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EFFECT OF GROWTH REGULATORS AND SUCROSE ON THE INDUCTION AND PRODUCTION OF FLAVONOIDS IN CALLUS OF *VITIS VINIFERA (L) IN VITRO*

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ABSTRACT

The study included the use of plant tissue culture technique in the induction of the callus of *Vitis vinifera* and to stimulate the production of flavonoids. The study was carried out in two stages after sterilization: The first stage was the establishment of callus by culturing a single-node on the MS medium which contained 2,4-D and IAA at different concentrations of 0, 1, 1.5, 2, 2.5mg/l with a fixed concentration of 0.5mg/L of BA in independent experiments. The second stage was implemented by cultivating the callus that was induced in the first stage on the MS medium which was provided with different concentrations of sucrose of 30, 60, 90, 120g/l. Results of the study showed that the auxin 2,4-D achieved the highest wet and dry callus weight of 1817.20 and 94.38mg respectively, while the IAA achieved a wet and dry callus weight of 660.07 and 66.43mg respectively. The MS medium that was provided with 2.5mg/l auxin achieved the highest wet and dry callus weight of 977.77 and 122.35mg respectively. The 2,4-D at the concentration of 2mg/l gave significantly higher wet and dry callus weight of 2722.50 and 146.50mg respectively compared with other treatments.

Keywords: grapes, callus, growth regulators, sucrose, flavonoids.

INTRODUCTION

Vitis vinifera (L.) is a member of the Vitaceae family, which has been cultivated in Asia, Africa and Europe (Bertamini et al., 2005). It is highly nutritious because it contains a high concentration of sugars. It is also rich in vitamins such as vitamins B and C, in addition to a good percentage of mineral elements such as potassium, calcium and sodium, and also compounds of therapeutic and nutritional value such as Flavonoids, phenols, anthocyanins and procyanidine (Maria and Hossien 2009).

Flavonoids, which have been studied in grapes as dilute organic compounds in water belong to the class of phenols with therapeutic importance, and in certain doses they work as anti-oxidant and anti-free radicals. The polyanthocyanins have been shown to enhance the level of good cholesterol and reduce bad cholesterol, which reduces the risk of coronary heart disease and maintains heart health (George and Sherrington 1993, Kanadaswami, et al., 2005). These compounds have been proven to prevent bleeding from the limbs or from the natural openings, prevent swelling of the legs due to water retention in the body, protection against retinopathy associated with diabetes and also protective role against high blood pressure (Asha and Bansal, 2014). Flavonoids, including Hasperdine, have been widely used in European countries in the treatment of alcohol-related liver disorders. Nargene has also played a role in cancer prevention by inhibiting the free radicals that cause DNA damage, which can lead to cancer. Quercetin has also been used to treat allergies, arthritis and asthma (Karuppusamy, 2009, Spatafora 2013, Abbas, *et al*., 2017).

In recent years, interest in practical applications of biotechnologies has increased. Plant tissue culture is one of the most important and most sophisticated of these technologies and it relies on simple methods which do not need complex and expensive laboratory equipment, It has many applications, the most important of which is the vegetative propagation due to the scarcity of some of them, the nutritional value of them, or the ability of some of them to produce substances of high medicinal value in the production of medicinal drugs (Al-Hadidy and Hussein 2000, Aubaid and Muhammed 2000, Karuppusamy 2009), The plants during the stages of growth make a group of metabolic substances used in growth and development such as glycoside, phenols, flavonoids, alkaloids and others. These compounds are important for the plant to survive and spread in the natural environment, and are mainly protective substances against pathogens, also as pharmaceuticals, food flavors, pigments, perfumes or pesticides. The plants are the constant source for the production of important secondary metabolites and are increasingly formed in the plant due to exposure to stress (Tripathi and Tripathi 2003, Vanisree et al., 2004). Stress is defined as the external effort on the cultivated cells, which affects the productivity of the secondary metabolites and varies according to the phylogenetic stage of the plant. The effect may be lack of water, light, metal elements, temperatures, chemicals or microorganisms (Grime, 1981). Matsumoto et al., (1971) indicated that sucrose was the best source of carbo-

hydrates for culture media, and that a concentration of 3% stimulated the production of phytochemicals and the production could be greater in response to increased concentration of sucrose. In a study conducted by (Andrej et al., 2004) on the effect of the concentrations of sucrose that was added to the medium for the cell suspension of grapes, the results showed 2-5 times increase in flavonoids concentrations with increased sucrose concentrations in the medium. In another study by (Gabriella et al., 2005) on the cell culture of *Camptotheca acuminate*, it was found that the addition of 9% sucrose to the medium achieved the highest content of anthocyanins with a value of 100mg/g dry weight in the dark and 65mg/g dry weight in light. Also (Cai et al., 2012) found that the addition of polysaccharides had significantly increased the production of anthocyanins in the grape cell culture as the concentrations of sugars added to the medium were increased. From this point and the importance of this plant from the nutritional and medical side, the aim of the research was to establish callus culture by cultivating a single-node on MS medium containing different types and concentrations of Auxins, and then stimulate these tissues to increase their production of secondary metabolic substances by exposing them to stress, and to detect the type and quantity of these compounds by using the high performance liquid chromatography (HPLC) on the cultured callus.

MATERIALS AND METHODS

Callus induction: New growth of 20-30cm was chosen from grape stems, the outside leaves surrounding, the explants were removed, and the stems were cut into small pieces of approximately 2 cm long with one node in each piece. The explants were sterilized with sodium hypochlorite at a concentration of 3% for 15 minutes. Then they were placed in glass bottles containing MS medium (Murashige and Skoog 1962) with the concentrations of 0, 1, 1.5, 2, 2.5mg/l of 2,4-D and IAA with a constant concentration of 0.5mg/l of BA in a separate experiment with 10 replicates per each concentration. The plants were incubated in the dark at 25°C ± 2°C for four weeks. The wet and dry weights criteria for the induced callus were used at this stage to determine the best concentration of auxien to induce callus for the subsequent experiment.

Effect of Sucrose: Due to the superiority of 2,4-D at 2.5mg/l with BA at 0.5mg/l concentration, 100 mg callus of this treatment was taken and implanted on MS medium with concentrations of 30, 60, 90, 120g/l sucrose. The cultures were incubated in the same conditions as in the previous experiment. Four weeks later, the extraction was carried out.

Extraction: The extraction was carried out as mentioned by Obouayeba and Bernard (2014). A known weight of the callus was taken and then dried until a constant weight was reached. Dried samples were grinded and 2ml of methanol were added with continuous stirring. The samples were then placed in the centrifuge at a rotation speed of 7500rpm for 15 minutes. Chloroform was added to the supernatant to get rid of some compounds such as fat and chlorophyll. The samples were placed in a rotary evaporator, supernatant was solved with a 1ml methanol and mixed by vortexing, the mixture was filtered with a 2.5µm filter and the supernatant was stored at 4ºC for use in subsequent analyses.

Quantitative and qualitative estimation of flavonoid compounds by using High performance liquid chromatography technique (HPLC): Flavonoids were separated from grapes callus extract according to (Suarez et al., 2005). 20µl of supernatant were taken and injected into the HPLC under the following conditions, Column: nucludar C18-DB, 3µm particle size (50/2.0mm L/ID), Mobile phase: 0.01M phosphate buffer: Methanol 60:40 v/v. Flow rate 1.2ml/min, and the concentration of flavonoids in the callus extracts was calculated according to the following formula:

Concentration of the unknown (µg/g) = Dilution times × number x concentration measurement × (sample package area) / (measurement package area).

Statistical analysis: All experiments were performed using the Complete Randomized Design (CRD) and global experiments. The results were analyzed using the statistical program (SAS 2002). The means were compared using the Least Significant Difference (LSD) at the probability level of 0.05.

RESULTS AND DISCUSSION

Effect of auxien: It is noticed from Table 1 that there is an increase in the rate of the wet callus weight with increased concentrations of auxien added to the nutrient medium. The concentrations of 1, 1.5, 2 and 2.5mg/l achieved a callus wet aver-age weight of 1065.65, 1366.40, 1783.34 and 1977.77mg respectively, while comparison treatment did not show any change in the average weight of callus. The same table shows that auxien 2,4-D treatment gave a mean callus wet weight of 1817.20mg compared to IAA, which resulted in a wet callus weight of 660.07mg. As for the effect of the interference, auxin 2,4-D at the concentration of 2mg/l resulted in the highest callus weight of 2722.50mg, while the IAA at 1mg/l concentration achieved the lowest wet weight of 530.80mg, in comparison with control treatments which did not record any change of callus weight for both types of auxin.

Table 1: Effect of auxien type and its concentration (mg/l) and their interaction on callus wet weight (mg)

average	2.5	2	1.5	1	0	Type of auxien
660.07	1300.15	844.18	625.20	530.80	0.0	IAA
1817.20	2655.40	2722.50	2107.60	1600.50	0.0	2,4-D
.2815	.6260					L.S.D
	1977.77	1783.34	1366.40	1065.65	0.0	average
	.4411					L.S.D

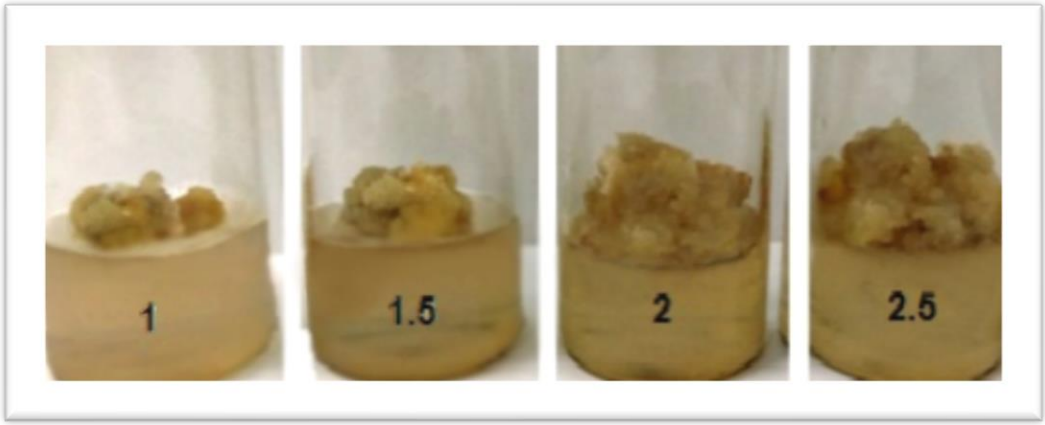

Figure 1: Effect of concentrations of 2,4-D (mg/L) on the induction of callus(mg)

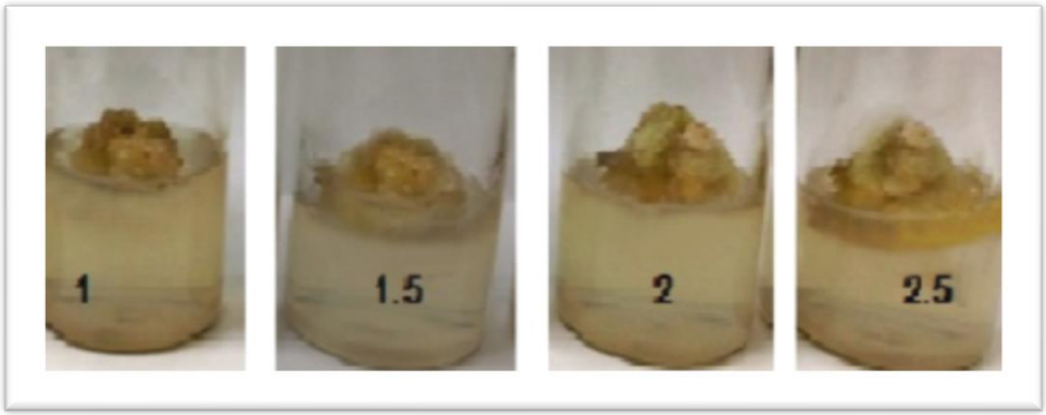

Figure 2: Effect of concentrations of IAA (mg / L) on the induction of callus (mg)

Effect of auxin type and interference among them on the dry weight (mg) of callus: Table 2 shows significant differences between the various concentrations of auxin, regardless of the type of auxien. The concentration of 2.5mg/l resulted in the highest rate of dry callus weight of 122.35mg, which significantly exceeded other concentrations, and the lowest dry weight of callus of 73.43mg was for the concentration of 1mg/l of auxien. The control treatment gave little response to callus induction. The same table shows that the superiority was for auxien 2,4-D when callus dry weight was 94.38 mg compared to IAA which gave a mean callus wet weight of 66.43mg. As for the effect of interference on the dry weight of the callus, auxien 2,4-D at the concentration of 2mg/l achieved the highest rate of 146.50mg while the IAA at the lowest concentration of 1mg/L achieved the lowest callus dry weight of 58.16mg, whereas comparison treatment did not give any significant weight change.

Table 2: Effect of auxien type and its concentration (mg/l) and their interaction on the dry weight (mg)

average	2.5	2	1.5	1	0	Type of auxien
66.43	108.20	90.64	75.15	58.16	0.0	IAA
94.38	136.50	146.50	100.20	88.70	0.0	2,4-D
.135	3.40					L.S.D
	122.35	118.57	87.68	73.43	0.0	average
	.230					L.S.D

The results of the previous tables have shown that the concentration of auxien has significantly affected the weight of the callus, and there was an increase in the rate of induced callus weight as the concentration of auxin added to the medium increased until reaching the optimal concentration of auxin when a constant level of cytokine was recorded. From this result it is concluded that the concentration of 2.5mg/l 2,4-D and 0.5mg/L BA were the best concentrations which resulted in the highest induced callus wet and dry weights. The increase in the weight of induced callus compared to control treatment could be attributed to the influence of growth regulators on cells division and expansion (Mohammed and Hassan 1998), as well as their effect on the central plate of cells which helps to widen the cellular wall until reaching the optimal concentration of auxin (Cellavopra and Honkariv 1984). (Goodwin 1985) says that the balance between Cytokinein and auxien is necessary for the induction of callus. Cytokinein works with auxien as a key to initiating cellular splitting. Adenine in the cytokinein molecule could be the part that plays a key role in cell division. The reason why the induction of the callus in the auxin-free medium did not have any response could be attributed to the role of auxien in the stimulation of cells division which leads to the formation of callus (George and Sherrington 1993). This is consistent with what (Infante et al., 2008, Beza 2010, Kurmi et al., 2011, Nadra et al., 2015) found when they cultured the node of *Vitis vinifera* on MS medium with different concentrations of auxin and found that the 2,4-D medium of 2.5mg/l was the best in achieving the highest callus wet and dry weights. Many researchers pointed out the importance of incubation in darkness (George and Sherrington 1993) and suggested that darkness has a role in preventing the oxidation of some light-sensitive compounds such as internal hormones like auxin. The light activates the enzyme IAA oxidase, therefore, the subculture of callus in new media and continued incubation in the dark increases the amount of callus induced from the explants. Incubation in the dark may also inhibit the oxidation of phenolic substances by light-induced oxidation enzymes. It is believed that the incubation of the explants in the dark leads to decreased cell walls thickness and increased permeability of substances like growth regulators to the implanted tissues and thus the response of explants to the callus induction (Compton and Gray. 2000). The results of wet and dry callus weights showed that the 2,4-D had significantly higher values than those obtained from IAA, and the reason for this superiority may be due to the effect of replacing the different groups in the ring or the effect of the side chain in the 2,4-D. It was found that the nature and location of substitution groups affected the activity of the compound, the side chain length of the acetate group of the first carbon atom of the phenyl ring also affected the activity of the auxin, and the two chlorine atoms associated with the second and fourth carbon atoms of the phenylphynoxy acetic acid increased the activity and effectiveness of auxin (Devlin et al. 1998) compared to the ring structure of the IAA compound which consists of two rings of phenyl and the side chain of the acetate group is connected to the first carbon atom of the phenyl ring. This is consistent with (Aurelia et al. 2005) who found that auxin 2,4-D in the growth medium prepared to induce callus from the ovulation of *Cannabis sativa* gave the best callus wet weight compared to other growth regulators such as NAA and IAA (Suat et al., 2010) on callus of *Centella asiatica* and (Kurmi et al., 2011) on the plant *Vitis vinifera*, had supported these results when they found that the presence of 2,4-D was important to induce callus compared with other growth regulators.

Effect of sucrose on the production of flavonoids in the callus of grape: It was found from the results in table 3 and figures 3, 4, 5, 6 obtained by high performs liquid chromatography(HPLC) that there was an increase in the amounts of flavonoids compounds extracted from grape callus in response to increased concentrations of sucrose added to the medium. The sucrose concentration of 120g/l resulted in the highest concentrations of Proanthocyanin, Nargnine, Hesperdine and Rutin with values of 43.38, 68.23, 55.32 and 89.54µg/g respectively, the MS medium with sucrose concentration of 90g /l resulted in the highest weights of Gintestnic and Quercetin with values of 80.86 and 113.62µg/g respectively, and MS with sucrose concentration of 30g/l resulted in the lowest concentrations of (Gintestnic, Rutin, Quercetin, Proanthocyanin, Nargn ine, Hesperdine) with values of 23.33, 38.21, 35.28, 7.89, 19.23 and 14.33µg/g respectively. The results showed that there was a gradual increase in the amount of flavonoids compounds in the grape callus extract with increased concentration of sucrose added to the medium; the reason for this increase could be due to that sucrose is the source that provides cells with the energy needed to grow and survive and the increase of sucrose concentration in the medium to the appropriate level causes an increase of osmotic pressure on the cells which leads to transferring water from the area in which the water voltage is high to the area where the voltage is low and thus affect the growth of callus and metabolism of some cellular compounds and, therefore, the sucrose added to the medium is a source of energy as well as maintains appropriate osmotic

pressure in the culture medium and is a significant factor in plant cell metabolism as it increases the production of secondary metabolic products (Akula and Ravishankar 2011). This is consistent with the findings of both (Matkowski 2004, Matsumoto et al., 1971) who exposed the callus tissue to stress conditions which led to the stimulation of its cells to produce secondary metabolites in larger quantities in comparison with the mother plant. The lack of responsiveness to Gintestnic and Quercetin in this medium may be due to increased voltage, which adversely affected these cells and caused the damage and a decrease of the efficiency of the enzymes responsible for the synthesis of secondary metabolism (Abdel Qader et al., 1982), or may be due to the fact that increased stress may cause the cells to be less able to absorb the nutrients they need for the production of primary metabolites and thus the production of secondary products, which are the final product of primary metabolism (Yassin 1992). The results of this study are consistent with the findings of (Zamboni et al., 2006) who found an increase in flavonoid compounds in grape callus with increased concentration of sucrose added to the medium, as well as with (Suat et al., 2010) who found an increase in flavonoids in the *Calentella asiatica* callus with increased concentration of sucrose added to the medium, also with (Cai et al., 2012) who recorded an increase by 9 folds in the production of anthocyanins with the increased concentration of polysaccharides added to the grape cell culture suspension compared with control.

Table 3: Effect of sucrose concentration (g/l) on the production of flavonoids compounds (µg/g)

Conc. of flavonoid compounds (µg/g)						Con. of
Hesperdine	Nargnine	Proanthocyanin	Quercetin	Rutin	Gintestnic	sucrose g/l
Retention time of flavonoid compounds						
1.7	3.1	3.9	4.0	4.9	5.6	
14.33	19.23	7.89	35.28	38.21	23.33	30
27.20	36.51	19.46	68.31	62.01	40.51	60
46.22	51.32	30.52	113.62	85.11	80.86	90
55.32	68.27	43.38	85.71	89.54	66.71	120
1.74	1.82	1.83	1.86	1.82	1.77	L.S.D

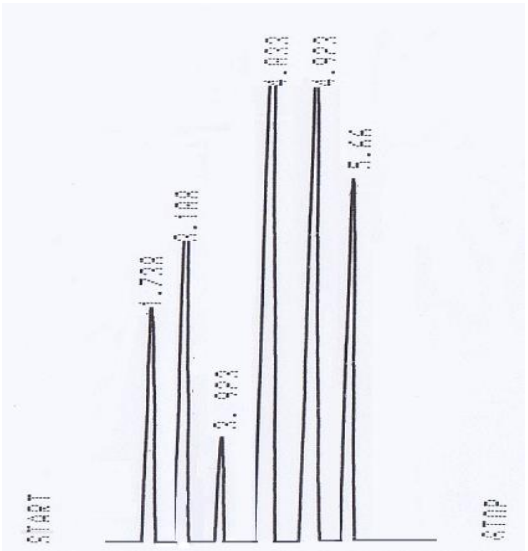

Figure 2: Effect of sucrose (60g/l) on Production of flavonoids compound

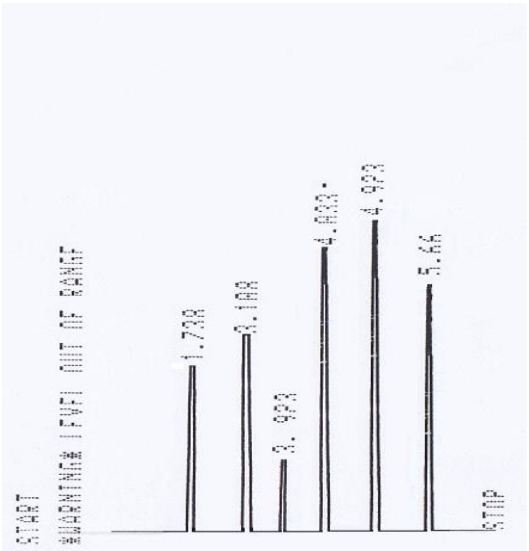

Figure 1: Effect of sucrose (30g/l) on production of flavonoids compounds

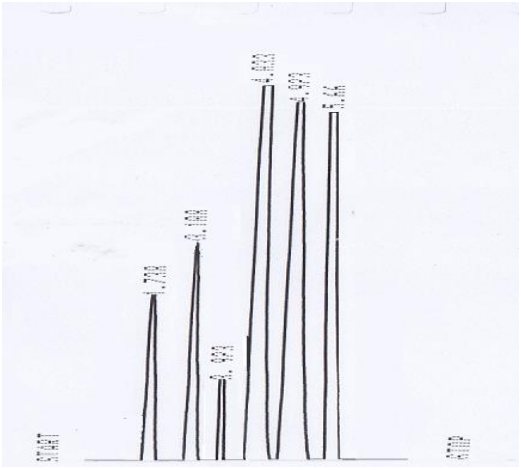

Figure 4: Effect of sucrose (120g/l) on Production of flavonoids compound

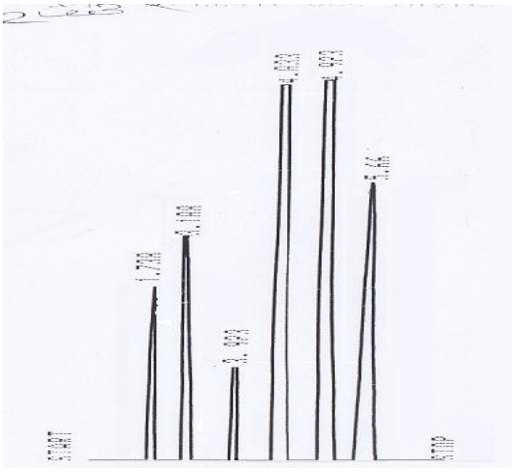

Figure 3: Effect of sucrose (90g/l) on production of flavonoids compounds

Conclusion

Results showed that the MS medium that was provided with a concentration of 120g/l sucrose gave significantly higher concentrations of Proanthocyanin, Nargnine, Hesperdine and Rutin with values of 43.38, 68.27, 55.32 and 89.54µg/g respectively, while the concentration of 90g/l sucrose gave the highest weights of Gintestnic and Quercetin with values of 80.86 and 113.62µg/g respectively compared to the comparison treatment.

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