Genetic Variability and Biotechnological Studies for the Conservation and Improvement of *Ensete ventricosum*

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To Eskinder, Hewan, Endashaw, Elfenish

and

Samuel

"Someone's sitting in the shade today because someone planted a tree a long time ago"

Abstract

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Enset (*Ensete ventricosum*) is a drought-resistant staple food crop which is grown in the south and south-western parts of Ethiopia. Due to lack of research on enset, no benefits have yet been gained from modern biotechnological approaches. This thesis deals with studies on genetic diversity in cultivated and wild enset, development of micropropagation and transformation procedures and identification of microbes associated with enset.

The genetic diversity in cultivated enset from nine enset-growing regions of Ethiopia was investigated using Random Amplified Polymorphic DNA (RAPD). All of the 111 enset clones studied showed unique band patterns. The genetic diversity observed in cultivated enset in a particular area appears to be related to the extent of enset cultivation and the culture and distribution pattern of the different ethnic groups than geographical distance. RAPD was also applied to document genetic diversity in 5 wild enset populations in Ethiopia together with some cultivated enset clones and *Musa* species. Both studies indicated that the genetic diversity within populations was higher than that of among populations. Our results suggest that the current cultivated clones have originated from a limited number of wild progenitors. The large gene pool observed in the species could be utilized for improvement of the crop.

Two studies focused on the development of efficient micropropagation and transformation procedures for enset in order to develop optimal systems for genetic improvement of the crop. The micropropagation procedure developed enabled the production of large numbers of propagules. Wounding the meristem and modifying the nutrients in the medium was essential to enhance the efficiency of micropropagation. In the transformation experiment, expression of β-glucuronidase (*gus*) gene was manifested in different explants of two enset clones, using both particle bombardment and *Agrobacterium*. *Agrobacterium* was shown to infect the monocot plant resulting in higher percentage (63%) of GUS expression than bombardment (18%), when shoot tips were sonicated. The frequency of GUS expressing explants appears to vary depending on sonication treatment, tissue type, co-culture period and transformation techniques.

Microbial contamination, in particular by apparently endophytic microbes that are resistant to antimicrobial agents was encountered during micropropagation work. This persistent problem prompted a microbiological study with identification of 16 bacteria, 7 yeasts and 1 unknown mycelial fungus from surface-disinfected *in vitro* and field-grown enset clones using 16S and 26S rDNA sequences. Some of the microbes identified could be pathogenic in field-grown enset.

Key words: Agrobacterium, endophytic contamination, genetic diversity, *Leuconostoc,* microorganisms, micropropagation, *Musaceae*, particle bombardment, *Pseudomonas reactans*, RAPD, rDNA, transformation, *Torulaspora*

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Appendix

Papers I-V

The present thesis is based on the following papers which will be referred to by their roman numbers

I. Birmeta, G., Nybom, H. & Bekele, E. 2002. RAPD analysis of genetic diversity among clones of the Ethiopian crop plant *Ensete ventricosum*. *Euphytica* 124: 315-325.

II. Birmeta, G., Nybom, H. & Bekele, E. 2004. Distinction between wild and cultivated enset (*Ensete ventricosum*) gene pools in Ethiopia using RAPD markers. *Hereditas* 140: 139-148.

III. Birmeta, G. & Welander, M. 2004. Efficient micropropagation of *Ensete ventricosum* applying meristem wounding: a three-step protocol. *Plant Cell Reports* 23: 277-283.

IV. Birmeta, G., Passoth, V., Roos, S. & Welander, M. 2004. Identification of bacteria and yeasts from *in vitro* cultures and from surface sterilized field samples of *Ensete ventricosum* by analysis of rDNA. *Biotechnology Letters* (in press).

V. Birmeta, G., Zhu, L.H. & Welander, M. 2004. GUS expression in the monocot crop *Ensete ventricosum* through particle bombardment and *Agrobacterium*-mediated transformation (submitted to *Plant Cell Reports*).

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Introduction

Enset (*Ensete ventricosum* (Welw.) Chessman, is a perennial, herbaceous, monocarpic and monocotyledonous crop in the family Musaceae (Westphal 1975). The corm and pseudostem of enset yield a highly carbohydrate-rich staple or co-staple food, for the quarter of the Ethiopian population that inhabits the south and south-western part of Ethiopia. Enset is a crop that tolerates prolonged drought periods, flooding and many diseases. Due to its drought tolerance, it is regarded as a priority crop in Ethiopia, where it makes a major contribution to the food security of the country. Regions where enset is used as staple food are usually less affected by the recurrent drought periods that occur in Ethiopia (Brandt *et al.* 1997). Enset is suitable for sustainable agricultural systems due to its contribution to soil fertility. In addition, enset can easily be stored without the need for refrigeration and is available throughout the whole year. It can be accessed at any time when there is food shortage and other crops fail as a result of drought, diseases or other factors. However, enset has been harvested so intensively during droughts that some important clones have become extinct, thereby reducing the genetic diversity of the crop.

Enset represents 65% of the total crop production in the southern regions of Ethiopia. Productivity is very high compared to other crops but varies depending on edaphic factors, altitude, cultural practices and varietal differences. Although estimating the productivity of enset has proved difficult, Bezunhe (1983) reports that enset plants grown 5 metres apart, or at 2000 plants/hectare, yield between 18.5 and 29.8 kg/plant or between 7,414 and 11,950 kg/ha/year. An integrated and comprehensive study of the biological, agricultural, ecological, social, and economic components that make up enset-based agricultural systems is needed in order to boost productivity and permit the distribution of enset products to non-enset growing regions of Ethiopia.

This thesis hopefully will provide a basis for the conservation of enset and the improvement of important agronomic traits through the determination of genetic diversity in wild and domesticated enset in Ethiopia and the development of micropropagation and transformation procedures. In addition, microbes that influence enset micropropagation and that may also affect field-grown enset plants are surveyed for the first time.

Ensete ventricosum — the enset crop

An enset plant has three main parts, an underground corm, an aerial pseudostem made of overlapping leaf sheaths, and several broad leaves (Figure 1). Enset is a large plant with a height of 12 m and a pseudostem that is dilated at the base and that has a width of up to 1 m. The growing point of the plant or meristem is located at the junction between the pseudostem and the underground corm near the soil surface. When the plant matures after 9–14 years, the true stem emerges through the leaf sheaths and produces inflorescences. The best time for harvesting is just after production of

inflorescences and seed set. However, enset sometimes can be harvested when only 3-4 years-old, depending on the clone and growing conditions. If not harvested, the whole plant falls down, shortly after seed set.

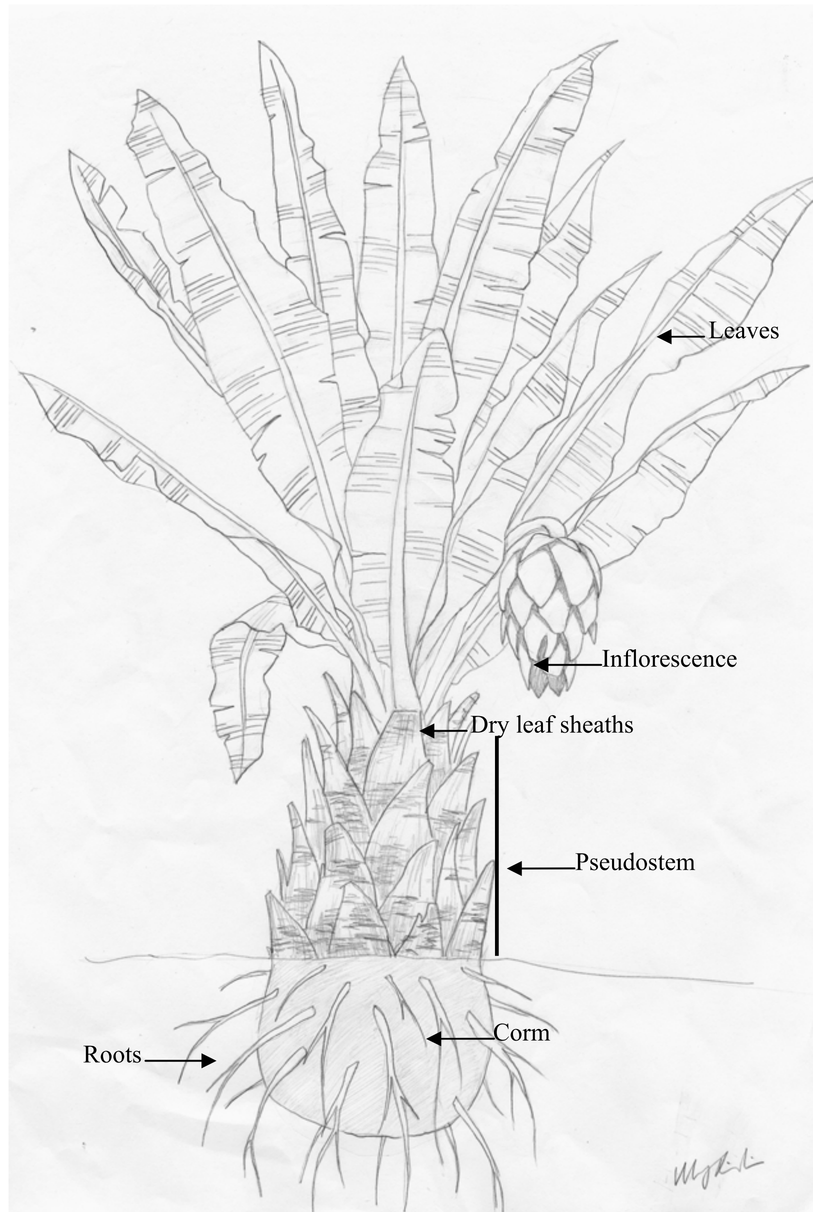

Fig. 1. An enset plant.

Why false banana?

In addition to other synonyms, enset is often known as false banana due to its physical resemblance to a banana plant. However, enset belongs to the genus *Ensete* in the banana family Musaceae while banana belongs to the genus *Musa*. Although enset produces banana-like fruits (Figure 2a) these fruits do not constitute the edible part, which is instead found in the underground corm and in the aerial pseudostem made up of overlapping leaf sheaths. Starchy food is produced by pulverizing the corm or scraping the pseudostem, followed by a short fermentation period. Unlike banana, enset is monocarpic and fruits only once in its life cycle. The fruits contain several seeds which are hard and about 1-2 cm long (Figure 2b). Sprouting occurs only when the main shoot with the meristem, is artificially decapitated at the junction between the pseudostem and corm at soil surface, while in banana sprouting occurs spontaneously. Enset is a diploid plant with the haploid chromosome number n = 9, whereas *Musa* species, including edible banana, have different ploidy levels and chromosome numbers (diploid, triploid or tetraploid), with n = 10 or n = 11 (Ude *et al.* 2002).

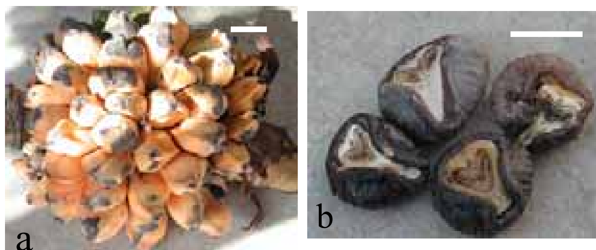

Fig. 2. a) Fruits of enset, b) Seeds of enset. Bars represent 1 cm.

Distribution of cultivated and wild enset

Of the six commonly recognized species of *Ensete*, *E. superbum* and *E. glaucum* grow wild in Asia, *E. perrieri* in Madagascar and *E. gilletii*, *E. homblei* and *E. ventricosum* in eastern Africa (Simmonds 1962). Some species of *Ensete* are also reported to grow in North America (Chessman 1947). *Ensete ventricosum* is considered to be the only wild species growing in Ethiopia (Simmons 1956).

Ensete ventricosum was previously cultivated only in the south and south-western parts of Ethiopia (Figure 3), but the recurrent droughts have led to the expansion of enset cultivation to other parts of the country. A wide adaptation within the species to altitude, soil and climate has allowed widespread cultivation in western Bale, south-western Oromia including south and east Shewa, Jima, Illubabor and Welega (Shank 1994). Wild enset grows at altitudes of 1200–1600 m above sea level while domesticated enset is

cultivated at altitudes of 1100–3100 m above sea level (Brandt *et al.* 1997). The optimal conditions for enset cultivation occur at 2000–2750 m with 1100–1500 mm rainfall, a temperature range of 10–21 ºC and a relative humidity of 63–80% (Brandt *et al.* 1997). Lack of sufficiently high humidity is more limiting for good growth than high temperatures (Alemu & Sandford 1996). Enset often grows best in acidic, heavy clay soils that retain high levels of organic matter when manured (Shank 1994).

Multipurpose crop

Enset is a multipurpose crop with all plant parts being utilized for human food, animal forage, medicinal or ornamental uses. Enset has high significance in day-today-life of the peasant households cultivating this crop as staple food. The peasants indicate that enset is their food, their cloth, their house, their bed, their cattle-feed and their plate (Brandt *et al.* 1997). Amongst its uses, enset produces a starchy, carbohydrate-rich food for human consumption. This is prepared in different ways by the ethnic groups that use enset as staple or co-staple food. However, three dishes are most common. A steam-baked flat-bread 'kocho' is prepared from the pulp of fermented pseudostem and corm. 'Bulla' is made from solidified starch obtained from the pseudostem and is used as porridge or as a flat-bread, while as 'amicho' where the corm is eaten boiled like potato. Nowadays, enset flour is also mixed with flour from cereal crops such as tef (*Eragrostis tef*) to make the traditional Ethiopian bread 'injera'. Although rich in carbohydrate and starch, enset foods are low in protein and vitamin A.

The fibre from the leaf and pseudostem is comparable to that of *Musa textilis* (Afza *et al.* 1996) and is used for construction and rope-making. The leaves are used as animal forage and for wrapping. The corm and pseudostem of some enset clones are used as traditional medicine to repair broken bones, facilitate abortions or discharge placentas after birth in both cattle and humans, and possess antimicrobial properties (Hölscher & Schneider 1998). Last but not least, the plant is an attractive evergreen ornamental which is being introduced to different geographical regions of Ethiopia and other neighbouring countries. The presence of 20–40 magnificent huge enset plants framing small huts and farmyards presents an impressive picture (Shank 1994).

As a result of its many uses, enset cultivation is deeply entwined in the economic, cultural and social life of different ethnic groups. Early travellers in the Wolayta region of southern Ethiopia called the human population and the region 'the enset culture' or 'the enset people' impressed with the importance apparently attached to this crop (Shank 1994).

Problems associated with enset cultivation

Although enset is thought to be resistant to many biotic and abiotic potential problems, the crop is now threatened by some destructive diseases such as bacterial wilt caused by *Xanthomonas campestris* pathovar (pv)

musacearum and fungal diseases caused by *Mycosphaerella musicola* and *Sclerotium rolfsi* (Stewart & Yirgou 1967; Quimio & Tessera 1996). Quimio and Tessera (1996) indicated the absence of effective control measures for the diseases, pests and mole rats that affect enset cultivation and that may cause the loss of some important clones. Bacterial wilt is a very destructive epidemic disease of enset and banana often reported in some east African countries. Hence farmers must be informed about measures that can be taken in order to counteract the mechanical transmission of this disease. In addition, the presence of resistance in enset clones needs more investigation. Research on the genetic diversity and distribution of *Xanthomonas campestris* pv *musacearum* in Ethiopia could also be a useful approach for the development of effective control measures. More pathogenicity tests are needed on microbes isolated from enset or reported as pathogens in other crops such as *Ralstonia solanacearum*.

Although enset foods are known to have high starch content, they are highly deficient in proteins and vitamin A. This has caused some malnutrition-related diseases in regions where the crop is used as the staple food and hence the introduction of other crops rich in vitamins and proteins to the daily diet is essential in order to improve the health status of these people, in particular, small children. Development of enset clones with enriched vitamins and/or proteins through biotechnological techniques and dissemination of the clones to these regions could be of great value.

Previous research on enset

To date, no research has been conducted on the genetic improvement of enset. One reason is the long regeneration time, which ranges from 9 to 14 years. The recalcitrant nature of enset seeds to germination may also have contributed to the lack of work using conventional plant breeding techniques. Most of the research on enset has been concentrated on agronomic studies of e.g. yield and productivity, plant density and intercropping (Bezunhe 1996).

Germplasm collection and *ex situ* conservation have been conducted by the Areka Research Station, where 77 and 128 enset accessions from Wolayta and Kembata administrative regions, respectively, are now grown *ex situ*. For *ex situ* conservation, back-up samples *in vitro* would, however, be very useful in order to avoid loss of accessions due to biotic and abiotic factors. Characterization of the germplasm of domesticated enset has been conducted using morphological traits (Tsegaye & Struik 2002). For a general estimate of genetic variability, modern molecular marker techniques are often preferred. Recently, an Amplified Fragment Length Polymorphism (AFLP) study was conducted to evaluate the germplasm of cultivated enset in some enset-growing regions of Ethiopia (Negash *et al.* 2002).

The presence of resistance/tolerance in enset clones to *Xanthomonas musacearum* has been screened in some enset clones; although no clone

was found to be fully resistant, varying levels of tolerance to the disease were encountered (Ashagari 1985). However more research is needed considering the various enset clones from the different enset-growing regions. Future use of molecular techniques could produce markers linked to tolerance in enset clones.

The deterioration of enset food products during storage is another major problem requiring investigation. Amongst others, bacteria of the genera *Leuconostoc* and *Lactococcus, Pseudomonas, Xanthomonas* are known to cause this deterioration in enset and other crops (Gashe 1987; Nigatu 2000, Todar 2004).

Traditionally enset has been considered by local farmers in Ethiopia, to have a medicinal value and the ability to heal a variety of illnesses. A substance called phenylphenalenone, which has antitumour, antibacterial, nematicidic and antifungal activity has been isolated from some clones (Hölscher & Schneider 1998). This finding may lead to the development of modern medicines for treatment of various human and animal illnesses and birth-related complications.

The traditional methods of processing enset are very labour-intensive and can also reduce the quality of enset food products and fibres. Some efforts have been made by the Institute of Agricultural Research (IAR), Nazeret, the Ministry of Agriculture and Awassa College of Agriculture, Awassa and Wolayta Rural Technology Centre of the Ministry of Agriculture to develop modern devices that enable easy decortication, pulverization and kneading (Belehu 1996). However, these devices are still on trial and have not yet been made available to the end users due to their high costs (Methzun & Abebe 1994).

Genetic diversity

Human beings in their struggle for survival have selected and domesticated crops to fulfill their immediate needs. Nowadays, many people have become aware of the importance of preserving genetic diversity for the survival and continuation of any form of life that exists on earth. Data on the relative genetic diversity within and among plant populations can have a major significance in the preservation of genetic diversity in crops and the improvement and maintenance of crop germplasm for sustainable agriculture. Several modern molecular techniques are now being applied together with morphological studies to investigate genetic diversity and relatedness in crops. The application of molecular markers has enabled studies on reproduction, speciation and spatial-temporal dynamics of populations in many species (Xu *et al.* 2002). Multilocus fingerprinting techniques, such as RAPD provide a cost-effective means to rapidly and simultaneously assess the genetic variability across many loci (Call *et al.* 1998; Weising *et al.* 2000). Use of a marker system such as RAPD, which has a relatively low cost, will make its wider-application more feasible. The questions on homology of RAPD bands and their reproducibility are now being answered. Reproducibility can be ensured if the experimental

conditions are carefully optimized prior to the full application of the technique. Furthermore, bands of the same size are usually homologous when samples from the same or closely related species are investigated (Rieseberg 1996). Lannért *et al.* (1996), investigated the homologous nature of RAPDs and showed that out of 250 RAPD-markers recorded, only 1.2% displayed lack of hybridization signal, indicating success in their application.

Tissue culture techniques

Plant tissue culture techniques involve the growing and multiplication of totipotent cells, tissues and organs of plants on defined solid or liquid media comprising nutrients under an aseptic and controlled environment (George 1993). *In vitro* techniques are used for cryopreservation, conservation of rare and highly endangered plants and production of secondary metabolites. Furthermore, *in vitro* regeneration and micropropagation methods are now widely used for the improvement of important crops through biotechnological methods such as genetic transformation. Development of improved crops through genetic transformation can only be achieved if an optimum procedure for regeneration of transformed explants is available.

Micropropagation is one of the tissue culture techniques used for the production of 'disease-free', high quality and uniform planting material. It enables production of large numbers of propagules within a relatively short time for commercial purposes, independent of season. However, the full application of these techniques depends on the cost of micropropagation. Micropropagation could be achieved either using axillary shoots, shoot buds or nodal segments, or from adventitious shoots using parts of leaves, petals, flowers or roots. Adventitious shoots can be obtained directly or indirectly through a callus phase. Micropropagation directly from axillary buds is more desirable for routine micropropagation, since it produces true to type propagules. Indirect regeneration through callus might be more suitable for genetic transformation, due to the disorganized, highly dividing nature of callus cells and thus the relative easiness to transfer DNA. Furthermore, regeneration of shoots from transformed callus usually results in complete transformed plants. By contrast, direct regeneration might result in the production of chimeric transgenic plants. However many crops, in particular monocots, are recalcitrant to the induction of callus and hence in such cases direct regeneration may be necessary. Furthermore, micropropagation of some crops is hindered by the recalcitrant nature of certain tissues, slow growth and loss of cultures as a result of endophytic microbe invasion and exudation of growth-inhibiting substances such as phenols (Zeweldu & Lüdders 1998). In order to overcome these problems, preparatory or preventative measures need to be devised in parallel to the micropropagation procedure. Diagnosis for endophytic microorganisms in the preparatory stage is important in order to prevent loss of cultures at a later stage and to ensure the production of 'disease free' plants.

Genetic transformation

Genetic transformation techniques, unlike conventional breeding, have more applicability in the improvement of crops with a long regeneration time. Development of crops with agronomically desirable traits through genetic engineering can be achieved directly through particle bombardment or electroporation or indirectly through *Agrobacterium*-mediated transformation. *Agrobacterium*-mediated transformation is the preferred method since only one or few copies of the transferred DNA (T-DNA) harbouring the foreign gene are transferred. Moreover, rearrangement of the introduced DNA occurs rarely in *Agrobacterium*-mediated transformation compared to direct gene transfer methods (Ke *et al.* 2001). Transformation of monocot plants through *Agrobacterium* has been thought to be unachievable due to the putative inability of *Agrobacterium* to infect monocotyledonous plants. However, recently, the ability of the bacterium to infect and transmit DNA has been demonstrated in many major monocot crops such as rice, barley and wheat (Hiei *et al.* 1994; Cheng *et al.* 1997; Tingay *et al.* 1997). *Agrobacterium* transformation and transfer of the T-DNA to the plant cell is a complicated process. This process is triggered by activation of a series of virulent (*vir*) genes residing on the tumour inducing (Ti) plasmid of the bacterium, through signals from the host plant cell. The activation of *vir* genes results in the generation of site-specific nicks within the T-DNA borders and production of linear single-stranded DNA molecules in the plant cell. The T-DNA is encoded in the Ti-plasmid and is defined by 25-bp conserved border sequences at both left and right ends. These border sequences are the only cis-or-trans acting elements needed for DNA transfer. Genes essential for DNA transfer are encoded within the *vir* region of the Ti-plasmid. Transcription of *vir* genes is regulated by *virA* and *virG* genes. An increase in the copy number of *virG* on a multicopy plasmid leads to an increase in *vir* gene expression which might in turn enhance the efficiency of DNA transfer in plants (Hansen *et al.* 1994). Once phosphorylated by *virA*, *virG* transcriptionally activates the other vir genes. Plants produce different compounds which result in different levels of gene expression in different hosts, thereby affecting their sensitivity to infection by *A. tumefaciens*. A low level of *vir* gene expression caused by low inducing ability of the host plant reduces the potential of the bacterium to infect the host plants (Hansen *et al.* 1994).

Objectives of this study

The overall objective of present study was to provide information that could be used to maintain the sustainability of enset, which is an important contributor to the food security of Ethiopia. The specific goals were:

- To perform RAPD analysis to estimate the magnitude and pattern of genetic diversity in relation to geographical distribution in cultivated and wild enset, and to study the genetic relationship between cultivated and wild enset in Ethiopia in order to facilitate the selection and identification of clones for future improvement and for the conservation of enset germplasm *in situ* in the field or *ex situ* in gene banks.
- To develop an efficient and cost-effective procedure for micropropagation of enset clones.
- To identify microorganisms that are intimately associated with *in vitro*-and field-grown enset.
- To develop genetic transformation procedures for enset using both microprojectile bombardment and *Agrobacterium*-mediated transformation.

Towards the improvement of enset (Papers I-V)

In this work, a basis has been established for future improvement of enset through modern biotechnological techniques. The thesis includes an investigation of genetic diversity in cultivated enset, a study of diversity in wild enset in Ethiopia, development of a micropropagation procedure, and genetic transformation procedures using both *Agrobacterium* and microprojectile bombardment. An analysis of the microflora associated with *in vitro* and field-grown enset was also conducted.

Genetic diversity was assessed in cultivated and wild enset germplasm growing in Ethiopia (**Papers I** and **II**). The information generated in this study can provide a basis for further investigations and research focusing on the conservation of enset germplasm and improvement of cultivated clones. DNA marker profiles can facilitate the selection of important genotypes with desirable traits and the identification of duplicates in gene banks.

The development of an optimal micropropagation procedure (**Paper III**) could have a major significance in the improvement of enset clones, in the introduction and dissemination of improved clones for instance, drought and disease-resistant clones, in particular to other non-enset growing regions of Ethiopia or other countries with food shortages.

The identification of microbes (**Paper IV**) associated with enset clones, could enable the selection of specific antimicrobial agents against contaminants of enset cultures. It also provides information about possible

pathogens that might affect productivity, lower the quality of enset food products and cause loss of important clones.

The transformation procedures (**Paper V**) have the potential to become instrumental in the development of genetically improved enset clones with desirable characters such as disease resistance, higher vitamin A and protein contents.

Evaluation of germplasm in domesticated and wild enset

Genetic diversity in cultivated enset clones (Paper I)

DNA-based markers play an important role in estimating diversity, identification of genotypes and tagging genes with desirable traits. RAPD was used in our study to estimate the genetic diversity and relatedness in 111 cultivated enset samples from 9 different geographical sites representing all the major enset-growing regions of Ethiopia (Figure 3). A total of 126 primers were tested for reproducibility. Of these 12, were selected for analysis in which they subsequently produced 72 repeatable and polymorphic bands. Each cultivated enset clone is propagated vegetatively, but the genetic diversity among the clones was found to be relatively high compared to that of most other outbreeding crops (Nybom & Bartish 2000). Several other studies have shown that variation among genotypes is comparable regardless of whether they reproduce vegetatively or not (Hamrick & Godt 1996). In this study, all the 111 enset clones investigated exhibited unique banding patterns. Our results are essentially in agreement with other reports on the diversity of enset based on AFLP (Negash *et al.* 2002) and on morphology and vernacular namings (Tesfaye & Lüdders 2003). More than 160 enset clones with different vernacular names have been recorded from the northern part of Omo river alone (Alemu & Sandford 1996). Our results indicated that the diversity and number of clones in enset cultivation regions could be as high as the number of vernacular namings used by local farmers. Shank (1994) also noted the presence of considerable variation within the species for characters associated with growth and adaptation. In this study, in addition to only sampling clones with different names, other factors may also have contributed to the high genetic variation that we observed. Among these factors are somatic variation, maintenance and protection of enset clones due to cultural reasons and perceived agronomic value (quality of food, fibre, medicine etc), introduction of new clones from other regions and occasional gene flow from wild enset possibly from other *Ensete* species too.

The genetic variability recorded for the 9 different sampling sites is summarized in Table 1. Our results indicated the presence of higher within-site diversity in the extreme western and southern parts of the enset-growing regions of the country. Partitioning the total variation demonstrated higher variation within the different sites (86%) than among

sites (14%). Principal co-ordinate analysis (PCO) showed that the north-eastern sites Answae, Seltae and Setunae were rather strongly differentiated from the other six sites (Figure 4). This variation in genetic diversity between sites could be due to climatic reasons, availability of germplasm or cultural history and the extent of dependency on enset as a food source. Genetic variability was not related to geographical distances but rather to the distribution pattern and extent of enset use in the various ethnic groups that cultivate this crop.

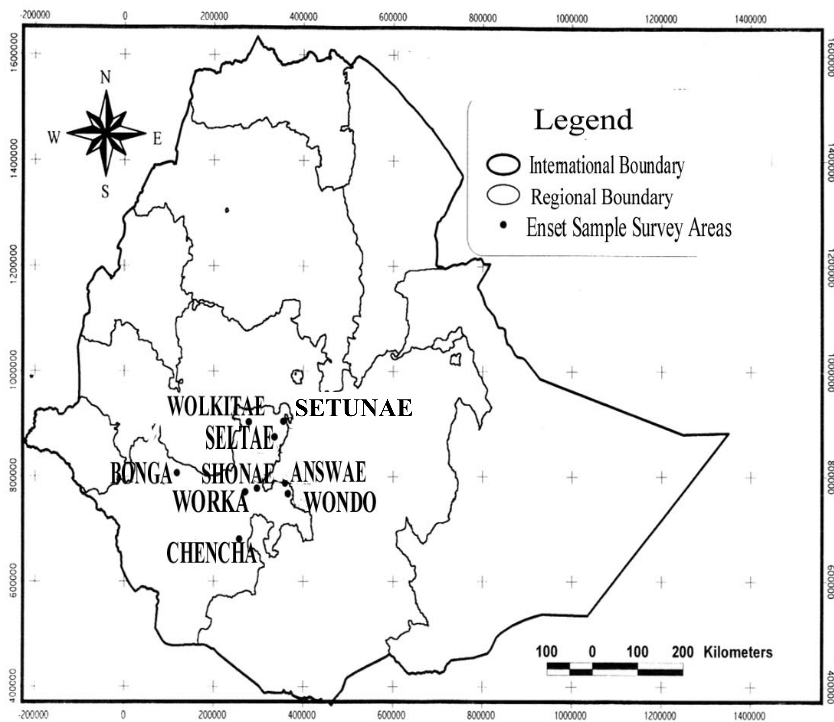

Fig. 3. The nine geographical regions in south-western Ethiopia where cultivated enset samples were obtained.

Table 1. *Collection sites for the 111 Ethiopian enset clones investigated, the province in which they are located, number of plants collected, percentage of polymorphic loci, mean Jaccard's similarity coefficient and mean Shannon-Weaver gene diversity index*

Site	Province	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	Woelayta	11	95.9	0.59	0.53

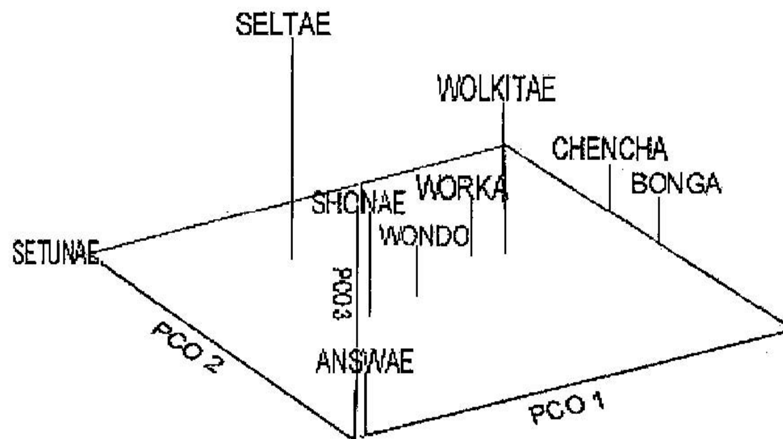

Fig. 4. Three dimensional PCO calculated from RAPD profiles in enset based on Nei's genetic distances among 9 collection sites in Ethiopia**.**

Genetic diversity in wild enset (Paper II)

RAPD markers were applied to investigate genetic diversity in wild germplasm in Ethiopia. This study was based on 48 wild enset plants from 5 different sites around the Bonga area, in the south-western part of Ethiopia. In addition, 9 representative cultivated clones and 8 *Musa*

accessions were also analysed. Genetic differentiation among the 5 wild enset populations was estimated with Analysis of Molecular Variance (AMOVA) and found to be very low in comparison with other outbreeding, perennial species (Nybom & Bartish 2000). In addition, there was no correlation between genetic and geographical distances among the 5 wild populations. This low differentiation could be attributed to the relatively small distribution area of wild enset in Ethiopia and/or the relatively short distance between the sampling sites, with the maximum distance between the sampling sites being 70 km. The within-population variation using Jaccard's similarity coefficients ranged from 0.65 to 0.69, with a mean of 0.67, and using the Shannon index from 0.58 to 0.67, with a mean of 0.63. These values were lower than the values obtained for cultivated enset (0.52–0.81 with a mean of 0.74). One reason for this low diversity in wild enset could be due to the low germination ability of enset seeds. Cluster analysis based on unweighted pair group method with artithmetic averaging (UPGMA) (Figure 5) and principal component analysis (PCA) demonstrated that the wild enset samples cluster separately from cultivated enset, suggesting that the present-day cultivated enset in Ethiopia originated from very few wild progenitors. Gene flow between cultivated and wild enset is probably very restricted. This is because enset has a mixed mode of reproduction in which the wild enset has an outbreeding system and reproduces through seeds, while cultivated enset is generally propagated vegetatively since the plant is harvested for food before seed set. Two populations located in a forest area showed somewhat higher diversity than the remaining three. This result suggests that populations with less human interference may harbour more diversity than populations close to human settlements.

Overall, the relatively high total genetic diversity observed in wild enset indicates the presence of a large gene pool which may harbour important genes that can be utilized in the improvement of enset clones. In addition, *Musa* species, as close relative of enset, may also constitute a valuable source of genes with desirable agronomic traits.

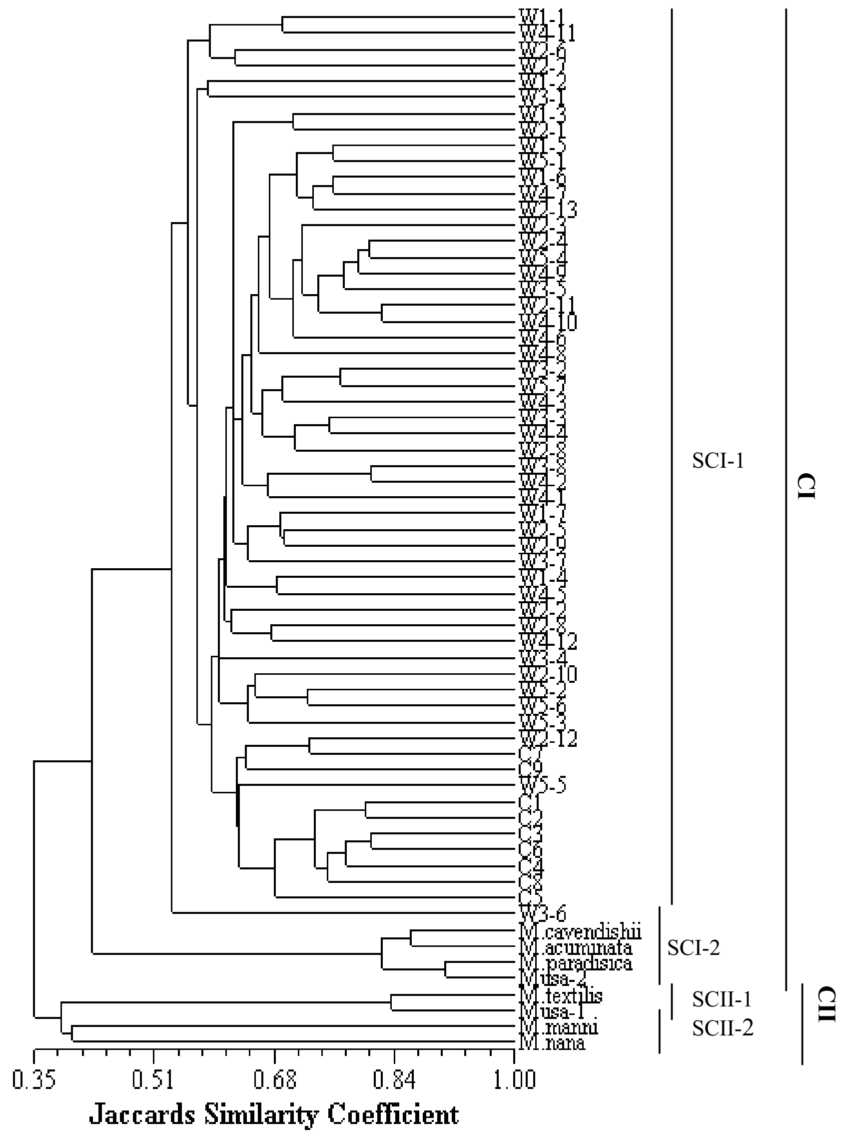

Fig. 5. Dendrogram based on UPGMA of RAPD profiles, showing phenetic relationships among 48 wild (W, numbers indicate the population and genotype, respectively) enset samples, 9 cultivated (C) enset clones, and 8 *Musa* accessions (M). Main clusters CI and CII are indicated, as well as subclusters SCI-1, SCII-1 and SCII-2.

Micropropagation of enset clones (Paper III)

Cultivated enset is generally propagated by production of suckers due to the long generation time during sexual reproduction and the recalcitrant nature of seeds to germination. Micropropagation could be highly useful for enset but has unfortunately proved to be exceptionally difficult due to phenol exudation, internal contamination and slow growth (Zeweldu & Lüdders 1998). In our study, the problem of phenol oxidation was minimized by adding activated charcoal to the medium. Prior to shoot multiplication, the cultures were screened for some common bacterial and viral diseases of enset and banana, as they often are cultivated in adjacent fields and are attacked by common pathogens. *Ralstonia solanacearum*, wilt causing bacterium, was identified from one enset clone using ELISA and this clone was discarded from further micropropagation. This bacterium was previously reported to be a pathogen of banana and was found in enset (Prior and Steva 1990), but no pathogenicity test was conducted.

In spite of the difficulty involved in enset culture, we developed an efficient micropropagation system for enset clones through meristem wounding, and modification of the MS (Murashige & Skoog 1962) nutrients according to the culture stage. The medium components, in particular the MS macronutrients, were modified based on the results of nutrient analysis of glasshouse-grown leaves from enset sprouts. In addition, three developmental stages were defined that enabled the production of 75 shoots from a single wounded explant in one subculture. The three main steps were initiation (Figure 6a), bud proliferation (Figure 6b and c) and shoot and root elongation (Figure 6d). Furthermore, wounding the meristem was found to be essential in order to obtain highly proliferating shoots. Unwounded explants produced only one or, in very rare cases, two shoots. Out of the four different media compared, two media which contained modified MS macronutrients with single or double strength micronutrients (EV-2MS), produced a higher number of propagules. Earlier, a procedure for micropropagation of enset has been reported by Negash *et al.* (2000), but only 2-3 shoots were produced from a single explant. Zeweldu & Lüdders (1998) reported their attempts to obtain shoot multiplication failed in unknown species of *Ensete* due to high oxidation of phenols which resulted in the death of explants. The procedure in our study involved direct regeneration from swollen corm with no intervening callus phase securing true-to-type propagules (Figure 6e). The different steps involved in micropropagation of enset are outlined in Figure 7.

Fig. 6. a) Initiation of enset cultures in growth cabinet, b) scanning electron micrograph of proliferating shoots on a corm piece, c) formation of multiple buds on swollen corm d) shoot elongation and rooting, e) shoot meristem originating from the internal part of a corm piece. Bars represent 1 cm in d and 0.3 mm in b, c and e.

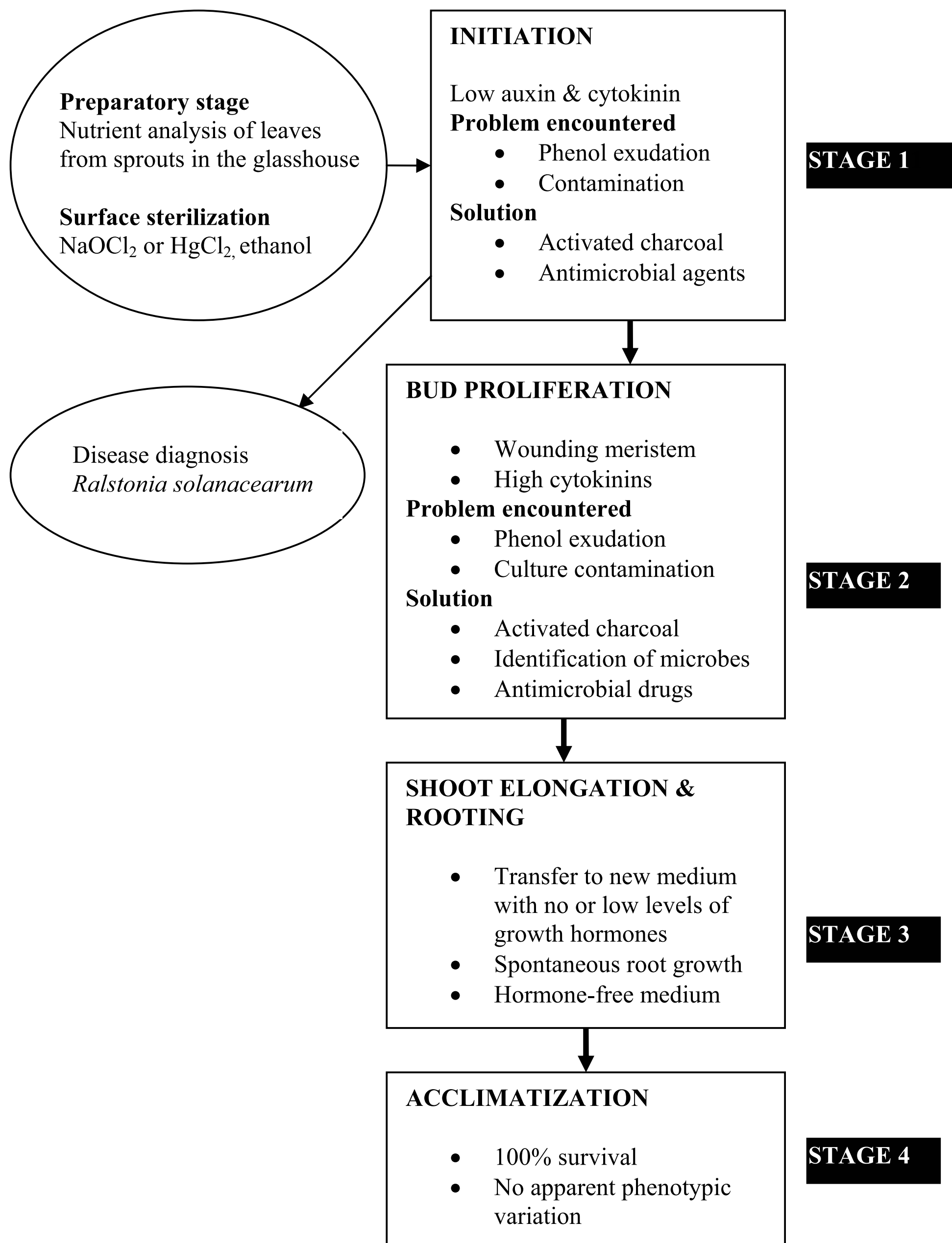

Fig. 7. The major steps and procedures followed in micropropagation of enset clones.

Identification of microbes using rDNA sequences (Paper IV)

Identification of microorganisms using modern molecular techniques such as DNA sequence information is more reliable and rapid than classification based on biochemical analyses (Sessitisch *et al.* 2002). The sequences of different regions of the genome are used for the identification of microbes. These include sequences that code for toxic or pathogenic factors, insertion sequences and conserved genes such as rRNA encoding sequences (16S, 23S and 26S). 16S rRNA sequence information has been used to identify endophytic bacteria from *in vitro* cultures of plants (Reed *et al*. 1995; Sessitisch *et al.* 2003) and from environmental and clinical isolates (Drancourt *et al*. 2000). The 26S rDNA (D1/D2 domains) has been used to identify yeasts in leaves of many plants (Inacio *et al.* 2003). The rDNA regions contain both conserved and variable regions which can be used for identification from species to kingdom level.

Bacterial and in particular, yeast contaminants, are reported to be highly destructive to plant tissue cultures (Leifert *et al.* 1989; 1990). The microflora associated with enset, in particular, endophytic microbes, is not well investigated. In this study, microorganisms that influence the micropropagation efficiency and that are also associated with field-grown enset were recorded using 16S/26S rDNA sequence analysis and comparison of the sequence identity with sequences in the database (EMBL). The microbes recorded were regarded as endophytic since they were not prevented by standard surface sterilization procedures or by addition of antimicrobial compounds in the medium. Ten bacterial and four yeast/fungal species were identified from *in vitro* enset cultures, while seven bacterial and five yeast/fungal species were identified from surface-sterilized field-grown enset clones (Tables 2 and 3). Figure 8 shows a scanning electron micrograph of some of the microbes recorded. The most frequently isolated microbes were the bacterium *Pseudomonas reactans* (6 isolates) and the yeast *Torulaspora delbrueckii* (10 isolates). Three bacterial and three fungal isolates had no close match with sequences from the data base, and may constitute new species. Their sequences have now been deposited in the EMBL database. Many of the bacterial species were gram positive (10 out of 17) and most had never been previously reported as *in vitro* contaminants of plants, which illustrates the uniqueness of the enset microflora. Many of the microbes recorded are noted to originate from humans, animals and compost (Table 2). Animal manure traditionally used as compost in enset cultivation, contains high organic matters which might have formed a suitable habitat to the growth of the various microbes. Some of the species identified in our study, such as *Klebsiella* spp., are reported to be fermentative, pathogenic and nitrogen fixers. *Leuconostoc* spp. are reported as fermentative and involved in spoilage of enset food products (Nigatu 1996). Similarly, the genera *Pseudomonas*, *Xanthomonas* and *Ralstonia* are known as regular components of microbial food spoilage (Todar 2004). *Pseudomonas* spp. are reported to be pathogenic and involved in food spoilage, while some strains are used as biocontrol agents

(Han *et al*. 1997; Gashe 1987). *Pseudomonas reactans* could also be a pathogen to enset as it is reported to cause blotch disease and discolouration in plants and mushrooms (Wells *et al.* 1996; Han *et al.* 1997), a common problem observed during storage of enset food products. Sneath *et al.* (1986) indicated that the nutrients in the plant culture media may favour microbial growth. This is also supported by our observation that organic compounds in the medium and during field-growth might encourage growth of the microbial contaminants. The frequently isolated yeast species *Torulaspora delbrueckii* and *Candida intermedia*, in this study, might also involve in the traditional fermentation of enset food products as they are reported to have such function in other crops in east Africa such as coffee (Masoud 2004). Our findings indicate the apparent endophytic and antimicrobial drug resistant nature of the contaminants and the need for a preparation stage in the glasshouse prior to *in vitro* introduction of the explants.

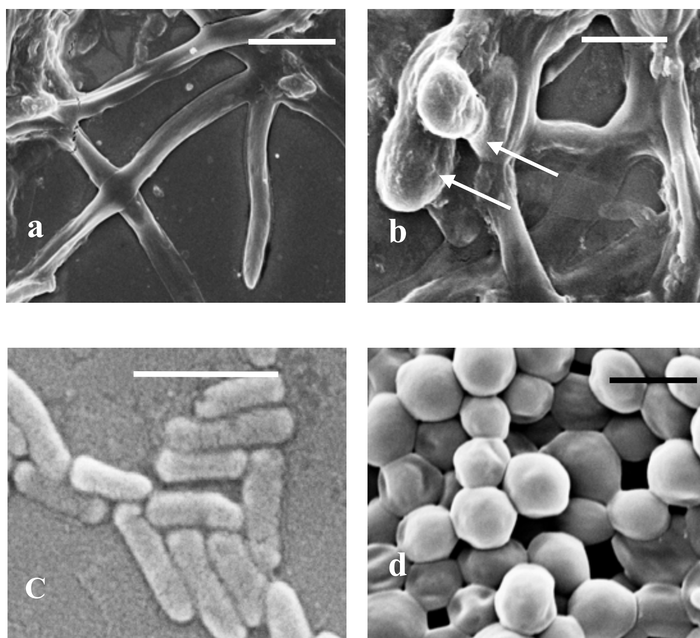

Fig. 8. Scanning electron microscope micrographs of (a) mycelia and (b) sporangium-like structures (arrows) of unknown fungus, (c) *Lactococcus lactis* bacteria and (d) *Torulospora delbrueckii* yeast. Bars represent 1µm in a, b and d and 0.5µm in c.

Table 2. *Bacterial species identified from in vitro and field-grown enset clones using 16S rDNA sequence identity, the number of isolates and known habitats*

Source	Gram reaction	Bacterial species	No/ Names of isolates	Known habitats (References)
In vitro	-	*Pseudomonas reactans*	6/GBb 7, 13, 14, 17, 20, 23	Soil, human, domestic animals, plant and mushroom pathogen (Han *et al*. 1997)
In vitro	-	*Sphingomonas* sp*.	1/GBb 6	Soil, plants, bioremediation (Pfaller *et al*. 1999)
In vitro	-	*Variovorax paradoxus*	1/GBb 2	Sewage, rhizosphere, soils (Bertrand *et al*. 2001)
In vitro	+	*Leuconostoc pseudomesenteroides*	2/GBb 1, 5	Roots, milk, fermentation, antibacterial, opportunistic pathogen (Hemme & Scheunemann 2004)
In vitro	+	*Lactococcus lactis*	1/GBb 3	Plants, opportunistic pathogen, antibacterial, drug resistant (Pytka & Bardowski 2004)
In vitro	+	*Micrococcus luteus**	1/GBb 4	Soil, plants, skin (Van den Houwe *et al.* 1998)
In vitro	+	*Microbacterium paradoxydans*	1/GBb 15	Soil, clinical (Kaul *et al*. 2004)
In vitro	+	unknown, highest identity to *Deinococcus grandis*	1/GBb 9	Soil, medical instruments (Rainey *et al*. 1997)
In vitro	+	*Kocuria palustris*	1/GBb 12	Rhizosphere, water (Burghardt *et al*. 1999)
In vitro	+	*Staphylococcus epidermidis**	1/GBb 11	Ubiquitous, milk, pathogen (Ingham *et al*. 2000)
Field	-	*Acinetobacter* sp*.	1/GBb 22	Compost, soil, water (Song *et al*. 2001)
Field	-	*Pseudomonas putida*	1/GBb 26	Soil, rhizosphere, water (Kaul *et al*. 2004)
Field	-	*Klebsiella ornithinolytica*	1/GBb 27	Environmental, antibiotic resistant (Jonas *et al*. 2004)
Field	-	*Klebsiella oxytoca*	1/GBb 24	Rhizosphere, opportunistic pathogen, resistant to cephalosporins (Jonas *et al*. 2004)
Field	+	*Microbacterium paradoxydans*	3/GBb 16, 18, 19	See above
Field	+	*Lactococcus lactis*	2/GBb 21, 29	See above
Field	+	*Leuconostoc citreum* IH22	1/GBb 28	Environment, opportunistic pathogen (Hemme & Scheunemann 2004)

Note, All enset clones were surface sterilized but only the *in vitro* isolates were treated with antibacterial\fungal chemicals. The symbol* denotes bacteria species that have been reported as contaminants of *in vitro* plant cultures in the literature

Table 3. *Yeast and fungal species recorded from in vitro and field-grown enset clones using sequence identity of D1/D2 regions of 26S rDNA*

Source	No./Names of isolates	Yeast/fungal species
In vitro	8/GBf 1, 4, 8, 9, 14, 18, 19, 24	*Torulaspora delbrueckii*
In vitro	3/GBf 2, 3, 6	*Hanseniaspora* spec.
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*

Genetic transformation of enset (Paper V)

Enset has a very long regeneration time which ranges between 9 and 14 years depending on the clone and altitude at which it is grown. Due to the long regeneration time and seed recalcitrance to germination, improvement of enset through conventional plant breeding appears to be impractical. 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 rolfisi* (Quimio & 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, we developed a transformation procedure for enset through both *Agrobacterium* and particle bombardment techniques as confirmed by *gus* gene expression. Two plasmid constructs were used for particle bombardment. The first plasmid, pC3301-1, carried the antifungal genes, β,1,3-glucanase, chitinase, the reporter gene, β-glucuronidase (*gus*), the selectable marker gene phosphinothricin-N-acetyltransferase (*bar*). The second plasmid, pC3301-2, carried all the above genes except the chitinase gene. For *Agrobacterium*-mediated transformation, the *Agrobacterium tumefaciens* strain EHA 105 harbouring pC3301-2 was used. All explant types used such as shoot tips, leaves, roots and zygotic embryos showed *gus* expression indicating that enset although a monocot, can be infected with *Agrobacterium.* The different explants expressing *GUS* expression after *Agrobacterium* infection and particle bombardment are shown in figure 9. Our procedure in particle bombardment involved sonication of

explants for 100 s, plasmolysis of tissues with 0.25 M sorbitol and mannitol each, for 2 hrs prior to transformation, and co-cultivation of infected tissues in the presence of acetosyringone (virulence inducing compound). The bombardment conditions were 3 cm target distance, 1100 psi rupture disc pressure and vacuum level at 25 inches Hg. *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 (Table 4). Particle bombardment gave a higher percentage of GUS expressing explants when sonicated leaves (60%) of clone Erba and shoot tips (49%) of clone Feresae were bombarded. Four days of co-cultivation was found to be optimum to obtain a higher percentage of GUS expressing explants with lower loss of cultures due to bacterial overgrowth as a result of long exposure time. In our experiments, shoot tips and leaf explants were the best tissues in both transformation techniques. In particle bombardment, pC3301-2, which is the smaller plasmid, showed a higher percentage of GUS expressing explants than pC3301-1. Our results indicate that the expression of *gus* gene is affected by sonication treatment, plasmid size, co-cultivation period and transformation methods. 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.

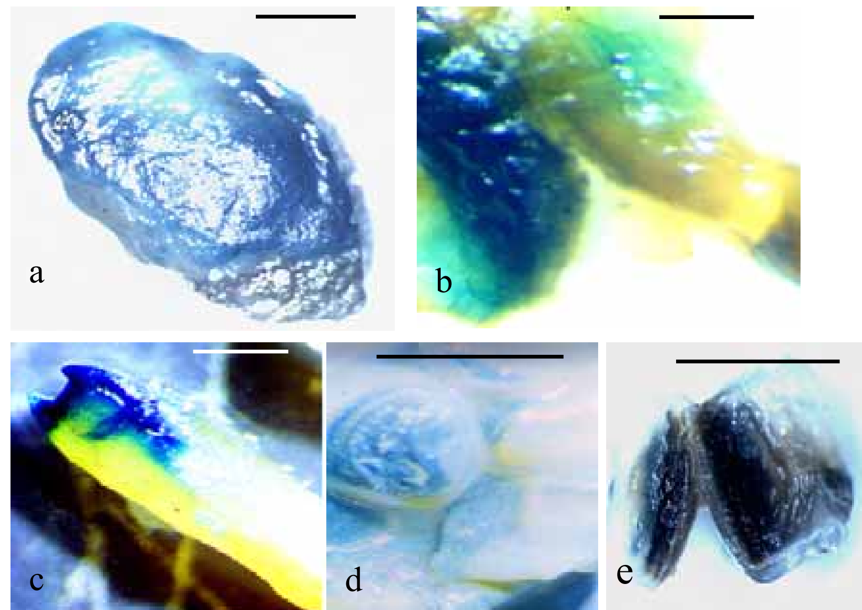

Fig. 9. Transient GUS expression detected after *Agrobacterium* and particle bombardment transformation experiments in the different *Ensete ventricosum* tissues. Fig a shoot tip, b leaves, c root piece, d zygotic embryo and e transverse section of zygotic embryo. Fig a, c, d and e after *Agrobacterium* infection and, b after particle bombardment. Bars denote 1 cm.

Table 4. *Summary of GUS expression in different explants after particle bombardment and Agrobacterium-mediated transformation, assayed after 4 days. In particle bombardment, plasmids pC3301-1 and pC3301-2 were used while for Agrobacterium transformation, A. tumefaciens strain EHA105 carrying plasmid pC3301-2 was used. The symbols-represent tissue types and treatments not performed.*

Factors	Clone	Percentage of GUS positive explants (No. of explants per treatment)			
		Shoot tips	Roots	Leaves	Zygotic embryos
Transformation methods					
Agrobacterium					
Plasmid (pC3301-2)					
Sonicated	Feresae	18±3.9 (50)	30±10.0 (30)	-	-
Non-sonicated	Feresae	57±11.1 (50)	23±5.8 (30)	-	-
Sonicated	Erba	63±5.8 (30)	-	50±10.0 (30)	12±2.9 (50)
Non-sonicated	Erba	46±5.8 (30)	-	0±0.0 (30)	13.±2.9 (50)
Particle bombardment					
Plasmid (pC3301-2)	-	-	-	-	-
Sonicated	Feresae	49±14.6 (25)	20±20.0 (15)	-	-
Non-sonicated	Feresae	29±15.2 (25)	47±11.6 (15)	-	-
Sonicated	Erba	40±20.0 (15)	-	60±20.0 (15)	12±2.9 (50)
Non-sonicated	Erba	40±20.0 (15)	-	40±20.0 (15)	10± 5.0 (50)
Plasmid (pC3301-1)					
Sonicated	Feresae	36±13.0 (25)	0±0.0 (15)	-	-
Non-sonicated	Feresae	24±12.6 (25)	0±0.0 (15)	-	-
Sonicated	Erba	0±0.0 (15)	-	20±20.0 (15)	5±5.0 (50)
Non-sonicated	Erba	20±20.0 (15)	-	40±20.0 (15)	10±5.0 (50)

Concluding remarks and future prospects

Population growth and limited access to arable land, in particular, in developing countries, make it necessary to maintain the existing crop germplasm and increase the productivity of important crops. Enset is one of the major crops of Ethiopia but it has not yet benefited from the advances in biotechnological research. In the present study, the genetic diversity of cultivated enset and wild enset was documented. A micropropagation procedure for the introduction of 'disease-free' planting materials to other regions was developed and a transformation procedure that could be utilized for the improvement of important clones was devised. Microbes associated with enset plant were identified. The outcomes of these investigations will contribute towards improving the livelihood of the peasant household, ensure the sustainability of the crop and benefit the country at large. Future research efforts should be directed to the following areas.

1) Wide-scale efforts on natural resource preservation and maintenance of the existing domesticated and wild enset germplasm in Ethiopia.

2) Investigation on seed germination for *in situ* or *ex situ* conservation purposes, development of early maturing enset clones in particular for the improvement of the crop through conventional breeding techniques.

3) Use of modern biotechnology to boost productivity of enset and maintain its sustainability.

4) Increasing the content of proteins and vitamin A in the plant to ensure the livelihood and health of peasant households.

5) Investigation of enset bacterial diseases and food spoilage microbes, pathogenicity testing of some of the microbes recorded in this study and other putative diseases, creating awareness among farmers on the diseases of the crop.

6) Preservation of the important indigenous production system of enset such as use of animal manure as compost.

7) Development of mechanical devices for processing of enset foods and making the devices available to the end users.

8) Investigations on the potential of enset leaves as a component of silage and livestock feed.

9) Further investigations on the potential of enset as medicinal plant, fibre, starch and ornamental.

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