# Effects of hypoxia, loganin and BAP on ajmalicine accumulation in cells of *Catharanthus roseus( Vinca rosea) In vitro*

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## ABSTRACT

**The Madagascar periwinkle (*Catharanthus roseus*) produces numerous indole alkaloids, several of which have an important pharmaceutical uses such as ajmalicine. The relationship between hypoxia and ajmalicine production in a cell medium culture of *Catharanthus roseus* were investigated during the cycle of cell culture, as well as the growth in fresh and dry matter. The results show that the lack of oxygenation in C20D cells provokes a very strong inhibition in accumulation of the alkaloids and of other possible substances. This can be explained by the absence of some downstream enzymes involved in the biosynthesis chain of alkaloids or by the blockage of the upstream terpenes to the production stage. Reoxygenation did not restore hypoxia effect on the alkaloid accumulation.**

**Moreover, the present study showed that the addition of the loganin in the 4[th] day, in the cell culture medium subjected to hypoxia restored the alkaloid production.**

**Also, the results show that the BAP increases the ajmalicine production. The effect of hypoxia was not shown in the presence of cytokinine. The BAP can without doubt decrease the effects of the hypoxia and increases the ajmalicine production.**

**Key words:** *Catharanthus roseus*, ajmalicine, Hypoxia, Cytokinine, Loganin.

# تأثيرات نقص الأوكسجين واللوغانين وهرمون BAP في تراكم الأجماليسين داخل خلايا الونكا الوردية Catharanthus roseus المزروعة مخبرياً

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## الملخص

تنتج الونكا الوردية Vinca rosea أو *Catharanthus roseus* العديد من أشباه القلويدات الاندوليـة مثل الـ ajmalicine ، وهى ذات استعمالات طبية مهمة تمت دراسة العلاقة بين إنتاج الـ ajmalicine ونقص الأكسجين في وسط المعلقات الخلوية لـ *Catharanthus roseus* خلال دورة حياة هذه الخلايا، وكذا معدل النمو بالنسبة للوزن الرطب والوزن الجاف. بينت النتائج أن نقص الأكسجين لخلايا C20 D أدى إلى تثبيط قوى لتراكم الـajmalicine وربما مركبات أخرى، كما أن إعادة التهوية لاحقاً لا تـؤدي إلى زوال هذا التأثير.

فضلاً عن ذلك فإن الـloganin استطاع أن يثير تراكم مادة الـajmalicine في المعلقـات الخلويـة المعرضة لعوز الأكسجين وبشكل أفضل في خلايا الشاهد.

أما تدخل هرمون BAP فقد أثار وبشكل ملحوظ تراكم مادة الـajmalicine .

**الكلمات المفتاحية:** نقص الأوكـسجين، أجماليـسين، الونكـا الورديـة، لوغـانين، سيتوكينين.

# 1. Introduction

The tropical plant *Catharanthus roseus* (L) G.Don is an apocynaceae which constitutes a model of laboratory as well for physiological studies as for studies on the secondary metabolism. The interest carried by the scientists with this plant is due to the presence of alkaloids with therapeutic significant activity such as ajmalicine.

The use of the cellular suspensions of *Catharanthus roseus in vitro* is a very powerful means to study the regulation of plant metabolism. Different research ways aims at optimizing the factors of the culture medium. Among the environmental and various nutritive factors which influence the production of secondary metabolisms in the cellular suspensions, as the nitrogen, the phosphate and the source of carbon (Mérrillon *et al.*,1982) as well as the phytohormones ( Berlin, J. 1988 ), the oxygen is a critical parameter since it is only the nutritive substance which one must provide continuously. To this effect, we thought that the *Catharanthus roseus* model was interesting to use for a study of the effects of oxygen. Various works suggest besides that oxygen intervenes on several levels in the biosynthesis of the indole alkaloids (Schlatmann *et al*.,1995; Ostrovsky *et al*., 1995; Ostrovsky *et al*., 1998). In the present article, we study the relation between the stop of agitation of the cells and the hypoxia on the one hand and the impact of this phenomenon on the growth and the alkaloid outputs of the cells on the other hand. Also, we want to know if this inhibiting effect could also appear in the presence of an alkaloid precursor (Loganin) and a production stimulative (Cytokinine).

# 2. Material and methods

## 2.1.Chemicals

Loganin, BAP was purchased from Sigma-Aldrich Chimie (L'Isle d'Abeau, France).

Other chemicals were from Merck ( Nogent-sur-Marne, France).

## 2.2. Plant material and growth conditions

Periwinkle (*Catharanthus roseus*) cell suspensions (line C20D) were maintained on a 7 day growth cycle in the B5 medium of gamborg (Gamborg & Miller, 1968) supplemented with 58 mM sucrose and 4.5 $\mu$M 2.5-dichlorophenoxy-acetic (2.4-D) (maintenance medium). The cells were cultured in 250 mL erlenmeyer flasks (with 50 ml culture) on a rotary shaker (100 rmp) at 24°C in the dark. The

cells were harvested by vacuum filtration (30µm nylon cloth) on the seventh or tenth day for growth evaluation and ajmalicine quantitation.

**2.3. Processing by hypoxia**

The hypoxia has been obtained by stopping the movement of the agitation tables on which rest vials of cultures. Sediment cells to the bottom of phials during all the hypoxia. The content of oxygen in the layer liquidates environment is reduced.

( Droual *et al*., 1997 ) Cells are submitted to the hypoxia 24 hours of agitation from the $5^{th}$ day of culture then we return to normal conditions during 10 days of culture.

**2.4. Evaluation of the growth**

***Weight of the fresh matter:*** It is determined by just cells weighing after they have been rinsed.

***Weight of the dry matter :*** Deep frozened cells are lyophilizes then weighed.

Obtained results are expressed by the average gap type of the three values.

**2.5. Ajmalicine quantitation.**

Lyophilizes cells are pulverized in a mortar. Twenty five mg of powder are extracted by 1ml of pure methanol during a night (continuous agitation).After centrifugation ( 5 min to 14000 rpm) 400 $\mu$ l of the remaining transferred in tubes of glass of 1 ml closed by cork screwing supplied by a septum in Teflon for dosage. The analysis of the alkaloid profiles is realized by CCM. 400 $\mu$ l of the remaining are appropriated and deposited on a slim frost of silica (Merck N° 5553 of 10x20 cm and 0.2 mm of thickness) by using an automatic deposer (CAMAG Automatic TLC SAMPLER III). A stallion range is realized by depositing known quantities of ajmalicine beside samples to dose. Fluorescent spotlights corresponding to the alkaloids are noticed with the help of a UV lamp. The dosage has been made by densitometry with the help of the scanner (CAMAG TLC SCANNER 3) in fluorescence by reflection.

# 3. Results and discussions

## 3.1 *Role of the hypoxia on the growth of cells C20D and their production of Ajmalicine.*

As mentioned before the hypoxia has been obtained by stopping the movement of the agited table on which rest phials of cultures have been made. Cells sediments were reduced to the bottom and the oxygen quantity as well. Results obtained in terms of growth showed that entire satisfaction was reached at the end of the culture. When cells were under hypoxia, light sag of the growth either for the fresh or the dry matter was observed. However; a correction of the growth was made during the following days of the growth (Fig. 1). In terms of alkaloid production when cells are cultivated under inductrice conditions (obtained by the suppression of 2.4-D in the culture medium), the alkaloid production normally reached in these conditions was totally inhibited. No observation of resumption of ajmalicine accumulation was made even to the $10^{th}$ day. This illustrate that the hypoxia pipe lead to a real inhibition of alkaloid synthesis and not to a simple production delay (Fig. 1) .

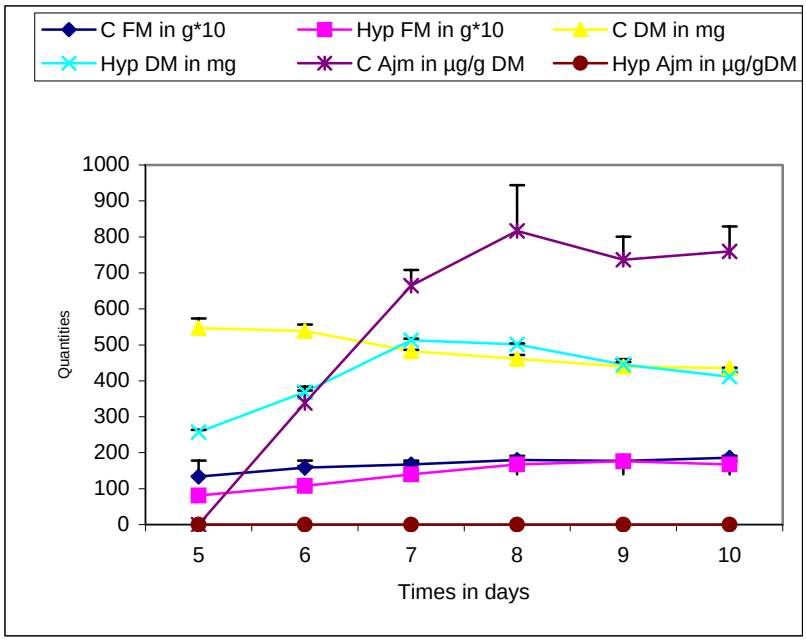


**Fig. 1. Effects of hypoxia on growth and alkaloid accumulation in *Catharanthus roseus* cells**

### 3.2 Study of the effect of the hypoxia

Preceding results have behaved us to deepen the effect of the deprivation of the oxygen. We have established that the effect inhibitor of the hypoxia has certainly to exert important manner upstream of the stage of the production of the Loganin (precursor of the biosynthesis of the ajmalicine). The results (Fig. 2) show that the hypoxia in the beginning of the culture cell C20D inhibits totally the alkaloid production and the addition in the 4$^{th}$ day of the Loganin in the cell culture medium subjected to hypoxia restored the alkaloid production.

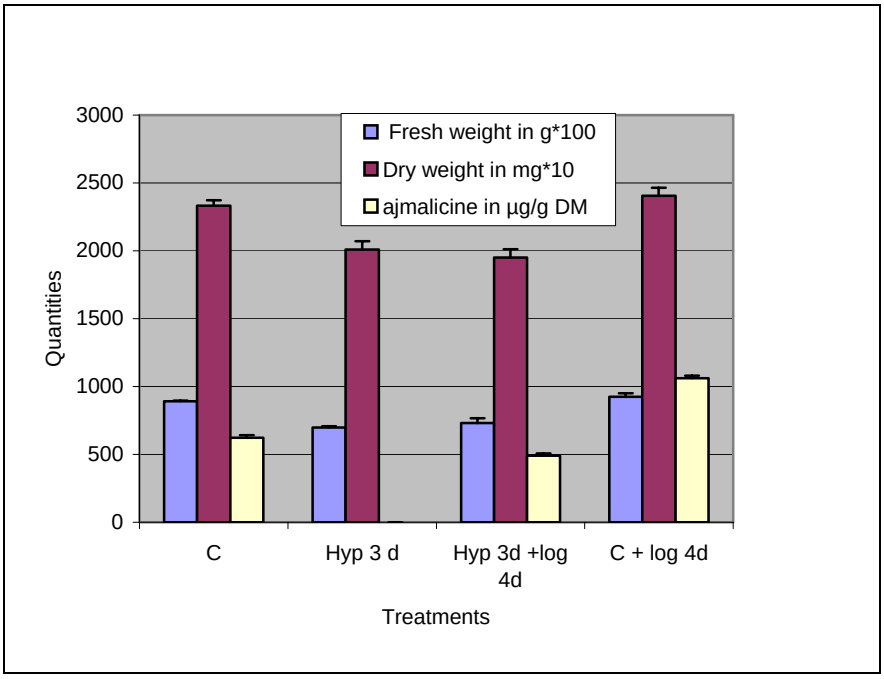


**Fig. 2. Effects of loganin on growth and alkaloid accumulation in *Catharanthus roseus* cells submitted to the hypoxia**

### 3.3 Effect of the hypoxia on stimulated cells by the BAP:

It was clearly shown that cultures placed in hypoxia conditions were unable to produce indolic alkaloids. To insure that hypoxia did not affect by slowing the kinetic of the alkaloid production; This

inhibitory effect was checked when cells were stimulated by cytokinine. The production was extended over 10 days of culture (Fig. 3). The results showed that the BAP increases the alkaloid production, since the condition N° 4 in product while the condition N°2 does not produce, but the condition N°4 has a weaker production than that the control. We notice therefore that the hypoxia is not shown in the presence of the cytokinine. The BAP can without doubt decrease in direct manner the effects of the hypoxia and increases the alkaloid production.

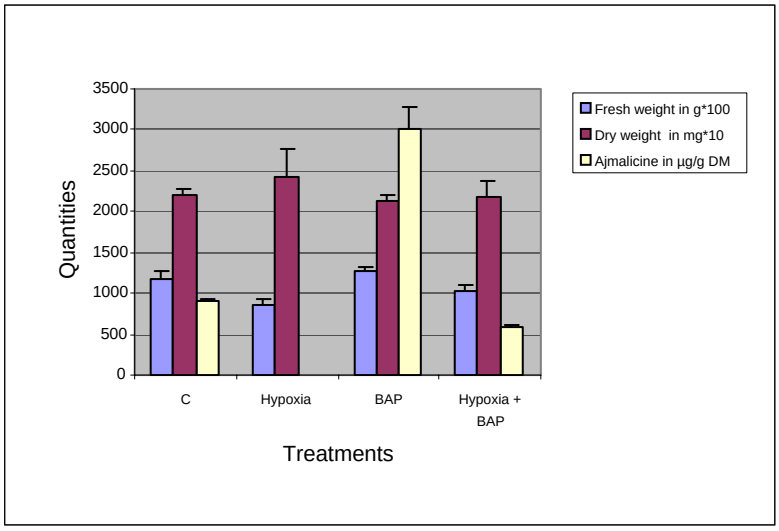


**Fig. 3. Effects of cytokinine on growth and alkaloid accumulation in *Catharanthus roseus* cells submitted to the hypoxia**

## 4 . Conclusion

Our results show that the stop of agitation of cells conduct to an oxygen privation than to a real inhibition of the ajmalicine production. This can be explained by a freezing in the way of upstream terpens at the stage of the production of the Loganine (Fig. 4) while the exogenous addition of the Loganine to the cells submitted to the hypoxia restores the biosynthesis of the ajmalicine. These results can be reproached to these obtained by other authors (Schlatmann *et al.*, 1994). Other possible hypothesis to explain the inhibition of the production of the ajmalicine is the absence of some enzymes

(enzymes of cytochrom P450) in endorsement in the biosynthesis chain of the ajmalicine (Meehan & Coscia, 1973; De Luca *et al*., 1986; Oudin *et al*., 1999) in condition of hypoxia. Our future effort is to elucidate the mediation of molecular regulation mechanisms.

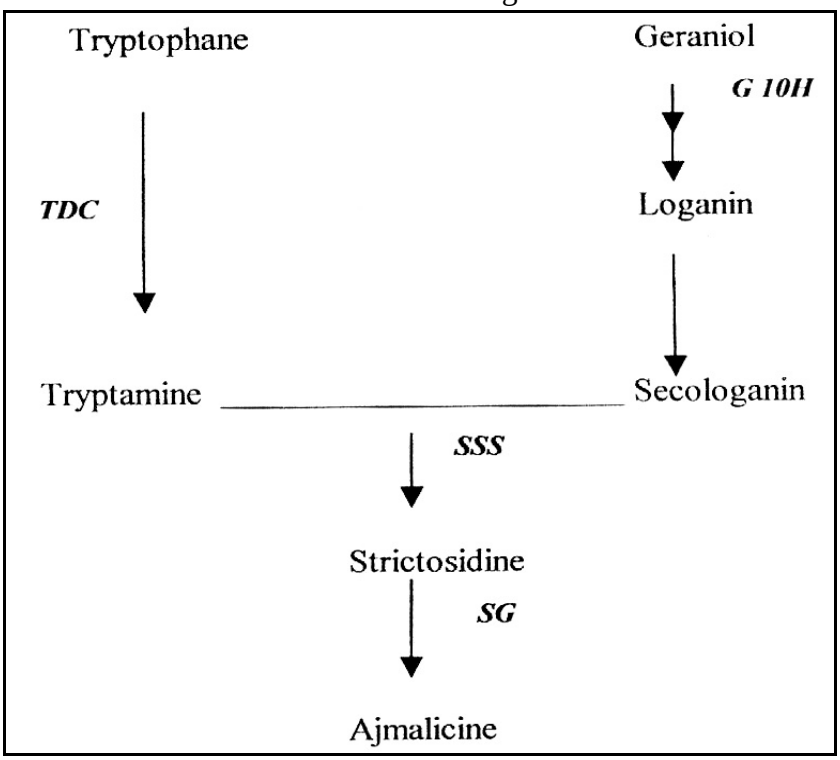


**Fig. 4. Intermediates in the biosynthesis of ajmalicine and some key enzymes catalyzing the reactions: Tryptophane décarboxylase (*TDC*), geraniol-10-hydroxylase (*G 10H*), strictosidine synthase (*SSS*), strictosidine glucosidase ( *SG* ).**

## Abbreviations

Log = loganin; BAP= 6-benzylaminopurine; 2,4 D= 2, 4 - dichlorophenoxyacetic acid ; hyp = hypoxia ; FM= fresh matter ; DM= dry matter C= control ; Ajm =ajmalicine ; d=day

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