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Callogenesis Induction on different types of explants of *Tetracera rosiflora* Gilg.

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ABSTRACT:

Tetracera rosiflora Gilg is a medicinal plant used in the Congolese pharmacopoeia for the treatment of various diseases such as diabetes, arthritis, dysentery, hepatitis etc. It represents an attic of active compounds, which have multiple pharmacological properties. The aim of the current research is to induce callus formation in *T. rosiflora* Gilg. Henceforth, it is interesting to apply biotechnology tools to plants such as this specie in order to improve their chemical composition in bioactive explants. Our findings showed that a high percentage of calli (80%) was obtained with stems without nodes in the M_4 medium and the stems with nodes gave neoformed buds. Obtained calli: friable whitish, whitish compact, whitish nodular, friable greenish and brittle brownish, were be produced according to the types of explants and the combination of growth regulators. The highest mass of fresh matter obtained was 92 mg in M4 medium and the lowest was 50 mg in M1 medium. The different combinations of growth regulators tested allowed the different explants to express their callogenic potentiality. However, the high proliferation rates have been obtained in the presence of 2,4-D that induced greater callogenesis of stems compared to leaves of *T. rosiflora*. The developed callogenesis system of *Tetracera rosiflora* is efficient and could give the possibility of production of the large scale of bioactive compound.

Keyword: *Callogenesis, Tetracera rosiflora, growth regulators*

INTRODUCTION:

Although plants are theoretically renewable resources, the lack of regulation regarding their exploitation is gradually leading to a destruction of their natural habitats that leads to a reduction in available resources. In addition, secondary metabolites extracted from medicinal plants are rather vulnerable to climate fluctuations, pathogens on crops [1, 2]. Among these medicinal plants is *Tetracera rosiflora* Gilg, a plant used in Congolese traditional medicine for the treatment of various diseases such as diabetes, dysentery and hepatitis. This liana is a promising source of anti-diabetic, anti-inflammatory, anti-microbial, antisickling and many pharmacological properties [3]. In fact, this plant contains different classes of secondary metabolites such as coumarins, irrodoids, flavonoids, tannins and anthocyanins [4, 5]. Therefore, the production of secondary plant metabolites through tissue culture technique has been a recognized approach since the early 1950s [6].Given the vulnerability of secondary metabolites produced by plants without considering climatic or socio-economic hazards, it is necessary to find an alternative solution for the production of bioactive molecules on a commercial and economically viable scale. The *in vitro* cultures of cells, and especially differentiated tissues of plants, because of their totipotency, offer an alternative for the *in vitro* production of secondary metabolites of interest. They make it possible to control the multiplication of biomass and the elicitation of biosynthetic pathways in a controlled environment unlike cultivation in the field or in the greenhouse [1, 7].

In order to improve the yield of these biotechnological processes in terms of production in bioactive compounds, numerous strategies have been implemented and biological, physical and chemical factors influencing crop quality have been defined. It is possible to mention the degree of differentiation and the type of tissue used, the composition of culture media, in particular at the level of the supply of growth regulators, the application of eliciting agents or the addition of biosynthetic precursors [1, 8, 9]. In higher plants, the organogenesis is performed by meristems consisting of undifferentiated cell masses that retain the ability to actively divide. They are totipotent cells that build all the organs (vegetative and reproductive) as well as the supporting tissues of the plant and these cells undergo a differentiation. On the contrary, the specialized tissues undergo a dedifferentiation in order to produce new meristematic massifs. The aim of the current research was to establish a reproducible protocol of the *in vitro* callogenesis induction in *Tetracera rosiflora* Gilg for a permanent production of secondary metabolites.

MATERIAL AND METHODS

1. Material

Different explants (stems without nodes, stems with nodes and leaves) were collected from *Tetracera rosiflora* Gilg plants growing naturally at Mont-Ngafula commune in Kinshasa province (D.R. Congo).

2. Methods

2.1. *Disinfection of explants*

The explants of stems and leaves were carefully separated. The stems were cut into fragments of about 5 mm length without nodes while the leaves were sliced into fragments of 5mm apart. The disinfection of explants before their culture were carried out in a laminar flow hood where they were soaked in alcohol 70% for 10 minutes under agitation and then were placed in $HgCl_2$ 0.125% solution for 5 minutes then they were washed 5 times with sterile distilled water.

2.2. *Experimentation*

For all the experiments carried out basal medium was used [10]. Five combinations were tested in which the control medium batch (M0) was free regulators, while the other media contained different concentrations growth regulators combinations (1 or 2 mg/ L) of 2,4-D auxin (Acid 2,4-Dichlorophenoxyacetic acid) or supplemented with coconut water; the addition of a cytokinin BAP (6-benzylaminopurine)

and auxin AIB or supplemented with coconut milk. Before autoclaving, the pH was adjusted to between 5.6 and 5.8. The media was distributed into different test tubes under a laminar flow hood in aseptic conditions. All media used were sterilized by autoclaving at 121 ° C under a pressure of a bar for 30 minutes. Table 1 gives the composition of used media.

Table 1. Media composition

Media	Concentration
M0	MS without growth regulators
M1	MS + 1 mg/ L BAP +1mg/ L AIB
M2	MS +1 mg/ L 2,4-D
M3	MS + 2 mg/ L BAP + 2 mg/ L AIB +100 mL of Coconut water
M4	MS + 2mg/ L 2,4-D + 100 mL of Coconut water

MS: Murashige and Skoog, BAP: 6-benzylaminopurine, 2,4-D, 2,4- Dichlorophenoxy acetic acid.

2.3. *Explants culture*

For each type of explant, 60 test tubes were used while 30 test tubes were incubated in the dark and another 30 were exposed in the continous light with 2000 lux of intensity. They were kept in the growth chamber at 26 ± 2 ° C. After four weeks, the induction rate of callogenesis was determined and the mass of the calli was weighed.

After disinfection, the drained explants were transferred in Murashige and Skoog (MS) medium supplemented with different combinations of growth regulators (see Table 1), and were placed in the culture chamber at 26 ± 2 ° C following two culture conditions in light and darkness.

2.4. *Determination of the induction rate of callogenesis*

The callogenesis response was calculated using following formula:

$$\text{Callogenensis (\%)} = \frac{\text{Number of explants with calli}}{\text{Total number of explants}} \times 100$$

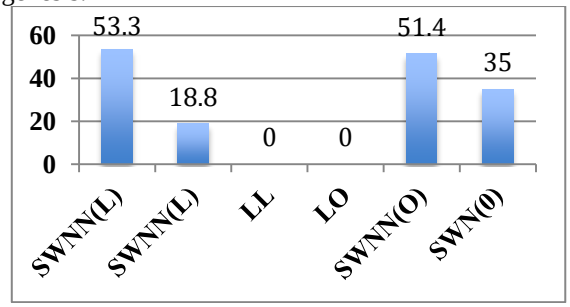
3. Data analysis

The different parameters studied were grouped as follows: three types of explants (leaves, stems without nodes and stems with nodes), two culture conditions (in darkness and in light) and five types of different culture media. The experimental device used is total randomization. ANOVA was performed using R version 3.1.1. The ANOVA model used is a mixed crossing over model with two classification criteria. If there is a significant difference, this analysis is completed by the paired multiple comparison test (LSD test). The probability threshold used is 5%.

RESULTS AND DISCUSSION

1. Media composition:

Callus initiation from the stem and leaf explants of *Tetracera rosiflora* Gilg was promoted by the presence of growth regulators in the medium. While the free regulators culture medium (Mo) is manifested by a complete absence of response callogenesis.



Legend: Stem with nodes under light: SWN (L); Stems with nodes in dark: SWN(O); Stems without nodes under light: SWNN(L), Stems without nodes in dark: SWNN(O); Leaves under light (LL), Leaves in dark (LO)

Figure 1. Percentage of calli in different medium cultures under light (L) and in dark (O)

The callogenesis rate varied from 30% to 80% depending to the types of culture medium. The M4 medium allowed the obtention of callogenesis with the rate of 80%, and the M1 medium allowed an induction of calli with the rate of 58.6%. Analysis of the variance showed that the effect of culture medium composition is highly significant (Figure 1).

M4 medium favored a callogenesis rate of 80% from stem explants without nodes and it was noticed that the addition of coconut water in the medium increased the callus regeneration capacity ranging from 6 to 10% and a shoot induction frequency of 55%. Previous studies on tissue culture reported that the exogenous application of a specific stimulator such as coconut water, promotes the induction process of callogenesi [11]. On the other hand, the media M1 and M2 gave the high rate of callogenesis, which were 56.4% and 68.6% respectively induction, which was observed in the medium without coconut water. Previous studies corborrated our obtained results [12, 13, 14, 15, 16, 17]. However, 2,4-D medium favored the induction of calli but from the *in vitro* culture of stem without nodes *Tetracera rosiflora* [18, 19].

3.2 Effect of growth regulators combinations on the morphology of calli

Depending on the media, the types of explants and the condition of cultivation used, calli from *T. rosiflora* differ in color and texture. Thus, the callus appearance changes with culture medium and culture condition (See table 1). The M1 medium gave the whitish calli (non chlorophyllian) compacts from the explants of stems without nodes while for the same types of explants gave in dark whitish callus compact.

The M2 medium, with the explants of stems without nodes gave friable green calli in light and whitish calli nodules or compact in darkness while those with nodes led to a neoformation of leafy shoots whatever the incubation condition. Leaf explants could not give callus in the same species (all explants) in the different culture media but in the stems, could not give the calli in light but whitish callus was induced in darkness. In the M3 medium, stems without nodes induced compact whitish calli and brownish calli that were friable in darkness, and greenish calli friable in light, while stems with nodes initiated neoformation of leafy shoots in light and friable brown calli in darkness.

In addition, the M3 culture medium promoted the onset of rhizogenesis and the appearance of roots. The M4 medium yielded greenish brittle calli with non-nodes stalk explants, whereas dark-free stalk explants initiated compact whitish calli, friable whitish calli, and friable brownish calli. On the other hand, the explants of stems with nodes could not give callus in the medium M4 under the two conditions of culture (Figure 2).

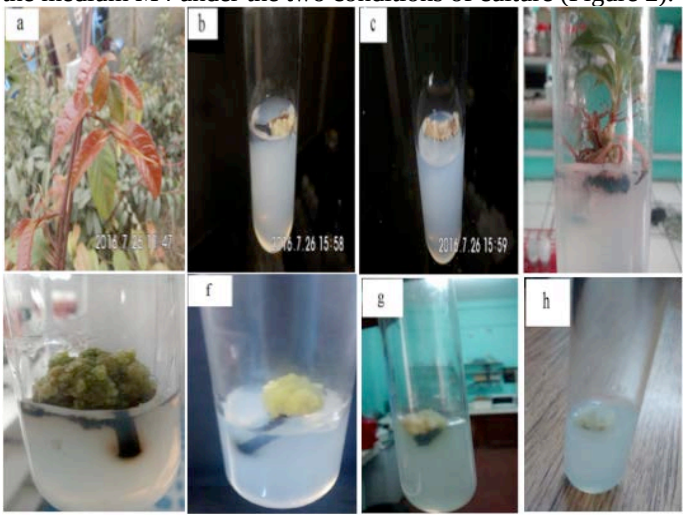


Figure 2. Plant, type of calli and newly formed buds of *Tetracera rosiflora* (a: *Tetracera rosiflora* plant, b: whitish callus, c: whitish nodular callus, d: bud neoformed, e: greenish callus, f: brownish callus: whitish friable callus h: whitish compact callus)

The effect of different concentrations of auxin and cytokinin on the induction of callogenesis from different types of *T. rosiflora* explants showed that stems and leaves show a variable response to their callogenesis ability and their morphogenetic response (appearance, color, texture). Several studies reported that the use of auxin and cytokinin, induces the triggering or induction of callus in a large number of plants such as: the oil palm: *Atriplex canescens* [20] ; *Atriplex halimus* [21,22]; *Atriplex nummularia* [23]; *Cola anomala* and *Cola acuminata* [24] ; banana and plantain [25] ; *Phoenix dactylifera* L. [26].

All parts of a plant are likely to undergo a process of dedifferentiation and generally it is accepted that leaves and young stems are more reactive. Our results corroborate those obtained by [21] on *Atriplex halimus*. The use of MS medium supplemented with AIB and BAP caused the emergence of roots from the stems of *Tetracera rosiflora*. The rooting phenomenon is also reported by [20].

3. Fresh material weight

The mass of fresh material was determined by weighing calli depending on the medium and conditions of culture. In fact, this mass was the highest on M4 medium (92 mg) and the lowest on M1 medium (50 mg). On M2 and M3 media, the average mass of calli was 68 mg

The results of the analysis of variance is presented in Table 2

Table 2. Comparison of means of different calli

	SWNN(L) - SWN(L)	SWNN(O)-SWN(O)	SWNN(L)-SWNN(O)	SWN(L)-SWN(O)
F	13.49	8.95	0.61	10.53
p-value	0.021	0.040	0.477	0.032
LSD	26.065	15.217	-	13.811

Legend: Stems with nodes (SWN), Stems without nodes (SWNN), light (L), Dark (O)

These results show that in the presence of light, SWNSN (L) has a significantly higher percentage of calli than SWN (L) ($p < 0.05$)

The same is true for dark experiment where SWNN (O) has a significantly higher percentage of calli than SWN (O) ($p < 0.05$). On the other hand, no significant difference is revealed by comparing the percentage of callus provided by the SWNN (L) in the presence of light and that presented by the SWNN (O) in dark ($p > 0.05$). Whereas for SWN callus percentages in darkness has a significant effect compared to the light ($p < 0.05$).

CONCLUSION

This study allowed establishing a reproducible protocol for the induction of callogenesis from stems explants of *Tetracera rosiflora*. After four weeks of *in vitro* culture, the induction of callogenesis was effective for all media except the control and the best average were obtained with M3 and M4 media for using fragments of nodeless stems. The present study consisted on the report for callus establishment in *Tetracera rosiflora*, which is under development. The variability of callogenesis towards explants depends on the type and concentration of growth regulators used and also on the genotype of the species. These results highlight the great ability of the callogenesis in MS medium containing 2 mg/ L of 2,4-D + 100 ml/L of coconut water. However, the use of cytokinin BA combined with auxinIAB appears to be important for induction and proliferation of calli. The callogenesis is always preceded by an increase in the volume of explants. These explants consist of differentiated tissues and their culturing in the MS medium supplemented with different combinations of AIB, BAP, 2,4-D and coconut water led to the dedifferentiation of the hormonal influence on the induction of callogenesis in *T. rosiflor*a.

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