Different Parameters for Alkaloid detection in *Catharanthus roseus* tissue culture

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Abstract

Tissue culture of Catharanthus roseus was established under many parameters to insure good results for detection of the alkaloids present in this plant . It was found that NItsch and Nitsch medium containing 8µM Benzyladeninpurine plus Naphalene acetic acid were the best and the callus of *C.roseus* left to grow in the dark and had much better influence for the production of Alkloids. The precursor phenylalanine showed a better result than other precursor(tryptophan) . Abscisic acid has an inhibitory effect on the production of Alkaloid.

Introduction

The genus *Catharanthus* is a member of the botanical family Apocynacyeae. *C.roseus* is one of the most important species endemic to Madagascar which has long been estimated as an ornamental plant. It is easily cultivated , seeds freely and in tropical countries becomes naturalized and spreads rapidly as garden escape , two color varieties , pink and white , are found in natural state , while a number of seed hybrids are commercially available . A great deal of confusion regarding the proper nomenclature of this plant has existed in the past. This periwinkle has been known variously as *Vinca rosea , Lochnera rosea, Catharanthus roseus , and Ammocallis rosea.* It has been recognized that the genera *Vinca* and *Lochnera* differ in 34 morphological characters and should not be used as synonyms (1).

C.roseus considered as the factory of some seventy five alkaloids, these include a number with demonstrable oncolytic activity and a few with actual clinical value in the treatment of cancer.

As a result of various extensive phytochemical efforts, only two alkaloids from this plant (Vincaleukeblastine and leurocristine) have antitumor activity. Diuretic, hypoglycemic and antiviral , were found to be associated with a number of alkaloids obtained from this plant .

Tissue culture of C.roseus crown gall are capable of producing small amounts of alkaloids(2).These were found both in the tissue and in the medium and vindoline was the major alkaloid produced . When Vindoline hypochlorite was added to a Catharanthus crown gall suspension , the tissue was able to effect several modifications of the vindoline molecule.

One of the earliest of the culture of normal C.roseus tissue was made by Deropp(3) , who worked with cambial tissue from stems .Some workers reported that catharanthus callus wauld be maintained without an exogenous auxin supply if certain inorganic salt plus Kinetin and inositol were present (4). While several different media were used for the culture of catharanthus callus, most workers have used the basic whites medium(5,6)or that of wood and braun(7).or some modification of these.

Anumber of growth factors or regulators were included in cultured media in order to stimulate callus growth of *C.roseus*. Babcock and carew (8) used 2,4-D in several concentrations as well as benzothiazole -2 oxyacetic acid(BTOA) ,Indol acetic acid (IAA) ,and Naphalene acetic acid (NAA).The level of growth factor is always important and different tissues may be more or less , sensitive to the same concentration. Further more, it is often necessary to used one concentration of growth factor to initiate callus formation and then a lesser amount to maintain callus growth. This situation was found to be true for C.roseus leaf callus cultures(9).

Experiments and results

Effect of nutrient media: Two types of media were used in this study. Murashige and skoog (MS1962) , and Nitsch and Nitsch (N.N.1969) (11) . To both types 2% sucrose were added and Banzyladninepurine (BAP) plus Naphalene acetic acid (NAA) In different concentrations were used .Agar 10g/l , pH were adjusted to 5.8±1 before autoclaving on 121 °C for 10min.

Explants used: Healthy leaves were harvested from plants growing in gardens (Authenticated by botanical garden in College of Science, University of Baghdad) . Leaves first washed in tap water then in distilled water, clean leaves were sterilized in 70% Ethanol for 3-5 min, then in 0.1% $HgCL_2$ for 15 min. Sterilized leaves were washed three times in sterilized distilled water 5 min each time. The leaves were dried on sterilize filter paper. Leaf discs 0.5 cm were made in sterilized cork borer , transferred to media prepared previously and cultured for 4 weeks . The results were summarized in tables 1 and 2.

These tables the results reveal that Nitsch medium had better response than MS medium and 8µM BAP plus NAA in Nitsch medium was the best concentration , so all latter experiments were carried out on this condition.

Test for the presence of alkaloids in cultures grown on Nitsch medium were shown in table 3.

Effect of light: Leaf discs were sterilized as before and cultured on Nitsch medium prepared previously with 8µM BAP plua NAA. Different light conditions were used. The results were summarized in table 4. In this table dark treatment had better alkaloid response than light treatment.

Effect of precursors: Leaf discs were cultured on Nitsch medium with 8µM BAP plus NAA in dark for a week then discs were transferred for 3 weeks to the same above medium plus

these different additives Phenylalanin and tryptophan as precursors for alkaloids and Abscisic acid (ABA) as an inhibitor for growth . The results were summarized in table (5).

Conclusion: Callus and cell suspension cultures of *C.roseus* are known to synthesize small amount of different Alkaloids(12). Hormonal and nutrition factors play an important role in controlling the synthesis of the desired product. Plant growth regulators have a dominant role in the induction and repressing of certain biosynthesis pathways leading to secondary plant products.(13,14,15) .

Different kinds and quantities of regulators were added to the media and the effects on cell yield and alkaloids production were observed .Nitsch and Nitsch medium containing BAP plus NAA8µM each were the best and this agreed with results obtained by zenk when he used a combination of auxin source and Benzyladnine in cell suspension cultures of *C.roseus*.(14).

The culture of *C.roseus* seems to have more alkaloids production in the dark and this will be more economic because no lighting facility needed . This higher level of accumulation suggests that the photodegredation of certain metabolites and /or enzyme may occur , although there is no direct evidence to substantiate this. Also increased alkaloids accumulation in darkness could be due to the repression of biodegradative processes which regulate the turnover of plant alkaloids (16, 17)

Metabolic precursors of the indole alkaloids were added to the medium . The technique of precursor stimulated product synthesis has was been proven to be successful in several cases (14,15) . But in our investigation, it was found that the precursor phenylalanine 100 PPM gives almost the same effect as the control (Nitsch medium plus BAP + NAA 8µM each) , while tryptophan showed that the an expected results (negative response) have completely different behavior than other precursors (18).

We are now able to design an alkaloid production medium which consists of the basal medium Nitsch and Nitsch described earlier plus BAP+NAA 8µM each and 100PPM phenylalanine (it is the better to try more than 100PPm such as 300,400,and 500PPM).

From this investigation, we hope to continue the study of *C.roseus* tissue culture by carrying on the phytochemical analysis to insure how many active indole alkaloids we have ? Especially serpentine , ajmalicine, vincristine, and vinblastine and then we would try to increase the yield of these alkaloids by modifying the different parameters mentioned before.

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Table (1): Fresh and dry weight and callus formation on MS medium and cultured for 4 weeks

Regulator Concentration	f.wt.per sample(mg)	Dry wt.per Sample (mg)	Callus formation
Control(No regulator)	11.629	1.888	No callus formed, discs turned brown
BAP+NAA2µM each	16.458	2.625	Very weak growth of friable callus were formed
BAP+NAA4µM each	15.956	2.304	Similar response as above
BAP+NAA32µM each	20.277	2.111	Little response were mformed and almost all samples turned brown after 3 weeks in cultures
BAP+NAA64µM	14.423	1.269	

Table (2): fresh and dry weight and callus formation on NItsch medium and cultured for 4 weeks

Regulator Concentration	f.wt.per sample	Dry wt. per Sample	Callus formation
Control(No regulator)	15.27	2.4	All samples turned brown And died later
BAP+NAA 2µM each	21.125	3.79	All sample turned brown after 3 weeks in cultures.
BAP+NAA4µM each	65.13	o.6	Very weak friable callus in edges of some samples
BAP+NAA8µM	91.66	o.9	Healthy friable callus formed in all samples , discs say green.
BAP+NAA32µM each	193.0	11.5	Good friable white callus were formed , but number of samples response less than in 8µM treatment.
BAP+NAA64µM	212.0	13.2	

Table (3): Alkaloids response on cultures grown on Nitsch medium for 4 weeks :

Regulator concentration	Total alkaloids results*
Control (No growth regulator)	Negative (No alkaloid)
BAP+NAA2µM each	+
BAP+NAA4µM each	+++
BAP+NAA8µM each	+++++
BAP+NAA32µM each	+++
BAP+NAA64µM each	+

Table(4): Responses of leaf discs grown on Nitsch medium and 8µ M BAP+NAA in different light conditions

Light condition	f.wt. mg	Dry wt. Mg	Alkaloid response
Dark treatment	53700	4092	+++++
Light treatment	40420	5394	++
Dark trt(15 day).—light trt.(15day)	4708	1095	+++

Table (5): Responses of leaf discs cultured for a week on Nitsch medium and 3 weeks in different concentrations of phenylalanine , tryptophan , and ABA

	Concentration	f.wt mg	Dry wt. Mg	Alkaloid response
Phenylalanine	50ppm	3385	325	++++++++
	100ppm	3088	380	
	200ppm	2910	302	
Tryptophan	50ppm	7440	717	No response
	100ppm	6383	570	
	200ppm	3495	334	
	400ppm	4167	410	
	600ppm	3187	170	
ABA	2ppm	7003	570	+++
	5ppm	5570	460	
	10ppm	5130	440	
Control BAP+NAA8µM each	-	91.66	0.9	+++++

دراسة بعض العوامل للكشف عن القلويدات في نبات عين البزون بالزراعة النسيجية

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الخلاصة

تم في هذه الدراسة استعمال عوامل مختلفة للكشف عن القاويدات الموجودة في اوراق نبات عين البزون المزروعة باستخدام تقنية الأنسجة النباتية . يظهر من النتائج ان الوسط الغذائي Nitsch and Nitsch والذي يحوي BAP+NAA µM8 هو افضل الاوساط لانتاج الكالس في الظلام .ان استعمال البوادئ مثل phenylalanine كان له نتائج افضل من tryptophan اما استعمال حامض الابساسيك اسيد فان له تاثير مثبط لانتاج القلويدات .