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IN VITRO PROPAGATION OF MYRTUS NIVELLEI BATT ET TRAB., A THREATENED SPECIES FROM THE SAHARAN REGION OF ALGERIA.

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Abstract: Myrtus nivellei Batt et Trab. is a shrub belonging to the Myrtaceae family. Our study aims to valorize this plant widely used in medicine, and to multiply and intensify its cultivation through in vitro culture methods to facilitate the extraction of medicinal substances, the pharmaceutical manufacturing process, and most importantly, to prevent its extinction.

We explored two techniques:
In micropropagation, Myrtus nivellei was cultured in MS and WPM media. MS medium enriched with 1 mg/L BAP and 0.132 mg/L GA3 showed high success rates, while WPM medium with 0.5 mg/L BAP, 0.25 mg/L BAP, and 0.2 mg/L IAA did not yield conclusive results.
In callogenesis, we studied the effect of MS medium on growth systems using leaves cultivated at 25°C in darkness, supplemented with ANA, 2,4-D, and kin. This study established an effective protocol for laboratory micropropagation through the culture of tender axillary buds.
Keywords: Myrtus nivellei, in vitro culture, micropropagation, callogenesis, conservation.

I. INTRODUCTION

Algeria, the largest country in Africa, is characterized by impressive plant diversity, resulting from its varied topography that includes coastal areas, mountains, steppes, oases, and the vast Sahara Desert. The latter hosts numerous unique plant species, some of which are threatened by extreme climatic conditions and human activities. Among these species, Myrtus nivellei Batt. and Trab., belonging to the Myrtaceae family, holds a special place. This aromatic and medicinal plant, endemic to the Saharan regions of Algeria and Chad, is recognized for its therapeutic properties and its use in traditional medicine.

Myrtus nivellei stands out for its exceptional ability to survive and adapt to extreme drought and heat conditions. However, this species is currently threatened by the degradation of its natural habitat,

exacerbated by climate change and anthropogenic pressures. The conservation of this rare and valuable plant has therefore become a priority.

In vitro culture emerges as an innovative and effective method for the conservation and multiplication of Myrtus nivellei. This technique not only helps to preserve the species from extinction but also enables the production of essential bioactive substances without relying on natural sources, which are often limited and vulnerable. By circumventing constraints related to climate, seasons, water availability, diseases, and pests, in vitro culture offers a sustainable solution to meet the growing demand for plant raw materials in the medicinal and pharmaceutical industries.

Our study focuses on optimizing the in vitro culture conditions of Myrtus nivellei, with specific objectives:

- ✓ **Conservation**: Preserve this threatened Saharan species by creating an ex situ genetic reserve.
- ✓ **Valorization**: Exploit the medicinal and aromatic potentials of Myrtus nivellei for industrial use.
- ✓ **Multiplication**: Ensure massive and controlled production of this plant to meet the demands of various industries while protecting natural populations.

The results obtained will not only contribute to the safeguarding of this unique species but also to the promotion of a sustainable and ethical use of Saharan plant resources.

II. MATERIAL AND METHODS

The study was conducted at the CRBT laboratory: Biotechnology Research Center of Constantine during the period (January - June 2023).

II.1. Presentation of the sampling area "The Tassili n'Ajjer"

The Tassili n'Ajjer is a vast mountain range located in the northeast of the Hoggar, covering an area of 7,200,000 hectares. Its expansive rocky plateaus are characterized by imposing cliffs that reach over 1,800 meters above sea level in the Djanet region **(Freulon, 1964)** (figure 1). This region is also renowned for its exceptional biodiversity adapted to the arid conditions of the Sahara, housing unique plant and animal species that have evolved to survive in extremely hot and dry environments. Due to its cultural, geological, and ecological significance, the Tassili n'Ajjer was inscribed on UNESCO's World Heritage List in 1982.

The climate of the Tassili is characterized by an arid desert with a low humidity rate of less than 26%, receiving approximately 50 to 100 millimeters of annual precipitation **(Verschuren, 1984)**. The species Myrtus nivellei Batt et Trab. was collected 146 km from the capital of the wilaya of Djanet, at a station located 94.8 km south of Ihrir, within the Tassili National Park.

Figure 1: Geographic map of the Tassili n'Ajjer region (Djanet) showing the collection site of the species M. nivellei Batt et Trab. (http://www.oopartir.com/photos/algerie-sud-carte,692.jpg).

II.2. Culture medium:

- **Composition of the tested media**

  For micropropagation: we tested two culture media: MS **(Murashige and Skoog, 1962)** and WPM: Woody Plant Medium **(Loyd and McCown, 1980)** (Table 1).

  For the callogenesis of Myrtus nivellei Batt and Trab., we only tested the MS medium **(Murashige and Skoog, 1962).**

Table 1: Composition of MS and WPM culture media (mg L-1).

	MS Medium	WPM Medium
Macroelements		
NH_4NO3	1650	400
CANO3	-	360
CACL2.2H2O	440	96
MGSO4.7H2O	370	180
KH2PO4	170	170
K2SO4	-	990
KNO3	1900	-
Microelements		
MNSO4H2O	22.3	22.3
ZNSO4.7H2O	8.6	8.6
H3BO3	6.2	6.2
KI	0.83	-
CUSO4.5H2O	0.025	0.025
NA2MOO4.2H2O	0.25	0.25
COCL2 6H2O	0.025	-
NaFe EDTA		
NAFE EDTA	37.25	37.25
FESO4.7H2O	27.85	27.85
Vitamins		
Nicotinic acid	0.5	0.5
Thiamine HCL	0.1	1
Pyridoxine HCL	0.5	0.5
Glycine	2	2

II.3. the tested hormonal combinations

- **For micropropagation**: Different concentrations of growth hormones BAP, IAA were added to the culture media (Table 2).

Table 2: Hormone compositions of the two media used in micropropagation.

Medium		Hormone Concentration (mg/L)	
		BAP	**IAA**
MS	**S_1**	1	-
WPM	**P_1**	0.5	-
	P_2	0.25	0.2

- **For callogenesis**: different concentrations of growth hormones kin, 2.4D, and ANA were added to the culture media with six combinations (Table 3).

Table 3: Hormone compositions of the two media used in callus induction.

	ANA	2.4D	KIN
M_0	Control (no hormones)		
M_1	0.01 mg/L	-	0.01 mg/L
M_2	-	0.5 mg/L	-
M_3	2.5 mg/L	-	2.5 mg/L
M_4	0.5 mg/L	-	0.5 mg/L
M_5	1 mg/L	-	1 mg/L

II.4. Methods

II.4.1. Preparation of stock solutions of culture media

Stock solutions are concentrated solutions of components used in the medium, which we dilute appropriately using precise volumes (Table 4).

Table 4: Composition for preparing stock solutions of MS and WPM culture media (mg L -1)

	Stock solution MS (gr L-1)	Stock solution WPM (gr L-1)	Volume
Macroelements			
NH4NO3	33	8	
CANO3	-	11.12	
CACL2.2H2O	8.8	1.92	
MGSO4.7H2O	7.4	7.4	50 ml
KH2PO4	3.4	3.4	
K2SO4	-	19.8	
KNO3	38	-	
Microelements			
MNSO4H2O	2.23	2.23	
ZNSO4.7H2O	0.86	0.86	
H3BO3	0.62	0.62	
KI	0.083	-	10 ml
CUSO4.5H2O	0.0025	0.025	
NA2MOO4.2H2O	0.025	0.025	
COCL2 6H2O	0.0025	-	
NaFe EDTA			
NAFE EDTA	3.725	3.725	10 ml
FESO4.7H2O	2.785	2.785	
Vitamins			
Nicotinic Acid	0.5	0.5	
Thiamine HCL	0.1	1	1 ml

Pyridoxine HCL	0.5	0.5
Glycine	2	2

II.4.2. Preparation of Fe-EDTA stock solution:

- Pour 600 ml of deionized water into a 1L beaker;
- Add a few drops of NaOH and heat until boiling;
- Remove from heat source;
- Add Na2 EDTA and stir until dissolved;
- Add FeSO4-7H2O;
- Transfer the solution to a 1-liter flask and bring to 1 liter with distilled water;
- Label the flask and store it in the refrigerator.

II.4.3. Preparation of vitamins stock solution:

- Pour 70 ml of deionized water into a 100 ml beaker;
- Weigh and dissolve the specified vitamins (D);
- Transfer the solution to a 100 ml flask and bring to 100 ml with distilled water;
- Label the flask and store it in the refrigerator.

II.4.4. Preparation of hormones stock solution:

- Weigh the desired growth regulators and dissolve them in a few drops of appropriate solvent;
- Dilute with a little water, check the state of solution, and add more solvent if needed;
- Transfer the solution to a 100 ml flask and bring to 100 ml with distilled water, then store it in the refrigerator.

The different stock solutions used are:

- Concentrated 20 times macroelement stock solutions.
- Concentrated 100 times microelement stock solutions.
- Concentrated 100 times Fe chelates stock solution.
- Concentrated 1000 times vitamin stock solutions.
- Growth hormone stock solutions (dissolved in a few drops of appropriate solvents).

All stock solutions are labeled, stored cold, and protected from light. Other hormones (IAA, BAP, KIN, GA3, 2-4 D, ANA) are already prepared at CRBT Constantine.

II.4.5. Preparation of final culture medium solutions

- **For micropropagation:** We prepared 1000 ml of each culture medium (MS and WPM) with continuous agitation using stock solutions of macroelements, microelements, Fe-EDTA supplemented with vitamins, sucrose, and Myo-inositol at required concentrations.

WPM: Divided into two (500 ml x 2), with addition of necessary concentrations of growth hormones. The pH of all culture media used was adjusted to between 5.6 and 5.8 using 1N HCl or NaOH solutions. The culture media were then heated and stirred, and solidified with 3.5 g of agar per solution until boiling (medium becomes clear). Finally, these media were transferred into appropriate containers and labeled.

- **For callogenesis:** We prepared 1500 ml of culture medium (MS) with continuous agitation using stock solutions of macroelements, microelements, Fe-EDTA supplemented with vitamins, sucrose, and Myo-inositol at required concentrations. Subsequently, the medium was divided into six parts (250 ml x 6), with addition of necessary concentrations of growth hormones. The pH of all culture media used was adjusted to between 5.6 and 5.8 using 1N HCl or NaOH solutions.

The culture media were then heated and stirred, and solidified with 1.75 g of agar per solution until boiling (medium becomes clear). Finally, these media were transferred into appropriate containers and labeled.

II.4.6. Sterilization:

a. Sterilization of laboratory instruments:

Metal instruments such as forceps and scalpels, as well as glassware like beakers and culture tubes, were sterilized in an oven at 121°C for 20 minutes. During handling, metal instruments are placed in a high-temperature sterilizer. It is crucial to have duplicate instruments to allow one to cool while the other is in use.

b. Sterilization of culture media:

MS and WPM media are sterilized using steam in an autoclave at 121°C and 1 bar pressure for 20 minutes to eliminate bacteria and molds (**Deore and Johnson, 2008; Purkayastha et al., 2010; Sarika and Menakshi, 2009**). After cooling, the sterilized media are distributed into Petri dishes and stored.

c. Disinfection of plant material intended for use:

Nodal stem segments with buds of the species Myrtus nivellei Batt et Trab., intended for micropropagation, and leaves of the same species intended for callus induction, were disinfected under a laminar flow hood using sodium hypochlorite according to the disinfection conditions outlined in Table 5. This process involved adding 2 drops of Tween 20 and treating with 70% ethanol for 30 seconds, followed by rinsing with sterile distilled water (table 5).

Table 5: Plant material disinfection conditions.

	Concentration	Soaking time
Sodium hypochlorite (NaClO)	10°	20 min
	12°	20 min

II.5. Micropropagation and callogenesis:

II.5.1. Micropropagation:

a. Method

After disinfection, the stems of Myrtus nivellei Batt et Trab. are dissected into nodal fragments under a laminar flow hood. These fragments are then seeded at a rate of 4-5 explants per culture jar. Each jar contains approximately 50 mL of MS culture medium (**Murashige and Skoog, 1962**) supplemented with 8 g/L agar and 30 g/L sucrose, along with hormones (BAP and GA3). Incubation: The jars are placed in a growth chamber at a temperature of 23±1°C under a photoperiod of 16 hours light and 8 hours darkness. After 3 weeks, explants are transferred from the culture tubes containing MS medium supplemented with BAP and GA3 to promote explant growth.

b. Culture multiplication:

After six weeks of culture, the Myrtus nivellei Batt et Trab. vitroplants are divided into nodal fragments and seeded into fresh MS culture medium in test tubes containing 1 ml of BAP. This process is repeated periodically until a sufficient stock of plant material is obtained for further experiments. Since the shoots root spontaneously, no additional treatment with auxins was necessary to promote rooting.

c. Acclimatization of vitroplants:

Vitroplants with well-developed roots are gently removed from the culture medium, washed with distilled water to remove all traces of nutrient medium, and transferred to plastic pots (7 cm in diameter) filled with a mixture of sand and peat (1:1). These pots are placed in an acclimatization chamber at a temperature of 23±2°C with a photoperiod of 16 hours light and 8 hours darkness, maintaining humidity at 70%. The seedlings are irrigated by spraying with distilled water 3 to 4 times a day during the first week, and then twice a week thereafter.

II.5.2. Callogenesis:

The culture initiation was conducted under a laminar flow hood. Leaves measuring 0.5 cm in length were dissected at the base using a scalpel in a sterile Petri dish, separating the blade and the petiole longitudinally. Leaf explants were superficially wounded to create small incisions to promote callus induction. Using forceps, they were delicately placed with the abaxial (lower) surface in contact with the culture medium surface, with four explants per Petri dish arranged in a triangular or quadrilateral pattern. Eight different hormone combinations were tested (six replicates for each hormone combination). Once cultures were established, Petri dishes were loosely sealed with Parafilm. Each dish was labeled with the culture date and hormone concentration.

Following culture initiation, the Petri dishes were placed in a dark culture chamber and incubated at a temperature of 25 ± 2°C.

III. RESULTS AND DISCUSSION

III.1. Results of sodium hypochlorite sterilization

Show that a significant number of explants are contaminated after sterilization with NaClO at 10° immediately following their culture, unlike jars containing NaClO at 12°. These observations are consistent with the findings of **Ruffoni et al., 2010**, who employed the same sterilization protocol using NaClO at 12°.

III.2. Results of Micropropagation

III.2.1. Production of vitroplants:

The first phase of this study focused on producing vitroplants from nodal fragments cultured on MS medium, supplemented with various hormonal combinations (BAP and GA3). The method of explant sterilization proved highly effective, enabling the establishment of aseptic cultures with a high rate of shoot proliferation.

III.2.2. Number of leaves

The response of Myrtus nivellei Batt et Trab. explants to the culture media used for micropropagation resulted in an increase in the number of newly formed leaves from the early stages of recovery. This increase varies depending on the species studied and the experimental culture medium (MS and WPM) (Figure 2).

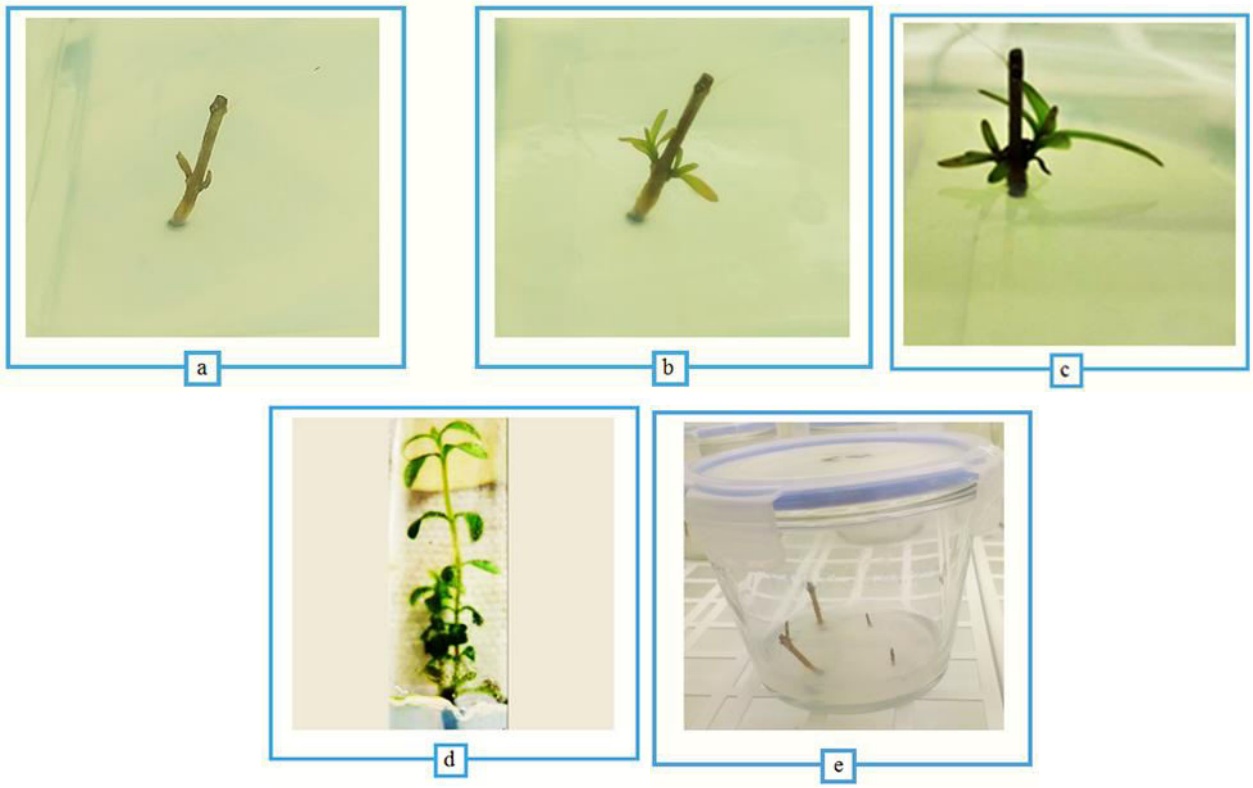

Figure 2: Effect of MS and WPM media on the number of leaves of Myrtus nivellei throughout the trial period (7 days, 15 days, 21 days, 40 days).

The effect of the MS medium is manifested by an increase in the number of leaves in the studied variety during the trial period (Figure 2, 3). Indeed, the number of leaves recorded after 7 days is on average 2 leaves (Figure 2a). After 15 days, a significant increase of more than 65% in leaf formation is observed (Figure 2b). After 21 days, the number of leaves continues to increase (Figure 2c), reaching 20 leaves after 40 days (Figure 2d).
The WPM medium did not produce any results (Figure 2e).

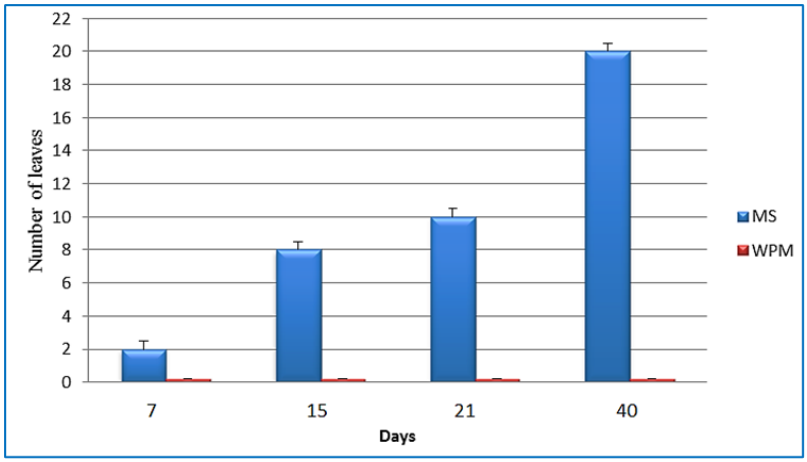

Figure 3: Effect of MS and WPM media on the number of leaves of Myrtus nivellei.

It is then observed that the two media, MS and WPM, show the following differences:

- The MS medium produces more leaves.
- Leaves do not develop in the WPM medium.

III.2.3. Stems length:

Stem elongation is one of the responses to the micropropagation of Myrtus nivellei Batt et Trab. in the culture medium (figure 4).

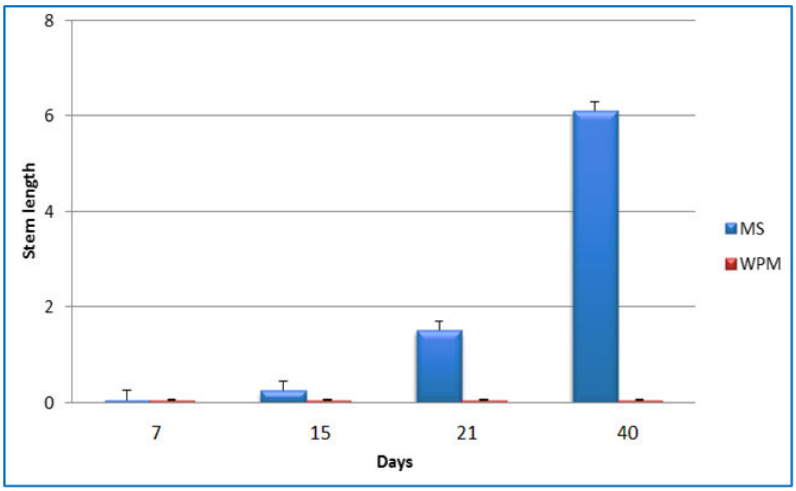

Figure 4: Effect of MS and WPM media on the stem length of M. nivellei Batt et Trab.

The effect is observed through stem elongation in the MS medium during the trial period (Figure 2). The stem length measured after 7 days is 0 cm (Figure 2a). After 15 days, the stem length reaches 0.25 cm (Figure 2b). After 21 days, the stem length reaches 1.5 cm (Figure 2c). Stem growth stops after 40 days at 6.1 cm (Figure 2d).

The presented results indicate that Myrtus can propagate rapidly in vitro.

III.3. Multiplication of vitroplants:

The results of vitroplant multiplication for the Myrtus nivellei Batt et Trab. species:

III.3.1. Number of leaves:

During multiplication, an increase in the number of leaves is observed. The average number of leaves recorded after 7 days is 6 (Figure 5f). After 21 days, the number of leaves further increases (Figure 5 g), reaching 13 leaves after 40 days (Figure 5 h).

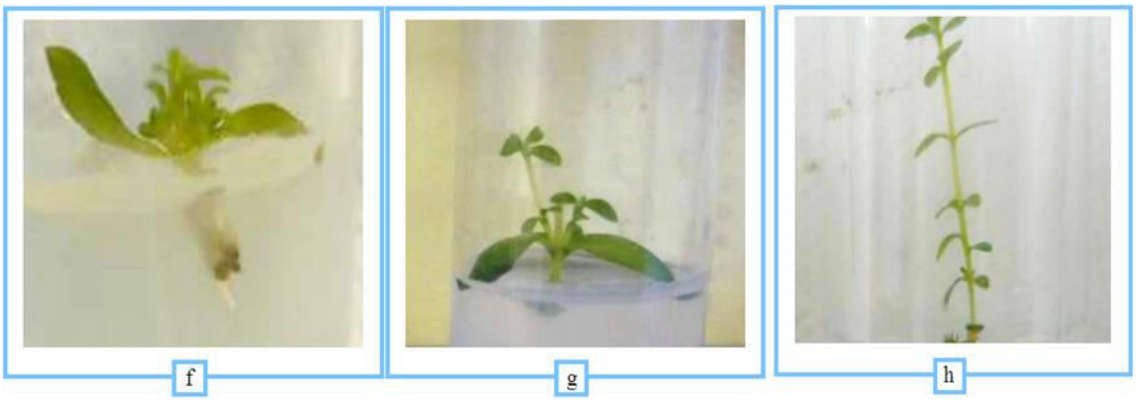

Figure 5: Effect of MS medium on the stem length of M. nivellei Batt et Trab.

The number of leaves increases over the weeks, reaching 13 leaves by the sixth week. This increase in leaf number leads to longer stem growth, consistent with findings by **Razaee et al., 2014** (figure 6).

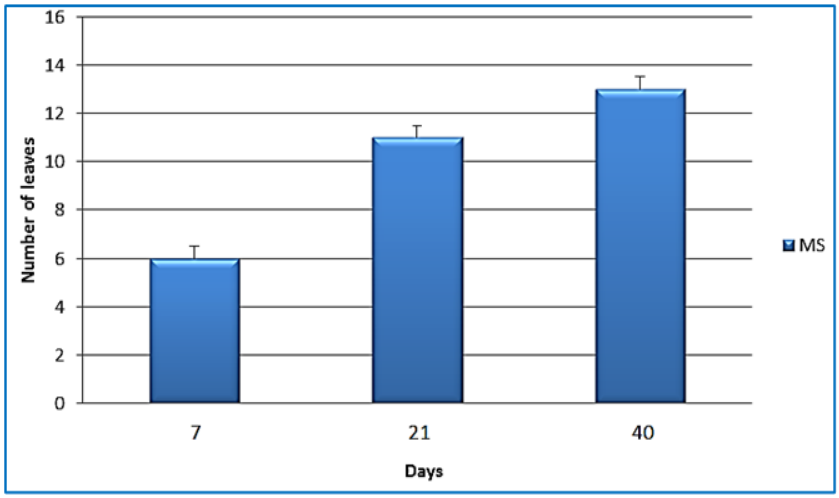

Figure 6: Effect of MS medium on the number of leaves of Myrtus nivellei

III.3.2. Length of stems:

Stem elongation during the trial period continues with slightly significant values. The maximum stem length is 1.06 cm after 7 days, 3.1 cm after 21 days, and 6.50 cm after 40 days (Figure 7).

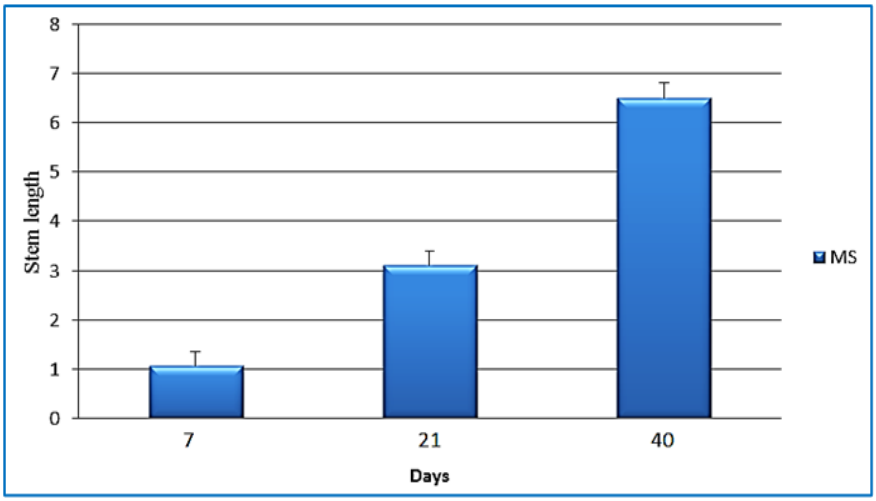

Figure 7: Effect of MS medium on the stem length of M. nivellei Batt et Trab.

III.3.3. Root length:

Rooting is a crucial stage in vitro culture, as it ensures successful acclimatization.

Figure 8: Root length of M. nivellei Batt et Trab.

All seedlings were rooted in MS medium.

The highest rooting rate is recorded after 6 weeks, with a length of 3.9 cm, which is close to the findings of **Ozhan et al., 2017**, who achieved a length of 5.20 cm (figure 9).

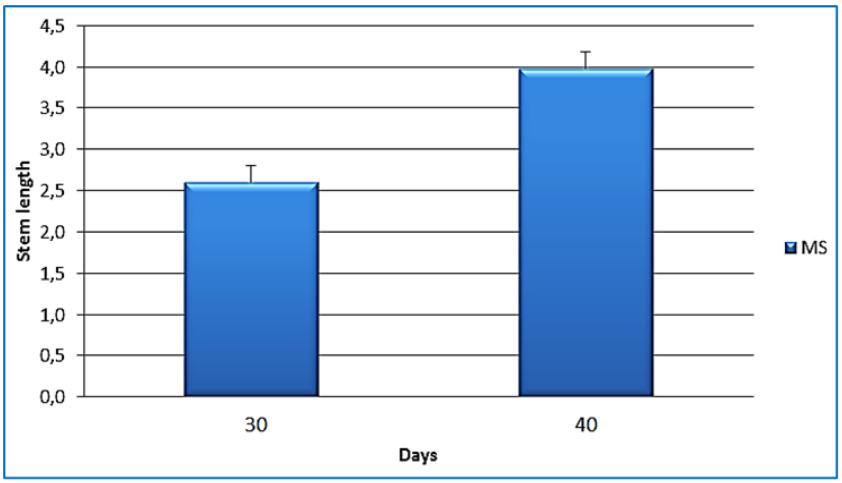

Figure 9: Effect of MS medium on the root length of M. nivellei Batt et Trab.

III.4. Callogenesis results:

The failure of callogenesis can be attributed to the disinfection method used. The high concentration of disinfectant causes continuous release of phenols, which, when oxidized, become toxic and lead to cell death (figure 10). Phenols can also disrupt the exchange between the explant and the culture medium **(Margara, 1989)**.

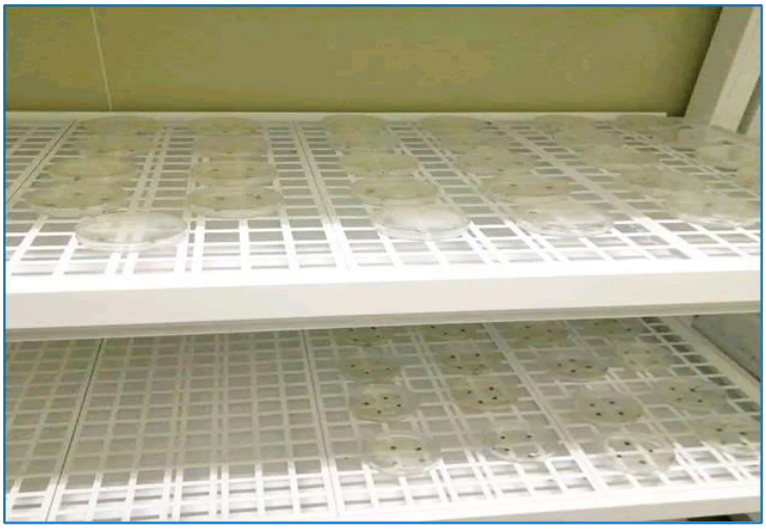

Figure 10: Callogenesis results of Myrtus nivellei Batt et Trab.

IV. CONCLUSION

In our study, we examined two plant propagation methods: micropropagation and callogenesis. Micropropagation proved effective, allowing rapid multiplication and the production of numerous healthy plants from a single initial specimen. However, callogenesis did not yield the expected results despite our efforts to induce callus formation and obtain viable plants. Challenges encountered included high disinfectant concentration, inappropriate growth conditions, or potential incompatibility with our plant line.

These observations underscore the importance of selecting the appropriate propagation method based on specific research objectives and the particular characteristics of the plant under study. Further research will be necessary to deepen our understanding of callogenesis and to optimize growth conditions, thereby improving success rates in our experimental system.

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