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BIOTECHNOLOGICAL STUDIES ON ENSET (*ENSETE VENTRICOSUM* (WELW.) CHEESMAN)), A FOOD SECURITY STAPLE FOOD CROP OF ETHIOPIA

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ABSTRACT: Several studies relevant for enset improvement and strategic conservation were carried out of which two studies focused on the molecular characterization and one on cultivated enset from nine regions of Ethiopia and the second on five wild enset populations around Bonga area. All the 111 cultivated enset clones were found distinct of each other. In both studies, the genetic diversity among populations was not related to the genetic distances indicating that the current cultivated clones were originated from a limited number of wild progenitors. In addition, the limited gene flow between cultivated and wild enset clones were due to many factors necessitating for *ex situ* and *in situ* conservation of wild enset. The third study centered on the development of an efficient micro-propagation protocol for enset that yield up to 75 propagules through wounding the meristem and growing them on modified nutrient medium. Another study developed transformation procedures for enset using both *Agrobacterium tumefaciens* and particle bombardment as revealed by ß-glucuronidase (Gus) gene expression. A study by the author also revealed identification of endophytes representing 16 bacteria, and 7 yeasts and the profile of cultureable yeasts and lactic acid bacteria from traditionally fermented enset food product, kocho using 16S/26S rDNA sequence analysis. It is interesting to note that *Candida ethanolica* known to control bacterial wilt and *Pantoea agglomerans* which infect plants, animals and humans were also identified amongst the microbes using sequence analysis of 16S/26S rDNA. Furthermore, the isolation of the spore forming *Bacillus anthrax*, the causative agent of anthrax, was critical emphasizing the importance of sanitation and hygiene in enset production, handling and processing. The information generated could be important to develop a starter culture which facilitates fermentation without compromising, kocho quality and the safety of kocho consumers.

Key words/phrases: Enset, Food spoilage, Genetic diversity, Micropropagation, Transformation.

INTRODUCTION

Enset (*Ensete ventricosum* (Welw.) Cheesman) belongs to the family Musaceae (Cheesman, 1947). Morphologically it resembles a banana plant that belong to the related genus *Musa*, and unlike banana, the edible part of enset is its corm and pseudostem where starch is stored. It grows wild in

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central, eastern and southern Africa (Baker and Simmonds, 1953), including Congo, Ethiopia, Mozambique, Tanzania, Uganda, and Zambia. However, it is cultivated as a crop only in the highlands of the southern, southwestern and central parts of Ethiopia, where more than twenty million people use it as a staple food. In addition, the whole plant is used for various purposes as animal feed, a source of medicine for humans and cattle, an ornamental, and for making ropes and mattresses out of the fiber.

The wild enset (*Ensete ventricosum*) populations grow mainly around Bonga (Kefficho administrative region) and the distribution is very restricted; to a smaller area around the Omo river (Gamo-Gofa administrative region), and mainly found in areas not disturbed by routine agricultural activities or other forms of human intervention (Gebre Yntiso, 1996) and where it may even be ritually protected (Shack, 1963). In a very sparsely situated it also occurs in other areas like Benshangul-Gumuz. It is regarded as food security crop as it is relatively drought resistant and is available when other crops fail due to drought or other natural calamities (Westphal, 1975). Enset culture is expanding and is being introduced into several regions in Ethiopia, thus promoting the diversification of farming systems for sustainable production (personal observation).

Enset makes a very substantial contribution to food security in Ethiopia, but production of enset is low for it depends entirely on unimproved varieties that are maintained through clonal propagation by local farmers. The production may decline as a result of genetic erosion due to drought, bacterial wilt disease and human over-population pressure (Almaz Negash *et al*., 2002).

Although Areka research station, in southern Ethiopia, has been maintaining enset germplasm in field banks since 1987, and with few diversity studies on landraces using farmer-based morphological parameters (Hildebrand, 2001), and genetic variation among cultivated enset clones (Almaz Negash *et al*., 2002; Genet Birmeta *et al*., 2002), Dagmawit Tobiaw and Endashaw Bekele (2011) and currently Temesgen Magule *et al*. (2015), Selamawit Getachew *et al*. (2014), there is still a dearth of information on its breeding and diversity. This necessitates characterization, maintenance of the existing germplasm in the wild populations, as well as introduction of genes from wild or related species into the cultivated clones to improve e.g. disease resistance and adaptation to enhance future food security of Ethiopia. Future plant improvements in enset, by the use of either conventional plant breeding or modern biotechnological techniques require reliable and

reproducible methods that can differentiate among clones and estimate their genetic relatedness.

Genetic diversity, geographical pattern of enset accessions

The various studies crucial for the improvement of enset carried out by the author include:

i. A study undertaken to describe the extent of genetic diversity in 111 representative samples from nine different enset growing sites in Ethiopia, and to investigate the presence of geographical patterns in this variation.

ii. A study to analyze the genetic diversity of wild enset in Ethiopia was carried out to provide information for conservation strategies as well as evidence of possible gene flow between the different gene pools (Genet Birmeta *et al*., 2004a).

iii. Identification of endophytic microbes that are resistant to surface sterilization procedures and antimicrobial drugs during a study on development of *in vitro* micro-propagation protocol for enset clones (Genet Birmeta et al., 2004b).

iv. Development of *in vitro* propagation protocol for enset clones (Genet Birmeta and Welander, 2004).

v. Development of genetic transformation procedures for two enset clones (Genet Birmeta, 2004).

vi. Identification of microbes that are involved in spontaneous fermentation of Kocho, a starchy enset food product and to identify those microbes involved in the spoilage of kocho (Genet Birmeta, submitted).

In addition to the above other studies that include draft genome sequence data, additional regeneration system for multiplication of clean planting material of *Ensete ventricosum*, SSR marker system based studies on genetic diversity assessment have been reported by Zerihun Yemataw *et al*. (2018), Temesgen Magule *et al*. (2015) and Selamawit Getachew *et al*. (2014).

DISCUSSION OF RESULTS

i) RAPD analysis to study genetic diversity among clones of *Ensete ventricosum* in Ethiopia

Genetic diversity

A RAPD analysis was conducted to characterize 111 individual plants of *E. ventricosum* cultivated at nine different enset growing sites in Ethiopia (Genet Birmeta *et al*., 2002). All the 111 clones analyzed were found to be genetically different from each other as shown using different statistical methods (Table 1). The results thus suggest that the different clones existing in Ethiopia could be at least as numerous as the named landraces, or even more since vernacular naming has its limitations. As enset is a multipurpose crop, each farmer generally grows several different clones for different types of food (cooking, bread making, and porridge, for fiber production, for medicinal purposes, as an ornamental etc. Thus, each farmer has his/her own miniature 'on farm' germplasm collection, with representation of the most important clones. The comparatively high diversity among enset genotypes observed in our study is in accordance to its outbreeding nature and also its diverse use and selection by farmers, mutations during vegetative propagation, introduction of new genotypes from wild populations and from other farming areas as well as maintenance of even inferior clones for social and cultural reasons (Shigeta, 1996).

Table 1. Collection sites for the 111 Ethiopian enset clones, the administrative regions in which they are located, number of collected plants, percentage of polymorphic loci, mean Jaccard's similarity coefficient and mean Shannon-Weaver gene diversity index (Genet Birmeta *et al*., 2002).

Site	Adminstrative region	No of plants	% polymorphic	Jaccard's coefficient	Shannon-Weaver index
Answae	Arsi	6	78.4	0.69	0.44
Bonga	Keffa	20	92.8	0.52	0.53
Chencha	Gamo-Gofa	25	87.6	0.58	0.49
Seltae	Shewa	8	85.6	0.61	0.49
Setunae	Shewa	7	81.5	0.65	0.46
Shonae	Kembatana-Haddya	9	88.7	0.64	0.49
Wolkitae	Shewa	13	92.8	0.53	0.55
Wondo	Sidama	12	88.7	0.83	0.50
Worka	Welayta	11	95.9	0.59	0.53

Partitioning of genetic variability

The study indicates that most of the genetic variability among enset clones can be attributed to within-site diversity (86%) whereas only a minor part (14%) is detected among sites. Comparatively low diversity was found for sites in the eastern parts of the collection area (Answae and Setunae) whereas sites with high diversity were found in the western parts (Fig. 1).

Bonga and Chencha in the extreme western and southern parts, respectively, of the enset growing area are the two most similar sites when enset clones are compared, and also appear to be rather different from other sites. Although 120 km apart, these two sites are inhabited by different but related ethnic groups, with a long tradition of ritually protecting wild enset, growing in their area (Shigeta, 1996). Consequently, gene/clonal flow may have taken place between wild and cultivated genotypes. Although part of the explanation for this pattern could be climatic in origin, or depends on the availability of indigenous germplasm, cultural history may also have played a role. The amount of within-site diversity may thus be affected by the degree of importance of this crop in a particular region as well as its livestock accumulation.

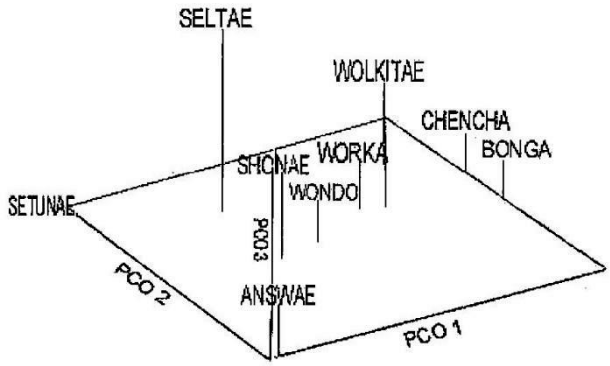

Fig. 1. Three-dimensional PCO calculated from RAPD profiles in enset, based on Nei's genetic distances among 9 collection sites in Ethiopia (Genet Birmeta *et al*., 2002).

Applicability and implications of this investigation

The study demonstrated the fact that much of the RAPD variation in enset was found within sites, suggesting that sampling from a few sites only may be sufficient for capturing a large proportion of the genetic variation. However, sampling should nevertheless cover a geographically wide area since there are significant differences in RAPD allele frequencies between sites. In addition, the distribution of RAPD variation may not necessarily reflect the pattern of variation in genes that control important agronomic characters like environmental adaptation. Special consideration should also be given to ethnical history and cultural patterns that may have affected the choice of enset clones grown in different sites.

ii) Distinction between wild and cultivated enset gene pools in Ethiopia using RAPD markers

Forty eight wild enset plants were from five wild population around Bonga area in south-west Ethiopia were used together with eight cultivated and eight *Musa* samples to analyze the extent of genetic diversity of wild enset using 16S rDNA sequencing.

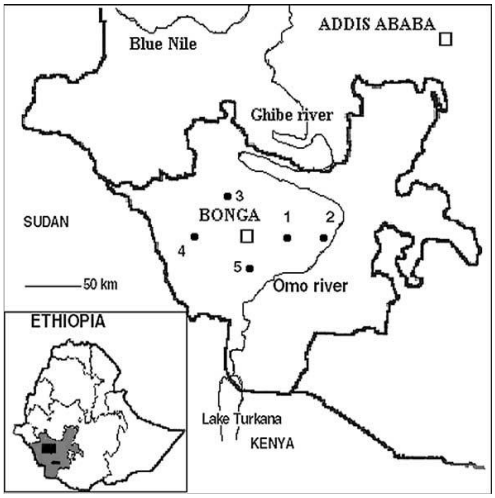

Fig. 2. A map showing the part of Ethiopia where wild and cultivated enset grows. In the large map, closed circles indicate the five sites at which wild enset populations were collected for this study, while the open squares represent cities. In the small map, the grey area shows the southern administrative zone of Ethiopia where enset cultivation is practiced, the larger black rectangle indicates the area where the wild enset populations were collected, and the smaller black rectangle represents the only other region where wild enset grows in Ethiopia, by the Omo river.

Within-population diversity in wild enset populations

In southwest Ethiopia, the cultivation area of *Ensete ventricosum* (enset) overlaps with the natural distribution area of this species. In this study, the extent of diversity was investigated in five wild populations in the Bonga area (Kefficho administrative region) using RAPD. Nine representative cultivated clones and eight *Musa* samples were included in order to provide information for conservation strategies as well as evidence of possible gene flow between the different gene pools (Genet Birmeta *et al*., 2004a). Values for within-population diversity were overall rather similar among the wild 5 populations (not shown). These estimators indicated that populations 2 and 3 are somewhat more diverse while especially population 5 was more homogeneous. It is interesting to note that populations 2 and 3 are located in dense forest with little influence by human disturbance while populations 1

and 5 are closer to human settlements and may thus have been influenced by human activities leading to decreased diversity.

Partitioning of genetic variation in wild enset

The Shannon index attributed most of the total diversity within populations rather than among populations. Among-population differentiation (10%) is quite low when compared with six outcrossing species (15-60%) (Bussel, 1999), and with 18 species (11-88%, and in another review (Nybom and Bartish, 2001). Furthermore, no significant correlation between geographical and genetic distances was observed. Although breeding system is critical, other factors like perennial life form, late successional status and having seeds that disperse by wind or animals (as in the case of enset), are also closely associated with low levels of between-population diversity (Hamrick and Godt, 1989). In our study, the PCA plot (Fig. 3), UPGMA clustering (Fig. 4) and Nei's genetic distance values indicated low differentiation and suggested that there is considerable gene flow between the five enset populations.

Wild enset compared to cultivated enset

The wild and cultivated enset samples in this study were genetically distinct from each other as indicated by their considerable differentiation in RAPD profiles and cluster analysis (not shown). PCA plot (Fig. 3) and dendrogram (Fig. 4) also demonstrated a clear separation between the cultivated clones and the wild enset samples, suggesting that cultivated enset may have been introduced into domestication from a limited number of wild enset genotypes in Ethiopia and that subsequent gene flow between the two forms may have been very restricted due to the different modes of propagation (clonal versus outcrossing) and harvesting of cultivated enset prior to flowering.

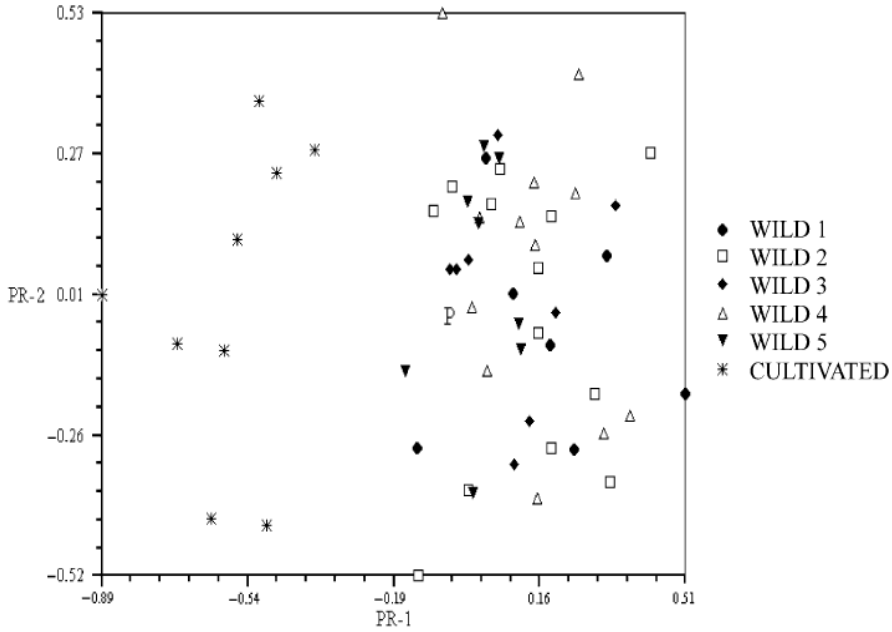

Fig. 3. PCA plot based on RAPD polymorphism, with the first two principal components showing phenetic relationships among 48 wild and 9 cultivated enset samples. Origin of the samples is indicated by their symbols.

Differentiation between *Ensete* and *Musa*

Cluster analysis (Fig. 4) showed the eight *Musa* samples group in accordance with the commonly accepted taxonomy based on morphology and data from molecular markers (Cheesman, 1947; Ude *et al*., 2002) except for *M. nana*, which clustered outside of the *M. acuminata* complex. From our results, it thus appears as if section *Eumusa* is closer to *E. ventricosum* than the other sections are. This may facilitate the transfer of genes into enset from well characterized banana germplasm, which mostly belongs to *Eumusa*.

Endashaw Bekele and Shigeta (2010) based on complete sequence of transcribed spacers and introns from trnT trnF region of chloroplast DNA from thirteen species of *Musa* and three species of *Ensete* including the cultivated and wild species of *Ensete ventricosum* revealed that *Musa beccari* represent ancestral forms of *Ensete* and *Musa*, *Ensete gilleti* or a species very close to it appears to be the ancestral species of *Ensete ventricosum*.

Preservation of genetic resources in enset

The distribution of cultivated enset in Ethiopia appears to be expanding. Unharvested mature enset plants in the field and enset products in the fermentation storage pit constitute a household security system, accessible whenever the need arises. However, enset cultivation is currently threatened by bacterial wilt (*Xanthomonas musacearum*), a very destructive disease in enset and banana. Wild enset as well as *Musa* species could be important sources of useful resistance genes. In addition, genes for desirable agronomic traits like resistance against other diseases, early maturation, higher nutritional quality and higher productivity would be beneficial for enset improvement. Unfortunately, deforestation due to villagization and construction of major refugee camp sites in the Bonga area, may lead to a decline in genetic diversity in the wild enset germplasm. In addition, disturbance of this environment may also affect wild animals like monkeys that are a primary means of seed dispersal and pollination in wild enset. Furthermore, the monocarpic life form, the absence of gene flow from cultivated enset due to early harvesting, and the recalcitrant nature of seeds could gradually lead to the extinction of wild enset. This calls for adopting strategies for both *ex situ* and *in situ* conservation to maintain the existing wild enset in Ethiopia. *In vitro* multiplication of enset clones (Genet Birmeta and Welander, 2004) and wild enset in combination with traditional propagation methods could be utilized for large-scale multiplication, transportation and introduction of this multi-purpose and sustainable crop to regions with food shortage. The information generated in this study, in combination with our previous study on cultivated enset (Genet Birmeta *et al*., 2002), can be utilized for strategic sampling, conservation of wild enset and introduction of enset cultivation to other regions with food deficit. The study has also shown that only a small portion of the genetic variation in wild enset is present in cultivated enset clones. Thus, the wild gene pool, which may for example contain genes conferring adaptation for growing at lower altitudes, could be utilized for the improvement of cultivated enset.

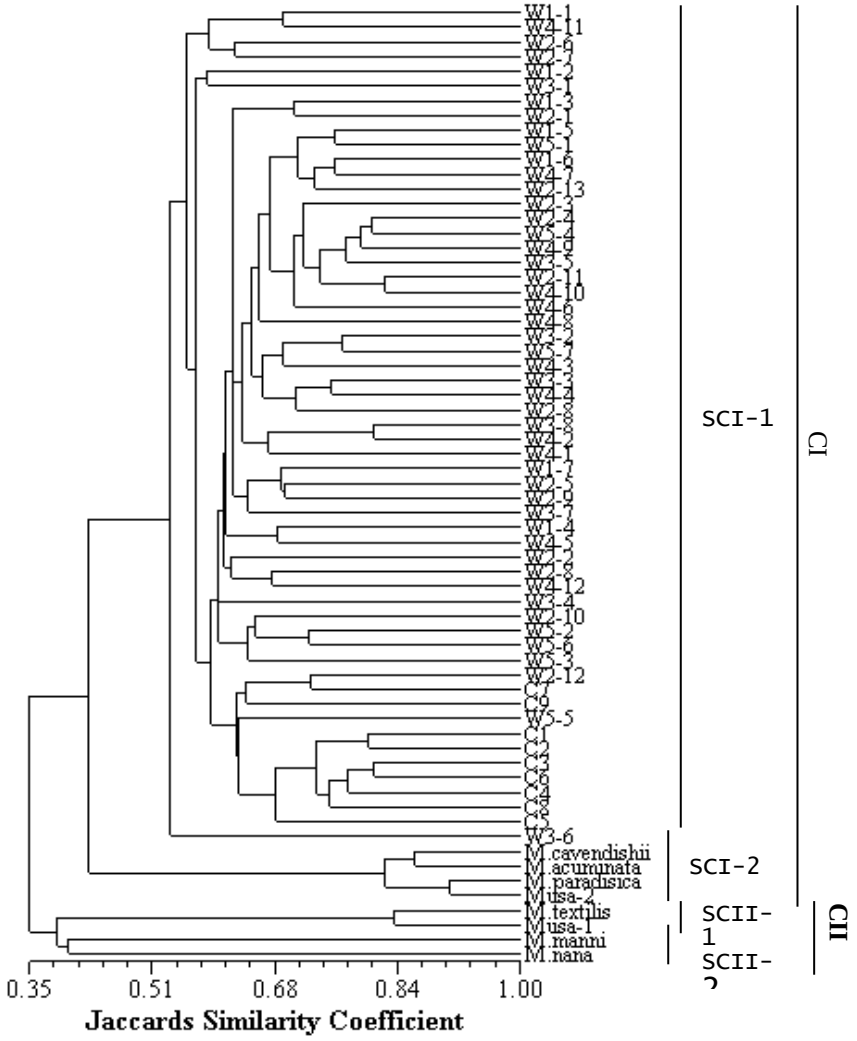

Fig. 4. Dendrogram based on UPGMA of RAPD profiles, showing phenetic relationships among 48 wild (W, numbers indicate the population and genotype, respectively) enset samples, 9 cultivated (C) enset clones, and 8 Musa accessions (M). Main clusters CI and CII are indicated, as well as sub-clusters SCI-1, SCII-1 and SCII-2 (Genet Birmeta *et al*., 2002).

Selamawit Getachew *et al*. (2014) reported transferability of 71 SSR loci from banana (*Musa accuminata*) to enset using 220 Enset accessions. Among the 71 SSRs tested, 12 (16.9%) were successfully transferable to enset. Eleven (16.9%) of these were used to examine the diversity of the 220 enset accessions that had been collected from eight different zones in Ethiopia. Their results showed that enset diversity was accumulated within

rather than between zones and accessions were not grouped into distinct clusters by zone of collection indicating a high rate of planting material exchange between distant zones. Their result is consistent with our previous studies of diversity of enset (Genet Birmeta *et al*., 2004a) using RAPDs, and Almaz Negash *et al.* (2002) using AFLP, Dagmawit Tobiaw and Endashaw Bekele (2011) using ISSR. It is of interest to observe that all markers used resulted to similar trends of conclusions. Temesgen Magule *et al*. (2015) developed genomic SSR markers for enset and reported a large proportion of these markers to be polymorphic and some were also transferable to related species of the genus *Musa*. This study also is consistent with the previous study and demonstrated the usefulness of the markers in assessing genetic diversity and structure in enset germplasm, and provides additional potentially useful information for developing conservation and breeding strategies in enset. Zerihun Yemataw *et al*. (2018) on the other hand reported Genome sequence data from 17 accessions of *Ensete ventricosum* and presented the first genome-wide sequence with a potential to exploit genetic diversity to generate markers to assist enset selection for key agronomic traits. Harrison *et al*. (2014) presented a draft genome sequence for enset (*Ensete ventricosum*). The sequence data of Harrison *et al*. (2014) suggest a genome size of approximately 547 mega bases for enset which is similar to the 523-megabase genome of the closely related banana (*Musa acuminata*). They also have reported that at least 1.8% of the annotated *M. acuminata* genes are not conserved in *E. ventricosum* with additional note that enset contains genes not present in banana.

iii) Identification of bacteria and yeasts from *in vitro* cultures and from surface sterilized field samples of *Ensete ventricosum* by rDNA analysis

An optimal micropropagation system, for crops like enset with a long life cycle and seeds recalcitrant to germination, is a pre-requisite for the improvement and production of disease-free planting material. Our attempts to establish new cultures from the growing apex situated in the underground corm of enset sprouts repeatedly failed due to microbial contamination. Elimination of endophytic contaminants, in particular from roots, bulbs and rhizomes is more difficult than that from aerial tissues (Kritzinger *et al*., 1998). The problem of endogenous microbial contamination of cultures is a major bottleneck during micropropagation. The microbes may not be pathogenic in the field but are very destructive under *in vitro* conditions. These microbes cause growth retardation or death of cultures as a result of overgrowth, competition for nutrients or change of pH and production of

toxic substances (Leifert *et al*., 1990). The standard tissue sterilization procedures and addition of antimicrobial drugs did not efficiently control microbial contamination, suggesting the endophytic and antibiotic-resistant nature of the contaminants. The difficulties in decontaminating explants originating from subterranean organs are frequently reported (e.g. Kritzinger *et al*., 1998). The aim of this study was to identify microbes that are contaminants of enset cultures, and microbes intimately associated with field materials. Identification of the microbes not only facilitates selection of effective and specific antibiotics for future *in vitro* control of the contaminants, but also indicates possible origins of the contaminants.

Bacterial and fungal contaminants of enset (*Ensete ventricosum*) cultures and microbes associated with surface-sterilized field material were identified by 16S and D1D2 regions of 26S rDNA sequencing for bacteria and yeast, respectively. Complete procedures and methods followed could be obtained from Genet Birmeta *et al*. (2004b). The identified bacteria showed a high diversity representing 15 species in the 26 isolates investigated (Table 2). Ten bacterial species were identified from *in vitro* cultures and seven in 10 isolates from field clones. Similarly, three yeast species and one filamentous fungus were identified as *in vitro* contaminants, whereas five yeast species were isolated from the field material (Table 3). The bacterium, *Pseudomonas reactans*, and the yeast, *Torulaspora delbrueckii*, were the most frequent *in vitro* contaminants. The difficulty in controlling the *in vitro* contaminants is due to their apparent endogenous nature and their resistance to antimicrobial drugs.

Only two bacterial species (*Microbacterium paradoxydans* and *Lactococcus lactis*) were found in both *in vitro* cultures and field materials, with *M. paradoxydans* representing the most frequent species associated with field tissues. Most of the bacterial species isolated from *in vitro* enset were Gram-positive and hitherto unrecorded as *in vitro* contaminants of plant cultures. Our results might therefore provide an indication of some unique characteristics of the enset microflora. The endophytic nature of the bacterial *in vitro* contaminants has been reported (Kritzinger *et al*., 1998). In our study, the few cultures that appeared ‘clean’ with no obvious contamination became contaminated when the meristem was wounded during the multiplication stage (results not shown), suggesting that the microorganisms are endogenous.

Table 2. Bacteria isolated from *in vitro* and field enset clones using the 16S rDNA sequencing (Genet Birmeta *et al*., 2004b).

Source	Gram reaction	Bacterial species	No/ Names of isolates
In vitro	–	*Pseudomonas reactans*	6/GBb 7, 13, 14, 17, 20, 23
In vitro	–	*Sphingomonas* sp.*	1/GBb 6
In vitro	–	*Variovorax paradoxus*	1/GBb 2
In vitro	+	*Leuconostoc pseudomesenteroide*	2/GBb 1, 5
In vitro	+	*Lactococcus lactis*	1/GBb 3
In vitro	+	*Micrococcus luteus**	1/GBb 4
In vitro	+	*Microbacterium paradoxydans*	1/GBb 15
In vitro	+	Unknown, highest identity to *Deinococcus grandis*	1/GBb 9
In vitro	+	*Kocuria palustris*	1/GBb 12
In vitro	+	*Staphylococcus epidermidis**	1/GBb 11
Field	–	*Acinetobacter* sp.*	1/GBb 22
Field	–	*Klebsiella ornithinolytica*	1/GBb 27
Field	–	*Klebsiella oxytoca*	1/GBb 24
Field	–	*Pseudomonas putida*	1/GBb 26
Field	+	*Microbacterium paradoxydans*	3/GBb 16, 18, 19
Field	+	*Lactococcus lactis*	2/GBb 21, 29
Field	+	*Leuconostoc citreum* IH22	1/GBb 28

All enset clones were surface-sterilized but only the *in vitro* isolates were treated with antibacterial/fungal chemicals. The symbol *denotes bacterial species that have been reported as contaminants of *in vitro* plant cultures in the literature. GB denotes the authors name used when submitting sequence information to NCBI database of microbes.

Current knowledge on the pathogens of enset plant is limited. Berhanu Abegaz Gashe (1987) identified *Leuconostoc*, *Pseudomonas*, *Bacillus*, *Erwinia* spp. and yeasts as causes of spoilage and discoloration of kocho. *Pseudomonas* spp., *Leuconostoc pseudomesenteroides* and *L. citreum* isolates identified in the present study might also involve in this process. Likewise, discoloration of enset food products is a common problem during storage, suggesting that *P. putida* and *P. reactans* could possibly be pathogens. The microfungal/yeast flora was less diverse than the bacteria. This was also observed during another study. Eight different yeast species were identified among the 25 isolates in comparison to 15 bacteria species from the 26 bacterial isolates (Table 2, Table 3).

As enset is a genetically diverse crop, the high diversity of its microbial flora, is anticipated to be more pronounced than recorded here. Surface-sterilization and antibiotic treatments exclude a substantial number of microorganisms from being isolated. However, as the identified microorganisms impair micro-propagation of enset, they have the highest importance in the frame of the current efforts to develop *in vitro* regeneration systems for future improvement and supply of disease-free enset planting material for cultivation. Many of the bacteria identified are directly or indirectly related to compost, or have human or animal origins

and might have been introduced due to traditional cattle-based agriculture and use of animal manure as compost. Our results suggest the need to transfer field plants to a glasshouse prior to introduction of explants to *in vitro* growth in order to reduce epiphytic microbes. Careful selection and incorporation of specific antibiotics in the culture medium are essential to eradicate endophytic microbes of animal and/or human origin. The identification of the contaminants will facilitate the selection of appropriate antibiotics for their prevention.

Table 3. Yeasts isolated from *in vitro* and field enset clones using D1D2 regions of 26S rDNA sequencing (Genet Birmeta *et al*., 2004b).

Source	No/Names of isolates	Yeast and filamentous fungi
In vitro	8/GBf 1, 4, 8, 9, 14, 18, 19, 24	*Torulaspora delbrueckii*
In vitro	3/GBf 2, 3, 6	*Hanseniaspora* sp.
In vitro	3/GBf 17, 20, 23	Unknown filamentous fungi
In vitro	1/GBf 7	*Candida pararugosa*
Field	5/GBf 13, 27, 29, 30, 31	*Candida intermedia*
Field	2/GBf 12, 15	*Pichia onychis*
Field	1/GBf 28	*Lodderomyces elongisporus*
Field	1/GBf 25	*Rhodotorula mucilaginosa*
Field	1/GBf 10	*Torulaspora delbrueckii*

GB denotes the authors name when submitting sequence information to NCBI database of microbes.

iv) Efficient micropropagation protocol for *Ensete ventricosum* clones through meristem wounding. A three step procedure

An efficient and optimum *in vitro* propagation system is crucial for the improvement of enset clones. To date, very few tissue culture studies have been reported for enset. Taye Bezuneh (1984), Afza *et al*. (1996), Almaz Negash *et al*. (2000) and Tesfaye Zeweldu and Ladders (1998) attempted to develop initiating *in vitro* regeneration of enset with very little success for large-scale micropropagation and they emphasized the major problems with growing and multiplying enset *in vitro*.

In our study, an efficient three-step protocol for *in vitro* propagation of *Ensete ventricosum* (enset) was developed that consisted of initiation, bud proliferation, and shoot elongation and rooting stages. Wounding the meristem region and dividing the corm tissue to induce sprout formation in the field, a traditional enset propagation practiced by local farmers in Ethiopia was also adopted in this work. Furthermore, since plant nutrient requirements vary at different growth stages under natural conditions, the three micropropagation steps that we adopted in our investigation ensured the formulation of different media components depending on the growth

stage of the cultures. The proportion of macronutrients is adjusted according to nutrient uptake observed in glasshouse-grown enset plants (Table 4). Detail procedures could be obtained from Genet Birmeta and Welander (2004).

Table 4. The ranges and average mineral contents in leaf tissue of sprouts of 24 clones of *Ensete ventricosum* grown in the glasshouse in comparison to essential nutrients considered adequate for higher plants in general. Information on higher plants adapted from Stout *et al.* (1951) (NI no information available) (Genet Birmeta *et al*., 2004c).

Mineral	Ranges of mineral content in enset leaves (mg/g DW)	Average mineral content in enset leaves (mg/g DW)	Average mineral content in higher plants (mg/g DW)
B	0.01–0.02	0.01	0.02
Ca	6.6–14.2	9.52	5
Cu	0.005–0.011	0.001	0.006
Fe	0.05–1.69	0.14	0.10
K	38–81	60	10
Mg	2.64–6.90	3.62	2
Mn	0.02–0.12	0.05	0.05
Mo	1.9–6.5[a]	3.8[a]	1[a]
Na	0.010–0.09	0.03	NI
N	24–35	30	15
P	3.6–7.7	5.3	2
S	2.03–4.05	2.8	1
Zn	0.003–0.097	0.018	0.02

[a] Unit of measurement for Mo is micrograms per gram dry weight

The main problems in enset culture initiation are phenol exudation and endophytic bacteria contamination. Afza *et al*. (1996) and Tesfaye Zeweldu and Ladders (1998) have also reported phenol exudation to be a major constraint in enset tissue culture in particular in many other tropical plants. Polyphenol exudation is exaggerated in response to wounding and high iron concentration, a problem which hinder shoots to elongate. The problem of phenol was overcome by adding 0.5–1% (w/v) activated charcoal together with dark incubation of the cultures. In addition, persistent endophytic bacterial infection inhibited *in vitro* growth resulting in the loss of some clones. This problem promoted another study which identified contaminants of enset cultures, which were resistant to various antimicrobial agents, (including some possible pathogens) and yeasts using rDNA sequencing (Genet Birmeta *et al*., 2004b). Shoot-tip necrosis, which is another constraint in enset micropropagation, caused by a shortage of calcium in the medium (for example, Mathew and Philip, 1996) was minimized by replacing calcium chloride with calcium gluconate monohydrate. Reducing ammonium content in the medium is one way of reducing toxicity.

The procedure developed provided a high propagation rate in spite of the various problems encountered (Fig. 5). In the protocol, more than 75 shoots were produced from a single wounded shoot tip, indicating the efficiency of this protocol. From an initial 12 shoots, clone Feresae produced 900 shoots in one subculture, indicating the potential of this clone for the production of

high number of shoots. The large differences in multiplication capacity observed between the different clones may be an indication of the specificity of different clones with respect to nutrient requirements. Differences in multiplication rate among clones have also been observed for banana and plantain (Vuylsteke, 1985). This protocol can be further optimized for other enset clones.

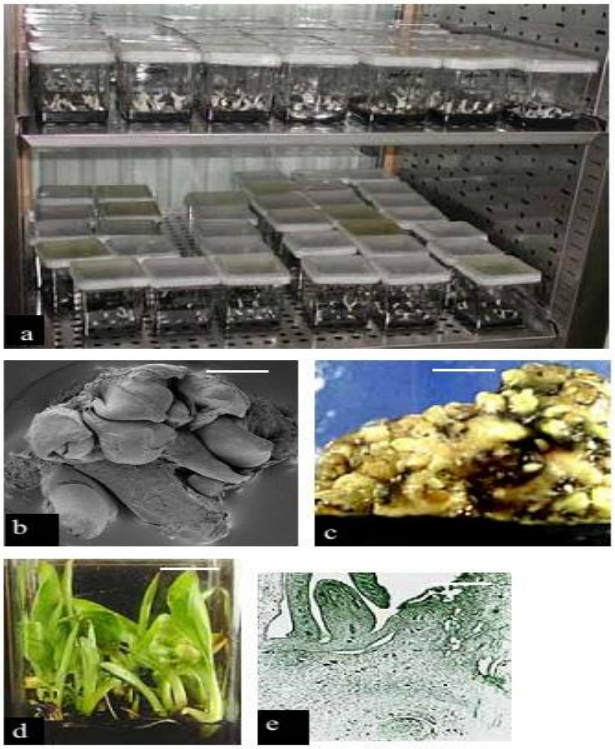

Fig. 5. a–i Micropropagation of *Ensete ventricosum*. a) Conical shaped shoots formed on initiation medium after 2 weeks, b) unwounded shoot with two adventitious shoots at the base, c) multiple buds formed on modified MS medium (EV-2MS) after 5 weeks on bud proliferation medium, d) multiple shoots formed on a small piece of corm tissue as seen with the scanning electron micrograph, e) light microscopy section showing corm. Arrow indicates an internal shoot meristem, f) multiple shoots formed on corm tissue in EV-MS medium, after 6 weeks, in elongation and rooting medium, g) multiple shoots formed on corm tissue in MS after 6 weeks in elongation and rooting medium, h) roots with well-developed secondary roots in elongation and rooting medium, i) plantlets after acclimatization in the glasshouse for 6 weeks. Bars: 1 cm (a–c, f–h); 0.3 mm (d,e); 3 cm (i). (Genet Birmeta and Welander, 2004).

Following our report, Mulugeta Diro *et al*. (2004) also reported that wounding the apical dome by splitting appears necessary to release lateral buds from apical dominance of the tip of the monopodial corm of enset during micropropagation. The greater response in production of multiple

shoots by zygotic seedlings cultured *in vitro* could be due to the absence of blackening and the juvenile nature of the explants. Recently, Tripathi *et al*. (2017) reported micropropagation system for enset cultivar 'Bedadeti' using corm discs containing intercalary meristems. The schematic flow diagram of Tripathi *et al*. (2017) showing various steps of regeneration of complete plants takes over twenty months while Genet Birmeta and Welander (2004) reported 75 shoots from corm tissues in 14 weeks while Tripathi *et al.* (2017) obtained only 36 complete plantlets per corm disc and with over extended time of twenty weeks. Although the number of plantlets could differ depending on the clones of enset used, the extended time observed from Tripathi *et al*. (2017) work needs further improvement.

v) Gus expression in the monocot crop *Ensete ventricosum* through particle bombardment and *Agrobacterium* transformation

Enset has a very long regeneration time ranging between 9 and 14 years depending on the clone and altitude at which it is grown. Due to the long regeneration time and recalcitrant seed to germination, improvement of enset through conventional plant breeding appears to be less viable. Although enset is known to have desirable traits such as high productivity, it has some drawbacks that require improvement to maintain the sustainability of the crop. For instance, enset is a simple basic starch crop, quite low in protein, and vitamin A, and is attacked by some destructive diseases such as bacterial wilt and fungal diseases like *Mycosphaerella musicola* and *Sclerotium rolfsi* (Quimio and Mesfin Tessera, 1996). Improvement of these traits in important enset clones could be achieved through modern biotechnological technique such as genetic transformation. Hence, development of transformation procedures for the improvement of these agronomically desirable traits in enset is essential.

In this study, a transformation procedure for enset was developed through both Agrobacterium and particle bombardment techniques as confirmed by gus gene expression (Genet Birmeta, 2004; Genet Birmeta *et al*., 2004c). All explant types used such as shoot tips, leaves, roots and zygotic embryos showed *gus* expression (Fig. 6). *Agrobacterium*-mediated transformation showed a higher percentage of GUS positive shoot tip (63%) and leaf (50%) explants in clone Erba, when the explants were sonicated prior to transformation. Our results indicate that the expression of *gus* gene is affected by sonication treatment, plasmid size, co-cultivation period and transformation methods. This study proved enset although a monocot crop could be infected by *Agrobacterium tumefaciens*. The transformation

procedure developed in this work could provide the basis to further optimize the procedures and develop clones with improved agronomic traits such as disease resistance.

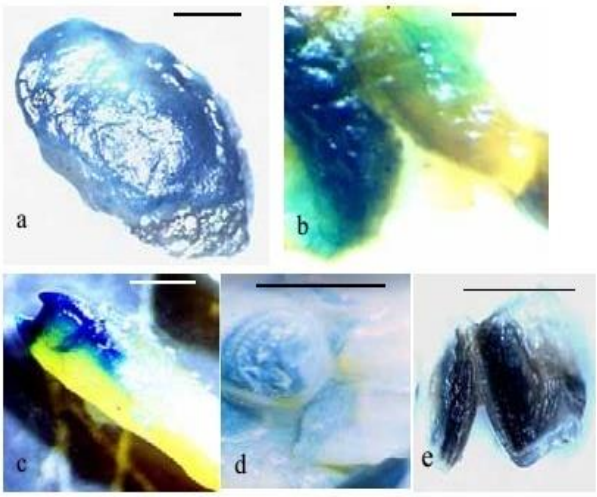

Fig. 6. Transient GUS expression detected after *Agrobacterium* and particle bombardment transformation experiments in the different *Ensete ventricosum* tissues. Fig a) shoot tip, b) leaves, c) root piece, d) zygotic embryo and e) transverse section of zygotic embryo. A, c, d and e after *Agrobacterium* infection and, b after particle bombardment. Bars denote 1 cm. (Genet Birmeta *et al*., 2004c).

vi) Yeasts and bacteria associated with kocho, an Ethiopian fermented food from *Ensete ventricosum*

Kocho, a traditionally fermented staple enset food is a food security crop as its food could be stored for a long period and is available whenever needed and when other crops fail. However, kocho quality, such as taste, colour (discoloration), texture, aroma (off-flavour), and its nutrition is often compromised due to the action of microbes in particular when they proliferate under favourable conditions. However, current knowledge on microbes involved in kocho fermentation and putative pathogens to humans is very limited. A microbial study was conducted on kocho to investigate the dynamics of the microbes across three fermentation stages to identify kocho spoilage microbes. The isolates were identified by sequencing the ITS and/or D1D2 26S rDNA and 16S rDNA regions for yeasts and bacteria respectively. Thirty one different endophytic, culturable yeasts, Lactic Acid Bacteria (LAB) and total aerobic bacteria were identified. Most of the microbes were known to involve in beneficial and harmful activities such as fermentation, contamination, food spoilage, pathogenicity. The identification of the strains responsible for spoilage can lead to information

that can help to eliminate the kocho spoilage microbes. Development of starter cultures using selected microbes facilitates fermentation in more predictable way, increase the nutritional value, and prevent the growth of unwanted (pathogenic) organisms and food spoilage. This is in accordance with the recent findings by the author (Genet Birmeta *et al*., 2018).

Yeasts associated with kocho

The yeasts predominantly isolated from 2 to 5 days (fresh), 3 to 4 months (mid-term fermented) and 7 to 12 months (long-term fermented) kocho are *Issatchenkia scutulata* var. *exigua* (25%), *Geotrichum silvicola* (36%), and *Candida silvae* (75%), respectively. Many of the yeasts identified including the three yeasts are involved in fermentation. The yeast *Geotrichum silvicola* was isolated across all the three fermentation periods, although it is less dominant in the long term fermented kocho. The study illustrated that the dynamics of yeasts and bacteria through time was different. The dominancy of yeasts decreases from fresh fermented to long-term fermented kocho, while the bacterial population in particular LAB was shown to increase with time (this trend was also observed in a previous study carried out by the author (Genet Birmeta *et al*., 2004b).

Lactic Acid Bacteria (LAB) associated with kocho

LABs are involved in fermentation, acid production and are often used as preservatives and may kill or avoid unwanted microorganisms including kocho spoilage organisms. LAB the most predominantly isolated from fresh, mid-term and long-term fermented kocho are *Lactobacillus plantarum* (95%), *Lactobacillus plantarum* (95%) and *Acetobacter pasteurianus* (42%), respectively. *Lactobacillus plantarum* was dominant and was isolated throughout all the fermentation stages. Similarly, *Leuconostoc mesenteroides, Leuconostoc pseudomesenteroides* were isolated from mid-term and long-term fermented kocho. In a previous study by the author, *Leuconostoc mesenteroides* was identified from *in vitro* enset material (Genet Birmeta *et al*., 2004b). Lactobacillus, Pediococcus and Leuconostoc species) are the major lactic acid bacteria in kocho, and in *Eragrostis tef*, an indigenous Ethiopian crop (Ayele Nigatu, 2000). *L. plantarum* isolated consistently in this study and *L. pseudomesenteroides* are known to have antimicrobial activity. Similarly, acetic acid producing bacteria, *Acetobacter pasteurianus* and *Acetobacter cerevisiae* which produce acetic acid were frequently isolated from long-term fermented kocho. When oxygen level is high, the production of acetic is increased leading for the inhibition of yeasts and moulds (Weinberg *et al*., 1993). The undissociated acids lactic acid,

acetic acid and propionic acid are known to inhibit growth of yeast (O'Kiely and Muck, 1992). Lactic acid fermentation of food has been found to reduce the risk of having pathogenic microorganisms grow in the food (Campbell, 1987). Production of bacteriocins by lactobacilli also reported to hinder the colonizing and competitive ability of many microbes (Hammes and Tichaczek, 1994). Aguirre and Collins (1993) pointed out LABs are safe for human consumption due to their common occurrence in foods and feeds together with their long-lived use which contributes to their natural acceptance as GRAS (Generally Recognized As Safe) for human consumption. Hence, the findings suggest enset fermentation in general by lactic acid bacteria such as *L. plantarum* and acetic bacteria such as *Acetobacter* may be safe practice and prevents kocho spoilage.

Kocho fermentation

Fermentation involving microbes is an ancient process ever known in Biotechnology. Fermentation process involves ethanol production by yeasts or organic acids by lactic acid bacteria through the action of their enzymes (Campbell, 1987). In traditional fermentation, fresh kocho contains a mixed microflora which starts fermentation (Abraham Bosha *et al*., 2016), and the microbes come from the raw material (Campbell, 1987). Many of the yeasts identified in this study have fermentative activity. *Candida silvae* (75%) and *Pichia jadinii* (17%) are isolated from long-term fermented kocho. *Candida* and *Pichia* spp. survive acidic condition created by LABs, as a result they were the only yeasts isolated from long-term fermented kocho. *Candida* sp. is lactate-utilizing genera and thrives under aerobic condition (Persson, 2015). During kocho fermentation, the practice of opening the pit regularly for mixing the kocho mass allows partial aerobic condition for microbes to thrive and consequently causing kocho spoilage. In addition, the fermentation of kocho, involving yeasts and LABs, has a major influence on the nutritional and food quality of enset food products (Abraham Bosha *et al*., 2016). LABs isolated frequently are *L. plantarum* and *Acetobacter* sp. suggesting their role in kocho fermentation.

Modern methods of gene-technology make it possible to design and develop starter cultures with specific qualities. The information generated here are relevant to develop a defined starter culture which facilitates kocho fermentation without compromising kocho quality and the safety of kocho consumers. Hence, more research is needed on candidate microbes. Modern methods of gene-technology make it possible to design and develop starter cultures with specific qualities.

Food spoilage

Foods are considered habitats for microorganisms. Most foods susceptible for yeast spoilage have low pH (5.0 or lower) which restricts the growth of competing bacteria and high sugar and organic acid content for easily metabolized carbon sources (Loureiro and Malfeito, 2003; Crawford, 2014). Kocho as traditional and spontaneously fermented food, is susceptible to spoilage, as it has favourable conditions for yeast growth such as low moisture, low pH range of 3-5, high concentration of soluble carbohydrates. Kocho, once removed from fermentation pits, becomes easily contaminated and spoilt by micro-organisms. In silage, the concentration of moulds and yeasts was found to be the major factor starting deterioration when it is exposed to air (Woolford, 1990). During fermentation and storage, if the population of yeast, using lactate as substrate exceed a certain level (105 cfu/g DM in silage), it is more likely that food product gets deteriorated (Jonsson and Pahlow, 1984). Microbes involved in fermentation also affect the nutritional quality of foods. Ohyama *et al*. (1975) indicated that aerobic deterioration of silage leads to a lower nutritional value due to the risk of proliferating deteriorating micro-organisms.

Pichia fermetans, *Pichia membranifaciens* and *Pichia jadini* (5 *Pichia* species isolated) and *Issatchenkia scutulata* identified in this study and many *Candida* sp., though fermentative yeasts, are recorded as frequent contaminants and food spoilage yeasts (Crawford, 2014) as they may proliferate under low pH produced by LAB which is inhibitory for most yeasts. Crawford (2014) also added that some strains of *Pichia* and *Candida* species produce zymocins, a yeast killer toxin, in agreement to the absence of other yeasts in long-term fermented kocho in this study. *Pichia jadinii* survived the acidic condition as it can utilize lactic acid as substrate. Thus the proliferation of *P. jadini*, in the long-term fermented kocho, under favourable condition may lead to kocho spoilage if it exceeds the set limit. In silage under aerobic condition and during inappropriate storage the dominating yeast genera are the lactate-assimilating *Candida* and *Pichia* sp. (Jonsson and Pahlow, 1984). This is in agreement with the study as in the long term fermented kocho, the yeasts *Candida* sp. and *Pichia* sp. are the only yeast species isolated. Oxygen availability appears to be a key parameter that influences spoilage of food caused by fermentative yeasts. Use of air tight sealed, oxygen-resistant packaging in particular during storage may minimize food spoilage as many yeasts and some bacteria proliferate in aerobic conditions.

Deak (2007) argued that compared with bacteria and molds, yeasts play a minor role in food spoilage. Berhanu Abegaz Gashe (1987) using a study of physiological characterization, identified bacteria such as *Leuconostoc*, *Pseudomonas*, *Bacillus*, *Erwinia* spp. isolated from slimy kocho samples and yeasts as causes of spoilage and discoloration of enset food products. In a previous study, the author has identified the bacterium, *Pseudomonas reactans*, and the yeast, *Torulaspora delbrueckii*, as the most frequent *in vitro* contaminants of enset cultures (Genet Birmeta *et al*., 2004b). *T. delbruecki* is recorded as food contaminant microbe. The numbers of bacteria in food can increase rapidly and soon become hazardous to health, particularly if the food has a favourable temperature and water content. Meskerem Elfu (2018) studied eight lactic acid producing bacteria isolated as potential starter culture organisms for enset fermentation preparation that would reduce fermentation time.

Harmful microbes associated with kocho

With regard to hygienic and health aspects, the study identified many microbes among others *Candida* sp., *C. silvae* and *Geotrichum silvicola* which are reported to be opportunistic or obligate pathogens. The observed dominance of spore forming bacteria of the genus *Bacillus* (3 species isolated), in particular *Bacillus anthracis* isolated from total aerobic bacterial medium is of critical health concern. Similarly, the bacteria *Pantoea agglomerans* (38%), isolated from total aerobic bacteria selective medium is reported to infect plants, animals and humans (Dutkiewicz *et al*., 2016). It is worth noting that enset based agriculture is a mixed agriculture which heavily relies on cattle manure as compost which may lead to introduction of pathogens from cattle. The study supports the significance of research around food-borne diseases as priority agenda. The study emphasises the paramount importance of sanitation and hygiene in enset production, handling and processing of enset and its food products.

Beneficial microbes associated with kocho

Many microbes such as *Candida ethanolica* are reported to have inhibitory or biocontrol activity, including against bacterial wilt. Some of the yeasts and LAB such as *L. plantarum* and *L. pseudomesenteroides* have antimicrobial activity which may be important to control the growth of unwanted food spoilage microbes and pathogens. Biotechnological and microbial research to unravel their potential as biocontrol agent including against unwanted food (kocho) spoilage organisms has crucial importance in eco-friendly and sustainable manner. Similarly, some of the organisms

identified are reported to have importance in biofertilizer (*Bacillus simplex*, *Candida ethanolica*, *Lactobacillus buchneri*), biogas (*Candida ethanolica*) production and thus more research should be geared towards unraveling their potential.

CONCLUSION AND RECOMMENDATIONS

The outcomes of these investigations will contribute towards improving the livelihood of the peasant households, ensure the sustainability of the crop and benefit the country at large. Future research efforts should be directed to the following areas:

- Wide-scale efforts on natural resource preservation and maintenance of the existing domesticated and wild enset germplasm in Ethiopia.
- Increasing the content of proteins and vitamin A in the plant to ensure the livelihood and health of peasant households.
- Investigation of enset bacterial diseases and food spoilage microbes, food-borne diseases, pathogenicity testing of some of the microbes recorded by the author and other researchers and other putative diseases.
- Awareness creation among farmers on the importance of sanitation and hygiene in enset production and processing.
- Further investigations on the potential of enset as medicinal plant, fiber, starch and ornamental.
- Development of a predictable starter culture and upgrading traditional fermentation of kocho to industrial level.
- Strengthening research to unravel the potential biotechnological uses of enset microbes as biocontrol, biofertilizer and biogas.

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