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Effect of plant growth regulators in inducing *Vitisvinifera* Callus and increased production of flavonoids in vitro

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Abstract

This study was aimed to use of plant tissue culture technology to induce callus from grapevine *Vitisvinifera* and stimulate it to increase the production of flavonoids. The study was carried out in two stages after the sterilization process was carried out: The first included establishing callus farms by cultivating cuttings containing a single node after completing their sterilization and cultivating them on MS medium containing 2,4-D and BA at different concentrations (0, 1, 2, 3,4) mg $L^{-1}$and BA in concentrations (0, 0.1, 0.2, 0.4) in independent experiments. The second phase included studying the effect of growth regulators on the concentrations of carbohydrates and flavonoids in the callus induced from the first experiment. The results of the study showed the superiority of auxien 2,4-D at a concentration of 2mg L^{-1}as it achieved the highest average fresh and dry weight of callus, reaching (3.32 and 1.03) mg respectively, while BA at a concentration of 0.2 mg L^{-1} achieved the highest average fresh and dry weight of callus was (1.91 and 0.51) mg respectively. The results also showed that the MS medium prepared with a concentration of 4 mg $L^{-1}$of 2,4-D was significantly superior in the average concentration of the compounds Hesperdine, Nargnine, Proanthocyanin, and Rutin, reaching (64.94, 62.56, 55.63, and 71.22)% respectively.While the medium prepared with a concentration of 3 mg L^{-1}outperformed 2,4-D achieved the highest concentration of Quercetin compounds reaching 75.36% compared to the neutral treatment, which achieved the lowest concentrations. The concentration of 0.4 mg L^{-1}BA achieved the highest concentration of the compounds Hesperdine, Nargnine, Proanthocyanin, Quercetin, and Rutin, reaching (52.65, 49.95, 47.65, 59.92, 56.70)% respectively.

Keywords: *Vitisvinifera*.calluses , flavonoids .Benzyladenine.

Introduction

The grape *Vitisvinifera* (L.) belongs to the grape family Vitaceae and is one of the late-maturing table grape varieties, quick to bear fruit and give, with excellent nutritional and medicinal value, and has a palatable taste and a striking aesthetic appearance. Its cultivation has spread in Asia, Africa and Europe [1]. Grapes are a fruit as

old as history itself. They are mentioned in the Torah, in myths, and in many tales of the ancients. It is almost difficult for us to enumerate all of their benefits and properties. Some nutritional scientists equate them with milk, and some of them confirm that grapes have properties that are not found in milk. How? This has been the case and it is established that grapes are among the most beneficial and profitable fruits and that they have an effective role in building the body. Some consider them to be a complement to enzymes, and others consider them to be an aid to growth and maintaining the best activity of the coverings of various organs, such as the liver and stomach, or for various functions such as vision and digestion [2]

Grapes are characterized by containing a good percentage of quickly absorbed sugars, as glucose and fructose are highly concentrated. They are also rich in vitamins such as vitamins C and B. They also contain a good percentage of mineral elements such as potassium, calcium and sodium. Therefore, grapes are given the value of a sugar multimineral salt because the presence of these Minerals activate ionization and thus increase the biological activity of these components. As for sugars, fatty substances and albumins, we find grapes also at the forefront of fruits, as they contain glucose and fructose, which in themselves constitute a nutritional element because they are easy to digest and are of high nutritional value because of the other substances associated with them, especially vitamin B1 and B2, which help nourish tissues and absorb sugar. Therefore, it is necessary for cardiac muscle function and the functions of the central nervous system, and it also contains flavonoids, phenolic compounds, anthocyanins, and procyanidine that have nutritional and therapeutic value [3] .Flavonoids, which have been carefully studied in grapes, are known to be organic compounds that are soluble in water. They belong to the category of polyphenolic with therapeutic power and have antioxidant and anti-free radical effects in certain doses. It has been found that proanthocyanin have a role in enhancing the level of good cholesterol and reducing bad cholesterol in the body. The body reduces the risk of coronary artery disease and maintains heart health [4,5].These compounds have also been proven through clinical trials conducted on humans to be effective, including the Rutin compound, in preventing bleeding from the extremities or from natural orifices, and preventing swelling of the legs as a result of water retention in the body. They also protect against retinopathy associated with diabetes and also protect against high blood pressure [6],Flavonoids have been widely used in European countries, including hesperidins in particular, to treat alcohol-related liver disorders. Nargnine also plays a role in cancer prevention, as it works to inhibit free radicals that cause damage to DNA, and this damage can lead to cancer. Quercetin has also been used to treat allergies, arthritis, and asthma [7, 8].Interest has increased in recent years in the practical applications of biotechnology. Tissue culture is one of the most important and most advanced of these technologies. It relies on simple methods that do not require complex and expensive laboratory equipment. It has many applications, the most important of which is laboratory vegetative propagation due to the scarcity of some of them, or for their nutritional value, or Due to the ability of some of them to

produce substances of high pharmaceutical value in the production of medical drugs [9, 10, 7],During their growth stages, plants build a group of metabolic substances that they use in growth and development. These substances include glycosides, phenols, flavonoids, alkaloids, and others. These compounds are important for the plant to survive and spread in the natural environment, and they are mainly defensive substances against pathogens. They are also Pharmaceuticals, food flavors, dyes, perfumes or pesticides, plants are a continuous source of production of important secondary metabolites.

From this standpoint and the importance of this plant from a nutritional and medicinal standpoint, the research aimed to establish tissue farms by cultivating the growing shoots on MS nutrient medium equipped with different types and concentrations of growth regulators to study their effect on callus induction and the production of secondary metabolism, and then detect quantitatively and qualitatively the compounds using a device High performance liquid chromatography (HPLC) for tissue cultures.

Materials and Methods

This study was carried out in the Plant Tissue Culture Laboratory of the College of Agriculture / University of Karbala. The study used the Helwani grape variety grown in plastic bags at two years of age.

Callus induction experiment

Stem cuttings 20-30 cm long were selected from recent growth. The outer leaves surrounding the nodes were removed and the cuttings were cut into small pieces 2 cm long so that each piece contained one node. The cuttings were sterilized with sodium hypochlorite at a concentration of 2% for 15 minutes, after which they were planted in glass bottles containing Medium prepared with different concentrations (0, 1, 2, 3, 4) mg/L of 2,4-D (0, 0.1, 0.2, 0.4) mg/L of BA in independent experiments with ten repetitions for each concentration The crops were incubated in the dark at a temperature of 25°C +_ 2 for four weeks. The fresh and dry weight standard of the induced callus was used at this stage to determine the best concentration of 2,4-D and BA for callus induction.

Extraction of flavonoids from grapevine callus

The extraction process was carried out according to what was mentioned in [12],taking a constant weight of the callus and then drying it until the weight was constant. The dried samples were ground and 2 ml of methanol was added to them with continuous stirring. The samples were then placed in the centrifuge at a rotation speed of 7500 rpm for 15 minutes. The filtrate was treated with chloroform to get rid of some compounds such as fats and chlorophyll. The samples were placed in a rotary evaporator. The filtrate was dissolved with 1 ml of methanol and mixed with a vortex

device. The mixture was filtered through a 2.5 um filter and the filtrate was stored at 4 C for use in subsequent analyses.

Qualitative and quantitative determination of flavonoids using a high-performance liquid chromatography device

The flavonoids compounds in the grape callus extract were separated according to what was mentioned [13].20 ml of the filtrate was taken and injected into the HPLC device under the following conditions: a separation column with dimensions (mmI.D. 4.6×50), and a mobile phase methanol: phosphate buffer in a ratio of 40:60. v/v, and a flow speed of 1.2 ml/min, and the concentration of flavonoids compounds in the callus extracts was calculated according to the following equation:-

Concentration of the unknown (g/µg) = number of dilutions x measurement concentration x (form package area)/(measurement package area).

Statistical analysis

All experiments were carried out using a completely randomized design (CRD) and factorial experiments. The results were analyzed using the SAS statistical program [14]and the means were compared according to the least significant difference (AFM) test at the 0.05 probability level.

Results and Discussion

Effect of 2,4-D and BA concentrations on the rate of fresh weight induced by the developing shoot

The results of Table (1) show that there are significant differences when adding 2,4-D in its different concentrations to the nutrient medium prepared for the induction of callus from the growing tops of grape plants. 2,4-D was superior at a concentration of 2 mg L-1 and provide the highest average weight. Callus freshness amounted to 3.32 mg, which differed significantly from the rest of the treatments, compared to the comparison treatment, which did not record any response. The results of the same table indicate that there are significant differences in the average weight of the callus with different concentrations. The BA added to the medium was significantly superior to the concentration of 0.2 mg L-1 in achieving the highest rate of 1.91 mg, compared to the comparison treatment which achieved the lowest rate of 1.68 mg. As for the effect of the interaction between the concentrations of auxien and cytokinines on the average fresh weight of callus, the concentration of 2 mg L-1 of 2,4-D and the concentration of 0.4 mg L-1 of BA achieved the highest rate of 3.85 mg, while the comparison treatment for auxien did not achieve the different concentrations of cytokinines No response mentioned.

Table (1): The effect of 2,4-D and BA concentrations on the fresh weight of callus induced from the growing shoot after 6 weeks of cultivation on the MS medium.

Con of 2,4-D mg l^{-1}	Con of BA mg l^{-1}				Mean
	0	0.1	0.2	0.4	
0	0	0	0	0	0
1	1.70	2.50	2.66	2.75	2.40
2	2.25	3.40	3.80	3.85	3.32
3	2.36	1.76	1.78	1.50	1.85
4	2.10	1.65	1.32	1.20	1.54
L.S.D.(0.05)	0.08				0.06
Mean	1.68	1.86	1.91	1.86	
L.S.D.(0.05)	0.02				

Effect of 2,4-D and BA concentrations on the dry weight rate induced by the shoot tip

The results of Table (2) show that there are significant differences in the dry weight rate of callus depending on the concentrations of 2,4-D added to the nutrient media. 2,4-D was superior at the concentration of 2 mg L-1 and provide the highest dry weight rate of callus, which reached 1.03 mg which differed significantly from the rest of the treatments, while comparison treatment did not record any response. The results of the same table indicate that there are significant differences in the average dry weight of callus depending on the concentrations of BA added to the nutrient medium. The concentration of 0.2 mg l^{-1} was significantly superior in achieving the highest rate of 0.51 mg, compared to the comparison treatment which achieved the lowest rate of 0.43 mg. As for the effect of the interaction between the concentrations of auxien and cytokinines on the average fresh weight of callus, the concentration of 2 mg l^{-1} of 2,4-D and the concentration of 0.4 mg $l^{-1}$of BA achieved the highest rate of 1.18 mg, while the comparison treatment for auxien did not achieve at different concentrations of cytokinines no response mentioned.

Table (2): Effect of 2,4-D and BA concentrations on the dry weight of callus induced from the shoot tip after 6 weeks of cultivation on the MS medium

Con of 2,4-D mg l^{-1}	Con of BA mg l^{-1}				Mean
	0	**0.1**	**0.2**	**0.4**	
0	0	0	0	0	0
1	0.40	0.48	0.88	0.96	0.68
2	0.71	1.05	1.17	1.18	1.03
3	0.78	0.40	0.40	0.23	0.45
4	0.27	0.16	0.09	0.03	0.14
L.S.D.(0.05)	0.03				0.02

Mean	0.43	0.41	0.51	0.48
L.S.D.(0.05)	0.01			

The reason for inducing callus on the surface of shoot tip may be attributed to the role of the growth regulator, which encourages the formation of callus and increasing its growth, as it is one of the Auxins that has an important role in the formation and growth of callus. Increasing the concentration leads to the formation of callus, reaching the optimum concentration, and increasing it beyond the optimum limit leads to adverse results if Adding growth regulators to the nutrient medium stimulates the continued division of the callus tissue after it is grown on the nutrient medium. It will be able to establish an internal hormonal system, and this system determines the direction of subsequent development by interfering with the growth regulators added to the medium, which are then responsible for maintaining the continuation of cell division activity [15]. These results are consistent with [16]. that Auxins 2-4-D in the growth medium prepared for callus induction from Datura plants provide the best callus weight compared to other growth regulators.

Effect of 2,4-D and BA concentrations on the concentration of Hesperdine

The results of Table (3) show that there are significant differences in the concentration of the Hesperdine compound that was estimated in grape callus with increasing concentrations of 2,4-D added to the nutrient medium 1, 2, 3, 4 mg l^{-1} reaching 36.56, 44.39, 58.38, and 64.99. mg l^{-1} respectively, while the comparison treatment gave the lowest concentration of 25.78 mg l^{-1}. The results of the same table also indicate that there are significant differences in the concentration of the same compound with increasing concentrations of BA added to the food media by 0.1, 0.2, and 0.4 mg l^{-1}, amounting to 43.50, 50.84, and 52.65 µ g^{-1} respectively, while the lowest concentration was achieved in the comparison treatment, amounting to 37.09 µ g^{-1}. As for the effect of the interaction between the concentrations of auxien and cytokinein on the concentration of the Hesperdine compound, the concentration of 3 mg l^{-1} of 2,4-D at the concentration of 0.4 mg l^{-1} of BA achieved the highest rate of 71.28 µ g^{-1}, while the comparison treatment achieved the lowest response of 20.12. µ g^{-1}.

Table (3): Effect of 2,4-D and BA concentrations on the concentration of Hesperdine compound (µg g^{-1}) of grapevine callus after 6 weeks of cultivation on MS medium

Con of 2,4-D mg l^{-1}	Con of BA mg l^{-1}				Mean
	0	0.1	0.2	0.4	
0	20.12	24.05	30.40	28.55	25.78
1	31.00	36.02	38.14	41.10	36.56
2	37.15	40.25	47.18	53.00	44.39
3	43.05	51.11	68.08	71.28	58.38
4	54.13	66.10	70.42	69.33	64.99

L.S.D.(0.05)	3.19				2.09
Mean	37.09	43.50	50.84	52.65	
L.S.D.(0.05)	1.87				

The effect of 2,4-D and BA concentrations on the concentration of Nargnine

The results of Table (4) show that there are significant differences in the concentration of the Nargnine compound that was estimated in grape callus with increasing concentrations of 2,4-D added to the nutrient medium 1, 2, 3, 4 mg l^{-1} which reached 34.01, 45.83, 58.05, and 62.56. µ g^{-1} respectively, while the comparison treatment provide the lowest concentration which amounted to 26.32 µ g^{-1} . The results of the same table also indicate that there are significant differences in the concentration of the same compound with increasing concentrations of BA added to the medium which amounted to 0.1, 0.2, and 0.4 mgl^{-1}. 67.05, 64.30, and 68.77 µ g^{-1}respectively, while the lowest concentration was achieved in the comparison treatment amounting to 50.21 µ g^{-1}. As for the effect of the interaction between the concentrations of auxien and cytokinein on the concentration of the Hesperdine compound, the concentration of 4 mg l^{-1} of 2,4-D at the concentration of 0.4 mg l^{-1} of BA achieved the highest rate of 68.77 µ g^{-1}, while the comparison treatment achieved the lowest response of 20.00 µ g^{-1}.

Table (4): The effect of 2,4-D and BA concentrations on the concentration of Nargnine (µg g^{-1}) of grapevine callus after 6 weeks of cultivation on MS medium.

Con of 2,4-D mg l^{-1}	Con of BA mg l^{-1}				Mean
	0	0.1	0.2	0.4	
0	20.00	26.70	28.33	30.15	26.29
1	30.63	34.06	35.27	36.09	34.01
2	38.00	42.15	53.17	50.00	45.83
3	46.08	54.40	67.00	64.75	58.05
4	50.21	67.05	64.30	68.77	62.56
L.S.D.(0.05)	5.46				3.23
Mean	36.98	44.87	49.55	49.95	
L.S.D.(0.05)	2.89				

The effect of 2,4-D and BA concentrations on the concentration of Proanthocyanin

The results of Table (5) show that there are significant differences in the concentration of the Proanthocyanin compound that was estimated in grape callus with increasing concentrations of 2,4-D added to the nutrient medium 1, 2, 3, 4 mg l^{-1} which reached 31.58, 41.78, 52.70, and 55.63. µ g^{-1} respectively, while the comparison treatment provide the lowest concentration of 23.43 µ g^{-1}. The results of the same table also indicate that there are significant differences in the concentration of the same

compound with increasing concentrations of BA added to the food media by 0.1, 0.2, and 0.4 mg l^{-1} amounting to 38.70, 43.58, and 47.65 micrograms g-1, respectively, while the lowest concentration was achieved in the comparison treatment, amounting to 34.16 µ g^{-1}. As for the effect of the interaction between the concentrations of auxien and cytokinein on the concentration of the Hesperdine compound, the concentration of 4 mg l^{-1} of 2,4-D at the concentration of 0.4 mg l^{-1} of BA achieved the highest rate of 66.00 µ g^{-1}, while the comparison treatment achieved the lowest response of 18.44. µ g^{-1}.

Table (5): Effect of 2,4-D and BA concentrations on the concentration of Proanthocyanin (µg g^{-1}) of grapevine callus after 6 weeks of cultivation on MS medium

Con of 2,4-D mg l^{-1}	Con of BA mg l^{-1}				Mean
	0	0.1	0.2	0.4	
0	18.44	23.13	25.00	27.15	23.43
1	27.19	30.20	32.17	36.77	31.58
2	35.23	38.08	45.63	48.20	41.78
3	41.60	52.08	57.00	60.13	52.70
4	48.37	50.01	58.14	66.00	55.63
L.S.D.(0.05)	1.23				0.61
Mean	34.16	38.70	43.58	47.65	
L.S.D.(0.05)	0.55				

The effect of 2,4-D and BA concentrations on the concentration of Quercetin

The results of Table (6) show that there are significant differences in the concentration of the compound Quercetin that was estimated in grape callus with increasing concentrations of 2,4-D added to the nutrient medium 1, 2, 3, 4 mg l^{-1} reaching 41.88, 56.91, 75.36, and 71.89. µ g^{-1} respectively, while the comparison treatment provid the lowest concentration of 26.45 µ g^{-1} . The results of the same table also indicate that there are significant differences in the concentration of the same compound with increasing concentrations of BA added to the food media by 0.1, 0.2, and 0.4 mg l^{-1} amounting to 52.69, 57.97, and 59.92 micrograms g-1, respectively, while the lowest concentration was achieved in the comparison treatment, amounting to 47.41 µ g^{-1}. As for the effect of the interaction between the concentrations of auxien and cytokinein on the concentration of the Hesperdine compound, the concentration of 3 mg l^{-1} of 2,4-D at the concentration of 0.2 mg l^{-1} of BA achieved the highest rate of 80.15 µ g^{-1}, while the comparison treatment achieved the lowest response of 20.85 µ g^{-1}.

Table (6): Effect of 2,4-D and BA concentrations on the concentration of Quercetin (µg g^{-1}) of grapevine callus after 6 weeks of cultivation on MS medium.

Con of 2,4-D mg l^{-1}	Con of BA mg l^{-1}				Mean
	0	0.1	0.2	0.4	
0	20.85	26.75	28.10	30.13	26.45
1	38.70	37.55	43.18	48.09	41.88
2	44.16	54.08	63.25	66.17	56.91
3	66.20	75.10	80.15	80.00	75.36
4	67.15	70.00	75.18	75.25	71.89
L.S.D.(0.05)	3.92				2.76
Mean	47.41	52.69	57.97	59.92	
L.S.D.(0.05)	1.25				

Effect of 2,4-D and BA concentrations on the concentration of Rutin

The results of Table (7) show that there are significant differences in the concentration of the Rutin compound that was estimated in grape callus with increasing concentrations of 2,4-D added to the medium 1, 2, 3, 4 mgl[1] reaching 37.37, 52.34, 70.00, and 71.22 µ g^{-1} respectively, while the comparison treatment provide the lowest concentration of 25.37 µ g^{-1}. The results of the same table also indicate that there are significant differences in the concentration of the same compound with increasing concentrations of BA added to the food media by 0.1, 0.2, and 0.4 mg l^{-1} amounting to 49.12, 54.91, and 56.70 µ g^{-1} respectively, while the lowest concentration was achieved in the comparison treatment amounting to 44.29 µ g^{-1}. As for the effect of the interaction between the concentrations of auxien and cytokinein on the concentration of the Hesperdine compound, the concentration of 4 mg l^{-1} of 2,4-D at the concentration of 0.4 mg l^{-1} of BA achieved the highest rate of 80.66 µ g^{-1}, while the comparison treatment achieved the lowest response of 20.85 µg g^{-1}.

Table (7): Effect of 2,4-D and BA concentrations on the concentration of Rutin (µg g^{-1}) of grapevine callus after 6 weeks of cultivation on MS medium.

Con of 2,4-D mg l^{-1}	Con of BA mg l^{-1}				Mean
	0	**0.1**	**0.2**	**0.4**	
0	21.25	25.18	26.75	28.30	25.37
1	34.00	38.26	39.05	38.18	37.37
2	43.18	47.00	60.05	59.13	52.34
3	60.14	67.05	75.55	77.27	70.00
4	62.92	68.12	73.18	80.66	71.22
L.S.D.(0.05)	3.28				2.64
Mean	44.29	49.12	54.91	56.70	
L.S.D.(0.05)	1.36				

From the results of the previous tables presented on the effect of adding different concentrations of growth regulators to the medium prepared to stimulate increased production of metabolic compounds, it was found that there is a significant effect of the growth regulators 2,4-D and BA in increasing the concentrations of flavonoids compounds. The reason may be due to the role of growth regulators. In increasing fresh and dry weight, as well as the concentration of carbohydrates, as it leads to an increase in nitrogen, the basic component for building amino acids, from which effective compounds are built through the processes of biosynthesis of essential amino acids, which are considered as initiators or raw materials, not their products. Alsothe role of growth regulators in the enzymatic activity of cells and maintaining stability. Cell membranes, increased CO_2 assimilation, and increased absorption of nutrients, minerals, and water, causing an increase in metabolic processes and the production of secondary compounds [17]. These results agreed with the results of [18] in that there was a significant increase in the concentration of alkaloids in the *Withania somnifera* plant with increasing concentrations of Plant growth regulators added to the medium and with the results of [19] which found that there was a significant superiority in the concentration of the volatile oils of the *Rosmarinus officinalis* plant by increasing the concentrations of growth regulators added to the medium. The study agreed with the findings of [20] in their study on the effect of growth regulators in increasing the production of flavonoids compounds in the callus of the plant (Nepetacataria), as well as with the findings of [21] when they studied the role of growth regulators in inducing callus and increasing the production of flavonoids compounds in the *Centellaasiatica* plant.

Growth regulators added to the media have a major role in inducing callus from the growing apex of grape plants, as well as in increasing the formation of luminous compounds that were estimated in grape callus using HPLC technology .

References

1) Huany, W. L., & Liu, L. F. (2002). Carbohydrate metabolism in rice during callus induction and shoot regeneration induced by osmotic stress. *Botanical Bulletin of Academia Sinica, 43*(2), 107-111.

2) Al-Saidi, I. H. (2000). *Grape production*. Ministry of Higher Education and Scientific Research, Faculty of Agriculture, University of Al Mosul.

3) Mohammed, A. A. (2008). Effect of low dose gamma irradiation on some phytochemicals and scavenger ability of in vitro culture *Eryngium foetidum* L. plantlets. *Medicinal and Aromatic Plant Science and Biotechnology*, *2*(1), 32-36.

4) Zang, W. C., Kikuchi, M., & Franco, C. (2002). Enhancement of jasmonic acid and light on anthocyanin biosynthesis in *Vitis vinifera* suspension cultures. *Plant Science, 162*(3), 459-468.

5) Cosmo, F. A., & Misawa, H. (1985). Eliciting secondary metabolism in plant cell cultures. *Trends in Biotechnology*, *3*(9), 318-322.

6) Bansal, A. J. Y. (2014). In vitro production of flavonoids: A review. *World Journal of Pharmacy and Pharmaceutical Sciences*, *3*(6), 508-533.
7) Strid, A., Chow, W., & Anderson, J. (1990). Effects of supplementary gamma irradiation on photosynthesis in *Pisum sativum*. *Biochemistry*, *1020*(1), 260-268.
8) George, E. F., & Sherrington, P. D. (1993). *Plant propagation by tissue culture* (2nd ed.). Exegetics Ltd.
9) Kanadaswami, C. L., Lee, P., Hwang, Y., & Huang, X. (2005). The antitumor activities of flavonoids. *In Vivo*, *19*(5), 895-909.
10) Karuppusamy, S. (2009). A review on trends in production of secondary metabolites from higher plants by in vitro tissue, organ, and cell cultures. *Journal of Medicinal Plants Research*, *3*(13), 1222-1239.
11) Herbert, D., Phipps, P. J., & Strange, R. E. (1971). Chemical analysis of microbial cells. In *Methods in microbiology* (Vol. 5, pp. 209-344). Academic Press.
12) Cellavapra, E. R., & Honkariv, R. (1984). Repetitive propagation of some medicinal plants through tissue culture. In *Plant tissue and cell culture: Application to crop improvement* (pp. 515-516). Czech Academy of Sciences.
13) Infante, R., Fiore, N., & Seibert, E. (2008). Preservation of grape fan leaf virus on callus culture of *Vitis vinifera* Cabernet Sauvignon. *Acta Psychopathologica Entomologica Hungarica*, *43*(1), 1.
14) SAS Institute Inc. (2002). *SAS/STAT user's guide for personal computers*. SAS Institute Inc.
15) Neumann, K. H., Kumar, A., & Imani, J. (2009). Plant cell and tissue culture: A tool in biotechnology, basic and application. In *Springer* (pp. 181-225).
16) Al-Hattab, Z. N., Al-Kateeb, E., Al-Quadhy, W. K., & Mahdi, G. (2000). Effect of growth hormones on tropane alkaloids production in *Datura metel* callus culture. *IBN AL-Haitham Journal for Pure and Applied Science*, *12*(1).
17) Verpoort, R., & Alfermann, A. W. (2000). Engineering of plant secondary metabolism. In *Metabolism* (pp. 3-8). Kluwer Academic Publishers.
18) Hamad, M. S., & Majid, N. B. (2017). Influence of explant and growth regulators on the induction of callus in *Annona muricata* in vitro. *Euphrates Journal of Agriculture Science*, *9*(4), 1-12.
19) Al-Tamimi, Z. M. A. H. (2018). The effect of magnetic fields and plant growth regulators on the multiplication and concentration of volatile oils of the *Rosmarinus officinalis* plant in vitro (Master's thesis). College of Agriculture, University of Baghdad.
20) Mohammed, A. N., Khayata, W., & Al Oqab, M. A. (2014). Effect of some plant growth regulators on callus, biomass, and cell suspension from *Nepeta cataria* and estimating their ability for production of some flavonoid compounds. *Research Journal of Aleppo University: Basic Science Series*, *69*, 29-47.
21)Suat, H. T., Musa, R., Ariff, A., & Maziah, M. (2010). Effect of plant growth regulators on callus, cell suspension, and cell line selection for flavonoid production

from *Centella asiatica* L. Urban. *American Journal of Biochemistry and Biotechnology*, *6*(4), 284-299.