IDENTIFICATION AND CONTROL OF SPOILAGE MOULDS IN TANZANIAN CURED VANILLA BEANS

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ABSTRACT
The aim of this study was to isolate, identify and control spoilage moulds in cured vanilla beans produced in Tanzania. The isolates were characterized by DNA sequencing of the internal transcribed spacer (ITS) region. The result showed that vanilla beans were contaminated with Aspergillus fumigatus, Aspergillus tubingensis, Aspergillus aculeatus, Byssochlamys spectabilis (Amanorph Paecilomyces variotii) and Penicillium polonicum. Sterile green vanilla beans were inoculated with the spores of A. tubingensis, A. aculeatus and B. spectabilis (Amanorph Paec. variotii), incubated overnight and disinfected by ethanol, followed by blanching and storage at tropical ambient temperature. The combination of blanching (65oC/3 min) and disinfection by ethanol (50% and 70%) completely inhibited mould growth and, therefore, could potentially be used to control spoilage by moulds and improve the quality of cured vanilla beans.

Keywords: Cured Vanilla Beans, Moulds, Molecular Identification, Quality.

1. INTRODUCTION
Vanilla (*Vanilla planifolia*) is the second most expensive spice after saffron, and is used to impart aroma and flavour in foods, beverages, pharmaceuticals and cosmetics. .. In 2020, world production of vanilla was 7,614 tonnes, led by Madagascar (39.1%) and Indonesia (30.3%) (FAOSTAT, 2020). In East Africa, vanilla is cultivated in Uganda, Kenya, Congo and Tanzania and contributes to improved livelihoods of vanilla value chain stakeholders (Muzanila and Assenga, 2022). Vanilla curing process is of long duration, cumbersome and requires skilled human resources in order to obtain quality products. In contrast with other vanilla producing areas in the world, in East Africa most of the vanilla is purchased as green beans and centrally cured by specialized processors (Fehr, 2010). For example, in Tanzania, most of vanilla is cultivated in the northwest around Lake Victoria in Kagera region, North Kilimanjaro and eastern parts of Morogoro but most of the curing is carried out centrally in Kilimanjaro region. Although vanilla production in Tanzania has increased significantly in the last five years, from 229.8 tons per year in 2015 to 1,949 tons in 2020 (Makoye, 2021)., processors encounter challenges including poor product quality (Maerere and van Noort, 2014) that undermine competitiveness on the world market (Havkin-Frenkel and Belanger, 2018). In the region, there is no standard procedure to control post-harvest mould contamination across the vanilla value chain, in contrast with other spices and fruits, for which fumigation is mostly applied. However, Sagoo *et al.* (2009) and Schaarschmidt *et al.* (2016) observed that microbial contamination in spices, including vanilla, could be reduced by use of best practices such as good agricultural

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practices (GMP) and good hygienic practices (GHP) along the value chain.

The use of agricultural chemicals to control moulds has raised global concerns about environmental pollution and health risks (Ponzo, *et al.,* 2018). In order to control post-harvest mould contamination in the spice industry, including vanilla, it is important to identify contaminants, control points and develop safe, effective, economica strategies compatible with commercial handling (Homaida *et al.,* 2017). Ethanol is generally recognized as safe (GRAS) (Ji *et al.,* 2019). It is a common food component with potent antifungal activity that has been used for a long time for treatment against many mould species that contaminate and spoil food products (Yuen *et al.,* 1995; Berni and Scaramuzza, 2013; Karabulut *et al.,* 2004). Various studies indicated that ethanol treatment controlled fungal post-harvest diseases in blueberries, table grape, mango, loquat, guava, (Akgun *et al.,* 2005; Chervin *et al.,*2005; Groot *et al.,* 2018; Ji *et al.,* 2019; Karabulut *et al.*, 2004; Lichter *et al.,* 2002; Pinto *et al.,*2006; Ponzo *et al.,* 2018; Romanazzi *et al.,* 2007). Karabulut *et al.* (2004) reported a complete inhibition of germination of *Botrytis cinerea* spores in table grapes by use of 30% ethanol at 50ºC/30 sec. Ponzo *et al.* (2018) observed significant reduction of anthracnose and severity on guavas by 40% ethanol for 2 min at 25ºC. Furthermore, Gabler *et al.* (2004) observed that 20% ethanol at 50ºC completely inhibited the germination of spores of *Rhizopus stolonifer, Aspergillus niger, Botrytis cinerea* and *Alternaria alternate*.

In developing contries, such as Tanzania, most of the processors of cured vanilla beans are unable to compete effectively on market due to a constraints including low product quality caused by spoilage moulds. There is no adequate information on causative spoilage moulds, low-cost, safe and effective hurdles for control of spoilage of vanilla beans. Therefore, the aim of this study was to isolate, identify spoilage moulds and assess the efficacy of heat treatment in combination with disinfection in controlling mould proliferation for improvement of the quality of cured vanilla beans.

2.MATERIALS AND METHODS

2.1 Sample collection

Twenty samples of cured vanilla beans from 20 different batches, each weighing about 50g, were collected from curing centres in Kilimanjaro region, Tanzania. The samples were aseptically collected in sterile polyethylene bags, labelled and transported to the African Seed Health Centre Laboratory, at Sokoine University of Agriculture (SUA), Morogoro, Tanzania and stored at 4ºC prior to further analysis.

2.2 Isolation of moulds

Three methods were used for isolation of moulds, namely the blotter test (Narayanasamy, 2017); direct plating of vanilla cuts onto potatoes dextrose agar (PDA) with incubation at 25±3°C and; washing off the surfaces of intact cured vanilla beans (Nega, 2014). The blotter test resulted into more mould recovery than the other methods and was subsequently used for isolation of moulds from the samples. Non-sterilized samples were evenly placed at a rate of four pieces per Petri dish at equal distance in each Petri dish on three layers of sterile moistened 9 cm diameter

Whiteman filter paper in sterilized Petri dishes. The plates were incubated at 25±3°C/7-15 days and then examined under microscope. The moulds that grew on samples were transferred to sterile PDA (HI media, India) for purification and identification (El-Gali, 2014; Toma and Abdulla, 2013). The isolates were maintained at 4°C until when used for DNA extraction.

2.3 Morphological identification of moulds

The sub-cultured isolates were investigated for pigment production and colony characteristics (Kim *et al.,* 2013). The conidia, hyphae, conidial head, conidiophores, spores, and colour were observed under ×400 microscope magnification (Leica GME, Switzerland) for morphological identification at the African Seed Health Centre, at SUA.

2.4 Identification of moulds by using molecular biological technique

2.4.1 DNA extraction

The isolates were transferred onto PDA and incubated at 25±3°C/ 5 days. Total DNA was extracted from mould mycelia according to the procedure described by Liu *et al.* (2000) with modification. Briefly, a small lump of mycelia was disrupted by using a sterile scalpel and added into a 150 µL Eppendorf tube containing 500 µL of lysis buffer (400 mM Tris-HCl, pH 8.0), 60 mM EDTA (pH 8.0), 150 mM NaCl,1% sodium dodecyl sulfate), and the tube was left at room temperature for 10 min. Thereafter, 150 µL of potassium acetate (pH 4.8); which was made of 60 mL of 5 M potassium acetate, 11.5 mL of glacial acetic acid, and 28.5 mL of distilled water, was added into the tube before being vortexed at >10 000 x g for 1 min The supernatant was transferred to another 150 µL Eppendorf tube and centrifuged again, as described above. After transferring the supernatant to a new 150µL Eppendorf tube, an equal volume of isopropyl alcohol was added and the tube was mixed by inversion briefly.The tube was centrifuged at >10 000 x g for 2 min, and the supernatant was discarded. The resultant DNA pellet was washed in 300 µL of 70% ethanol. After the pellet was centrifuged at 10 000 rpm/ 1 min, the supernatant was discarded. The DNA pellet was air dried, re-suspended in 100 µL ddH_2O and stored at -20ºC (Lin *et al.,* 2014) until further analysis.

2.4.2 PCR Amplification and Sequencing

The PCR amplifications were carried out in a total volume of 25 µL, containing 10 ng template DNA, 10X PCR buffer premix (puReTaq Ready-To-Go™ PCR kit, Germany) and 10 pmol of ITS1/ITS4. The sequences of the ITS1 and ITS4 primers were 5‘-TCCGTAGGTGAACCTGCGG-3‘and5-TCCTCCGCTTATTGATATGC-3, respectively (Hussain *et al.,* 2018).

The PCR amplification was carried out according to the following temperature profile as was described by (Khare *et al.,* 2018) with modification. In brief, the polymerase chain reaction was performed in 25 µL of reaction mixture using template DNA isolated by the method described earlier. The template DNA was amplified in Applied Bio system (GeneAmp PCR system 9700, Singapore) with 30 cycles, each cycle at 94ºC for 3 min for denaturation, 0.45 min at 55ºC for annealing, 1.25 min at 72ºC for extension and a 10 min final extension at 72ºC.

The amplified PCR products were resolved by gel electrophoresis in a 1.5% agarose (Model: 08-

1214, Rated 150V, 100 mA Class II, Galileo Bioscience) gel stained in 0.5 mg/mL ethidium bromide in TBE buffer at 100V for 45 min. The DNA bands resolved on agarose gel were visualized in UV transilluminator and photographed. The sizes of the amplicon was estimated after comparing with a commercial 100 bp DNA ladder on agarose gel. The PCR products were stored at 4ºC till sequencing (Olagunju *et al*., 2018).

The DNA sequencing was carried out at Southern African Centre for Infectious Disease Surveillance (SACIDS), SUA, Morogoro) and five amplicons were sequenced. The sequences were assembled, edited and aligned by using the Geneous software (Version no.10.2.3, Biomatters Ltd, New Zealand) then blasted against known sequences in the GenBank using BLAST to find regions of local similarity between sequences in order to identify the species (Bechem and Afanga, 2017).

Table 1: The ITS primer pair used in this study

Primer	Sequence 5’ ⟶ 3’
ITS 1 (Forward)	TCCGTAGGTGAACCTGCGG
ITS 4 (Reverse)	TCCTCCGCTTATTGATATGC

2.5 Control of spoilage moulds by ethanol

2.5.1 Inoculation of green vanilla beans

From the culture in PDA medium incubated at 25°C for 7±1 days, spores of three fungal species identified before (*A. tubingensis, A. aculeatus, B.spectabilis (*anamorph: *Paec. variotii)* were collected by flooding the surface of the plates with sterile saline solution (NaCl, 9 g per litre of water containing Tween 80 (0.1% vol/vol); Prolabo, Paris, France) and the spore suspensions was counted on a haemocytometer using a compound microscope before standardization to $1x10^5$ spores/mL.

Green beans were inoculated according to the procedure described by Karabulut *et al.,* (2004) with modification. Briefly, the green vanilla bean was washed three times by dipping in a sterile ddH_2O for 2-3 min. then dried under laminar flow (Holten LaminAir, type HH 1.2 basis, Denmark) at ambient temperature. Artificial inoculation of green vanilla was done by puncturing the beans (3 mm deep approximately on both apex and blossom ends) with a sterilized scalpel and directly inoculated by dipping into a beaker containing 500mL spore suspension of each fungal specie ($1x10^5$ spores/mL).

Inoculated green vanilla beans were wrapped with sterile aluminium foils and left overnight under a sterile laminar air flow cabinet for fungi sticking on punctured beans. Three replicate units were used per treatment for each fungus. The treatments were as follows (i) inoculated green vanilla beans were dipped into sterile distilled for 5 and 10 min. and (ii) Inoculated: green vanilla beans were dipped into ethanol solution of 30, 50, and 70% (v/v) for 5 and 10 min each. After treatment, all samples were blanched at 65ºC/ 3 min. The blanched vanilla beans were incubated at 48±2ºC/72 h before storage at 30±2ºC for drying to approximately 25% moisture content.

2.6 Statistical Analysis

Experiments were performed using a completely randomized design. All statistical analyses were performed with SPSS software (version 21, IBM Corporation, New York, USA). The data were analysed by two-way analysis of variance (ANOVA). Mean separations were performed by Boniferon range tests. Differences at $P < 0.05$ were considered as significant.

3. RESULTS AND DISCUSSION

3.1Morphological identification of spoilage moulds

In this study, various mould mycelia were observed on the surface of vanilla cuts and the isolated moulds were examined on the basis of molecular and morphological characteristics. The moulds identified were *Aspergillus aculeatus, Aspergillus fumigatus Aspergillus tubingensis, Byssochlamys spectabilis* (anamorph: *Paecilomyces variotii*) and *Penicillium polonicum.*

The morphological charactristicsof *A. tubingensis* included a black colour colonies with whitish colonies on the top and pale-yellow colonies on the reverse while the features of *A. aculeatus* were yellow to dark brown/grey tones colour on the top and pale yellow on the reverse (Silva *et al.,* 2011). *B. spectabilis* (anamorph: *Paec. variotii*) colour of the colonies was pale yellow and white margins closely similar to those of *A. aculeatus*. The *A. fumigatus* colonies were dark green with white mycelia at the edges although white thick mycelia formed under the colonies and green yellow on reverse, while *Penicillium polonicum* had white bluish colour and yellowish cream on reverse. All of these characteristics were observed on PDA.

3.2 Molecular identification of the isolated moulds

Five mould isolates were identified on the basis of their molecular characteristics. The amplification of 18S rRNA with ITS1 and ITS4 primers was successfully carried out and 18S rRNA gene was chosen as a target for PCR amplification as is widely used in the molecular analysis (Hussain *et al.,* 2018). The ITS region of the rRNA gene is generally believed to represent a convenient target for the molecular identification of specific species of fungi (Zhang *et al.,* 2011).

Based on the sequence similarity with corresponding sequences in the GenBank, all the five isolates were identified to species as shown in the Table 2.The sequence analysis of the ITS regions of the nuclear encoded rDNA showed significant alignments of 81.96-100 % with the isolated mould species. Of all isolates identified, *B. spectabilis (*Anamorph*: Paec. variotii)*, *P. polonicum* and *Aspergillus fumigatus* raised food safety concern as they could potentially produce mycotoxins in foodstuffs. *B. spectabilis* and *A. fumigatus* are among the heat-resistant fungi important in the food industry because they can resist heat treatments used for food processing and can grow and spoil the products during storage at room temperature (Moreira *et al.,* 2018).

Table 2: Identification of mould isolates from vanilla beans by ITS region of rRNA gene sequence

Identified fungal species	Length (bp)	Identity (%)	Coverage (%)	Access number (GenBank)
Aspergillus fumigatus	602	100	100	MH892837.1
Aspergillus tubingensis	603	100	100	MH045586.1
Aspergillus aculeatus	577	100	100	MN187974.1
Byssochlamys spectabilis (Paecilomyces variotii)	615	100	99	KC157706.1
Penicillium polonicum	592	81.96	92	MH382817.1

These species are ubiquitous, thermo-tolerant, commonly found in food products (including pasteurized), soil, indoor air environments and woods (Houbraken *et al.,* 2008). In general, *B. spectabilis (*Anamorph*: Paec. variotii)* can survive considerable periods of heat above 85°C and can grow under very low oxygen conditions and produce mycotoxins such as viriditoxin and deoxynivalenol (Houbraken *et al.,* 2005; Casas-Junco *et al.,* 2018; Urquhart *et al.,* 2018). *P. polonicum* has been reported by (Núñez *et al.,* 2000; Polizzi *et al.,* 2012) as an important food spoilage and airborne fungus found in indoor environments; grows better at a_w of 0.99, and extreme temperatures, such as 4, - 37ºC and relative humidity of 97–100% and produce secondary metabolites including the potent neurotoxin verrucosidin and nephrotoxic compounds. So far, contamination of *B. spectabilis (*Anamorph: *Paec. variotii)* in cured vanilla was not reported before; however these species cause food (Moreira *et al.,* 2018). During curing process at a stage of sweating (fermenting), incubation temperature, relative humidity and time are about 45-50ºC, RH of 95-100% and 48-72 h, respectively (Röling *et al.,* 2001). However, regardless of high temperature, fungal growth on blanched vanilla beans has been observed at this stage. Probably identified moulds *A. fumigatus, B. spectabilis (*Anamorph: *Paec. variotii)* and *Penicillium polonicum* are among of the species that grow on blanched vanilla beans during sweating "fermentation" process.

The isolation of *Aspergillus* and *Penicillium* spp in this study corresponds to other reports on their presence in spices including vanilla. For example, El-Gali (2014) observed that *Aspergillus* and *Penicillium* genera were more frequently detected than other genera of moulds in spices. Röling *et al.* (2001) reported that black *Aspergillus* and green *Penicillium* strains were the major moulds found on vanilla beans during curing. In addition, Kumar and Balamohan (2013) indicated that vanilla beans were susceptible to contamination by storage moulds, such as, *Aspergillus, Fusarium* and *Penicillium,* due to harvesting of immature beans, improper killing and drying and high relative moisture content in the beans. In addition, Sarter (2011) isolated *P. lividum, P. vanillae, P. rugulosum A. niger, A. oryzae* and *A. amstelodami* in vanilla beans from Madagascar and Comoros. Therefore, the isolation of *A. tubingensis (*which was misidentified as *A. niger* by microscopic identification*), A. aculeatus, A. fumigatus* and *P. polonicum* in this study confirmed that *Aspergillus* and *Penicillium* genera are the most common spoilage and probably mycotoxicogenic contaminants in cured vanilla beans. This is because *Aspergillus* and *Penicillium* spp are most xerotolerant or xerophilic moulds and, have ability to grow within a

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wide range of temperature (Berni and Scaramuzza, 2013). Xu et al (2020) reported that some of the aspergilli may dominate under the whole curing stages, and played an important role in the flavour formation of vanilla beans.

3.3 Efficacy of ethanol on mould growth control

As indicated in Table3, immersion of inoculated green vanilla beans in 50% and 70% ethanol for 5 and 10 min followed by blanching at 65ºC/ 3 min completely inhibited the proliferation of *A. tubingesis, A. aculeatus* and *B. spectabilis*. This suggested that ethanol inhibited all development stages of the fungi including the spore germination, germ tube elongation as well as sporulation (Yuen *et al.,* 1995). On the other hand, treatment of vanilla beans with 30% ethanol was less effective for inhibition of the proliferation of the moulds, but resulted into significantly lower spores counts (P>0.05) compared to control treatment.

Gurtovenko and Anwar (2009) observed that the interaction of ethanol with biological membranes at concentrations below 30.5% (v/v) induced expansion of the membranes together with a reduction of their thickness, as well as causing disorders and enhancement of the inter-digitation of lipid acyl chains. However, the bilayer structure of the membranes was maintained. Lichter *et al.* (2002) observed that 30% ethanol reduced the survival of *B. cinerea* spores, and 40% ethanol completely inhibited spore germination. Sequeira *et al.* (2017) observed that short term exposure of *P. chrysogenum* spores to ethanol resulted into significant reduction of conidia germination and mycelia development in samples treated with 70% and 100% than in 0 and 30% ethanol. According to Gurtovenko and Anwar (2009) .high ethanol concentration is efficient in the control of moulds because it is associated with expansion and reduction of membrane thickness along with increasing hydrophilicity of the membrane interior due to accumulation of ethanol molecules which make the lipid/water interface unstable and prone to formation of defects (Gurtovenko and Anwar, 2009).

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Table 3: Efficacy of ethanol in controlling growth of spoilage moulds in cured vanilla beans*

Time (min)	*A. tubingesis*				*A.aculeatus*				*B. spectabilis*			
	Control	30%	50%	70%	Control	30%	50%	70%	Control	30%	50%	70%
5	4.55±2.06[a]	1.33±1.53[a]	0	0	4.11±1.76[a]	1.33[a]±1.53	0	0	7.11±2.09[a]	1.67± 0.58[a]	0	0
10	4.55±2.29[a]	1.33±0.58[a]	0	0	3.67±2.54[a]	1.67[a]±2.08	0	0	6.22±2.38[a]	1.33 ± 1.53[a]	0	0

*Values with same superscript letter across columns are significantly different (P<0.05).

4. CONCLUSION

The results of this study indicated that spoilage of cured vanilla beans could be due to contamination by moulds including *Aspergillus fumigatus, Aspergillus tubingensis, Aspergillus aculeatus, Byssochlamys spectabilis* (Amanorph *Paecilomyces variotii*) and *Penicillium polonicum*. The combination of blanching and disinfection by ethanol completely inhibited mould growth and, therefore, could be used as simple strategy to control spoilage by moulds and improve the quality of cured vanilla beans.

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