1 week after the start of post harvest treatment.
• *Conclusions* As recently shown, there are specific differences in the chemical composition of differentially processed coffee beans. It is concluded that these substantial differences are the consequence of the differential expression of germination processes, i.e. they are the result of differences in the corresponding metabolic activities. The coherence of germination-related metabolism and of expression-specific coffee qualities establishes the basis for a novel approach in coffee research.

Key words: *Coffea arabica*, coffee processing, germination, post-harvest treatment, isocitrate lyase, β-tubulin.

INTRODUCTION

It is well known that the mode of coffee processing, i.e. the wet or the dry method, determines the quality of the corresponding green coffees, and establishes the characteristic flavour differences. The classical explanation for this phenomenon was based on the fact that, in general, only mature fruits are used for wet processing, whereas the dry processing includes fruits from various maturation stages. By demonstrating that these quality differences are still evident, even when identical coffee samples were processed by the two methods in parallel, the customary explanation was rebutted (Selmar *et al.*, 2002). Likewise, the composition of low molecular flavour precursors, i.e. carbohydrates (Knopp *et al.*, 2006) and free amino acids (Selmar *et al.*, 2002; Bytof *et al.*, 2005) is different in differentially processed green coffees. From this, it can be reasoned that the metabolic status of differentially processed coffees must be different. Quite recently, evidence was presented that germination processes are induced during the course of post-harvest treatment of coffee (Selmar *et al.*, 2006). However, the scheme depicted was rather qualitative. To quantify the differences in the metabolic reactions in differentially processed coffee seeds, more sophisticated methods, e.g. competitive RT-PCRs and flow cytometry, must be applied.

Coffee seeds (*Coffea arabica*) are classified as having an intermediate storage behaviour situated between orthodox and recalcitrant seeds (Ellis *et al.*, 1990). Coffee seeds can be dried down to a water content of $<10\,\%$ and viability declines upon further drying. The water content of the seeds inside the ripe fruit is relatively high [about $52\,\%$ on a fresh weight basis (Wintgens, 2004, Eira *et al.*, 2006) and the maturation drying is not very pronounced (Hinniger *et al.*, 2006)]. The seeds are able to germinate on water when isolated from yellowish-green fruits at around 225 d after anthesis, indicating an inhibition of precocious germination when the seed is still in the fruit (Eira *et al.*, 2006). Precocious germination within the fruit is most likely prevented by the joint effect of abscisic acid and the osmotic potential of the fruit flesh (Bewley and Black, 1994). In the course of wet processing, the fruit flesh (pulp) is mechanically removed, permitting initiation of seed germination. Confirmation that coffee seeds do, indeed, germinate during the course of processing was established recently on the basis of the expression of the germination-specific isocitrate lyase (ICL) and an increase in the abundance of β-tubulin, a marker for cell division or elongation (Selmar *et al.*, 2006). Surprisingly, germination-related ICL expression also takes places during dry processing, although in this case the pulp remains around the seeds. Obviously an endogenous induction of germination overcomes the pulp-related suppression of these processes.

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However, the extent and progression of germination should be different in coffees processed by these two methods. Based on competitive RT-PCR and flow cytometry analyses, the related metabolic processes in differentially processed green coffees could reliably be quantified for the first time. This paper unveils a clear picture of the different extent and time course of germination and suggests *Coffea arabica* as a promising object for the examination of the transition from embryogenesis to germination in a non-orthodox seed.

MATERIALS AND METHODS

Experimental processing

The experimental processings were performed using coffee cherries (*Coffea arabica* L., 'Acaiá') that had been shipped from Brazil by air to the laboratory of the Institute for Plant Biology, TU Braunschweig. For this, mature and sound coffee fruits were transferred directly after harvesting into Styrofoam boxes equipped with some ordinary ice bags. This procedure did not affect viability or germination capacity. The fruits arrived not later than 36 h after harvest and were used for the laboratory processing experiments. Identical material was used for both dry and wet processing. The procedures applied corresponded to the conditions in processing factories in the green coffee-producing countries.

For determination of the initial status (0 – unprocessed seeds), coffee seeds were shock frozen in liquid nitrogen promptly after detaching the fruits in the greenhouse. In all cases, prior to processing, the fruits had been manually sorted very carefully.

Wet processing

For wet processing, the fruits were manually de-pulped and the mucilaginous parchment beans were transferred into 5-L Erlenmeyer flasks adding an excess of fresh water. The coffee was fermented under the ambient conditions of the laboratory (21 °C) for 36 h during which the water was changed three times. The resulting parchment coffee was dried in a laboratory drying oven at 35–40 °C until the desired water content of 12 % (wet basis) was achieved. The beans were then manually de-hulled.

Dry processing

For dry processing, the mature coffee cherries were dried in a laboratory drying oven at temperatures of 35–40 °C until the desired water content of 12 % (wet basis) was achieved. The beans were then manually de-husked.

Expression studies

Based on known ICL sequences of various plants, redundant primers were created to generate a homologous probe for the ICL of *Coffea arabica*. A corresponding 480-bp fragment was cloned into a bacterial vector (TOPO TA), transformed into *Escherichia coli* (Cell DH 5α), amplified and used both as a probe for northern blots and for sequencing. Based on the sequence elucidated, specific ICL primers were created: ICL forward (3′-gggattgggacctgcctagaacc-5′) and ICL reverse (3′-agaggaaaggaaccatgaggttga-5′), which were used to produce a 424-bp PCR product.

Extraction of RNA

Coffee seeds were shock frozen, ground in liquid nitrogen and 200 mg powder were extracted using 2 mL peqGold-RNA-pure solution (peqLab) according to the corresponding instruction leaflet. To remove all carbohydrates, an additional purification step with peqGold OptiPure (peqLab) was performed. Then the DNA was removed by DNase I treatment. The complete DNA degradation was checked by PCR. The quality of the RNA was verified by electrophoretic analysis.

RT and PCR reaction

The RT reaction was performed with 100 ng total RNA, using the RevertAid™ H Minus First Strand cDNA Synthesis kit (MBI Fermentas). Instead of oligo dT, a specific ICL RT primer (3′-ccatgaggttgacacact-5′), was used (0·25 μL of 10 μM). To quantify reliably the transcript concentration, an internal RNA standard was added to the RT assay. The standard was constructed as follows: the vector containing the ICL probe (424 bp) was digested by *Mva*1269I and *Nde*I resulting in a linearized vector, containing both primer annealing sites, where the corresponding ICL fragment was reduced from 424 bp to 187 bp. Into this vector, a 942-bp fragment from pBR 322, also cut by *Mva*1269I and *Nde*I, was integrated. The construct was transcripted into RNA and purified. The corresponding PCR product resulted in a 1129-bp fragment, which could be easily differentiated on the gel from the ICL PCR product (424 bp). For competitive RT-PCR assays, 1, 10, 25 and 50 pg of the internal standard were added. For each sample, two independent sets of competitive RT reactions were performed.

PCR was performed according to the standard procedure using 1 μL of the RT incubation. To enhance the stringency, 3 % DMSO were added. The annealing temperature was 63 °C with 29 cycles. For each sample, three sets of PCR reactions were performed, resulting in an overall number of six RT-PCR sets, each performed with four concentrations of internal standard. PCR products were analysed by agarose electrophoresis (1·2 %) and stained with ethidium bromide. Quantification was performed using a Scion Image computer program.

β-Tubulin as a marker for cell division

Embryos were extracted out of the coffee seeds using a scalpel and homogenized in Modil-buffer (pH 6·8) according to de Castro *et al.* (1998), containing a protease-inhibitor cocktail (Complete Mini Protease Inhibitor, Roche®). Proteins were separated by SDS–PAGE (10 %) and blotted to a nitrocellulose membrane. β-Tubulin was detected using specific β-tubulin antibodies (TUB 2·1; Sigma). The secondary antibody was an anti-mouse IgG,

conjugated with alkaline phosphatase. Detection was performed using BCIP and NBT. Quantification was performed using Scion Image computer program.

Flow cytometry

Embryos were extracted out of the coffee seeds using a scalpel. Five embryos were transferred into 200 μL of TBS buffer (pH 7·3) containing a protease-inhibitor cocktail (Complete Mini Protease Inhibitor®, Roche) and chopped with a razor blade. Another 500 μL of TBS buffer, containing 5 μL RNase, were added to the embryo-fragment suspension. After 10 min incubation (RT) the suspension was filtered through 88-μm mesh nylon gaze; 5 μL aqueous propidium iodide (1 mg mL^{-1}) were added and incubated for 20 min (RT), before being used for flow cytometric analyses. The flow cytometer was a DakoCytomation CYAN and the software used was Summit (v. 4·0) and ModFit LT (v. 3·1; Verity Software House).

RESULTS

The onset of germination and the quantification of its progress can be assessed in several ways; for instance, by the determination of germination specific enzymes or their corresponding transcripts, and by the analysis of cell cycle activity. As coffee seeds are rich in fats, the expression of the isocitrate lyase (ICL), the key enzyme of the glyoxylate cycle, was chosen for examination (Zhang *et al.*, 1993; Reynolds and Smith, 1995). Moreover, ICL is regarded suitable to determine the transition from late embryogenesis to germination (Goldberg *et al.*, 1989). However, since a reliable, monofactorial estimation of the onset of germination is quite problematic in recalcitrant and intermediate seeds, the resumption of cell cycle activity in the coffee embryo was also analysed. For this, the presence of β-tubulin as well as the ratio of 4C to 2C nuclei, measured by flow cytometry, was quantified to register ongoing cell cycle activity as previously applied to germinating seeds of various plant species (de Castro *et al.*, 1995, 1998; Hilhorst *et al.*, 1998; Jing *et al.*, 1999; Vázquez-Ramos and Sánchez, 2003).

The overall expression of ICL in coffee seeds during early germination was too low for reliable northern-blot evaluation (Selmar *et al.*, 2006). Consequently, the expression of ICL was estimated by RT-PCR using homologous primers based on the sequence of the coffee ICL. For solid quantification, analyses were performed as competitive RT-PCRs using an internal standard created by insertion of an external DNA fragment, resulting in an elongated PCR product. These analyses revealed that ICL is expressed in coffee seeds during the course of wet processing as well as during dry processing. However, the corresponding time frames of expression were quite different. Whereas in the course of wet processing, maximal expression occurred only 2 d after the start of post-harvest treatment (Fig. 1A), in dry-processed coffee seeds the highest transcription level was detected about 1 week after the commencement of processing (Fig. 1B).

The resumption of cell cycle activity was monitored by flow cytometric analysis and by the estimation of β-tubulin formation. β-Tubulin, a major constituent of the cytoskeleton, was quantified on the basis of western-blot analyses of embryo protein extracts. During the course of post-harvest treatment, β-tubulin concentration increased significantly (Fig. 2). In wet-processed seeds, the highest β-tubulin accumulation was detected 2 d after the start of treatment (Fig. 2A), corresponding to the first day of drying of the parchment coffee. In contrast, during dry processing, the strongest signals were detected 6 d after initiation of processing, albeit at comparable maximum levels as in the wet-processed seeds.

The flow cytometric data for coffee embryos, extracted from differentially processed coffee seeds revealed a pattern similar to that elaborated from the β-tubulin analyses (Fig. 3A, B). Maximal 4C content was found in

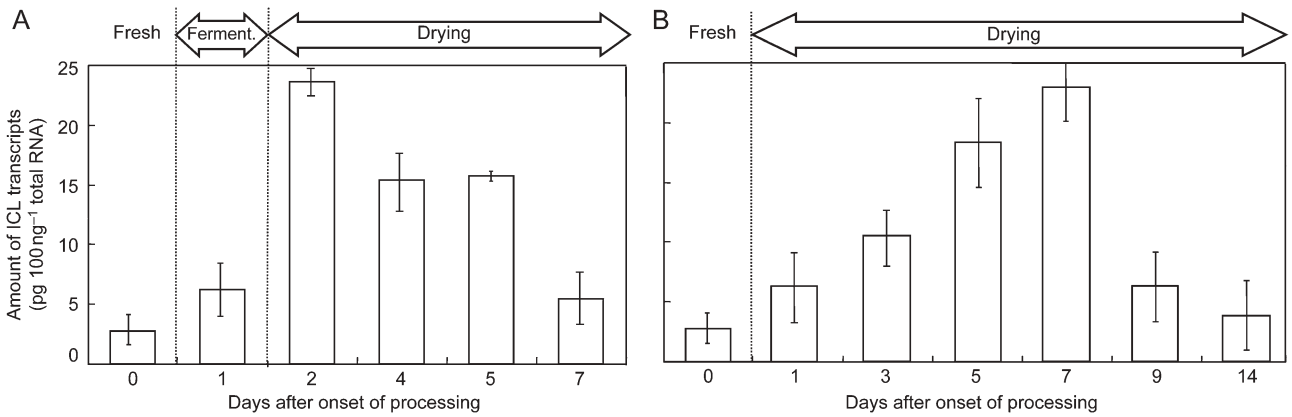

FIG. 1. Expression strength of ICL in fresh and differentially processed coffee seeds. For each sample, three independent sets of RT reactions, using 100 ng RNA each, were isolated from fresh and differentially processed coffee seeds. The corresponding DNA was used in an overall number of six RT-PCR assays, each performed with four concentrations of internal standard. 'Fresh' stands for fully ripe coffee beans. In the case of wet processing, fermentation (ferment.) of depulped beans lasted about 1 day, followed by drying at 30 °C in a hot-air cabinet; consequently, wet 1 d stands for a sample taken directly out of the fermentation tank, and wet 2 d corresponds to a sample, which was dried for about 1 d after fermentation. For dry processing, the entire coffee cherries were deposited directly in the hot-air cabinet. Error bars indicate s.d.

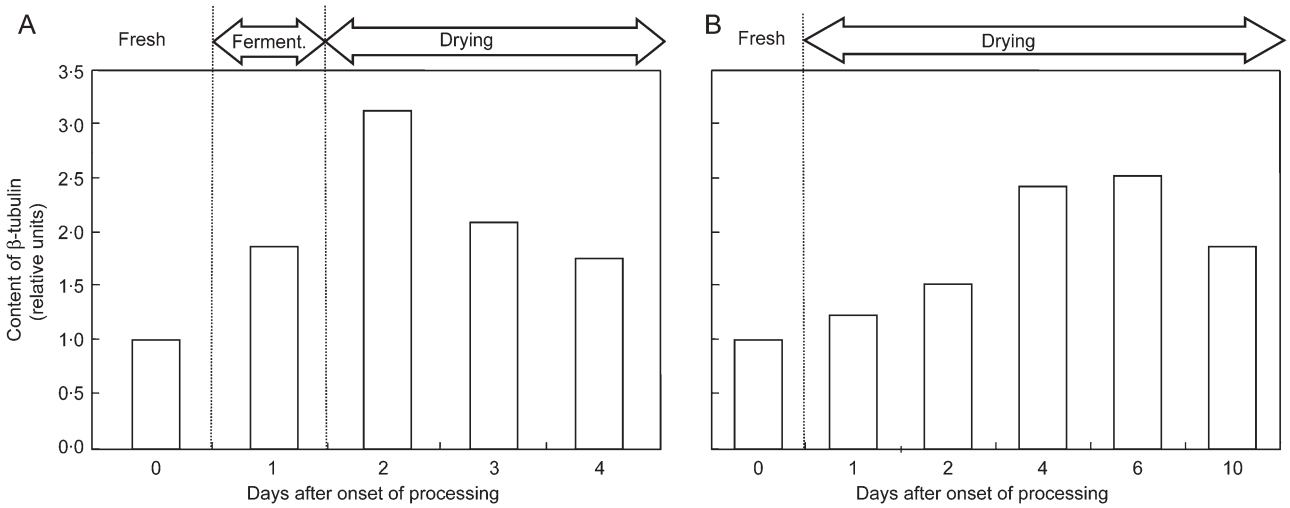

FIG. 2. Abundance of β-tubulin in embryos, isolated from fresh and differentially processed coffee seeds. For each sample, ten embryos were homogenized using 150 μl buffer. Of this extract, 10 μL was used for electrophoresis. After blotting and immunological detection, visualization was achieved by BCIP and NBT. For quantification, the Scion Image computer program was applied. Classification is as described in Fig. 1.

those samples that were also characterized by maximal β-tubulin content. The rate of DNA replication (increase in the frequency of 4C nuclei) was faster in embryos from wet processed beans compared with those from dry processed ones. However, the maximum frequency of 4C nuclei was altogether markedly lower in the dry-processed beans.

DISCUSSION

The hypothesis that the differences in differentially processed coffees are a consequence of differences in the metabolic status of seeds (Selmar *et al.*, 2002) was confirmed by the present work. In particular, the pattern and time courses of germination were specifically associated with the mode of post-harvest treatment.

ICL

The transient increase of ICL expression during wet processing reflected the onset of germination as a consequence of the removal of the pulp. However, an increase in enzyme expression was also observed in dry processed seeds, i.e. without removal of the covering structures. In this case, the increase started later but reached about the same maximum level. Apparently detachment from the mother plant or the onset of drying is enough to trigger transcription of the ICL gene. Either the presence of potential inhibiting factor in the fruit tissue, or the relatively lower moisture level of the seeds, seem to retard ICL transcription relative to the fully imbibed seeds during wet processing.

At first sight, it was surprising that various analyses of fresh coffee seeds also revealed minor ICL signals,

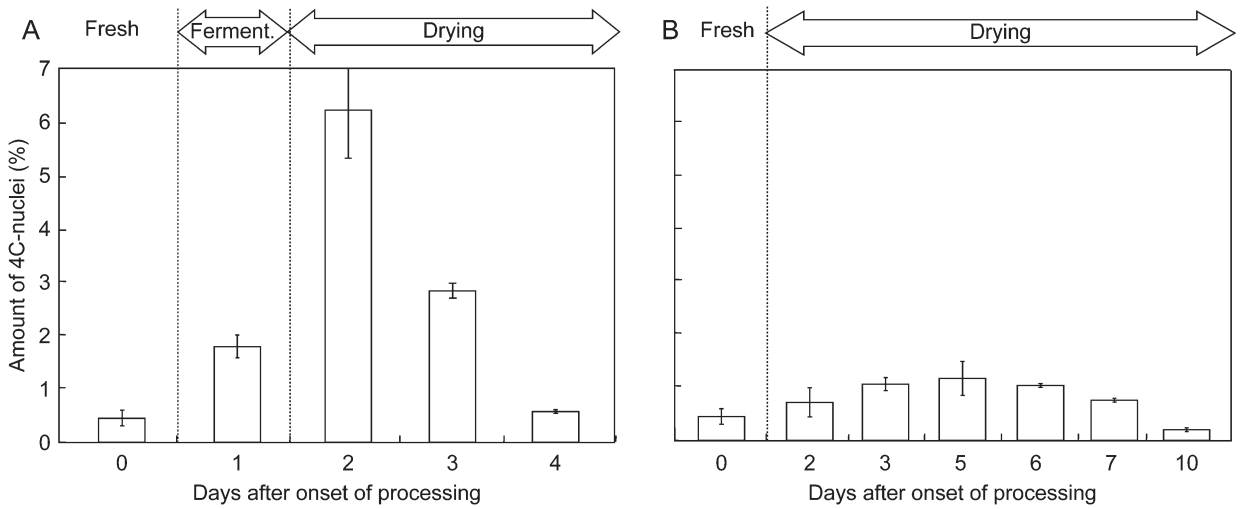

FIG. 3. Changes in the relative 4C-nucleus content in embryos, isolated from fresh and differentially processed coffee seeds. In each case, the nuclei of five embryos were released in 700 μL of TBS buffer and stained with propidium iodide. Error bars indicate s.d.

although germination should not yet have been unlocked in these fruits. In this context, it should be pointed out that those samples classified as untreated, fresh fruits could not be analysed earlier than 36 h after harvest in the plantation in Brazil – i.e. the time needed for the cooled transport to the laboratory in Germany. In contrast, coffee fruits that were harvested in the greenhouse in Braunschweig could immediately be shock-frozen in liquid nitrogen. These samples did frequently exhibit negligible or not even detectable ICL signals (data not presented). This again indicates that picking of the coffee fruit is enough to initiate ICL transcription. Oxygen may play a role in this. Montavon *et al.* (2003) reported that mature beans contain relatively low amounts of oxidized phenols and apparently the fruit plus silver skin protects the seed from oxygenation. In the same article, those authors showed that aerobic incubation of milled coffee bean tissue triggers the fragmentation or digestion of the 11S seed storage protein and the release of free amino acids. Interestingly, degradation of the 11S storage protein is one of the markers for seed priming and germination, e.g. with sugar beet (Job *et al.*, 1997).

Resumption of cell cycle activity

The overall pattern of the ICL expression was confirmed by the time courses of the resumption of cell cycle activity, estimated by the abundance of β-tubulin. However, for the flow cytometric data this was not the case. Whereas the time courses for the 4C : 2C ratio were similar to those of β-tubulin and ICL, the magnitudes were quite different. This difference in pattern compared with the equal levels of β-tubulin accumulation might be related to a requirement for higher seed moisture levels for initiation of DNA replication compared with the initiation of β-tubulin accumulation, as previously observed during priming of tomato seeds (Groot *et al.*, 1997). The analysis of cell cycle activity showed a decline in the frequency of 4C nuclei, a phenomenon which has never been reported with for instance tomato seeds, during drying after a priming treatment. The decline can be due to the occurrence of mitosis during drying, driving G_2 phase nuclei with 4C DNA levels to the G_1 phase with 2C DNA levels. It cannot be excluded that, after drying, 4C nuclei are more sensitive to the sample preparation causing nuclear membrane disruption and poor detection by the flow cytometer. However, in that case one would expect the flow cytometric profiles to show broader peaks for the 4C nuclei from dried embryos, which was not the case. Therefore with regard to cell cycle activity, it was most likely that drying of the seed after wet processing also induces the seed to mimic the seed maturation phase.

It is interesting to note that with both type of processing β-tubulin levels declined during further drying of the seeds. This decrease is in contrast to the situation observed in tomato seeds that are dried before finalizing the germination process in the frame of priming treatments (de Castro *et al.*, 1995). However, it resembles the decline in β-tubulin activity during tomato seed maturation (de Castro and Hilhorst, 2006). While coffee seed biology is similar to that of tomato seeds with respect to many aspects and *Coffea arabica* and *Solanum lycopersicum* (=*Lycopersicon esculentum*) share common gene repertoires (Lin *et al.*, 2005), the decline of β-tubulin observed would represent a clear difference.

It has been clearly established that coffee seeds undergo a germination-related metabolism during processing and that the extent of this metabolism strongly depends on the mode of post-harvest treatment. These findings for the first time establish a solid scientific basis to explain the occurrence of the specific differences in the chemical composition of differentially processed coffee beans and thus to provide their characteristic quality differences. This coherence to germination-related metabolism and its impact on processing-specific coffee qualities opens the door for a quite novel approach in modern coffee research.

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