Biochemical Insights into Coffee Processing: Quality and Nature of Green Coffees are Interconnected with an Active Seed Metabolism

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SUMMARY

Aroma potential and thereby coffee qualities are strongly influenced by the mode of processing. In the past, we have postulated that these processing-related quality variations are due to specific differences in biochemical reactions occurring within the viable coffee beans during processing. In this paper, unequivocal evidence is presented that various biochemical processes occur within the coffee seeds and that the coffee seeds are able to respond to changes in their environment. Also, evidence is presented that germination is initiated and proceeds during coffee processing. The germination-related metabolism was monitored by both the expression of germination-specific enzymes (i.e. the isocitrate lyase) and the resumption of cell cycle activity / cell division (estimated by ß-tubulin accumulation). The extent and time course of these germination processes depend on the mode of processing. Apparently, the removal of the pulp provides the stimulus for activating the preliminary, hardly perceptible steps of the germination process. Surprisingly, also in dry process seeds, germination-related metabolism occurs, probably due to endogenous triggering. However, here the germination processes reveal a time course different from that of wet process coffees.

Apart from germination, other metabolic processes have been found to occur in coffee beans. The most significant phenomenon is the accumulation of γ-aminobutyric acid (GABA), a typical response to drought stress. However, GABA is exclusively accumulated in dry process, and not in wet process, green coffees. The reason for these differences is the different time frame for the stress-related metabolism. In dry processed beans the stress-related synthesis of GABA is likely to take place over several days, leading to a large accumulation of this non-protein amino acid. In contrast, the induced stress metabolism in wet process beans is terminated before there is a significant accumulation of GABA, as the drying procedure is much faster in wet than in dry process beans. Only in the case of a prolonged drying period, wet process coffees also reveal high levels of GABA.

The data presented in this paper demonstrate the high relevance of the fact that green coffee seeds represent vital and living organisms, in which various metabolic reactions occur during processing, e.g. a germination-related metabolism and a stress metabolism. The extent of these processes depends on the conditions during processing and thereby influences coffee quality. Consequently, specific alterations of these conditions should influence these metabolic processes and affect coffee quality.

Moreover, the new insights can be used to explain potential quality differences of coffees resulting from classical wet and new progressive processing methods with mechanical mucilage removal, e.g. the aquapulping-methods. We deliberately introduced modifications of these progressive methods by adding intermediate storage phases in which the metabolic processes associated with the fermentation stage in wet processing could proceed. Sensory analyses suggested that the introduced modifications led to perceptible changes in the flavour profile.

INTRODUCTION

The question why wet process coffees reveal cup qualities different from those of dry process coffees may be as old as the introduction of the wet processing itself. However, a conclusive answer is still lacking. Wet process coffees are characterized by pronounced and distinctive aromas and a fine and pleasant 'acidity'. In contrast, dry processed coffees are characterized by their fuller body. In the past, these differences had been attributed exclusively to the facts that wet processing required much more accuracy and diligence, and that only fully ripe cherries were used. In order to monitor the intrinsic effects of processing, standard processings with identical starting material have to be performed in order to exclude any effect other than the mode of processing.

From plant physiological considerations it is obvious that more complex biological aspects should also be involved in this characteristic differentiation. In this context we have to consider that coffee beans – even after processing – are viable seeds, which is evident in the ability of fresh green seeds to germinate and grow. This means that processed coffee beans are able to respond metabolically to changes in their environment. Hence, we have postulated that internal metabolic factors – in addition to external factors – should be responsible for the observed differences in wet and dry process green coffees (Bytof et al., 2000; Selmar et al., 2002).

To elucidate the metabolic differences between wet and dry process coffees, we have performed numerous processing trials under carefully controlled conditions. In order to exclude any other effect than the mode of processing, it was necessary that absolutely identical starting material was used for the experimental processings.

EXPERIMENTAL

Model processings

Most of the experimental processings were performed in Brazil, Tanzania, Mexico and Colombia. In all cases – due to a very careful manual sorting – only fully ripe red cherries (*Coffea arabica* L.) were used as "identical starting material" for both, dry and wet processing trials in parallel. The resulting green coffees were shipped by air to the Institute for Plant Biology, TU Braunschweig and were analyzed both sensorially and biochemically. In addition, experimental laboratory processing trials were conducted. In all cases, fresh and sound red coffee cherries were used for both types of processing.

Sensoric assessment

The sensoric assessments were performed by professional sensory boards of the industrial partners, mainly from Tchibo and Kraft Foods.

Quantification of free amino acids

Coffee beans were 'shock' frozen and ground in liquid nitrogen. Amino acids were extracted with sulphosalicylic acid (4% w/v). After derivatization with *o*-phthaldialdehyde / 2-mercapthoethanol (OPA/MCE), they were separated by HPLC on a C18 column and detected fluorometrically (λ_{ex} = 334 nm; λ_{em} = 425 nm). For quantification, norvaline was added as internal standard.

Quantification of soluble sugars

The composition of low molecular carbohydrates was analyzed by a HPAEC system on a DIONEX® PA20 column with a sodium hydroxide gradient. For detection, a pulsed amperometric detector (PAD) was used.

Expression studies of isocitrate lyase using RT-PCR

Based on the alignments of known isocitrate lyase (ICL) sequences of various plants, redundant primers were created to generate a homologous probe for the isocitrate lyase of *Coffea arabica*. A corresponding 480 bp-fragment was cloned into a bacterial vector (TOPO TA). The modified plasmid was transformed into *E. coli* (Strain DH 5α), amplified and used for both, as probe for the detection of ICL-messengers in Northern blots and for sequencing. Based on the elucidated sequence, specific isocitrate lyase primers were created, and RT-PCR analysis was performed using RNA isolated from fresh and differently processed coffee seeds (for details see Selmar et al., 2004).

ß-tubulin as marker for the resumption of cell cycle activity and cell division

Isolated coffee embryos were homogenized with Modil-buffer (pH 6.8) containing a protease-inhibitor cocktail according to De Castro et al. (1998). The protein extracts were separated by SDS-gel electrophoresis. After blotting on a nitrocellulose membrane, the proteins were detected immunochemically using specific ß-tubulin antibodies (TUB 2.1, Sigma) in a dilution of $1{:}10^{-6}$. Secondary antibody was an anti mouse IgG, conjugated with alkaline phosphatase. Detection was performed using BCIP (5-bromo-4-chloro-3-indolylphosphate) and NBT (nitroblue tetrazolium).

RESULTS AND DISCUSSION

Sensorial differences of wet and dry process coffees

The sensorial assessments confirmed the corresponding results which have already been published in part (Selmar et al., 2002): the known differences in the cup quality of dry and wet process coffees are also evident when identical starting material is used for both types of processing. Thus, we have proved unequivocally that these quality differences are – at least partly – intrinsic to the mode of processing itself. In other words: there are substantial differences in the differentially processed coffee beans, which are principally generated in the course of postharvest treatment. The question arises as to whether these differences could be correlated with definite differences in the metabolic status of the differentially processed coffee seeds.

Chemical differences of wet and dry process coffees

In order to determine such metabolic differences, we chose the composition of free amino acids and the content of soluble carbohydrates as related markers. Both represent important precursors for coffee aroma compounds. Extensive and detailed data on the composition of free amino acids have been published by Bytof et al., (2004) and Selmar et al. (2002). In this paper, just the overall results are summarized: in all model and experimental processing trials with identical starting material, the total concentration of free protein amino acids in wet processed seeds is markedly higher than in dry processed seeds (Table 1).

Table 1. Total content of free protein amino acids in dry and wet processed green coffees.

model- and experimental green coffee production by traditional wet and dry processing in parallel	free protein amino acids [mg / kg f.w.]		
	dry processing	wet processing	difference (%)
plantation (Brazil, A)	3,570	4,310	20,9
plantation (Brazil, B)	4,030	4,360	8,2
plantation (Colombia)	2,760	2,850	3,3
laboratory (Brazil - Germany)	5,050	5,400	6,9
laboratory (Peru - Germany)	3,710	3,940	6,2
laboratory (Kenia - Germany)	3,250	3,570	9,8

The variations between single trials, even between processed samples from the same plantation but using different batches, were extremely high. These variations offer an explanation for the failure to detect differences in former studies. It is absolutely necessary to use the same plant material for both processing trials. Only an arduous manual sorting, which allows the use of completely identical plant material for both processings, enables the detection of quantitative differences of substantial factors, which *per se* are highly variable. Moreover, it is also necessary that for laboratory trials realistic processing conditions are applied. Unfortunately, in the work of Arnold and Ludwig (1996) the conditions chosen for the dry process did not match the real dry method but rather resembled the “semi-washed” process.

The major sugar present in coffee beans is sucrose, where, between samples, the variation in its content is nearly as high as that of the amino acids. We detected distinctive and reliable differences in the contents of other sugars that correlated with the mode of processing applied. In Figure 1, the glucose and fructose contents in wet and dry process coffees are compared. The contents of glucose and fructose are about five to ten times higher in dry processed than in wet processed beans.

The comparison of the glucose and fructose levels in fresh and processed coffee seeds reveals that fresh and dry processed beans have nearly the same content. In contrast, in wet processed coffees the corresponding levels are low, indicating that during wet processing the content of these sugars decreases. We suggest that this loss originates from an enhanced glucose turnover due to anaerobic fermentation in the endosperm tissue; however, also a leaching cannot be excluded. The data on the differences in the contents of free protein amino acids

and soluble sugar identify for the first time definite differences between wet and dry processed coffee beans, and thereby indicate the occurrence of metabolic changes.

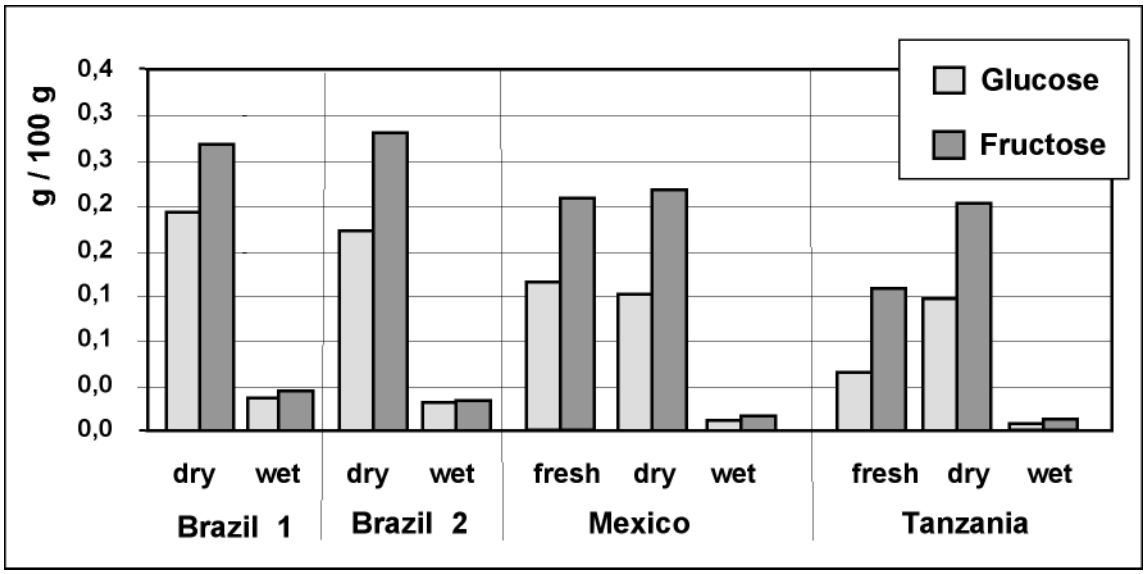

Figure 1. Glucose and fructose contents in wet and dry process green coffees from experimental processing trials using exclusively fully ripe coffee cherries.

Biochemical causales for the differences of wet and dry process coffees: germination

What might be the biochemical causales of the observed metabolic differences? As outlined earlier (Selmar et al., 2002), the recalcitrant coffee seeds, in principle, are able to germinate within the fruit. However, germination is inhibited by active principles located in pulp (phytohormones, high osmotic potential, germination inhibitors). Metabolism is unlocked, as soon as the pulp is removed such as in wet processing. This induction should alter the composition of substances present in the seeds and thus influence the coffee quality. In contrast with dry processing the pulp remains around the seeds and the metabolism should not be activated until endogenous unlocking occurs.

The exact point of time when germination starts cannot easily be determined, especially in recalcitrant seeds. We chose the expression of putative germination-specific enzymes and the resumption of cell cycle activity and cell division as reliable germination markers. One of the first enzymes expressed in germinating seeds is the isocitrate lyase (ICL), the key enzyme of the glyoxylate cycle, which is responsible for the conversion of stored lipids into carbohydrates. We investigated the expression of ICL during green coffee processing. Unfortunately, related Northern blot analyses revealed that the number of transcripts, even in young seedlings, is very low. Consequently, the related expression studies were performed using the more sensitive RT-PCR technique.

In RT-PCR experiments it could be clearly shown that in fresh coffee seeds ICL is not expressed (Figure 2). In contrast, in wet processed green coffees, a positive signal of the ICL was detected. Surprisingly, also in dry processed coffee seeds, transcripts for ICL were found (Selmar et al., 2004). However, the time course of its expression is different.

In Figure 2, the experimental data of RT-PCR gels from about ten independent experiments are summarized. Whereas in wet processed seeds maximum expression of isocitrate lyase was

detected about one day after the start of the processing, the corresponding maximum in dry processed beans was achieved several days later.

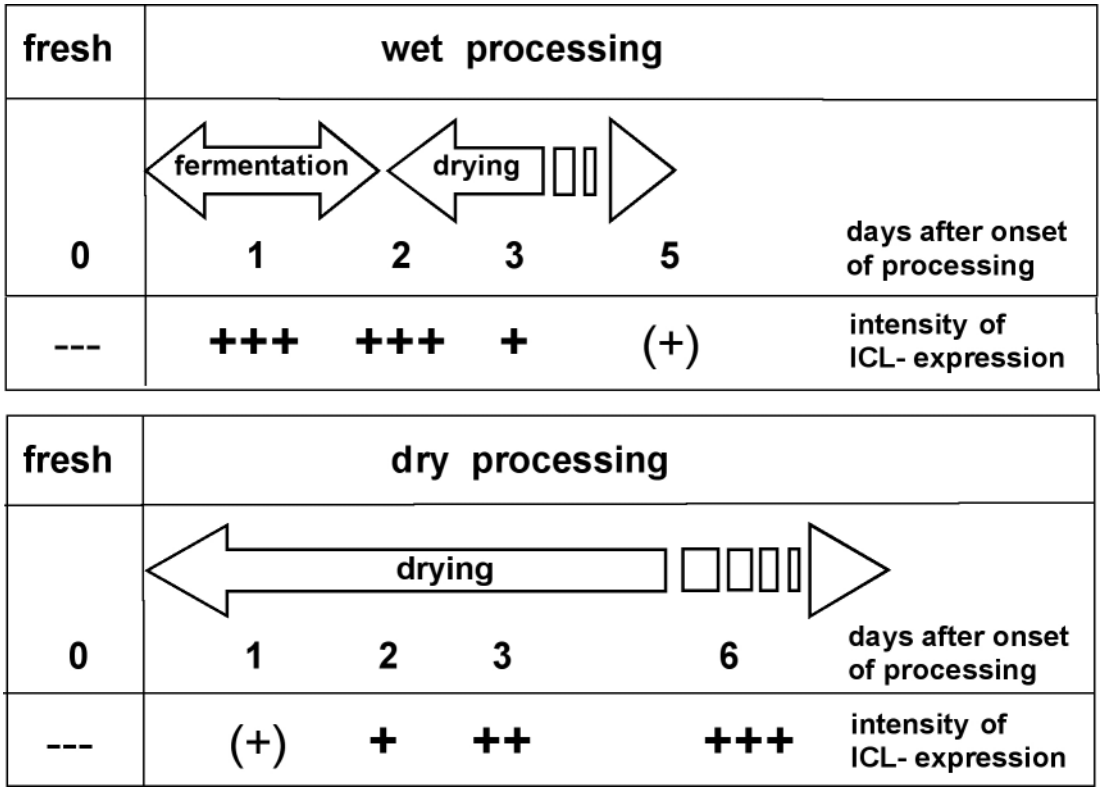

Figure 2. Expression pattern of ICL in fresh and processed green coffees.

A further suitable marker for germination is the resumption of cell cycle activity and cell division. To monitor these events, we analyzed the occurrence of ß-tubulin, the major constituent of the microtubules, which are necessary for cell division. The occurrence of this small protein is clear evidence for cell cycle activity and is considered to be a reliable marker for the onset of germination in seeds.

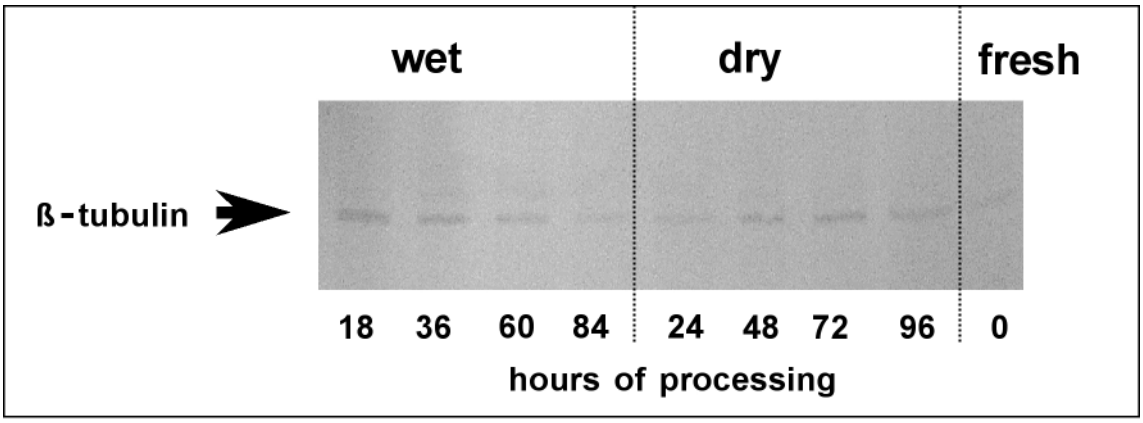

Figure 3. Western blot analysis of ß-tubulin in fresh and wet and dry processed green coffees. Equal amounts of plant material in each lane.

As the embryo is the organ where these processes occur first, we prepared embryo protein extracts, separated them electrophoretically, and, after subsequent blotting on a nitrocellulose membrane, analyzed the proteins by using anti-ß-tubulin antibodies.

In embryos prepared from fresh coffee seeds, no ß-tubulin was detectable. In contrast, in wet processed seeds, significant amounts of ß-tubulin were present. ß-Tubulin was determined also in dry processed seeds; however, the time courses of accumulation were quite different. In wet processed coffee seeds, maximum ß-tubulin accumulation was achieved within the first day of processing, shortly after pulp removal, whereas in dry processed seeds, maximum ß-tubulin accumulation was found not earlier than several days after the onset of processing.

The data of the ß-tubulin accumulation fully confirmed the corresponding data from the isocitrate lyase expression studies. Thus, these experiments prove without any doubt that in coffee seeds, during the course of processing, germination-associated processes are initiated and that the extent of the related changes depends on the mode of processing.

Biochemical causales for the differences between wet and dry process coffees: stress metabolism

In addition to the germination processes mentioned, further metabolic reactions take place in the coffee seeds during postharvest treatment. In dry process coffee, high amounts of γ-aminobutyric acid (GABA) are accumulated, whereas only small amounts of this non-protein amino acid are present in wet process beans. Untreated fresh seeds contain only traces of GABA. In plants, GABA is considered to be a stress metabolite, typically accumulated in tissues that are exposed to drought stress.

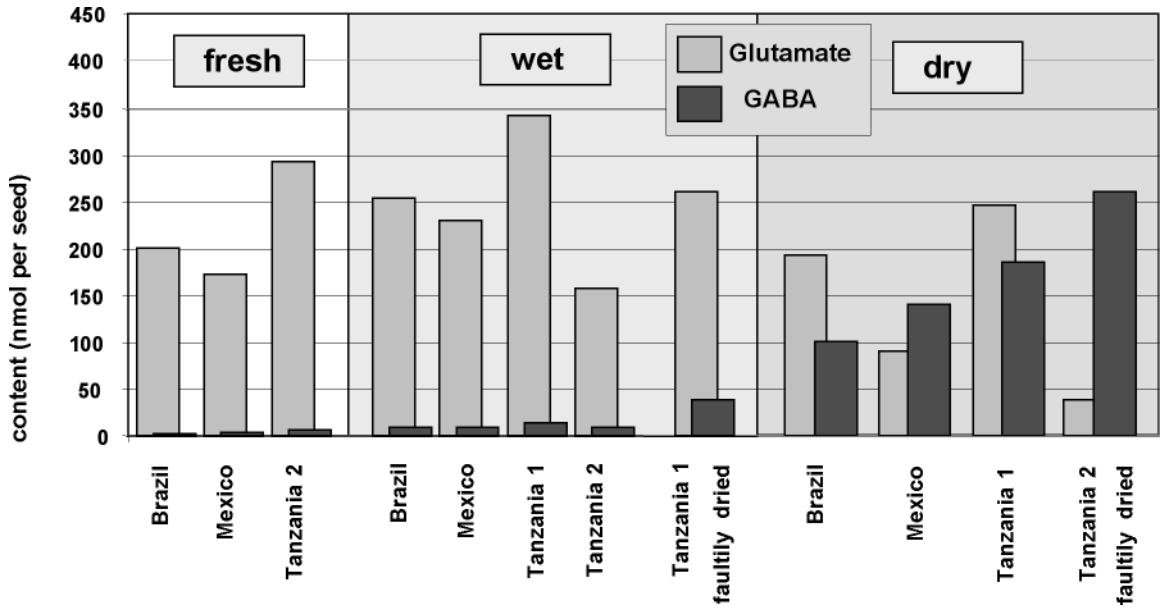

Figure 4. Occurrence of GABA in fresh and differentially processed green coffees.

It is apparent that the living and viable coffee seeds, which are dried from an initial moisture content of 50% down to about 12%, suffer massive drought stress and express a related metabolism. Yet, it still has to be explained why a stress related GABA accumulation occurs only in dry process seeds even though wet process seeds are also dried down to the same final moisture content (12%). In this context, the time frames for the drying become relevant. If it is assumed that stress reactions are elicited at about 45% and that all metabolic reactions cease at about 25% moisture content, then in the case of dry processing, an average time span of 4 to 8 days results, in which an active stress metabolism can take place. In wet processing, the drying procedure requires only 2 to 4 days to reduce the moisture content from 50% down to 12%. Consequently, the time window for an elicited stress metabolism corresponds only 1 to 2 days. Assuming that the synthesis and accumulation of GABA starts about 1 to 2 days after

stress induction, it becomes plausible that GABA can be accumulated in response of the drying step only during the dry and not during the wet process.

If indeed the different time frames of the stress-related metabolism are responsible for the observed differences in GABA accumulation, then changes in the time course of the drying procedure should alter the extent of GABA accumulation.

In Tanzania, we performed a field experiment in which the drying period of wet process beans was extended by artificial moistening. In this case of faulty drying, the amount of GABA in wet processed beans was significantly higher than in the control sample (Figure 4). This effect can be much more impressively demonstrated by artificially extending the whole dry process by moistening. In this way, the accumulation of GABA in the dry processed beans is tremendously enhanced. These data confirm the assumption that indeed the GABA accumulated in processed coffees is the result of an active stress metabolism, and that the metabolic processes in green coffees can be modulated by changing the processing conditions.

In plants, GABA is produced by decarboxylation of glutamic acid by the action of a glutamate decarboxylase. Consequently, GABA accumulation should be associated with a decline in the glutamate content. The comparison of the glutamic acid levels in fresh and processed seeds (wet and dry) derived from identical starting material revealed that the stress-related increase in the GABA concentration was accompanied by a corresponding decrease of glutamic acid. This negative correlation is exhibited clearly when the drying time in dry processing is artificially prolonged (faultily dried).

These data suggest that the GABA: glutamate ratio represents a reliable marker for the distinction of differentially processed green coffees. GABA is not accumulated in conventionally wet processed seeds; enhanced ratios point to drying failures. High levels of GABA are indicative of dry processing.

The data presented in this paper prove that green coffee seeds represent vital and living organisms, in which various metabolic reactions occur during processing, i.e. a germination-related metabolism and a stress metabolism. Furthermore, the extent of these processes depends on the conditions during processing and affects coffee quality. Consequently, specific alterations of processing conditions should influence these metabolic processes and thereby offer a promising tool to influence and control coffee quality.

Practical applications

In order to illustrate the benefit of these insights, one feasible practical application should be mentioned. At the present, due to aggravated environmental directives, especially for wastewater management, and due to increasing cost pressure, rising amounts of green coffee are being produced by new progressive methods, which utilize mechanical removal of the mucilage. It has been reported that the quality of the coffees produced by such progressive methods is different from that of coffees produced by classical wet processing (Puerta-Quintero, 1999).

There is no doubt that also in the case of these progressive methods, germination and stress-related metabolism will be activated; however the substantial differences originate – again – from different time frames for the corresponding metabolism. In the case of classical wet fermentation, the entire time span from the initiation of the germination-related processes until their termination takes about 2 to 4 days (about 1 to 2 days for washing, sorting and

fermentation and about 1 to 2 days of drying time until the critical water content of 25% is reached – a stage at which metabolic processes virtually cease). In contrast, the time window for the related reactions within seeds which are produced using mechanical mucilage removal is only about 1 to 2 days because the 1 to 2 days allocated for the washing and fermentation steps are omitted.

According to our understanding of an active seed metabolism and its influence on coffee quality, we assume that the extension of the time period of the active metabolism should influence quality. Consequently, in a field experiment in Colombia, we introduced an interim storage phase under moist conditions. The sensorial analyses indeed displayed some positive flavour differences in the samples from the extended process as compared to the control samples. However, when the storage phase time was further extended (more than 24 h), negative effects on flavour were perceived. Present investigations are emphasizing the metabolic differences with regard to germination and stress metabolism in green coffees that result from such modified progressive processing methods.

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REFERENCES

Arnold, U.; Ludwig, E. (1996) Zeitschr. Lebensm.Unters. Forsch. **203:** 376-384.

Bytof, G.; Selmar, D.; Schieberle, P. (2000) J. Appl. Bot. **74**: 131-136.

Bytof, G.; Knopp, S-E.; Schieberle, P.; Teutsch I.; Selmar D. (2004). Europ. Food Res. Technol., *in press.*

De Castro, R.D.; Hillhorst, H.W.M.; Bergervoet, J.H.W.; Groot, S.P.C.; Bino, R.J. (1998) Phytochem. **47:** 689-694.

Puerta-Quintero, G.I. (1999) Cenicafe (Colombia) **50:** 78-88.

Selmar, D.; Bytof, G.; Knopp, S-E. (2002). *In* 19ième Colloque Scientifique International sur le Café, Trieste. ASIC, Paris,.

Selmar, D.; Hunecke, D.; Junghärtchen, I.; Breitenstein, B. ; Bytof, G. ; Knopp S-E. (2004) 20ème Colloque Scientifique International sur le Café, Bangalore. ASIC, Paris.