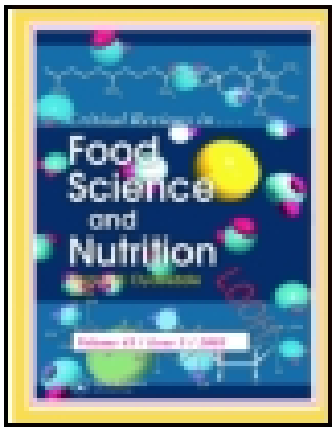

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Biochemistry, Germination and Microflora Associated with Coffea arabica and Coffea canephora Green Coffee Beans

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Biochemistry, germination and microflora associated with *Coffea arabica* and *Coffea canephora* green coffee beans

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Abstract

The germination process of coffee beans occurs while these non-orthodox seeds are still inside the cherry. A range of metabolic reactions related to germination have already been induced in harvested coffee seeds at this time. The germination process is asynchronous and the evolution of germination depends heavily on how the beans are processed post-harvest.

The microflora associated with the coffee cherry surface comprises several bacterial, yeast and fungal species and greatly affects the processing of coffee beans. In particular, indigenous (or applied) lactic acid bacteria and yeast are essential to ferment the coffee berry mucilage after de-pulping. On the other hand, the fungi negatively affect coffee quality, especially regarding food safety, spoilage, off-tastes and mycotoxin contamination.

The first part of this review gives an overview of *C. arabica* and *C. robusta* germination. Additionally, plant growth hormones contributing to the process are also discussed. The second section deals with microflora associated with the beans during harvesting and processing exploring its effects on coffee quality and safety. Biological control strategies for improving the microbial safety of coffee beans are also discussed.

1. Introduction

Coffee represents one of the most important crops in the world and is ranked second on international trade exchange markets, only after petrol or oil (Privat et al., 2008). The majority of the coffee plants are from the Rubiaceae family and genus *Coffea*. There are around 100 species and the two which are of most economic and industrial importance are both cultivated for the production of coffee beverages. *Coffea arabica* and *Coffea canephora* (*robusta*) represent about 70 % and 30 % of the total coffee traded in the world, respectively, with Brazil providing 30 % of the total world market share (Coltro et al., 2006; de Castro and Marraccini, 2006). *C. arabica* produces a better quality coffee beverage than *C. canephora*, which is often used in a blend with the former (de Castro and Marraccini, 2006). Due to the economical and agricultural importance of coffee, there have been many studies into coffee bean harvest, processing and roasting. Germination has the potential to improve the quality of coffee beans from the perspectives of extractability, nutritional properties and flavour enhancement of green coffee (Selmar et al., 2006). Warm and humid conditions are employed to promote fast germination. Conversely, this also encourages an acceleration of fungal spoilage and potential mycotoxin production. Mould growth and mycotoxin production is a problem which is frequently addressed in food related research literature (FAO, 2006; Noonim et al., 2008; Silva et al., 2000; Taniwaki, 2006; Velmourougane and Bhat, 2009).

The objective of this paper is to review our knowledge of the biochemical composition and alterations during coffee bean germination and fermentation. Herein, we will address the contribution of natural and artificially introduced microflora to the fermentation process. The

biochemical and quality changes evolving in the coffee bean due to external factors will also be discussed. The emphasis will be on environmental conditions (heat, light, moisture, chemicals etc.), internal biochemical progressions and the microbial load (fungi and bacteria etc.) of coffee beans present during fermentation and involved in the subsequent germination.

1.1. Coffee tree life cycle

C. arabica is an allotetraploid (4 chromosomes) and mostly autogamous (self-fertilising) tree. In contrast, *C. canephora* is diploid and may be a progenitor of *C. arabica* (Clarindo and Carvalho, 2009). The life cycle of the coffee tree (*Coffea* sp.) has been described using the extended BBCH (**B**iologische Bundesantalt, **B**undessortenamt and **CH**emische Industrie, Germany) scale (Arcila-Pulgarín et al., 2002). Coffee trees can sometimes bear fruit from the first year up to twenty or more years and will have greatest productivity between years 5 to 10 (Arcila-Pulgarín et al., 2002).

The BBCH scale does not describe the true timing of coffee seed development in the field, which takes at least 50-60 days (*C. arabica*) (Maestri and Vieira, 1961) however, it does accurately illustrate its growth stages (Arcila-Pulgarín et al., 2002). Briefly, coffee seed growth begins with germination and after approximately one week in optimal conditions, root formation is evident with radicle protrusion through the seed coat. When the shoot emerges from the soil, cotyledons unfold and a pair of coffee tree leaves is exposed. The appearance of new leaves continues (stage 1) until after approximately three weeks when branch formation becomes apparent (stage 2). The branches are elongated (stage 3) and inflorescence emerges after just over seven weeks of growth. Flowers are visible but still closed until the eighth or ninth week when the first blossoms

begin to open (stage 5). This continues (stage 6) until week ten when fruits become visible (stage 7). *C. arabica* anthesis can occur on one day or over a few days with more than one flowering period possible within a single harvest cycle (Wormer, 1964). The small yellowish berries develop to their full size throughout the next few weeks (stages 7-8). Then the fruit is ripe for picking and leaves begin to wither with senescence extending upwards until only the uppermost shoots have berries. By now, harvesting should be finished (Arcila-Pulgarín et al., 2002).

Mature coffee fruit is a red or yellow drupe with a pulpy mucilaginous flesh surrounding a lignified endocarp (Figure 1), consisting of two elliptical shaped seeds with a longitudinal furrow on their surface (Dedecca, 1957). The endosperm is enveloped by the pericarp and is made of three distinct layers; the outermost exocarp, the middle mesocarp and the innermost endocarp (parchment) which is in contact with the silver skin containing the embryo (Figure 1) (Krug and Carvalho, 1939; Mendes, 1941). A number of current reviews exist on the detailed physiology and cytology of the green coffee bean (DaMatta et al., 2007; de Castro and Marraccini, 2006; Eira et al., 2006).

Growth conditions are very important in the cultivation of a productive commercial coffee tree. Overall, drought and suboptimal temperatures are the major limitations for coffee production. Generally, it is accepted that *C. arabica* requires a wet tropical highland climate at high altitudes (600 and 1600m) and *C. robusta* is often grown under the same conditions but it can grow at sea level also (FAO, 2006). Despite efforts to propagate coffee plants from vegetative states, they are still primarily grown as seedlings produced directly from seeds (Wintgens and Zamarripa, 2008). According to the Brazilian authorities, seedling production is completely dependent on

temperature (optimal 18-21°C) and rainfall (optimal 1200-1800 mm) (Alègre, 1959; Coste, 1993; de Castro and Marraccini, 2006; Weitbrecht et al., 2011). Humidity contributes to the water requirements for coffee growth and Robusta coffee can grow successfully at almost 100% humidity (Coste, 1993).

2. Coffee seed: dormancy, germination and processing

Coffee beans are neither orthodox; going through a desiccation period and metabolic dormancy before germination, nor recalcitrant; having a high water content at the end of maturity with germination commencing immediately. Instead, they have been categorised into a new group between those of orthodox and recalcitrant seeds. They are intermediate (nonï orthodox) seeds and low temperature damage to dry seeds is a notable feature (Ellis et al., 1990; Roberts, 1973). Another characteristic of intermediate seed dormancy is defined as a block in the progression of germination to a state of completion under favourable conditions, which has evolved differently across species to allow adjustment to the established environment (Hilhorst, 1995). Release from dormancy leads to germination (Bytof et al., 2000; Finch-Savage and Leubner-Metzger, 2006).

Different coffee species illustrate contrasting levels of desiccation sensitivity, which is related to their ecological origins. This is shown by Arabica coffee which has intermediate seed storage behaviour and is native to the dry and cool regions of Ethiopia, while *C. liberica* (not of industrial importance) shows more recalcitrant seed storage behaviour and is native to the hotter and more humid regions of Liberia (Hong and Ellis, 1995). Optimum storage conditions of 10-11% water content and approximately 10°C extend the viability of green beans from Arabica coffee (Hong and Ellis, 1992). It was also reported in the same study, that *C. arabica* desiccation

sensitivity increases after imbibition for 3-10 days, which insinuates that high-moisture-processing could play a negative role in these beans viabilities (Hong and Ellis, 1995). In addition to *C. arabica* and *C. robusta* (Ellis et al., 1990; Hong and Ellis, 1995), other seeds such as *Elaeis guineensis* (oil palm seeds) (Ellis et al., 1991b), *Carica papaya* L. (papaya seeds) (Ellis et al., 1991a), and *Zizania palustris* (wild rice) (Kovach and Bradford, 1992) also show intermediate seed storage behaviour.

As a member of the Rubiaceae family and containing a spatulate axile embryo (Figure2), coffee species are regarded as being physiologically dormant (Baskin and Baskin, 2004; Finch-Savage and Leubner-Metzger, 2006). This state of arrest occurs within the fruit, likely by the joint effects of abscisic acid (ABA) and the osmotic potential of the fruit flesh (Bytof et al., 2000). Various factors can affect the process of coffee bean germination, such as processing and storage. For instance, in wet processing, the coffee fruit flesh is removed upon harvesting and thus, germination suppression is neutralised (Selmar et al., 2006). The fruit remains intact in dry processing nevertheless, it has been shown that germination progresses in these coffee beans, but to a lesser extent (Bytof et al., 2007). However, once inhibition is alleviated, the germination process can proceed similarly for coffee beans processed in any manner. In wet processing, germination related metabolism is observed from day 1 with maximum levels on day 2. In contrast, dry processed beans germinate after 2-3 days and signature metabolites peak on days 4-5 (Selmar et al., 2006). After approximately 2-3 months, a seedling will emerge from the soil in optimal temperature and moisture conditions (Maestri and Vieira, 1961). In nature, the fruits fall from the tree when they are ripe and the indigenous microbes present ferment the flesh, thus freeing the coffee beans which will germinate during favourable environmental conditions. In

general, coffee beans are asynchronously stimulated to germinate during post-harvest processing. The non-uniform initial metabolic state of the germinating beans affects, in turn, the attributes of the coffee cup (Bytof et al., 2000; Eira et al., 2006).

The germination process can be accelerated *in vitro* by removing the bean husk (endocarp), which is severely inhibitory (Valio, 1980) and following imbibitions, the bean swells to double its original size (Arcila-Pulgarín et al., 2002; Ellis et al., 1990; Valio, 1976). Throughout germination, from the start of imbibition to radical protrusion, many events occur. This complex process results in embryo growth and weakening of the endosperm to allow emergence in the form of a radicle. Seed development can be monitored through the intimately connected evolution of various proteins, enzymatic activities, hormones and carbohydrate reserve mobilisation. Under field conditions, the germination process is said to continue until the seedling emerges through the soil (da Silva et al., 2004). In *in vitro* tests, germination *sensu stricto* is said to be complete upon emergence of the radicle (Figure 3, day 6).

Storage, moisture and temperature are all factors which greatly influence the germination capacity of coffee seeds. After drying and storage, coffee seeds lose vigour and cannot be used to propagate seedlings. In order to preserve the germination capacity of the beans, the beans must be maintained at 10-12% moisture content during storage (da Rosa et al., 2011). Germination is also less frequent under exposure to light and thus darkness should be maintained during storage of viable beans (Resende et al., 2009; Valio, 1976). Additionally, the germination of *C. arabica* takes around 3 weeks at its optimum temperature of 30-32°C, where the germination time is increased to 3 months at 17°C (Läderach et al., 2011; Moraes, 1963).

2.1. Green coffee biochemistry and germination

2.1.1. Carbohydrate and sugar mobilisation

The major storage compounds in coffee beans are cell-wall polysaccharides which encompass 48-60% (dry weight) and primarily consist of galactomannans and arabiongalactan-proteins (Clifford, 1985; Wolfrom and Patin, 1965). Other major storage compounds present include lipids (10-16%); with triglycerides and free fatty acids making up this portion of the green coffee beans, and proteins (~11%), sucrose (4-8%) and chlorogenic acids (6.5%) also present (Joët et al., 2010). The total polysaccharide content and structure of the Robusta and Arabica GM are quiet similar (Nunes and Coimbra, 2002) however, the content of water extractable AG from Robusta green coffee was higher than that extracted from Arabica coffees (Fischer et al., 2001; Nunes and Coimbra, 2001). The major monosaccharide residues of the high molecular weight material from hot water extracted Ugandan *C. robusta* green coffee were galactose (47 mol %), arabinose (23 mol %), mannose (21 mol %), and glucose (7 mol %) (Nunes and Coimbra, 2002).

Recently published literature has provided detailed descriptions of the structural features of galactomannans, arabinogalactan-protein (Type II AG) carbohydrates and cellulose isolated from coffee, in particular *C. arabica*, by hot water extraction methods (Bradbury and Halliday, 1990; Fischer et al., 2001; Nunes and Coimbra, 2001, 2002; Oosterveld et al., 2004). (Galacto-)`mannans are composed of ɓ-1,4-linked mannose residues, which can be un-substituted or substituted with single galactose *O*-6 linked units at a high frequency thus increasing solubility (Galactose/Mannose (G/M) 1:2) (Marraccini et al., 2005), or rather sparingly (G/M 1:130) thereby decreasing solubility (Bradbury and Halliday, 1990). In summary, these research teams

have shown that an arabinogalactan-galactomannan (AG-GM) mixture occurred as the main hot-water soluble polysaccharides. The *C. robusta* AG galactose residues were (1, 3)-, (1, 3, 6)-, (1, 6)-, or terminally-linked in an approximate 10:7:1:2 ratio, respectively and arabinose residues were terminally and (1,5)-linked in a 13:7 ratio, with these values indicate highly substituted AG, with terminal arabinose residues. *C. arabica* AG-MG structures were similar however, the (1, 3)-Gal/(1, 3, 6)-Gal linkages are in a 1:1 ratio. The GM from Robusta coffee was shorter than the Arabica extracted chains and the high molecular weight GM substitutions were similar for both coffee types. Robusta green beans contain more GM with a higher degree of branching and the amount of AG extracted from a Robusta green coffee was higher than the amount extracted from Arabica coffee.

2.1.2. Storage proteins, amino acids and nitrogenous components

In coffee and other seeds, proteins are stored in organelles for protection against uncontrolled proteolysis. Upon germination, organised enzymatic hydrolysis of these reserves commences and peptides/amino acids are translocated for use by the growing embryo (Callis, 1995). In cereals, the main storage proteins are prolamines and glutelins whereas in legumes, globulins are the primary storage proteins (Muntz, 1998). Other coffee seed nitrogenous compounds include caffeine and trigonelline which are present at ~0.9-2.2 and 1%, respectively (Table 1). Two homologous (>98% sequence homology) legumin-like seed storage proteins (11S storage globulins) were first isolated from *C. arabica* endosperm using acidic aqueous buffers (Acuña et al., 1999). Similar storage proteins have also been identified in other *Coffea* species at the DNA level. The protein subunits are 55kDa in the native form composed of two polypeptides of 24 and

33kDa. Immunocytochemistry studies have revealed that the legumin storage protein is stored in cytoplasm-located vacuoles and it accounted for most of the cell volume (Acuña et al., 1999).

A comprehensive study, into protein changes in germinating coffee beans over a 6 week period, revealed that the overall levels remained constant but the total free amino acids, which are correlated with flavour and colour formation in coffee, were reduced over time (Shimizu and Mazzafera, 2000). Generally, *C. robusta* has a higher concentration of amino acids than *C. arabica* (Murkovic and Derler, 2006). There was a reduction in storage proteins (legumin-like proteins) in later weeks (Shimizu and Mazzafera, 2000) as expected due to mobilisation of reserves by proteolysis during seed germination (Callis, 1995). The primary amino acids observed during germination, all of which were reduced during the process, were; asparagine, glutamic acid, aspartic acid, alanine, lysine, serine and glutamine (Shimizu and Mazzafera, 2000). Alanine, which is known to be involved in energy production, human liver function and muscle carnosine enhancement, is the most prominent amino acids found in coffee beans with Robusta and Arabica species having an average of 1200 µg/g and 680 µg/g, respectively, followed by arginine which is present in average concentrations of 800 µg/g and 360 µg/g, respectively (Murkovic and Derler, 2006; Prodolliet et al., 1995). Phenylalanine, an essential amino acid, is also quiet abundant being present in concentrations of 180-780 µg/g (Murkovic and Derler, 2006). Tyrosine was the only amino acid which was detected in increasing levels as germination progressed (Shimizu and Mazzafera, 2000). The major amino acids present in the soluble protein fraction was glutamine/glutamic acid and glycine, as well as smaller but significant amounts of leucine, aspartic acid/asparagine, alanine, lysine and serine (Shimizu and Mazzafera, 2000).

2.1.3. Lipids, vitamins and minerals

In addition to the important sugar-based and proteinaceous components, green coffee also has lipids, vitamins and minerals. The exact compositions of green coffee beans are summarised in Table 1. The 10-17% (dry weight) of lipid components, which are typically present in green coffee, harbour fat-soluble vitamins and have been studied in addition to the water-soluble vitamins. Nicotinic acid is the most abundant micronutrient in green (and roasted) coffee but the major fat-soluble vitamin present is vitamin E and its levels depend on post-harvesting processing and sample age (Macrae, 1988). Tocopherols, known for their antioxidant properties, are also present in green coffee but, like all fat-soluble nutrients, are not passed to the final roasted brew (Macrae, 1988).

Water-soluble vitamins, which are destroyed during roasting, have also been detected in green coffee but in far lower amounts than is physiologically relevant. The B vitamins are present at levels of; B_1 (0.002% dry weight), B_2 (0.002% dry weight), B_6 (0.001% dry weight) and B_{12} (trace amounts) (Macrae, 1988). Niacin (0.02% dry weight), pantothenic acid (0.01% dry weight), folic acid (0.0001% dry weight) and ascorbic acid (0.46-0.61% dry weight) are also present, but are heat-labile and so destroyed during roasting (Macrae, 1988).

The minerals detected in green coffee beans include primarily potassium, magnesium and calcium and others such as, sodium, iron, manganese, rubidium, zinc, copper, strontium, chrome, vanadium, barium, nickel, cobalt, lead, molybdenum, titanium and cadmium (Clark, 2003).

2.1.4. Coffee enzymes and their roles

Turgidity in the green coffee bean embryo and endosperm cells increase upon imbibition. This causes greater osmotic pressure on the cell walls thus, preparing the embryonic axis for expansion. A proportion of water (imbibition fluid) is absorbed but cell wall extensibility is limited by mechanical restraint of the endosperm (da Silva et al., 2004). The biochemical changes which ensue in the seed lead to an increase in enzymatic activity, thus weakening the endosperm cap and increasing its penetrability (da Silva et al., 2005; da Silva et al., 2004). Changes in plant growth regulators are associated with enzyme induction (da Silva et al., 2005; Giorgini and Comoli, 1996). Restricted enzymatic hydrolysis of the cells surrounding the embryo creates space to increase flexibility and plasticity needed for embryo expansion and later for carbohydrate reserve mobilisation (da Silva et al., 2005; da Silva et al., 2008). After 5-7 days, embryo growth inside the bean results in a protuberance on the endosperm cap (Figure 3). The hydrolytic enzyme activities plateau as the protuberance enlarges and increase again before the endosperm is punctured allowing the radicle to emerge through the endosperm cap (Eira et al., 2006). After 7-10 days, degradation of the lateral endosperm commences, thereby mobilising the seeds reserves to facilitate the growing embryo (da Silva et al., 2004). Germination *sensu stricto* has now ceased and the resulting seedling continues to grow.

Various green coffee bean-derived enzymes, particularly endo-mannanase (*β-mann*), and proteins, such as storage proteins, have been isolated and studied from biochemical and physiological perspectives. Their roles in the development and germination of the coffee seed from pre-harvest, post-harvest processing, and germination will be discussed in the following sections. Additionally, the overall protein compositions and amino acid profiles of *C. arabica* and *C. canephora* will be considered with regards to their relevance in the germination process.

Green coffee bean polysaccharides represent approximately 50% of the total dry weight (Bradbury, 2001). Mannans (50%), in particular galactomannans, are the primary cell wall constituents (20-30%) in green coffee beans (Bradbury and Halliday, 1990; Clifford, 1985; Wolfrom et al., 1961), in addition to arabinogalactan (30%), cellulose (15%) and pectins (5%). Studies have shown that the G/M ratio is highest in early endosperm development and decreases upon further maturation of the tissue (Marraccini et al., 2005; Redgwell et al., 2003). This change is consistent with displacement of perisperm tissue by endosperm tissue (Rogers et al., 1999) and hardening of the seed during green coffee development. This leads to the conclusion that the degree of substitution of GM is developmentally regulated (Redgwell et al., 2003). It is hypothesised that Ŭ-D-galactosidase is responsible for the removal of galactose from GM (Redgwell et al., 2003) and acts synergistically with endo-ɓ-mannanase (E.C. 3.2.1.78) (*β-mann*), ɓ-mannosidase (E.C. 3.2.1.25) (*β-mano*) and other accessory enzymes to mobilise carbohydrate reserves during the germination process (Reid and Meier, 1972). The main product of mannan hydrolysis is mannose which is released through synergistic enzyme hydrolysis.

Man activity was first discovered in coffee beans by Takaki et al. (1979). Since then, two enzymes (*manA* and *manB*) have been isolated and biochemically characterised (Marraccini et al., 2001). The two *man* enzymes had 56% homology with each other and shared high sequence identity with other mannan endohydrolases from various sources and the enzyme transcripts actively hydrolysed mannans constituted of five or more mannose units. Many isomers were found throughout germination and *man* activity was not observed before imbibition and transcripts were not identified during grain maturation or in coffee tissue outside the seed endosperm (Marraccini et al., 2001).

During coffee bean germination very low endo-ϐ-mannase activity was detected before radicle emergence, probably contributing to the weakening of the endosperm at the site of radical protrusion, and increases several-fold reaching its peak 14 days later (Marraccini et al., 2005). The bulk of *β-mann* activity, and thus the mobilisation of galactomannan in *C. Arabica* seeds, is essentially post-germinative (Giorgini and Comoli, 1996). However, mannanase transcription is positively correlated with giberellic acid (GA) uptake during coffee bean germination. It has been hypothesised that mannose degradation, by *β-mann* and *β-mano*, allows GA-mediated inhibition of coffee bean radical protrusion (Takaki et al., 1979), but this is in conflict with later reports where *β-mann* is attributed with a role in endosperm cap softening (Marraccini et al., 2005). Mannose has been shown to inhibit ATP synthesis and hexose metabolism (Herold and Lewis, 1977). However, there are conflicting reports about the effect of mannose on germination inhibition, with studies saying that both may be unrelated (da Silva et al., 2005) or inter-dependent (Takaki and Dietrich, 1979; Takaki et al., 1979; Valio, 1976).

In contrast to GM hydrolysing activities, cellulase enzymes are detected before normal coffee bean germination commences (Takaki et al., 1979), which may insinuate a role in endosperm cap softening before radical protrusion. The concentrations of this enzyme are enhanced upon addition of GA_3 during initial coffee bean imbibition (Takaki et al., 1979).

Coffee derived Ŭ-D-galactosidase (E.C. 3.2.1.22) (Ŭ-gal), like other galactohydrolases, is able to cleave Ŭ-1,6 links between galactose units from the N-terminal end of GM seed storage tissue (Buckeridge and Dietrich, 1996). Ŭ-Gal deficient seeds have restrained germination (Mathew and Balasubramaniam, 1987; Shen et al., 2009). In the perisperm-dominant stage of coffee seed

development, Ŭ-gal activity is below the limits of detection. However, as endosperm dominates in the latter stages of green coffee seed maturity, Ŭ-Gal can be detected in the fruit flesh (which is discarded during pulping or fermented) and at even higher levels in the seed endosperm tissue corresponding to rapid seed expansion and hardening (Marraccini et al., 2005).

Molecular and Ŭ-gal-specific antibody-directed research also revealed that Ŭ-gal is extracellularly located (Marraccini et al., 2005). To maintain a high G/M ratio and solubility (for coffee bean intracellular transport) in *de novo* synthesised GM and to allow endosperm hardening via galactose removal, Ŭ-gal activity is necessary ócon-siteô (Marraccini et al., 2005). Additionally, there are amino-acid differences in the *C. arabica* and *C. robusta* species, none of which are implicated in enzyme-substrate interaction or specificity (Marraccini et al., 2005). It is also interesting to note that the patterns of *α-gal* and ɓ-mannanase expression are not synchronous, with levels of the former continually increasing after initial detection and the latter dropping dramatically after reaching its maximum (Marraccini et al., 2005). In coffee, Ŭ-gal is known to transglycosylate cyclodextrins, with 6-*O*-Ŭ-galactosyl Ŭ-cyclodextrin being the first isolated from green coffee beans (Kitahata et al., 1992). This enzyme has two known isoforms in germinating coffee beans and, in addition to its role during germination, is of interest due to its contribution to cyclodextrin branching which has applications industrially and in the medical field (Shen et al., 2009).

Very limited study has been completed on green coffee proteases/peptidases thus far. One research group isolated a number of endo-peptidases, from *C. arabica* and *C. robusta*, which were capable of hydrolysing proteins without releasing single amino acids (Ludwig et al., 2000).

They were found to be resistant to serine and pepsin inhibitors but susceptible to inactivation by heavy metals and iodacetamide (Ludwig et al., 2000). However, no activity profile or data regarding the enzyme roles during germination were hypothesised or proven.

2.1.5. Hormones and growth regulators

In addition to the nutrients and enzymes necessary for coffee seed germination, there are five main classes of hormones involved in the growth of all plants: auxins, cytokinins, gibberellins (GAs), abscisic acid (ABA) and ethylene (Arteca, 1996; Davies, 1995). The functions of these growth control compounds are generally similar across a broad range of plant species and tissues, with specific effects also being seen under certain circumstances. In general, auxins promote cell growth and division (Darwin and Darwin, 1881), cytokinins promote cell division (Haberlandt, 1913; Van Overbeek et al., 1941), GAs are involved in cell elongation regulation (Davies, 1995; Mauseth, 1991; Raven et al., 1998; Salisbury and Ross, 1992), ABA inhibits cell division (Salisbury and Ross, 1992) and ethylene controls fruit ripening and can inhibit the growth and development of plant cultures (Mauseth, 1991; Salisbury and Ross, 1992).

Previous research into coffee seed or embryo development, specifically *C. arabica*, has shown how hormones stimulate various reactions. In celery (Jacobsen et al., 1976), pepper (Watkins et al., 1985) and tomato seeds, GAs were shown to induce endosperm degradation by stimulating hydrolytic activity in the endosperm cell walls (da Silva et al., 2005). In coffee seeds, GA_{4+7} was responsible for the stimulation and inhibition of germination with two sensitivity thresholds from 0-1 µM and between 10-100 µM. This is possibly, but unlikely, caused by heterogeneity in the population and is probably due to two mechanisms/sites of inhibition (da Silva et al., 2005).

These results indicated that coffee seed germination is dependent on *de novo* synthesis of GAs, like other species. Contrastingly, coffee is the only species where germination inhibition results from physiological concentrations of GA (da Silva et al., 2005). GAs influence coffee seed germination by effecting both the embryo and surrounding endosperm tissue with exogenous GA causing germination inhibition in *C. arabica* (Valio, 1976). GA_{4+7} imbibition by coffee seeds increased cell wall turgidity and decreased levels of *β-mann* and ϐ-*mano* activity were produced (da Silva et al., 2005). In summary, exogenous GA_{4+7} was shown to negatively impact *C. arabica* seed germination contrasting the essential positive impact of *de novo* endogenous GA synthesis during embryo elongation, radicle protrusion and endosperm cap weakening (da Silva et al., 2005; Valio, 1976). Application of exogenous GA_{4+7} resulted in cell death and loss of embryo vitality (da Silva et al., 2005). GA_3 application to pre-germination *C. arabica* seeds inhibited radical protrusion (Maestri and Vieira, 1961; Takaki et al., 1979; Valio, 1980).

ABA regulation of embryo growth potential and endosperm cap weakening during germination was illustrated in *C. arabica* (ǿRubiǾ) seed germination (Silva et al., 2004). In a later study by the same group, ABA was shown to negatively regulate coffee germination by inhibiting both the embryo cell expansion and early cell division events (da Silva et al., 2008).

Kinetin has been shown to reverse germination inhibition by both exogenous GA and ABA (Valio, 1976). Cytokinin-like hormones were also shown to have a positive effect on *C. arabica* germination (Valio, 1976).

2.2. Green coffee production

2.2.1. Post-harvest processing

To prepare green coffee beans which suitable for storage, transport and roasting, the outer layers of the coffee fruit, including the skin, mucilage and pulp, must be removed from the seeds (Jones and Jones, 1984). There are two primary methods; dry (natural) or wet processing, and a third procedure called semi-dry processing (Figure 4).

Dry processing is generally favoured for Robusta coffee beans. In this process, the beans are mechanically non-discriminatingly picked when most of the cherries are fully matured. This allows inclusion of varying proportions of green and over-ripe cherries as well as raisins and dry shrivelled fruits (Silva et al., 2000). Following harvesting, dry processing involves the fermentation of sun-dried coffee on platforms (typically made from cement, concrete, tarmac or earth), in ~10 cm layers, which are heaped at night and spread each morning for 10-25 days (Silva et al., 2000). Wet processing is more labour intensive coffee fruit is selectively hand-picked, mechanically de-pulped and then washed for 24-48 h where fermentation occurs, in large water tanks, thus removing the mucilage remaining (Silva et al., 2000). The beans are then machine dried (or sun-dried; semi-dry processing) to ~12% moisture content, followed by mechanical removal of any remaining dried skins. This type of processing is typically used for Arabica coffee and is necessary in some situations for example, when there is a lack of predictability in sunshine hours or sun temperature etc. Semi-dry processed cherries are de-pulped and then fermented and normally dried on platforms. Dry and semi-dry processing methods are used in Brazil (Silva et al., 2008) and Ethiopia, with wet processing being favoured in Colombia, Central America, Hawaii and Indonesia (Schwan and Wheals, 2003).

The microbial flora present on coffee fruits, and transferred to the coffee beans during processing or through other damage to the protective pulp, is hugely variable. The biodiversity depends on many factors such as the coffee variety, processing methods, environmental factors, harvesting and storage. Indeed, it has been noted that during natural fermentation heavy rainfall caused an increase in the microbial load (Silva et al., 2000). Similarly to the processing method, microflora effects coffee fermentation and can impact characteristics of the final coffee beverage (Vilela et al., 2010). However, the main function of the microflora is pectinolytic, and probably cellulolytic, coffee bean mucilage fermentation.

2.2.2. Commensal microflora

Bacterial species are usually dominant on coffee cherries and beans, followed by filamentous fungi and yeasts, with considerable variation between farms and years of harvest (Silva et al., 2000). Examples of the diversity in green coffee are illustrated herein.

Bacteria isolated from dry-processed *C. arabica* var. Mundo Novo grown in the Sul de Minas region of Brazil included 754 isolates comprised of gram-negative species; *Aeromonas, Pasedomonas, Enterobacter* and *Serratia* genera, and gram-positive species; *Bacillus, Cellulomonas,* and in lesser numbers, *Arthobacter, Microbacterium, Brochothrix, Dermabacter* and *Lactobacillus* genera (Silva et al., 2000). Of the 107 yeasts isolated most were fermentative and came from the genera *Pichia, Candida, Arxula* and *Saccharomycopsis*. There were also 292 fungal isolates from the genera *Cladosporium, Fusarium, Penicillium* and to a lesser extent *Aspergillus, Beauvaria, Monilia, Rhizoctonia* and *Arthrobotrys* (Silva et al., 2000).

In a comprehensive study of the microbial population of dry-processed Brazilian farmed *C. arabica* L. species, it became apparent that the ecological profile of this harvest was very different from dry-processed beans (Vilela et al., 2010). Bacteria dominated the microbiota in the first 24 h and yeast also increased during this time, however the filamentous fungi were less well represented in the population and reduced further after 96 h (Vilela et al., 2010). In total, there were 728 isolates with 382 yeasts, 286 bacteria and 60 filamentous fungal species. Previous work on Brazilian farmed Arabica coffee, which was dry processed, had a higher incidence of filamentous fungi as the fermentation progressed (Silva et al., 2008). The authors suggest that semi-dry processing may provide a mechanism for the control of fungi, especially mycotoxogenic species, during fermentation.

2.2.3. Coffee bean fermentation

In spite of the presence of yeasts during green coffee bean fermentation, it was shown in a study of Arabica coffee farmed in Brazil, that these are not pectinolytic species (Avallone et al., 2001). However, evidence to the contrary has also been reported for Robusta coffee harvested in India (Agate and Bhat, 1966). Additionally, acidification by LAB during coffee fermentation has also been proposed as the agent responsible for mucilage hydrolysis (Avallone et al., 2002). However, it is generally accepted that degradation of the pectin-rich mucilage adhering to coffee beans is the combined role of yeasts and LAB.

During the fermentation process, yeast counts increase while fungal and bacterial species remain unchanged. The role of these fermentative microbes is to produce pectinolytic and other hydrolytic extra-cellular enzymes capable of breaking down the pulp-derived pectin

carbohydrate chains present in the mucilage and pulp, which is an obstacle to drying and storage (Avallone et al., 2002). This can be done chemically or using purified enzyme preparations, both of which are not as cost-effective or convenient during fermentation (Avallone et al., 2002). The microbial hydrolysis results in the formation of pectin-sugars, ethanol and carboxylic acids (Silva et al., 2000). During prolonged fermentation, combined high humidity and increased temperatures can encourage fungal spoilage. This, in turn, can cause a shift in the microbiota to allow a predominance of mycotoxigenic microbes which is a serious health concern and additionally, fungi are also associated with the production of off-flavours.

Wet and dry processing both give way to very different natural microbial communities, primarily due to environmental and substrate differences. It has been suggested that the actual numbers of various microbes present are not critical but the physiological roles played by the species present is of more relevance (Silva et al., 2000). Wet processed coffee has mucilage-covered coffee bean as the substrate and high moisture primarily anaerobic conditions, whereas dry processing involves the pulp covered coffee bean as a substrate combined with a lower moisture environment (Silva et al., 2000). In wet fermentation, the coffee bean is exposed to machinery directly, without the protection offered by the pulp in dry processing, thus rendering it more susceptible to contamination during de-pulping (Jones and Jones, 1984). In dry processing, the most serious contamination threat comes from the daily re-spreading and heaping of the coffee bean piles which increases aeration and potentially introduces contaminating microbes. Additionally, the effect of rainfall and the high ground temperatures in dry processing lead to differences in the predominant microflora (Silva et al., 2000).

2.2.4. Bacteria and yeast

Lactic acid bacteria (LAB), and other Gram-negative/-positive bacteria, have been isolated from coffee of different origins and processed in various ways. The LAB species *Erwinia herbicola, Klebsiella pneumonia* and *Lactobacillus brevis* have been isolated from wet, dry, and semi-dry fermented green coffee (Avallone et al., 2002; Avallone et al., 2001; Vilela et al., 2010). Research on wet processed *C. arabica*, showed that the most strongly pectinolytic species present during fermentation was the enterobacter *E. dissovens*, which is unable to hydrolyse highly methylated coffee pectin, and other species were not pectinolytic at the pH of fermentation. This, in combination with microscopic observations of intact polysaccharides post-fermentation, led the authors to suggest that mucilage degradation is correlated with LAB-induced acidification (Avallone et al., 2002). Mixed LAB inocula is suggested to limit off-flavour development, standardise coffee quality, improve polysaccharide degradation and limit secondary fermentation during storage (Avallone et al., 2002). Additionally, the role of LAB and yeasts in the biological control of coffee is also discussed in the following section. However, previous fermentation studies of Kona coffee cherries have shown that, in this case at least, the fermentation of mucilage was successful using bacterial species with little change in the pH (Frank et al., 1965). The authors also confirmed the isolates ódemucilagingô ability showing that only 44 *E. dissolvens* species were ódemucilation-positiveô of the 174 tested.

In a study on *C. arabica* farmed in Tanzania, *P. kluyveri, P. anomala* and *H. uvarum* were the yeast species found to dominate during different stages of wet coffee processing (Masoud et al., 2004). These species are fermentative and the latter two species are able to grow on pectin as a

sole carbon source (Masoud et al., 2004). It was thus deduced that these yeast species could play a role in coffee fermentation, however their synergy or interactions with pectinolytic LAB or acetic acid bacteria was not investigated.

The evolution of bacterial species in a population of dry processed Brazilian farmed *C. arabica* var. Acaiá, was studied resulting in the isolation of 940 microbial colonies comprised of bacteria, yeast and fungi (Silva et al., 2008). The authors noted that population diversity was mostly influenced by moisture content which impacted substrate competition and colonisation by various species. *Bacillus* species were predominant in the study and its cellulolytic ability was correlated to cellulose hydrolysis in the mucilage, particularly by the *B. cereus* group (*B. polymyxa* and *B. subtilis*) (Silva et al., 2008). *E. aerogenes, E. cloacae* and *Klebsiella* isolates were also isolated and *T. ptyseos, P. putrefaciens, E. aerogenes, Acinetobacter sp*. and *P. mirabilis* were found to produce pectin lyase, indicating a likely role in the degradation of the highly methylated coffee pulp pectin thus accelerating the fermentation process (Silva et al., 2008).

2.2.5. Fungal contaminants/mycotoxins

Green coffee beans, like other harvested beans, cereals and fruits, are regularly contaminated and often colonised by fungal species during growth, harvesting, processing, transport and storage. These infections are unwanted and the extent of their negative contribution primarily depends on environmental factors such as humidity, temperature, farming practices and storage. Research has shown that the main fungi present in coffee cherries which are subject to dry/ semi-dry/ wet processing are species of; *Aspergillus, Penicillium, Cladosporium, Fusarium, Pestalotia*,

Paecelomyces, and to a lesser extent; *Cylindrocarpon, Eurotium, Fusariella, Geotrichum, Mucor, Phoma* and *Ulocladium* (Silva et al., 2008; Vilela et al., 2010). The predominant toxigenic genera, which are present at all stages of the green coffee production process are represented by *Aspergillus, Penicillium* and *Fusarium* (Batista et al., 2003; Batista et al., 2009; Silva et al., 2000; Taniwaki, 2006). Fungal toxins associated with coffee are Ochratoxin A (OTA), Aflatoxin $B_{1/2}$ ($AFB_{1/2}$), Aflatoxin $G_{1/2}$ ($AFG_{1/2}$), Fumonisin B_2 (FB_2), Patulin, (PT) and Sterigmatocystin (STC), the most common being OA, AFB_1 and FB_1 (Batista et al., 2003; Bokhari and Aly, 2009b; Noonim et al., 2008; Taniwaki, 2006) and will be discussed more in detail.

Ochratoxin A (OTA) is a hepatoxic, nephrotoxic, teratoxigenic, immunosuppressive and carcinogenic toxin which is found in coffee and originates from *Aspergillus* and *Penicillium* species (Bucheli et al., 2000; Pitt, 2000; Taniwaki et al., 2008). It was first reported in green coffee by Levi *et al*. (1974) and in roasted coffee by Tsubouchi *et al*. (1987). The level of OTA present in green and roasted coffee varies considerably and there is little known about which conditions induce isolates to produce OTA (Batista et al., 2003). However, it is know that *A. ochraceus* and *A. carbonarius* are the most potent OTA producers in coffee, with production levels of 5-13 mg/kg^{-1} attributed to these species (Bucheli and Taniwaki, 2002). Far lower levels have been reported in other studies; 20-80 $\mu g/kg^{-1}$ (Levi et al., 1974), 0.2-15 $\mu g/kg^{-1}$ (Micco et al., 1989), 0.1-48 $\mu g/kg^{-1}$ (Romani et al., 2000), a maximum of 1.8 $\mu g/kg^{-1}$ in severely infected coffee beans from Vietnam (Leong et al., 2007) and 0.5-11.9 $\mu g/kg^{-1}$ was recently reported in roasted coffee available in the French market (Tozlovanu and Pfohl-Leszkowicz, 2010). In one study, the OTA producers isolated were species of *A. Ochraceus, A. sulphreus, A. elegans, A.*

sclerotiorum, A. auricomus, A. insulicola, A. petrakii and *A. sulphureus* (Batista et al., 2003). Additionally, the *A. flavus* species present produced AFB_1 and AFB_2 (Batista et al., 2003) and *Penicillium* species isolated all tested negative for OTA and $AFB_{1/2}/G_{1/2}$ production. Importantly, OTA was only found in 5 of 40 samples tested and the offending fungi were only isolated from the coffee bean surface and not internally (Batista et al., 2003).

A second type of mycotoxin occurring in coffee at various stages of development and production are the Aflatoxins. These include Aflatoxin B_1, B_2, G_1 and G_2 ($AFB_{1/2}$ and $AFG_{1/2}$) and are highly toxic potent carcinogenic and teratogenic *Aspergillus*-produced ketide-derived metabolites (Bokhari and Aly, 2009a; Magnoli et al., 2008). AFB_1 is the most common of these found in coffee and has been given status as a class 1 human carcinogen (Cancer, 1993). A study on *A. flavus and A. parasiticus* species isolated from Argentinean-stored Columbian-derived *C. arabica* green coffee samples revealed levels of 9.5-17.5 ng/mL and 11.5-48.0 ng/mL AFB_1, respectively; and 6.0-28.0 ng/mL AFG_1 from *A. parasiticus* (Magnoli et al., 2008). In one study, *Aspergillus* species were isolated from Arabic green coffee and the levels of AFG_1 produced (2.8-17 ng/g) were measured *in vitro* and varied depending on media, temperature and other additives present (Bokhari and Aly, 2009a). The naturally occurring AFB_1 levels were measured in the green coffee samples in another study from the same research group and levels between 0-219.0 $\mu g/kg^{-1}$ were measured with an average of 110.5 $\mu g/kg^{-1}$ and 83% of the 18 samples tested were AFB_1 positive (Bokhari, 2007). Levels of up to 13.44 $\mu g/kg^{-1}$ of OTA were also measured in this study (Bokhari, 2007).

Fumonisin B_2 (FB_2) is a carcinogenic mycotoxin normally associated with *Fusarium*, but also *Aspergillii* species, and has been detected in coffee beans (Noonim et al., 2009). In coffee, the black *Aspergillus niger* (13 species from *C. arabica* and *C. robusta* beans) were found to produce FB_2 in levels up to 9.7 $\mu g/kg^{-1}$. Low levels of other known coffee contaminating mycotoxins, such as sterigmatocystin and patulin, have also been found in green coffee beans (Bokhari and Aly, 2009b).

The European Union prohibits the sale of foods and beverages with contaminants above certain specific thresholds due to health concerns, with the current World Health Organization/Food and Agricultural Organization Joint Expert Committee on Food Additives (Joint FAO/WHO Expert Committee on food additives, 2007) setting a Provisional Tolerable Weekly Intake of 100 ng/kg bw/week for OA, for example. Many surveys have been undertaken on coffee present in various countries and National limits have also been imposed for mycotoxins in all foods (van Egmond et al., 2007). Encouragingly, fungal contamination is largely preventable through good agricultural practices, careful post-harvest processing (Bucheli et al., 2000), and appropriate storage (Bucheli et al., 1998) of separated/graded green coffee beans, thus omitting defective beans and adulterating husk (Federation, 2005).

2.2.6. Green biological control

Due to the impact of fermentative microbes on the final beverage, it is important to control the microflora present, thus preventing negative impact by fungi, especially mycotoxigenic species. Due to the increasing demand for organic coffee, it has become apparent that biological control is a viable and economical solution to the coffee leaf rust problems left in the wake of non-

pesticide farming. In a recent Brazilian study, biological control was readily accepted by farmers to support a more sustainable and ecologically sound crop production (Haddad et al., 2009). It can also be deduced that the same acceptability would be afforded to biological control agents able to act in a fungicidal manner throughout coffee fruit development and more critically during green coffee bean fermentation and germination processes.

Some LAB and yeast species are natural fungal antagonists and, as such, have been employed in green coffee as biological control agents during fermentation (Chalfoun, 2010; Velmourougane et al., 2011). LAB with antifungal activity represents a promising alternative to chemical preservation and have been employed in many foods (Rouse and van Sinderen, 2008; Schnurer and Magnusson, 2005), including; malting and brewing cereals (Lowe and Arendt, 2004; Rouse and van Sinderen, 2008), bakery products (Lavermicocca et al., 2003; Ryan et al., 2011) and fruit products (Trias et al., 2009).

LAB and yeast secondary metabolite research has lead to the identification and characterisation of food-grade anti-fungal agents which are currently in use in non-coffee food production (Lavermicocca et al., 2003; Lind et al., 2005; Rouse and van Sinderen, 2008; Ryan et al., 2011; Schnurer and Magnusson, 2005; Strom et al., 2005). Additionally, a study into *Lactobacillus* plantarum sp. antagonism of *A. carbonarius*, a frequently isolated coffee contaminating mould, revealed the anti-fungal activity of this LAB towards the ochratoxigenic black *Aspergilli* (Djossou et al., 2011). Research into *A. ochraceus* coffee mould showed yeast antagonism using species of *P. anomala, P. kluyveri* and *H. uvarum*, with the former two also preventing OTA production (Masoud and Kaltoft, 2006). The first study into the on-farm biocontrol of *A. ochraceus* was published recently (Velmourougane et al., 2011). The affordable and readily

available *Saccharomyces cerevisiae* commercial bakersô yeast was used as a fungal antagonist and resulted a reduced incidence of ochratoxigenic moulds and OTA contamination during green coffee processing (Velmourougane et al., 2011).

It has become clear that teaching small coffee farmers, who are the most common green coffee producers, in preventative measures would be a progressive step. In addition to employing good farming and processing practices, the domination of fungal contaminants can be curtailed using green methods. Educating farmers would allow a natural acceptance of new biological control measures, which are currently broadly viewed with skepticism. This approach is the way forward.

3. Conclusions and future prospects

This review has discussed the *C. arabica* and *C. robusta* green coffees with emphasis on their biochemical evolution during processing. Emphasis was placed on internal biochemical progressions during germination and the microbial disaccord during the fermentation step of post-harvest processing. The critical steps of fermentation and germination have a number of associated problems, including contamination by mould and the presence of mycotoxins. This topic was discussed from a biological control perspective and thus far, research into food applied anti-fungal LAB and yeast has been fast-growing. In spite of the growth in knowledge and ease of application of food grade bio-protective species, in particular LAB and yeast, this technology has had a low implementation rate in coffee production. However, with an increase in coffee consumption, a lack of consumer acceptance of chemical preservatives and an industry preference for economically viable food processing, alternatives are becoming more sought after.

Government and private sector food technologists are creating joint ventures to ensure that the scientific progress and necessary farm implementation can be accomplished. Therefore, a renewed and growing interest in the concept of food biopreservation is expected. In future green coffee production, this concept would allow fermentation during post-harvest processing to progress to completion without fungal contamination, provide an extended storage capacity, and enhanced safety from a toxin perspective, using naturally present or added microflora and/or their antimicrobial products.

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Table 1 Chemical composition of Green coffee beans

Compounds	***C. arabica*** **(% dry weight basis)**		***C. robusta*** **(% dry weight basis)**		
Caffeine	1.2	0.9*	2.2	1.7*	
Trigonelline	1.0	1.0*	0.7	0.9*	
Ash	4.2		4.4		
Acid components					
Chlorogenic acids	6.5		10.0	5.0-8.0[§]	
Aliphatic acids	1.0		1.0		
Quinics	0.4		0.4		
Sugar/ Carbohydrate/ Fibre components					
Sucrose	8.0	8.4*	4.0	6.4*	7.0-11.0[§]
Reducing sugars	0.1		0.4		
Polysaccharides	44.0		48.0		
Lignin	3.0		3.0		
Pectin	2.0		2.0		
Proteins	11.0		11.0	11.0-15.0[§]	
Free amino acids	0.5		0.8		
Lipids	16.0		10.0	13.0-17.0[§]	

Adapted from (Francis, 2003)
*Results obtained using LCMS (Perrone et al., 2008)
[§] Results adapted from (Joët et al., 2010)

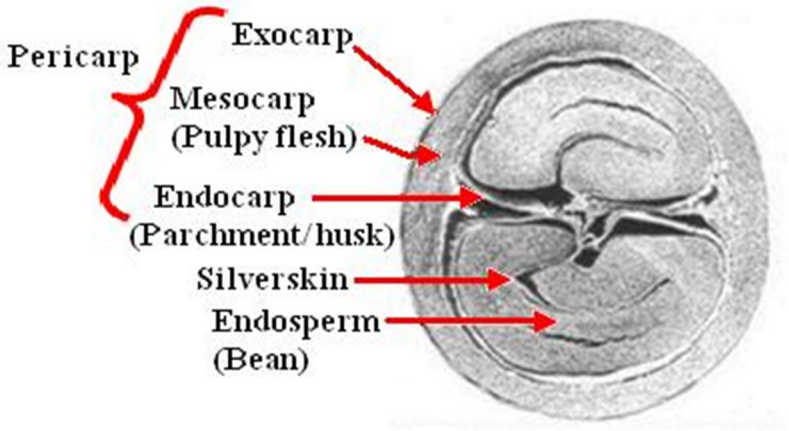

Figure 1 Two elliptical shaped coffee beans present inside the mature coffee fruit cherry (220-250 days after flowering) (de Castro and Marraccini, 2006).

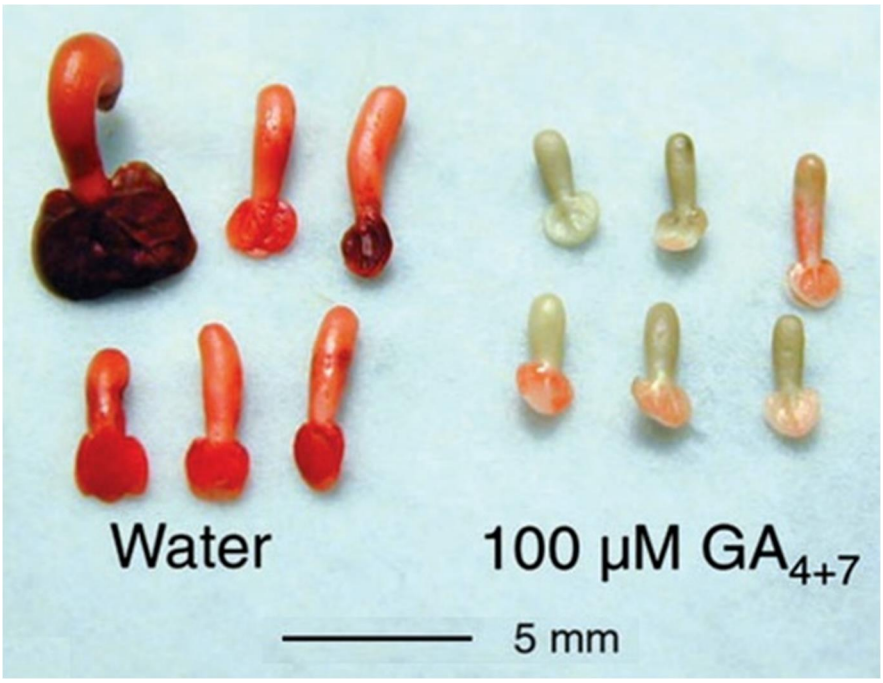

Figure 2 This figure is taken from a research paper by da Silva *et al*. (2005). It shows tetrazolium-stained spatular-shaped coffee embryos (red stain shows germinative capacity, unstained signifies cell death) from seeds 14 days after they were imbibed in water (control) or in 100 µM of GA_{4+7}.

Figure 3 Germination of coffee beans over 45 days after imbibition by water at 30 °C (unpublished pictures, University College Cork).

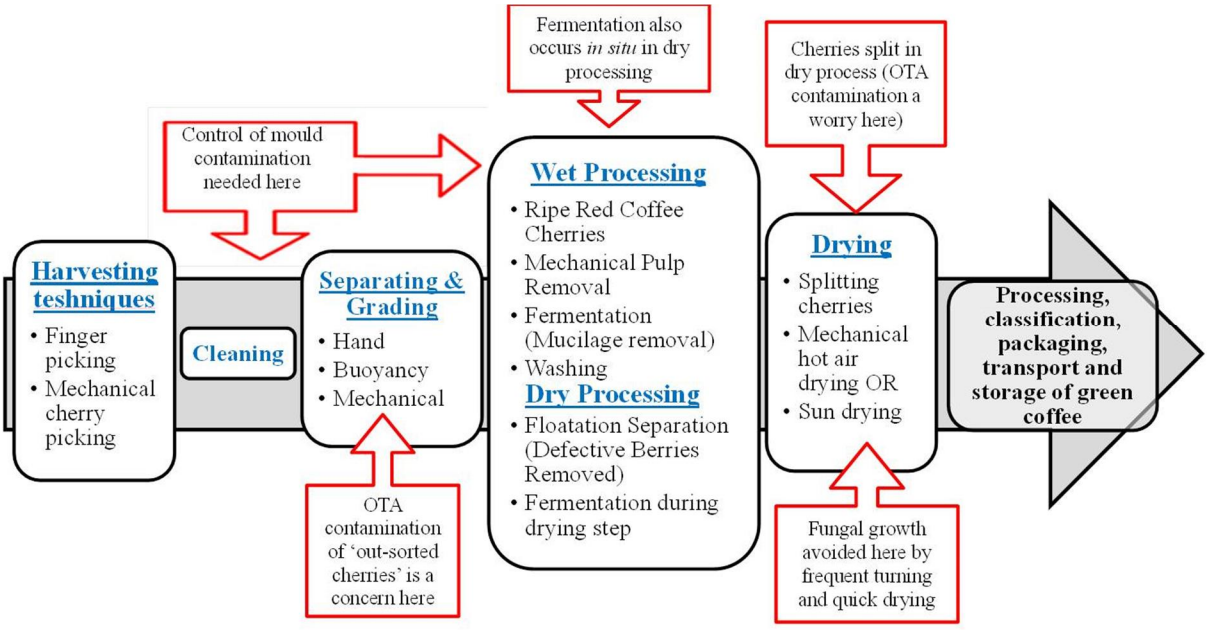

Figure 4 Coffee processing by the wet and dry methods.