INCREASING (PHENOLYIC AND FLAVONIODS COMPOUNDES OF CICER ARIETINUM L. FROM EMBRYO EXPLANT USING TITANUM DIOXIDE NANOPARTICLE IN VITRO

*Hashim K. Mohammed AL-oubaidi and Nada M. Kasid*

Biology Department, College of Science, Al- Mustansiriyah University, Baghdad Iraq.

ABSTRACT

The present study is aimed to increase the secondary metabolite production (phenolic and flavonoids compounds) in Cicer arietinum L. in vitro. Secondary metabolites was estimated qualitatively and quantitatively using high performance liquid chromatography HPLC compared with the mother plant. In order to increase the production of secondary metabolites, titanium dioxide nanoparticle used at concentrations (con, 0.5, 1.5, 3, 4.5, 6) mg/l. The results revealed that the concentrations (4.5, 6) mg/l TiO2 nanoparticle caused highly significant production in most of the secondary metabolites from callus embryo of Cicer arietinum L.

KEYWORDS: callus induction, plant tissue cultures, Cicer arietinum, secondary metabolites, titanium dioxide nanoparticle.

INTRODUCTION

Cicer arietinum L. Chickpea is the third most important crop production next to dry bean its legume crops and it has been important crops for human nutrition grown in over 50 countries around the world (Rao et al., 2002).

Chickpea enriched with carbohydrates and proteins also its cholesterol free and a good source of dietary fibers, various importance of chickpea consumption including cardiovascular disease (CVD), coronary heart disease (CHD) and cholesterol control, increased consumption of soluble fiber from foods results and has an inverse correlation with coronary heart disease mortality treatment of bronchitis, leprosy, skin diseases, blood disorders and biliousness (Belino et al., 2015).
Flavonoids and isoflavonoids are one of the most popular groups of secondary metabolites found in plants. Many legume seeds have been reported to be rich sources of these secondary metabolites (Heiras-Palazuelos et al., 2013).

Plant cell cultures are an preference source to whole plant for the production of high-value secondary metabolites which are usually acting lower role than primary metabolites in the plant (Karuppusamy, 2009).

Tissue culture techniques are utilized to enhance yield of secondary metabolites by trigger stress response like using elicitors, precursors and biotransformation, variation in environment conditions, change in medium constituents (Radman et al., 2003).

Plant tissue cultures are exposed to stresses and stress consociation that they may not have encountered in nature in their long evolution. It is exceptional reflection on the plasticity of the plant genome that it could been converted and respond to novel in vitro stresses (American journal of plant physiology, 2011).

Nano science is one of the most superior research and development in modern science, nanotechnology is now frequently used throughout the pharmaceutical industry, medicine, and tissue engineering, the use of nanoparticle (NP) materials offers many advantages due to their unique size and physical properties (Faraji et al., 2010).

**AIMS OF THE PRESENT WORK**

Recent study have reflected increasing interest in using chemical elicitors in plant tissue cultures techniques for increasing secondary metabolites. This has intended to use the nano chemical compound TiO2 on explant embryo in chickpea plant aiming at increasing the secondary metabolites.

Results of secondary metabolites produced from callus treatment with elicitors are compared with the secondary metabolites obtained from the mother plant which was not subjected to elicitation.

**MATERIALS AND METHODDDS**

**Plant material and sterilization**

The explant of C.arietinum L. from seeds were collected from the local market in Baghdad, Iraq on 1/9/2014. Then rinsed with running tab water for 3 hr. Transferred to laminar air flow
cabinet and submerged in (99)% ethanol for one minute, washed and sterilized DDH2O, then rinsed with sodium hypochlorite at the concentration (2.0)% for (10) min. Then washed with DDH2O three times for five minutes and planted in vials of Agriculture (universal tubes) (pierik, 1987).

MEDIUM FOR CALLUS INDUCTION
The sterilized explants (embryos) were dissected and cultured on culture vessels containing Murashige and Skoog, (1962) (MS) medium with different concentrations of the auxin 2,4-dichlorofenoxy acetic acid (0, 1, 2, 3 or 4) mg/l, Table (1), then distributed into 10 replicates for each concentration which incubated at dark period at a temperature 25 ± 1 °C f. The percentage of callus formation was recorded after 30 days (Ramawat, 2008).

Fresh and dry weight of callus measurements
The fresh weight of callus induced from explant embryos was measured by using a sensitive balance, then the callus was dried using an electric oven at 70 °C for 24 hrs, then measured by a sensitive balance (Hopkins and Huner, 2004).

2 mg/l of 2,4-dichlorophenoxy acetic acid it was the best concentration for callus induction, titanium dioxide nanoparticle was used with different concentrations (con,0.5,1.5,3,4.5,6) mg/l.

Extraction and analysis of phenolic and flavonoids compounds from C.arietinum L.
A quantity of 1 g (dry weight) was weighed and powdered in a pestle-mortar followed by suspending fine crushed sample in 5 ml of ethanol: water (80:20 v/v). These samples were collected in screw–capped specimen tubes (10 ml) and the suspension was subjected to ultrasonication by Branson Sonifiers 450 (Branson Ultrasonic Crops, Danury CT, USA) for 15 min at 4 C° followed by centrifugation at 7,500 g for 15 min. The clear greenish supernatant was subjected to charcoal treatment to remove pigments from each sample and was then transferred to glass tubes. The residue was re-extracted twice and the supernatant was pooled prior to evaporation under vacuum (Buchi Type Rotavapor). Dried samples were re-suspended in 1.0 ml HPLC grade methanol by vortexing and filtered through membrane filter (pore size 0.45 Mm, Millipore) and stored at 4 C° for for further analysis by HPLC.

Samples analyses were performed with the HPLC system equipped with two shimadzu LC-10 AT equipped with binary delivery pump model LC-10A shimadzu, the eluted peaks were
monitored by UV-Vis 10 A- SPD detector shimazu SPD-10AVP and C-R6A chromatopack data processors, the standard phenols were obtained from Sigma Chemical Co (Tiwari et al., 2008). all the solvents used in this investigation were of HPLC grade. And Rheodyyne model 7725 in factor with a loop size of 20 MI, and integrator and CLASAA-VP software for data recording and processing (Shimadzu). Reverse phase chromatographic analysis was carried out in isocratic condition using C-18 reverse phase HPLC colum (150 X 4.6 mm i.d., particle size 5 Mm) Luna 5 M C-18 (Mauricio et al., 2007), Phenomenex, Torrance USA at 25 C, flow rate 1.0 ml/ min, injection volume 20 MI and detection at 290 nm. Sample was injected in the sample loop and the means of the peak areas of individual compounds were taken for quantification, the compound present in the sample were identified by comparing retention time (Rt.) of the standards .Amounts of individual compound were calculated by comparing peak areas of reference compound with those in the sample run under similar elution conditions.

The concentration of samples was measured by comparing the area of sample with the area of the standard multiply by concentration of standard which was 25 mg/l under the same conditions by using the following formula: (Budhiraja, 2004).

\[
\text{Concentration of sample (µg/ml)} = \frac{\text{Area of sample}}{\text{Area of standard}} \times \text{concentration of standard} \times \text{dilution factor}
\]

**Experimental design and statistical analysis**

A completely randomized design (CRD) was used. Least significant differences (LSD) were calculated. The difference between the test average compared according to the least significant differences (LSD) at the probability of 5% (Salkind and Ramsey, 2007).

**RESULTS**

**Effect of Titanium dioxide (TiO2) nanoparticle on fresh and dry weight of embryo callus on MS media after four weeks of culture in dark condition.**

From table 2 the fresh weight show high significant differences with treats (4.5,6) mg/l TiO2 nanoparticle up to the concentration (762,769) µg/ml respectively and had no significant between them and the treats (0.5,1.5) mg/l.

Dry weight show high significant differences recorded with treat 4.5 mg/l TiO2 nanoparticle up to the concentration 94.7 µg/ml and had no significant difference from 6 mg/l TiO2.
nanoparticle, the lowest concentration observed with control treatment reported 46.9 mg/l TiO2 nanoparticle and no difference from the treats (0.5, 1.5, 3, 6).

Effect of different concentrations of TiO2 nanoparticle (mg/l) on secondary metabolites production

From table 3 adding TiO2 nanoparticle resulting in significantly highly differences gallic acid reached up to (100.2,103.0) µg/ml in concentrations (4.5,6) mg/l TiO2 nanoparticle respectively and no difference between these treatment and the treats (cont,0.5,1.5,3) mg/l nanoparticle.

Chlorogenic acid show high significant difference with treat (3,4.5) mg/l TiO2 nanoparticle recorded with ( 118.8,130.2) µg/ml respectively and had no significant difference than treat (1.5,6) mg/l TiO2 nanoparticle ,the lowest concentration recorded in mother plant reached to 0.3 µg/ml.

O-coumaric acid gave high significant difference with treat 6 mg/l TiO2 nanoparticle reached to 199.8 µg/ml, while mother plant gave the lowest concentration reached up to 2 µg/ml.

Tannic acid gave high significant difference with treat 6 mg/l TiO2 nanoparticle recorded 561 µg/ml and the lowest concentration recorded with mother plant 2 µg/ml.

Cinnamic acid show high significant difference in the presence of (4.5,6)mg/l TiO2 nanoparticle reached up to (112.0,82.9) µg/ml respectively and had no significant differences than treats (0.5,3) mg/l, the lowest concentration of cinnamic acid recorded with mother plant the value reached to 0.8 µg/ml.

Table (1): The medium of callus induction components

<table>
<thead>
<tr>
<th>NO</th>
<th>Components</th>
<th>Concentration (mg/l.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MS</td>
<td>Full strength</td>
</tr>
<tr>
<td>2</td>
<td>Sucrose</td>
<td>30000</td>
</tr>
<tr>
<td>3</td>
<td>L- Asparagine</td>
<td>150</td>
</tr>
<tr>
<td>4</td>
<td>Glycine</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>Kinetin</td>
<td>0.2</td>
</tr>
<tr>
<td>6</td>
<td>2,4-D</td>
<td>0,1,2,3,4</td>
</tr>
<tr>
<td>7</td>
<td>Agar-Agar</td>
<td>8000</td>
</tr>
<tr>
<td>8</td>
<td>Nicotinic acid</td>
<td>0.5</td>
</tr>
<tr>
<td>9</td>
<td>Myoinositol</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>Thiamin</td>
<td>0.2</td>
</tr>
<tr>
<td>11</td>
<td>Pyridoxine</td>
<td>0.5</td>
</tr>
</tbody>
</table>
Table (2): Effect of different concentration of TiO2 nano particle on fresh and dry weight of callus embryo on MS media after four weeks of culture in dark condition.

<table>
<thead>
<tr>
<th>Characters</th>
<th>Concentrations of TiO2 (mg/l)</th>
<th>LSD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cont</td>
<td>0.5</td>
</tr>
<tr>
<td>Fresh weight</td>
<td>587</td>
<td>664</td>
</tr>
<tr>
<td>Dry weight</td>
<td>46.9</td>
<td>59.7</td>
</tr>
</tbody>
</table>

Table (3): Effect of different concentrations of TiO2 nanoparticle (mg/l) on secondary metabolites of embryo callus of Cicer arietinum.

<table>
<thead>
<tr>
<th>Secondary Metabolites</th>
<th>Concentration of TiO2 NPs (mg/l)</th>
<th>mother plant</th>
<th>L.S.D 0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cont.</td>
<td>0.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>55.7</td>
<td>50.2</td>
<td>59.4</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>51.7</td>
<td>54.5</td>
<td>99.5</td>
</tr>
<tr>
<td>O-coumaric acid</td>
<td>81.8</td>
<td>109.5</td>
<td>72.8</td>
</tr>
<tr>
<td>Tannic acid</td>
<td>143</td>
<td>152</td>
<td>199</td>
</tr>
<tr>
<td>Cinnamic acid</td>
<td>44.9</td>
<td>49.7</td>
<td>43.2</td>
</tr>
</tbody>
</table>

Table (4) Shows equipments and apparatus were used throughout the experimental work:

<table>
<thead>
<tr>
<th>No.</th>
<th>Device</th>
<th>Company and origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Autoclave</td>
<td>Labtech (Korea)</td>
</tr>
<tr>
<td>2</td>
<td>Distillator water</td>
<td>Germany (GFL)</td>
</tr>
<tr>
<td>3</td>
<td>Incubator</td>
<td>RGX-25 (Korea)</td>
</tr>
<tr>
<td>4</td>
<td>Laminar Air flow cabinet</td>
<td>LN090 (Denmark)</td>
</tr>
<tr>
<td>5</td>
<td>Oven</td>
<td>Fisher (USA)</td>
</tr>
<tr>
<td>6</td>
<td>pH meter</td>
<td>(USA) Oakino</td>
</tr>
<tr>
<td>7</td>
<td>Sensitive balance</td>
<td>Kern (USA)</td>
</tr>
<tr>
<td>8</td>
<td>Refrigerator</td>
<td>Concord (Lebanon)</td>
</tr>
<tr>
<td>9</td>
<td>Hot plate with magnetic stirrer</td>
<td>Ikamage (Germany)</td>
</tr>
<tr>
<td>10</td>
<td>High performance liquid chromatography (HPLC)</td>
<td>Royaltion (Japan)</td>
</tr>
<tr>
<td>11</td>
<td>Soxhlet</td>
<td>Sigma Aldrich (U.S.A)</td>
</tr>
<tr>
<td>12</td>
<td>High performance liquid chromatograph</td>
<td>Shimadzu Coporation (Japan)</td>
</tr>
<tr>
<td>13</td>
<td>Titanium oxide Nanoparticle</td>
<td>Nanoshel (USA)</td>
</tr>
</tbody>
</table>
HPLC curves of C.arietinum using TiO2 NPs as elicitor.

**FIG. (2):** HPLC for 0.5 mg/l of TiO2 NPs. 

**FIG. (1):** HPLC for control treatment of TiO2 NPs.

**FIG. (4):** HPLC for 3 mg/l of TiO2 NPs. 

**FIG. (3):** HPLC for 1.5 mg/l of TiO2 NPs.

**FIG. (6):** HPLC for 6 mg/l of TiO2 NPs. 

**FIG. (5):** HPLC for 4.5 mg/l of TiO2 NPs.

Fig 6: effect of TiO2 nanoparticle on Cicer arietinum callus

**DISCUSSION**

The results showed that there were high significantly differences with fresh and dry weight by adding TiO2 nanoparticles, Nano-Tio2 improved light absorbance and promoted the
activity of Rubisco activase thus accelerated Spinach growth. Nano-Tio2 improved plant
growth by enhanced nitrogen metabolism that promotes the absorption of nitrate in spinach
and accelerating conversion of inorganic nitrogen into organic nitrogen, thereby increasing
the fresh weights and dry weights (Nair et al., 2010). there is no doubt that TiO2
nanoparticles could dramatically increase callogenesis, the size of calli Viana et al., (2010).
The effect of treatments (cont, 0.5,1.5,3,4.5,6) mg/l NPs on increasing secondary metabolites
from callus embryo by HPLC technique was very high significant comparing with the mother
plant TiO2 nanoparticles increase aloin content in aleo vera plant due to its effect on gene
expression, Aloe Vera L has a different secondary metabolites and the most important of
them is Aloin Mona et al., (2014).

CONCLUSION
Adding titanium dioxide nanoparticles for callus medium caused highly significant increase
in all the studied secondary metabolites (phenolic and flavonoids compounds) of Cicer
arietinum L. with concentrations (4.5,6) mg/l.

RECOMMENDATION
Utilize another elicitor to increase (phenolic and flavonoid compounds) of C.arietinum L.

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