Production of Procyanidin compound in seeds , callus and in regenerated plants from *Vitis vinifera* L.

إنتاج المركب الفينولي Procyanidin من البذور والكالس والنباتات المتولدة من الكالس في نبات العنب *Vitis vinifera* L

Karem T. K. AL-Hatemy
College Of Science/ Kufa Univ

كريم طالب الحاتمي
كلية العلوم/ جامعة الكوفة

Abstract

This experiment was conducted in Science and Agriculture College labs, Kufa University, on 2008 to describe methods for extraction and purification of Procyanidin compound from each of grape seeds mother plant(*Vitis vinifera* L.) ,callus induced from Axillary buds by using MS medium supplemented with 2,4-D at concentration of (1,2,3)mg/l and from leaves of regenerated from callus by using MS medium supplemented with (IAA)at concentration of 2mg/l and (BA) benzyl adenine at concentration of 0.2 mg/l and from cocoas powdered (control) as well these Procyanidin. The content of Procyanidin were done by using HPLC (high performance liquid chromatography) and compare of the quantitatively of Procyanidin with these seeds in mother plant and callus tissue. This study include alcohol extraction by using mixture of ethanol, methanol, D.W and HCL. The result revealed that, the superiority of Procyanidin in seeds content than the content in callus and regenerated plant and increase the content of these compound in the leaves of regenerated plant with the content in callus tissue.

المستخلص

أجريت هذه الدراسة في مختبرات كلية العلوم وكلية الزراعة / جامعة الكوفة عام 2008 وتضمنت استخلاص وتنقية مركب إلـ Procyanidin من مستخلص بذور العنب *Vitis vinifera* L ومن الكالس المستحث من البراعم الجانبية (Axillary's buds) باستخدام وسط MS المجهز بمنظم النمو-2,4 D(Dichlorophenoxy acetic acid) تركيز 3,2,1 ملغم/لتر وكذلك من النموات المتولدة من أنسجة الكالس المستحث من البراعم الجانبية لنبات العنب باستخدام وسط MS المجهز بمنظمي النمو IAA(Indol acetic acid) تركيز 2 ملغم/لتر ول BA (Benzyl adenine) تركيز 0.2 ملغم/لتر .جرى استخلاص كحولي للبذور والكالس والنموات المتولدة خارج الجسم الحي باستخدام مزيج من مذيبات الميثانول 80٪ وال الايثانول80٪ والماء المقطر وحامض HCL. تم التحري والكشف عن المركب الفينولي Procyanidin باستخدام كاشف خلات الرصاص 1٪.بينت النتائج بان المستخلصات الكحولية للعينات النباتية المدروسة قد أعطت كشفا ايجابيا مع خلات الرصاص وكانت نتائج التقدير الكمي لمركب Procyanidin قد بينت تفوق بذور العنب بمحتوى المركب تفوقا معنويا على محتوى انسجه الكالس مع تفوقا معنويا للمركب في المعاملة2,4-D تركيز 2 ملغم/لتر مقارنة بمحتواه بالمعاملة 2,4-D تركيز1 , 3 ملغم/لتر. نفذت التجربة باستخدام اختبار T (T– test) في مقارنة كمية المركب إلـ Procyanidin في الأجزاء النباتية المدروسة .

Introduction

A large proportion of the drugs used in modern medicine are either directly isolated from plants or synthetically modified from a lead compound of natural origin. Plant produced more than 3000 chemical compound that widely used in diverse applications, including pharmaceuticals, foods color, dyes. Function in plant includes attraction of pollinating insects and protection against pests and pathogens [1].Grape plant (*Vitis vinifera* L) is one of the most studies among higher plants because of its importance as medicinal species [2]. Procyanidin or condensed tannins, are polymeric flavan-3-ol compounds are the most abundant Phenolic compound in plant originating mainly from the seeds of black grape (*Vitis vinifera* L) that is valued for its ability to help the body adopted to cold and consider as anti-oxidant agent scavenging the free radical from human body [3]. Phenolic phytochemical are important for human nutrition [4]. Are the most widely distributed secondary metabolites, ubiquitously present in the plant kingdom. Among Grapes plants has the highest content of Phenolic compounds reaching up to 6% (w/w) in some varieties [4]. Procyanidin (condensed tannins) originally classified as anti-nutritional factors, may have health benefits for humans [5,6].

Some flavanols (Procyanidin) have particular therapeutic interest because of their anti-tumor activity. .Proanthocyanidins (Procyanidin)extracts demonstrate a wide range of pharmacological activity, their effects include increasing intercellular vitamin C levels, decreasing capillary permeability, scavenging oxidants and free radicals, and inhibiting destruction of collagen, the most abundant protein in the body and prevents oxidation of low density lipoprotein [3] and may inhibit the growth of several viruses including the human immunodeficiency virus 1 (HIV-1) [7,8]. The anti-oxidant activity of Procyanidin have been reviewed in numerous publication in recent years may help provide protection against the many diseases by contributing, together with other natural compounds scavenge free radicals (antioxidant activity). Interestingly, Procyanidin display high antioxidant activities related to their Phenolic content [9]. Moreover, some grapes seeds varieties have higher antioxidant activities than the most important sources of natural antioxidants such as E and C vitamins [5].

Some secondary metabolite in plant cell culture technology have been reported to be accumulation with a higher titer compound with those in parent plant and has several advantages as methods of production useful plant-specific metabolites and is not limited by environmental, ecological or climatic conditions and can thus proliferate at higher growth rates than whole plants in cultivation. The capacity for plant cell culture , tissues, and organ culture to produce and accumulate many of the same valuable chemical compounds as the parent plant have been recognized almost since the inception of *in vitro* technology, in many cases, a wide range of controlled plant *in vitro* culture can generate differentiation processes in grapes tissue culture and about secondary metabolites [2].

Due to environmental and genetic factors, the content of Procyanidin found only in seeds, therefore, in order to obtain constant amounts of standerilzed quality Procyanidin, it seems appropriate to employ tissue culture technologies for its production, since then several papers have been published on the effect of various

growth factors on callus initiation. There is a series of distinct advantage to producing a valuable secondary product in plant cell culture, rather than *in vivo* in whole crop plant. These include, Production can be more reliable, simpler, and more predictable and isolation of the phytochemical can be rapid and efficient, as compared to extraction from complex whole plants, also, compounds produced in vitro can directly parallel compounds in the whole plant and cell culture, can yield a source of defined standard phytochemical in large volumes and finally, cell culture can be radio labeled, such that the accumulated secondary product, then provided as feed to laboratory animals, can be traced metabolically.

Material and methods

Plant material

Grap plants (*Vitis vinifera* L.) axillaries buds were collected from one year mature plant which washed with distilled water twice and soaked in 30 ml of 70 ethanol for 2 min followed by dipping in 2% Sodium hypochlorite for 3 min and rinsed 3 times with sterilized distilled water [10].

Callus induction

Axillary buds were cultured in Murashige- Skoog (MS) [11] basic nutrient medium as described in Table(1), medium supplemented with (1,2,3) mg/l (2,4-D), callus initiated after 4 weeks grown in culture media Fig(1). Callus were transferred to fresh medium containing (2)mg/l of (IAA) and (BA) benzyl adenine at concentration of (0.2) mg/l, then maintained in this medium for about a 4 weeks and incubated under light luminescent 3000 Lu×24/day at 24±1 C to regenerate shoots from callus Fig (2)

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Fig(1): callus induced from Axillary buds of (*Vitis vinifera*)

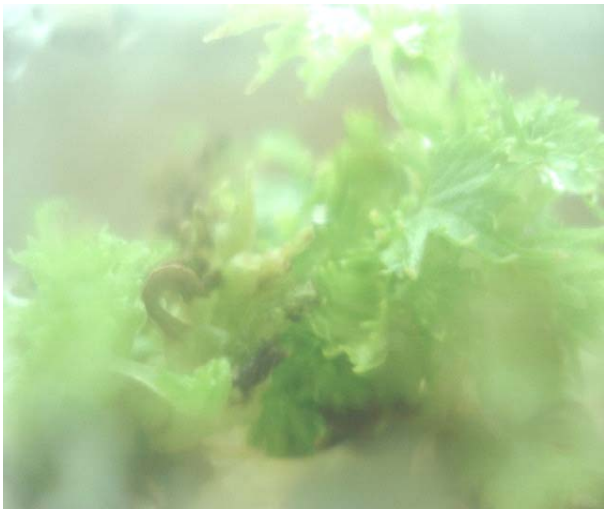

Fig (2): regenerated shoots from Callus (*Vitis vinifera*)

Table (1): MS media composition

Concentration Mg/l	Components	
Power	salt MS	1
0.1	Thiamine – HCL	2
0.5	Pyridoxine - HCL	3
0.5	Nicotinic acid	4
2.0	Glycine	5
100	Inositole	6
30000	Sucrose	7
8000	Agar	8

Extraction and Determination

Plant samples (seeds, callus, leaves of regenerated shoots) were freeze –dried, then powered and homogenized in mixture of solvents MeOH: EtOH: D.W:HCL 69:20:10:1 (v/v/v/v) for 24 hours, then solvent were evaporated under reduced pressure, the final volume of extract was 40 ml. pH was adjusted to 9 by adding NH4OH 10٪ and extracted with 30 ml x3 chloroform(CHCL3) the upper layer which contain the Phenolic compound were separated, and used for determination of Procyanidin. Normal-phase HPLC analysis of grape seeds and (cacao powder) standard was carried out under conditions similar to those described by [12] and supplied 5 concentration (1, 0.8, 0.6, 0.4, 0.2) injected in Uv- spectrophotometer in 280 nm.

Procyanidin detection and quantification

HPLC grade water at flow rate of 1 ml/min for 16 minutes. The analysis of Procyanidin in grape seeds is complex and has relied on reverse-phase HPLC with UV detection at 280 nm.(13–16). The binary mobile phase consisted of solvent A composed of methylene chloride/methanol/water/acetic acid (82:14:2:2 v/v/v/v), and solvent B composed of methanol/water/acetic acid (96:2:2 v/v/v). Separation was performed using a linear gradient at a flow rate of 0.2mL/ min−1 as follows: time for 16 minutes.

Statistical analysis

The data analysis by using (T-test) [13].

Results and discussion

The Phenolic compound isolated from alcoholic extracts of the different tissue, seeds, callus and regenerated shoots showed positive reaction to lead acetate indictor. Results, shown in figure(3) illustrates the differences in Procyanidin content affected with the concentrations of plant hormone in callus culture media, the presence of 1mg/L 2,4-D affected positively the Procyanidin production from callus,[14] observed the lowest hyosciamine/hyoscine ratio in callus grown on nutrient medium supplemented by 1mg/L(2,4-D). However, Procyanidin production from callus was more considerably affected, in callus grown on higher auxin supply (3mg/L 2,4-D) the content of Procyanidin was approximately three time lower than that of callus cultured grown on auxin (2mg/L 2,4-D). The data as show in the same figure that the quantitative yield of Procyanidin in different culture media was independent of cell

mass production [15] and is dependent of morphogenesis in (*Vitis vinifera* L) tissue culture , callus have the highest percentage of its compound production after 4 weeks in media containing 2,4-D at concentration of 2 mg/l it was about 0.30 mg/g dry weight compare with the other (1, 3) mg/l concentration of auxin present in MS media, This percentage in same of that repotted for *Datura metel* and *Datura innoxia* callus culture grown in MS media with 2,4-D at 2 mg/l [10].

They repotted *Datura metel* , callus cultures grown in media supplemented with 2,4-D at concentration of 0.1 mg/l enhanced the production of tropane alkaloids (0.51 mg/ dry weight) compared with leaves of parent plant [16].

Also, result show in figure(4) that After 4 weeks later of regenerate grown in media have a wide range variation in Procyanidin content and have significantly differed in their content with content of these compound in callus tissues grown in MS media with 2mg/L, but showed a wide range of significantly differed in Procyanidin content with callus grown in MS media with 1 and 3mg/L figures (5,6) and obtain increase about(0.8, o.12) mg/g dry weight compare with treatment of(2mg/l) 2,4-D and about seeds content. Shoots regenerated from callus tissue culture significantly differed in their Procyanidin content characteristics from seeds of donor plants, Also, Procyanidin content was diminished in their seeds and have differed substantially compare with content in other explants.

The main Procyanidin detected in shoot culture was much lower than that in seeds and more than in callus tissues. Shoots obtained from callus culture often display variable traits, the causes of this are not clear. It has been suggested that in the process of regeneration, genetic selection may occur [17]. Cell varying in ploidy level have been observed in callus culture employed for induction of organogenesis. The effect of auxin on callus initiation from Axillary buds of crop plant have been studied by numerous publications [4,18,19], found that auxin from the apex of parent plant does not enter the bud, suggested that the cytokinins transported from root and increase in the buds which promoting the branching later, in this cause the concentration of cytokinins used in tissue culture to regenerated plant from callus which lower than the concentration of auxin. BA(benzyl adenine) in combination with auxin in culture medium enhanced the production of Procyanidin from regenerate shoots. Al-Hatemy [10] observed that increased of tropane alkaloids production from callus tissue of *Datura Metel* and Datura innoxia with increase of BA in medium supplemented by cytokinins (BA). Therefore, substances occurring in the intact plant may not detectable in callus culture. Cell culture from (*Vitis vinifera)* are capable of production Phenolic compounds when morphologically differentiated, however, these substances could be detected again. In contrast, it could be shown in the present study that morphological differentiation is a prerequisite for Procyanidin production. This lead to the conclusion that auxin (2,4-D) is abundant and obviously a limiting factor in Procyanidin production. These results show that tissue culture are capable of production Procyanidin in quantities well above the limit of detection, depending on nutrient medium, auxin and cytokinins ratio and cell line (plant of origin/type of explants). However, production and extraction have to be increased manifold before large-scale production can be considered seriously. Also, these result revealed that the

biosynthesis of Phenolic compounds in plant which is subsequently deaminated by enzyme phenylalanine ammonia lyase (PAL), and the activity of these enzyme has been associated with the accumulation of anthocyanin and other Phenolic compounds [15]. And associated with the response to growth regulators in culture medium [15,19]. PAL activity has been detected in the green shoots and leaves.

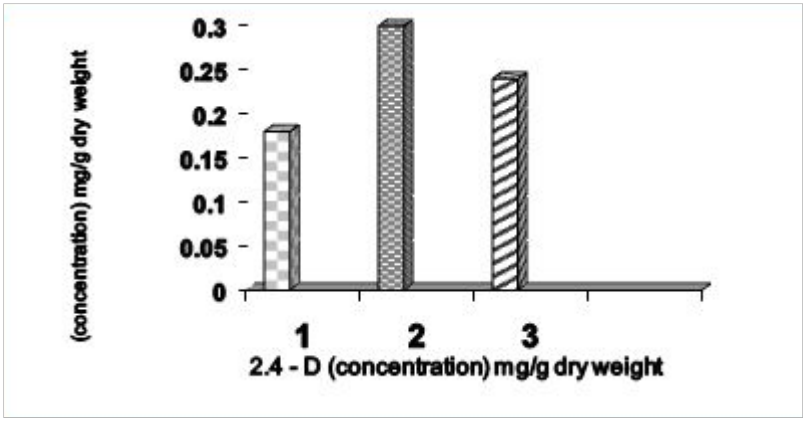

Fig (3): Effect of 2.4-D concentration on Procyanidin production from callus grown in (2mg/L) (*Vitis vinifera*).

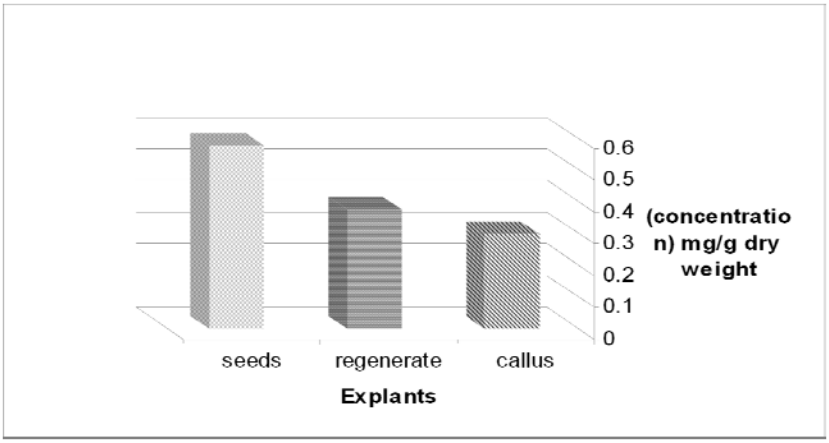

Fig (4) : Procyanidin content in (seeds) , Procyanidin production from callus grown in (2mg/L) (*Vitis vinifera*).

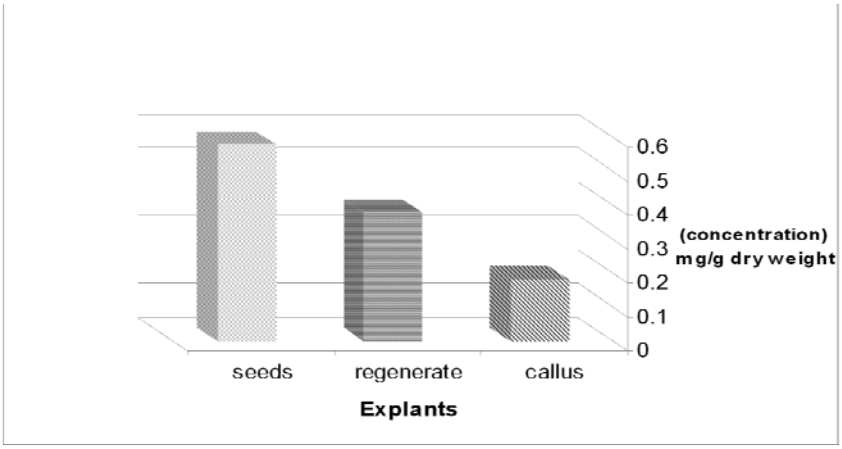

Fig (5): Procyanidin content in (seeds) , regenerate

plant and callus grown in 2.4-D 1mg/L

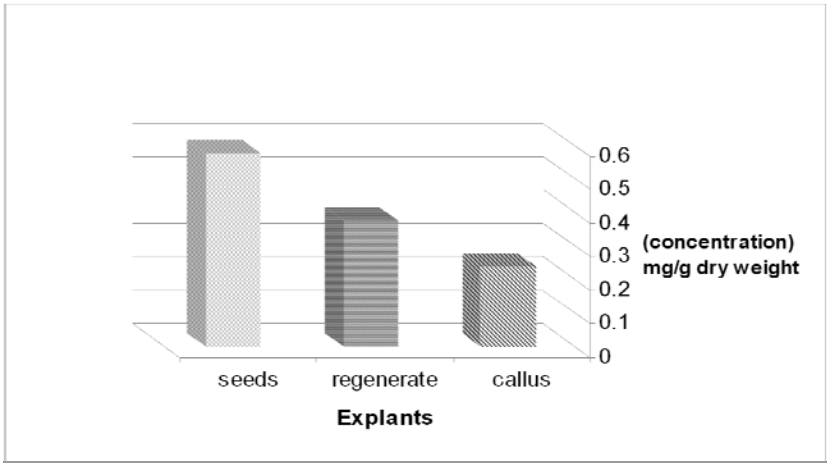

Fig (6): Procyanidin content in (seeds), regenerate plant and callus grown in 2.4- D(3mg/L)

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