Physical elicitation of *Dillenia indica* callus for production of secondary metabolites

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Abstract: Plant tissue culture technique combined with elicitors is required to produce plant *in vitro* secondary metabolites. The effect of some physical elicitors on the production of some secondary metabolites from callus cultures of *Dillenia indica* was investigated. Elicitors including two ultraviolet irradiation powers (15 and 30 watt) for two times (1 and 2 hours), two microwave radiation powers (100 and 200 watt) for two times (10 and 20 sec) and six light incubation conditions (dark, 3000 lux of white light, green, blue and red light). Using Clorox at 15% (v/v) for 15 min recorded the highest significant decontamination percentage. Subjection leaf disc explants to 2.0 mg/L NAA + 2.0 mg/L BAP resulted in the highest callus formation. Callus cultured on medium supplemented with 2.0 mg/L 2,4-D produced the highest significant callus fresh weight (3.085 g). The highest significant fresh weights (5.96 and 5.58 g) were obtained when callus culture was irradiated with ultraviolet at 30 watts for one hour, and callus incubated under green light, respectively while the lowest significant fresh weight (2.318 g) was detected when callus culture was incubated under full darkness. The highest phenolic compounds content (231.158 mg/100g f.w) was estimated in callus treated with 200 watts of microwave irradiations for 20 seconds. The highest significant flavonoid content (ranging from 840.833 to 1071.667 mg/100g fresh weight) could be detected in callus treated with 200 watts of microwave irradiation for 10 sec, and all ultraviolet irradiation treatments. The highest significant antioxidant activity (98.98 %) was obtained from extract of fruits. There was no significant difference among all elicitation treatments except that of UV at 15 watt for 1 hr which recorded less significant antioxidant activity.
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1. Introduction

The genus *Dillenia* has 60 species, of which *Dillenia indica* Linnaeus (Family: Dilleniaceae) is the most common edible species. Originally from Indonesia, this evergreen tropical tree is now found from India to China. The common names include Chulta (Bengali, Hindi), Bhavya (Sanskrit) and Elephant apple (English). It is a spreading tree and has beautiful white fragrant flowers, toothed leaves, and globose fruits with small brown seeds (Janick and Poull, 2008). The leaf, bark, and fruit of this plant are used as traditional medicine. The juice of *D. indica* leaves, bark, and fruits are mixed and given orally (5-15 ml, two to five times daily) in the treatment of cancer and diarrhea (Sharma *et al*., 2001).

The use of plant cell and tissue culture methodology as a means of producing medicinal metabolites has a long history (Rout *et al*., 2000; Verpoorte *et al*., 2002). Since plant cell and tissue culture emerged as a discipline within plant biology, researchers have endeavored to utilize plant cell biosynthetic capabilities for obtaining useful products and for studying the metabolism (Misawa, 1994; Verpoorte *et al*., 2002). In recent decades, interest in chemo-preventive plant natural products has grown rapidly. The etiology of several degenerative and aging-related diseases has been attributed to oxidative stress, and many studies have been undertaken to search for the most effective antioxidants (Halliwell, 1995; Soobrattee *et al*., 2005).

The objectives of this study were to investigate callus formation and production of phenols, flavonoides, and anti-oxidants from *Dillenia indica* trees using tissue culture techniques.

2. Material and Methods

This investigation was conducted at Tissue Culture Res. Lab., botany and microbiology department, faculty of science, Al-Azhar University and Tissue Culture & Germplasm Conservation Laboratory, Horiculture Research Institute, Agricultural Research Center, Giza, Egypt, during the years from 2010 to 2014.

2.1. Plant materials

The mother plant of *Dillinia indica* authorized in Herbaria Horti Boanici Ormani by Prof. Dr. Therese Labib. And it was founded in Zoo Garden, Giza, Egypt.

The mother plant was used as a source of plant materials (leaves explants).

2.2. Culture medium and incubation conditions

The basal salts mixture of MS medium (Murashige and Skoog, 1962) supplemented with 30 g/L sucrose and pH adjusted to 5.7±1 then it was solidified with 7 g/L agar. All the culture treatments were incubated in growth rooms under controlled conditions, where temperature was maintained at 25 ± 1°C day/night schedules and illumination intensity of 3000 lux day light at the top of cultures level from white fluorescent lamp (120 cm long 40 watts), the photoperiod was 16/8 day/night controlled by electronic timer.

2.3. Callus initiation and growth

2.3.1. Explant disinfection

2.3.1.1. Effect of sodium hypochlorite (Clorox) and mercuric chloride on disinfection and survival percentages of *Dillenia indica* L.

Leaf disc explants (about 1 cm^2) were initially subjected to hygienic soapy (Septol) water solution for 30 min, then to savlon solution (3 %) for 40 min and rinsed with running tap water for one hour.

Under aseptic condition in safety cabinet, leaf disc explants were immersed in ethanol (70 %) for one min. thereafter; they were surface disinfected with one of the disinfectant substances of Clorox (Sodium hypochlorite 5.25 %) or mercuric chloride ($HgCl_2$) as follow:

- Clorox at 15 % for 10 or 15 min.
- Mercuric chloride at 0.1 % for 10 or 15 min.

All of the above used disinfectant solutions were provided with a few drops of tween-20 (polyxyethelen sorbite monolaurate) as an emulsifier (wetting agent). After the disinfection treatments, the explants rinsed in sterilized distilled water for three times to remove all traces of disinfectant substances, then they explanted vertically (one explants/tube) for four weeks into autoclaved MS hormone free medium .At the end of the incubation period (four weeks) for each disinfection treatment, the following measurements were recorded:

- Disinfection percentage (%).
- Survival percentage (%).

Each treatment contained five replicates and 10 explants for each replicate.

2.3.2. Initiation of callus from leaf explants

Sterilized leaves from *Dillenia indica* under study, were cut into approximately 1 cm^2 segments, and used as explants. They were cultured on basal MS medium (control treatment) in addition of MS medium supplemented with different concentrations of an auxin and combinations of an auxin and a cytokinin as study the effect of growth regulators on callus formation as follows:

- 2,4-D at 1.0, 2.0 and 3.0 mg/L.
- NAA at 1.0, 2.0 and 3.0 mg/L.
- 2,4-D at 1.0 mg/L plus BAP or kin at 1.0 mg/L.
- 2,4-D at 2.0 mg/L plus BAP or kin at 2.0 mg/L.
- NAA at 1.0 mg/L plus BAP or kin at 1.0 mg/L.
- NAA at 2.0 mg/L plus BAP or kin at 2.0 mg/L.

All cultures were examined after 5 weeks of incubation at 25±2 ºC under 16 hrs. light and 8.0 hrs. Dark provided by cool florescent light intensity of 2500 lux to record the following data:

2.3.3. *In vitro* callus growth

2.3.3.1 Effect of plant growth regulators, different light conditions and number of subcultures on callus fresh weight and callus dry weight (g) of *Dillenia indica* L. cultures

Callus initiated from sterilized leaves explants of *Dillenia indica*, were cut into 1 gm segments, and used as explants. They were cultured on the surface of MS medium supplemented with different concentrations of an auxin, a cytokinin and combinations of an auxin and a cytokinin as study the effect of growth regulators on callus formation.

In this stage, one type of culture media ingredients (MS media full strength), three plant growth regulators treatments and four subcultures of explants on *in vitro* are used. In each subculture 1 gm of callus transferred in each jar.

- 2,4-D at 2.0 or 4.0 mg/L plus 2.0 mg/L BA.
- NAA at 2.0 or 4.0 mg/L plus 2.0 mg/L BA.

In this experiment three different conditions of light 1500 lux, 3000 lux and dark conditions provided by cool florescent light are used to study the effect of light conditions on callus fresh weight and dry weight.

For number of callus subculture, the formed fresh callus tissues resulting from each one of the above tested plant growth regulators treatments were aseptically removed, divided to one gram weight and recultured into fresh culture media of the corresponding components. These procedures were successively repeated for four subcultures cycles of callus tissues at 5 weeks of incubation at 25±1 ºC. Each treatment consisted of ten replicates.

2.4. Physical elicitation treatments

In this stage, one type of culture media ingredients (MS media full strength) and two plant growth regulators treatments on *in vitro* are used. 1 gm of callus transferred in each jar.

- NAA at 4.0 mg/L plus 2.0 mg/L BA.

2.4.1. Ultraviolet irradiation

The callus cultures were exposed to 15 or 30 watts by using one or two UV-C lamps for 30 or 60 min. The source of ultraviolet rays was Philps TuV, 15W, 54 V, 0.34 A. Model G15T8 ultraviolet UV-C lamp (45 cm long and 2.8 cm diameter and contains mercury (Hg) 2.0 mg, water air disinfection, 253.7

nm – 254 nm UV-C, shortwave germicidal linear tube.

2.4.2. Microwave irradiation

Callus cultures were microwaved with 100 or 200 watts for 10 or 20 sec. The used microwave apparatus is a single phase grounding 1.3 kw out-put 650 w at a frequency of 2450 MHz (Model: WP 100 AP 30-2).

2.4.3. Light quality

Callus cultures were incubated under different types of light including: Full dark, 3000 white light, green light, blue light and red light.

At the end of elicitation treatments, callus fresh and dry weight were recorded and the dried callus were subjected to chemical investigations.

2.5. Chemical analysis

2.5.1. Extraction procedures

Air dried powdered samples (0.5 g, each) of the *in vitro* grown calli and microshoots; leaves and fruits from the mother tree were separately mixed with 70 % methanol (3 X 10 ml, each) and shacked for 24h. Methanolic extract of each sample was filtered, evaporated under reduced pressure and the residue was used for determination.

2.5.2. Total Phenols

Total phenolic compound contents were determined by the Folin-Ciocalteau method (Slinkard and Singleton, 1977). The extract samples (0.5 ml of different dilutions) were mixed with 2.5 ml of 0.2N Folin-Ciocalteau reagent for 5 min and 2.0 ml of 7.5 % sodium carbonate were then added. The absorbance of reaction was measured at 760 nm using spectrophotometer (V-530- UV/VIS, JASCO Corp., Japan) after 2 h of incubation at room temperature. Results were expressed as gallic acid equivalents mg / 100 g dry weight.

2.5.3. Total flavonoids

Total flavonoids were determined using method of Woisky and Salatino (1998). 0.5 ml of 2 % $AlCl_3$ methanol solution was added to 0.5 ml of sample. After 1 hour at room temperature, the absorbance was measured at 420 nm using spectrophotometer. Total flavonoid contents were calculated as quercetin from a calibration curve mg / 100 g dry weight.

2.6. Antioxidant study

2.6.1. Free radical scavenging activity (DPPH – RAS method)

The free radical scavenging activity of methanol extracts was measured by α, α-diphenyl-β-picryl- hydrazyl (DPPH) using the method described by Brand-Williams et al. (1995). The 0.1 mmol/l solution of DPPH in methanol was prepared and 1 ml of this solution was added to 3 ml of extracts solution at concentration of 0.1 to 1.5 g/100ml. After 30 min absorbance was measured at 517 nm using spectrophotometer. Radical scavenging activity was expressed as inhibition percentage and was calculated using the formula:

$$\% \text{ Radical scavenging Activity} = \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \text{X } 100$$

3. Results and Discussion

3.1. *In vitro* callus formation of *Dillenia indica*

3.1.1. Leaf disc explants disinfection

3.1.1.2. Effect of mercuric chloride and Clorox on decontamination and survival percentage of leaf disc explants of *Dillenia indica*

As shown in table (1), the highest significant contamination percentage (90.91 %) was recorded when $HgCl_2$ was applied for 10 min, while the lowest significant contamination percentage (9.09 %) was detected when Clorox was used at 15 % for 10 min. The data revealed also that the contamination percentage was reduced with deceasing duration of sterilization from 15 to 10 min. As for survival percentage, data indicated that the highest significant survival percentage (90.91 %) was recorded when Clorox was applied at 15 % for 10 min. while, the lowest significant survival percentage (9.09 %) was detected when mercuric chloride was used at 0.1 % for 10 min. Data in table (1) disclosed that the survival of explants increased from 9.09 to 40.09 % when the time of sterilization with 0.1 % of $HgCl_2$ was increased from 10 to 15 min. Whereas, it was decreased from 90.91 to 77.27 % when the time of application with 15 % Clorox was increased from 10 to 15 min.

As above mentioned data, the variability's in the decontamination rates of *Dillenia indica* leaf disc explants as influenced by the various tested sterilization treatments under trials are corresponding with those earlier reviewed by numerous scientists:- Relating to the effect of the types of disinfectant substances, Awad (1993) remarked that subjecting the shoot tip explants of *Psidium guajava* to 0.5 % of NaOCl yielded the highest contamination percentage, while applying 0.1 % $HgCl_2$ gave zero % contamination. Oppositely, Abd El-Kader and Hammad (2012) on *Dillenia indica;* and Salamma and Rao (2013) on *Croton scabiosus,* pointed out that the disinfection rates were higher for explants sterilized with Clorox than those treated with $HgCl_2$.

Referring to the effect of sterilization duration, El-Sayed (2005) reported that exposing the *Sequoia sempervirens* explants to Clorox at 30% for a longest period of sterilization (10 min) produced the highest survival rate (96.4 %), while the shortest time gave the minimum value (58.6 %). Badoni and Chauhan (2010) demonstrated that after sterilizing the *Solanum tuberosum* for three durations; 2, 5 and 8

min, the mortality rates were 70, 90 and 90 %, respectively in case of using $HgCl_2$, while they were 80 %, 40 % and 50 %, respectively in case of using 1.0 % NaOCl.

Table 1. Effect of surface sterilization with different concentrations of NaOCl solution and $HgCl_2$ on the survival percentage of *D. indica* leaf disc explants

Treatments	Conc.	Duration (min)	Contamination (%)	Survival (%)
$HgCl_2$	0.1gm %	10	90.91 a	9.09 d
$HgCl_2$	0.1gm %	15	59.09 b	40.91 c
Clorox	15 % (v/v)	10	9.09 d	90.91 a
Clorox	15 % (v/v)	15	22.73 c	77.27 b

Means having the same letter(s) in each column are not significantly different from each other at 5 % level according to L.S.D. test

3.1.2. Effect of plant growth regulators on callus formation

As shown in table (2), and Fig (1) callus formation was varied according to the type and concentration of plant growth regulators used. Using combination of NAA (2.0 mg/L) and BAP (2.0 mg/L) leads to callus formation at the highest frequency while using the combination of 2,4-D (1.0 mg/L) and kin (1.0 mg/L) failed to induce callus (zero callusing). The data also revealed that using 2,4-D or NAA individually at different concentration (1.0, 2.0 and 3.0 mg/L) induced leaf explants to from callus at the same frequency. Using the cytokinin BAP with NAA (at 1.0 mg/L each) was more effective than kin at the same concentration in inducing callus formation. Also, BAP exhibited the same trend when it was used with NAA at 2.0 mg/L it was more effective than kin at the same concentration. In this respect, the effect of plant growth regulators on callusing rates of explant, when they were applied individually or in combination forms at different concentrations, have been earlier experimented by several scientists.

Table 2. Effect of plant growth regulators on callus initiation from leaf disc explants of *Dillenia indica*

Treatments (MS+)	Callus formation
1.0 mg/L 2,4-D+	+
2.0 mg/L 2,4-D	+
3.0 mg/L 2,4-D	+
1.0 mg/L NAA	+
2.0 mg/L NAA	+
3.0 mg/L NAA	+
1.0 mg/L NAA + 1.0 mg/L BAP	++
1.0 mg/L NAA + 1.0 mg/L Kin	+
1.0 mg/L 2,4-D + 1.0 mg/L BAP	+
1.0 mg/L 2,4-D + 1.0 mg/L kin	-
2.0 mg/L NAA + 2.0 mg/L BAP	+++
2.0 mg/L NAA + 2.0 mg/L Kin	+
2.0 mg/L 2,4-D + 2.0 mg/L BAP	++
2.0 mg/L 2,4-D + 2.0 mg/L Kin	+

- No callusing + Low frequency ++ Medium frequency +++ High frequency

Amongst different concentrations of auxin 2,4-D which used in an individual form, it was affirmed that the most effective levels for inducing the callus formation in the maximum rates were 0.1 mg/L 2,4-D for *Echinacea purpurea* (Patan *et al.,* 1996). Moreover, Abdellatef and Khalfalla (2010) on *Moringa oleifera* Lam., found that the explants failed to produce callus tissues in MS medium free of 2,4-D, they attributed that to the presence of 3,4-D was capable to induce callus tissues. They remarked also that, among the 2,4-D concentrations tested (0.1-3.0 mg/L), using 0.1, 0.5, 1.0 or 2.0 mg/L gave 100 % of callusing. Increasing the 2,4-D level to 3.0 mg/L significantly reduced the callusing rate to 84 %.

Relating to the effect of the individual or combination form, Youssef (1997) on *Ceratonia siliqua*, pointed out that, after treating the explants with individual form of NAA or 2,4-D at 10.0 mg/L and their combination with BAP or kin, remarked that the highest callusing response (86.0 %) was recorded for

explants subjected to combination form of 2,4-D plus BAP, while the lowest value (42.3 %) was determined when the individual form of NAA was used. Similarly, El-Shamy *et al.* (2007) on *Tanacetum parthenium,* subjected the explants to 2,4-D, NAA. BAP alone or in combination in different concentrations (0.0, 0.5 or 1.0 mg/L) and recorded that applying the combination of 1.0 mg/L NAA plus 1.0 mg/L BAP resulted in the highest rate of callusing (91.67 %). Recently, Rawat *et al.* (2013) submitted the explants of *Aconitum violaceum* to 2,4-D at 0.5 – 1.0 µM or kin at 0.25 - 2.0 µM and remarked that employing the combination 5.0 µM 2,4-D plus 0.5 µM kin obviously increased the callus formation in the highest rates.

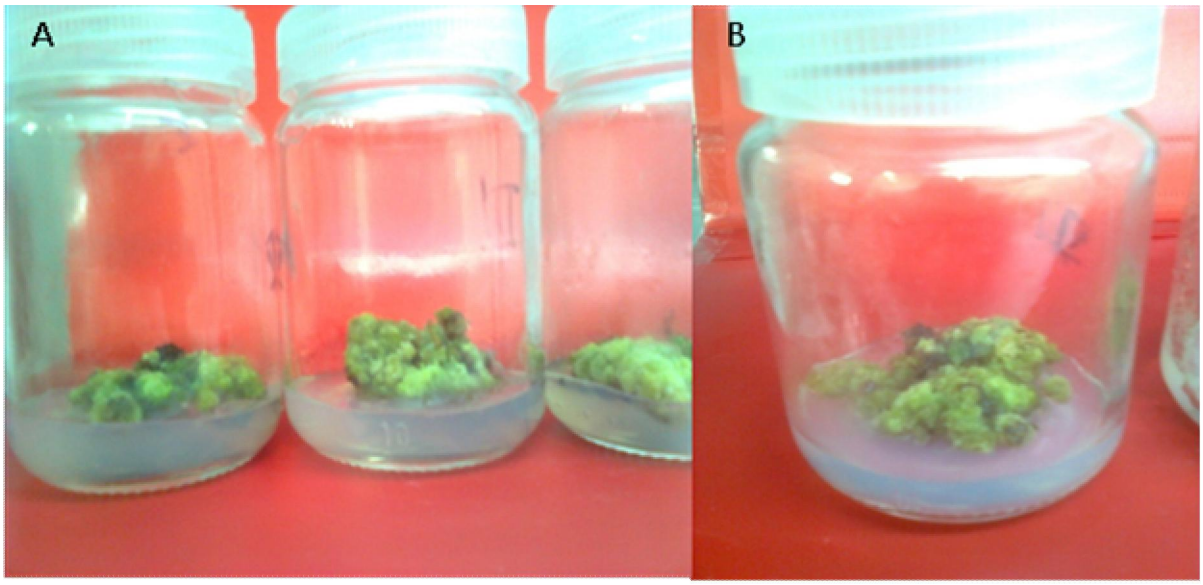

Figure 1 (A-B). Callus induction from leaf explant after four weeks of culturing on MS medium supplemented with NAA (2.0 mg/L) and BAP (2.0 mg/L), respectively

3.1.3. Effect of plant growth regulators and light intensity on callus fresh weight during four successive subcultures

As shown in table (3), the effect of plant growth regulators differed according to light conditions and the number of subculture. During the first subculture, data presented in Table (3) showed that there are no significant differences among plant growth regulators tested in callus cultures incubated in full dark. Whereas, callus cultures incubated under 1500 and 3000 lux affected significantly according to type and concentration of plant growth regulator. Callus cultured on medium supplemented with 2.0 mg/L 2,4-D produced the highest significant callus fresh weight (3.085 g) while, increasing the 2,4-D subculture were higher than in first subculture on the same plant growth regulators treatments. With increasing light intensity from 1500 to 3000 lux, the callus growth in term of fresh weight takes a different direction. The highest significant fresh weights (3.902 and 3.775 g) were recorded in medium supplemented with 2.0 mg/L NAA and 4.0 mg/L 2,4-D, respectively. While the lowest ones were recorded for medium amended with 2.0 mg/L 2,4-D or 4.0 mg/L NAA. It could be concluded that callus growth concentration up to 4.0 mg/L significantly reduced the callus growth to 1.378 g. Using NAA at 2.0 mg/L had a positive effect on callus growth to record the highest significant fresh weight (2.872 g) under 3000 lux light intensity. On the same condition (3000 lux) 4.0 mg/L 2,4-D also reduced callus growth to minimum growth (1.602 g).

During second subculture, callus growth different trend. In the dark condition, using medium augmented with 4.0 mg/L NAA recorded the highest significant fresh weight (5.379 g) while the other plant growth regulators treatments produced callus fresh weights less than NAA at 4.0 mg/L and there in no significant differences among them. Generally, callus fresh weights in dark conditions in second under 3000 lux increased with increasing concentration of 2,4-D from 2.0 to 4.0 mg/L and takes an opposite direction with NAA.

During third and fourth subcultures under all light intensity conditions (0.0, 1500 and 3000 lux) the most suitable plant growth regulator which induced callus growth to reach the maximum values was MS medium supplemented with 4.0 mg/L NAA plus 2.0 mg/L BA.

Table 3. Means of callus fresh weight of *D. indica* explants as affected by different growth regulator and different light condition after first, second, third and fourth subcultures at 25±1 ºC

Treatments mg/L	Callus fresh weight/g											
	1st Subculture			2nd Subculture			3rd Subculture			4th Subculture		
	Dark	1500 Lux.	3000 Lux.	Dark	1500 Lux.	3000 Lux.	Dark	1500 Lux.	3000 Lux.	Dark	1500 Lux.	3000 Lux.
	Means ± SE	Means ± SE	Means ± SE	Means ± SE	Means ± SE	Means ± SE	Means ± SE	Means ± SE	Means ± SE	Means ± SE	Means ± SE	Means ± SE
2 2,4-D + 2BA	1.281 ± 0.128^{a}	3.085 ± 0.485^{a}	2.186 ± 0.161^{ab}	3.765 ± 0.654^{b}	2.278 ± 0.198^{b}	2.359 ± 0.284^{b}	2.189 ± 0.122^{b}	2.132 ± 0.256^{b}	3.524 ± 0.255^{bc}	2.189 ± 0.122^{c}	1.703 ± 0.0652^{b}	1.849 ± 0.338^{ab}
4 2,4-D + 2BA	1.279 ± 0.153^{a}	1.378 ± 0.277^{b}	1.602 ± 0.154^{b}	3.197 ± 0.545^{b}	3.041 ± 0.293^{ab}	3.775 ± 0.354^{a}	1.462 ± 0.179^{b}	2.069 ± 0.536^{b}	2.802 ± 0.309^{c}	1.831 ± 0.584^{c}	1.973 ± 0.0834^{b}	1.417 ± 0.0620^{b}
2 NAA + 2 BA	1.587 ± 0.256^{a}	1.696 ± 0.152^{b}	2.834 ± 0.185^{a}	3.872 ± 0.188^{b}	3.929 ± 0.239^{a}	3.902 ± 0.404^{a}	2.283 ± 0.306^{b}	3.031 ± 0.550^{ab}	4.177 ± 0.560^{ab}	4.553 ± 0.528^{b}	2.085 ± 0.175^{b}	2.226 ± 0.504^{ab}
4 NAA + 2 BA	1.725 ± 0.438^{a}	1.692 ± 0.351^{b}	1.899 ± 0.127^{ab}	5.379 ± 0.415^{a}	3.419 ± 0.105^{ab}	2.158 ± 0.153^{b}	4.413 ± 0.394^{a}	3.682 ± 0.446^{a}	4.910 ± 0.346^{a}	6.385 ± 0.612^{a}	3.833 ± 0.244^{a}	2.869 ± 0.201^{a}
F ratio	2.975	2.975	2.975	5.882	5.882	5.882	1.881	1.881	1.881	5.052	5.052	5.052
P value	*	*	*	**	**	**	*	*	*	**	**	**

Columns with similar letters are not significantly different according to Holm-Sidak. NS=non-significant, * = significant at P < 0.05, ** = significant at P < 0. 01, (Note: each Colum of means compared according to different treatments showed in first Colum)

3.1.4. Effect of plant growth regulators and light intensity on callus dry weight during four successive subcultures

As presented in table (4), callus dry weight significantly affected by plant growth regulators under the three light intensity examined (0.0, 1500 and 3000 lux). During the first subculture under all light intensities tested there was no significant difference due to effect of plant growth regulator. During the second subculture and under 0.0 lux the highest significant dry weight of callus (0.490 g) was achieved on medium enriched with 4.0 mg/L NAA plus 2.0 mg/L BA, while under 1500 and 3000 lux the greatest dry weights of callus (0.293 and 0.290 g) were recorded with medium supplemented with 2.0 mg/L NAA plus 2.0 mg/L BA, respectively. During the third and fourth subcultures, the highest significant callus dry weights were recorded for medium amended with 4.0 mg/L NAA plus 2.0 mg/L BA under all light intensities tested.

3.2. Secondary metabolites production

3.2.1. Effect of light quality and microwave and UV irradiation on callus growth (fresh and dry weight)

Data presented in table (5) showed the effect of elicitation treatments (including light quality, microwave irradiation and ultraviolet irradiation) on callus growth in terms of fresh and dry weights.

As for fresh weight, data showed that the highest significant fresh weight (5.96 g) was obtained when callus culture was irradiated with ultraviolet at 30 watts for one hour, while the lowest significant fresh weight (2.318 g) was detected when callus culture was incubated under full darkness. By comparing the effect of light quality on callus fresh weight, incubation the callus culture under green light had a positive effect and recorded the highest significant value (5.581 g) followed by red light (4.123 g), while incubation the cultures in full dark reduced the callus growth to the lowest value (2.318 g). The power of microwave irradiation and exposure time also affected the callus growth. When callus cultures irradiated with 200 watts of microwave, the callus fresh weight decreased with increasing exposure time from 10 to 20 seconds. Whereas, when 400 watts is used the callus fresh weight decreased with increasing exposure time.

Ultraviolet radiation significantly affected callus growth in term of fresh weight. The exposure time had a positive effect on callus fresh weight in the two powers used (15 and 30 watt). Concerning to the effect of ultraviolet irradiation, Ehsanpour and Razavizadeh (2005) have been exposed the callus of *Medicago sativa* to UV-C for 10, 30 or 60 min. It was found that irradiation of UV for 150 min increased fresh weight of callus up to 10 %. When callus exposed to UV light for 30 min the callus growth increased to 17.3 %. Applying UV light for 60 min increased fresh weight of callus up to 37.96 %. Meanwhile, Ramani and Jayabaskaran (2008) on *Catharanthus roseus,* demonstrated that when four day old culture is irradiated for 5 min with UV-B, the

fresh weight of the callus decreased gradually. While, when six day old culture is irradiated with UV-B for 5 min, there was no decrease in fresh weight till 24 h.

Table 4. Means of callus dry weight of *D. indica* explants as affected by different growth regulator and different light condition after first, second, third and fourth subcultures at 25±1 ºC

Treatments mg/L	Callus dry weight/g											
	1st Subculture			2nd Subculture			3rd Subculture			4th Subculture		
	Dark	1500 Lux.	3000 Lux.	Dark	1500 Lux.	3000 Lux.	Dark	1500 Lux.	3000 Lux.	Dark	1500 Lux.	3000 Lux.
	Means ± SE	Means ± SE	Means ± SE	Means ± SE	Means ± SE	Means ± SE	Means ± SE	Means ± SE	Means ± SE	Means ± SE	Means ± SE	Means ± SE
2 2,4-D +2 BA	0.187 ± 0.0133^{a}	0.310 ± 0.0488^{a}	0.216 ± 0.0183^{a}	0.343 ± 0.0595^{b}	0.169 ± 0.0148^{b}	0.176 ± 0.0212^{ab}	0.176 ± 0.0182^{b}	0.185 ± 0.0282^{a}	0.354 ± 0.0256^{a}	0.176 ± 0.0182^{c}	0.155 ± 0.00603^{b}	0.168 ± 0.0307^{a}
4 2,4-D + 2BA	0.131 ± 0.0185^{a}	0.138 ± 0.0280^{a}	0.161 ± 0.0156^{a}	0.291 ± 0.0497^{b}	0.226 ± 0.0219^{ab}	0.281 ± 0.0263^{a}	0.119 ± 0.0110^{b}	0.188 ± 0.0490^{a}	0.255 ± 0.0284^{a}	0.170 ± 0.0510^{c}	0.180 ± 0.00745^{b}	0.122 ± 0.0122^{a}
2 NAA + 2BA	0.162 ± 0.0262^{a}	0.170 ± 0.0153^{a}	0.285 ± 0.0188^{a}	0.353 ± 0.0172^{b}	0.293 ± 0.0175^{a}	0.290 ± 0.0302^{a}	0.211 ± 0.0195^{b}	0.245 ± 0.0305^{a}	0.259 ± 0.122^{a}	0.424 ± 0.0711^{ba}	0.190 ± 0.0162^{b}	0.137 ± 0.0195^{a}
4 NAA + 2BA	0.162 ± 0.0454^{a}	0.170 ± 0.0354^{a}	0.187 ± 0.0130^{a}	0.490 ± 0.0379^{a}	0.254 ± 0.00788^{ab}	0.160 ± 0.0113^{b}	0.384 ± 0.0263^{a}	0.334 ± 0.0404^{a}	0.360 ± 0.0453^{a}	0.529 ± 0.0579^{a}	0.346 ± 0.0196^{a}	0.241 ± 0.0209^{a}
F ratio	1.652	1.652	1.652	8.305	8.305	8.305	1.353	1.353	1.353	4.516	4.516	4.516
P value	*	*	*	**	**	**	*	*	*	**	**	**

Columns with similar letters are not significantly different according to Holm-Sidak. NS=non-significant, * = significant at P < 0.05, ** = significant at P < 0. 01, (Note: each Colum of means compared according to different treatments showed in first Colum)

Table 5. Fresh & dry Weight of callus of *D. indica* after treatment by U.V, Microwave and light quality

Elicitor	Treatment	Fresh weight.	Dry weight.
		Means ± SE	Means ± SE
Light quality	3000Lux	3.652±0.682^{c-e}	0.319±0.0539^{bcd}
	Dark	2.318±0.367^{e}	0.199±0.0280^{d}
	Blue light	3.263±0.724^{de}	0.336±0.0392^{abcd}
	Green light	5.581±0.458^{ab}	0.441±0.0387^{ab}
	Red light	4.123±0.586^{a-e}	0.428±0.0612^{abc}
microwave	100 watts for 10 sec	4.057±0.888^{b-e}	0.349±0.0717^{abc}
	100 watts for 20 sec	3.406±0.806^{de}	0.302±0.0720^{cd}
	200 watts for 10 sec	3.672±0.117^{c-e}	0.324±0.0557^{bcd}
	200 watts for 20 sec	4.247±0.854^{a-d}	0.394±0.0350^{abc}
U.V.	15 watts for 2 h	3.863±0.531^{c-e}	0.327±0.0122^{bcd}
	15 watts for 1 h	5.417±0.373^{a-c}	0.436±0.0422^{abc}
	30 watt for 2 h	5.472±0.370^{a-c}	0.358±0.0409^{abc}
	30 watt for 1 h	5.96±0.566^{a}	0.464±0.0116^{a}
P value		**	**
F ratio		2.908	2.367

Columns with similar letters are not significantly different according to Holm-Sidak. NS=non-significant, * = significant at P < 0.05, ** = significant at P < 0. 01, (Note: each Colum of means compared according to different treatments showed in first Colum)

Relating to the effect of microwave treatments, Moeller (1992) mentioned that microwave are electromagnetic waves that cause a clear effect on cells and tissues growth of several plant species. On *Satureja bachtiarica,* Vishki *et al.* (2012) disclosed that a significant decrease in fresh and dry weight was observed in comparison with control. Exposure to electromagnetic field can lead to

cell death as a result of increase in free oxygen radicals and DNA damage (Lakobashvil and Lapidot, 1999).

As a number of researchers mentioned light had significant effect on callus growth and morphogenesis (park *et al*., 2003), studied the Production of camptothecin (CPT) from callus cultures of *Camptotheca acuminata* Decne was affected by light and culture conditions. The highest cell growth was obtained in dark and green light condition, respectively.

3.2.2. Effect of light quality and microwave and UV irradiation on total phenolics, total flavonoids and antioxidant activity

3.2.2.1. Total phenolic compounds

The presented data (Table 6) indicated significant variations in the total phenolic compounds as a result of the tested treatments. The highest phenolic compounds content (231.158 mg/100g f.w) was estimated in callus treated with 200 watts of microwave irradiations for 20 seconds, while the lowest phenolic contents (51.662 and 63.316 mg/100g f.w.) were detected in callus incubated in 3000 lux and full darkness, respectively. It is clear quite from data that all the elicitation treatments including type of light, microwave and U.V. irradiations increased the phenolic content in callus cultures compared with un treated callus. Red light had the most inducing effect for production of phenolic compounds compared to other light conditions. The total phenolic increased with increasing tiome of espouser when 200 watts of microwave irradiation is used, while it was decreased with increasing time when 400 watts is used. The total phenols increased with time of exposure under the two powers of UV irradiation.

3.2.2.2. Total flavonoids

It is clear quite that the total flavonoids significantly affected by different treatments under trail. The highest significant flavonoid content (ranging from 840.833 to 1071.667 mg/100g fresh accumulation was accompanied by an increase in the phenolic compound deposition in cell walls and intercellular space and by deposition of a lignin like material on the surface of callus cultures. The strain characterized by an increased formation of phenolic compounds was more resistant to UV-B radiation as compared to that with lower phenolic productivity. Moreover, Ahmed (2010) reported that callus cultures of *Cleome droserifolia* exposed to microwave at 65 watt for 60 sec or ultraviolet irradiation for 24 hrs gave the highest value of flavonoids compared with control and other treatments.

Lighting conditions are well-known factors affecting primary and secondary metabolism in plant weight) could be detected in callus treated with 200 watts of microwave irradiation for 10 sec, and all ultraviolet irradiation treatments. While the lowest significant one (370.0 mg/100g fresh weight) was estimated for microshoots. The greatest flavonoid value (1071.667 mg/100g fresh weight) represents 2.9-, 2.8-, 1.7-, 2.8- and 1.9- fold higher than those detected for microshoots, fruits, leaves of mother tree, callus grown under darkness and callus grown under 3000 lux, respectively.

3.2.2.3. Antioxidant activity

From mentioned data in table (6), the highest significant antioxidant activity (98.98 %) was obtained from extract of fruits, whereas the lowest significant one (92.345 %) was recorded for extract of callus exposed to Ultraviolet at 15 watt for one hour. There was no significant difference among all elicitation treatments in antioxidant activity except the treatment of UV with 15 watt for one hour which was significantly less than other treatments.

Relating the effect of ultraviolet and microwave irradiation treatments, flavonoids are the classical UV-B-regulated compounds in plants, and their biosynthesis and its regulation have been very thoroughly explored (Shirley, 1996). Phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS) are enzymes in the flavonoid biosynthetic pathway which have long been known to be UV-B inducible, but at least in parsley *(Petroselinum crispum*) genes for several other enzymes of importance for early steps in flavonoid biosynthesis, such as glucose 6-phosphate dehydrogenase, 3-deoxy-D-arabino heptulosonate 7-phosphate synthase, and S-adenosyl-homocysteine hydrolase, are also under UV control (Logemann *et al.*, 2000). Zagoskina *at al.,* (2003) investigated the effect of ultraviolet (UV-B) radiation on the accumulation and tissue localization of phenolic compounds in two strains of callus cultures of tea plant (*Camellia sinensis* L.). UV-B radiation promoted the accumulation of soluble and polymeric forms of phenolic compounds, such as lignin. This cell cultures (Ramakrishna and Ravishankar, 2011).Light is an important factor affecting growth, organogenesis and the formation of plant products including both primary andsecondary metabolites. The stimulatory effect of light on the formation of compounds, including flavonoid and anthocyanins has been shown in plants (Krewzaler and Hahlbrock, 1976 ; Zhong *etal.*,1991). Certain wavelengths of lights have been reported to increase antioxidative actions against abiotic challenges. In broad bean leaves, for instance, significantly increase of catalase activity under red light contributed to scavenging of hydrogen peroxide generated by *Botrytis cinerea* infection (Islam *et al*., 2011).

Table 6. Total phenols, flavonodes and total antioxidant in all callus treatment, new plant and mother plant

Treatments		Total phenols % mg/100g fresh weight	Total flavonoids % mg/100g fresh weight	Antioxidant Inhibition %
		Means ± SE	Means ± SE	Means ± SE
Light quality	3000Lux	51.662±5.969 ^{f}	378.333 ± 9.280 ^{de}	94.474 ± 0.497^{b}
	Dark	63.316±8.687 ^{f}	551.667 ± 43.429 $^{c-e}$	94.197 ± 0.107^{bc}
	Blue light	73.318±6.955 ^{ef}	568.333 ± 52.626 $^{c-e}$	95.026 ± 0.420^{b}
	Green light	116.390±9.321 ^{de}	657.667 ± 43.594 ^{bc}	94.797 ± 0.584^{b}
	Red light	200.184±17.582 $^{a-c}$	562.333 ± 50.054 $^{c-e}$	95.653 ± 0.162^{b}
microwave	100 watts for 10 sec	181.31±18.689 $^{a-c}$	918.333 ± 34.591 ^{a}	94.730 ± 0.302^{b}
	100 watts for 20 sec	231.158±35.389 ^{a}	412.500 ± 17.017 $^{c-e}$	95.503 ± 0.460^{b}
	200 watts for 10 sec	196.566±9.402 $^{a-c}$	525.000 ± 39.686 $^{c-e}$	95.390 ± 0.308^{b}
	200 watts for 20 sec	168.128±37.829 $^{b—d}$	568.333 ± 37.924 $^{c-e}$	95.188 ± 0.440^{b}
U.V.	15 watts for 2 h	154.761±6.050 $^{b-d}$	1001.667 ± 59.325 ^{a}	95.424 ± 0.228^{b}
	15 watts for 1 h	147.686±9.409 ^{cd}	990.833 ±70.657 ^{a}	92.354 ± 2.413^{c}
	30 watts for 2 h	197.396±16.846 $^{a-c}$	840.833 ± 72.260 ^{ab}	94.681 ± 0.111^{b}
	30 watts for 1 h	153.332±7.574 $^{b-d}$	1071.667 ± 55.252 ^{a}	94.298 ± 0.402^{bc}
Fruit from mother plant		185.712±20.292 $^{a-c}$	380.833 ± 44.119 ^{de}	98.980 ±0 .058^{a}
Leaves from mother plant		211.707±3.325 ^{ab}	619.167 ± 44.119 $^{b-d}$	96.048 ± 0.120^{b}
In vitro shoot		201.613±50.828 $^{a-c}$	370.00 ± 31.225 ^{e}	95.384 ± 0.283^{b}
F ratio		1. 6.819	25.369	3.747
P value		***	**	***

Columns with similar letters are not significantly different according to Holm-Sidak. NS=non-significant, * = significant at P < 0.05, ** = significant at P < 0. 01, (Note: each Colum of means compared according to different treatments showed in first Colum)

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