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International Centre for Genetic Engineering and Biotechnology
United Nations Industrial Development Organization

Collaborative Research Programme

TERMINAL EVALUATION REPORT

UNIDO contract # 90/019

ICGEB ref. #: CRP/NIG 88-04

Project initiation: 2/11/90

Project termination: 30/6/94

International Centre for Genetic Engineering and Biotechnology
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TERMINAL EVALUATION REPORT

Part 1

Title of Project

Mass Propagation of Selected Trees for Wood Pulp and Paper Industry in Nigeria.

Keywords: In vitro Clonal Propagation. Multiple Shoots. Rooting and hardening. Protocol Development. Nursery Establishment.

UNIDO contract # 91/019 **ICGEB ref. #:** CRP/NIG 88-04

Project initiation: 2/11/90 **Project termination:** 30/6/94

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Abstract:

Protocols were developed for the in vitro multiplication of Gmelina arborea and Pinus caribaea through the culture of explants on full or reduced strength MS, B5, SH or AE media. Several factors e.g. activated charcoal, carbohydrate source, type and concentration of auxins and cytokinins, were screened for their enhancing effects on multiple shoot production, elongation and rooting. Shoot-regeneration potential of induced callus was also studied. In G. arborea, major successes (up to 19 shoots/explants) were achieved using seeds and nodal segments as explants on B5 supplemented with 5-10 mg l^{-1} BAP. Shoot elongation was enhanced by 0.5% activated charcoal and 2% sucrose or maltose while rooting was optimal in supplements of 0.08-0.1 mg l^{-1} IBA or NAA. For P. caribaea, optimal multiple shoot production (17/explant) was on 25% strength AE medium; elongation was enhanced by 0.5% charcoal and 2% sucrose and rooting was best on 25% strength AE medium supplemented with 0.1 mg l^{-1} IBA. 20-30% of the in vitro -produced shoots were successfully hardened and a pilot plot of P. caribaea was established.

OBJECTIVES/METHODOLOGY
(proposed at the time of the submission of the research proposal)

The project was designed to produce ample tissue culture-generated vegetative propagative seedlings, to ensure the establishment of large-scale plantation of Pinus caribaea and Gmelina arborea, for the production of pulp and paper. The research was planned in three stages:

i) Laboratory assay of the response of explants of Pinus and Gmelina cultured on various media. This would enhance the determination of callusing and budding potentials, and plantlet regeneration on suitable media.

ii) Laboratory multiplication of plants and transfer into greenhouse nurseries as potted plants, and

iii) establishment of plants in adequately fertilized pilot field plots.

G. arborea is a recognised source of short-fibre pulp while the tropical coniferous plant P. caribaea, provides long pulp fibre. Since neither of the two plants is grown in sufficiently large numbers to satisfy all the industrial requirement (in fact, seed of P. caribaea is scarce and very costly) the project was intended to use tissue culture procedures to raise plantlets in order to maximise the provision of seedlings for establishing plantations and minimize the need for importing wood pulp.

Two methods of raising plantlets in vitro were envisaged.

i) induction of multiple shoot production through stimulation of bud differentiation from embryo and other explants. Such shoots would then be isolated, elongated and rooted in appropriate media. After hardening, the plants could be established in nurseries and innoculated with mycorrhizal fungi. This is necessary for fast and effective development of the pine plantlets into trees.

ii) micropropagation through plantlet regeneration from induced callus. By suitable manipulation of the culture medium, somatic embryogenesis could be achieved, whereby embryos similar to the sexually-produced (zygotic) embryos are obtained in culture from subcultured undifferentiated callus cells. Such embryos could then be isolated, rooted, hardened and transferred into potted soil and grown as above, into mature plants.

RESULTS
(compare against the set objectives)

1. Plantlet Regeneration through Multiple Bud Proliferation

During the first two years, research progressed faster than initially planned. Major successes were achieved in this part of the study. For G. arborea, seeds and nodal segments used as explants produced up to 19 shoots/explant on B5 medium supplemented with 5–10 mg l^{-1} BAP while up to 17 shoots/explant were obtained for P. caribaea on 25% strength AE medium supplemented with 0.1–0.5 mg l^{-1} BAP.

Various factors were subsequently screened for their enhancing effects on shoot production, elongation and rooting. Stunted shoots produced initially were best elongated on B5 medium (for G. arborea) or 25–50% strength AE medium (for P. caribaea) supplemented with 0.5% charcoal and either 2% glucose or maltose. In these media 9-fold elongation was achieved within 4 weeks. Rooting of elongated shoots of G. arborea was best in B5 medium supplemented with 0.08–0.1 mg l^{-1} IBA or NAA where 80–100% of the shoots rooted without callusing. Similar results were obtained for P. caribaea in 25% strength AE medium supplemented with 0.1 mg l^{-1} IBA.

Initially, hardening of plantlets posed some problems with only 2–5% success obtained. By the beginning of the third year, this had been increased to 20–30% for both plants. The increase was due to two reasons: (1) improvement in the control of dessication in the fragile plants while exposing them to the outside environment for the first time and (2) control of fungal attack by spraying the plants forthnightly with 1g l^{-1} benlate (active substance, benomyl).

After the establishment of plantlets as potted plants in the nursery, the plants were inoculated with mycorrhizae fungi by adding infested soil scooped from the surroundings of the roots of mature pine plants to the culture bags. Some of the inoculated plants have now been established in a pilot plantation located at the University of Nigeria, Nsukka.

2. Studies on Somatic Embryogenesis

Callus was induced in G. arborea using seed and nodal segments as explants in MS and B5 media supplemented with 2mg l^{-1} kinetin, 2mg l^{-1} 2,4-D or 2mg l^{-1} NAA + 2mg l^{-1} BAP. All types of explants (embryo, hypocotyl, root, cotyledon) tried in P. caribaea produced callus but the response varied with explant and type of hormonal supplements employed. Thus for embryos, callus induction was best on MS supplemented with 2mg l^{-1} 2,4-D or 2mg l^{-1} NAA. Combinations of NAA at 2mg l^{-1} and kinetin at 1mg l^{-1}; 2mg l^{-1} 2,4-D and 1mg l^{-1} kinetin; and 2mg l^{-1} 2,4-D and 1mg l^{-1} BAP also gave good results for all explants. Callus growth was best on MS and B5 media supplemented with 2mg l^{-1} kinetin; 2mg l^{-1} 2,4-D or 2mg l^{-1} NAA + 2mg l^{-1} BAP. Several transfers of calluses into factorial combinations of the phyto-hormones failed to produce plantlets although many of the calluses looked green and embryogenic especially for P. caribaea. Work is still progressing in this direction.

Work plan and time schedule (originally envisaged)

The major constraint to paper production in Nigeria has been the provision of locally-sourced fibres especially long fibres. The resulting shortage in wood pulp for newsprint and other purposes could be addressed by appropriate planning, research and development. Gmelina arborea is a recognized source of short-fibre pulp while Pinus caribaea provides long-fibre pulp. However, seeds needed for the establishment of plantations of these plants are in short supply.

It was intended, in this project, to use tissue culture procedures to raise numerous plantlets from P. caribaea and G. arborea. Micropropagation is widely used in many countries for both mass propagation and as a tool for genetic conservation. The technique is based on the pioneering studies on somatic embryogenesis performed in the late 1950s and early 1960s by Steward and his collaborators at Cornell University and by Reinert and his co-workers in Germany. The workers demonstrated that the phenomenon of totipotency found in plant cells could be exploited by inducing tissues and cells cultured on appropriate nutrient media to form large masses of undifferentiated parenchyma tissue or callus. By suitable manipulation of the medium, the callus cells can be made to regenerate asexual somatic embryos (embryoids) virtually from every cell of the callus, which develop into plantlets. Thus numerous embryoids could be produced from a small piece of plant callus obtained from ary part of the plant. These are then isolated, reared into plants, rooted, hardened and transferred into potted soil.

The second method envisaged in the project was to raise plantlets from multiple shoots of pine and Gmelina (organogenesis) induced through the stimulation of bud differentiation under appropriate media conditions. This method is recommended for forest plants especially trees since it eliminates the appearance of somaclonal variants whose origin could be environmental (epigenetic-induced by culture conditions); genetic (changes in nuclear genes) cytoplasmic or chromosomal (producing polyploids, aneuploids and even structural chromosomal variants - inversions, interchanges, etc.).

The project was envisaged to last for three years, as follows:

Year 1: The collection of plant material and the screening of explants on various tissue culture media and additives (auxins, cytokinins, etc.) in order to stimulate callus or bud growth.

Year 2: The working out of protocols for shoot elongation, rooting and hardening and transfer of rooted plants into greenhouse nurseries as potted plants.

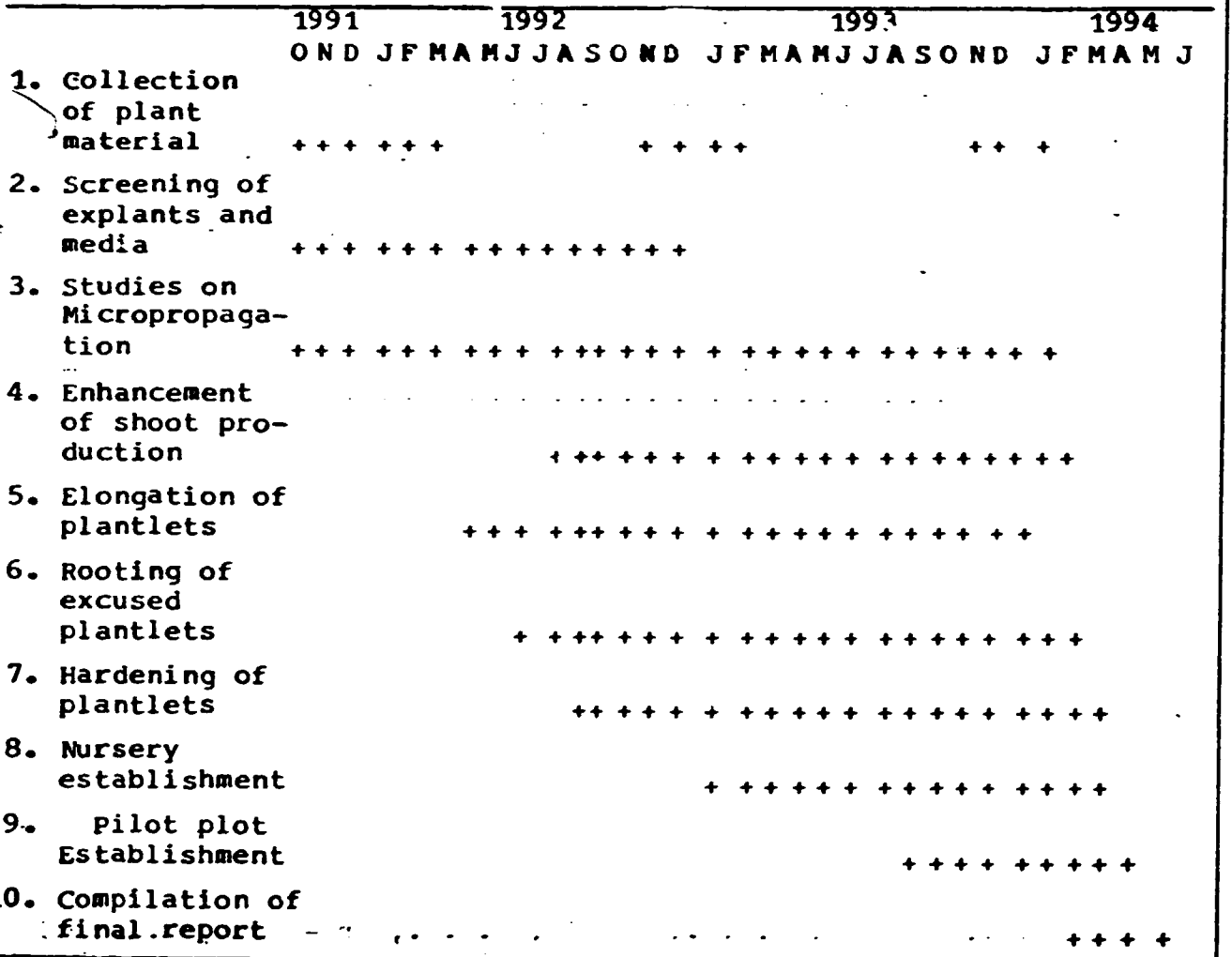
Year 3: Finalising of protocols and the establishment of pilot field plots.

Postgraduate students were also to be admitted and trained as part of the project.

Work plan and time schedule (actual)

Project landmarks, duration of individual tasks (use bar charts); evaluation criteria (publications, patents, services, training)

Most of the objectives of the project were achieved within the time frame envisaged. In fact, work progressed faster than planned, during the first two years. The actual time-schedule is shown below:



The summary above spans the commencement of work in Oct. 1991 with collection and screening of explants on four media (MS, B5, SH and AE) used in variable strengths; development of protocols for callus induction and growth as well as for plantlet production from multiple shoots. Optimal additives to the basal media which varied according to requirements included vitamins, casein hydrolysate, myoinositol, phytohormones (NAA, 2,4-D, IBA, and IAA), gibberellins (GA_2 and GA_3) and cytokinins (Kinetin and BAP). The studies on enhancement and optimization of multiple shoot proliferation, elongation, rooting and hardening were followed with nursery and pilot plot establishments in the third year. (including mycorrhizal inoculations)

Training of Postgraduate Students and Investigators

Three postgraduate students have been sponsored under the project.

The investigators also attended the following workshops/training (see attached):

NETWORKING

The project has enabled researchers to establish good working relationship with the following workers and laboratories:

1. The Biotechnology Laboratory, International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria.
2. The Laboratory for Tropical Crop Husbandry, The Katholieke Universiteit Leuven, Belgium.
3. Dr. E.B. Esan, Cocoa Research Institute, Ibadan, Nigeria.
4. The Tissue Culture Laboratory, Root Crop Research Institute, Umudike, Umuahia, Nigeria.
5. The Tissue Culture Laboratory, National Institute for Oil Palm Research (NIFOR), Benin City, Nigeria.

PUBLICATIONS

Ameh, G.I., Okonkwo, S.N.C., Okezie, C.E.A., Ene-Obong, E.E. and Okoro, O.O. (1992). Response of Pinus caribaea explants in vitro. Proceedings of the African Regional Symposium on Biotechnology for Development. ILRAD, Nairobi, Kenya.

Ihemere, U.E., Okonkwo, S.N.C., Ene-Obong, E.E., Okezie, C.E.A. and Okoro, O.O. (1992). In vitro studies of Gmelina arborea Roxb. through nodal segments. Proceedings of the African Regional Symposium on Biotechnology for Development. ILRAD, Nairobi, Kenya.

Ene-Obong, E.E., Ihemere, U.E., Ameh, G.I., Okezie, C.E.A., Okoro, O.O., and Okonkwo, S.N.C. (1993). Mass clonal propagation of trees for renewable reforestation in Africa: species studies of Gmelina arborea Roxb. and Pinus caribaea Mor. Proceedings, Seminaire Regional sur L'Afrique au defi des biotechnologies vegetables. IIRSDA, Abidjan, Cote D'Ivoire.

Workshops/training attended by Investigators:

i)	S.N.C. Okonkwo, Ene-Obong and Okezie	- African Regional Symposium on "Biotechnology for Development, Feb. 17-21, 1992. Nairobi, Kenya.
ii)	E.E. Ene-Obong, Okezie, Ihemere, Ameh and Okoye	- 6th National Conference of Botanical Society of Nigeria. March, 1992. Jos, Nigeria.
iii)	C.E.A. Okezie	- Workshop on Enzyme Engineering. Sfax, Tunisia (1992).
iv)	S.N.C. Okonkwo	- 4th General Conference of TWAS. Nov. 1992, Kuwait.
v)	S.N.C. Okonkwo, Ene-Obong, Okezie	- Seminaire Regional sur L'Afrique au defi des biotechnologies vegetales. April 1993, Abidjan, Cote D'Ivoire.
vi)	S.N.C. Okonkwo and Ene-Obong	- National Workshop on Biotechnology. April, 1993, Lagos, Nigeria.

Part 4

STATEMENT OF EXPENDITURES

To be filled by ICGEB		To be filled by the Affiliated Centre	
Budgets as per original proposal		**Summary of expenditures** *	
1) Capital equipment	US$	1) Capital equipment	US$ 20,000.00
2) consumables	US$	2) consumables	US$ 10,000.00
3) training	US$	3) training	US$ 21,00.00
4) literature	US$	4) literature	US$ 1,500.00
5) miscellaneous	US$	5) miscellaneous	US$ 7,500.00
TOTAL GRANT	US$.....	TOTAL	US$ 60,000.00

Please itemize the following budget categories (if applicable)

Capital equipment

	Description	Qty Ordered	Qty on hand
1.	MKV Shaker C/W TACHO-300	1	1
2.	Plate form 42 x 250ml	2	2
3.	Fisher Benchtop Sterilizer 10" 230 V50 H014	1	1
4.	Microtome Histocut 820-II	1	1
5.	Oven 150 MDL 507G 230V	1	1
6.	Electronic Balance EL PM400	1	1
7.	Air pump P/V 230V 50HZ	1	1

Training (provide names, duration of training, host laboratory)

1. Dr. E.E. Ene-Obong (Part airfare) - Practical Course on Plant Transformation, ICGEB, New Delhi, 1991. (3 weeks).
2. Several Conferences and Workshops by researchers and students. (Please see section on Workplan - actual).
3. Three postgraduate students sponsored. Two have completed the M.Sc. programme. The third is still working for a Ph.D.

Literature

Nigerian Journal of Botany - 3 years subscription.

* Please do not send invoices, receipts etc.; these should be kept by the Affiliated Centre for future reference and sent to ICGEB upon request.