Project Update: October 2022

Period: August 3rd – October 3rd, 2022

Progress of activities of the first main objective:

1. Development of protocols for the generation of seedlings of *Myrica parvifolia* and other forest species *in vitro* and in nursery

Related projects:

Tentative Title	Seed germination and *in vitro* establishment of *Morella* sp (Myricaceae) from the Tambillo Community Protected Area
Institution:	Universidad Técnica de Manabí
Modality:	Undergraduate thesis
Principal Researcher:	Ph.D. Liliana Corozo - Researcher and Lecturer Ph.D. (c) Fátima Macías - Researcher
Students	Laleska Cevallos Jailene Loor

Tentative Title	Establishment of a micropropagation protocol for *Caesalpinia* sp for use in a future ecosystem restoration and conservation plan
Institution:	Universidad Politécnica Salesiana
Modality:	Research project
Principal Researcher:	M.Sc. Mateo León - External researcher Mg. Myriam Mancheno – Researcher and Lecturer
Students	Santiago Rojas José Solórzano Tatiana Muñoz

Important note: in the previous report (35230-1 August 2022) the undergraduate thesis topic was "In vitro multiplication of woody species for reforestation purposes in the Tambillo Community Protected Area", due to unforeseen factors this topic was modified. The new thesis topic is "Seed germination and *in vitro* establishment of *Morella* sp (Myricaceae) from the Tambillo Community Protected Area".

1.1. Germination of seeds of *Morella* sp.

The mature seeds of *Morella* sp. were collected from the Tambillo Community Protected Area in June 2022. 200 seeds with better characteristics were used, which were subjected to a pre-germination process, which consisted of removing the waxy layer (outer cover), placed in boiling water for 2 min, then, the seeds were left to stand in GA3 solutions for 24 and 48 h at 20 °C. Finally, germination trays with 50 cells were used for sowing and the substrate was peat (Image 1).

1.2. *In vitro* establishment of *Morella* sp. cultures

The results on the effect of sodium hypochlorite and quaternary ammonium

concentrations on the *in vitro* establishment of *Morella* sp. apices, and the time of immersion in the disinfectant agents for the survival of the explants were evaluated in 30 days.

1.3. *In vitro* propagation of *Caesalpinia* sp.

1.3.1. Location of the seed source

In Jima Parish and the Carmen de Zhipta Community there are two specimens of *Caesalpinia* sp., commonly called tara or guarango. People from Jima parish consider this plant species as the emblematic tree of the place (Image 2).

1.3.2. Collection of biological material

The mature pods of *Caesalpinia* sp. have a red-orange color and the seeds are round, flattened and brown or dark brown, this is the main characteristic to consider at the time of collecting the fruits (Images 3 – 4).

In the Biotechnology Laboratory of the Universidad Politécnica Salesiana, pre-germinative treatments were carried out, with the objective of breaking dormancy and stimulating seed germination, these pre-treatments were: placing seeds in boiling water, soaking the seeds in water at room temperature, softening the external covering by rubbing on a rough surface.

1.3.3. Disinfection protocol

In a laminar flow chamber, the following were used for seed disinfection: a solution of Tween 20, 70% ethanol and a solution of sodium hypochlorite. Subsequently, the aseptic seeds were placed in sterile Murashige & Skoog (MS) culture medium supplemented with Gamborg's vitamins, sucrose and agar. Finally, the samples were placed in the growth chamber of the Universidad Politécnica Salesiana, and the growth conditions were photoperiod 12 h light - 12 h dark and temperature 20 °C ±2.

1.3.4. In vitro establishment

Explants of approximately 1 to 2 cm were obtained from donor seedlings developed under in vitro conditions. They were placed in MS culture medium supplemented with Gamborg's vitamins, sucrose, agar and benzyl aminopurine (6-BAP). The growth conditions were mentioned above. Finally, after 30 days, the new shoots were evaluated (Image 5).

1.3.5. In vitro multiplication

Explants with new shoots were transferred to a new MS culture medium supplemented with Gamborg's vitamins, sucrose, agar, growth regulators, such as: 6-BAP and naphthaleneacetic acid (NAA). The growth conditions were mentioned above. Finally, after 30 days the new shoots were evaluated.

1.3.6. In vitro rooting

In this part, shoots longer than 2 cm were placed in MS culture medium supplemented with Gamborg's vitamins, sucrose, agar, growth regulators such as indolbutiric acid (IBA)

and NAA. The growth conditions were mentioned above. Finally, after 60 days the new roots were evaluated.

1.4. Placement of new *Morella* sp. seedlings in black polyethylene bags.
Morella sp. seedlings germinated from seeds (see February_2022 report), which are 11 months old, were placed in black polyethylene bags with a substrate mixture (black soil, rice husk, peat and sand) for nursery development.

Partial results

2. Development of protocols for the generation of seedlings of *Myrica parvifolia* and other forest species in vitro and in nursery

2.1. Seed germination of *Morella* sp.
The effect of GA3 solutions on *Morella* sp. seed germination was positive, germination increased 2.7 times in 24 h and 2.33 times in 48 h (Image 6).

2.2. In vitro establishment of cultures of *Morella* sp.

2.2.1. Effect of sodium hypochlorite and quaternary ammonium concentrations on the *in vitro* establishment of *Morella* sp. apices
The effect of the concentrations of the two disinfecting agents on the percentage of contamination and the percentage of morphogenic response were evaluated. The best results related to culture contamination were 45.75% and 45.25% with sodium hypochlorite and quaternary ammonium both at 1.5%, respectively. On the other hand, the best results for the percentage of morphogenic response were 45.75% and 41.75% with 1% sodium hypochlorite and 1.5% quaternary ammonium, respectively (Image 7).

2.2.2. Effect of immersion time on the disinfection of *Morella* sp. explants
The immersion time together with the different concentrations of the disinfecting agents directly influence the death of the explants in the in vitro establishment, the best results were 29% and 20.75% with sodium hypochlorite and quaternary ammonium both in 10 min.

Note: the complete results of the undergraduate thesis will soon be available in the digital library of the Universidad Técnica de Manabí.

2.3. *In vitro* propagation of *Caesalpinia* sp.

2.3.1. Disinfection protocol
The percentage of contamination was 50%.

2.3.2. In vitro establishment
The different explants generated an average of three new shoots, achieving a response in 90% of the plant material (Image 8).

2.3.3. In vitro multiplication

The different explants generated an average of six new shoots, achieving a response in 70% of the plant material (Image 9).

2.3.4. *In vitro* rooting

The new shoots achieved 50% rooting (Image 10).

2.4. Placement of new *Morella* sp. seedlings in black polyethylene bags.

At least 500 new *Morella* sp. seedlings will be placed in black polyethylene bags, which will be kept in nursery conditions (Image 11).

Image 1: Undergraduate students of the Universidad Técnica de Manabí sowing seeds of *Morella* sp in germination trays with peat substrate. © Liliana Corozo

Image 2: *Caesalpinia* sp specimen located in Jima parish. © Mateo León

Image 3: Ripe fruits of *Caesalpinia* sp. © Mateo León

Image 4. Mature seeds of *Caesalpinia* sp. © Mateo León

Image 5: *In vitro* establishment of C*aesalpinia* sp explants. © Mateo León

Image 6: Effect of GA3 on the germination of *Morella* sp. © Liliana Corozo

Image 7: *In vitro* establishment of *Morella* sp apices. © Mateo León

Image 8: Results of *in vitro* establishment of *Caesalpinia* sp explants. © Mateo León

Image 9: Results of *in vitro* multiplication of *Caesalpinia* sp shoots. © Mateo León

Image 10: Results of *in vitro* rooting of *Caesalpinia* sp explants. © Mateo León

Image 11: Placement of new *Morella* sp seedlings in black polyethylene bags with organic substrate. © Mateo León

3. Intensify actions that involve the Jima Community and the teams of the collaborating universities (researchers – lecturers and students)

Related projects:

Title	Reforestation of areas degraded by forest fires in the Tambillo Community Protected Area
Institution:	Universidad de Cuenca
Modality:	Social Outreach Hours
Principal Researcher:	Ph.D. Juan Pablo Iñamagua – Researcher and lecturer

Tentative Title	Evaluation of the production of essential oils of *Morella* sp in three stages of development, from the Tambillo Community Protected Area
Institution:	Universidad de Cuenca
Modality:	Undergraduate thesis
Principal Researcher:	Ph.D. Juan Pablo Iñamagua – Researcher and Lecturer M.Sc. Mateo León – Researcher
Student:	Jessica Sigua

3.1. Local trips to the Tambillo Community Protected Area (TCPA)

August 4th – 5th 2022: a field visit was conducted with PhD Juan Pablo Iñamagua, thesis student Jessica Sigua, and undergraduate students of agronomy at the Universidad de Cuenca. The objectives were a) to identify individuals of *Morella* sp. in three stages of development, b) to verify the geolocation of the individuals considered and the characterisation of the mother trees of the species *Morella* sp., *Clusia* sp., *Hedyosmum* sp., and *Weinmannia* sp. (Image 5), c) to collect seeds of *Morella* sp., *Clusia* sp. and *Hedyosmum* sp. to obtain new seedlings in nursery.

August 13th – 14th 2022: a field visit was conducted with the thesis student Jessica Sigua and undergraduate students of agronomy at the Universidad de Cuenca. The objectives were a) to geolocation and collect botanical samples of *Morella* sp. in three stages of development for preliminary trials of essential oil extraction (Image 6), b) to collect mature seeds of *Morella* sp. to obtain new seedlings in nursery.

September 10th, 2022: a field visit was conducted with the thesis student Jessica Sigua and undergraduate students of agronomy at the Universidad de Cuenca. The objectives were a) to geolocation and collect botanical samples of *Morella pubescens* in three stages of development for preliminary trials of essential oil extraction (Image 7), b) to collect mature seeds of Morella sp. for obtaining new seedlings in nursery.

September 16th, 2022: a field visit was conducted with Ph.D. Juan Pablo Iñamagua, Ph.D. Pablo Borja, and undergraduate students of agronomy at the Universidad de Cuenca. The objectives were a) verify the meteorological sensors (Image 8 - 9), b) verify the geolocation of the individuals considered and the characterisation of the mother trees

of the species *Morella* sp., *Clusia* sp., *Hedyosmum* sp., and *Weinmannia* sp. (Image 10), c) explore the soil conditions in areas degraded by the forest fire (Image 11).

3.2. Evaluation of essential oil production of *Morella* sp. at three stages of development.

3.2.1. Characterisation of *Morella* sp. plants in three stages of development
With the help of the members of the Cooperativa de Desarrollo de la Comunidad Jima Ltda, the areas where *Morella* sp. individuals are found were selected. Five trees were identified for each stage of development, in which variables such as: diameter at breast height, total height, crown height, crown diameter, number of branches and number of leaves per branch were considered.

Developmental stages are understood as the differences in the height of trees in the study area. Individuals with total height between 1 - 2 m, 2.1 - 5 m, and 5.1 - 10 m were considered for the first stage, second stage, and third stage, respectively.

3.2.2. Obtaining of essential oils from *Morella* sp.
The botanical material was collected from different previously selected individuals. The samples were placed at rest at room temperature and under shade.

The extraction of essential oils was carried out by the steam entrainment technique, using a laboratory scale vertical glass distillation equipment (watch video). The mixture of essential oil and hydrolate was collected in polyethylene tubes, then centrifuged and the pure essential oil was recovered to measure its yield. Finally, the samples were stored in amber glass vials and kept refrigerated.

Partial results

4. Intensify actions that involve the Jima Community and the teams of the collaborating universities (researchers - professors and students).

4.1. Local trips to Tambillo Community Protected Area
Between August and September 2022, six additional trips were made. In line with the plan, 77% more additional trips were made.

4.2. Evaluation of the production of essential oils from *Morella* sp. in three developmental stages

4.2.1. Obtaining of essential oils from *Morella* sp
The greatest number of essential oils was obtained from individuals in the second stage of development (Figure 12).

Note: the complete results of the undergraduate thesis will soon be available in the digital library of the University of Cuenca.

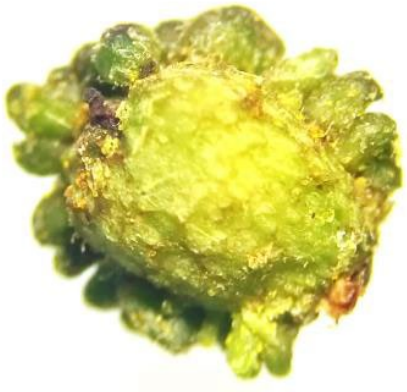

Image 1: Left) *Morella* sp tree. Top right) Leaves and shoots. Middle right) Mature seeds. Bottom right Commercially mature seed with part of the waxy layer removed traces of wax can be seen. © Mateo León

Image 2: Left) *Clusia* sp tree. Top right) Fruit at commercial maturity. Middle right) and Bottom right) Immature fruit. © Mateo León

Image 3: Left) Tree of *Hedyosmum* sp. Top right) Fruit at commercial maturity. Middle right) and Bottom right) Immature fruit. © Mateo León

Image 4: Left) *Weinmannia* sp tree. Top right) Leaves. Middle right) Leaves and fruits, Bottom right) Immature fruits. © Mateo León

Image 5: Characterization of mother trees of Hedyosmum sp. © Mateo León

Image 6: Collection of plant samples from *Morella* sp by Jessica Sigua undergraduate student. © Mateo León

Image 7: Collection of plant samples from *Morella* sp by Jessica Sigua undergraduate student. © Mateo León

Image 8: Verification of meteorological sensors. © Mateo León.

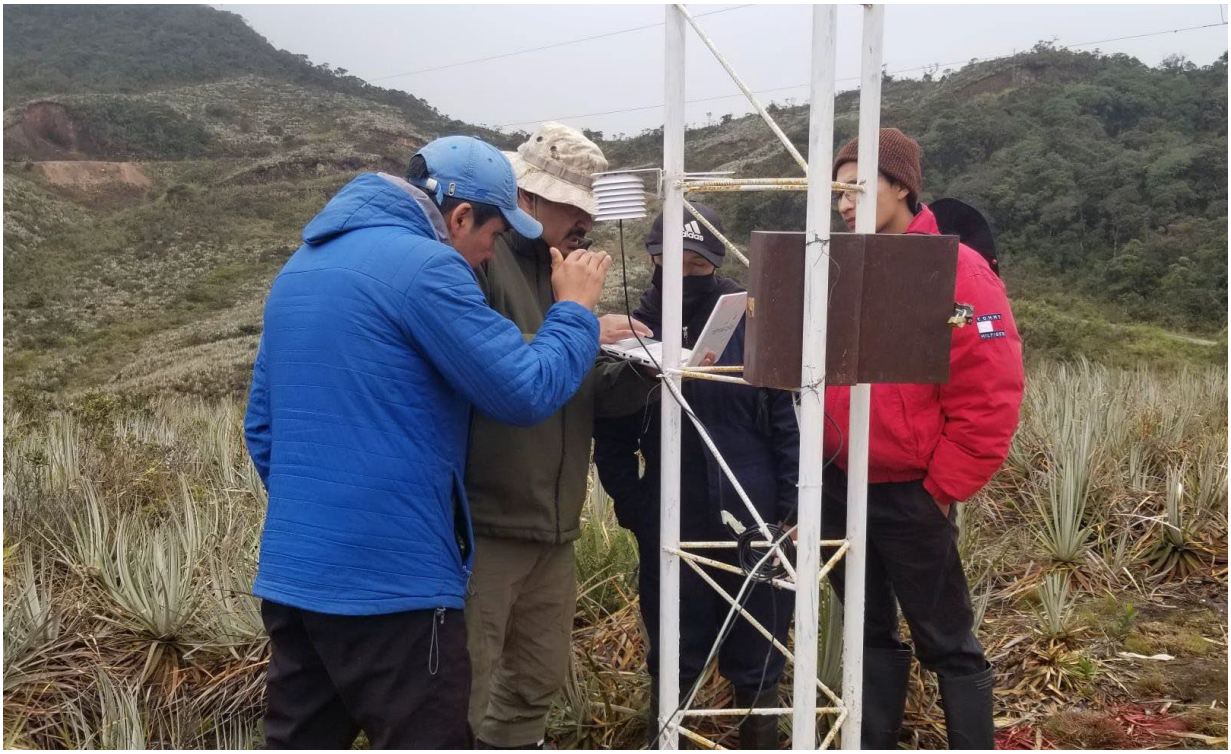

Image 9. Verification of meteorological sensors. © Mateo León.

Image 10: Characterization of mother trees of *Hedyosmum* sp. © Mateo León

Image 11: Exploration of soil conditions in areas degraded by wildfire. © Mateo León

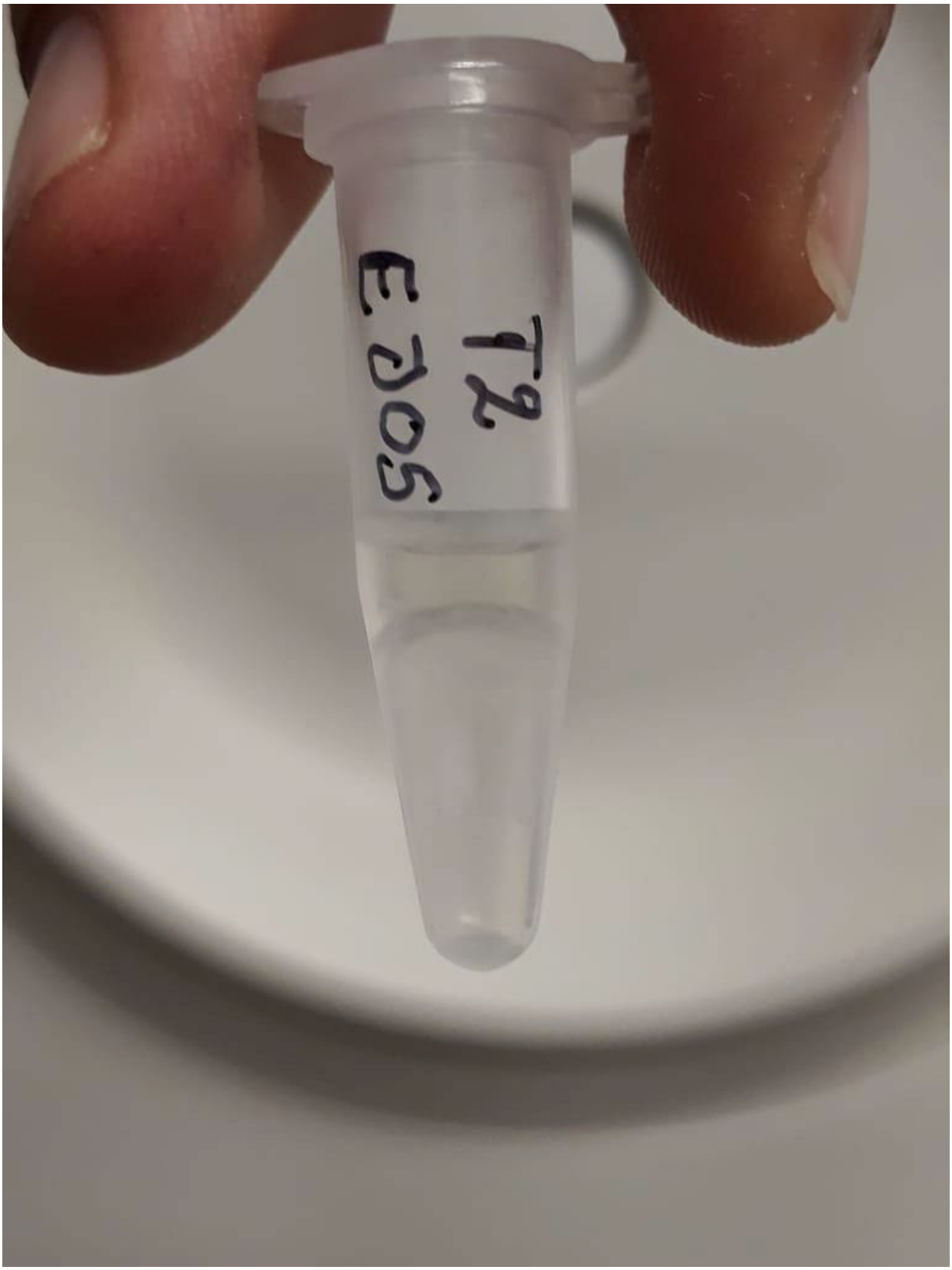

Image 12: Mixture of essential oil and hydrolate of *Morella* sp. © Mateo León