INFLUENCE OF EXPLANT AND GROWTH REGULATORS IN THE INDUCTION OF CALLUS OF *Annona Muricata* IN VITRO

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ABSTRACT

This study was conducted in the laboratory of plant tissue culture Department of Horticulture and Landscape , College of Agriculture – University of Baghdad during 2015-2016. The study includ using of plant tissue culture technology in the induction of callus from young explants (Leaf and node) planted in the MS media containing different concentrations of growth regulators, with the use of different auxins (Picloram, NAA and Cytokinin BA) at different concentrations for the purpose of induction callus. The results showed, that the young leaf was excelled on the young node which was planted in the MS media with a concentration of (1.2 mg.L^{-1}) picloram and (0.6 mg.L^{-1}) BA , the highest fresh and dry weights of callus were obtained reached of (212.25, 22.43 mg) respectively, compared to the young node which gave, (94.00, 11.43 mg) at the same concentration of fresh and dry weight, respectively. The young leaf was excelled on the young nods for the of *Annona muricata* in the MS media at a concentration of (1.0 mg.L^{-1}) NAA and (0.6 mg.L^{-1}) BA, which it gave the highest fresh and dry weights of the callus (185.00, 21.43 mg), respectively, compared to young nods which gave, (140.43, 14.86 mg) at the same concentration, of fresh and dry weight of callus respectively.

*** Research from the thesis of Master for the second Author**

تأثير الجزء النباتي ومنظمات النمو في استحثاث الكالس لنبات القشطة خارج الجسم الحي

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المستخلص

نفذت الدراسة في مختبر زراعة الانسجة النباتية التابع لقسم البستنة وهندسة الحدائق – كلية الزراعة – جامعة بغداد خلال عامي 2015 – 2016 وتضمنت الدراسة استخدام تقنية زراعة الانسجة النباتية في استحثاث الكالس من الاجزاء النباتية (ورقة فتية , عقدة فتية) المزروعة في وسط MS الحاوي على تراكيز مختلفة من منظمات النمو اذ استخدمت الاوكسينات المختلفة من الـ Picloram , NAA والسايتوكانين BA وبتراكيز مختلفة لغرض استحثاث الكالس . بينت النتائج تفوق الورقة الفتية على العقدة الفتية المزروعة في الوسط الغذائي المجهز بتركيز 1.2 ملغم لتر$^{-1}$ من الـ Picloram و0.6 ملغم لتر$^{-1}$ من الـ BA واعطت اعلى معدل وزن طري وجاف للكالس بلغ (212.29 , 22.43)ملغم على التوالي مقارنة بالعقدة الفتية التي اعطت عند التركيز نفسه (94.00 , 11.43)ملغم وزن طري وجاف للكالس على التوالي . تفوقت الورقة الفتية على العقدة الفتية لنبات القشطة في الوسط الغذائي MS المجهز بتركيز1.0ملغم.لتر$^{-1}$ من الـ NAA و0.6 ملغم.لتر$^{-1}$ من الـ BA اذ اعطت اعلى معدل وزن طري وجاف للكالس بلغ (185.00 ,21.43) ملغم على التوالي . مقارنة بالعقدة الفتية التي اعطت عند التركيز نفسه (140.43 , 14.86) ملغم وزن طري وجاف للكالس على التوالي .

***البحث مستل من رسالة ماجستير للباحث الثاني**

1. INTRODUCTION

Annona muricata, it is an evergreen or semi-deciduous plant, which is from a tropical fruit or under tropical, belongs to the Annonaceae family, which includes 130 genus and 2300 species. *Annona muricata* is called by several names, including graviola, soursop, guanabana. The original country varies by species, some of which India, it is original country, including tropical America, Brazil, Thailand, Ecuador and Peru, Callus production depends on the type of explant used and the components of the food media, through the cultivated explant different aspects of the calcareous are obtained, It may be stiff or brittle textures, sometimes the Callus appears yellow, white, or green depending on the plant segment used to inducing callus [1]. The (4) indicated to the importance of placing callus in the dark, As darkness has an important and effective role in preventing the oxidation of some light-sensitive compounds such as auxins, darkness also inhibits the appearance of phenolic substances by light-induced oxidation enzymes. Darkness leads to increased thinness of callus wall and reduce their thickness, leading to increased growth regulators into the tissue. And then increase the response of the explant to the induction of callus. The selection of the explant for tissue culture is associated with the target required to be accessed, and depending on the response of the explant to the farming process [5]. Based on the previous information, the importance of research is illustrated by the induction of callus from different parts of the plant of the *Annona muricata* and the extraction of some of the alkaloids of acetogenins for its known medical importance.

2. MATERIALS AND METHODS

This study was conducted in the Laboratory of Plant Tissue Culture - Graduate Studies - college of Agriculture - University of Baghdad, for the period (9/3/2015 - 20/6/2017) in order to stimulate callus and evaluate the active substances in it.

1. Preparation of Nutrient Media

The prepared MS media used [6] with a weight of (4.33 $g.L^{-1}$) produced by (the Dutch company Duchefa) at all propagation stages. Table (1) shows concentrations of inorganic salts in the MS nutrient media. Both vitamins and plant growth regulators were added to the nutritional media after they were prepared as base solutions, auxins (picloram, NAA) and cytokinines (BA) has been tested during the implementation of the research and according to the different stages of the study, and ensures the nutritional media these organizations with the required concentrations according to each experiment. Sucrose and myo-inositol were added in quantities as shown in Table (2) with reference to the use of iodized distilled water in the preparation of base solutions and nutrient media, after adding all the components of the nutrient media, complete the volume to 800 ml. pH was adjusted to 5.7 (by adding 1 mol of NaOH as a drops) or 1 mol of HCl, which is suitable for hardening of the nutrient media as required, As well as, availability mineral elements for absorption from implanted part using a pH meter, then complete the final size to 1000 ml and added 7 $g.L^{-1}$ of the Agar to hardened of the media, and for dissolving the Agar and mix the components media well. The nutrient media was heated on the thermal magnetic Stirrer (Hot plate Magnetic Stirrer) and after it became homogeneous, distribute the nutrient media in the culture tubes with 10 ml per tube and covered with appropriate covers, it was sterilized by the autoclave at 121°C and 1.04 $kg.cm^2$ for 15 minutes, the culture tubes were removed from the autoclave after the sterilization stage was completed and placed in the agriculture room and left to cool.

2. Surface Sterilization of Explant

The explant was sterilize after washing with running water and cleaning fluid for 5 minutes to get rid of dust and materials suspended on the surface, and placed in 200 ml bottles, it was moved in the Laminar Air Flow Cabinet to perform sterilization, the explant to be sterilized were submerged without cutting with concentration (3, 2, 1)% of sodium hypochlorite and adding two droplets of diffuse material (Cleaning fluid) for 10 minutes with continuous shaking, then washed with sterilized distilled water three times for five minutes each time to get rid of the residue of sterile material.

3. Callus induction:

After obtaining the best concentration of sterilization, the explants (part of the young leaf and young nodes) were planted in the prepared MS nutrient media, whose components are shown in Table (2) and by 10 replicates for each explant and concentration. The plants were incubated in total darkness and the fresh and dry weight measurements were taken after 6 weeks of planting.

4. Measuring The Fresh and Dry Weight of The Callus :

The fresh and dry weight of the callus was measured after six weeks of planting using a sensitive balance, after removing the remnants of the glue attached to it using surgical blade, the callus cutting was dried in oven at 70°C and weighed at constant weight, the fresh and dry weight standard for induced callus was used at the stage of evolution to determine the best type and concentration of plant growth regulators used.

5. Statistical Analysis:

Study experiments were designed as factorial experiments using Completely Randomized Design (CRD). The results were analyzed using the statistical program Genstat Discovery Edition for 2012, and the averages were measured by the Least Significant difference (L.S.D) under 5% probability level, with 10 replicates per plant fraction and treatment [3].

Table 1: Concentration of inorganic salts used in MS media

Name of Compound	Chemical Formula	Concentration (mg. L^{-1})
Ammonium nitrate	NH_4NO_3	1650
Potassium nitrate	KNO_3	1900
Magnesium Sulfate Heptahydrate	$MgSO_4.7H_2O$	370
Manganese Sulfate Monohydrate	$MnSO_4H_2O$	16.9
Zinc sulfate heptahydrate	$ZnSO_4.7H_2O$	8.6
Copper sulfate pentahydrate	$CuSO_4.5H_2O$	0.025
Calcium chloride dihydrate	$CaCl_2.2H_2O$	440
Cobalt Chloride Hexahydrate	$CoCl_2.6H_2O$	0.025
Potassium iodide	KI	0.83
Boric acid	H_3BO_3	6.20
Dipotassium phosphate	KH_2PO_4	170
Sodium Molybdate Dihydrate	$Na_2MoO_4.2H_2O$	0.25
Iron sulfate heptahydrate	$FeSO_4.7H_2O$	27.84
ethylenediaminetetraacetate	$EDTA.Na_2$	37.25

Table 2: The components of the MS nutrient media for the study experiments

Materials	The components of the MS nutrient media (mg. L^{-1})
MS salts	Full power
Pyridoxine – HCl	0.5
Glycine	2.0
Nicotice acid	0.5
Thiamine – HCl	0.1
Myo-insitol	100
NAA	1.0 ,0.6 ,0.2 ,0.0
BA	1.0 ,0.6 ,0.2 ,0.0
Picloram	1.2 ,0.8 ,0.4 ,0.0
Sucrose	30000
Agar	7000

3. RESULTS AND DISCUSSION

The results of Table (3) showed significant differences when the explant was overlapped with Picloram concentrations and BA concentrations, The young leaf was excelled that cultivated in MS media with a concentration of 1.2 mg L^{-1} of Picloram and 0.6 mg L^{-1} of BA, And gave the highest rate of fresh weight of callus reached 212.29 mg. The young node achieved the rate of fresh weight for the callus of 94.00 mg at the same concentration of Picloram and BA. Table (4) showed significant differences when the explant overlapped with different concentrations of Picloram and BA. The young leaf that cultivated in MS media, was excelled with a concentration of 1.2 mg L^{-1} of Picloram and 0.6 mg L^{-1} of BA by giving it the highest dry weight of 22.43 mg. It was significantly excelled than the rest of the other treatments compared to the young node, which gave the rate of 11.43 mg of dry callus at the same concentration. The control treatment was not given any weight of callus for the young leaf and the young node. Also for concentration of 0.4 mg L^{-1} of Picloram in interaction with concentrations of (0.6, 0.2) mg / L^{-1} of BA. Table (5) indicates significant differences in the interaction between explant with different NAA and BA concentrations. The young leaf of the *Annona muricata* that cultivated in MS media, was excelled with a concentration of 1.0 mg L^{-1} of NAA and 0.6 mg L^{-1} of BA by giving it the highest fresh weight of callus reach of 185.00 mg. It was significantly excelled than the rest of the other treatments compared to fresh callus induction from the young node for *Annona muricata*, which gave the rate of fresh weight reach of 11.43 mg at the same previous interaction of NAA and BA. Table (6) shows significant differences in the interaction between explant with different NAA and BA concentrations. The young leaf of the *Annona muricata* that cultivated in MS media, was excelled with a concentration of 1.0 mg L^{-1} of NAA and 0.6 mg L^{-1} of BA by giving it the highest dry weight of callus reach of 21.43 mg. It was significantly excelled than the rest of the other treatments compared to fresh callus induction from the young node for *Annona muricata*, which gave the rate of dry weight reach of 14.86 mg at the same previous interaction of NAA and BA.

Table 3: Effect of triple -interaction between explant, Picloram, and BA in the fresh weight (mg) of callus induced from the *Annona muricata*

Explant	**Picloram mg.L^{-1}**	BA **mg.L^{-1}**		
		0.2	**0.6**	**1.0**
Young leaf	0.4	0.0	0.0	34.0
	0.8	23.86	109.29	64.14
	1.2	85.43	212.29	145.29
Young node	0.4	0.0	18.86	25.00
	0.8	31.14	63.71	64.00
	1.2	46.41	94.00	86.14
LSD 0.05	**3.97**			

Table 4: Effect of triple -interaction between explant, Picloram, and BA in the dry weight (mg) of callus induced from the *Annona muricata*

Explant	**Picloram mg.L^{-1}**	BA **mg.L^{-1}**		
		0.2	**0.6**	**1.0**
Young leaf	0.4	0.0	0.0	4.86
	0.8	3.29	12.00	7.57
	1.2	10.43	22.43	15.86
Young node	0.4	0.0	3.43	4.71
	0.8	4.57	8.43	6.57
	1.2	7.00	11.43	10.29
LSD 0.05	**0.51**			

Table 5: Effect of triple -interaction between explant, NAA, and BA in the fresh weight (mg) of callus induced from the *Annona muricata*

Explant	**NAA mg.L^{-1}**	BA **mg.L^{-1}**		
		0.2	**0.6**	**1.0**
Young leaf	0.2	3.29	31.71	22.9
	0.6	14.86	75.29	64.14
	1.0	75.71	185.0	196.00
Young node	0.2	17.00	34.71	42.14
	0.6	53.29	71.86	85.17
	1.0	55.57	140.43	133.71
LSD 0.05	**4.09**			

Table 6: Effect of triple -interaction between explant, NAA, and BA in the dry weight (mg) of callus induced from the *Annona muricata*

Explant	**NAA mg.L^{-1}**	BA **mg.L^{-1}**		
		0.2	**0.6**	**1.0**
Young leaf	0.2	0.57	4.71	3.43
	0.6	2.57	8.57	7.57
	1.0	9.00	21.43	23.29
Young node	0.2	3.14	5.00	5.86
	0.6	7.00	8.57	10.57
	1.0	6.71	14.86	13.71
LSD 0.05	**0.53**			

The results of tables (3) (4) (5) (6), it is shows that auxins in general is necessary for the induction of callus tissue from explant cultivated in vitro, the sources indicate that the treatment of auxin affect the physiological state of the cells and change the pattern of differentiation in the cells that respond to him, It was found that the treatment of auxins has made the differentiated cells of the explant suffer from the case of loss of differentiation and accelerate the division to formation of callus tissue [7][8]. The cause may be due to these effects (Increase the soft and dry weight of callus) to Cytokinines, especially benzyl adenine, encourage the absorption of nutrients into the treated cells and stimulate cell division as well as inhibit protein degradation and stimulate photosynthesis enzymes which reflected in increased cell volume and encourage the process of the division especially when reach the ideal equilibrium state. It also increases the build-up of RNA, proteins and enzymes within the cell [9]. The increase in the fresh and dry weight of callus is a reflection of the changes in the different contents of the cultivated explant cells based on its growth in the used nutrient media, which is based mainly on the growth regulators added, the process of dividing the callus cells is accompanied by an increase in the important contents of the maintenance of division and growth such as carbohydrates, proteins and amino acids with internal changes leading to cell division [10]. Many researchers have reported that cytokinines, particularly BA, in tissue culture are Is due to the fact that they are stable compounds for not being easily analyzed it and efficient in stimulating cell division and increasing the production of nuclear acids [11][12]. The high levels of auxins and cytokinines cause a decrease in growth rates and cell division due to disruption of biological processes within tissues, As a result of the hormonal imbalance leading to the formation of ethylene, which in turn inhibits the metabolic activities and low rates of division and growth of plant parts [13][14]. Therefore, the balance between growth regulators in the nutrient media leads to the induction of callus at different rates and the velocity of the callus induction depends on the concentration of the internal growth regulators found in the explant. The phenomenon of the formation of callus stimulated by growth regulators is a strong evidence of the process of allocating cells and not lose their potential energy, which leads to the stimulation of cellular division. These results agree with [16], that the induction of the callus depends on the type and source of the vegetative part and on the type and concentration of the growth regulators of the nutrient media. Several researchers have indicated to the importance of the incubated in dark, which is due to the role of darkness in preventing the oxidation of some light-sensitive compounds such as auxins, Darkness may therefore inhibit oxidation of phenolic substances by light-induced oxidation enzymes, It is also believed that the incubation of the explant in the dark lead to increased permeability of materials, especially growth regulators into the tissues and then the transplantation of these parts to stimulate callus.

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