# Optimisation of the *in vitro* Regeneration and Callogenesis of *Eucalyptus sideroxylon*, a Natural Resource of Eucalyptol

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***Abstract***

*The use of biotechnological techniques for the large-scale production of plant secondary metabolites is increasingly recognized as a promising alternative, especially in light of the ongoing climatic changes that are significantly reducing the arable lands. Eucalyptus sideroxylon, is a medicinal, industrial and ornamental tree species with significant economic and ecological potentials. The foliage of this species provides a natural bioresource for the production of essential oils with Eucalyptol content exceeding 85%. In this study the in vitro regeneration and callogenesis of this species were assessed based on various experimental conditions. The initiation of axenic in vitro cultures revealed a 100% response on Murashige and Skoog culture medium phytohormone-free. The meta-topoline riboside (mTR) concentration 2.5 µM engendered the best multiplication result with a response of 100%, the highest number of microshoots per explant (9.17) and microshoots length (0.90 cm).*

*The effect of indole-3-butyric acid (IBA) on rhizogenesis after 4 weeks of in vitro culture revealed that the concentration 5 µM resulted in the highest rooting response (95%), the highest roots number (6.33) and the highest roots length (1.95 cm). The initiation of callus from leaves, stems and roots resulted in a 100% response with both applied phytohormones, 2,4-D and Picloram, at concentrations of 5 µM and 10 µM. A variation in the colour (green, red-pink, yellow) and texture (compact, friable) of the induced calli was observed according to the applied experimental conditions. Further investigations are required to improve the in vitro regeneration protocols for this species, thereby enhancing the sustainable biotechnological production of high-value secondary metabolites.*

**Keywords:** *Eucalyptus sideroxylon*, *In vitro* regeneration, Callogenesis, Phytohormone, Medicinal plant.

## Introduction

The plant species *Eucalyptus sideroxylon* Cunn. ex Woolls (*Myrtaceae* family), commonly referred to as Red Ironbark, is known for its medicinal and aromatic properties. The distinguishing features of this species include its deeply fissured, hard and dense bark resembling black iron, along with its large branches and attractive pink or red flowers.[21] While considered native to Australia, this species has been introduced to several regions worldwide where it has successfully grown under different bioclimatic conditions. In Tunisia, Red Ironbark has been introduced and primarily cultivated for its valuable timber, pulp and extracts which possess medicinal properties and find therapeutic applications.[9]

This species is commonly cultivated as a shade tree and appreciated for its ornamental value. The flowers of this species attract bees, making it a reliable source for honey and nectar production. Additionally, it serves as an excellent winter habitat, providing sustenance for birds, squirrels and sugar gliders. The wood derived from *E. sideroxylon* is highly valued for its exceptional quality and durability, making it suitable for various uses, such as construction, sleepers, posts, piers and even boatbuilding.[21] This species is recognized as one of the most significant short-rotation hardwoods and is extensively planted worldwide to meet the demands of the pulp and paper industry.[24] Traditionally, the barks of this species have been utilized as a valuable source of medicines, materials and energy. Today, they are also considered a promising resource for bio-refineries.[20]

Moreover, this tree species holds great significance due to its valuable secondary metabolites. The foliage and bark of *E. sideroxylon* are abundant sources of terpenes, phenolics, flavonoids and tannins, as evidenced by scientific studies.[4,21,26] The extracts of *E. Sideroxylon* revealed a wide range of biological properties including their potential for managing multidrug-resistant bacterial infections[26], antifungal activity, cytotoxic effects on MCF7 cells[3], anti-inflammatory properties[4], antiviral activity[25], antioxidant capacity[21], as well as acaricidal and repellent effects.[29]

The essential oils derived from *E. sideroxylon* serve as a natural source of Eucalyptol (1,8-cineole), with reported contents up to 76% in Tunisia[9] and 86% in Morocco.[18] 1,8-cineole exhibits a wide range of pharmacological properties including anti-inflammatory and antioxidant effects as well as therapeutic benefits against respiratory and cardiovascular diseases among others.[8] Therefore, the concentrations of 1,8-cineole in eucalyptus essential oil determine its medicinal value.[9]

Although *E. sideroxylon* holds significant medicinal and economic values, it has been noted that climate change had adverse effects on this species. According to previous reports, *E. sideroxylon* is projected to face an increased risk of habitat loss in the future.[31] Thus, the optimisation of *in vitro* culture techniques for the sustainable production of high-value secondary metabolites and the conservation of the genetic resources of this species is required. Cell, tissues and organ *in vitro* culture technologies, offer wide application in the sustainable production of plant secondary metabolites under controlled and optimal conditions, independent of external climate fluctuations.

Furthermore, *in vitro* culture offers the opportunity to enhance the production of valuable secondary metabolites.[28] This was possible through the optimisation of *in vitro* culture conditions and the employment of elicitation strategy[7]. This controlled approach ensures a consistent and sustainable supply of high value secondary metabolites with wide application in pharmaceutical, cosmetics and agrochemical industries.[34]

Taking into account the preceding considerations, this study focuses on developing an experimental protocol for the *in vitro* regeneration of *E. sideroxylon*, using seeds as initiation explants. Additionally, this investigation aims to induce callus formation from various explants including leaves, stems and roots by applying different phytohormones at various concentrations. The obtained results will be useful for the sustainable *in vitro* production of high-value secondary metabolites from *E. sideroxylon*, which have extensive industrial applications. Furthermore, the obtained findings will provide a pivotal starting point for both the conservation and the large-scale propagation of *E. sideroxylon*.

## Material and Methods

**Plant material:** The mature fruits of *E. sideroxylon* were randomly gathered from a well-established mother tree situated in the Korbous Arboretum, located in the sub-humid bioclimatic zone in North-Eastern Tunisia (Cap Bon region). The tree thrives in a limestone soil type, with an annual rainfall measuring approximately 500 mm per year. Its specific geographical location lies at an altitude of 400 meters. Seeds collection took place in March 2022, which corresponds to the species' fruiting period. The samples were dried in a shaded area under a dry and well-ventilated environment.

**Initiation of *in vitro* axenic culture:** To initiate axenic *in vitro* cultures, the seeds obtained from the studied species' fruits were carefully extracted and rinsed with tap water followed by surface treatment using 70% ethanol for 1 minute. Subsequently, they underwent sterilization in a solution composed of commercial bleach (90%) and tween 20 (0.01%) during 20 minutes. This process was followed by rinsing five times with sterile distilled water, each lasting for 5 minutes. Murashige and Skoog[23] MS medium, phytohormone free, was employed for the initiation of the *in vitro* culture. The pH of the medium was adjusted to 5.8 prior to solidification using 0.8% agar. The medium was then autoclaved at 121°C for 20 minutes. The seeds were gently pressed into the culture medium in pre-sterilized tubes using sterilized forceps. All these procedures were conducted under a laminar flow hood to maintain aseptic conditions.

Following inoculation for 24 hours in darkness, the tubes were placed in a culture room under controlled conditions including a photoperiod of 16/8 hours and a temperature of 25 ± 1°C. After four weeks of *in vitro* culture, the multiple axenic shoots were divided into two separate batches. One group was utilized for testing and evaluating the *in vitro* regeneration using nodal segments as explants, while the second group was designated for assessing callogenesis induction assays.

***In vitro* multiplication:** The *in vitro* culture of nodal explants was assessed in glass jars containing 80 mL of MS culture medium supplemented with different concentrations of the cytokinin meta-topoline riboside (mTR): 0, 2.5, 5 and 7.5 µM. After 4 weeks of *in vitro* culture, the effect of the different hormonal conditions was assessed. The evaluation involved measuring the average number of microshoots, the average microshoots length (in centimetres) and callogenesis rates.

**Rhizogenesis:** To induce root formation, the regenerated microshoots were inoculated in MS culture medium supplemented with various concentrations of indole-3-butyric acid (IBA). The tested concentrations were 0, 2.5, 5 and 7.5 µM. After four weeks of *in vitro* culture, the root induction rates, as well as the average number and length of the formed roots, were recorded.

**Callus induction:** To initiate *in vitro* callus, each type of explant (leaves, stems, roots) was placed in sterile Petri dishes containing 10 mL of MS culture medium. The medium was enriched with two types of auxins, each at two concentrations: 2,4-D (5 µM and 10 µM) and Picloram (5 µM and 10 µM). After inoculation, the Petri dishes were kept in darkness for a period of 7 days. Following this initial dark phase, a photoperiod of 16/8 hours was adopted, along with a temperature of 25 ± 1°C. The effect of the different phytohormones and concentrations on the inoculated explants was evaluated after four weeks of *in vitro* culture. This assessment included determining the percentage of callus induction, as well as evaluating the morphology and the colour of the induced calli.

**Data analysis:** The variations of the studied parameters under different experimental conditions were assessed using one-way analysis of variance (ANOVA). Mean values were compared using Duncan's test at a significance level of 0.05. The statistical analyses were conducted using the software IBM SPSS Statistics version 28.0 for Windows.

## Results and Discussion

***In vitro* culture initiation:** The first part of this study aimed to develop a simple, rapid and efficient micropropagation protocol for the species *E. sideroxylon* using seeds as initial explants. The obtained results show that the initiation rate of axenic cultures in a hormone-free MS culture medium is 100% for the studied species (Fig. 1). *In vitro* germination allowed the *in vitro* regeneration of seedlings with an average aerial part length of 1.44 cm and an average root length of 4.12 cm. The average number of leaves and newly formed nodes reached 7.30 and 3.65 per vitroplant respectively.

***In vitro* multiplication of *E. sideroxylon*:** The *in vitro* multiplication phase involved the cultivation of multiple shoots in the MS culture medium, either with or without concentrations of the cytokinin mTR. The obtained results revealed variations in terms of shoots induction rates, callogenesis rates, as well as the number and length of the induced microshoots (Fig. 2). Except for the MS medium supplemented with mTR at a concentration of 7.5 µM, which exhibited a shoots induction response of 60%, a 100% response was observed for the remaining mTR concentrations (Table 1).

It is noted that the mTR concentration 2.5 µM resulted in the highest number of microshoots per vitroplant (9.17) and the highest microshoots length (0.9 cm). The concentration 7.5 µM of the phytohormone mTR was the only concentration that did not lead to callus formation at the base of microshoots. However, callogenesis rates of 85% and 60% were observed following the addition of 2.5 µM and 5 µM mTR respectively. The obtained results showed that high concentration of mTR resulted in a negative impact on the *in vitro* multiplication of *E. sideroxylon* microshoots.

**Table 1**
**Effect of meta-topoline riboside on the regeration of *E. sideroxylon* microshoots after 30 days of *in vitro* culture. Values followed by different letters are significantly different (P ≤ 0.05)**

| Treatments | Response (%) | Microshoots number | Microshoots length (cm) | Callogenesis (%) |
|---|---|---|---|---|
| Control | 0 [a] | - | - | - |
| mTR (2.5 µM) | 100 [c] | 9.17 [c] | 0.90 [c] | 85 [c] |
| mTR (5 µM) | 100 [c] | 6.50 [b] | 0.70 [b] | 60 [b] |
| mTR (7.5 µM) | 60 [b] | 4.33 [a] | 0.53 [a] | 0 [a] |

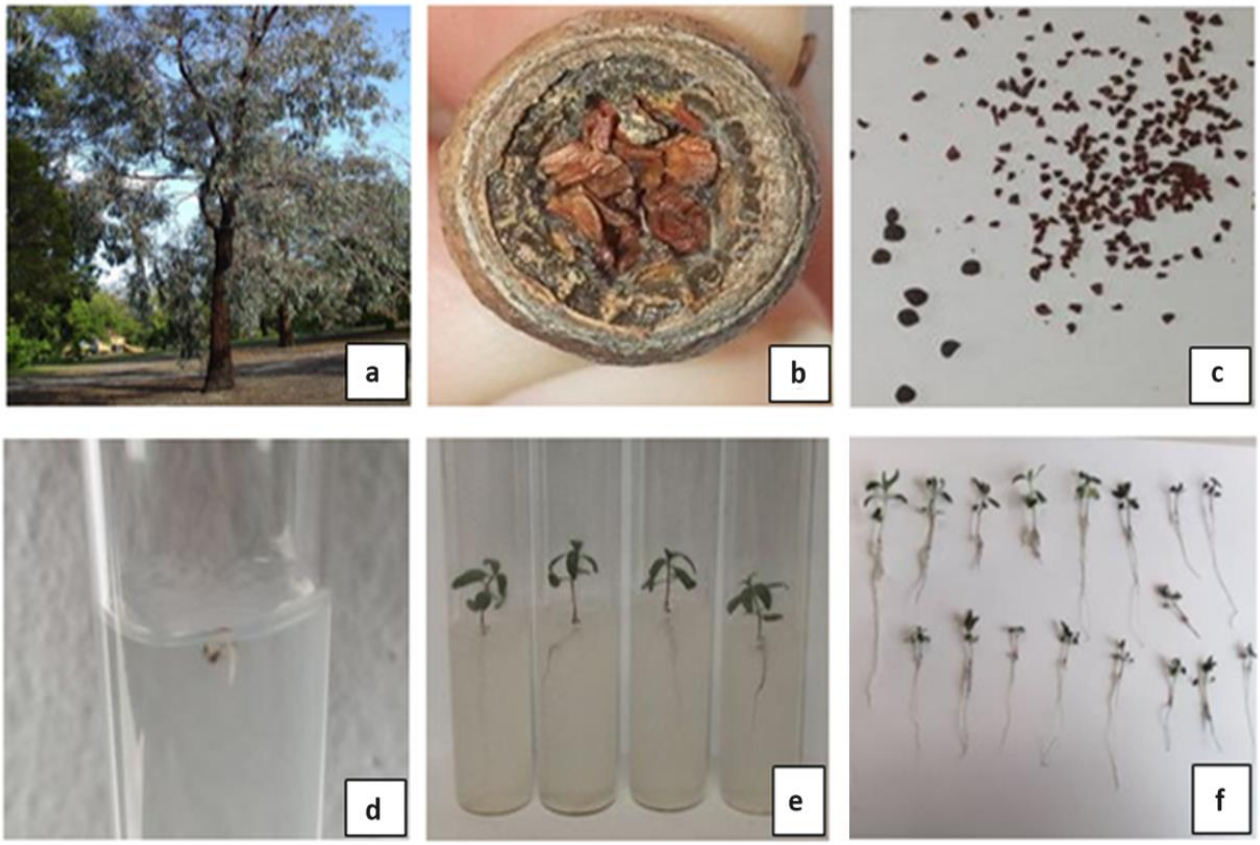


**Figure 1: Initiation of *in vitro* axenic cultures from *E. sideroxylon* seeds. a: mother tree, b: mature fruit, c: seeds, d: *in vitro* inoculation, e, f: axenic *in vitro* seedlings after one month of *in vitro* culture**

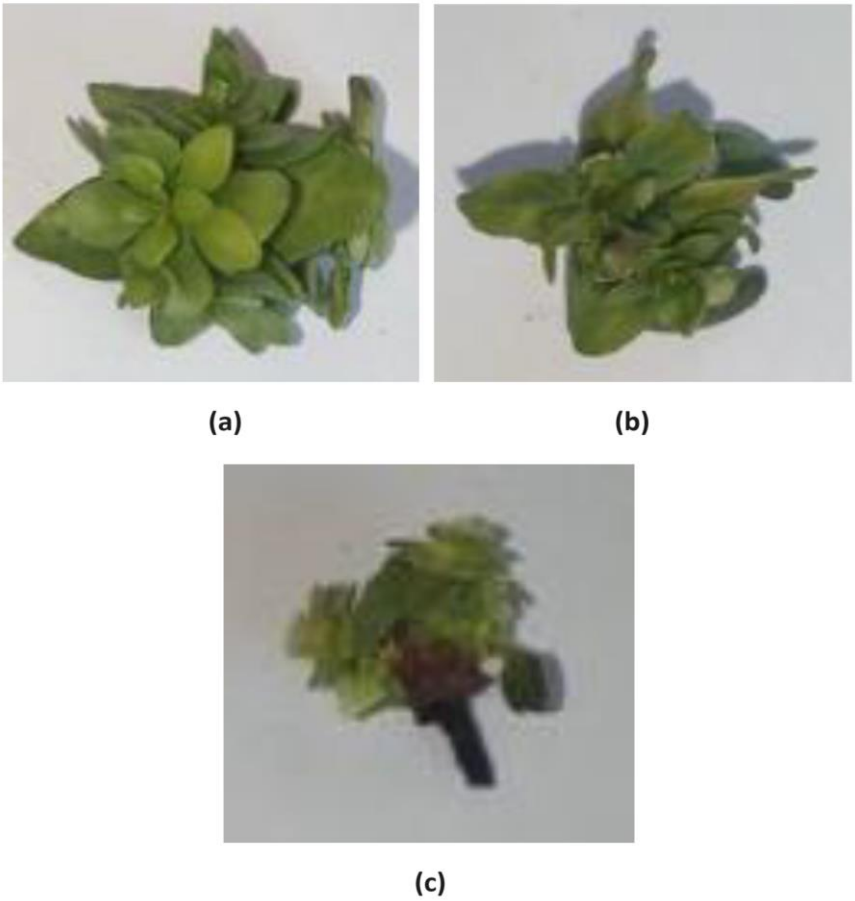


**Figure 2: *In vitro* multiplication of *E. sideroxylon.* (a) 2.5 µM mTR, (b) 5 µM mTR, (c) 7.5 µM mTR**

**Table 2**
**Effect of IBA concentration on the rooting responses after 4 weeks of *in vitro* culture. Values followed by different letters are significantly different (P ≤ 0.05)**

| Treatments | Rooting response (%) | Roots number | Roots length (cm) |
|---|---|---|---|
| Control | 0 [a] | - | - |
| IBA (2.5 µM) | 60 [b] | 1.25 [a] | 0.50 [a] |
| IBA (5 µM) | 95 [d] | 6.33 [c] | 1.95 [c] |
| IBA (7.5 µM) | 75 [c] | 2.60 [b] | 1.50 [b] |

Although N6-benzyladenine (BA) is a widely utilized cytokinin due to its efficacy and cost-effectiveness, its application in plant micropropagation has been associated with various issues such as hyperhydricity, apical necrosis, senescence, rooting inhibition and acclimatization problems.[16] In the recent years, there has been a notable surge in the utilization of meta-topolin (mT) and its derivatives, presenting a promising alternative particularly in the refinement of micropropagation techniques for woody plant species.[30] In comparison to BA, topolin phytohormones have exhibited higher shoot proliferation rates per explant and facilitated easier rooting during both shoot proliferation and rooting phases.[2]

Study by Baroja-Fernandez et al[5] has indicated that cytokinin mTR is nearly twice as effective as others cytokinins in stimulating shoot growth. Furthermore, its efficacy in shoot bud differentiation has also been documented.[17] Similarly, other research has shown that shoot length was significantly enhanced in mediums containing mTR compared to N6-benzyladenine.[6,33]

***In vitro* rhizogenesis of *E. sideroxylon*:** Indole-3-butyric acid (IBA) was tested to allow vitroplants to develop a functional roots system. To induce rhizogenesis, the regenerated microshoots were transferred to MS culture medium supplemented or not with different concentrations of IBA (0, 2.5, 5 and 7.5 µM). Results were recorded after four weeks of *in vitro* culture. The obtained results revealed no detected rhizogenesis for the microshoots transferred to an IBA-free MS culture medium. However, variable percentages of rooting induction were recorded for the three used concentrations of IBA (Table 2).

The highest rooting induction response (95%) was observed with the MS culture medium supplemented with 5 µM IBA.

This phytohormonal condition yielded the highest average number of roots (6.33) and the highest root length (1.95 cm). The concentration 7.5 µM IBA showed the second-best rooting response (75%) with a mean root number of 2.60 and an average root length of 1.50 cm. The concentration 5 µM IBA proved to be the most favourable concentration for rhizogenesis of *E. sideroxylon* microshoots.

Rhizogenesis is a crucial step towards cloning of plant species of economic importance. IBA has been identified as the most effective auxin for initiating root formation in various plant species. Studies have shown that supplementing the growth medium with IBA increases rooting percentage, root number and root length in microshoots.[12] Scientific investigations indicated that IBA surpasses other auxins like NAA and IAA in inducing roots with a higher number per shoot across different plant species[1]. This auxin is widely utilized in the nursery industry worldwide for rooting numerous plant species.[32] Notably, IBA demonstrates significant potential for enhancing root initiation, exhibiting low toxicity and offering improved stability compared to NAA and IAA.[27]

**Callogenesis:** After 30 days of *in vitro* culture, a cluster of undifferentiated cells was developed leading to the formation of diverse callus structures which varied based on the type of phytohormones, their concentrations and the used explants. Culturing different explants on media enriched with 2,4-Dichlorophenoxyacetic acid (2,4-D) or Picloram resulted in callus induction originated from the incisions, then spreading and invading the entire surface of the explant. The initiation of callus from the different explants was successfully induced by the presence of both auxins 2,4-D and Picloram in the MS medium. The MS culture medium auxin free did not allow callogenesis. The callogenesis rate reached 100% for the two applied concentrations of 2,4-D and Picloram (Table 3). However, a variation in the volume, colour and texture of the obtained callus was observed according to the used explants, auxin type and concentrations.

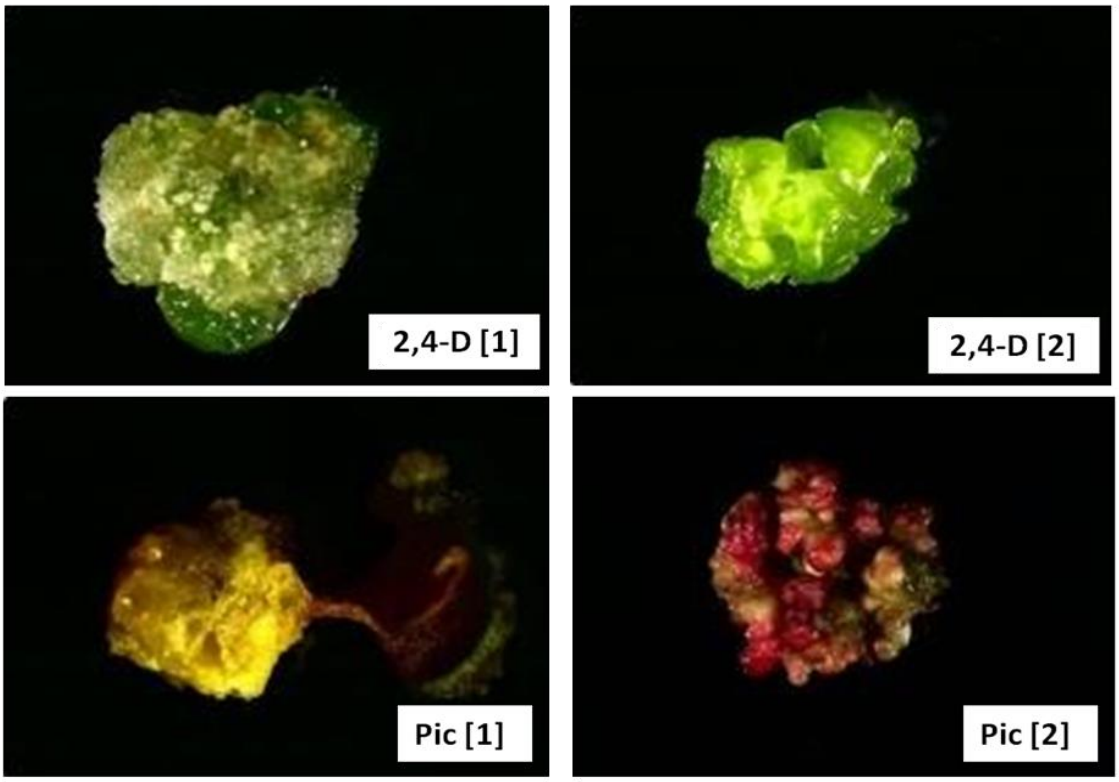


**Figure 3: Variation of colour and texture of calli induced from leaves explants according to the phytohormone type and concentrations. [1] = 5 µM, [2] = 10 µM**

**Table 3**
**Callus induction rates, colour and texture of calli derived from different *E. sideroxylon* explants**

| | | Leaves | | Stems | | Roots | |
|---|---|---|---|---|---|---|---|
| **Treatments** | **Callogenesis (%)** | **Color** | **Texture** | **Color** | **Texture** | **Color** | **Texture** |
| Control | 0 | - | - | - | - | - | - |
| 2,4 D (5 µM) | 100 | Green | Friable | Yellow | Friable | Yellow | Friable |
| 2,4 D (10 µM) | 100 | Green | Friable | Green | Friable | Yellow | Friable |
| Picloram (5 µM) | 100 | Yellow | Compact | Yellow | Friable | Yellow | Friable |
| Picloram (10 µM) | 100 | Red-Pink | Compact | Yellow | Friable | Yellow | Friable |

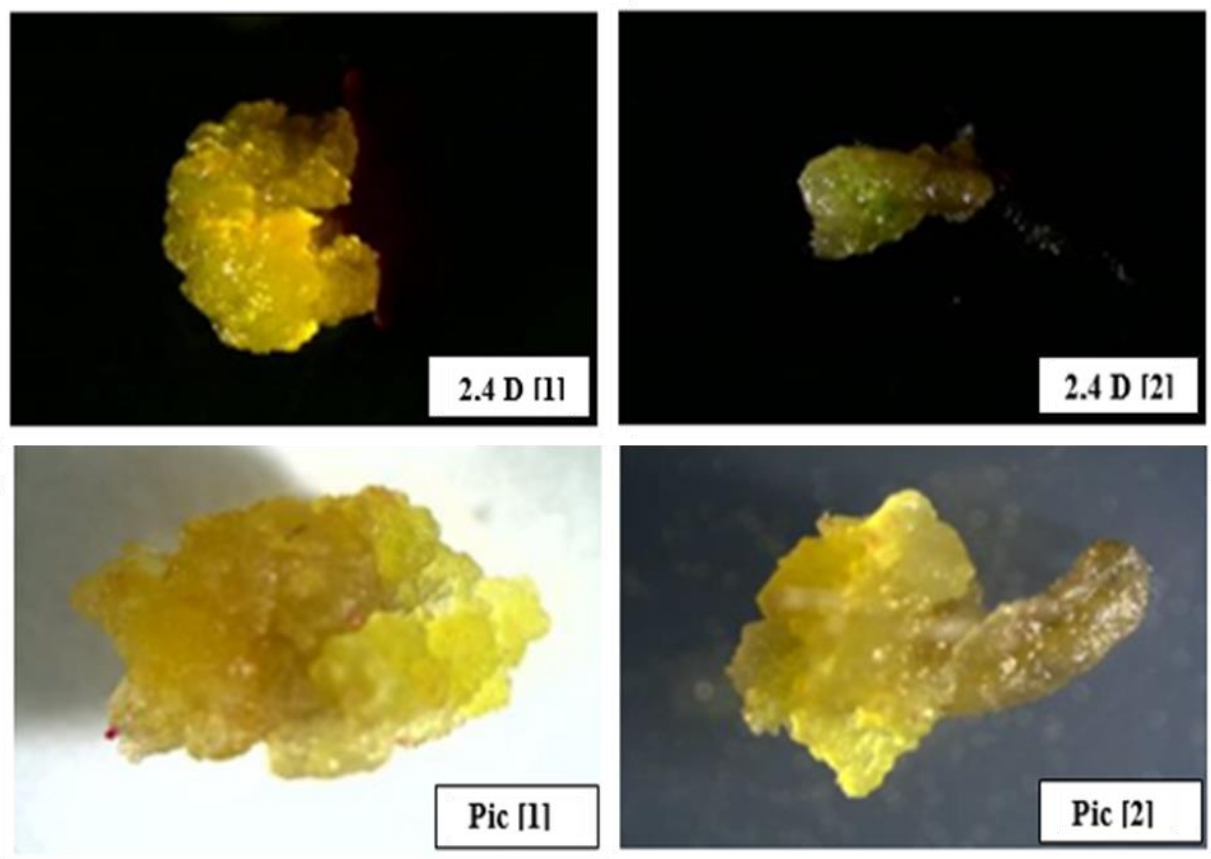


**Figure 4: Variation of colour and texture of calli induced from stems explants according to the phytohormone type and concentrations. [1] = 5 µM, [2] = 10 µM**

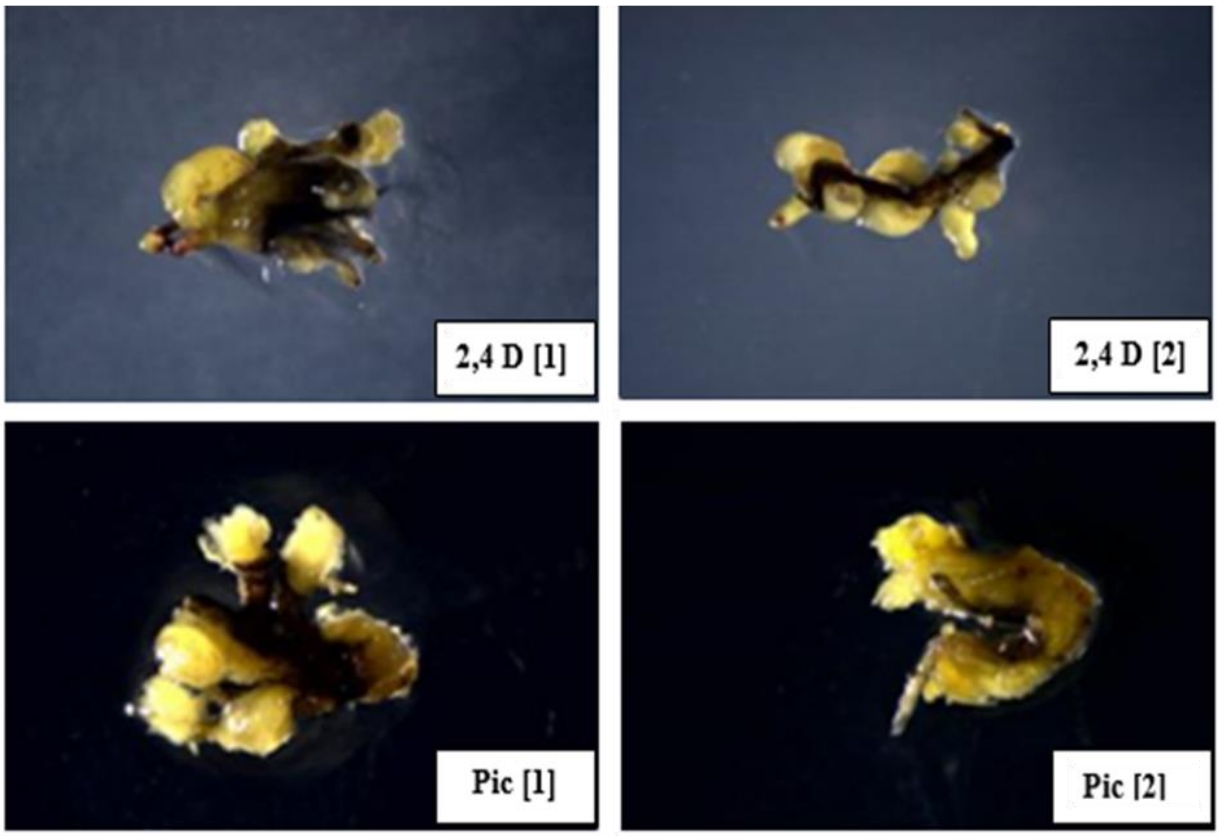


**Figure 5: Variation of colour and texture of calli induced from roots explants according to the phytohormone type and concentrations. [1] = 5 µM, [2] = 10 µM**

The obtained results showed that the application of 5 µM 2,4-D on leaves explants allowed the initiation of green callus with friable texture while the concentration of 10 µM 2,4-D allowed the initiation of light green and friable callus (Fig. 3). The application of 5 µM Picloram on leaves explants produces yellow callus with compact texture. Interestingly, 10 µM Picloram, enabled the initiation of reddish-pink and compact callus from leaves. Concerning stems explants, the regenerated callus with 5 µM 2,4 D was yellow with friable texture and large volume while 10 µM 2,4 D engendered green and friable callus (Fig. 4). Following application of 5 µM Picloram, the induced callus from stems explants was yellow with a friable texture and large volume.

The second concentration of Picloram (10 µM) enabled the initiation of yellow and friable callus. The two tested concentrations of 2,4-D and Picloram on roots originated explants allowed the proliferation of yellow colored calli with friable texture (Fig. 5). Callus culture stands out as an effective method for generating valuable secondary metabolites, particularly in tree species.[15] Optimizing *in vitro* callus culture serves as a tool to produce high value phytochemicals or to facilitate indirect somatic embryogenesis.[11] The initiation of *in vitro* callus is influenced by various factors including the type of explant and phytohormones, as well as their concentrations, all of which play crucial roles.[10] Beyond biomass generation, plant growth regulators (PGRs) impact plant metabolism and the synthesis of biomolecules which hold significance in pharmaceutical, cosmetics, food and agrochemical industries.[19]

The synthesis of secondary metabolites through *in vitro* induced callus appears to be affected by numerous factors including the type and concentration of plant growth regulators.[14] Among plant growth regulators, 2,4-D is widely utilized to induce callus in various plant species[22]. In the recent years, Picloram (4-amino-3,5,6-trichloro picolinic acid), a hormone with auxin-like activity, has emerged as a selective inducer of considerable callus formation[13]. Interestingly, this phytohormone induces colored callus in the studied Eucalyptus species, enhancing its potential for the *in vitro* production of flavonoids and anthocyanins secondary metabolites with wide applications in natural pharmaceutical, cosmetics and dye industries.

## Conclusion

The optimal conditions for *in vitro* regeneration of *E. sideroxylon* microshoots and callus were investigated using Murashige and Skoog (MS) culture medium supplemented with various phytohormones at different concentrations. Results demonstrated that the most effective induction and multiplication of microshoots occurred at a concentration of 2.5 µM of meta-topoline riboside (mTR). Additionally, the highest response for rhizogenesis was observed when the MS medium was supplemented with 5 µM of indole-3-butyric acid (IBA). The application of both 2,4-D and Picloram led to a 100% callogenesis response for the three types of explants: leaves, stems and roots.

Notably, the obtained calli displayed variations in texture and colour, with coloured calli holding particular interest due to their potential for flavonoid and anthocyanin compounds production. Further investigations are required to optimize the experimental protocols and elucidate the factors influencing the *in vitro* accumulation of high-value secondary metabolites in this multipurpose species.

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