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1 **The effects of yeasts involved in fermentation of *Coffea arabica* in East Africa**
2 **on growth and ochratoxin A (OTA) production by *Aspergillus ochraceus***

3

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1 **Abstract**

2 The effects of *Pichia anomala*, *P. kluyveri* and *Hanseniaspora uvarum* predominant
3 during coffee processing on growth of *Aspergillus ochraceus* and production of
4 ochratoxin A (OTA) on malt extract agar (MEA) and on coffee agar (CA) were
5 studied. The three yeasts were able to inhibit growth of *A. ochraceus* when co-
6 cultured in MEA and CA. Growth inhibition was significantly higher on MEA than
7 on CA. Furthermore, *P. anomala* and *P. kluyveri* were found to have a stronger
8 effect on growth of *A. ochraceus* than *H. uvarum*. The three yeasts were able to
9 prevent spore germination of *A. ochraceus* in yeast glucose peptone (MYGP) broth.
10 In yeasts free supernatant of MYGP broth after an incubation period of 72 h, spores
11 of *A. ochraceus* were able to germinate with very short germ tubes, but further
12 development of the germ tubes was inhibited. The three yeasts decreased the pH of
13 MYGP broth from 5.6 to a range of 4.4 to 4.7, which was found to have no effect on
14 spore germination of *A. ochraceus*.

15 *Pichia anomala*, *P. kluyveri* and *H. uvarum* were able to prevent production of OTA
16 by *A. ochraceus* when co-cultured on MEA. On CA medium, *P. anomala* and *P.*
17 *kluyveri* prevented *A. ochraceus* from producing OTA. *Hanseniaspora uvarum* did
18 not affect production of OTA by *A. ochraceus* on CA medium.

19 Key words: Coffee, *Pichia anomala*, *Pichia kluyveri*, *Hanseniaspora uvarum*,
20 *Aspergillus ochraceus*, OTA.

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1 **1. Introduction**

2 Wet processing of coffee is mainly used for *arabica* coffee, where the ripe coffee
3 cherries are pulped followed by fermentation and drying (Fowler et al., 1998). The
4 main goal of fermentation is to degrade the slimy mucilage adhering firmly to coffee
5 beans by pectolytic enzymes produced by natural occurring microbiota (Illy and
6 Viani, 1995). At all steps of coffee processing, Gram-negative and Gram-positive
7 bacteria, yeasts and filamentous fungi are present at high levels (Silva et al., 2000).
8 In a previous study (Masoud et al., 2004) on the yeasts community of *arabica* coffee
9 in East Africa, we found that the total yeasts counts were in a range of 4.0×10^4 to 5
10 $\times 10^7$ cfu / g with an increase during fermentation. *Pichia anomala*, *P. kluyveri* and
11 *Hanseniaspora uvarum* were the three predominant yeasts during the different stages
12 of processing (Masoud et al., 2004).

13 Ochratoxin A (OTA) is a secondary metabolite of toxigenic species of *Aspergillus*
14 and *Penicillium* which has been detected in foods such as cereal products, wine,
15 beer, coffee, spices and grape juice (EC No 472 / 2002). In a literature survey, Varga
16 et al. (2001) reported that in temperate regions OTA is mainly produced by
17 *Penicillium* species, whereas in tropical and subtropical areas OTA is produced by
18 *Aspergillus* species. Several studies have reported the occurrence of both OTA
19 producing fungi and OTA in green coffee beans (Levi et al. 1974; Levi, 1980;
20 Mislivic et al., 1983; Micco et al., 1989; Studer-Rohr et al., 1995; Nakajima et al.,
21 1997; Romani et al., 2000; Ottender and Majerus, 2001; Pittet and Royer, 2002).
22 Taniwaki et al. (2003) isolated *A. ochraceus*, *A. carbonarius* and *A. niger* from
23 Brazilian coffee cherries and beans and found that 3, 75 and 77 % of *A. niger*, *A.*

1 *ochraceus* and *A. carbonarius* isolates produced OTA, respectively. A survey on
2 stored green coffee beans from various origins has shown that coffee samples from
3 African origin have significantly higher levels of OTA than those from America and
4 Asia (Pardo et al., 2004). Little is known about the origin of OTA in coffee and
5 when exactly it is produced along the coffee processing chain.

6 Growth of yeasts and moulds together on same substrate can lead to positive or
7 negative interaction. During ripening of the blue mould cheese Danablu, growth of
8 *P. roqueforti* has been found to be stimulated by *Debaryomyces hansenii*; while
9 *Candidum geotricum* and *Yarrowia lipolytica* were found to inhibit growth of *P.*
10 *roqueforti* under same conditions (van den Tempel and Nielsen, 2000; van den
11 Tempel and Jakobsen, 2000). Furthermore, *D. hansenii*, *Candida sake* and *P.*
12 *anomala* were reported to control growth of some plant pathogenic fungi (Droby et
13 al., 1989; Vinas et al., 1998; Walker et al., 1995; Masih et al., 2000). Biological
14 control of OTA producing fungi during the different stages of coffee processing
15 might help to reduce the accumulation of OTA in green coffee beans. Petersson et al.
16 (1998) found that *P. anomala* significantly reduced growth and production of OTA
17 by *P. verrucosum* in malt extract agar as well as in wheat.

18 The aim of the present study was to investigate the effects of *P. anomala*, *P.*
19 *kluyveri* and *H. uvarum* predominant during coffee processing on growth and OTA
20 production by *A. ochraceus* in laboratory substrates including coffee based agar
21 medium.

22 23 **2. Materials and methods**

1 **2.1. Cultures**

2 Yeasts used in this study were obtained from coffee samples collected from Arusha
3 region, Tanzania (Masoud et al., 2004). They included six strains of *P. anomala*
4 (S12, S13, S14, S15, S16, S17), four strains of *P. kluyveri* (S4Y3, S7Y1, S8Y4,
5 S13Y4) and two strains of *H. uvarum* (S3Y8, S15Y2). In addition, two strains of *A.*
6 *ochraceus* (B677, B722) with the potential to produce OTA were studied. They were
7 also isolated from coffee samples collected from Arusha region, Tanzania (Institute
8 of Hygiene and Toxicology, Federal Research Centre for Nutrition and Food,
9 Karlsruhe, Germany).

10

11 **2.2. Culture media**

12 Malt yeast glucose peptone medium (MYGP) was prepared by dissolving 3 g malt
13 extract (Difco, Detroit, MI, USA), 3 g yeast extract (Difco), 5 g Bactopeptone
14 (Difco) and 10 g D(+)-Glucose monohydrate (Merck, Darmstadt, Germany) in 1 l
15 distilled water and the medium was adjusted to pH 5.6 by 1 M NaOH (Merck) . For
16 malt extract agar (MEA), 20 g of malt extract (Difco), 10 g D(+)-Glucose
17 monohydrate (Merck), 5 g Bactopeptone (Difco) and 20 g of agar (Difco) were
18 dissolved in 1 l distilled water and the medium was adjusted to pH 5.6 by 1 M
19 NaOH (Merck). Diluent saline peptone (SPO) was prepared by dissolving 8.5 g
20 NaCl (Merck), 0.3 g disodium hydrogen phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$) (Merck) and
21 1 g bactopectone (Difco) in 1 l distilled water. SPO was adjusted to pH 5.6 by the
22 addition of 1 M HCl and 1 M NaOH. Coffee agar (CA) was prepared by adding 20

1 g of grounded green coffee beans (Levi Farm, Arusha, Tanzania) and 20 g of agar
2 (Difco) to 1 l distilled water.

3

4 **2.3. Effect of yeasts predominant in coffee on growth of *A. ochraceus***

5 Strains of *P. anomala*, *P. kluyveri* and *H. uvarum* were propagated in 25 ml of
6 MYGP broth at 25 °C for 48 hours. After propagation cells were harvested by
7 centrifugation at 3000 x g for 10 min, and resuspended in SPO. Cell concentrations
8 were estimated by microscopy using a counting chamber (Neubauer) and the
9 suspensions were diluted to final concentrations of 10⁴ and 10⁶ cells / ml.
10 Suspensions of yeasts were mixed with 20 ml of melted MEA and poured in Petri
11 dishes, which were left for 2 h to solidify. Spores of *A. ochraceus* were harvested
12 from MEA plates and suspended in SPO. Spore concentration was estimated by
13 microscopy as described above and the suspension was diluted in SPO to 10⁶ spores
14 / ml. After solidification of the MEA plates inoculated with yeasts, spots of 10 µl of
15 *A. ochraceus* spore suspension (10⁶ spore / ml) were placed on three sites of each
16 plate. Spots of *A. ochraceus* spore suspension were also placed on three sites of
17 yeast free MEA plate, which was used as a control. The plates were incubated at 30
18 °C for 7 days where after, growth of fungi was determined by measuring the fungal
19 colony diameter. The experiment was done in triplicates. The same experiment was
20 done on CA medium.

21

22 **2.4. Effects of yeasts cells and yeasts free supernatant on germination of *A.*** 23 ***ochraceus* spores**

1 In this assay, the effects of the six strains of *P. anomala*, the four strains of *P.*
2 *kluuyveri* and the two strains of *H. uvarum* on spore germination of *A. ochraceus*
3 B722 were investigated. Spores of *A. ochraceus* B722 (10^6 spores / ml) were
4 inoculated together with 10^6 cells / ml of each yeast in 10 ml MYGP broth (pH 5.6)
5 and incubated at 30 °C. Spores of *A. ochraceus* were also inoculated in yeasts cells
6 free supernatant, which was obtained by propagation of each yeast in 25 ml of
7 MYGP broth at 25 °C for 24 hours. Where after yeasts cultures were centrifuged at
8 3000 x g for 10 min and the supernatant was filtered through a 0.22 µm nitro-
9 cellulose filter (Osmonics, Minnetonka, MN, USA). The pH of supernatant was
10 determined. Spores of *A. ochraceus* inoculated in MYGP broth (pH 5.6) was used as
11 a control. Furthermore, spores of *A. ochraceus* were inoculated in MYGP broth
12 adjusted to pH 4.4, 4.5, 4.6 and 4.7. After 24, 48 and 72 h of incubation at 30 °C,
13 germination of the fungal spores was inspected by microscopy where five regions of
14 each sample with about 10 to 20 spores in each region were inspected. The
15 experiment was carried out in triplicates.

16

17 **2.5. Effects of yeasts on production of OTA by *A. ochraceus***

18 The ability of the two strains of *A. ochraceus* B722 and B677 to produce OTA when
19 co-cultured with the six strains of *P. anomala*, the four strains of *P. kluuyveri* and the
20 two strains of *H. uvarum* on MEA and CA plates was investigated. Yeasts were co-
21 cultured with *A. ochraceus* on MEA and CA plates as described above. *Aspergillus*
22 *ochraceus* was also inoculated in yeast free MEA and CA plates, which were used as
23 controls. After an incubation period of 7 days at 30 °C, production of OTA was

1 estimated by thin layer chromatography (TLC) (Samson et al., 2002). Agar plugs
2 were aseptically removed from mould colonies on MEA and CA plates and one drop
3 of chloroform / methanol mixture (1:2) was added to each plug. The plug was placed
4 onto a TLC plate silica gel 60 (Merck Art 5721) with mycelium side towards the gel.
5 OTA of 10 µg / l in toluene / acetic acid (99:1) was used as a standard. Then the
6 TLC plates were developed in toluene / acetone / methanol (5:3:2) and left to dry in
7 a fume hood for 10 min. The TLC plates were examined visually under UV light at
8 366 nm wave length.

9

10 **3. Results and Discussion**

11 **3.1. Effect of yeasts predominant in coffee on growth of *A. ochraceus***

12 The effect of six strains of *P. anomala*, four strains of *P. kluyveri* and two strains of
13 *H. uvarum* at 10⁴ cells / ml on growth of *A. ochraceus* B722 on MEA and CA media
14 is shown in Fig. 1. The three yeasts were found to inhibit growth of *A. ochraceus*
15 when grown together. On both MEA and CA media, strains of *P. anomala* and *P.*
16 *kluyveri* were found to have stronger effect on growth of *A. ochraceus* compared to
17 *H. uvarum*. On CA medium, the levels of growth inhibition of *A. ochraceus* by the
18 two strains of *H. uvarum* was extremely low. The two strains of *P. anomala* S12 and
19 S17 were found to have the highest percentages of inhibition against *A. ochraceus*.
20 The percentage of fungal growth inhibition caused by *P. kluyveri* S13Y4 was lower
21 than those caused by *P. anomala* and the other three strains of *P. kluyveri*. Small
22 differences in the degree of inhibition among the remaining strains of *P. anomala*
23 and *P. kluyveri* were observed. Increasing concentration of yeasts to 10⁶ cells / ml

1 increased growth inhibition of *A. ochraceus* (results not shown). Same findings on
2 the effects of yeasts on growth of *A. ochraceus* B677 were obtained (results not
3 shown).

4 *Pichia anomala* was reported to inhibit a number of fungi like *Botrytis cinerea*
5 (Masih et al., 2000), *P. roqueforti*, *A. candidus* (Petersson and Schnürer, 1995) and
6 *P. verrucosum* (Petersson et al., 1998). *P. kluyveri* and *H. uvarum* were found to
7 produce killer toxins against other yeasts (Zorg et al., 1988; Abranches et al., 1997).
8 However, the antagonist activities of those two yeasts against filamentous fungi have
9 not been investigated. A good understanding of the mode of action of the antagonist
10 activity will help to clarify the mechanism behind it. In the present study, the degree
11 of inhibition was found to be dependent on the yeast species and the substrate used.
12 On both MEA and CA media, strains of *P. anomala* and *P. kluyveri* were found to
13 have stronger effect on growth of *A. ochraceus* compared to *H. uvarum*. On CA
14 medium, the levels of growth inhibition of *A. ochraceus* by the two strains of *H.*
15 *uvarum* was significantly lower. For all yeasts, inhibition of fungal growth was
16 significantly higher on MEA compared to that on CA medium. The three yeasts
17 showed less growth on CA compared to MEA medium (results not shown), which
18 might explain the lower inhibition of fungal growth by the three yeasts on CA. The
19 CA medium may contain less specific nutrients essential for growth of yeasts. On
20 the other hand, *A. ochraceus* showed very good and equal growth in both yeasts free
21 plates of CA and MEA (results not shown).

22

1 **3.2. Effects of yeasts cells and yeasts free supernatant on germination of *A.***
2 ***ochraceus* spores**

3 Germination of *A. ochraceus* B722 spores when co-cultured with *P. anomala* S12 in
4 MYGP broth and when inoculated in the cell free supernatant of *P. anomala* S12 is
5 shown in Fig 2. Co-culture of *P. anomala* S12 with *A. ochraceus* B722 totally
6 inhibited fungal spore germination after 24, 48 and 72 h of incubation (Fig 2 D, E,
7 F). Similar results were obtained for the effect of the other strains of *P. anomala*, *P.*
8 *kluyveri* and *H. uvarum* on spore germination of *A. ochraceus* B722 (results not
9 shown). In yeast free supernatant, spores of *A. ochraceus* did not germinate after 24
10 h (Fig 2 G). Spores of *A. ochraceus* started to swell after 48 h, but germ tubes were
11 not observed (Fig 2 H). After 72 h, some spores germinated with very short germ
12 tubes (Fig 2 I) compared to the control (Fig 2 A, B, C). The same observations were
13 obtained for the other five strains of *P. anomala*, the four strains of *P. kluyveri* and
14 the two strains of *H. uvarum* (results not shown). The pH of the yeasts free
15 supernatant was determined; it was found that the pH decreased from 5.6 to a range
16 of 4.4-4.7 by the three yeasts species. Germination of *A. ochraceus* spores in MYGP
17 broth at pH values 4.4-4.7 was not affected (results not shown). It seems that
18 inhibition of spore germination in yeasts free supernatant was not due to changes in
19 the pH of medium caused by the yeasts.

20 Depletion of the amounts of glucose in MYGP broth by the investigated yeasts
21 might result in reduction of spore germination of *A. ochraceus*. The three yeasts
22 might also produce extra cellular metabolites toxic to *A. ochraceus* which cause
23 reduction of spore germination in yeasts free supernatant. Spadaro and Gullino

1 (2004) reported that the mechanisms behind the antagonist activity of yeasts against
2 fungi responsible for fruit diseases can be competition for nutrients and space,
3 adhesion of the antagonist cells to the mycelium of the fungi or by inducing
4 resistance in the host tissue. Droby et al. (1989) suggested that the mechanism of the
5 antagonist activity of *D. hansenii* against *P. digitatum* in grapefruit might be due to
6 competition for nutrients because the antagonist activity was overcome by the
7 addition of exogenous nutrients to grapefruit. The yeast *Metschnikowia pulcherrima*
8 was found to inhibit growth of postharvest pathogenic fungi of apple fruit; it was
9 suggested that the antagonist activity seems to be due to a combination of
10 competition for nutrients and production of toxic metabolites *in vitro* (Spadaro et al.,
11 2002). Strains of *P. anomala*, *P. kluyveri* and *H. uvarum* used in this study have
12 been found to be strong producers of some volatile compounds, mainly ethyl acetate,
13 acetate, 2-phenylethyl acetate, ethyl propionate and isoamyl alcohol (unpublished
14 results). The effect of ethyl acetate on growth of *P. roqueforti* was studied by
15 Fedlund et al. (2004), who found that only high concentrations of ethyl acetate
16 reduced fungal growth. It has been reported that growth of a number of plant
17 pathogenic fungi can be inhibited by volatile compounds produced by the
18 endophytic fungi *Muscodora albus* (Strobel et al., 2001, Mercier and Jiménez, 2004)
19 and by *Gliocladium* spp. (Stinson et al., 2003). Other non volatile metabolites toxic
20 to *A. ochraceus* might also be produced by the three investigated yeasts.

21

22 **3.3. Effects of yeasts on production of OTA by *A. ochraceus***

1 Of the most important aspects during coffee processing is to prevent production of
2 OTA. *Aspergillus ochraceus* B722 was found to produce OTA when grown on yeast
3 free MEA and CA plates (Fig 3 and 4). When the six strains of *P. anomala* were co-
4 cultured with *A. ochraceus* B722, OTA was not detected on both MEA and CA
5 plates (Fig 3). *P. kluyveri* also prevented OTA production by *A. ochraceus* on both
6 MEA and CA media (Fig 4). However, the two strains of *H. uvarum* did not prevent
7 OTA production on CA medium; it was only prevented on MEA (Fig 4). The same
8 observations were made for *A. ochraceus* B677 (results not shown). Although the
9 yeasts did not inhibit growth of *A. ochraceus* completely (Fig 1), they were able to
10 prevent production of OTA. It has been found that *P. anomala* reduced both growth
11 of *A. verrucosum* and OTA production when co-culture together on MEA or on
12 wheat (Petersson et al., 1998). Reduction of OTA might be as a result of its
13 degradation or adsorption by yeasts. It has been reported that *S. cerevisiae* and *S.*
14 *bayanus* adsorbed about 45 % of OTA present in synthetic grape juice medium
15 (Bejaoui et al., 2004). Production of extra cellular compounds by the three yeasts
16 might also inhibit production of OTA by *A. ochraceus*. *Streptococcus lactis* was
17 reported to produce a heat-stable low molecular weight compound that inhibits
18 production of aflatoxin by *A. flavus* in vitro (Coallier-Ascah and Idziak, 1985).
19 Mellon and Moreau (2004) found that a class of polyamine conjugates inhibited
20 aflatoxin B₁ biosynthesis in *A. flavus* but they did not reduce growth of that fungus.
21
22 In the present study, the two strains of *A. ochraceus* showed very good growth and
23 production on of OTA in both MEA and CA media. *Pichia anomala*, *P. kluyveri* and

1 *H. uvarum* were found to reduce growth of *A. ochraceus* and prevent biosynthesis of
2 OTA on MEA medium. On CA medium, *P. anomala* and *P. kluyveri* were able to
3 reduce growth of *A. ochraceus* and prevent production of OTA. For the purpose of
4 preventing production of OTA in coffee, the present work indicated the possibility
5 of using *P. anomala* and *P. kluyveri* in biological control of OTA producing fungi
6 during coffee fermentation. Further studies on the effects of *P. anomala* and *P.*
7 *kluyveri* on other OTA producing fungi present in coffee are needed. The
8 mechanisms behind the antagonist activity of those yeasts need to be clarified. In
9 addition, studies of interactions between those two yeasts and OTA producing fungi
10 *in vivo* i.e. during coffee processing have to be conducted.

11

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Legends to figures

Figure 1. Growth inhibition of *A. ochraceus* B722 on MEA and CA plates inoculated with six strains of *P. anomala* (S12, S13, S14, S15, S16, S17), four strains of *P. kluyveri* (S4Y3, S7Y1, S8Y4, S13Y4) and two strains of *H. uvarum* (S3Y8, S15Y2). Inhibition is expressed as the percentage of reduction of the fungal colony diameter compared to the control (fungal colony diameter on free yeast plates). Bars represent standard deviations.

Fig 2. Germination of *A. ochraceus* B722 spores incubated at 30 °C when inoculated in MYGP broth without yeast after 24 h (A), 48 h (B), 72 h (C); when co-cultured with cells of *P. anomala* S12 in MYGP broth after 24 h (D), 48 h (E), 72 h (F); when inoculated in MYGP yeast free supernatant after 24 h (H), 48 h(I), 72 h (J).

Figure 3. Production of OTA by *A. ochraceus* B722 co-cultured with strains of *P. anomala*. Lane numbers from 3-8 refer to *A. ochraceus* co-cultured on CA with: lane 3, *P. anomala* S12; lane 4, *P. anomala* S13; lane 5, *P. anomala* S14; lane 6, *P. anomala* S15; lane 7, *P. anomala* S16; lane 8, *P. anomala* S17. Lane numbers from 10-15 refer to *A. ochraceus* co-cultured on MEA with: lane 10, *P. anomala* S12; lane 11, *P. anomala* S13; lane 12, *P. anomala* S14; lane 13, *P. anomala* S15; lane 14, *P. anomala* S16; lane 15, *P. anomala* S17. *A. ochraceus* grown on CA with out yeasts (lanes 1, 2) and on MEA (lanes 16, 17) were used as controls. OTA standard 10 µg / l (lane 9).

Figure 4. Production of OTA by *A. ochraceus* B722 co-cultured with strains of *P. kluyveri* and *H. uvarum*. Lane numbers from 3-8 refer to *A. ochraceus* co-cultured on CA with: lane 3, *H. uvarum* S3Y8; lane 4, *H. uvarum* S15Y2; lane 5, *P. kluyveri* S4Y3; lane 6, *P. kluyveri* S7Y1; lane 7, *P. kluyveri* S8Y4; lane 8, *P. kluyveri* S13Y4. Lane numbers from 10-15 refer to *A. ochraceus* co-cultured on MEA with: lane 10, *H. uvarum* S3Y8; lane 11, *H. uvarum* S15Y2; lane 12, *P. kluyveri* S4Y; lane 13, *P. kluyveri* S7Y1; lane 14, *P. kluyveri* S8Y4; lane 15, *P. kluyveri* S13Y4. *A. ochraceus* grown on CA with out yeasts (lanes 1, 2) and on MEA (lanes 16, 17) were used as controls. OTA standard 10 µg / l, (lane 9).

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