Annex C.6

Delayed Processing Trials

The several iterations of the delay before processing experiments were carried out over two seasons and in different collaborator countries, adjusted to conform to their capacities and interests. It comes as no surprise to find that the results do not appear to be entirely consistent. There is plenty of evidence that the course of any given coffee process has a tendency, but only a tendency, to follow some 'usual' course, and in a proportion of instances will diverge strikingly from the expected.

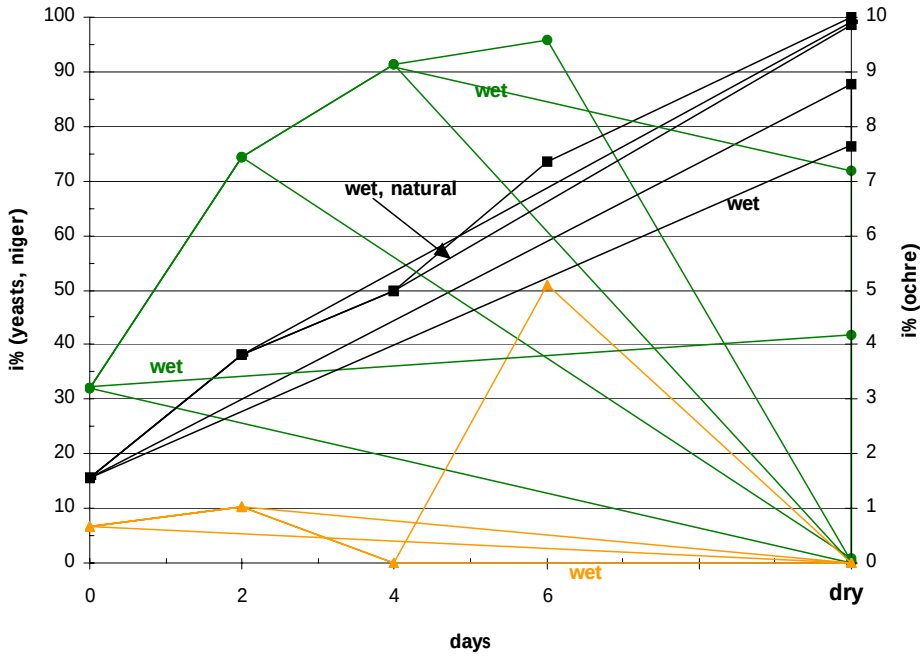
It has not been possible to discern subtle effects because the sampling/analysis systems present data with large variation around the mean. In fact, even fairly large apparent differences have been shown to fall short of statistical significance. Nevertheless, the results are meaningful if interpreted in light of experience with both the measurement and the biological systems involved and against clearly enunciated hypotheses.

In this Annex the data on fungal communities of the seed are presented and discuss ed in light of OTA production, which can be taken as an indication of growth of OTA-producing fungi.

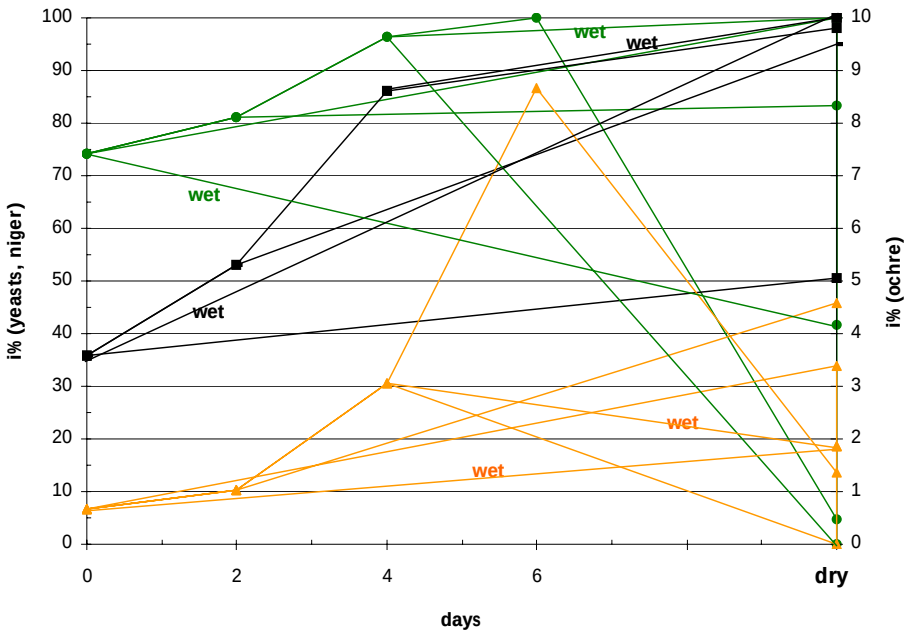
Comparing two runs from the Côte d'Ivoire 2003-4 season shows the impact of a difference of initial conditions. The same pattern is discernable though the magnitudes of the populations are different and, in some cases, this produces a partially different outcome.

Figures A & B: Changes in infection rate of robusta coffee beans (i-infection) by yeasts (green), niger group aspergilli (black) and ochre group aspergilli (orange). At Day 0 three treatments were established: cherry drying; wet processing by fermentation; sacking. At Day 2 a sub-sample was removed for cherry drying. At Day 4 two sub-samples were removed: wet processing by fermentation; cherry drying. After 6 days of sacking the remaining fruit was dried as cherry. On the graph, all the lines represent cherry processing except where 'wet' is superimposed. Note that the slopes of all lines to the determination at dryness ('dry') are not numerically significant since this parameter is not measured with days. Note that ochre aspergilli are graphed against a second y-axis.

Run 1, Delayed processing



Run 2, Delayed processing



The coffee in the second run had a higher initial infection rate of yeast and niger aspergilli. Excepting the persistence of the yeasts in the beans of the 4-day delayed wet processed treatment and the persistence of yeasts in immediately dried cherry coffee, the initial high population of yeast in the second run did not significantly affect outcomes.

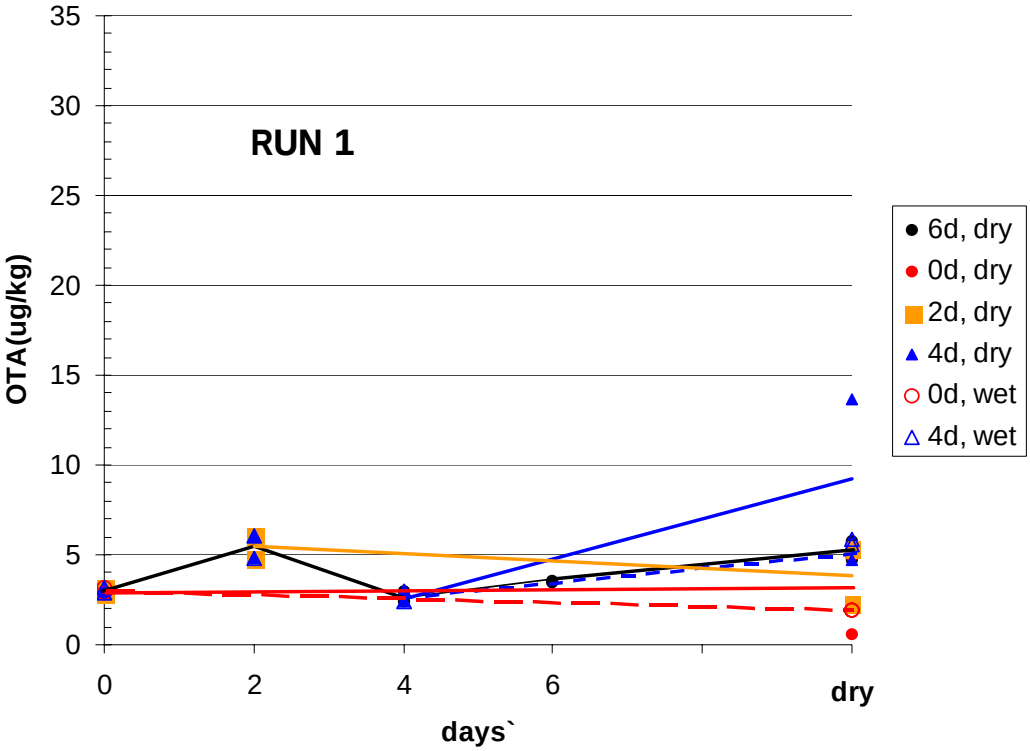
Similarly, with the niger aspergilli, outcomes are very comparable, except that the immediately dried cherry developed fewer infections during drying when the initial infection rate was higher. This could have been due to the exceptionally high yeast population.

Ochre aspergilli frequency was initially the same (0.5%) in both runs. In the second run the ochre aspergilli persisted through most treatments (all except 4-day delayed cherry processing) whereas it was not detected after drying in the first run. The actual differences are too small to be considered statistically significant but it is clear that in the second run, the fungus survived processing and it was this run where some probable increases in OTA were observed.

During sacking, a numerical increase in ochre infection was recorded that corresponds to a period during which it is clear there is no increase in OTA. This could signify that there was little increase in biomass, whether or not there was an actual increase in infection, or that the fermentation conditions prevented *net* accumulation: possibly the observed super-optimal temperatures and undoubted very low O_2 levels prevented OTA production, or perhaps there was higher gross production but enzymatic or respiratory breakdown took place during this fermentation.

Table A summarizes the differences of the second run; there are none in the first run. Both wet processing treatments (with and without a 4-day delay) and direct drying are the only treatments that may have produced a change in OTA content, in this case an increase.

Figures C & D: OTA levels from samples taken during processing and rapidly oven-dried. Solid lines represent dry processing treatments; broken lines indicate wet processing treatments. Red is no delay; amber 2d delay; blue 4d delay; black 6d delay. The data are graphed on identical axes. Note that the slopes of all lines to the determination at dryness ('dry') are not numerically significant since this parameter is not measured with days.



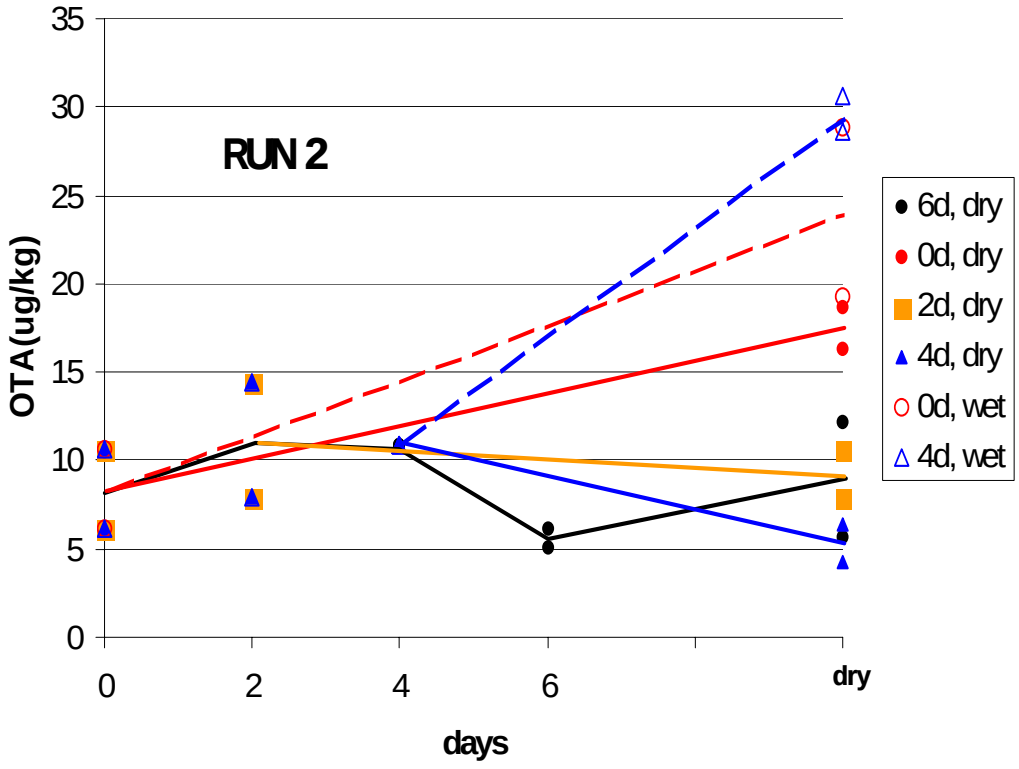


Table A: An analysis of the performance of the six treatments in the second run of the delayed processing experiment with respect to the two measured parameters. There is not enough replication for a statistical test but standard error is typically between 75 and 150%.

Final ochre infection (max) [initial = 0.5%]	Treatment	OTA (ppb) [initial = 8]
→ 2% (3%)	4d delay, wet	>> (29)
→ 2% (2%)	0d delay, wet	>> (24)
→ 3.5% (3.5%)	0d delay, dry	+ (18)
- No effect on OTA content -		
→ <0.2% (3%)	4d delay, dry	Between 5 and 9
→ 1% (8.5%)	6d delay, dry	
→ 4.5% (4.5%)	2d delay, dry	

The experiment was refocused for the 2004-5 season on a 4-day delay period in cherry processing followed by drying on different surfaces.

The fungal communities of the two runs were distinct. The first run initially had a high level of yeast infection (86%), little niger aspergilli and a significant occurrence of ochre aspergilli at 7%. Nine of 12 replicate samples showed detectable ochre aspergilli (Table C). Initially in Run 2 yeast infection was much lower (19%) but niger aspergilli much more prominent at 37%. Ochre aspergilli were detected in only 3 of 12 replicates, giving an average occurrence of 3%. A distinction of this community is that 'other aspergilli' and *Penicillium* collectively produced almost 70% of the infection rate. *Fusarium* was not found in significant numbers in either run.

Unlike the previous season, yeast infection rates fell over four days of sacking as did that of niger aspergilli infection in the second run, but not in the first where it increased. Ochre aspergilli frequency was below detection limit after sacking in the second run but apparently unaffected in the first run. Despite this, it was the second run that showed high OTA levels, not the first run where this OTA-producer was present throughout.

None of the numerical differences between the drying surfaces in respect of the development of the fungal communities inhabiting beans could be said to be significant. In the dried product ochre aspergilli were rare in the second run but relatively common in the first. This represents a familiar pattern where an initial ochre aspergilli infection above a certain level, 5-10%, tends to persists through processing; below this level it will tend to disappear.

The highly replicated design of this study presents an opportunity to examine variation in sampling/analysis (the source of the variation cannot be distinguished

between these two) of relatively uncommon taxa as experienced in these studies. Table C sets out the complete data set for infection rates of ochre aspergilli in the first run.

Table B: Bean infection rates of cherries by selected fungi sampled before, during and after robusta cherry processing. Almost all the samples showed complete infection so this measure is omitted as are the minor fungi. Fresh and Day 4 determinations are replicated 12 times and each treatment, 4 times.

	Run 1				Run 2			
Taxonomy	**Fresh**	**Day 4**	**Dry 0d Delay**	**Dry 4d Delay**	**Fresh**	**Day 4**	**Dry 0d Delay**	**Dry 4d Delay**
	Cement				**Cement**			
Yeasts	86%	2%	3%	33%	19%	8%	2%	1%
Niger aspergilli	4%	40%	86%	47%	37%	10%	75%	83%
Ochre aspergilli	7%	5%	9%	<1%	3%	<0.1%	0%	0.2%
Other aspergilli	0%	34%	28%	26%	36%	92%	99%	75%
***Penicillium* spp.**	3%	29%	6%	1%	32%	20%	22%	14%
	Tarpaulin				**Tarpaulin**			
Yeasts			0%	21%			1%	5%
Niger aspergilli			94%	49%			81%	61%
Ochre aspergilli			4%	8%			0%	0%
Other aspergilli			30%	33%			100%	77%
***Penicillium* spp.**			0%	1%			10%	12%
	Bamboo				**Bamboo**			
Yeasts			8%	0%			1%	0%
Niger aspergilli			81%	65%			99%	93%
Ochre aspergilli			3%	11%			0%	0%
Other aspergilli			21%	78%			100%	100%
***Penicillium* spp.**			12%	9%			1%	9%

All replicates of the initial sampling represent one mean and all replicates of the 4 day sacking treatment represent another since in neither case have the other treatments, different drying surfaces, been applied. There are fewer high infection rates after sacking, though the number of non-zero analyses is higher than in the fresh cherry analysis. The means of the two treatments are close at 7% and 5% respectively and the standard error comes down after sacking to 76% from 124% in the fresh cherry. This may be coincidence or could reflect a property of the distribution adjusting to more uniform conditions in sacks.

Table C: Complete data set of ochre aspergilli infection rate from Run 1 of the delay of processing experiment. The arrows reiterate the relationship between the initial and final conditions.

		Replicates				Surfaces
Initial 0		11%	0%	19%	0%	Cement
		29%	6%	6%	0%	Tarpaulin
		7%	1%	6%	1%	Bamboo
	Initial 4	2%	7%	7%	4%	Cement
		1%	2%	6%	4%	Tarpaulin
		11%	9%	3%	0%	Bamboo
Dry 0		6%	26%	0%	6%	Cement
		0%	1%	16%	0%	Tarpaulin
		4%	0%	4%	3%	Bamboo
	Dry 4	2%	0%	0%	0%	Cement
		7%	21%	0%	1%	Tarpaulin
		10%	9%	19%	9%	Bamboo

Table D: Means and standard errors for ochre aspergilli bean infection rates (i-analysis) calculated from the values in Table C, above.

	Mean	Standard Error	Overall Mean	Overall Standard Error
Fresh	7%	124		
Delay 4	5%	72		
Cement	10%	120		
Tarpaulin	4%	185	6%	143
Bamboo	3%	69		
Cement	1%	200		
Tarpaulin	7%	133	7%	115
Bamboo	12%	41		

In studies with fewer replicates, it is clear that the interpretation is indicative: when 12 sub-samples can have a mean of 7%, a maximum of 29% and three sub-samples showing an occurrence below detection, small differences are insignificant. A single infection from a sample relates to 1 to 2% depending on sample size, and on this data, standard error is about 100%. Two or three replicates supporting a mean value adds much significance to the mean, but distinguishing small differences is not justified.

None of the determinations of the treatments fall outside the range of values in the initial analysis so there is no likelihood that the treatments produced a statistical change in ochre infection rate. Once again, this is not a measure of biomass but argues there is no de novo infection or mortality. By looking at the frequency of high and low values it could be argued that sacked, dried on cement, caused some mortality and sacked, dried on bamboo mat, caused some de novo infection.

OTA analysis shows that the first run had less OTA initially and less at the end of processing in a pattern not apparently related to that of ochre aspergilli occurrence. Standard deviation ranges from about 40% of the mean to well over 200%, but most are about 100% of the mean. There is unlikely to be any statistically significant differences in this data set but certain trends should be drawn out for discussion.

Numerical trends will be described first, followed by a discussion of possible significance. The fresh cherry contained about 1.5ppb OTA in Run 1 and about ten fold that amount in Run 2. After four days in sacks, the OTA content had increased to 7.6 and 27ppb, respectively.

The cherry that was immediately dried contained about 4ppb at the end of drying with no differences produced between the cement, plastic tarpaulin and bamboo mat surfaces. In the second run immediately dried cherry, dried on cement, contained about 26ppb, dried on tarpaulin 12.5ppb, and dried on bamboo mats almost 10ppb.

Coffee that had spent four days in sacks before drying contained about 6ppb after drying on cement and tarpaulin and about 9ppb after being dried on bamboo matting in the first run. In the second run, the cement and tarpaulin treatments contained about 12ppb after drying, and about 6ppb on bamboo matting.

Table E: Numerical analysis table for Runs 1 and 2, ppb of OTA

Time	Surface	Run 1		Run 2	
0		1.5	(Sacked)	15	(Sacked)
4d			7.6		27
Dry	Cement	4	6	26	12
	Tarpaulin	4	6	13	12
	Bamboo	4	9	10	6

The data set for the two runs only shows four clear outliers as defined in the legend of Table F, below. In the first run only one sample from the 4-day sacking sampling is an outlier and the effect of removing it is to reduce the mean from 7.6 to 5ppb and the standard error from 125% to 50%.

In the second run the mean value of initial OTA contamination falls from about 16 to 12ppb and the standard error from 90% to about 50%; the content after four days in sacks falls from 27 to about 9ppb and the standard error from over 230% to 100%; and the immediately dried on cement treatment final determination falls from about 26 to about 12ppb while the standard error falls from around 115% to less than 30%.

Aside from a numerical rule, the other evidence that these values are outliers is that the trends they would represent, had they been 'real', are not borne out by the related samples, i.e. the hypothesis they require is not supported by other evidence.

Table F: Complete OTA analysis of two runs of the delay of processing protocol from the 2004-5 season. Values in blue are outliers defined by the value approaching or exceeding (next largest value + 2 x standard dev). C=cement; T=plastic tarpaulin; B=bamboo matting on tables. The arrows reiterate the relationship between the initial and final conditions.

Run 1	Replicates				Surface
Initial 0	1.4	1.0	0.9	1.3	Cement
	1.1	1.3	1.1	3.8	Tarpaulin
	1.6	1.8	2.4	0.6	Bamboo
Initial 4	6.1	3.5	10.7	1.9	Cement
	4.9	6.9	6.7	3.0	Tarpaulin
	4.3	3.9	36.7	3.1	Bamboo
Dry 0	5.0	3.3	1.9	5.1	Cement
	3.5	9.1	2.8	1.9	Tarpaulin
	2.1	4.1	6.7	3.8	Bamboo
Dry 4	4.5	3.8	5.0	11.9	Cement
	9.3	1.5	6.9	4.6	Tarpaulin
	10.5	8.9	14.7	3.0	Bamboo
Run 2					
Initial 0	5.0	6.5	56.1	19.3	Cement
	24.4	14.0	17.9	7.1	Tarpaulin
	9.0	7.3	17.2	6.6	Bamboo
Initial 4	4.3	225.7	5.8	9.3	Cement
	5.1	16.1	7.5	35.3	Tarpaulin
	5.7	4.5	3.1	5.5	Bamboo
Dry 0	70.6	15.2	11.1	8.8	Cement
	6.3	9.7	28.9	5.3	Tarpaulin
	10.4	9.3	13.7	4.8	Bamboo
Dry 4	13.9	6.6	18.3	9.4	Cement
	12.4	25.0	2.4	10.4	Tarpaulin
	5.7	2.7	11.1	4.1	Bamboo

In the first run, indications are that an increase in OTA takes place through processing since none of the 12 samples are above 4ppb, whereas 5 of 12, after immediate drying, and 9 of 12 after sacking and drying, are above this level. The samples are otherwise thoroughly over-lapping so there is no effect of sacking or of the drying surface.

Table G: Collated means with and without outliers. The initial samples at 0 and 4 days can each be averaged since the treatments are not enacted at the time of sampling. 'B1' and 'B2' = two different sources of cherries; two processing chains (sacked and immediately dried) are dried on each of three surfaces: cement; plastic tarpaulin; bamboo matting on tables.

Including all data

	Run 1				Run 2				
	All	By source		St dev	All	By source		St dev	Surface
		B1	B2			B1	B2		
0	1.5	1.4	1.7	0.8	15.9	11.0	20.7	14.1	
4	7.6	4.9	10.3	9.5	27.3	43.6	11.1	63.1	
0 to Dry	3.8	4.1	3.5	1.5	26.4	42.9	10.0	29.6	Cement
	4.3	6.3	2.4	3.3	12.5	8.0	17.1	11.0	Tarpaulin
	4.2	3.1	5.2	1.9	9.6	9.8	9.3	3.7	Bamboo
4 to Dry	6.3	4.5	4.4	3.7	12.0	13.9	12.4	5.2	Cement
	5.6	6.7	4.2	3.3	12.6	12.4	13.7	9.4	Tarpaulin
	9.3	10.5	11.8	4.9	5.9	5.7	6.9	3.7	Bamboo

Excluding outliers

	Run 1				Run 2				
	All	By source		St dev	All	By source		St dev	Surface
		B1	B2			B1	B2		
0	1.5	1.4	1.7	0.8	12.2	11.0	13.6	6.6	
4	5.0	4.9	5.1	2.5	9.3	7.2	11.1	9.3	
0 to Dry	3.8	4.1	3.5	1.5	11.7	15.2	10.0	3.2	Cement
	4.3	6.3	2.4	3.3	12.5	8.0	17.1	11.0	Tarpaulin
	4.2	3.1	5.2	1.9	9.6	9.8	9.3	3.7	Bamboo
4 to Dry	6.3	4.5	4.4	3.7	12.0	13.9	12.4	5.2	Cement
	5.6	6.7	4.2	3.3	12.6	12.4	13.7	9.4	Tarpaulin
	9.3	10.5	11.8	4.9	5.9	5.7	6.9	3.7	Bamboo

The second run is quite different. Ignoring the outliers, the initial sampling is scattered, five are above 12ppb (the overall average), averaging 18ppb, and 6 are below the overall average, averaging 7ppb. After four days sacking, only two of 11 samples are above 12ppb; immediate drying produces three samples of 11 above the 12ppb level; drying after sacking has 4 of 12 samples, average 17ppb, above the initial or control level with 8 samples, averaging 7ppb. The two lowest values in the data set appear in the dry 4-day sacking group.

The effect of removal of the outliers is disproportionately to reduce variation as against changing the mean, and thereby clarifies where the 'weight' of the results lies. It does, however, remove a sense of the high (and, potentially, low) extremes that occur in sampling of a heterogeneous system such as mycotoxins in grains of a good size. Standard error, a measure of sample variation, does not increase above control levels as a result of the treatments so the presence of a few outliers (they are few by definition), especially as they appear in both control and outcome samplings, is not an indication of an upward trend in variation through the application of the treatments.

Other delay studies have indicated that coffee with higher initial OTA was less stable and more susceptible to subsequent OTA production during processing. The implication was that higher OTA related to higher standing biomass of mycotoxigenic fungi and therefore quicker growth once conditions improve with respect to their eco-physiological capacity. Here it appears that if there are any changes through processing, it is the less contaminated coffee of the first run that changed. The mycological data shows the second run to have had a lower contamination rate of ochre aspergilli throughout the run, assuming the category 'other aspergilli' doesn't contain a proportion of such OTA-producers. This is contrary to expectations borne of the higher OTA levels and some of the other studies.

Similar delay of processing experiments were conducted in Meru and Thika, Kenya, although neither dry processing nor delay of processing are common in Kenya.

The means of selected fungal taxa are presented in Tables H and I, below. There was very little in the way of OTA-producers and no ochre aspergilli was detected in either of the two Meru trials. One sample that had experienced a 6-day delay before cherry drying, and another that had been fermented on the day of harvest had ochre aspergilli at or around the detection limit. This is broadly consistent with the picture of OTA content.

One of 24 samples from Meru showed OTA at quantifiable levels (0.1 ppb), this from four day delayed cherry coffee. From Thika, three samples contained enough OTA to quantify and all were less than 0.75ppb. All four of these were from the cherry coffee treatments, one each from no delay, 4-day delay and 6-day delay treatments. The latter treatment is one of the two that also contained ochre aspergilli at detectable levels. The OTA analysis data set is not included here.

Infection by yeasts reliably increases over the six days of delay reflecting the increase in yeasts in the x+m analysis. Their high numbers and dominance fell away during drying, whether or not the coffee was fermented. Niger aspergilli are not very common in arabica coffee, but sacking did allow this group to become important in one of the repetitions. Generally, however, their numbers were low but significant in the cherry coffee but never above 5% in the parchment.

Fusarium was generally present and sometimes became important but generally did not show much tendency to increase during sacking. *Penicillium* also was occasionally numerically important and, like the niger aspergilli, showed some tendency to increase during sacking.

Table H & I: Means of infection frequency (i - analysis) and mesocarp + external community (x+m - analysis) of arabica cherry coffee held in sacks before either wet or dry processing. Two Runs were conducted in Thika district and two in Meru district, Kenya.

Periods of delay are in days (0, 4 or 6). 'Final' represents the analysis of the dried product, prepared as described in the columns of the table. Parchment was prepared only after 0 and 4d delay, all 6d delay was prepared as cherry coffee. i – analysis in % infection of beans (could be >100%). x+m – analysis is presented as total c.f.u./cherry, followed by the proportion of this total represented by the respective taxon.

Thika - Run 1

	Initial			Final			Initial x+m		
Cherry	0d	4d	6d	0d	4d	6d	0d	4d	6d
Total	44%	93%	85%	18%	59%	90%	9.7E+02	5.2E+04	1.3E+05
Yeasts	5%	41%	43%	0%	2%	1%	0.61	0.88	0.89
Niger grp	0%	3%	3%	0%	8%	6%	0.00	0.00	0.00
Ochre grp	0%	0%	0%	0%	0%	2%	0.01	0.00	0.00
Penicillium	3%	22%	47%	0%	23%	19%	0.08	0.02	0.04
Fusarium	30%	41%	62%	2%	24%	45%	0.04	0.04	0.05

	Parchment	
Total	25%	16%
Yeasts	9%	5%
Niger grp	1%	0%
Ochre grp	1%	0%
Penicillium	1%	0%
Fusarium	6%	7%

Thika - Run 2

	Initial			Final			Initial x+m		
Cherry	0d	4d	6d	0d	4d	6d	0d	4d	6d
Total	53%	85%	99%	20%	28%	57%	6.9E+02	5.3E+0	1.1E+05
Yeasts	1%	48%	40%	0%	0%	0%	0.41	0.97	0.93
Niger grp	14%	6%	48%	1%	15%	45%	0.04	0.00	0.03
Ochre grp	0%	0%	0%	0%	0%	0%	0.01	0.00	0.00
Penicillium	1%	7%	8%	1%	3%	2%	0.06	0.00	0.00
Fusarium	21%	18%	35%	5%	6%	7%	0.12	0.01	0.00

	Parchment	
Total	25%	26%
Yeasts	5%	4%
Niger grp	1%	5%
Ochre grp	0%	0%
Penicillium	2%	3%
Fusarium	12%	5%

Meru - Run 1

	Initial			Final			Initial x+m		
Cherry	0d	4d	6d	0d	4d	6d	0d	4d	6d
Total	34%	92%	99%	20%	12%	98%	6.1E+03	2.2E+05	6.6E+05
Yeasts	14%	63%	71%	9%	9%	1%	0.73	0.97	0.93
Niger grp	0%	1%	90%	0%	5%	94%	0.00	0.00	0.03
Ochre grp	0%	0%	0%	0%	0%	0%	0.00	0.00	0.00
Penicillium	1%	2%	3%	1%	3%	8%	0.03	0.01	0.02
Fusarium	9%	6%	5%	3%	2%	3%	0.05	0.01	0.00

	Parchment	
Total	29%	26%
Yeasts	12%	24%
Niger grp	0%	5%
Ochre grp	0%	0%
Penicillium	0%	2%
Fusarium	2%	2%

Meru - Run 2

	Initial			Final			Initial x+m		
Cherry	0d	4d	6d	0d	4d	6d	0d	4d	6d
Total	13%	61%	77%	14%	32%	47%	7.6E+02	2.4E+04	9.5E+03
Yeasts	0%	28%	14%	0%	0%	0%	0.49	0.94	0.88
Niger grp	0%	0%	3%	1%	6%	4%	0.00	0.00	0.00
Ochre grp	0%	0%	0%	0%	0%	0%	0.00	0.00	0.00
Penicillium	1%	4%	23%	0%	3%	12%	0.03	0.00	0.03
Fusarium	4%	16%	36%	6%	10%	18%	0.17	0.01	0.02

	Parchment	
Total	31%	21%
Yeasts	5%	3%
Niger grp	0%	5%
Ochre grp	0%	0%
Penicillium	9%	1%
Fusarium	8%	2%

Other iterations of the basic design of looking at different periods of delay in sacks were conducted in Uganda. Mesophilic fungi would be expected to be inhibited under the conditions of sacking but the niger aspergilli, especially in robusta coffee, has been shown to be able to increase its infection rate in sacks. This is demonstrated here as well as an indication the flavi aspergilli (potentially aflatoxin producers) can also increase infection rates over the first four days of delay.

Ochre aspergilli appear at low frequency in the dried product of both runs and after 2 or 4 days of sacking in the second run. On this evidence one could hypothesise that

two and four days fermentation in sacks favours these fungi and direct drying and 6 days fermentation does not.

Surprisingly, yeasts are not reported to infect the coffee in these experiments. This could be due to unfamiliarity with their often subtle growth since they do not spread as filamentous organisms do, making identification more difficult.

There is little evidence in the mycological measures of the impact of sacking on outcome, all of which look the same. This is partly due to the strong association of the niger aspergilli with robusta cherry: most is highly infected, regardless of its history. An important clarification to this is that the measure is of ***frequency of occurrence*** and not ***biomass***, so frequency might reach the maximum quite quickly leaving scope for differences in growth, which we cannot measure, to arise. No OTA analysis is available for this work.

Table J: Changes in the fungal community inhabiting beans of cherries exposed to progressive delays (held in sacks) in spreading and sun-drying and the outcome of their eventual drying. Changes between the days of sacking can be taken to represent a fermentation time-course.

Run 1	Period delay before drying				Dry 0d	Dry 2d	Dry 4d	Dry 6d
	0	2	4	6				
Total infection (%)	10	95	82	63	78	100	100	96
Yeasts	0	0	0	0	0	0	0	0
Black aspergilli	1	41	60	41	74	100	100	96
Ochre aspergilli	0	0	0	0	0	4	6	0
Flavi aspergilli	0	32	55	6	0	19	22	7
Others	10	11	3	3	12	16	3	22

Run 2	Period delay before drying				Dry 0d	Dry 2d	Dry 4d	Dry 6d
	0	2	4	6				
Total infection (%)	13	96	99	100	100	100	100	100
Yeasts	0	0	0	0	0	0	0	0
Black aspergilli	8	48	99	100	100	100	100	100
Ochre aspergilli	0	1	2	0	0	1	3	0
Flavi aspergilli	0	4	1	0	2	5	2	0
Others	3	5	1	0	20	11	7	8

In a second experimental design, a 4-day sacking period was the focus with an additional treatment added to assess any impact of drying sacked cherry on plastic tarpaulin as compared to what is often taken as best practice, viz. cement surfaces.

Again, there were no differences in outcome between the treatments as measured by frequency of fungi and no ochre aspergilli were detected (Table K, below). Despite this, OTA was present in the fresh cherry. *A. carbonarius* was also not detected but it is possible it went undetected since it is very difficult to distinguish against a high background of *A. niger*-type isolates. Yeast infection, the only instance in these two studies, developed in the coffee dried on tarpaulin.

Despite the ochre aspergilli being present below detection limit, if at all, the coffee contained some OTA at harvest. Results are presented in Table L, below. The standard error is good for this type of system, usually around 75%. Even so, none of the treatments produce a statistically significant increase in OTA.

Looking at numerical differences, two of the four repetitions of this treatment went up but two, including the largest change, went down. The possibility that sacking predisposes the cherry to forming OTA during drying when the moisture content becomes more favourable for the growth of OTA producers also is not convincingly supported. Three of the eight cases actually fall during drying. While these differences, like the five increases, are not statistically significant, they cannot be construed as increases. In general the standard error tends to about 75%, in other words, 33% of values fall outside of the mean ± ¾ of the mean. On this measure, too, there is no consistent difference between treatments.

Table K: Changes in the fungal community (selected taxa in %) inhabiting beans of cherries exposed to a four-day delay (held in sacks) in spreading and sun-drying and the outcome of their eventual drying on cement or plastic tarpaulin.

	Fresh	4d sacked	Cement		Tarpaulin	
			Normal	Delay	Normal	Delay
Total infection	56	86	98	100	97	100
Yeasts	0	0	0	0	8	8
Black aspergilli	35	79	98	100	89	92
Ochre aspergilli	0	0	0	0	0	0

Table L: OTA production in beans of cherries exposed to a 4-day delay (held in sacks) in spreading and sun-drying and the outcome of their eventual drying on cement or plastic tarpaulin. Means are of six determinations taken from six experimental units, in ppb.

Run 1		Tarpaulin		Cement	
		Fresh	Dry	Fresh	Dry
0d delay	**Mean**	**3.0**	**2.3**	**1.0**	**2.0**
	Std error	91	55	159	84
4d delay	**Mean**	**1.4**	**6.4**	**1.7**	**1.8**
	Std error	101	71	79	81

Run 2		Tarpaulin		Cement	
		Fresh	Dry	Fresh	Dry
0d delay	**Mean**	**2.2**	**3.9**	**5.5**	**4.0**
	Std error	133	35	22	29
4d delay	**Mean**	**3.9**	**2.8**	**0.0**	**1.9**
	Std error	39	117		64

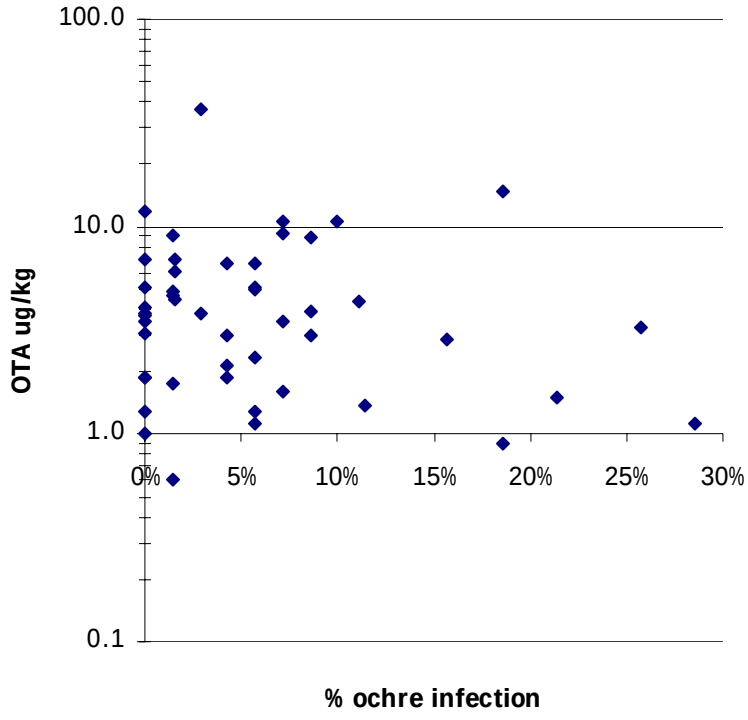
With the presence of *A. ochraceus* and *A. carbonarius* unconfirmed it is possible that another species has produced the observed OTA. Some isolates of *A. niger* can

produce OTA. However, *A. niger*'s success in the experiment would suggest that the OTA level would have gone up markedly if this was the case. It would be difficult to imagine that so much infection could be accomplished without significant growth.

Correspondence between the presence of what is undoubtedly the most important OTA-producer, *A. ochraceus*, and OTA is not close as Figure E shows. Some samples have high OTA and little or no ochre aspergilli isolated others have little OTA and high OTA infection. Of course there are many samples that suggest some correspondence too but, as a population, OTA appears to be independent of ochre aspergilli.

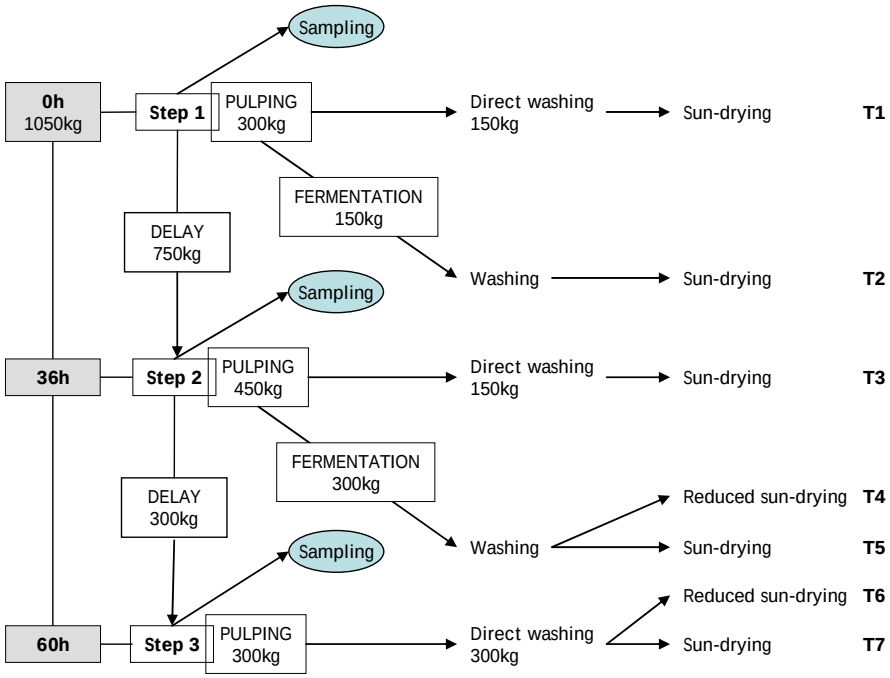
There are many factors available to intervene to explain the lack of quantitative correspondence such as the difference between presence (infection) and growth (development of biomass). Also the fact that only a proportion of this species produce the toxin and that due to practical problems in identification, some of the 'ochre aspergilli' are undoubtedly non-producing species of the section. Importantly, it must be remembered that the error of single determinations of either parameter is very high.

Figure E: Scatter plot of samples for which OTA and ochre aspergilli were determined in an experiment in which both OTA and ochre aspergilli were readily detected.



The design of these experiments, conducted in Colombia, provides for direct comparisons between traditional fermentation, mechanical washing and the 'solid state' fermentation that takes place in the sacks. Figure F summarises the protocol, which is somewhat complicated. There was no significant OTA produced in these studies, though OTA producers were detected.

Figure F: Diagram of the Colombian version of the delay of processing experiment run in May and September 2004. The labels 'T1' to 'T7' locates the treatments in the procedure as is given in subsequent tables.



Contrary to mucilage analysis which showed yeast populations higher in May than September (data not shown here), the bean infection by yeasts was more pronounced in September than in May. Both show that traditional fermentation conditions (T2) are more conducive to this than the sacking fermentation (T3) but the yeast infection increases again with further delay (T4/5 and T6/7). In this case the effect of the traditional fermentation is lost, i.e. the lack of distinction between T4/5 and T6/7. Perhaps this is a pH effect since bacteria rapidly reduce the pH to about 4.5 (close to the *Lactobacillus* minimum), and yeasts are then capable of further reductions.

Ochre aspergilli were isolated regularly and at levels where OTA has been found in other regions. The taxon was more in evidence in May than September. Consistent with other studies, ochre aspergilli were detected during the sacking period and often persisted through drying. In May, the highest level (12%) was reached in traditional fermentation where there was a remarkably low level of yeast infection. The ochre aspergilli were distributed more or less evenly, with no discernable reduction, in all of the products.

Apparently, there are no treatment effects on infection rate of ochre aspergilli. Of course, it is possible that this fungus could grow and produce OTA in some of the treatment conditions without any impact on infection rate.

Table M: Internal fungal communities during processing and in the final product with delayed processing – Colombia studies.

May 2004 Run

Time	Treatment	Yeast	Ochre	Niger	Flavi	*Penicillium*	*Fusarium*	*Cladosporium*
0	**T1**	22.4	0.0	0.0	0.0	25.5	0.0	0.0
36	**T2**	14.3	12.2	23.5	2.0	64.3	0.0	2.0
36	**T3**	3.1	2.0	13.3	2.0	90.8	0.0	0.0
60	**T4/T5**	57.1	3.1	3.1	0.0	26.5	0.0	0.0
60	**T6/T7**	61.2	0.0	3.1	0.0	39.8	0.0	0.0
Dry	**T1**	17.5	2.1	2.1	0.4	56.1	32.1	3.9
Dry	**T2**	9.6	1.1	1.4	0.4	38.6	24.3	2.5
Dry	**T3**	3.6	1.4	0.0	0.7	40.7	8.9	1.1
Dry	**T4**	3.6	1.1	0.0	0.7	76.8	6.4	0.4
Dry	**T5**	27.1	2.9	0.0	0.0	93.2	5.7	0.0
Dry	**T6**	60.4	0.4	0.7	2.1	53.9	3.6	0.0
Dry	**T7**	72.1	0.7	0.0	0.4	99.6	0.7	0.7

September 2004 Run

Time	Treatment	Yeast	Ochre	Niger	Flavi	*Penicillium*	*Fusarium*	*Cladosporium*
0	**T1**	31.6	0.0	0.0	0.0	41.3	13.3	1.5
36	**T2**	83.7	0.0	1.0	0.0	12.2	4.6	0.5
36	**T3**	31.6	2.6	2.6	3.1	52.0	3.1	3.1
60	**T4/T5**	53.9	0.4	0.2	1.4	46.6	37.1	6.6
60	**T6/T7**	50.9	1.6	0.0	2.5	58.4	27.9	3.2
Dry	**T1**	99.6	0.0	0.0	0.0	95.4	73.9	0.0
Dry	**T2**	43.6	0.3	0.0	1.4	65.0	74.6	1.4
Dry	**T3**	97.9	0.0	0.0	1.1	81.4	33.2	0.4
Dry	**T4**	96.1	0.4	0.0	2.5	35.0	58.6	2.9
Dry	**T5**	11.8	0.4	0.4	0.4	58.2	15.7	10.4
Dry	**T6**	97.5	1.8	0.0	5.0	37.9	41.4	1.4
Dry	**T7**	4.3	1.4	0.0	0.0	78.9	14.3	5.0

OTA, however, was only detected in a few samples at <0.3ppb with one exception. 3.2ppb was reported in from a treatment in September, run independently by the collaborators which was parchment produced under Cenicafé's GMP regime, comprising 24 hours fermentation, with several hand sorting steps.

Unexpectedly, *Fusarium* was only detected in the final product, which goes against other data that exists on the dynamics of this fungus through processing. The apparent success in both runs of *Penicillium* during the sacking period, and its persistence during drying, has been seen sporadically in other studies and the persistence of the yeasts during drying here is also notable.

Drying conditions in May were very poor, and those in September were good, but the two aspects of the mycology that diverge in comparison are that yeasts and *Fusarium* are at higher levels in the products, regardless of the treatment. In other

words, there are more powerful factors than the treatments exerting an influence on the outcome.

A second form of delay, holding under water (or 'soaking') was also studied and, in one trial in India, compared to sacking, fermentation and dry processing over two seasons. The initial study was restricted to soaking referenced to traditional fermentation.

Table N: Mycological data from individual runs and the means of three runs of delayed arabica processing by soaking. All data is % internal bean infection. '<' = not detected; 'floats' = floats coffee, was processed as cherry coffee; 'final' data is from the dried product; only the cupping for the first run was completed.

	Initial fresh			48h soaked			Fermented		
	1	2	3	1	2	3	1	2	3
Total infection %	20	63	63	68	56	60	92	86	95
Ochre aspergilli	<	<	<	<	<	<	<	<	<
Niger aspergilli	<	<	<	<	<	<	20	2	<
Fusarium	<	<	<	<	<	<	<	<	<
Yeast	<	47	43	<	38	43	<	24	46
Cladosporium	3	1	8	24	2	<	4	10	<
others	17	15	12	44	16	17	68	50	49
	Final parchment			**48h soaked**			**Floats**		
	1	2	3	1	2	3	1	2	3
Total infection %	100	28	40	100	40	48	100	96	98
Ochre aspergilli	<	<	<	<	<	<	6	2	<
Niger aspergilli	32	18	10	15	24	42	83	16	16
Fusarium	47	<	6	54	8	4	6	<	<
Yeast	11	4	18	<	4	2	<	78	80
Cladosporium	<	<	2	15	<	<	6	<	2
others	11	6	4	15	4	<	<	<	<
Bleached	0.67			22.67			15.14		
Bean density	1.36			1.17			1.26		
Cup quality	FAQ+			FAQ			FAQ to FAQ-		

Means	Initial			Final		
	Fresh	Soaked	Fermented	Parchment	Parchment soaked	Floats
Total infection %	49	61	91	56	63	98
Ochre aspergilli	<	<	<	<	<	3
Niger aspergilli	<	<	7	20	27	38
Fusarium	<	<	<	18	22	2
Yeast	30	27	23	11	2	53
Cladosporium	4	9	5	1	5	3

The second and third runs share common initial mycological conditions while the first run shows a very low initial infection rate that includes no detectable yeast infection. Yeast fails to develop through any of the treatments in this run, except in the parchment during drying. In the other runs, yeast infection is steady through the wet portion of processing at around 40% but falls, as expected, during drying.

Ochre aspergilli are only detected here in the floats coffee of the first two runs at low levels. Niger aspergilli arise during drying, though in the yeast-less first run it emerges during fermentation but not soaking. It is not common for this group to be so well represented in arabica parchment. In the floats coffee, which was prepared as cherry coffee, either yeast dominates the flora or niger aspergilli do.

Fusarium, as with the Colombian studies, develops during drying, a period when it normally falls in infection rate. *Cladosporium* provides a steady background which could suggest a degree of lab contamination by this troublesome air-borne fungus, although there is no doubt that it occurs in coffee. During fermentation and some of the soaking replicates, miscellaneous moulds provide the largest collective component of infective flora. These largely disappear during drying.

There were two runs of the more complicated delay of processing experiment conducted in the Indian 2005 robusta season (Table O). In the second run a very high level of niger aspergilli bean infection from fresh fruit through all of the treatments *except* drying, suggests an analytical problem.

The high occurrence of *Cladosporium* at 97% and the ephemeral appearance of unidentified *Aspergillus* species at 70%, both in the fresh coffee, seem likely to be spurious results.

Table O: Robusta bean infection rates (% of beans by taxon) of the delay of processing robusta cherry and parchment from 2 runs. < = below detection limit.

% i	Initial							
Treatment	Fresh		Ferment		Soaked		Sacked	
Run #	1	2	1	2	1	2	1	2
Total i %	99	100	74	100	23	100	17	100
Aspergillus sp	4	70	<	<	1	7	7	<
Niger	<	33	1	100	<	100	1	100
Ochre	<	<	<	<	<	<	<	<
Cladosporium	97	<	1	<	3	<	4	<
Fusarium	17	<	<	<	<	<	4	<
Yeast	<	1	71	<	20	<	1	<

Table O contd.: Robusta bean infection rates (% of beans by taxon) of the delay of processing robusta cherry and parchment from 2 Runs. < = below detection limit.

% i	Final											
Treatment	Cherry		Parchment		Parchment soaked		Parchment sacked		Cherry sacked		Floats	
Run #	1	2	1	2	1	2	1	2	1	2	1	2
Total	46	54	86	64	84	82	88	100	100	100	86	96
Aspergillus **sp**	4	4	2	2	4	4	2	10	<	14	<	16
Niger	18	10	6	<	14	<	10	24	64	46	20	34
Ochre	<	<	<	<	<	<	<	4	<	2	<	2
Cladosporium	4	<	4	4	4	4	<	8	<	8	<	4
Fusarium	12	26	16	6	10	8	58	12	28	12	32	18
Yeast	8	14	58	52	56	66	58	42	28	18	34	22

In the products, ochre aspergilli were isolated at low levels from the second run only from both parchment and cherry coffee that had been sacked, along with floats coffee processed as cherry. Niger aspergilli contamination was elevated in sacked cherry coffee from both runs with the levels comparable to the arabica trials above. Parchment coffee, mycologically speaking, was indistinguishable whether it had been traditionally prepared, soaked or sacked, and except for the slightly elevated higher niger aspergilli, the sacked cherry coffee was the same as its comparator, directly dried cherry.

Some OTA analysis was done on these samples, though without replication. Two of the four floats samples contained significant amounts of OTA. In arabica Run 2, only the floats coffee had detectable OTA; in arabica Run 3 floats coffee was below detection and all but one of five other treatments contained OTA. In this trial the initial sample contained about 5ppb of OTA, numerically higher than any of the treatments though, undoubtedly, none of these differences are significant. Once again, none of the delay treatments showed any indication of promoting OTA production.

Table P: OTA content of samples generated in the Indian delay of processing experiments. OTA reported in ppb. BDL = below detection limit; empty cells = no determination.

Treatment	Arabica		Robusta	
	Run 2	Run 3	Run 1	Run 2
Floats	4.7	BDL	10.2	BDL
Initial		5.6		
P	BDL	2.4	3.3	BDL
P so24		2.7		
P so36		1.4		
P so48	BDL	BDL	5.6	BDL
P sk48			2.6	BDL