Micropropagation, Transformation and Genetic Diversity of *Hagenia abyssinica* (Bruce) J.F. Gmel.

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Abstract

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The tree species *Hagenia abyssinica* belongs to a monotypic genus in the family Rosaceae. It grows on the highlands of tropical Africa, where it is seriously endangered principally due to high timber demands. Therefore, the establishment of fast and efficient biotechnological methods to propagate and improve different traits of interest is much needed. The objectives of this study were to establish micropropagation and *in vitro* regeneration protocols and to transform this species with the *rolB* gene to improve the rooting efficiency. In addition, a study of genetic diversity was needed to provide information for *in situ* and *ex situ* conservation.

A micropropagation protocol has been developed using explants from both juvenile and mature tree sources. These explants were initiated, multiplied and rooted using either WPM or MS medium and different combinations of BAP and IBA. Using leaves from *in vitro* grown shoots of juvenile or mature tree origin, an *in vitro* regeneration protocol has been developed based on using TDZ alone or in combination with NAA or 2,4-D. Regeneration efficiency was also studied using TDZ or BAP in combination with IAA. The effects of wounding, kanamycin and cefotaxime on regeneration have been studied. TDZ was found to be the best cytokinin for regeneration of *H. abyssinica* at low concentrations since it promotes direct shoot regeneration, as well as at high concentrations since it promotes callus induction and shoot regeneration although it inhibits shoot elongation. All auxins appeared to be toxic to *H. abyssinica,* particularly at higher concentrations. Based on the results of these regeneration experiments, *H. abyssinica* was transformed with the *rolB* gene. One clone of the transformants exhibited 77% rooting while no roots were produced by any of the untransformed control shoots. Kanamycin was found to be the main factor inhibiting the recovery of transformants whereas cefotaxime is toxic only at high concentrations. Since somaclonal variation of micropropagated plants is highly undesirable, genetic stability was tested on 80 micropropagated plants, 40 of axillary origin and 40 from adventitious shoots. These plants had been grown *in vitro* for over two years when they were screened with randomly amplified polymorphic DNA (RAPD) markers. Only one plant, originating from an axillary shoot, deviated by showing two extra bands thus indicating that *H. abyssinica* is relatively stable *in vitro*. A genetic diversity study based on inter simple sequence repeats (ISSR) was conducted on a total of 120 samples representing 12 populations from different parts of Ethiopia. This study showed that within- and between-population variability in *H. abyssinica* is typical of an outcrossed, perennial, late-successional species. Two recently planted populations and one indigenous primary forest-population showed the highest diversity. An autocorrelation analysis between genetic and geographic distances indicated that gene flow takes place over only about 30 km and this is likely to decrease even further because of genetic isolation among populations brought about by the current logging practices.

Key words: Hagenia, *in vitro* regeneration, inter simple sequence repeat (ISSR), micropropagation, *rolB* gene, rooting, transformation

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To Genet and Abenezer

"To every *thing there is* a season, and a time to every purpose under the heaven"
Ecclesiastes 3:1

Contents

Appendix

Papers I-V

This thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Feyissa, T., Welander, M. & Negash, L. 2005. Micropropagation of *Hagenia abyssinica*: a multipurpose tree. *Plant Cell ,Tissue and Organ Culture* 80, 119-127.

II. Feyissa, T., Welander, M. & Negash, L. 2005. *In vitro* regeneration of *Hagenia abyssinica* (Bruce) J.F. Gmel. (Rosaceae) from leaf explants. *Plant Cell Reports* 24, 392-400

III. Feyissa, T., Zhu, L.H., Negash, L. & Welander, M. Regeneration and genetic transformation of *Hagenia abyssinica* (Bruce) J.F. Gmel. (Rosaceae) with *rolB* gene. (Submitted).

IV. Feyissa, T., Welander, M. & Negash, L. Genetic stability, *ex vitro* rooting and gene expression studies in *Hagenia abyssinica*. (Submitted).

V. Feyissa, T., Nybom, H., Bartish, I. V. & Welander, M. Analysis of genetic diversity in the endangered tropical tree species *Hagenia abyssinica* using ISSR markers. (Submitted).

Introduction

Background

Forests are very important to the world economy as well as for the preservation of global ecosystems. Consequently propagation and genetic improvement programmes directed to the conservation and development of forests are extremely important (Tzfira, Zuker & Altman, 1998). Commercially, forest trees have a wide range of uses. The ever increasing global demands for wood and wood products, combined with the zooming human population have increased the pressure on existing forests. In developing countries, this scenario has resulted in a progressive deterioration of natural forests and land degradation, leading to unsustainable agriculture systems.

Ethiopia is a developing country with a total area of 1.13 million sq km located between the geographic coordinates 3° and 15° N, and 33° and 48° E. The altitude ranges from 125 m below sea level at the Danakil depression up to 4620 m at Mt. Ras Dejen. The landscape is characterized by plateaus, mountains, hills, deep gorges, rivers, incised valleys, undulating plains and sharp cliffs. The world's longest rift valley, about 6000 km, extends from Syria in the north passing through the Red Sea to Mozambique in the south, thus effectively dividing the country into two halves. Agriculture is the backbone of Ethiopian economy; about 85% of the nearly 77 million inhabitants depend on agriculture for their livelihood.

One of the most important reasons why agriculture has become unsustainable and unproductive in Ethiopia and other East African countries is the extensive deforestation of huge expanses of mountainous regions (Negash, 1995). Due to rapid population growth, forests have been cleared in the last century mainly to obtain land for agriculture and grazing as well as to be used for fuel and construction. The total forest coverage of Ethiopia had decreased from the original 35% to 16% by 1952, to 3.6% by 1980, to 2.7% by 1987, and to about 2.4% by 1990 (Rodgers, 1992) and is still declining. Lack of an effective reforestation programme and sufficient efforts to maintain the vegetation cover are among the reasons for the deforestation in Ethiopia (Taddese, 2001). At the end of 19th century, efforts were made to satisfy the fuel and construction needs of the nation by importing *Eucalyptus* from Australia. A large area has been planted with this tree species since it grows very fast and many people have become dependent on it for energy and construction. However, recently the plantation of exotic trees in general and *Eucalyptus* trees in particular has raised the question of ecological risks. The research findings with respect to plantation of these trees in Ethiopia are inconsistent and sometimes controversial. Lemenih, Olsson & Karltun (2004) reported that the soil fertility under *Eucalyptus* forest is even poorer than the soils subjected to continuous farming. Currently, the country suffers an environmental crisis due mainly to deforestation and overgrazing which has resulted in soil erosion and desertification. Forest plantations with exotic trees can be useful for restoring the soil on degraded farmlands, but the extent, rate and direction of changes in soil attributes depend on the particular tree species used (Lemenih, Olsson & Karltun, 2004). Therefore, when selecting exotic species for

afforestation or reforestation of degraded farmlands long-term effects on soil properties should be considered. By contrast, indigenous trees are well adapted to the soil and climate in the country, and are therefore the best candidates for afforestation and reforestation, though some of them are slow growing. *H. abyssinica* is one of the many indigenous trees of Ethiopia that can be used for reforestation purposes.

Hagenia abyssinica: description, ecology and distribution

Hagenia abyssinica (Bruce) J.F. Gmel. belongs to a monotypic genus in the family Rosaceae. It is commonly known as *kosso* in Ethiopia. Taxonomically, it has also been treated as *Brayera anthelmintica* Kunth and *Banksia abyssinica* 'Bruce' ex Steud. Although Lemordant (1972) claims that Godinho, a Portuguese priest, described the use of this plant as a vermifuge in Ethiopia in 1645, it was James Bruce in 1790 who described the species scientifically and directed the attention of Europeans to this remarkable tree (Bruce, 1790 in Abegaz *et al.*, 1999).

The species was once abundant in the semi-humid mountain woodlands of Ethiopia within an altitudinal range of 2450 to 3250 m.a.s.l. (Hedberg, 1989). Friis (1992) reports a wider altitudinal range: 1850 to 3700 m. At present the tree is sparsely distributed in mountainous central, central-west and southeastern parts of Ethiopia (Negash, 1995). The species is also found in Kenya, Tanzania, Uganda, Sudan, Democratic Republic of Congo, Malawi, Burundi and Rwanda. This tree is characterised as a typical example of Afromontane endemism since it is confined to the mountains of tropical Africa with rainfall ranges between 1000 and 1500 mm/year (Friis, 1992).

The species grows in undifferentiated Afromontane forest mixed with *Podocarpus* or *Juniperus-Podocarpus* forest. It also occurs as dry single-dominant Afromontane forest or grows in forests dominated by *Juniperus*. It is often associated with *Schefflera abyssinica*, *Schefflera volkensii*, *Galiniera saxifraga*, *Rapanea melanophloëos*, and/or with the mountain bamboo *Arundinaria alpina*. Sometimes it is deliberately left as an isolated tree in farmland or derived grassland and occasionally planted by local people (Friis, 1992).

It is a dioecious species with separate male and female trees that are distinguished with certainty only after flowering. The height reaches up to 20 m with short trunk and thick branches. The bark is usually red-brown, flanking raggedly. The leaves are pinnately compound with 11 or 13 leaflets, measuring up to 40 cm in length. Leaflets are sessile or subsessile, opposite or subopposite, narrowly lanceolate, pubescent above, and densely silvery villous beneath. Rachis usually has small orbicular secondary leaflets, 2-10 cm long, between the main ones. Petioles measure about 13 cm long and winged with adnate stipules for almost their whole length (Hedberg, 1989; Amin, 1990). The individual flowers of the female tree are small and inconspicuous. They do, however, form bright and reddish inflorescences unlike other wind-pollinated flowers which are usually dull in colour (Negash, 1995). The inflorescences are conspicuous and decorative, and can be seen from a distance when they are in full bloom since they reach up to half a meter or more in length. They have branched drooping terminal panicles with 2-

3 rounded bracts. Sepals are eight or ten in two series. Flowers are red with a diameter of up to 1.75 cm. The outer sepal lobes are unequal, oblong, linear, inner lobes smaller, ovate; stigmas captitate; petals 0, 4 or 5 (Dale & Greenway, 1961). The male fluorescence is relatively smaller in size and dull-cream in colour as compared to female (Fig. 1a, b and c). Stamens are eight to ten or more. The larger size of female inflorescence is advantageous for receiving wind-born pollen grains from male inflorescence. The female and male flowers produce abundant nectar and pollen respectively (Negash, 1995).

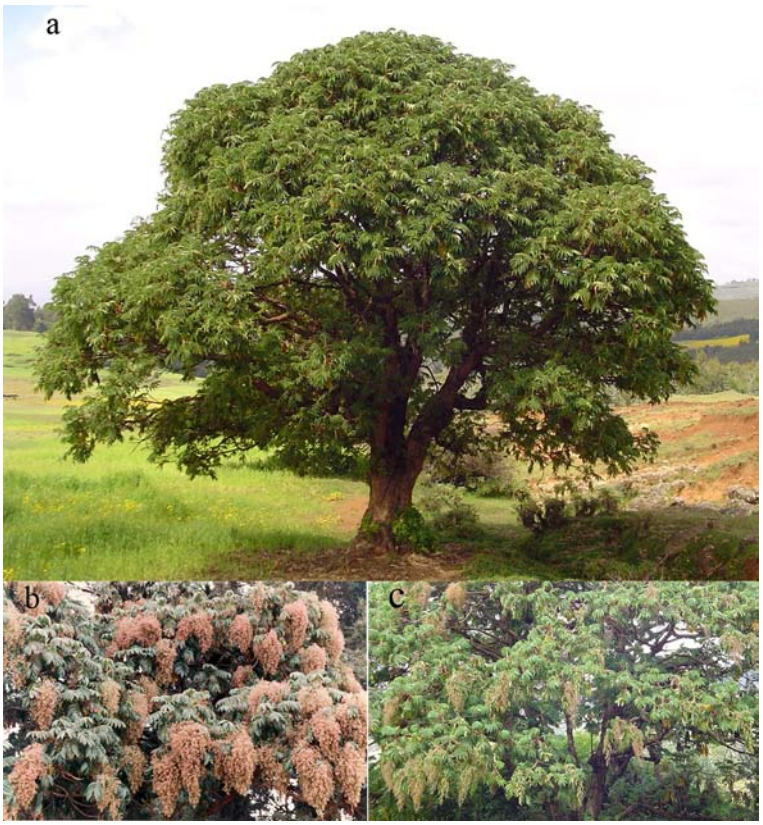

Fig. 1. *Hagenia abyssinica* tree (a); Inflorescences of female tree (b) and male tree (c).

Pollination is reported to be effected by wind (Negash, 1995). Seed germination is very poor, and many fruits do not contain any seeds at all (Feyissa, unpubl. results). Possibly the lack of viable seeds is, at least in part, due to insufficient pollination, caused by long distances between male and female trees. To achieve successful wind pollination in the tropics, a large number of male and female individuals must grow close to one another. This is because nearly all wind-borne pollen grains fall within a hundred meters of the parent plant and the large amount of pollen that are blown by the wind reach the vicinity of the ovules only by chance (Raven, Evert & Eichhorn, 2005). Hence, if individual *H. abyssinica* plants are widely scattered, the chances that a pollen grain will reach a receptive stigma

are very low. As a result, the success of good quality seed production in this species is rather limited.

Significance and uses of *Hagenia abyssinica*

Every part of this tree is used for different purposes such as medicine, timber, firewood, poles, mulch, green manure, and as an ornamental plant. In Ethiopia, the traditional consumption of raw meat commonly leads to contraction of tape worm (*Taenia saginata* Goeze) which uses cattle as an intermediate host and encysts there. Therefore, the dried female inflorescence has been widely used for its potent anti-tapeworm activity. The dosage varies depending on the genotype of the female tree, and age and sensitivity of the patient. For an adult patient it ranges from 8 to 16 g (Negash, 1995). In North Africa and Near East, *H. abyssinica* has long been used as an anthelmintic drug under the name 'kousso', 'kosso' or 'kusso'. In the 19th century, it was included in most European pharmacopoeias as an effective drug against intestinal worms, making it one of the most famous African plants at that time (Fluckiger & Buri, 1874; Leichsenring, 1894; Lounasmaa, Widen & Huhtikangas, 1973). Johnston (1844) briefly described the ecology, morphology and uses of this tree and appreciated its appearance and beauty. According to his opinion it would grow very well in England. However, he was apprehensive about the consequences of the toxic effects of this plant to be used as a remedy.

High quality timber is produced from *H. abyssinica* for making furniture, cabinets, floors, veneer, and truck body construction. As the tree is characterized by lots of branches and a short bole, several trees have to be cut down in order to provide the required amount of timber. This tree species has also been used in traditional apiculture where peasants use the branches of the tree for keeping and stabilizing their beehives. Although it is easy to split the wood of this tree into pieces, it has to dry while the tree is still standing, either through girdling or through natural death to be used for fuel after some years. The wood is fine-grained, fairly hard and heavy. It appears to have a high calorific value and is fragrant during burning (Negash, 1995).

As *H. abyssinica* is one of the species that constituted the natural forests of mountainous regions, it has extremely valuable biological attributes in fertile soil formation, soil conservation, as well as rainwater conservation. The large number of branchlets and leaves allows the tree to intercept and reduce the kinetic energy of a stormy rainfall, thus protecting against soil erosion. It also helps to conserve water within the aquifer system of the underlying geological formations. The leaf production, senescence, abscission and decomposition rates are fast so that the species can produce a great amount of litter per unit time, thus making it an effective nutrient pump (Negash, 1995).

Research on *Hagenia abyssinica*

Despite its economic and ecological importance, only a few studies have been carried out on *H. abyssinica* and almost all research has focused on its medicinal

properties. The first crystalline substances, called Kosins, were produced from female inflorescence in 1870 by Merck of Germany (Abegaz *et al.*, 1999). Subsequently the interest for kosso increased and the species became incorporated into the European Pharmacopoeia. Fluckiger & Buri (1874) and Leichsenring (1894) isolated the toxic substance in kosso and investigated its effectiveness against tapeworm. Studies on the structure of the chemical compounds responsible for medical and toxic effects, namely kosotoxin, protokosin and α- and β-kosins have continued but interest has decreased over the years. The currently accepted molecular structures were reported by a Finnish research group (Lounasmaa, Widen & Huhtikangas, 1973, 1974). According to these reports, kosso flowers (*Flos koso*) contain phloroglucinol derivatives similar to those in *Dryopteris* ferns. Some effects of *H. abyssinica* against *Moniezia* spp. in sheep have also been reported (Mesfin & Obsa, 1994). However, no effects were observed on nematode (*Haemonchus contortus*) egg counts in lambs after treatment with preparations from female inflorescences of *H. abyssinica* (Githiori *et al.*, 2004).

Very few studies have been performed on propagation and genetics of *H. abyssinica*. Negash (1995) studied seed germination under different treatment conditions and suggested two alternative propagation methods; (1) germinating seeds on Petri dishes and (2) sowing directly on seedbeds, and transplanting the seedlings. In the shade, seedlings of *H. abyssinica* have the highest growth rate but the lowest survival rate when compared with three other indigenous trees, *Olea europa* subspecies *cuspidata*, *Podocarpus falcatus* and *Juniperus procera* (Fetene & Feleke, 2001). Wubet *et al.* (2003) reported of the mycorrhizal status in several indigenous tree species of Ethiopia and found that *H. abyssinica* trees are dominated by *Arum*-type arbuscular mycorrhizas.

So far, there are no major publications on the genetics of this species although there are few preliminary studies showing *e.g.* that *H. abyssinica* has 40 chromosomes (Hailu, 1998). Further studies are, however, needed to confirm the chromosome number and to determine the ploidy level. Some topics that need to be investigated concern *e.g.* the amount and distribution of genetic diversity, development of sex-specific molecular markers, genome mapping, and information about genes responsible for specific traits of importance for the conservation and improvement of this species.

In line with maintaining and increasing the forest cover for sustainable agriculture of the country and protection of the ecosystem, it is very important to establish sustainable commercial tree propagation and planting in order to provide forest products to meet the demand of the nation. Instead of destroying the naturally occurring forests and exacerbating the loss of genetic diversity of forest species, it is very important to select elite genotypes for propagation, thus maintaining useful characters consistently and/or improving the traits of interest. However, tree improvement by conventional breeding is a slow process because of a long juvenile period and high heterozygosity (Singh *et al.*, 2002). Therefore, modern technologies such as tissue culture and genetic transformation can be used as an alternative or complementary to traditional breeding. The potential of biotechnology for overcoming the limitations of conventional breeding and for accelerating forest-tree-breeding programs can be realized at several levels (Tzfira,

Zuker & Altman, 1998): (1) clonal propagation of superior genotypes using tissue culture techniques; (2) somatic cell techniques such as somatic hybridisation using protoplasts, haploidisation and exploitation of somaclonal variations; (3) the use of induced mutations; (4) molecular breeding to complement traditional breeding such as marker-assisted selections; (5) direct rapid introduction of specific traits via genetic engineering of forest tree-species.

Plant tissue culture

Plant tissue culture is the science or art of growing plant cells, tissues or organs on artificial media by isolating them from the mother plant (George, 1993). Historically, the science of tissue culture development is linked to the discovery of cell and subsequent cell theory, which states that the cell is the basic structural unit of all living things. Plant tissue culture is based on the cell doctrine that states a cell is capable of autonomy and is potentially totipotent. In 1902, the German botanist Gottlieb Haberlandt developed the concept of *in vitro* cell culture. He isolated single cells from palisade tissue of leaves, pith parenchyma, epidermis and epidermal hair of various plants and cultured on Knop's salt solution containing glucose and peptone. In his cultures, cells that synthesized starch and increased in size survived for several weeks though none of them divided. He predicted the requirements for cell division under experimental conditions that have been proved through time. Therefore, Haberlandt is considered as the father of plant tissue culture. Following Haberlandt, many workers continued working on plant tissue cultures. In 1939, Gautheret cultivated cambial tissues of carrot root, Nobecourt (carrot), and White (tobacco) for prolonged periods of time. In strict sense, these were the first true plant tissue cultures (Chawla, 2002).

By using plant tissue culture techniques, complete new plants can be obtained from different explants through direct or indirect morphogenesis and through somatic embryogenesis. Direct morphogenesis is the production of shoots from explants without passing through callus (unorganised tissue) phase while indirect morphogenesis refers to induction of shoots through callus phase. The culture in which an organised form of growth can be continuously obtained is referred to as organ culture (George, 1993). The most important kinds of organ cultures used for micropropagation are meristem cultures, shoot cultures, embryo cultures and isolated root cultures. Callus cultures, suspension or cell cultures, protoplast cultures or anther cultures are grouped as unorganised tissue cultures.

Although the basic nutritional requirements of *in vitro* cultured plant cells are very similar to those utilized by plants, the nutritional composition varies depending on the type of cells, tissues, organs, protoplasts and the plant species. There is also a difference in nutritional requirement among genotypes or cultivars of the same species. A nutrient medium is defined by its composition of mineral salts, carbon source, vitamins, plant growth regulators and other organic supplements. A particular medium is identified by its salt composition unless otherwise specified. Other additives such as amino acids, vitamins, growth regulators or organic supplements can be added in any concentrations to a given salt composition to get the desired results. Although several media have been

developed, the MS medium of Murashige & Skoog (1962) is very widely used in different plant tissue culture systems. Organic additives and the type and concentrations of growth regulators that are added to the basic media are generally considered to be the most important factors that affect the response of plant tissues in culture. However, other additives such as activated charcoal and polyamines and exogenous factors such as temperature, light intensity and quality, are important in determining the responses of some tissues in culture (Mathias & Boyd, 1986). If cultures are maintained for long time, abnormal phenotypes could be observed. Most of those changes are physiological and temporary, but some are genetic and affect the manner in which the plants grow, flower and fruit so that changing the economic and aesthetic value (George, 1996). Therefore, due to these changes it is important to carefully evaluate the performance of tissue cultured plants as they grow to maturity in greenhouse, garden or field.

Plant cell and tissue culture are used for clonal propagation, production of disease-free plants, haploid production, triploid production, *in vitro* pollination and fertilization, embryo rescue, somatic hybridization and cybridization, somaclonal and gametoclonal variant selection, germplasm conservation, secondary metabolite production, and genetic transformation.

Although living cells are considered potentially totipotent, only some cells that are competent divide and give rise to complete plant in tissue culture. Furthermore, not all plant species are equally amenable to tissue culture. Although production or improvement of perennial plants, both woody and herbaceous, using tissue culture especially for cloning and genetic engineering seem very attractive, the complex seasonal cycles and life cycles of those plants complicate the control of their growth in tissue culture (McCown, 2000). The early establishment of shoot cultures for these perennial plants is one of the important approaches. Stabilized shoot cultures are excellent sources of cells, tissues and organs that can be used in further complex procedures such as protoplast generation (McCown, 1988; Russell, 1993), gene insertion and transclone recovery. If shoot cultures cannot be readily established, these advantages cannot be realized. Therefore, a major cause of recalcitrance in perennial plants is the inability to establish fully stabilized shoot cultures. Establishing shoot culture involves multiple steps. Murashige (1974) generalized these steps into three stages; aseptic culture establishment, multiplication of propagula and preparation for reestablishment of plants in soil. Similarly, Debergh & Maene (1981) generalized into four stages. Isolation stage that involves decontamination and growth of preformed buds, stabilization stage that involves uniform and continuous shoot growth, optimization stage involving refining medium and environmental conditions, and acclimation/rooting of the micropropagules. It is difficult to generate stabilized shoot cultures for plants that have seasonal growth dynamics dominated by strong episodic or determinant shoot growth. The relatively slow growth rate of perennials in culture also complicates the tissue culture procedures as many perennial tissues release high content of phenolic compounds into a culture medium. In some cases, some technical approaches can overcome those limitations in tissue culture. However, development of a deeper understanding of physiological bases of such genetically predetermined phenomena is important (McCown, 2000).

Genetic transformation

Plants are genetically engineered by introducing a gene(s) into plant cells that are growing *in vitro* or *ex vitro*. The production of transgenic plants is based on the stable insertion of foreign DNA into the plant genome, regeneration of these transformants to produce the whole plant and expression of the introduced gene(s). *Agrobacterium*-mediated transformation has provided a reliable means of producing transgenics in a wide variety of species that can be cultured and regenerated *in vitro*. Recently, some plants such as *Arabidopsis thaliana* have also been transformed by *Agrobacterium*-mediated transformation by dipping the young buds of flowers of *ex vitro* growing plants (Rakoczy-Trojanowska, 2002). This method is known as infiltration or, in general, *in planta* transformation. Other methods of gene-transfer systems include particle gun bombardment, electroporation and membrane permeabilization using chemicals. Of these, particle gun bombardment has proved to be successful with plants that are less sensitive to *Agrobacterium* infection, such as cereals and legumes (Walden & Wingender, 1995). However, recently, *Agrobacterium*-mediated transformation has become the method of choice for these plants (Nadolska-Orcyzyk, Orczyk & Przetakiewicz, 2000). The development and optimization of several regeneration protocols, efficient vector constructs and availability of defined selectable marker genes and different methods of transformation have resulted in the production of transgenic plants in more than 100 species (Babu *et al.*, 2003; Wimmer, 2003). These transgenic plants include many important crops, fruits and forest plants. The plant transformation technology is not only used to improve plants but also a versatile platform for studying gene function in plants. Plant genetic transformation technology has a great potential in increasing productivity through enhancing resistance to diseases, pests and environmental stresses and by qualitative changes such as chemical composition of the plant. Plants can also be used for high volume production of pharmaceuticals, nutraceuticals and other beneficial chemicals. Transgenic plants might be used as drug delivery devices, with vaccines being synthesised in plants (Hansen & Wright, 1999). Many plant species previously considered to be recalcitrant to transformation, with advances in tissue culture combined with improvements in transformation technology, have now been transformed.

Agrobacterium tumefaciens and *Agrobacterium rhizogenes* induce crown gall tumour and hairy root disease at wound sites on dicotyledonous plants respectively. *A. tumefaciens* causes the crown gall tumour by transfer of a part of the tumour-inducing (Ti) plasmid DNA, T-DNA (transferred DNA), into the plant genome while *A. rhizogenes* causes the hairy root disease by transferring T-DNA region(s) of the root inducing (Ri) plasmid. Most studies of the mechanism by which T-DNA is transferred from *Agrobacterium* to plant cell have been carried out with *A. tumefaciens*. The T-DNA region is flanked by 25 bp imperfect repeats termed the right (RB) and left (LB) borders (Hellens *et al.*, 2000). Genes in T-DNA are responsible for the neoplastic properties of the transformed cells and for the synthesis of opines, which can serve as carbon and nitrogen sources for the bacteria. These bacteria respond to certain plant phenolic compounds that act to induce the activity of the virulence (*vir*) genes that are encoded on the Ti plasmid

outside of T-DNA region. By removing tumour-inducing genes in T-DNA and inserting the gene of interest, the Ti plasmids are used to deliver and insert this gene into the genome of susceptible plant cell. This modified T-DNA has been cloned into a plasmid that can exist stably in *Escherichia coli* or *A. tumefaciens*. The latter is used as a delivery system to transfer this T-DNA into plant cells. In the binary vector system, the shuttle vector with the gene of interest is introduced into an *A. tumefaciens* strain that carries a compatible plasmid with *vir* genes that are essential for transferring a T-DNA region into a plant cell. In a cointegrate vector system, the shuttle vector is introduced into *A. tumefaciens* and recombines with the *vir* gene containing, disarmed Ti plasmid to give a single plasmid that has both functions (Glick & Pasternak, 1998). The developments of binary Ti vectors and a range of *Agrobacterium* strains are the key advances that have made *Agrobacterium* transformation the method of choice (Hellens & Mullineaux, 2000).

Different kinds of explants and tissue culture techniques can be used in the production of transgenic plants. The potential totipotency, and *in vitro* competence of some plant cells paved the way in making these cells the target for transformation as well as to be cultured *in vitro*. Since different explants and genotypes differ in their response to transformation and regeneration, there is no universally applicable method of culture for all species (Walden & Wingender, 1995). For every explant of each genotype there should be reliable culture and regeneration protocol to perform transformation. Each of these steps usually requires separate media. To avoid the formation of chimeras during transformation, it is preferable to use the culture technique that involves callus production. In several cases, the lack of efficient and reliable regeneration systems is a major draw back preventing the development of gene transfer technologies for perennial plants (Yepes & Aldwinckle, 1994). Increasing the regeneration efficiency and development of an effective system for gene transfer that permits efficient DNA delivery, selection of transformants, and recovery of transgenic plants are critical factors during transformation experiments. Plants have been transformed with several genes conveying traits of interest to them among which the root locus (*rol*) genes are the one. Four *rol* genes referred as *rolA*, *rolB*, *rolC*, and *rolD* have been identified in the TL-DNA of the Ri plasmid, of which the *rolB* gene is the most effective in promoting rooting in different plant species (White *et al.*, 1985; Spena *et al.*, 1987; Capone *et al.*, 1989).

Future developments in transformation

The increasing public and scientific concern regarding the proliferation of antibiotic and herbicide resistance genes in the environment, especially those used only as selectable marker in plant transformation, led to the development of strategies for the regeneration of selectable marker free transgenics. Furthermore, the presence of selectable marker makes it difficult to transform the plant with new genes since the same marker cannot be used more than once. Different techniques are available for removal of these genes especially when using *Agrobacterium*-mediated transformation (Glick & Pasternak, 1998; Mattew *et al.*, 2001). These techniques include co-transformation of plants with two separate DNAs, one

carrying the marker gene and the other carrying the gene of interest. Using traditional breeding methods the two genes are separated by chromosome segregation during a few rounds of crossings. Using plant transposable elements is also another alternative. These methods are time consuming to apply in slow growing trees. Recently, *dao1* gene encoding D-amino acid oxidase (DAAO) could also be used for either positive or negative selection, depending on the substrate. For positive selection, D-alanine and D-serine are used. These amino acids are toxic to plants, but are metabolised by DAAO into non-toxic products. For negative selection, D-isoleucine and D-valine are used which have low toxicity, but are metabolised by DAAO into toxic products (Pilone, 2000; Erikson, Hertzberg & Näsholm, 2004). These progresses show the high potential of producing marker-free transgenic plants in the future.

Genetic diversity

Selection, domestication and cultivation of plants is based on the genetic diversity of the species. Therefore, information on distribution, preservation, variation and relations is extremely important for several purposes. Studies of genetic diversity in plants have greatly enhanced our understanding of modes of speciation, adaptation, and population dynamics. Such studies have important applications in *in situ* and *ex situ* conservation strategies as well as in plant breeding (Bussell, 1999; Nybom & Bartish, 2000). Genetic diversity of a species depends on factors like phyletic group, life form, geographic range, regional distribution, breeding system, seed dispersal mechanism, mode of reproduction and successional status (Hamrick & Godt, 1989; Bhat, Babrekar & Lakhanpaul, 1999). Several techniques, including morphological, biochemical and molecular methods, have been used to measure genetic diversity in plant species.

The most widely used PCR-based DNA marker systems are random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and recently simple sequence repeats (SSRs) or microsatellites (Staub, Serquen & Gupta, 1996; Gupta & Varshney, 2000). The low reproducibility of RAPD, high cost of AFLP and the need to know the flanking sequences to develop species-specific primers for SSR polymorphism are the main drawbacks of these methods. The inter simple sequence repeat (ISSR) technique overcomes most of these limitations (Gupta *et al.*, 1994; Zietkiewicz, Rafalski & Labuda, 1994; Reddy, Sarla & Siddiq, 2002). This PCR-based technique amplifies DNA segments that are flanked by two inversely oriented identical microsatellite repeat regions (Reddy, Sarla & Siddiq, 2002). A single primer is used, which usually is 16-25 base pair long and contains di-nucleotide microsatellites (Wang *et al.*, 1994; Moreno, Martin & Ortiz, 1998). The technique combines the advantages of AFLP and microsatellite analysis with the universality of RAPD. Longer primers (16–25 mer) permit the use of high annealing temperatures (45–60 °C), which is probably the reason for the improved reproducibility of ISSR compared to RAPD where 10-mer primers are used (Reddy *et al*., 2002).

Objectives

The aim of this thesis has been to establish protocols for tissue culture, to carry out genetic transformation with the *rolB* gene using modern biotechnological approaches, and to evaluate the molecular genetic diversity of this species to provide a basis for its conservation and improvement. The specific objectives were:

-To develop an efficient micropropagation protocol
-To establish an efficient *in vitro* regeneration protocol
-To carry out genetic transformation with the *rolB* gene to increase the rooting efficiency and establishment of plantlets
-To evaluate the genetic stability of micropropagated plants using randomly amplified polymorphic DNA (RAPD) markers
-To evaluate molecular genetic diversity among and between populations collected from different parts of Ethiopia using inter simple sequence repeat (ISSR) DNA markers

Summary of results and discussion

Tissue culture, transformation and genetic diversity of *Hagenia abyssinica* (Papers I-V)

This study has scientific and practical significance in propagation, conservation and improvement of *Hagenia abyssinica*. Micropropagation (Paper I), *in vitro* regeneration (Paper II), and genetic transformation (Paper III) protocols have been developed for *H. abyssinica* for the first time. Pioneer work has also been undertaken on genetic stability studies and *ex vitro* rooting (Paper IV), and on molecular genetic diversity studies (Paper V) for this endangered tree species.

Micropropagation of *Hagenia abyssinica* (Paper I)

A micropropagation protocol has been developed for *H. abyssinica* using explants from both juvenile and mature material origin. Seedlings for culture initiation were obtained by sowing seeds on Petri dishes. Seedlings of length 0.5 to 1 cm were sterilized and cultured on either MS or WPM medium without growth regulators for culture initiation. No performance differences were observed between these two media (Fig. 2a). After five weeks on shoot initiation medium, the roots were removed and the shoots were cultured on MS or WPM medium containing different concentrations of BAP in combination with IBA.

Meristems were isolated from five to seven-month-old greenhouse grown plants and cultured on medium I consisting half strength of MS macronutrients, Nitsch micronutrients, Jacquiot vitamins (Gautheret, 1959), 4.4 μM BAP, 0.0045 μM 2,4-D, 0.29 μM GA_3 or on medium II consisting of MS medium containing 4.4 μM BAP in combination with 0.49 μM IBA. Medium I was more suitable for meristem initiation than medium II. On medium I, 21 of the 30 meristems developed into shoots while only 9 of 78 meristems developed into shoots on medium II.

Shoots from greenhouse grown plants were also sterilized and initiated on WPM containing 4.4 μM BAP in combination with 0.49 μM IBA and 1% activated charcoal. Shoots grew slowly at the beginning, but on the second transfer onto the same medium without activated charcoal, growth was relatively fast. The positive effect of activated charcoal is probably due to the adsorption of phenolic compounds released from the explants though it affected the production of more shoots at initiation stage, presumably by adsorbing growth regulators and by lowering the pH of the medium (Maene & Debergh, 1985; Nissen & Sutter, 1990; George, 1993)

Shoots were also collected from the bases of mature trees and sterilized with 0.15% mercuric chloride and initiated on the medium based on shoots from juvenile plants.

Shoots from explants of seedling origin were used to optimise nutrient medium and growth regulators concentration for shoot multiplication (Fig 2b). The best result was obtained from shoots subcultured on either MS or WPM medium

supplemented with 4.4 μM BAP in combination with 0.49 μM IBA. Shoots subcultured on MS medium with concentrations higher than 4.4 μM BAP and 0.49 μM IBA, containing agar and gelrite as a gelling agent showed stunted growth, folded abnormal leaves and swelling of stems. Shoots subcultured on MS media containing 0.7% agar supplemented with growth regulators concentration lower than 2.2 μM BAP and 0.25 μM IBA were normal but with lower multiplication rates and some were smaller and thinner. All shoots subcultured on WPM were normal. The initiated shoots from greenhouse plants, meristem, and mature trees origin were multiplied on this optimum medium (Fig. 2c).

The position effect was investigated using shoot explants from three different positions (starting from the base of the trunk up wards) of one female tree. The rate of shoot multiplication was evaluated from the first to the seventh subculture. Shoots obtained from the lowest base performed best at the fourth subculture and least at the sixth subculture while the explants obtained from the second and third position showed no significant differences in performance throughout the subcultures.

Rooting of shoots was achieved using MS medium containing macronutrients at one-third strength supplemented with 4.9 μM IBA. The cultures were kept in dark for four days and transferred to medium of the same composition but containing 0.3% activated charcoal without growth regulators. Up to 100% rooting was achieved depending on the genotype. Supra-optimal concentration of IBA promoted callus formation, which agrees with literature reports (George, 1996). Shoots multiplied on MS medium rooted better than those multiplied on WPM (Table 1). Similarly, Morini & Concetti (1985) reported similar results where shoots of peach rootstock elongated on MS responded better to rooting than those elongated on WPM. There were differences in rooting ability among genotypes as has been reported for other species (Welander, 1994). Plantlets were transferred to pots containing a mixture of soil and perlite in a 2:1 ratio, respectively, and were maintained in the greenhouse. The survival rate in the greenhouse was 93%. Further observations of more than 600 plants in the greenhouse showed no aberrant phenotypes (Fig. 2d, e and f). Histological studies of the *in vitro* shoots revealed that the shoots were axillary in origin (Fig. 2g).

Table 1. *Percentage and the number of roots produced by shoots derived from different explants of* H. abyssinica *and the effect of multiplication media (WPM and MS) on rooting. Data given as means ± SD.*

Group	Explant/ Genotype	Rooting %		No. of roots per shoot	
		WPM	MS	WPM	MS
Juvenile	Seedlings	73	87	3.3 ± 2.5^{b}	14.3 ± 6.8^{a}
Juvenile	Shoots	47	80	2.4 ± 2.1^{b}	7.3 ± 5.5^{b}
Juvenile	Meristems	47	93	5.2 ± 3.2^{a}	13.7 ± 5.3^{a}
Mature	G1B2	67	87	3.8 ± 3.5^{b}	8.8 ± 4.3^{a}
Mature	G2B1	97	100	8.9 ± 5.3^{a}	10.3 ± 4.0^{a}
Mature	G3B1	87	93	3.0 ± 1.9^{b}	8.3 ± 3.8^{a}
Mature	G4B1	63	87	2.7 ± 1.8^{b}	4.9 ± 2.8^{b}
Mature	G5B1	50	73	4.1 ± 2.8^{b}	5.7 ± 3.7^{b}

Means in the same column within the same treatment group followed by different superscript (a, b) are significantly different at 5% probability level

Effect of irradiance on micropropagated plants after a 2-month exposure to irradiances of 75, 150, 300 μmol $m^{-2}s^{-1}$ showed that increased irradiance reduced stem and leaf lengths, increased branch number, shorter internodes and compact leaflets. However, there was no significant difference in the number of leaflets and leaves among the treatments.

Fig. 2. Culture initiation from seedlings of *H. abyssinica* on MS medium (left) and WPM (right) (a); Shoot multiplication from seedlings (b) and mature tree origin (c); Plants obtained from explants of juvenile sources; seedling, meristem and shoot from left to right (d); Plants obtained from explants of different mature trees (e); Micropropagated plants; after six months in greenhouse (f); Longitudinal section of a shoot showing axillary origin of a shoot (g). Bars, a, b and c = 1.25 cm; d and e = 11 cm; g = 100 μm

In vitro regeneration of *Hagenia abyssinica* (Paper II)

In vitro regeneration is a prerequisite for genetic transformation of plants. A protocol for *in vitro* shoot regeneration of *H. abyssinica* has been developed using leaf explants from *in vitro* shoots of seedling and mature tree origin. The explants were cultured on MS medium containing various concentrations of NAA and TDZ. Callus was formed at the cut surfaces and several shoot primordia were observed on explants with lower concentration of TDZ (0.05, 0.1 μM) alone or in combination with NAA (0.01, 0.1, 0.5 μM) after three weeks (Fig. 3a). Callus induction was directly related to the concentration of growth regulators. NAA and

2,4-D promoted callus induction but high concentrations were toxic to the explants. High concentration of TDZ promoted callus induction and shoot regeneration (Fig. 3b) but inhibited shoot elongation. TDZ at concentrations less than 1 μM promoted direct shoot regeneration.

The rate of regeneration was significantly different among explants of seedling origin and mature trees. Regeneration of 96% to 100% was obtained between 1.0 and 10 μM TDZ from explants of mature origin. The highest number of shoots per explant was 8.4 ± 4.8 at 1.0 μM TDZ (Table 2). Complete leaf explants performed best in both shoot regeneration and number of shoots per explant among different explants tested. The five genotypes showed strong significant variation in rate of regeneration in the range of 1.3% to 98.8%. Shoots were multiplied on multiplication medium (Fig. 3c and d). From histological studies, active cell divisions and xylem differentiation were observed in two weeks culture. Differentiated cells formed meristematic regions that could be observed from 5-week-old cultures onwards. Ninety seven percent of shoots of seedling origin and 93% of shoots of mature tree origin rooted with no significant difference. After two months, more than 90% of the plantlets of both origins survived in the greenhouse (Fig. 3e).

Table 2. *Percentage of regeneration and number of shoots per explant of mature origin of* H. abyssinica *at different concentrations of TDZ and NAA*

NAA (μM)	TDZ (μM)	Entire leaf		Leaf segments	
		% regeneration	No. of shoots/explant	% regeneration	No. of shoots/explant
0.0	1.0	98.8^{a}	8.4 ± 4.8^{a}	79.2^{b}	5.2 ± 2.9^{ab}
0.0	5.0	96.3^{a}	7.0 ± 3.5^{ab}	91.7^{a}	5.5 ± 3.0^{a}
0.0	10.0	100.0^{a}	5.4 ± 3.2^{bc}	75.0^{b}	4.4 ± 2.1^{abc}
1.0	0.5	52.5^{bcd}	2.6 ± 1.6^{cd}	13.3^{f}	1.7 ± 0.8^{d}
1.0	1.0	47.5^{cde}	3.4 ± 1.8^{cd}	28.3^{de}	2.5 ± 1.5^{cd}
1.0	2.5	42.5^{de}	2.2 ± 1.1^{d}	30.0^{de}	2.2 ± 0.9^{cd}
1.0	5.0	60.0^{bc}	2.1 ± 1.1^{d}	33.3^{d}	2.1 ± 1.3^{cd}
1.0	10.0	37.5^{e}	3.2 ± 2.0^{cd}	28.3^{de}	2.5 ± 0.9^{cd}
1.0	20.0	62.5^{b}	2.7 ± 1.4^{cd}	50.0^{c}	1.8 ± 0.9^{d}
5.0	0.5	10.0^{f}	2.5 ± 0.6^{d}	11.7^{f}	2.2 ± 1.1^{cd}
5.0	1.0	15.0^{f}	2.8 ± 1.0^{cd}	10.0^{f}	3.4 ± 1.0^{abcd}
5.0	2.5	35.0^{e}	2.8 ± 1.7^{cd}	28.3^{de}	2.9 ± 1.7^{bcd}
5.0	5.0	37.5^{e}	2.0 ± 1.1^{d}	20.0^{ef}	4.4 ± 2.9^{abc}
5.0	10.0	50.0^{bcde}	3.9 ± 1.2^{cd}	21.7^{def}	4.0 ± 2.9^{abcd}
5.0	20.0	35.0^{e}	3.7 ± 3.2^{cd}	15.0^{f}	3.0 ± 1.3^{bcd}

Means within each column followed by the same superscript, a-f, are not significantly different at 5% probability level

In our study, the main factors that affected morphogenesis were growth regulators concentrations, type of explants, age of donor plant and genotype. In many woody plants, including recalcitrant species, callus induction and plant regeneration have been achieved using TDZ (Huetteman & Preece, 1993). The inhibitory effect of TDZ on shoot elongation has been reported by many researchers recommending that TDZ concentration must be reduced and/or other cytokinins or combinations of cytokinins and auxins must be used for further

shoot elongation (Bates *et al.*, 1992). NAA promoted callus induction and shoot elongation, but at higher concentrations, the calli turned brown and died. The lower regeneration rate of explants of seedling origin as compared to mature tree origin indicates that explants of different age require different concentrations of growth regulators. This protocol is significant for tissue culture and genetic transformation research on *H. abyssinica* and to understand the mechanisms that control the regeneration process in this species. The toxic effect of exogenous auxins needs to be studied further.

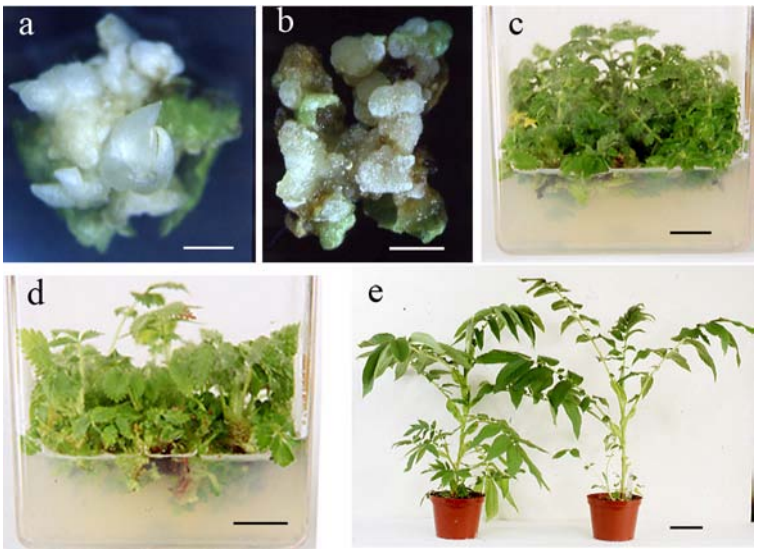

Fig.3. H. abyssinica shoot primordial developing by direct regeneration after three weeks (a); Callus formation after five weeks (b); Shoots of seedling origin (c) and mature tree origin (d) on multiplication medium; Four-month-old plants in greenhouse (e), mature tree origin (left) and seedling origin (right). Bars, a = 2.5 mm; b = 5 mm; c and d = 1 cm; e = 11 cm.

Genetic transformation of *Hagenia abyssinica* (Paper III)

In this paper, *H. abyssinica* has been transformed with *rolB* gene to improve the rooting efficiency of this species. As efficient regeneration is a prerequisite for transformation protocol, it is necessary to optimise conditions for regeneration and identify factors that affect transformation both during regeneration and transformation experiments. This study showed that IAA, BAP, TDZ and wounding affect callus induction and shoot regeneration of *H. abyssinica* from leaf explants. Callus induction was faster when the explants were wounded as compared to unwounded explants. However, wounding negatively affected shoot regeneration by turning the calli or explants brown especially at high concentration of IAA. Up to 100% regeneration were obtained from unwounded explants cultivated on MS medium containing 1 μM IAA and 1 or 10 μM TDZ. Explants are wounded to be infected by *Agrobacterium* during transformation experiments and reports showed wounding enhances regeneration (Goh, Lakshmanan & Loh, 1994; Miranda *et al.*, 1999) as well as earlier induction of

somatic embryogenesis (Santarem, Pelissier & Finer, 1997). Increasing the concentration of BAP and TDZ promoted callus induction and shoot regeneration. However, high concentration of TDZ inhibited shoot elongation, which is in agreement with other reports (Fasolo, Zimmerman & Fordham, 1989; Preece & Imel, 1991; Bates *et al.*, 1992; Bhagwat, Vieira & Erickson, 1996). IAA failed to promote callus induction and at higher concentrations shoot regeneration was negatively affected.

The effect of cefotaxime and kanamycin on regeneration was studied and cefotaxime at concentrations less than 500 mg l^{-1} was not toxic to explants. Several researchers reported the positive effect of cefotaxime in enhancing morphogenesis (Mathias & Boyd, 1986; Predieri & Fasolo Fabbri Malavasi, 1989; Pius *et al.*, 1993). Kanamycin is routinely used to select transformants in several transformation experiments. In our study, only 6% of explants treated with 50 mg l^{-1} kanamycin produced very small callus after six weeks but did not result in shoot regeneration.

Leaf explants were used for transformation experiments and kanamycin resistant calli and roots were observed after six weeks of culture. Up to 17% kanamycin resistant and 9% *gus* positive calli as well as up to 23% of *gus* positive roots from transformed calli could be obtained after six weeks of culture. The *gus* assay showed that the transgene could efficiently be inserted into cells of the host plant and the cells could divide and multiply. The expression of the *gus* gene at the tip of the roots showed that the root was transformed. This is in line with the results of Welander *et al.* (1998) who showed that the *gus* gene was highly expressed in the root tip of apple rootstock M26. This is due to the fact that *rolB* promoter is tissue and cell specific and therefore is only expressed in the root meristem and not in root elongation region. Continuous application of 50 mg l^{-1} kanamycin failed to produce any transformed shoots. The transformed shoots were obtained only by excluding kanamycin from the selection medium after six weeks of culture. This indicates that kanamycin inhibits the recovery of transformants. The transformants were selected later during shoot multiplication using 100 mg l^{-1} kanamycin and three transformed shoots, one labelled as T1, was obtained from a medium containing 5 μM TDZ and two shoots, labelled as T2.1, T2.2, produced from one callus, were obtained from a medium containing 5 μM IAA in combination with 5 μM TDZ. The veins of leaves obtained from T1 showed *gus* positive expression whereas the leaves from the other two transformants (T2.1 and T2.2) and untransformed control shoots were *gus* negative. PCR analysis confirmed that T1 contained all the three genes, *nptII*, *rolB* and *gus*, whereas T2.1 and T2.2 contained only *nptII* and *rolB* genes (Fig. 4a). Further analysis with Southern blotting confirmed the presence of these genes with one strong band and one very weak band of *nptII* and *rolB* genes and one strong band of *gus* gene in T1. One band of *nptII* and *rolB* genes was observed in T2.1 and T2.2 clones (Fig. 4b). RT-PCR revealed strong expression of *rolB* gene in clone T1 but not in others (Fig. 4c). The undetectable expression level of the *rolB* gene in the other two clones might indicate a lower expression of *rolB*.

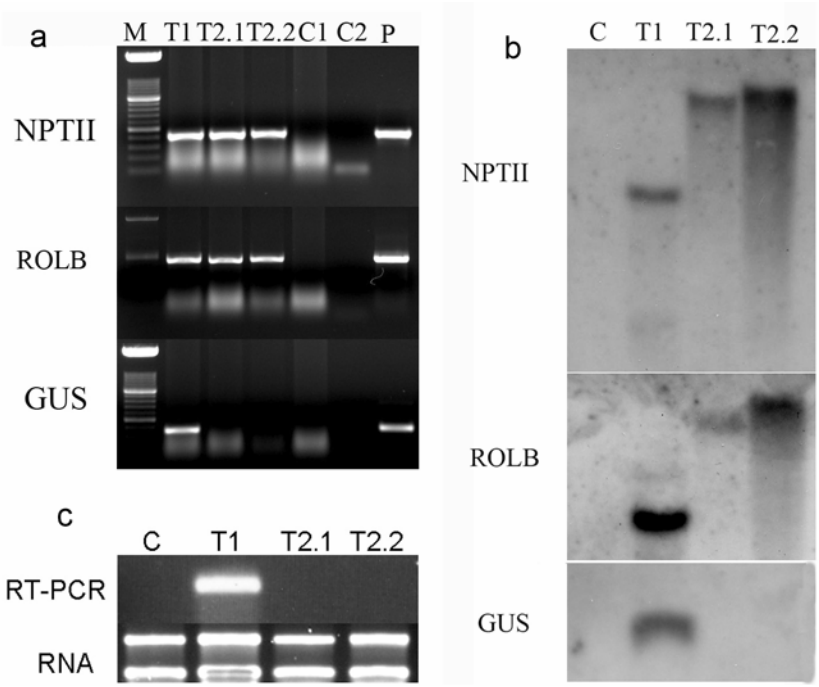

Fig 4. PCR analysis of the *nptII*, *rolB* and *gus* genes (a); Southern blot hybridization (b) and RT-PCR (c) of the clones (T1, T2.1, T2.2) and control plant (C1 or C) of *H. abyssinica*. C2 = negative control, P = plasmid DNA, M = 1 kb molecular marker.

It is well known that *rolB* gene expression increases the sensitivity of cells to auxin (Maurel *et al.*, 1990; Welander *et al.*, 1998), consequently increasing the rooting efficiency. In this study, the three transformed clones and untransformed control shoots were cultured on growth regulators free rooting medium. T1 resulted in 77% rooting with 6.8 roots per rooted shoot. T2.1 and T2.2 resulted in 50% and 57% rooting with 4.3 and 4.5 roots per rooted shoot respectively. The control shoots did not produce any root. This shows significant increase in the rooting efficiency of *H. abyssinica*. As this species does not propagate by cutting, the cuttings from transformants may root, permitting vegetative propagation of this species making the possibility of getting uniform genotypes easy, within relatively short time.

Genetic stability studies and *ex vitro* rooting of *Hagenia abyssinica* (Paper IV)

In vitro clonal propagation of trees is an attractive alternative for obtaining large number of elite genotypes (Bindiya & Kanwar, 2003). However, somaclonal variation of the micropropagated elite genotypes is a potential draw back. On the other hand stable somaclonal variations of specific type may be advantageous for improvement of certain traits (Antonetti & Pinon 1993; Bindiya & Kanwar, 2003). Randomly amplified polymorphic DNA (RAPD) markers were used to assess

genetic stability of 80 micropropagated *H. abyssinica* plants, 40 from axillary shoot origin and 40 from adventitious shoot origin. The shoots were isolated from the same mother tree and micropropagated for over two years. Among the 83 deca-mer primers screened, 16 gave reproducible band patterns. These 16 primers produced 115 bands for each plant. One plant from axillary origin showed two unique bands with primer OPC-11. All other plants showed identical band patterns. Several reports showed that plants of adventitious shoots origin are more subjected to somaclonal variation than those of axillary shoots. This is not the case in this study. However, as it is only one plant out of 40 axillary shoots showed variation, it cannot be concluded that axillary shoots are more subjected to somaclonal variation. Using RAPD technique, several authors have reported the absence of somaclonal variation in long-term micropropagated trees (Valles *et al.*, 1993; Goto, Thakur & Ishii, 1998). On the other hand, several investigators reported the presence of genetic variations in micropropagated plants (Rani, Parida & Raina, 1995; Munthali, Newbury & Ford-Lloyd, 1996; Hashmi *et al.*, 1997; Bindiya & Kanwar, 2003).

Ex vitro rooting was also carried out using microshoots or cuttings from different sources and dipping them in IBA, IAA and NAA for 1, 5 or 10 minutes. Rooting of 40% was obtained from IAA treated shoots for 5 min. Plant tissues rapidly oxidize IAA (Epistein & Ludwig-Muller, 1993; De Klerk, Ter Brugg & Marinova, 1997) and higher concentrations have to be applied as compared to other auxins such as IBA. Therefore, IBA is preferable to IAA. However, our study showed IAA is preferable to IBA for *ex vitro* rooting of *H. abyssinica*. IAA treated shoots grew faster with good appearance followed by IBA treated shoots, but NAA treated shoots developed poorly. Among micropropagated and non-micropropagated greenhouse grown plants that had been treated for *ex vitro* rooting, significant differences were observed both in percentage of rooting and root number per shoot. The highest percentage of rooting (45%) and maximum mean root number per cutting (6.2) was obtained from non-micropropagated plants that were grown in the greenhouse for 10-12 months. Cuttings from micropropagated mature plant origin failed to produce any root.

Using a complementary DNA representational difference analysis fragment (cDNA RDA14) clone, adventitious rooting related oxygenase (*ARRO-1*) gene was highly expressed in IBA treated stem discs of apple (*Malus domestica*) (Butler & Gallagher, 1999). *ARRO-1* is 1282 bp long and is represented in apple by a number of gene copies encoding a 2-oxoacid-dependent dioxygenase (2-ODD). This gene is specific to root development process and Southern blot analysis showed that it also hybridizes strongly with several fragments in pear (*Pyrus communis*) and plum (*Prunus domestica*) genomes. This suggests that *ARRO-1* is relatively conserved within the Rosaceae, but not in more divergent species such as birch (*Betula pendula*) (Butler & Gallagher, 2000). In our study, the *ARRO-1* gene is not expressed in *H. abyssinica* using the 216 bp RDA14 probe. This indicates that probably there is no homologous sequence of this probe in this species as it is relatively divergent species in Rosaceae and confined to mountains of tropical Africa.

Genetic diversity of *Hagenia abyssinica* (Paper V)

Genetic diversity within and among 12 populations of *H. abyssinica* collected from different parts of Ethiopia was examined by analyzing 120 plant samples with eight inter simple sequence repeat (ISSR) primers. Two of the populations, Sigmo and Gedo, had been planted 15 years earlier within a reforestation programme whereas the remainder represented more or less well preserved primary forest habitats. A total of 104 clearly scorable bands were generated of which 84 (81%) were polymorphic although some bands were polymorphic only in certain populations. Number of polymorphic bands within populations varied from 52% to 87%, with a mean of 73%. The Jaccard similarity coefficient was calculated among all the 120 individuals and ranged from 0.30 to 0.88 while average similarity within populations ranged from 0.53 to 0.66. Shannon's information index ranged from 0.30 to 0.50 and Nei's gene diversity from 0.21 to 0.35.

In general, ISSR- and RAPD-based estimates appear to be very similar when obtained in the same material (Nybom, 2004; Weising et al., 2005). Comparison of ISSR data for *H. abyssinica* with values obtained for 60 RAPD-based studies compiled in Nybom (2004) can thus be informative. Mean within-population gene diversity for *H. abyssinica* (0.30) was most similar to the RAPD values for long-lived perennials (0.25) that are outcrossed (0.27), dispersed by wind or water (0.27) and belong to late successional species (0.30) - all of which is typical for *H. abyssinica*.

A considerable amount of overall differentiation between populations was detected by both Shannon's information index (0.26) and G_{ST} (0.25), indicating that gene flow is rather limited in spite of both wind pollination and the production of light, and wind-dispersed seeds. When compared to RAPD-based G_{ST} and F_{ST} values, the presently obtained G_{ST} (0.25) is most similar to values obtained for long-lived perennials (0.19 and 0.25) that are outcrossing (0.22 and 0.27), wind or water-dispersed (0.17 and 0.25) and late sucessional (0.22 and 0.23).

Levels of relatedness among populations were calculated from the Jaccard similarity matrix and varied from 0.50 to 0.72. A principal coordinate (PCO) analysis of all samples (Fig. 5) as well as a cluster analysis (UPGMA) for the 12 populations (Fig. 6) indicated the existence of a rather weak association between geographical and genetic distances. Populations collected from the southeastern highlands of the country (Kersa, Adaba, Dinsho-1, Dinsho-2, Goba) grouped closely together both in the dendrogram and in the PCO. Interestingly, these populations were also the ones that had the lowest within-population diversity. Populations collected from the south-western and central-western parts of the country (Bonga, Sigmo, Gedo, Jibat) grouped loosely together in the PCO but showed no similarities in the dendrogram. Three of these populations were overall very variable, in two cases (Sigmo and Gedo) probably at least partly due to their recent originations from a comparatively heterogenous plant material. Three populations collected from the central part of the country (Menagesha, Intoto, Chilalo) took intermediate positions in the PCO and clustered loosely with the group from the southeastern area. They also showed intermediate levels of within-

population diversity. A Mantel test showed that there was a significant association between genetic and geographic distances, whereas an autocorrelation analysis indicated that there was significant evidence of gene flow only over distances up to 30 km.

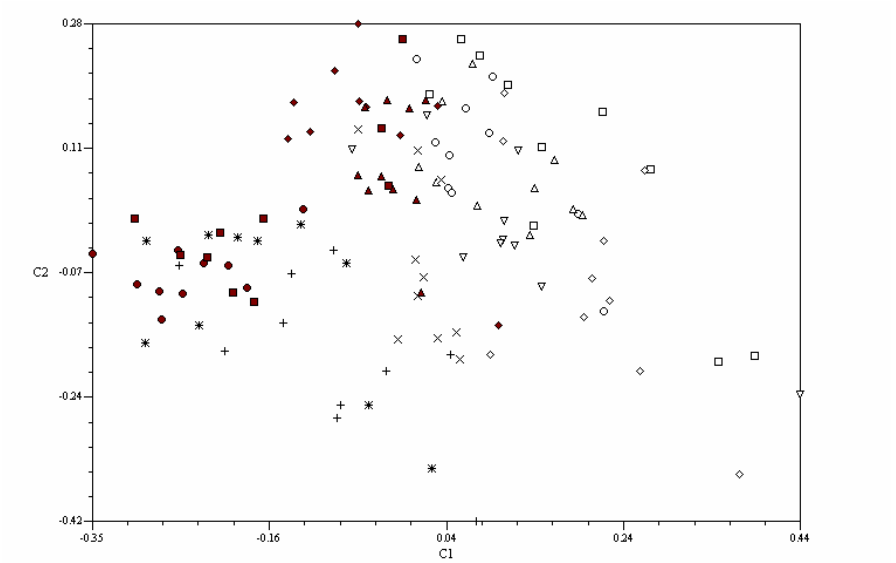

Fig. 5. Principal coordinate analysis of 120 individual samples of *H. abyssinica* investigated with ISSR: Open circle = Bonga, solid circle = Adaba, open square = Sigmo, solid square = Dinsho-1, open diamond = Gedo, solid diamond = Dinsho-2, open triangle pointing upwards = Jibat, solid triangle pointing upwards = Goba, open triangle pointing downwards = Menagesha, cross = Intoto, plus = Chilalo, star = Kersa

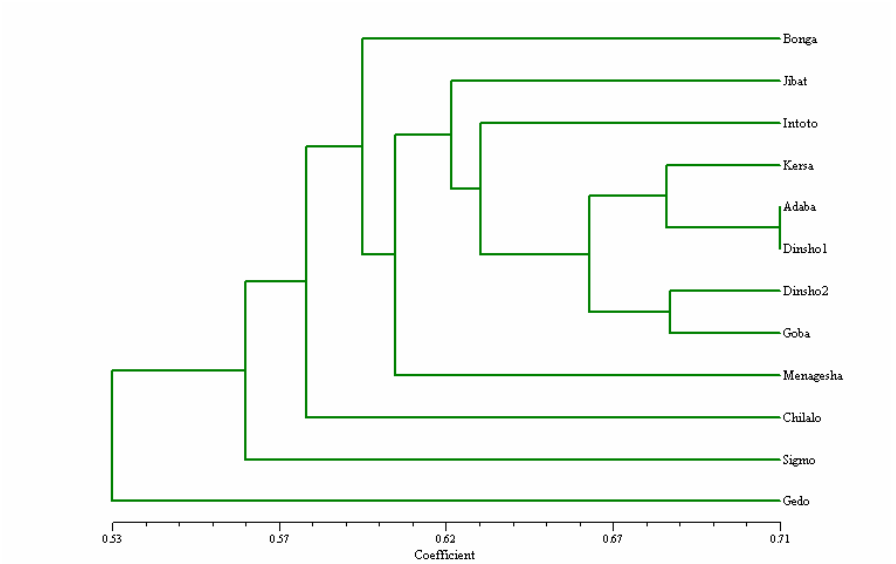

Fig. 6. ISSR-based UPGMA dendrogram for 12 populations of *H. abyssinica*.

Conclusions and future perspectives

Forests are among the most important natural resources both economically and ecologically to preserve the ecosystem. However, with rapid population increase, particularly in developing countries, huge amount of forests are destroyed every year resulting in land degradation and desertification, which consequently led to unsustainable agriculture. Research on application of biotechnology and studies on genetic diversity contribute a lot in conservation and improvement of these species.

The micropropagation protocol has been developed using explants from juvenile material and mature trees. Using explants of mature tree origin for micropropagation is more advantageous than explants of juvenile origin because the phenotype, and in the case of *H. abyssinica* and other dioecious plants, the sex of the mother plant is known. Therefore, this protocol can be used for propagation of this species that consequently contributes to its conservation and improvement. Furthermore, the shoots from these cultures could be effectively used for *in vitro* regeneration and genetic transformation.

Reliable *in vitro* regeneration is a prerequisite for transformation, thus *in vitro* regeneration protocol of *H. abyssinica* has been developed. This protocol can be used for transformation experiments as well as for the improvements of this species using *in vitro* experiments. The main factors that affected the rate of regeneration in our study were the concentrations of growth regulators, the type of explant, the age of the donor plant and the genotype. TDZ is the best growth regulator in promoting callus induction and shoot regeneration at high concentration although it inhibits shoot elongation. Therefore, TDZ should be excluded or its concentration should be reduced from the medium after callus indution for further shoot elongation. NAA and 2,4-D promoted callus induction but not shoot regeneration and were toxic to explants. The toxicity of auxins to *H. abyssinica* and the factors involved must be studied further.

Our studies on genetic transformation with *rolB* gene showed that kanamycin inhibited the recovery of transformants. Transformed shoots were obtained only after exclusion of kanamycin from the selection medium after six weeks of culture. When shoots had developed they could be selected on shoot multiplication medium with kanamycin. Therefore, our study showed that emphasis should be given in selection and optimization of selectable marker during genetic transformation of *H. abyssinica*. Since *rolB* gene promotes rooting ability the transformed shoots could be multiplied and rooted easily. However, the degree of rooting efficiency is affected by the position of the transgene in the genome of the plant. In nature, *H. abyssinica* does not propagate vegetatively. Further studies and field evaluation of *rolB*-transformed plants are important to investigate if the transformants can propagate vegetatively

It is important to have genetically stable clones during plant tissue culture. The results of genetic stability study of micropropagated plants using RAPD showed that all plants were genetically stable except one from axillary origin that showed

two additional bands. This indicates that micropropagated plants of *H. abyssinica* are relatively stable in culture.

H. abyssinica has recently decreased in Ethiopia and is regarded as endangered tree species. So far, a reduction in overall genetic diversity is not evidenced by our study of its genetic diversity. However, this may be due to the long generation times of the species.

Information about within-population variability can be used to identify sites for *in situ* conservation and for collection of material for establishment of gene banks and plant improvement programmes. Particular attention should be given to the Bonga population, as it grows in undisturbed primary forest and, at the same time, showed the highest within-population variation.

The obviously spatially-independent differentiation of populations at distances above 30 km, revealed by our study, suggests that gene flow is sparse between most of the *H. abyssinica* populations in Ethiopia. The presently available data do not allow us to draw any strong conclusions regarding the observed genetic structure. Gene flow is, however, likely to decrease even further because of genetic isolation of the extant populations due to the increasing range fragmentation brought about by current logging practices. These findings should be taken into account when conservation management policies for the species are developed.

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