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UV and Laser Diode as a Source of Flavonoids Stimulation Production in Marigold *Calendula officinalis*

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Abstract. *Calendula officinalis* is an important herbal plant as it is a source of value compounds, and it is a convenient model for plant micro propagation. The aim of this study was micro propagation of *C. Officinalis* and evaluation of the efficiency of Laser diode and UV-C irradiation, in the stimulation of flavonoids biosynthesis and accumulation in vitro culture. BA 1.5mg.L^{-1} had a significant effect on the number of explants, reached 6.7 shoots.explant^{-1}. While the nodes, BA 1.mg.L^{-1} had the highest effect on the average shoot length explants, which increased to 2.9 cm. UV exposure resulted in an increase in the shoots and number of leaves of explants treated by BA 1.5 mg/L. After 60 min of UV exposure, the accumulation of flavonoids Rutin in marigold increased to 343.458 ppm /g compared to the control 21.735 ppm/g. Galic acid decreased to 0.122 ppm/ g. The laser diode caused more changes in flavonoids, the Rutin was 4.742ppm /g while Gallic acid reached 50.825 ppm/g, about 5-fold. This stimulation by laser can act as an actor to stimulate one compound and reduce the others, it seems the plant's secondary compounds compete with each other's and sometimes with the same type, like Rutin and Galic acid.

Keywords. *In vitro* cultures, Marigold *Calendula officinalis*, Flavonoids.

1. Introduction

1.1. Plant Characterization Calendula Officinalis

Calendula officinalis L. is a part of the botanical family of Asteraceae. The genus of Calendula includes 15-20 species [1]. Because the Calendula has a long flowering period, its name comes from the Latin word calends, which means the first day of every month.). In many countries, also in British Columbia in Canada, the plant is used for the treatment of many medical conditions such as wounds and scratches on the skin, dyspepsia, and diarrhoea [2]. The flowers were made into extracts, and balms and applied directly to the skin to soothe damaged and inflamed skin and to help heal wounds. Also, marigold was used to treat fevers, treat cancer and promote menstruation [3], and was used, for jaundice, and as an antispasmodic [4]. Moreover, there are plenty of medical products derived from *C. officinalis* applied in the treatment of various skin tumours, ulcers, dermatological, lesions, and swellings [5]. Plant in vitro cultures are not only an important tool for applications in new modern agriculture technauqe, such as plant improvement andmodification , or in protection of plant biodiversity, as clonal propagationand germplasm storage , but also offer a useful tools system for understanding regulatory mechanisms of plants development. Several approaches have been

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successfully used to improve biological compounds activity and enhance secondary compounds of marigold.

1.2. Plant in Vitro Cultures as a Source of Flavonoids

In vitro cultures technique, many plants have been mentioned as biological matrices for large-scale production of important and valuable and essential natural compounds, belonging mainly to so-called secondary plants metabolites [6]. *In vitro* culture, explant can be stimulated by some external elicitors. Due to the advantage of its practical feasibility and activeness, elicitation by UV is one of the most commonly applied strategies for enhancing and accumulating the production of important compounds (particularly secondary metabolites gradients) in plant vitro culture and biotechnology. Currently, it is widely accepted in many fields of agriculture technique that In vitro propagation through tissue culture methods is the best way means of mass production of large numbers of plants in a short span of time [7]. Indeed, stimulation of plant compounds, particularly secondary plant metabolism (regarded as a mechanism involved in defence reactions in wild plants) is a well-known phenomenon in plant tissue technique.

2. Materials and Methods

The seed was washed with distilled water, the mature embryos were excised from the seeds and soaked in water in a flask for 12 min. Obtained embryos were put in a refrigerator for up to two weeks. Then, chosen seeds were sterilized with a commercial hypochlorite preparation diluted with water (1:3, v/v), on Petri dishes with different concentrations. To keep seeds sterility and to reduce contaminations, the procedures performed with the sterilized seeds and the forthcoming steps of in vitro culture technique initiation and maintenance were made in the vertical laminar airflow cabinet installed in the special culture plants room. *C. officinalis* seedlings were obtained from sterilized embryos and grown on medium MS (solid agar) during the first 4 days in the dark and then in the growth chamber. On the fifth day of cultivation, the obtained seedlings were moved to an incubator (a growth chamber). The seedlings grew in a growth chamber in a 16 h light/8 h dark photoperiod under 200 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR; daylight and warm white 1:1, LF-40W), day/night temperature of 24°C/22°C and 70% relative humidity. The explant of *C.officinalis* shoot tips and nodes were cut into 1.0 cm lengths from the seedlings and subjected to the procedure of growing on MS media with hormones BA at concentrations (0.5, 1.0, 1.5, 2.0) mg L^{-1} [8].

The plants were dried (control and treated samples) for five days at room temperature and then ground by using grinding mortar, then extracted by Soxhlet extractor using pure ethanol 99%, followed by evaporation of the solvent by a rotary evaporator, The extracts were removed to round-bottom flasks and evaporated to dryness at 40°C under reduced pressure in a rotary evaporator Laborota 4000, Heidolph. Samples were measured according to Shimadzu HPLC (High-Performance Liquid Chromatography).

2.1. Effect of UV Radiation

Calendula officinalis explant were exposed to UV-C radiation for 60 min. Samples were placed at a distance of 40 cm from a UV-C tube (Alpina, 15 W, 90 μW/cm) emitting radiation at λ=254 nm. After laser and UV treatment, the tested samples and untreated controls (in 3 replicates each) were incubated in typical conditions (darkness, 24ºC,) for subsequent 7 days.

2.2. Elicitation with Laser Diode

A laser diode was used to elicitate C. Officinalis explant (energy: 5 mw, distance: 20 cm, time: 60 sec. with 650 nm wavelength). The period of laser exposure 60, was applied for elicitation of samples, prepared as described previously.

3. Results and Discussions

Results of the present study revealed that explants of *Calendula officinalis* affected by different concentrations of BA. So it is considered plant phytohormones are among the most critical physiological factors that can affect the explants' induction of plants in vitro culture. Many studies

have been reported regarding the effects of plant growth regulators in branches, nodes, leaves and callus induction of different plants [9].

3.1. Effect of BA on Shoot Tips Multiplication of C.officinalis L cultured on MS Medium

The results of Calendula officinalis in Table (1) showed that the concentrations of BA used had a significant effect on the number of shoots formed on the individual explant of the marigold, all concentrations of BA increased the average of these explants to reach 6.7 shoots. explant^{-1} when using 1.5 mg.L^{-1}of BA. Also, the results showed that the average shoot length where low compared to the control and the average number of leaves increased significantly by using 1 mg.L^{-1} of BA to reach 7.6 leaves. explant^{-1}. These results are similar to the study by [10], the explant treated with a concentration of 1.0 mg L^{-1} of BA gave the highest averages in micropropagation of Chrysanthemum Indicum L after four weeks of cultivation in vitro culture.

As can be seen in the results, the presence of cytokinin in the MS medium, at concentration 1.5 mg/L concentration of BA was sufficient for increasing the number of branches. The reason for these results may be due to the fact that BA is a cytokinin that one of its main physiological effects is to eliminate the apical dominance and enhance the growth of lateral shoots and the formation of the vegetative shoots [11], and it can influence more growth of explants' branches and leaves in vitro culture.

Table 1. Effect of BA concentrations on initiation and multiplication of *Calendula officinalis* shoot tips cultured on MS medium.

BA (mg.L^{-1})	Shoots Number	Shoots Length (cm)	Leaves Number
0.0	1.95	3.78	7.1
0.5	4.47	2.16	6.75
1.0	5.9	2.32	7.60
1.5	6.7	2.6	7.20
2.0	5.7	1.9	6.65

3.2. Effect of BA on Nodes Multiplication of C.officinalis L cultured on MS Medium

The results in Table 2 infer that the addition BA was effective in the multiplication and establishment stages nodes explant in terms particualy of the number of leaves from explant, at concentration 1. BA mg/L produced shoot length 2.9 cm and 8.70 leaves. explant $^{-1}$ numbers of leaves, compared to control 2.7 cm and 8.7 leaves. explant $^{-1}$ respectively. This result is in agreement with the study by (12) showed that cultivating the branches of *Calendula officinalis* in MS medium prepared with concentrations of 0.0, 0.1, 1.0 or 3.0 mg /L of h of BA or Kin showed that a concentration of 1.0 mg / L BA gave the highest average number of branches 2.33. shoots.explant^{-1}.The results of adding phytohormons usually, show the importance of Cytokinins and auxin hormones and their role in cell division, differentiation of cells and the emergence of shoots on the cultured explant on MS media [13].

Table 2. Effect of BA concentrations on initiation and multiplication of Calendula officinalis nodes cultured on MS medium.

BA (mg.L^{-1})	Shoots Number	Shoots Length (cm)	Leaves Number
0.0	2.95	2.7	7.3
0.5	2.7	2.40	7.6
1.0	3.2	2.9	8.70
1.5	3.0	2.7	7.1
2.0	2.4	1.75	4.9

3.3. Elicitation Explants (Shoot Tips and Nodes) with UV Radiation, Laser Diode and Interaction with Phytohormones

After 60 min of UV exposure resulted in an increase in the shoots and number of leaves of explants treated by BA 1.mg/L, by 4.7 shoots.explant-1 and 6.5 leaves. explant -1 for a number of leaves,

compared with the explants control. The explants (shoot tip) stimulated by UV, produced the highest number of shoot and number of leaves in this experment. UV exposure with the explants treated with BA showed the highest increase all shoot tips explant ,except shoot length samples treated with concentration BA 2mg.L. which decresed lower than control. While the nodes explant, the results showed, the highest increased growth explants noticed at concentration 1.mg.L. Out of all BA concentrations used in this study, one combination containing UV and BA 1. mg/L, with UV radiation showed to be the most efficient in promoting shoot development from cotyledon explants. It was interesting to find that shoots occurred at concentrate these combinations, where the amount of cytokinin was fit with interaction of UV radiation exposure. These results were in agreement with the study by [14], the effect of the type of cytokines, used in the micropropagation of the belladonna plant, the obtained results show the clear predominance of BA other hormones, in increasing rates of shoot number, shoot length and number of leaves.

Table 3. Elicitation explants with ultraviolet (UV) radiation and interaction with the concentration of BA ($mg.L^{-1}$) on initiation and multiplication shoot tip of Calendula officinalis shoot tips cultured on MS medium.

BA ($mg.L^{-1}$)	Shoots Number	Shoots Length (cm)	Leaves Number
0.0	1.95	3.7	7.1
0.5	4.9	3.75	7.75
1.0	6.9	3.8	8.70
1.5	7.96	3.9	10.60
2.0	6.7	2.9	7.95

Table 4. Elicitation explants with ultraviolet (UV) radiation and interaction with the concentration of BA (mg.L-1) on initiation and multiplication nodes of Calendula officinalis nodes cultured on MS medium.

BA ($mg.L^{-1}$)	Shoots Number	Shoots Length (cm)	Leaves Number
0.0	2.95	2.7	7.3
0.5	3.2	2.80	7.6
1.0	4.2	3.1	9.3
1.5	3.5	2.9	7.7
2.0	3.0	1.5	4.9

Table 5. Elicitation explants with ultraviolet (Lasre) radiation and interaction with the concentration of BA ($mg.L^{-1}$) on initiation and multiplication shoot tip of Calendula officinalis shoot tips cultured on MS medium.

BA ($mg.L^{-1}$)	Shoots Number	Shoots Length (cm)	Leaves Number
0.0	1.95	3.78	7.1
0.5	3.8	3.1	7.5
1.0	6.59	3.52	8.2
1.5	6.9	3.9	10.40
2.0	6.5	2.7	7.55

Table 6. Elicitation explants with ultraviolet (Lasre) radiation and interaction with the concentration of BA ($mg.L^{-1}$) on initiation and multiplication nodes of Calendula officinalis nodes cultured on MS medium.

BA ($mg.L^{-1}$)	Shoots Number	Shoots Length (cm)	Leaves Number
0.0	2.95	2.7	7.3
0.5	3.2	2.20	4.6
1.0	4.0	2.9	8.70
1.5	3.5	1.9	6.8
2.0	3.0	1.3	4.4

3.4. Effect of UV Radiation and Laser Diode on Flavonoids Production in Marigold Calendula Officinalis

The accumulation of flavonoids was elevated in *C. officinalis* explants, Two flavonoid compounds were measured in *C. Officinalis*, Rutin, and Galic acid. The Rotain seemed to be more susceptible to this elicitation. After 60 min of UV exposure, the accumulation of flavonoids rutin in marigold increased to 343.458 ppm /g compared to the control 21.735 ppm/g. In turn, the content of Galic acid showed a significant decrease after elicitation by UV.

After exposure to 60 min of UV, the accumulation of Galic acid decreased to 0.122 ppm/g, while the content of Galic acid in the control samples was 10.025 ppm/ g. laser diode after 60 sec of exposure caused more changes in flavonoids, the Rutin was 4.742ppm /g while Galic acid reached 50.825 ppm/g compared to the control 10.025 ppm/ g. The application of UV light radiation has often been reported to influence the compounds accumulation, accumulation of flavonoids elevated in *C. officinalis* particularly Rutin when compared to the control samples was so clear, however, all plants treated with UV seemed to be more susceptible to this elicitation and production of compounds protective against this potentially harmful irradiation on plants, such as anthocyanins [15]. The significant increase of Rutin in marigold explants reached 16-fold in this study, Many reports indicated the beneficial effect of some elicitations on plant secondary metabolism. Results of a study by [16], UV-C radiation caused an increase in the content of plant secondary compounds in hairy root tissue and stimulates the active compounds in Calendula officinalis secretion up to 8.5-fold. laser diode after 60 sec caused more changes in flavonoids, the Rutin Galic acid. This stimulation by laser can act as an actor to stimulate one compound and reduce the others, it seems the plant's secondary compounds compete with others and sometimes with the same type, like Rutin and Galic acid. These results it is similar to results found by [17], of elicitation, the effect of elicitation with Ethylene on steroid synthesis of hairy root culture seemed to be opposite to the extracted oleanolic acid.

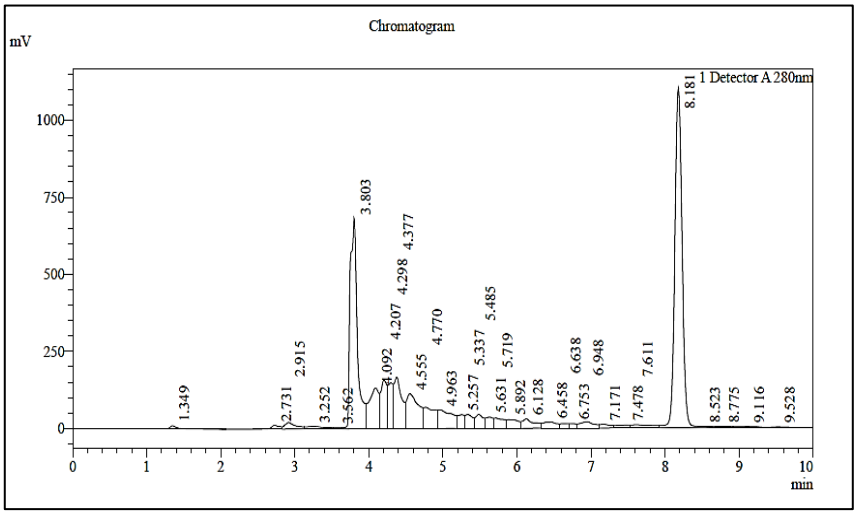


Figure 1. Chart of HPLC (High Performance Liquid Chromatography)after elicitation with UV.

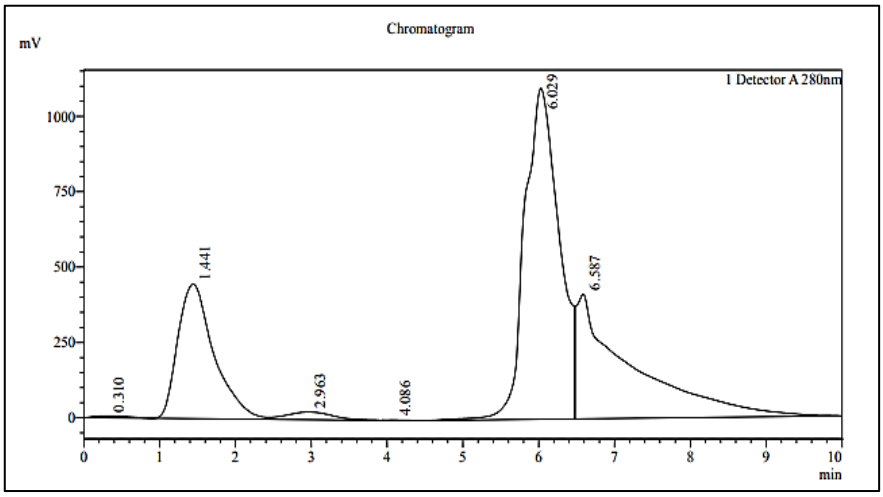


Figure 2. Chart of HPLC (High Performance Liquid Chromatography)after elicitation with Laser diode.

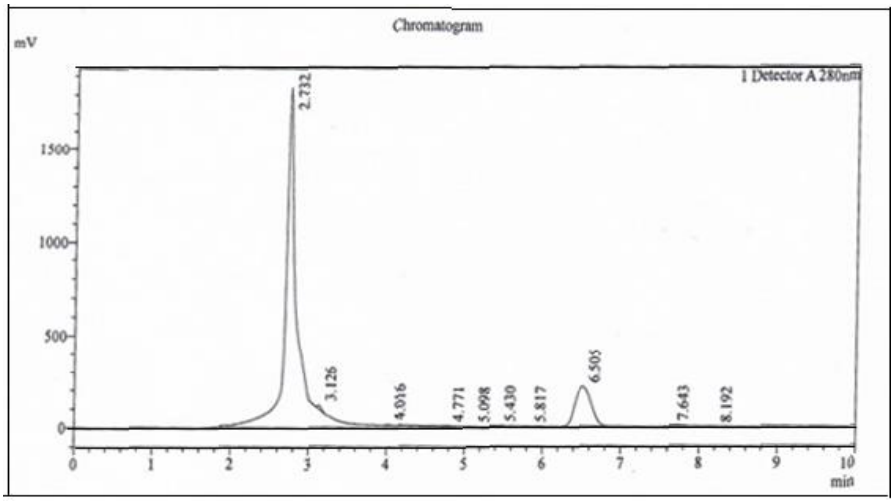


Figure 3. Chart of HPLC (High Performance Liquid Chromatography) control.

Conclusion

Out of all BA concentrations used in this study, one combination containing UV and BA 1.5 mg/L, with UV radiation showed to be the most efficient in promoting shoot development from explants. It was interesting to find that shoots occurred at concentrate these combinations, where the amount of cytokinin fit with the interaction of UV radiation exposure

The best concentration used to obtain the highest average shoot number was 1.5 mg L^{-1} of the BA (6.7 shoots.explant^{-1}). the highest value for the average length of the shoot reached 2.6 cm at the same concentration, Whereas the number of leaves reached to 6.7 leaves explant^{-1}. at concentration 1. mg L^{-1} of BA. Using nodes explant and the BA at a concentration of 1.0 mg L^{-1} produced 3.2 shoots.explant-1, 2.9 cm shoot length, and 8.7 leaves. explant $^{-1}$ number of leaves per explant respectively. while the number of shoots, shoot length, and leaves at the highest concentration was 2.4, 1.75, and 4.9 respectively. 60 min of UV exposure resulted in an increase in the shoots and number of leaves of explants treated by BA 1.mg/L, by 4.7 shoots.explant^{-1} and 6.5 leaves. explant^{-1} for a number of leaves, compared with the explants control. After 60 min of UV exposure, the accumulation of flavonoids rutin in marigold increased to 343.458 ppm /g compared to the control 21.735 ppm/g. In turn, the content of Galic acid showed a significant decrease after elicitation by UV. After exposure to 60 min of UV, the accumulation of Galic acid decreased to 0.122 ppm/g. laser diode after 60 sec caused more changes in flavonoids, the Rutin was 4.742ppm /g while Galic acid reached 50.825

ppm/g. This stimulation by laser can act as an actor to stimulate one compound and reduce the others, it seems the plant's secondary compounds compete with others and sometimes with the same type, like Rutin and Galic acid.

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