Effect of Growth Regulators and Irradiation and Methyl Jasmonate in the Induction and Production of Flavonoids in Callus of Vitis Vinifera In-Vitro

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Abstract

Plant tissue culture technology was used in the induction of callus from the node of the Vitis vinifera and thus stimulated to increase the production of flavonoids. The study was carried out in three stages after the sterilization of the explant: The first consisted of the establishment of callus cultivars by culturing the node on the MS medium, which is equipped with different concentrations and types of growth regulators. The second stage was performed by exposing the induced callus tissue to different doses of gamma radiation, while the third stage was performed by Cultivation the irradiated and unirradiated callus on the MS medium which was provided with different concentration of methyl Jasmonate. The results of the study showed that auxien 2, 4-D had the highest wet and dry weight of (1817.20 and 94.38) mg respectively, while NAA achieved a wet and dry weight of (677.02 and 71.16) mg respectively, The concentration of 2.5 mg / L of auxien resulted in the highest rate of wet and dry weight of callus of (1991.75 and 124.75) mg, respectively. The irradiation treatment was significantly higher in the concentrations of Gintestnic, Rutin, Quercetin, Proanthocyanin, Nargnine, and Hesperdine of (133.08, 149.44, 157.82, 48.03, 89.42, and 72.75) and μg / g respectively were significantly higher than those obtained from the comparison treatment which achieved the lowest concentration rates.

Keywords: Callus, flavonoids, grapes, methyl Jasmonate, irradiation.

Introduction

Vitis vinifera (L.) is a member of the Vitaceae family, which has been cultivated in Asia, Africa and Europe [1]. It is highly nutritious because it contains a high concentration of sugars. It is also rich in vitamins such as vitamins B and C. It also contains a good percentage of mineral elements such as potassium, calcium and sodium, as well as compounds of therapeutic and nutritional value such as Flavonoids, phenols, anthocyanins and procyanidine [2].

Flavonoids, which have been studied in grapes as dilute organic compounds in water, belong to the class of phenols with therapeutic strength and have anti-oxidant and anti-free radicals in certain doses. 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 [3,4]. 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 [5]. Flavonoids have been widely used in European countries, including Hasperdine, 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 and this damage can lead to cancer. Quercetin has also been used to treat allergies, arthritis and asthma [6, 7].

The production of the by-products of the plant is increased due to the exposure of stress [8]. Their composition and concentrations of this compound are also influenced by the environmental and agricultural conditions of the plants. As a result of these differences and instability in the quantity of secondary products, the
Researchers have been using modern technologies to increase the production of secondary metabolism. Plant tissue culture from various plant parts found to be a good source of secondary products [9]. In many cases they have shown a high production of secondary metabolites compared to the original plant and have been described as bio-conversion plants from low-value compounds to high-value products [10]. This production can be regulated by the use of plant growth regulators [11]. MeJA is a plant hormone widely used in the plant kingdom and plays a major role in much plant physiological processes, including photosynthesis, flowering and aging [12].

Many researchers such as [13, 14, 15] pointed out that the addition of certain stressors such as Methyl jasmonate to plant cell suspensions or plant branches may stimulate gene expression for the biological production of enzymes that control the construction of secondary compounds. In a study on the effect of MeJA in increasing the production of secondary compounds, [16] found that feeding the cell cultures of Vitis vinifera with MeJA stimulated accumulation of anthocyanin production. In 2011, [17] and his group noted that the increased concentration of MeJA added to cell cultures of grape had stimulated an increase in phytoalexin production by 35% compared to neutral treatment. Radiation is defined as a physical phenomenon in which energy is transferred into the spaces without the need for a physical medium [18].

Ionizing radiation affects the body of the organism and the type of effect is related to the dose at which the plant is exposed. It may be activating, inhibitory or deadly [19]. The results of most researchers have shown that low doses of gamma rays improve the physiological characteristics of the plant and produce high amounts of secondary metabolites [20,21,22] mentioned that when the seeds of the capsicum annum plant were exposed to different radiation doses ranging from 20-160 gray as well as comparison treatment, the results showed that low doses increased the efficiency of photosynthesis and production of the capsaicin, which reached four times more than the comparison treatment. There was also a significant increase in the production of carotenoid and ascorbate which is an important factor for the development of resistance. When the seeds of Eryngium foetidum were exposed to low doses of gamma, [23] found that there was an increase in seed germination rate, seedling growth and plant content of chlorophyll and sugars. The gray 40 treatment showed significant increase in ascorbic acid, phenolic, flavonoids, Tannin, and saponin compared with neutral treatment. Based on the advance informations, the aim of this study was to stimulate the callus from the nodes of grape plant and then stimulate it to increase the production of flavonoids by using physical and chemical stimulation methods.

Materials and Methods

Callus Induction

New growth of 20-30 cm 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 [24] with the concentrations of (0, 1, 1.5, 2, 2.5) Mg / L of 2,4-D and NAA with a constant concentration of 0.5 mg / 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 criterion for the induced callus was used at this stage to determine the best concentration of auxien to induce callus for subsequent experiments.

Figure 1: The explants used to stimulate callus
Irradiation Effect on Callus

After determining the best type and concentration of auxien depending on the results of the previous experiment, a constant weight of 100 mg of the growing callus on the MS medium and is equipped with 2.5 mg / L 2,4-D and 0.5 mg / L BA was taken and exposed to gamma dose of (15, 30 and 45) gray as well as comparative treatment, and all cultures were incubated in the same conditions. Four weeks later, the extraction was carried out.

Effect of Methyl Jasmonate

Due to the superiority of 45 gray dose treatment, callus from this treatment was taken for the next experiment as well as for a comparative treatment. 100 mg of this callus were taken and implanted on MS medium with concentrations of (0, 1, 2, 3) mg / l methyl Jasmonate. 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 [25]. A known weight of the callus was taken and then dried until the weight was constant. Dried samples were grinded and 2 ml of methanol were added with continuous stirring. The samples were then placed in the centrifuge at a rotation speed of 7500 rpm 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 1 ml methanol and mixed with vortex, the mixture was filtered with a 2.5 um filter and the supernatant was stored at 4c⁰ for use in subsequent analyses.

Quantitative and Qualitative Estimation of Flavonoid Compounds by Using High Performance Liquid Chromatography Technique (HPLC)

Flavonoids were separated from the extract of grapes callus according to [26]. 20 ul of supernatant were taken and injected into the HPLC under the following conditions, Column: nucludar C18-DB, 3µm particle size (50*2.0mm ID), Mobile phase: 0.01M phosphate buffer : Methanol 60:40, v/v. Flow rate 1.2 ml/min, and the concentration of flavonoids in the callus extracts was calculated according to the following formula:

\[
\text{Concentration of the unknown (g / μg) = Dilution times \times number \times concentration measurement \times (sample package area) / (measurement package area).}
\]

Statistical Analysis

All experiments were performed using the Randomly Randomized Design (CRD) and global experiments. The results were analyzed using the statistical program [27]. The means were compared using the Least Significant Difference (LSD) and the probability level was 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 2.5 mg / L achieved a wet average weight of callus of 1085.15, 1357.35, 1800.30 and 1991.75 mg 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 wet weight of 1817.20 mg compared to NAA, which gave a wet weight of 677.02 mg. As for the effect of the interference, auxien 2,4-D at the concentration of 2 mg / L resulted in the highest weight of 2722.50 mg, while the NAA at 1 mg / L concentration achieved the lowest wet weight of 571.80 mg, while comparison treatments did not record any change of callus weight for both types of auxien.

<table>
<thead>
<tr>
<th>Type of auxien</th>
<th>concentration of auxin (mg/l)</th>
<th>average</th>
<th>2.5</th>
<th>2</th>
<th>1.5</th>
<th>1</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAA</td>
<td></td>
<td>677.02</td>
<td>1328.10</td>
<td>878.10</td>
<td>607.10</td>
<td>571.80</td>
<td>0.0</td>
</tr>
<tr>
<td>2,4-D</td>
<td></td>
<td>1817.20</td>
<td>2655.40</td>
<td>2722.50</td>
<td>2107.60</td>
<td>1600.50</td>
<td>0.0</td>
</tr>
<tr>
<td>L.S.D</td>
<td></td>
<td>28.07</td>
<td>62.78</td>
<td>1357.35</td>
<td>1085.15</td>
<td>0.0</td>
<td>average</td>
</tr>
</tbody>
</table>

Table 1: Effect of auxin type and its concentration (mg / l) and their interaction on callus wet weight (mg)
Effect of Auxien and Interference on the Dry Weight (mg) of Callus

Table (2) shows significant differences between the different concentrations of auxien, regardless of the type of auxien. The concentration of 2.5 mg / L and gave the highest rate of dry callus weight of 124.75 mg, which significantly exceeds other concentrations, and the lowest dry weight of callus for 1 mg / L concentration was 77.85 mg. 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 NAA of a mean callus wet weight of 71.16 mg. As for the effect of interference on the dry weight of the callus, auxien 2,4-D at the concentration of 2 mg / L achieved the highest rate of 146.50 mg while the NAA at the lowest concentration of 1 mg / L achieved the lowest callus dry weight of 67.00 mg, whereas comparison treatment did not give any significant weight change.

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

<table>
<thead>
<tr>
<th>Type of auxien</th>
<th>Concentration of auxien(mg/l)</th>
<th>0.0</th>
<th>1.5</th>
<th>2.0</th>
<th>2.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAA</td>
<td>71.16</td>
<td>115.00</td>
<td>98.70</td>
<td>77.10</td>
<td>67.00</td>
</tr>
<tr>
<td>2,4-D</td>
<td>94.38</td>
<td>136.50</td>
<td>140.50</td>
<td>100.20</td>
<td>88.70</td>
</tr>
<tr>
<td>L.S.D</td>
<td>1.56</td>
<td>3.48</td>
<td>124.75</td>
<td>122.60</td>
<td>88.65</td>
</tr>
<tr>
<td>L.S.D</td>
<td>average</td>
<td></td>
<td>2.46</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The results of the previous tables have shown that the concentration of auxien has a significant effect on the increase in the weight of the callus induced by the grape node and there was an increase in the rate of induced callus weight by increasing the concentration of auxien added to the medium to the optimal concentration of auxien with a constant level of cytokine. From this result it is concluded that the concentration of 2.5 mg / L 2,4-D and 0.5 mg / L BA was the best concentration that gave the highest weight of the induced callus which was (199.75 and 124.75 mg / kg dry and dry) respectively.

This increase in the weight of induced callus compared to control treatment may be to the influence of growth regulators in encouraging cells to divide and expand [28], as well as its effect on the central plate of cells which helps...
to widen the cellular wall until reaching the optimal concentration of auxien [20,30] says that the balance between cytokinein and auxien is necessary in the induction of callus.

Cytokinein works with auxien as a key to initiating cellular splitting. Adenine in the cytokinein molecule which may be the part that plays the key role in cell division. The reason for the non-response of the plant parts to the induction of the callus in the auxien-free medium is due to the role of auxien in the stimulation of cells division, which leads to the formation of callus [31].

This is consistent with what [32-35] found when they cultured the node of Vitis vinifera on MS medium with different concentrations of auxien, the 2.4-D medium of 2.5 mg / L which was the best in achieving the highest wet and dry weight of the callus. Many researchers pointed out the importance of incubation in darkness [36]. This is due to the role of dark in preventing the oxidation of some light-sensitive compounds such as internal hormones like auxien. The light activates the enzyme IAA oxidase.

Therefore, the sub cultures of callus in new media and continued incubation in the dark increases the amount of callus that 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 decrease cell walls thickness which leads to increased permeability of materials, especially growth regulators, to the tissues that are implanted and thus the response of explants to the callus induction [37].

The results showed that the 2,4-D was significantly higher than the NAA in the rate of wet and dry weight of callus, The reason for the 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 had an effect on the activity of the compound, or the length of the side chain of the acetate group associated with the first carbon atom of the phenyl ring increased the activity of the auxien and the two chlorine atoms associated with the second and fourth carbon atoms of the phenyl phynoxy acetic acid increased the activity and effectiveness of auxien [38], compared to the ring structure of the NAA compound which consists of two rings of phenyl and relate the side chain of the acetate group is the first carbon atoms of the phenyl ring.

This is consistent with [39] who found that auxien 2,4-D in the growth medium prepared to induce callus from the ovulation of Cannabis sativa gave the best wet weight of callus compared to other growth regulators such as NAA and IAA. [40] on callus of Centella asiatica and [34] on the plant Vitis vinifera, had supported these results when they showed that the presence of 2,4-D was better to induce callus compared with other growth regulators.

**Effect of Gamma Rays on the Production of Flavonoids**

It was noticed from the results shown in Table (3) and Figures (4, 5, 6, 7) that the superiority of gamma rays was at the dose 45 mg gray resulted in a remarkable increase in the concentration of flavonoids (Gintestnic, Rutin, Quercetin, Proanthocyanin, Nargnine, Hesperdine) with values of (90.36, 94.93, 98.11, 26.32, 60.38, 28.62) µg / g respectively, while comparative treatment recorded the lowest rate in the concentrations of the same flavonoids with values of (23.33, 38.21, 35.28, 7.89, 19.23, 14.33) respectively.

In general, the data mentioned in Table (3) there is a significant increase in the rate of production of flavonoids with an increase in the concentration of radiation dose, and therefore the treatment of radiation dose of 45 gray achieved the highest rates in the concentrations of flavonoids. That is why in this study this dose was chosen as well as a comparison treatment to complete the subsequent stimulation experiment.

<table>
<thead>
<tr>
<th>Flavonoids Compound (µg / g)</th>
<th>Gamma ray (gray)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hesperdine</td>
<td>Nargnine</td>
</tr>
<tr>
<td>1.7</td>
<td>3.1</td>
</tr>
</tbody>
</table>

Table 3: Effect of gamma ray on the production of flavonoids (µg / g) in callus of grapes
<table>
<thead>
<tr>
<th>14.33</th>
<th>19.23</th>
<th>7.89</th>
<th>35.28</th>
<th>38.21</th>
<th>23.33</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>18.41</td>
<td>48.67</td>
<td>18.13</td>
<td>80.32</td>
<td>79.43</td>
<td>61.92</td>
<td>15</td>
</tr>
<tr>
<td>22.71</td>
<td>58.11</td>
<td>20.12</td>
<td>93.61</td>
<td>86.67</td>
<td>68.23</td>
<td>30</td>
</tr>
<tr>
<td>28.62</td>
<td>60.38</td>
<td>26.32</td>
<td>98.11</td>
<td>94.93</td>
<td>90.36</td>
<td>45</td>
</tr>
<tr>
<td>1.81</td>
<td>1.82</td>
<td>1.82</td>
<td>1.82</td>
<td>1.82</td>
<td>1.82</td>
<td>L.S.D</td>
</tr>
</tbody>
</table>

Figure 4: Effect of 0 gray on the production of flavonoids compound

Figure 5: Effect of 15 gray on the production of flavonoids compounds

Figure 6: Effect of 30 gray on the production of flavonoids compound

Figure 7: Effect of 45 gray on the production of flavonoids compound
Effect of Irradiation and the Methyl Jasmonate and their Interaction in the Rate of Production of Flavonoids

The results in Table (4) shows a significant increase in the level of concentrations of flavonoids compounds (Rutin, Quercetin, Nargnine) by increasing the concentrations of methyl jassont added to the medium. The data indicated that the concentration of 3 mg / L achieved the highest rate of flavonoids with values of (131.93, 145.42, 75.45) μg/ g respectively.

The concentration 90 mg / L of methyl jasmonate achieve the highest weight of Proanthocyanin and Hesperdine with values of 52.64 and 71.90 μg / g respectively, while no significant difference was observed at both concentrations of 2 and 3 mg / L methyl Jasmonate in regard to the concentration of Gintestnic and the values were 113.04 and 113.67 μg / g, respectively. As to the free methyl jasmonate treatment the lowest rate of flavonoids for (Gintestnic, Rutin, Quercetin, Proanthocyanin, Nargnine, Hesperdine) with values of (56.85, 66.57, 66.70, 17.11, 39.81, 21.48) μg / g were recorded respectively.

As the results showed the treatment of irradiation gave higher rate of concentrations of the same flavonoids with values of (133.84, 149.44, 157.82, 48.03, 89.42, 72.75) (μg / g) respectively, compared with unirradiated treatment which has achieved the lowest rate of flavonoids with values of (57.46, 72.77, 78.12, 21.17, 32.80 and 35.25) (μg / g) respectively. As for the effect of dual Interference, the results showed that the irradiation treatment combined with a concentration of 2 mg / L methyl Jasmonate resulted in the highest rate of flavonoids with values of (158.72, 170.36, 182.21, 70.32 and 110.56) (μg / g) respectively, while the lowest rate of concentrations of flavonoids were obtained from unirradiated and free methyl jasmonate treatment with mean values of (23.33, 38.21, 35.28, 7.89, 19.23 and 14.33) μg/g respectively. Generally, it was noted from the results in table (4) that the different concentrations of methyl Jasmonate added to the MS medium have led to clear increase in the rate of flavonoids, The reason may be due to the role of methyl jasmonate, as a widespread plant hormone in the plant kingdom, in regulating many plant Physiological processes, including photosynthesis, which may increase plant effective material in plant [41].

This is consistent with [42,43] who found that the concentration of 3 mg / L of methyl Jasmonate led to an increase in the production of anthocyanins by 25 times compared with the comparative treatment in grape cell cultures. The increase in flavonoids with the increase in the levels of the methyl jasmonate supplemented to the medium may stimulate all genes that are involved in the structural pathways of the anthocyanins compound.

This result also agrees with a result of [44] who found that the accumulation of Gintestnic and Quercetin compounds was eight times more in the cell cultured in medium with methyl jasmonate as a supplement compared with medium without it, and agrees with a result of [45] who noted in a study on the effect of methyl jasmonate concentration and red light on the production of Proanthocyanin compound in the cell suspension cultures of the grape, that the concentration of 3 g / L resulted in a significant increase in the compound concentration which reached nine times that obtained from a comparison treatment, while the production of the compound decreased when exposed to cell suspension culture of grapes exposed to red light alone.

Also [46] found an increase in the production of Hesperdine, Rutin and Proanthocyanin, flavonoids which was 9 times more than comparison treatment, in response to the increase of the concentration of methyl Jasmonate to 2 mg / to the medium of the cell suspension culture of the grapes. Based on the previous results shown in table 4 and the figures, it is noted that irradiation has an effective role in stimulating the increase in the production of flavonoids; the reason may be that radiation had stimulant rather than inhibitory and deadly effects. The type of effect is related to the dose which the plant was exposed to.

The low dose activates or stimulates the growth of most plants [47,48] Explained that the causes of activation could be due to the direct effect of radiation in the genetic material of the nucleus and the transmission of the effect of radiation to next generations, also low doses of radiation could lead to the removal of some inhibitory enzymes of some life processes in the plant. Another explanation is that radiation activation leads to a change in the physiological characteristics
of cytoplasm and this causes an increase in the physiological processes and therefore the life of the plant. [49] Also attributed the stimulus to photons of gamma radiation which have enough energy to increase the elasticity or break down the chemical bonds of the material, thereby causing photochemical reactions and thus have biological effects. This result agrees with [50] that low-doses of gamma stimulated production of the shikonin compound in the callus of Lithospermum erythrorhizon. as well as with [51] who found that the dose of 40 gray led to an increase in the total content of amino acids and sugars and increased the total content of flavonoids and phenolic compounds in the callus of Rosmarinus officinalis.

Also the results of the study by [52] recoded an increase the production of secondary metabolism in medicinal plant Phyllanthus odontadenius when seeds were exposed to different doses of gamma rays and they found that dose of 50 gray was the best because it achieved an increase in the production of secondary metabolic compounds Saponins, Tannins, Anthocyanin, Polyphenols, Steroids compared with the comparison treatment.

Table 4: Effect of methyl Jasmonate (mg / L) and gamma ray (gray) on the production of flavonoids (μg / g) in the callus of grapes

<table>
<thead>
<tr>
<th>Flavonoids compound (μg/g)</th>
<th>Con.of methyl jasmonate (mg/l)</th>
<th>Retention time of Flavonoids compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hesperdine</td>
<td>1.7 3.1 4.9 5.7</td>
<td>21.48 39.81 17.11 66.70 66.57 56.85</td>
</tr>
<tr>
<td>Nargnine</td>
<td>59.72 60.54 123.94 125.24 113.04</td>
<td></td>
</tr>
<tr>
<td>Proanthocyanin</td>
<td>71.90 68.65 134.72 131.93 131.32</td>
<td></td>
</tr>
<tr>
<td>Quercetin</td>
<td>62.92 75.45 145.42 131.93 110.56</td>
<td></td>
</tr>
<tr>
<td>Rutin</td>
<td>1.62 1.62 1.63 1.63 1.62</td>
<td></td>
</tr>
<tr>
<td>Gintestnic</td>
<td>1.21 1.21 1.22 1.22 1.22</td>
<td></td>
</tr>
<tr>
<td>Cobalt</td>
<td>28.62 60.38 26.32 98.11 94.93 90.36</td>
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<tr>
<td>1.87 1.88 1.88 1.88 1.87</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Conclusion

The results show that concentration of 3 mg / L methyl Jasmonate achieving the highest rate of Rutin, Quercetin and Nargnine of (131.93, 145.42, 75.45) μg / g respectively, while the concentration of 2 mg / L was the highest for Proanthocyanin and Hesperdine (52.64, 71.90) μg / g respectively. In the treatment for the effect of interference between the irradiation treatment with the concentration of 2 mg / L methyl Jasmonate, the results showed higher concentrations of Gintestnic, Rutin, Quercetin, Proanthocyanin, Nargnine and Hesperdine (157.72, 170.36, 182.21, 70.32, 110.56, 98.67) respectively.
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