Determination of Anthocyanins Content in *Prosopis farcta* L. Callus Cultures
تقدير محتوى الانثوسيانين في مزارع كالس نباتات الخرنوب *Prosopis farcta* L.

Rana Tariq Yahya **Hanaa Saeed Al-Salih**
College of Science/ University of Mosul
رنا طارق يحيى **هناء سعيد الصالح**
كلية العلوم/ جامعة الموصل

Abstract

The current study succeeded to initiate callus cultures of *Prosopis farcta* L. from hypocotyls stems which induced on the agar-solidified MS* medium supplemented with 4.0 mg/ L TDZ alone or with addition of 1.0 mg/ L NAA. Callus initiation percent reached 100% after 7 days of culture when hypocotyls stem segments were cultured on MS medium with addition of both TDZ and NAA. Whereas when TDZ alone were added initiation percent reached 60%. The results revealed detection of high amount of Anthocyanins which reached 440.98 µm/gm of fresh weight of callus cultures, grown on MS* medium supplemented with 1.0 mg/ L NAA and 4.0 mg/L TDZ, then 182.24 µm/gm of fresh weight of callus grown on MS with addition of 4.0 mg/L TDZ alone.

MS* : MS medium with increasing KNO_3 to 2000 mg/ L, Thiamine-HCl to 0.5mg/ L, Pyrodoxine-HCl to 1.0 mg/L

Key words: *Prosopis farcta* plants, callus cultures, anthocyanins content

المستخلص

نجحت الدراسة الحالية في إنشاء مزارع كالس لنباتات الخرنوب *Prosopis farcta* L. من قطع السيقان تحت الفلقية على وسط MS* الصلب مدعماً بإضافة 4.0 ملغم/ لتر TDZ لوحده او مع 1.0 ملغم/ لتر NAA. وبلغت نسبة استحداث الكالس 100% بعد سبعة ايام من الزراعة على وسط MS الصلب مضافاً اليه كل من NAA و TDZ . بينما عند اضافة 4.0 ملغم/ لتر TDZ لوحده اعطى نسبة استحداث للكالس 60%. وأظهرت نتائج الدراسة أن اعلى كميات للانثوسيانين بلغت 440.98 مايكروغرام/غرام وزن طري للكالس النامي على الوسط MS* مدعماً بإضافة 1.0 ملغم / لتر NAA و 4.0 ملغم / لتر TDZ ، يليه 182.24 مايكروغرام/غرام وزن طري للكالس النامي على وسط MS* مضافا اليه 4.0 ملغم/ لتر TDZ لوَحدهِ.

MS*: وسط MS مع زيادة تركيز KNO_3 الى 2000 ملغم/ لتر، Thiamine-HCl الى 0.5 ملغم/ لتر، Pyrodoxine-HCl الى 1.0 ملغم/ لتر.

الكلمات المفتاحية : نباتات الخرنوب ، مزارع الكالس ، محتوى الانثوسيانين

Introduction

Plant secondary metabolites have found applications in pharmaceutical industries, cosmetics, biopesticides and agrochemicals, flavours or food additives and natural pigments [1]. Flavonoids' is a class of polyphenolic compounds commonly found in plants. There are over 4000 compounds that have been characterized as flavonoids [2].

Anthocyanins are the largest group of water-soluble pigments in the plant kingdom and belong to a family of compounds known as flavonoids. Anthocyanins are responsible for most of the red, blue, and purple colors of fruits, vegetables, flowers, and other plant tissues or products. Several studies have shown that anthocyanins display antioxidant activity [3]. Anthocyanins have much interest among researchers considered as secondary metabolites [4]. Currently, plant biotechnology such as plant tissue culture which considered as an alternative source for the production of many natural products among which anthocyanins, which produced by plant cell and tissue cultures had been reported for many species including *Crataegus sinaica* BOISS [5], *Catharanthus roseus* [6], *Daucus carota* L. [7]. *Prosopis farcta* L. which is a member of the family Leguminosae [8].

This study aimed firstly, production of large amount of callus cultures from hypocotyls stem segments of *Prosopis farcta* L. and secondly, detection the production of anthocyanins in callus cultures of this plant, and quantitative determination of the content of anthocyanins in callus cultures.

materials and methods

Sterile seedlings production:

Seeds of *Prosopis farcta* L. were obtained from mature fruit of natural grown plants near Hay Al-Arabi in Mosul city . The seeds were washed in running water for five minutes, soaked two minute in ethyl alcohol 96% then in 1:2 (V/V) sodium hypochlorite (NaOCl): water with shaking for five minutes, and rinsed thoroughly in sterilized distilled water three times [9]. Then seeds were placed on the surface of agar-solidified MS [10] medium with 0.8% agar and supplemented with 3% sucrose, pH adjusted to 5.8 before autoclaving. Cultures maintained in culture room at 25 ±2°C in dark. After seven days germination started, so they transferred to the light condition with 16 hour light at 1500 lux.

Callus cultures initiation

Excised 2.0 cm of hypocotyl stems after 21 days of growth were placed on the surface of agar-solidified MSO (control) and MS medium modified by increasing the concentration of KNO_3 to 2000 mg/L, Thiamine-HCl to 0.5 mg/L and Pyrodoxine- HCl to 1.0 mg/L [7], the medium was supplemented with different concentrations of NAA and TDZ each alone or together.

Determination of anthocyanins content in fresh callus

Anthocyanins content in callus cultures of *Prosopis farcta* L. was determinate according to the method of [11], for that 0.5 gm of fresh callus after 60 days of growth on solidified MS medium (with addition of NAA and TDZ each alone or with combination) was weighed in 15 ml plastic centrifuge tube and divided to small pieces using forceps. Five ml of methanol containing 1% concentrated HCl at 4°C was added to the sample. The tubes were vortexed then the samples were centrifuged at 15000 g for 20 min at 4°C. Absorbance of the clear supernatant was measured at 528 nm. Anthocyanins content was calculated according to the extinction coefficients: ($E1^{1\%}$ cm = 680 at 528 nm) where cm: cuvette width. Total anthocyanins yield was expressed as (µg/gm) fresh weight of callus.

Results and Discussion

Callus cultures production:

Table (1) showed easily callus initiated in all tested media used in this study, except MSO medium which did not stimulated callus formation the explants turned yellowish and died after 15 days from culture.

The results explained that TDZ played an essential role for callus formation either alone or when interacted with NAA in MS* especially the concentration 4.0mg/L of TDZ Table (1). The better medium for callus initiation was MS* supplemented with 1.0mg/ L of NAA+ 4.0mg/L of TDZ in which callus initiation percent recorded to be 100%, with steadily enhanced callus fresh weight, the callus grown on this medium was friable and yellow-green in color Figure (1-A). Callus divided into small pieces and transferred to a new medium with the same composition all are succeeded to grown again forming callus cultures Figure (1-B). MS* medium supplemented with 4.0 mg/L TDZ alone encouraged callus growth but less than the medium mentioned above. This callus was friable type and yellowish-green in color Figure (1-C)

Table (1): Efficiency of NAA and TDZ in MS* media for callus induction from hypocotyl stem explants of *Prosopis farcta* L. seedlings

Induction medium (mg / L)	Fresh weight (gm) 30 day	60 day	Callus initiation (%)	Period of initiation (days)
MSO	0	0	0	0
MS*+1.0 NAA	0.13	0.16	10	30
MS*+1.0 NAA+ 2.0 TDZ	0.52	0.7	20	15
MS*+1.0 NAA+ 4.0 TDZ	6.52	18.82	100	7
MS*+ 2.0 TDZ	0.6	1.13	30	12
MS*+ 4.0 TDZ	1.68	3.67	60	10

ten explants cultured / treatment

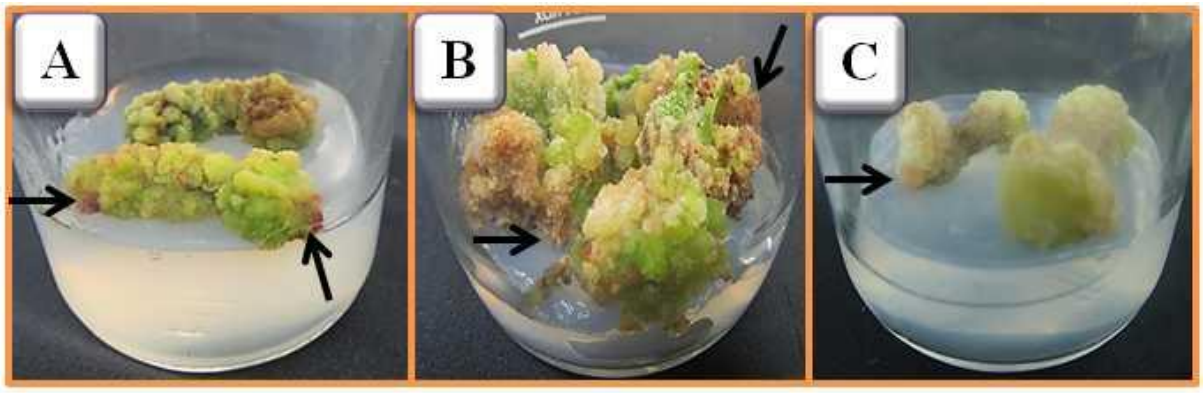

Fig. (1): Callus initiation and growth from hypocotyl stem segments of *Prosopis farcta* L.
(A) Callus initaiated from hypocotyl stems after 20 days grown on MS*+1.0 mg/L NAA+ 4.0 mg/L TDZ
(B) Callus culture from (A) grown for 60 days
(C) Callus initiated from hypocotyl stem segments after 30 days of growth on MS* + 4.0 mg/L TDZ
Arrows: refers to the red color of callus

Other media in Table (1) tested in this study did not enhanced great division of callus cells and the callus grown on these media were weakly in growth.

The high activity of TDZ were relative to purine cytokinins ,which may stimulated de novo synthesis of auxin [12], and had been attributed to its extreme stability both in a free and conjugated form within the plant tissues [13]. The low concentration of auxin with high concentration of TDZ in the medium enhanced callus initiation [14]. NAA in low concentration was reported to play a role in rapid cell division [15]. In our study when NAA applied together with TDZ there was significant increasing rate in callus induction and growth, this due to the effect of cytokinin which was mentioned to enhance cell division [16]. This was better than in the case of adding of 2.0 mg/L TDZ alone or with 1.0 mg/L NAA

Anthocyanins content:

Data showed successful anthocyanins production in all types of callus produced in this study with different content dependent on the concentration of plant growth regulators added to the media.

The obtained results in Table (2) expressed the high anthocyanins content in callus that grown on the agar solidified MS* with 1.0 mg/L NAA and 4.0 mg/L TDZ Figure (2-A), in which anthocyanins content reached up to 440.98 µm/gm fresh weight. This high content of anthocyanins improved the red color of callus Figure (2-B) represented the color appeared on callus tissue growing on the above medium Figure (1-A). Whereas anthocyanins detected in the callus grown on MS* medium containing 4.0 mg/L TDZ decreased to reach 182.24 µg/gm ,it represents in red-color which appeared on callus tissue Figure (1-C).

Table (2): Anthocyanins content in callus tissues of *Prosopis farcta* L. after 60 days of growth on MS* supplemented with NAA and TDZ

Samples	Anthocyanins (µg /gm)
MS*+1.0 NAA	**82.28**
MS*+1.0 NAA+ 2.0 TDZ	**80.24**
MS*+1.0 NAA+ 4.0 TDZ	**440.98**
MS*+ 2.0 TDZ	**88.74**
MS*+ 4.0 TDZ	**182.24**

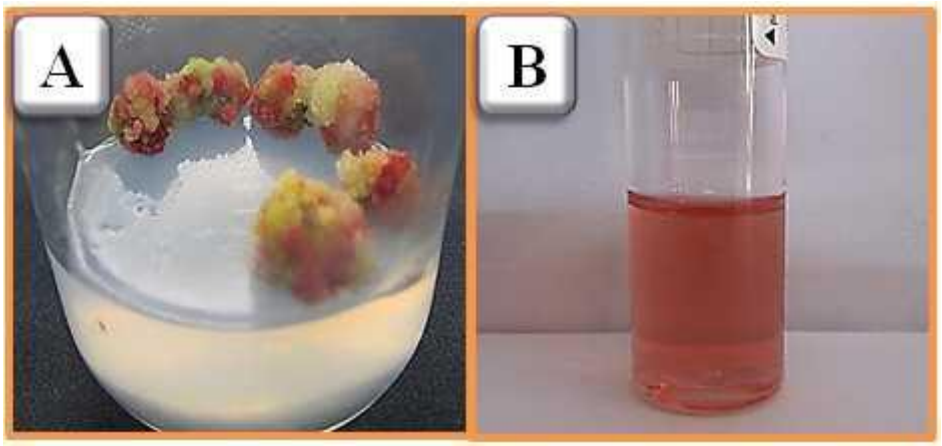

Fig. (2): Callus tissues from *Prosopis farcta* L. grown on MS*+1.0 mg/L NAA+ 4.0 mg/L TDZ(A) and its anthocyanins extract (B)

The difference in Anthocyanins content may be due to the structural genes encoding the enzymes of the anthocyanins biosynthetic pathway which are conserved among different plant species and their expression is regulated by several regulatory genes [17]. TDZ a cytokinin causes an increase in anthocyanins accumulation in cultures and in parts of intact plants as in Rosa [18], *Daucus carota* L. [7]. Addition to the treatments with TDZ were performed for induction of anthocyanins synthesis in cells and the effect of cytokinins on flavonoids pathway and anthocyanins accumulation was well demonstrated in *Arabidopsis* plants[19,20].

References

1. Ramachandra Rao, S. and Ravishankar, G.G. (2002). Plant cell cultures: chemical factories of secondary metabolites. Biotechnol Adv. 20:101–53.
2. Lentini, A., Forni, C., Provenzano, B. and Beninati, S. (2007). Enhancement of transglutaminase activity and polyamine depletion in B16-F10 melanoma cells by flavonoids naringenin and hesperitin correlate to reduction of the in vivo metastatic potential. Amino. Acids., 32: 95-100.
3. Mazza, G. (2007). Anthocyanins and heart health. Ann Ist Super Sanita. 43: 369-374.
4. Peterson, J. and Dwyer, J. (1998). Flavonoids: Dietaryoccurrence and biochemical activity. Nutr. Res., 18: 1995-2018.
5. Maharik, N., Elgengaihi, S. and Taha, H. (2009). Anthocyanin production in callus cultures of *Crataegus sinaica* Boiss. Internat. J. Acad. Res., 1:30-34.
6. Taha, H. S., Abd El-Rahman, R. A., Fathalla, A., Abd-El-Kareem, M. and Aly, U. E. (2008). Successful Application for Enhancement and Production of Anthocyantn Pigment from Calli Cultures of Some Ornamental Plants . Aust. J. Basic and App. Sc. 2: 1148-1156.
7. Al-Anzy, A. A. M. (2013). Conservation of *rol-genes* in carrot (*Daucus carota* L.) Plants Regenerated From its Transformed Tissues by *Agrobacterium rhizogenes* R1601. Ph.D. Thesis, College of Science, University of Mosul, Iraq.
8. Kumar, K and Singh, N. (2009). Micropropagation of *Prosopis cineraria* (L.) druce – A multipurpose desert tree. Res. 1,28-32.
9. Yahya, R. T. and Al-Salih, H. S. (2013). Induction of callus cultures from stems and cotyledonary leaves of *Prosopis farcta* L. by using some plant growth regulators. Accepted in Raf. J. S.
10. Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol. Plant. 15: 473-479.
11. Mori, T., Sakura , I. M., Sekim, M. and Furusaki, S. (1993). Use of auxin and cytokinin to regulate anthocyanin production and composition in suspension cultures of strawberry cell. J. Sci. Food Agric. 65:271-276.
12. Neumann, K., Kumar, A. and Imani, J. (2009). Plant Cell and Tissue Culture – A tool in Biotechnology. Springer, Berlin, Germany.
13. Murch, S.J. and Saxena, P.K. (2001). Molecular fate of thidiazuron and its effects on auxin transport in hypocotyls tissues of *Pelargonium* × *hortorum* Bailey. Plant Growth Regul. 35:269-275.
14. Chaudhary, Z., Afroz, A. and Rashed, H. (2007). Effect of variety and plant growth regulators on callus proliferation and regeneration response of three tomato cultivars *(Lycopersicon esculentum*). Pak. J. Bot. 39, 857-869.

15. Juan , L., Lihua, W., jing, L. and Junhui,W. (2010). Effect of different plant growth regulators on callus induction in *Catalpa bungei*. African J. Agri. Res. 5: 2699-2704.
16. Gaba, V.P. (2005). Plant Growth Regulators in Plant Tissue Culture and Development. In: Plant Development and Biotechnology. Trigiano, R.N. and D.J. Gray (Eds.). CRC Press, Washington DC. USA.
17. Quattrocchio, F., Wing, J., Woude, K., Souer, E., Vetten, N. , Mol, J. and Koes, R. (1999). Molecular analysis of the *anthocyanin2* gene of Petunia and its role in the evolution of flower color. The Plant Cell. 11 :1433-1444.
18. Talebi, S., Mortazavi, S. N., Naderi, R. and Sharafi, Y. (2013). Role of nitric oxide and thidiazuron on changes of pigments during postharvest in Rosa (Cv. 'Sensiro'). Internat. J. Agron. Plant Prod. 4:121-126.
19. Wade. H. K., Sohal, A. K. and Jenkins, G. I. (2003). *Arabidopsis* ICX1 is a negative regulator of several pathways regulating flavonoid biosynthesis genes. Plant Physiol. 131: 707-715.
20. Deikman, J. and Hammer, P. E. (1999). Induction of anthocyanin accumulation by cytokinins in *Arabidopsis thaliana*. Plant Physiol. 108: 47-57.