Ex-situ Conservation of *Prunus Africana* through Tissue Culture Techniques.

By Fiona Mwaniki. Tree Biotechnology Project. 30th August 2005.

Introduction

The medicinal value of the extract from *P africana* bark for the treatment of benign prostatic hyperplasia, a common condition in elderly men has led to international trade worth approximately USD 220M per year in 1997, in the final pharmaceutical product. Approximately 4000t of bark is collected annually through destructive harvesting of trees from natural stands to meet this demand. This bark exploitation has caused serious damage to wild populations of Prunus leading to concerns on the long term sustainability of harvesting and conservation of this tree species (Cunningham, *et al*.1997). This overexploitation has sparked conservation concerns, resulting in the species being listed in Appendix II of CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) in 1994, becoming effective in 1995 in order to monitor species in International trade (Cunningham and Mbenkum, 1993).

This research aims at conserving the Prunus (ex-situ) through provenance collections of overexploited Prunus germplasm, particularly where commercial bark harvesting is taking place and their subsequent conservation in conservation stands. It also aims at the rapid multiplication of selected *Prunus africana* germplasm through tissue culture techniques for on farm planting by farmers. This will provide an alternative to the destructive sampling of wild stocks as well as help arrest the rapid erosion of overexploited *Prunus* germplasm.

Multiplication through tissue culture techniques will be done for selected trees which are, high active-ingredient yielding, fast growing, and of good form, using indigenous knowledge.

The expected outputs from the research are conservation of selected overexploited Prunus germplasm from various provenances, through the establishment of gene banks, distribution of already characterized and evaluated, tissue culture multiplied germplasm for planting by farmers and documentation of the results and information for dissemination to stakeholders.

Justification

To date successful plantations and enrichment plantings have been limited to Kenya where over a period of 90 years, *Prunus africana* has been planted by the Forest department for timber. However, their planting material is often of unknown origin and of narrow genetic base. They are therefore a limited utility for conservation purposes. (Eyog et al, 2001).

A study was carried by Cunningham and Mbenkum, 1993, to investigate the economic feasibility of different planting systems enrichment planting, small scale farming and plantations for *Prunus* as an alternative to the current overexploitation of wild populations in Cameroon. Some of the recommendations made were that low technology propagators at field sites would boost the number of young seedlings being produced, and research needs to be conducted for selection of fast growing, high active yielding *Prunus africana* cultivars.

If more attention is placed on the origin and genetic diversity of material, enrichment plantings and plantations can serve a useful conservation function and act as a source of planting material for other users (Eyog *et al,* 2001).

This research therefore aims to select in natural forests and farmer's fields overexploited *Prunus* germplasm, and plant them in gene banks, for their conservation. Due to the intermediate nature of the seed, propagating the tree through seeds may be difficult. Furthermore, it takes 15 years for the trees to seed. Propagating *Prunus* through tissue culture is a rapid method of multiplying the *Prunus africana* and is a faster way of achieving our objectives of conservation.

Literature review

Prunus Africana is popularly known as Pygeum, and is a large evergreen tree that grows in the afromontane of Africa. It is the only species of Genus *Prunus n*ative to Africa and can grow to a height of up to 40 meters. It has pendulous branches with thick oblong- shaped, leather like, mat colored leaves and creamy white flowers. The fruit (drupe) resembles a cherry when ripe.

Traded internationally and harvested from the wild, the *Prunus* is hardly a minor forest product. The tree is valued for its medicinal extract from its bark for the treatment of

benign prostatic hyperplasia, a common condition in elderly men. Annually, approximately 4000t of bark are harvested primarily from Cameroon, Equatorial Guinea, Kenya and Madagascar to meet this demand (Cunningham, *et al*.2002).This bark exploitation has caused serious damage to wild populations of *Prunus* including trees inside forests of high conservation value, leading to concerns on the long term sustainability of harvesting and conservation of this tree species. Diversity of other uses of this tree include making of handles for axes and hoes, firewood and poles (Cunningham, *et al*.1997).

A serious constraint in the expansion of on farm tree planting is seed availability. Here a key difficulty is the intermediate nature of the seed. Furthermore seed yields fluctuate widely between years. Seed shortage is likely to be exacerbated in future as the size of natural populations diminishes. The approximate time to first flowering and fruiting is 15 years. Raising *Prunus* through tissue culture techniques will therefore accelerate their production.

The transfer of germplasm from the wild into on farm niches helps to preserve valuable genetic resources particularly if attention is paid to the origin and diversity of cultivated material. In addition, cultivation of endangered forests species takes pressure off their natural resource base, thus promoting the conservation of natural forests (Engles *et al*, 2000).

Objectives of the project

- To gather through explorations and collection expeditions in different ecological zones, overexploited Prunus germplasm as well as meaningful and rich stock of Prunus with superior characteristics.
- Rapid multiplication (through Tissue culture techniques) of both the heavily exploited germplasm and the Prunus with superior characteristics, for establishment of conservation stands and their distribution to farmers.

Hypothesis

Conservation of Prunus africana can be enhanced through tissue culture techniques.

Expected out puts

- Conservation of the overexploited Prunus germplasm of different provenances, in established conservation stands
- Continued availability of the selected Prunus germplasm through their continuous multiplication.
- Documented research results for ease of dissemination and retrieval.

Selection

Three sites have been chosen to be areas in which selection of Prunus africana will be done. These are Mt. Kenya forest, Mt. Elgon forest and the Abardares. These sites were chosen as they are areas in which the tree is known to occur naturally and is heavily overexploited.

Random selection of trees will be done in the chosen respective provenances with the help of forest officers and farmers in the communities with indigenous knowledge. Selection will be done for trees that are high ingredient yielding, fast growing, early seeding, and have good form i.e. trees that are straight, with no mechanical damage no forking or kinking, no dwafing or diseased. Germplasm will be collected from trees which have passed selection criteria as well as from trees that have been overexploited and are becoming extinct.

Measurements to be taken for the selected trees are diameter at breat height and height of the trees. Other data to be noted on site include, approximate age and location of the tree.

Branches or coppices will be collected from 25 randomly chosen trees per provenance using secateurs. They will be packed according to provenances and carefully labeled using marker pens. The packaging will consist of polythene bags containing water, which will be stored in cooler boxes. Seeds will be collected from 25 randomly chosen and supposedly unrelated individuals using a seed harvester, packed in khaki bags which will also be labeled using a marker pen.

The collected coppices will be brought to the lab for propagation through tissue culture techniques.

Bark samples will also be collected from the selected trees and packed in Khaki bags, which will also be labeled. The bark samples will also be taken to selected laboratory for chemical analysis.

Collected seeds will be germinated in seed beds and then transplanted into potted tubes containing soil media. They will be planted in conservation stands once they are ready for planting.

Field trial design (Conservation stands)

The sites for planting the propagated material for both seed and tissue culture from the tree provenances will be identified. The land will be cleared, ploughed and planting holes dug. The sites will be at isolation belts of between 300 to 500 meters from other natural vegetation that have Prunus trees.

The propagated material ready for field planting, will be planted out in the field during the onset of the rains, using local labour.

The material from the three provenances will be planted in each of the provenance. Each provenance will have a total of 533 trees and will be replicated 3 times across the trial design. Each replicate will therefore have 133 trees from each provenance. There will be 4 replicates for each of the provenance. Replicates will be used for planting prunus propagated from seed.

The site will be visited twice a year for monitoring and maintenance i.e. weeding. Data will be collected once a year. This includes data for survival, height, root collar, diameter and diameter at breast height for 10 randomly selected trees per plot of 133 trees. The data will be analysed at the nursery. Thinning will also be done when necessary.

Tissue Culture Procedures

The explants from the field will be stored in a refrigerator, while still in the polybags with water to keep them fresh. Tissue culture will be done progressively, one provenance at a time

The explant to be used here are shoot tips form the *Prunus*.

Media composition and Preparation

The Murashige and Skoogs (MS) media will be used. (Appendix I)

Preparation of growth regulators will be prepared as in Appendix II

However, a protocol will have to be worked out through trial and error, to establish which media combination will be successful in the propagation of the *prunus.*

The culture media should have a pH of 5.7 +/- 0.1, before agar is added. This pH is achieved by measuring the media pH using the pH meter and adding HCL or NaOH to adjust the pH accordingly.

Agar is always added last, and it should be mixed well, using heat, preferably in a microwave

The prepared media will be discharged into test tubes (5mls per tube) using a syringe. The test tubes will be covered and packed in an autoclavable tray and placed in the autoclave. The autoclave will be set to 15 minutes at slow exhaust. When the autoclave cycle is complete and the pressure has reached zero, and temperature below 100^{oc}, the autoclave door will be opened and the tray with tubes removed.

Surface disinfection of Explant material

The explants will be trimmed to size convenient to handle and placed in a container with little quantity of water to maintain the moisture until processing. The water in the container is then discarded. The material is then put into 70% ethanol for 25 minutes, followed by 5-10% sodium hypochlorite solution with few drops of tween for 20 minutes. The material is then rinsed with 3 changes of sterile distilled water. The explants are then kept in the last change of water. If the material is heavily contaminated, a double disinfection process with sodium hypochlorite may be used

The explants are now ready to take to the transfer chamber. This last step will be performed inside a laminar flow hood.

starting the Explants (this is done in the transfer/ innoculation room)

The transfer chamber should be ready with the walls and workspace sprayed and wiped with 70% ethanol. Your hands should also have been washed with soap and water till the elbows, first, then sprayed with 70 % ethanol.

There should be a container of 70% ethanol to sterilize and rinse the instruments, I.e the blade (no 10) and forceps.

Immerse the forceps and scapel for 30 seconds or more in the 70% ehanol then burn off the etanol over the flame from the bunsen burner. With the forceps place an explant tip on the petri dish. Place the forceps in the other hand to hold the tip while the first hand uses the scalpel to cut off 1 cm of the basipetal end of the shoot tip. Place the scapel in the70% ethanol, move the forceps to the other hand. Grab a sterile test tube of medium with the other hand, hold it by the base. With the little finger of the hand holding the forceps, remove the cap and cradle it there while you use the forceps to firmlty lay the bud on the medium. Recap the test tube and seal with a piece of scotch tape (or Para film).

Growing

Place the test tubes in the planter tray (or other appropriate holder) and place the tray on a shelf under fluorescent light which is 8 - 10 inches above the top of the tubes, in the incubation room. Room temperature is o.k. Continous light is acceptable, but 16 hours light and 8 hours darkness is standard. Check daily for cotaminants. If any are found, sterilize tube and contents before discarding the contents in an autoclave

Transfer the explant every two weeks or so until it is actively growing. In one to two months you should be able to divide the culture into two pieces, each of which is about 0.5 cm in diameter. Continue to divide and transfer until you have enough plantlets. The platlet should be singulated as you transfer to pre-rooting medium which has no hormone (or only IAA).

Transplanting

With the plantlets begin to root, perhaps two to four weeks, transplant them to a light artificial soil mix, such as peat/pearlite, in a seedling tray. Cover with clear plastic and place on a lighted shelf or in a shaded greenhouse. After two or three weeks, begin leaving the plastic off for a period of time each day. The time the plantlets are left uncovered

should get longer each day, until after about a week, the cover can be left off completely. (Tissue cultured plantlets are more delicate than seedlings as the stomates remain open until they slowly adjust to normal humidity and light).

Calendar of Events

Activity	**Duration of Activity in Months**
Selection	**1**
Tissue culture	**6**
Hardening	**3**
Land preparation for trials	**1**
Planting in trials	**1**
Total	**12**

Estimated Budget

	Cost (USD)
Equipment	
Laminar air flow-benchtop horizontal flow 'O' series	**5000.00**
Autoclave portable stainless stell (4OL)	**1878.00**
Water still Bibby model W14S single distilation wall bench mounting	**1750.00**
Sub total	**8628.00**
Expendable Supplies	

Reagents NH_4NO_3, KNO_3, $MgSO_4.7H^2O$, $CaCl_2.2H_2O$, $FESO_4$, NaEDTA, $ZnSO47H_2O$, $CUSO_4.5H_2O$, KI, COCl26H2O, H3BO4, Na2MoO42H2O, Sucrose, hormone, vitamins	**2150.00**
Sub total	**2150.00**
Local Travel	
Travel to Mt. Kenya, Mt. Elgon and Abardares by road	550.00
Sub total	**550.00**
Extra Manpower	
Fieldwork, installations of equipments and laboratory assistant	450.00
Sub total	**450**
Other Costs	
Hardening in greenhouses and field testing of plantlets	222.00
Sub total	**222**
Total Project Budget (USD)	**12,000**

Appendix I. Murashige and Skoog's Stock Solution.

Preparation of stock solution

Stock solution I.

	g/litre	**Storage**
NH4NO3	**16.5**	**Refigerator, 4oc**
KNO3	**19.0**	
CaCl2.2H2O	**4.4**	
KH2PO4	**1.7**	
MgSO4.7H2O	**3.7**	

Stock Solution II	**g/litre**	**Storage**
KI	**0.16**	**Refigerator, 4oc**
H3BO3	**1.24**	
MnSO4.4H2O	**4.46**	
ZNSO4.7H2O	**1.72**	
Na2MoO4.2H2O	**0.05**	
CuSO4.5H2O	**0.005**	
CoCl2.6H2O	**0.005**	

Stock Solution III

	g/litre	**Storage**
FESO4	**2.78**	**Refigerator 4oc**
Na2.EDTA.2H2O	**3.73**	

Stock IV

Vitamins stock solution	**g/litre**	
Myo-Inositol	**20g**	**Refigerator 4oc**

Nicotinic acid	**0.1**	
Pyridoxine-HCl	**0.1**	
Thiamine-HCl	**0.1**	
Glycine	**0.4**	

Appendix II

Preparation of growth regulators stock solution

1. weigh 10mg of a growth regulator
2. Place in a 100 ml beaker
3. Add a few drops of 0.5N HCL or NaOH (depends on the type of growth regulator) or 20-30 ml of 96% ethanol.

   Cytokinins are always dissolved in NaOH or HCL and auxins in ethanol.
4. mix until completely dissolved
5. Add some quantity of double distilled water (DDW)
6. Pour the content into a 100ml volumetric flask. Rinse the beaker with a small quantity of DDW and pour it into the volumetric flask.
7. Add DDW up to the mark on the flask
8. Cover the flask or seal the flask with para film
9. Mix well
10. Write a label indicating the name of the growth regulator, concentration and date of preparation.
11. This stock solution will give you 0.1mg of the growth regulator for each ml you pipette. If the concentration of growth regulator is too concentrated, dilute the original stock by 10 or 100 times as desired.

References

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