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Extraction of secondary metabolites responsible for biological activities from the leaf callus of *Tetracera rosiflora*

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ABSTRACT

Introduction

Tetracera rosiflora Gilg is a plant used in traditional Congolese medicine for its multiple virtues, including antidiabetic effects. However, this species is threatened with extinction due to habitat destruction and overexploitation of its natural resources.

Purpose

To address this issue, it is essential to explore alternative methods for producing its bioactive secondary metabolites, such as through in vitro culture.

Methods

This study aimed to extract the secondary metabolites responsible for biological activities (antidiabetic and anti-amylase) and compare their concentrations in both the wild plant and in vitro-derived callus. A phytochemical screening was conducted on the leaves and various extracts of *T. rosiflora*.

Results

In vitro culture of *T. rosiflora* yielded higher callogenesis rates for stem explants on MS + 2,4-D medium (48%) and for leaf explants on MS + AIB + BAP medium (50%). Quantification of polyphenols revealed that the wild plant contained significantly higher levels (28.13 ± 1.18 mg gallic acid equivalent/g dry mass) compared to the callus, which showed a maximum of 1.41 ± 0.0045 mg gallic acid equivalent/g dry mass.

Conclusion

This study contributes to the potential amplification of valuable secondary metabolites from endangered wild species through callogenesis techniques.

INTRODUCTION

Diabetes is a serious and complex chronic disease that is evolving significantly across all regions of the world. In some parts of the world, before the advent of insulin injections and other pharmaceutical preparations, healers often used medicinal and dietary resources considered and proven to be antidiabetic. In this context, more than 1,200 plants have been experimentally used in the treatment of diabetes (Alarcón et al., 1998; Kang et al., 2005; Scopici et al., 2004).

Nearly 10.5% of the global population is affected by diabetes, and its prevalence is increasing at an alarming rate. According to data from the World Health Organization (WHO), approximately 783.2 million people are projected to be diabetic by 2045 (Ansari et al., 2022b). This disease is responsible for approximately 4 million deaths each year, nearly half of which occur before the age of 70, due to hyperglycaemia (Geiss et al., 2014; Guariguata et al., 2014; International Diabetes Federation [IDF], 2017). The mortality rate is notably higher in low-income countries compared to high-income countries. Among all continents, Africa appears to exhibit the most significant progression of this disease (Bommer et al., 2017). In the Democratic Republic of the Congo (DRC), there are approximately 4 million diabetics, with nearly 50,000 deaths annually, according to WHO statistics from 2017 (WHO, 2017).

Despite encouragement and recommendations by the World Health Organization to promote research on diabetes and the evaluation of traditional medicine (WHO, 2006), only a few of these medicinal plants have been the subject of scientific studies. This area of research remains one of the least prioritised and most underfunded within public health research.

The DRC possesses a rich heritage of medicinal and dietary resources traditionally used to manage several diseases, including diabetes, cardiovascular conditions, and other pathologies (Bellakhdar et al., 1991; Eddouks et al., 2002; Ziyyat et al., 1997).

Tetracera rosiflora is a medicinal plant traditionally used in Congolese medicine for its antidiabetic and other therapeutic properties. However, due to habitat destruction and overexploitation, this species is now threatened with extinction. To ensure a sustainable supply of its bioactive compounds, alternative production methods such as in vitro culture and callus induction must be explored. This study examines the secondary metabolites present in wild *T. rosiflora* and callus cultures to assess their potential biological activity (Hammiche et al., 2006; Hmamouchi et al., 1999; Muanyishay et al., 2018). Traditionally, an aqueous decoction of the plant is taken orally, either alone or mixed with other medicinal plants. To date, no scientific studies have been conducted on the pharmacological properties of *T. rosiflora*.

The scientific literature indicates that *T. rosiflora* contains saponins, including an isolated and partially identified triterpene glucoside saponin (Sandberg et al., 1960, 1962; Segal et al., 1969). Literature data show that some saponins isolated from plants significantly reduce blood glucose levels (Abdel-Hassan et al., 2000; Abdel-Zaher et al., 2005; Nojima et al., 1998). Additionally, saponins have been attributed with various biological activities, including immunostimulant, cytotoxic, and antitumour properties. However, due to its endangered status stemming from habitat destruction and resource overexploitation, there is a pressing need for the in vitro cultivation of this species to produce secondary metabolites of therapeutic interest (Muanyishay et al., 2018).

On one hand, *Tetracera rosiflora* is a vine whose vegetative propagation is seasonal, limiting its availability. On the other hand, its recognised therapeutic potential has led to its abusive exploitation, putting the species at risk of extinction. To address this issue, in vitro propagation techniques offer a viable alternative strategy. These include callus induction, which can increase biomass and allow for the rapid and large-scale production of secondary metabolites.

This study aims to:

i. extract and identify secondary metabolites responsible for antidiabetic and anti-amylase activity in *T. rosiflora*;
ii. compare metabolite content between wild plant material and in vitro callus cultures; and
iii. evaluate the potential of callus culture as a sustainable alternative for metabolite production.

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Therefore, this work aims to compare the secondary metabolite content of *T. rosiflora* to that of its in vitro-derived callus (undifferentiated plant cells), to establish callus cultures of *T. rosiflora* on synthetic medium, and to quantify the compound families responsible for the biological activities observed in both wild and cultured plant material.

METHODS

Plant Material

Plant material for in vitro culture

Young leaves and stems of *Tetracera rosiflora* were harvested on 3 May 2018 in Cité Pumbu, Mont Ngafula Commune, Kinshasa, Democratic Republic of the Congo. The plant was identified at the Herbarium of the National Institute of Agronomic Studies and Research (INERA), housed at the Faculty of Science, University of Kinshasa (UNIKIN), by the botanist Blaise Bikandu. The herbarium number is currently under review.

Methods

Leaves of *T. rosiflora* were cleaned and dried at room temperature (±27°C) for three weeks. The crushed material constituted the dry matter used to extract total aqueous and methanolic extracts of polyphenols, tannins, and flavonoids. Fresh leaves and callus cultures of *T. rosiflora* were subjected to phytochemical screening using standard qualitative tests for saponins, flavonoids, tannins, anthocyanins, quinones, and coumarins.

Tannins were extracted using the method described by Bruneton (2002). Flavonoids were extracted following the method of Markham (1982), with modifications based on the protocol of Bruneton (1993).

Callus induction was performed on Murashige and Skoog (MS) medium supplemented with various phytohormones (2,4-D, IBA, and BAP) to optimise growth conditions. Among the tested media, M1 and M2 yielded the highest organogenesis rates, even in the absence of coconut milk, consistent with previous findings (Rani et al., 2004; Usman et al., 2005). Furthermore, 2,4-D was effective in promoting callus induction from in vitro cultures of nodeless *T. rosiflora* stems (Handaji et al., 2005; Singh et al., 2006). The nature, age, position, and orientation of the explants, as well as their specific nutrient requirements and endogenous hormone content, significantly influenced callogenesis response (Mathews, 1987).

Growth regulators were used at low concentrations, which varied depending on physiological stage and plant part. Auxins such as IBA stimulated cell division and elongation, vascular tissue differentiation, root initiation, apical dominance, and fruit development. Cytokinins like BAP stimulated cell division and differentiation, enhanced bud formation, and counteracted rhizogenesis, contributing to callus induction.

The total polyphenol content in wild and callus samples was quantified using the Folin–Ciocalteu method, and callogenesis rates were recorded.

The treatments applied in this study were the presence or absence of growth regulators and the type of explants. The parameters studied included two explant types (leaves and stems), two culture conditions (total darkness and continuous light photoperiod), and five different media. A completely randomised experimental design was used. Analysis of variance (ANOVA) was conducted using R software version 3.1.1. A mixed crossover model with two classification criteria was employed. In the event of significant differences, results were further analysed using the Least Significant Difference (LSD) multiple comparison test at a 5% significance level.

RESULTS AND DISCUSSION

Phytochemical screening confirmed the presence of saponins, steroids, flavonoids, tannins, quinones, and coumarins in both wild and callus extracts of *Tetracera rosiflora*. The wild leaves of *T. rosiflora* showed significantly higher total polyphenol content (28.13 ± 1.18 mg GAE/g dry mass) compared to the callus cultures (1.4055 ± 0.0045 mg GAE/g dry mass).

In Vitro Culture of Tetracera rosiflora Gilg

A few days after the initiation of culture, callogenesis was observed as swelling at the site of the explant **(Figure 1)**. This was followed by profuse cell proliferation, resulting in the complete coverage of the explant, which eventually became unrecognisable **(Figure 2)**. Twenty days after culture, the initial swelling indicated callus initiation. After a further 25 days, continued proliferation of callus tissue led to complete enveloping of the original explant.

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Figure 1:
A few days after culture, callogenesis was manifested by a swelling of the explant

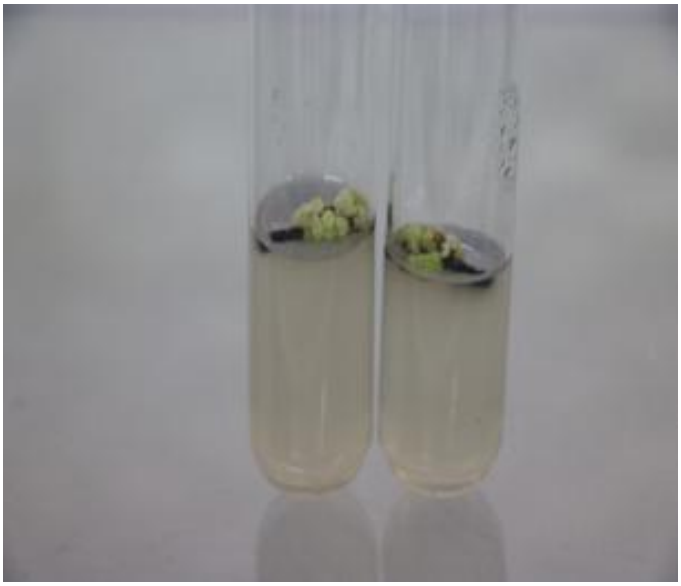
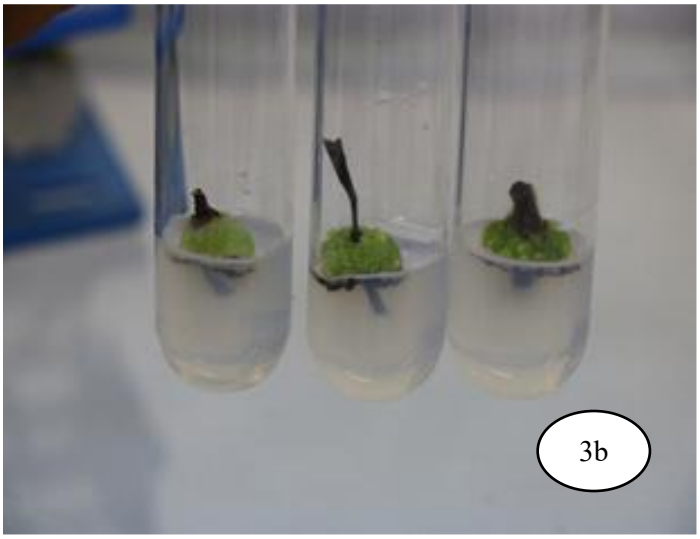


Figure 2:
A proliferation of cells completely recurving the explant which became unrecognizable

Callus Morphology Under Light Conditions

On day 64, a non-compact, whitish callus was observed on MS medium supplemented with IBA and BAP under light conditions **(Figure 3a)**. A day later, a greenish callus of similar texture was observed under the same medium and conditions **(Figure 3b)**. This suggests progressive differentiation under the influence of auxins and cytokinins in light exposure.

Figure 3:
On the left 64-day-old non-compact whitish callus obtained on MS + AIB+BAP medium (culture condition: light) and on the right 65-day-old greenish callus obtained on MS +AIB+BAP medium (culture condition: light)



Effects of Hormone Composition, Explant Type, and Culture Conditions on Callus Growth

Table 1 presents the results of callogenesis across different media compositions, types of explants, and culture conditions.

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Table 1:
Callogenesis results by growth condition, medium composition, and explant type

Growing conditions	Types of medium according to the growth regulators used	Nature of explant	Callogenesis results
Light	Medium M0 (MS without phytohormones)	Stems without nodes	Absence of callus
		Leaflets	Absence of calstigo
	Medium M1 (MS + 2,4-D)	Stems without nodes	Non-compact whitish callus
		Leaflets	Absence of callus
	Medium M2 (MS +AIB +BAP)	Stems without nodes	Non-compact whitish callus
		Leaflets	Absence of callus
Obscurity	Medium M0 (MS without phytohormones)	Stems without nodes	Non-compact whitish callus
		Leaflets	Absence of callus
	Medium M1 (MS + 2,4-D)	Stems without nodes	Non-compact whitish callus
		Leaflets	Absence of callus
	Medium M2 (MS +AIB +BAP)	Stems without nodes	Non-compact whitish callus
		Leaflets	Absence of callus

These findings align with earlier studies, which reported that callus induction is influenced by the presence and combination of growth regulators and the type of explant used (Handaji et al., 2005; Mathews, 1987; Rani et al., 2004; Singh et al., 2006; Usman et al., 2005). Stems without nodes were more responsive to callogenesis than leaflets across all tested media and conditions.

From **Table 1**, it appears that on the M0 medium (Murashige and Skoog [MS] without phytohormones), no cellular activity was recorded for either leaf or stem explants throughout the entire culture period. Incubation of explants on this medium resulted in a complete absence of callus formation in both continuous light and continuous darkness conditions.

The addition of 2,4-dichlorophenoxyacetic acid (2,4-D) at 1 mg/L to the culture medium allowed for callus induction in stem explants without nodes only, with a callus induction rate of 48% under continuous light and 30% under continuous darkness at day 60 of culture. Whitish, non-compact (non-chlorophyllous) calluses were obtained in both light and dark conditions.

The highest callogenesis rate (50%) was recorded on MS medium supplemented with indole-3-butyric acid (AIB) and 6-benzylaminopurine (BAP). The passage through callus formation (callogenesis) is a crucial phase in in vitro selection, as it induces genetic variability (somaclonal variation) by increasing the levels of endogenous phytohormones. It serves as a genetic material reservoir, unlike wild plants whose fluctuating levels of endogenous phytohormones are often influenced by intrinsic factors such as variety, age, organ type, and size.

Muanyishay et al. (2018) reported that callus induction was successful using media M1 and M2, containing BAP with AIB and 2,4-D, respectively, yielding organogenesis rates of 56.4% and 68.6%. They also observed a reduction in callus induction frequency without the addition of coconut milk, as shown in previous studies (Usman et al., 2005; Rani et al., 2004). Furthermore, 2,4-D has been demonstrated to promote callus induction in in vitro cultures of node-free stems of *Tetracera rosiflora* Gilg (Handaji et al., 2005; Singh et al., 2006).

Phytochemical Study on the Extracts

The experimental results presented in Table 2 show the presence or absence of the tested phytochemical groups.

Table 2:
Results of phytochemical tests carried out on the extracts of the leaves of *Tetracera rosiflora*

Chemical group		EE	ED	EET	EM	EA
Polyphenols	Flavonoids	-	+	+	+	+
	Tannins	+	+	+	+	+
	Anthocyanins	-	-	+	-	+
	Linked quinones	-	-	+	+	-
	Coumarins	-	-	-	-	+
Saponins		-	-	+	+	+
Steroids		+	-	+	-	-

Legend: EE: Ethereal extract; ED: Dichloromethane extract; EET: Ethanolic extract; EM: Methanolic extract; EA: Aqueous extract; +: Present; -: Absent

From this **Table**, it can be seen that tannins are present in all extracts. Flavonoids are present in all extracts except the ethereal extract. Only the ethanolic and aqueous extracts contain anthocyanins. Coumarins were detected only in the aqueous extract. Steroids were found in two extracts: ethereal and ethanolic. Saponins were found in three extracts: ethanolic, methanolic, and aqueous. Linked quinones were observed only in the ethanolic and methanolic extracts.

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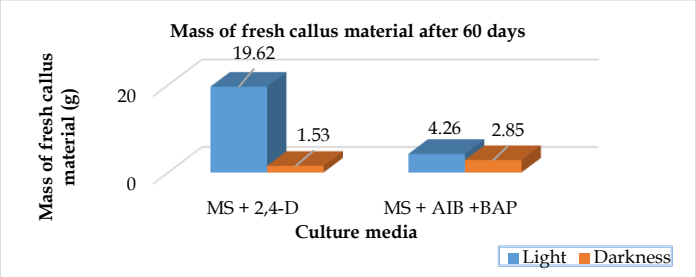
Figure 4:
Variation of callogenesis rate (%) with culture duration for stem explants grown on M1 medium (MS + 2,4-D)



On the other hand, the use of a hormonal combination of an auxin (AIB) and a cytokinin (BAP) at the tested concentrations (1 mg/L each) enabled callus formation in both types of explants used. However, for leaf explants cultured under continuous darkness, incubation resulted in a complete absence of cellular activity. Greenish, non-compact callus was observed under permanent light, with a callogenesis rate of 50% on day 60 of culture. For stem explants without nodes, greenish, non-compact calluses were formed under continuous light with a callogenesis rate of 18.75%, while whitish, non-compact calluses formed under continuous darkness showed a callogenesis rate of 46.67% at day 60 of culture.

Figure 5:
Variation of callogenesis rate (%) with culture duration for leaf explants grown on M2 medium (MS + AIB + BAP).

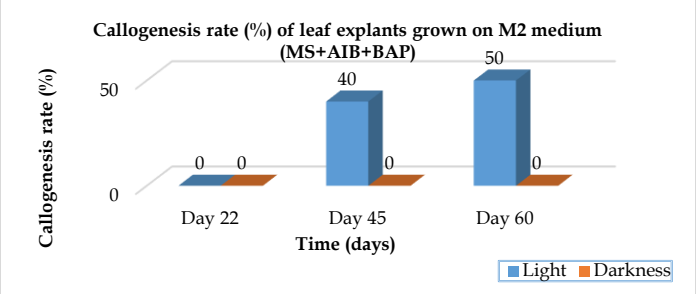
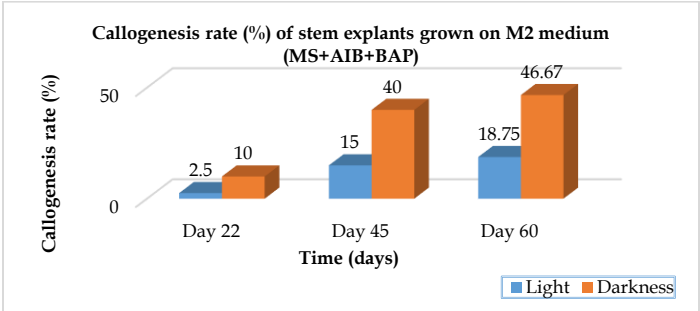


Figure 5. Variation of callogenesis rate with culture duration for leaf explants grown on M2 medium (MS + AIB + BAP).

Figure 6 shows the callogenesis rate (%) of stem explants grown on M2 medium (MS + AIB + BAP).

Figure 6:
Variation in callogenesis rate of stem explants grown on M2 medium (MS + AIB + BAP)



From **Figures 5** to **9**, it is evident that callus growth exhibits variability in response depending on the nature of the explant (stem or leaf), the chemical factors involved (such as the growth regulators used), and the culture conditions. These results are comparable to those obtained by Muanyishay (2018), whose study also reported a complete absence of cellular activity on Mo medium (MS without phytohormones). In particular, incubation of cultures with leaf explants resulted in a complete absence of callus for all types of media used (MS + 2,4-D at 1 mg/L and MS + AIB 1 mg/L + BAP 1 mg/L).

Callus formation (callogenesis) is a crucial stage in *in vitro* selection, as it induces genetic variability (somaclonal variation) by increasing the quantity of endogenous phytohormones in the plant. It can therefore be considered a reservoir of genetic material, unlike wild plants whose fluctuating stock of endogenous phytohormones is influenced by various plant-specific factors such as variety, age, organ type, and size.

Muanyishay et al. (2018) induced callus using M1 and M2 media containing BAP with AIB and 2,4-D, respectively. These media yielded the highest organogenesis rates of 56.4% and 68.6%, respectively. However, a reduction in the frequency of callus induction was observed in the absence of coconut milk, as previously shown by Usman et al. (2005) and Rani et al. (2004). Additionally, 2,4-D promoted callus induction in *in vitro* cultures of node-free stems of *Tetracera rosiflora* Gilg (Handaji et al., 2005; Singh et al., 2006).

Several studies have demonstrated that the use of auxins and cytokinins triggers or induces callus formation in a wide range of plant species. These include oil palm and

plantain (Strosse et al., 2003), as well as date palm (*Phoenix dactylifera* L.) (Sané et al., 2006; Asemota et al., 2007).

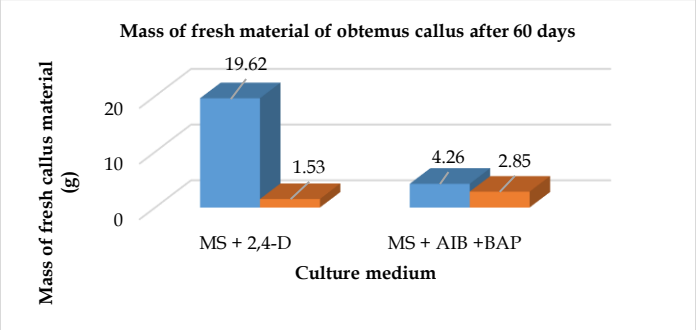
All parts of a plant are capable of dedifferentiation, and it is generally accepted that leaves and young stems are more reactive (Augé et al., 1989). Our results align with those obtained by Benrebiha (2005) in *Atriplex halimus*.

Mass of Fresh Callus Material Obtained

The mass of fresh callus material depends on the callogenesis medium and the culture conditions **(Figure 7)**. Specifically, the mass was higher for cultures maintained under permanent light and lower for those kept in continuous darkness. Notably, the callus mass was significantly greater on medium M1 (MS + 2,4-D) compared to medium M2 (MS + AIB + BAP). After 60 days of culture, a fresh mass of 19.62 g was obtained for cultures grown on M1 under permanent light, compared to 1.53 g under permanent darkness. On M2, the fresh callus mass was 4.26 g under permanent light and 2.85 g under continuous darkness.

Figure 7 illustrates the mass of fresh callus material grown over 60 days on M1 (MS + 2,4-D) and M2 (MS + AIB + BAP) media.

Figure 7:
Mass of fresh callus material grown for 60 days on M1 (MS + 2,4-D) and M2 (MS + AIB + BAP) media



These results are comparable to those reported by Muanyishay et al. (2018), who obtained fresh callus masses of 68 g on M1 (MS + 2,4-D) medium and 50 g on M2 (MS + AIB + BAP) medium.

Effect of Coconut Water on the Mass of Fresh Callus Material

To increase callus mass for efficient phytochemical screening, two additional media were prepared: medium M3 (MS + 2,4-D + coconut water) and medium M4 (MS + AIB + BAP + coconut water). Callus mass was measured prior to the addition of coconut water and throughout the period following callus transplantation into M3 and M4, in order to assess the effect of coconut water on callus growth. After 56 days of subculture, it was observed that the addition of coconut water significantly influenced callus mass. For medium M3 (MS + 2,4-D + coconut water), a substantial increase in callus mass was recorded under both light and dark incubation conditions (see **Figure 8**). Specifically, callus mass increased from 19.62 g to 30.10 g under permanent light and from 1.53 g to 3.15 g under continuous darkness.

In contrast, for medium M4 (MS + AIB + BAP + coconut water), a more pronounced increase in callus mass was observed under dark incubation than under light conditions (see **Figure 9**). Callus mass increased from 2.85 g to 3.33 g under permanent light and from 4.26 g to 10.34 g under continuous darkness.

Figure 8:
Mass of callus obtained on M3 medium (M1 + coconut water)

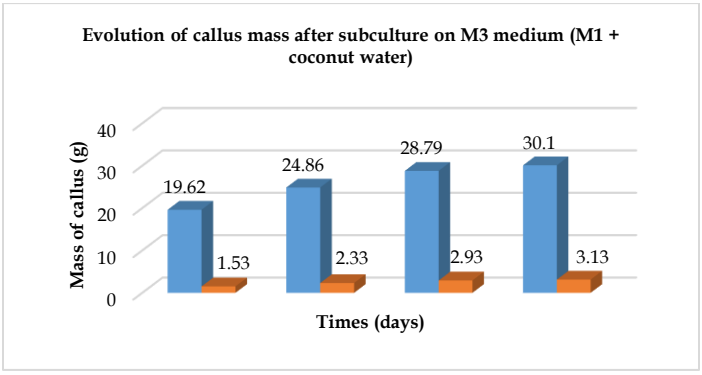
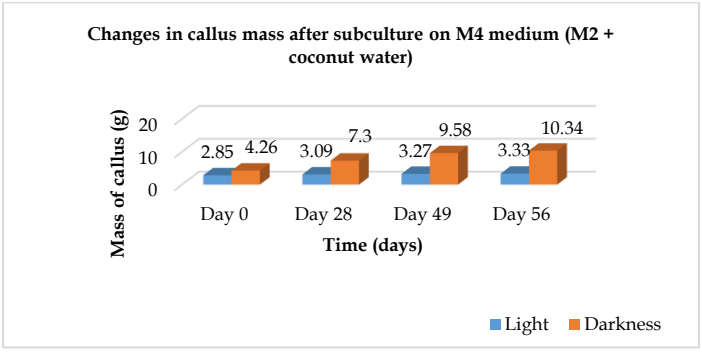


Figure 9 illustrates the evolution of callus mass after subculture on M4 medium (M2 + coconut water).

Figure 9:
Evolution of callus mass obtained on M4 medium (M2 + coconut water)



This variation in callus weight demonstrates the stimulating effect of coconut water. These results are comparable to those reported by Muanyishay (2018), who observed a 25% increase in the rate of callogenesis following the addition of coconut water.

Indeed, several studies have confirmed the stimulatory action of coconut water (Steward & Caplin, 1951; Rabéchault et al., 1972; Fotso et al., 2002). Coconut water may help prevent mineral deficiencies by providing additional macro- and microelements (Rabéchault et al., 1972).

Results of the Dosage of Total Polyphenols

Table 3 presents the results of the total polyphenol content in powder derived from the callus and wild *T. rosiflora* leaves.

Table 3:
Results of the dosage of total polyphenols

	Powder from callus obtained on different culture media				Powder of T, wild rosiflora
	Medium M3 *(conditions: light)*	**Medium M3** *(conditions: dark)*	**Medium M4** *(conditions: light)*	**Medium M4** *(conditions: dark)*	
Total polyphenol content (mg EAG/g dry extract)	0,6555 ± 0,0045	0,7185 ± 0,00075	1,4055 ± 0,0045	0,7245 ± 0,00075	28,13 ± 1,18

From this **Table**, it is evident that calli obtained through in vitro culture possess a lower polyphenol content than wild *T. rosiflora*. Among the calli, the highest total polyphenol content was recorded in those cultured on M4 medium (MS + AIB + BAP + coconut water) under continuous light. Phytochemical screening confirmed the presence of saponins, steroids, flavonoids, tannins, quinones, and coumarins in both wild and callus extracts. Wild *T. rosiflora* leaves contained significantly more polyphenols (28.13 ± 1.18 mg GAE/g dry mass) compared to callus cultures (1.4055 ± 0.0045 mg GAE/g dry mass).

These findings suggest that the culture media used were not optimal for polyphenol production. Growth regulators are among the crucial factors influencing the biosynthesis of secondary metabolites in plants (Tshiruvengadam & Chung, 2015).

CONCLUSION

Modern science, in studying and analysing the therapeutic effects of plants, does not aim to diminish their value; rather, it seeks to specify, compare, and classify their various properties—whether beneficial or not.

This study highlighted the potential of callus culture as a technique for obtaining secondary metabolites from *T. rosiflora*. However, the significantly lower polyphenol content in callus cultures suggests the need for further optimisation. Future research should focus on enhancing metabolite accumulation in vitro through elicitor treatments and metabolic engineering. This work contributes to the sustainable conservation and utilisation of *T. rosiflora* as a medicinal resource.

In Memoriam: The authors of this work dedicate these results in memory of the late Professor Diamuini Ndonfunsu.

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Ethical Approval: Nil required.

Conflicts of Interest: None declared.

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