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IN VITRO INDUCED CALLUS OF *THEVETIA NERIIFOLIA* JUSS.

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Abstract

This study was carried out to investigate to acquisition of a highest fresh and dry weight of the callus tissue of the *Thevetia neriifolia* Juss *in vitro*. To obtain this target, the explant (seeds) were cultivated to induction the callus on the, Murashige and Skooge medium (MS), with the effect of two plant growth regulators, Benzyl Adenine (BA) in concentration 0.5, 1, and 1.5 mg/L and Dichlorophenoxy acetic acid (2,4-D) in concentration (0.5, 1 and 1.5 mg/L), that added to MS cultured medium. The experiment showed that the maximum fresh weight (2.39 g) was obtained by combination (1mg /LBA + 1mg /L 2,4-D) and the largest dry weight (0.226 g) by the combination (1mg /L BA + 1mg /L 2,4-D. The current study has shown the superiority of the combination (1mg/L BA + 1mg/L 2,4-D) in the induction of highest fresh and dry weight of the callus tissue of the *Thevetia neriifolia* Juss *in vitro*, as in statistical tables (1 and 2).

Key words **:** *Thevetia neriifolia* Juss, Seeds, Callus, Benzyl Adenine**,** 2,4-D.

Introduction

The plant *Thevetia neriifolia* Juss is called yellow oleander, due to the yellow color of its flowers, is belonging to the Apocynaceae family, which has medicinal and ornamental importance, and distributed worldwide in tropical and subtropical regions (Joshi, 2000 and Zavaleta, 2012). *Thevetia neriifolia* Juss is named to commemorate the explorer Andre Thevet (1502-1590), who discovered it as genuine plant during his trip to Brazil and transported it to Europe (Kishan *et al.,* 2012). Most parts of plant are used in folk treatments, because they contain natural products that facilitate their pharmaceutical uses in the treatment of heart failure, microbial inflammatory, anti-cancer and rheumatism, etc. (Nesy *et al.,* 2015 and Save *et al.,* 2015). This plant has been introduced into Iraq in the last 15 years as ornamental plant in nurseries and gardens without knowledge of its medicinal importance (Salim *et al.,* 2016). In recent years, there has been a clear interest in the cultivation of medicinal plants and their investment in the acquisition of therapeutic or pharmacological substances rather than chemically processed substances, experiments have shown that the active, laboratory-manufactured material does not perform the physiological effect of the same active substance extracted from medicinal plants (Dornenburg and Knorr, 1995). The technology of plant tissue culture allows to produce secondary substances with high medical value and their production is fast without relying on the season in which these plants grow without the need to allocate large areas for the purpose of cultivation and use these areas to grow crops economically important (Kumar and Sopory, 2010). Due to the importance of *Thevetia neriifolia* Juss plant, which contains important secondary compounds involved in the pharmaceutical industry compared to the demand and the increasing need for these compounds, it is necessary to use plant tissue culture technology and the use of hormones in the nutrient medium MS to increase the production of effective secondary compounds resulting from the induction of callus tissue where Its textile plantations are continuous throughout the year in the production of pharmaceutical compounds (Ghorbanpour *et al.,* 2013).

Materials and Methods

Preparation of Solutions and Medium

1) Growth regulators solutions:-

50 mg of Dichlorophenoxy acetic acid (2,4-D) in 1 ml of absolute ethyl alcohol to ensure complete solubility and complete the volume to 50 ml with distilled water to have a concentration solution 1mg /L. Benzyl Adenine (BA) was prepared by dissolving 50 mg of BA In 1 ml hydrochloric acid (N 1) to ensure solubility and complete the volume to 50 ml with distilled water to make a concentration solution 1 mg/L.

2) Preparing the nutrient medium of Thevetia seeds :

Use the Murashige and Skooge medium (MS), with additive different combination of growth regulators to induce Thevetia callus from seeds (Murashige and Skooge, 1962). Dissolved (4.41 g) of prepared MS medium per liter of distilled water and sugar additive at 30 g / L and growth regulators (2,4-D) at (0.5, 1, and 1.5) mg / L with BA concentration at (0.5, 1, and 1.5) mg / L and then put on the (hot plate magnetic stirrer device) for 5 minutes to ensure complete solubility of sugar and nutrient medium. pH meter was adjusted to (5.8 degree) with 1 N (NaOH) or hydrochloric acid and then added 7g/L (agar-agar) and placing the medium on the (hot plate magnetic stirrer) until boiling, to dissolve the agar. When the solution became transparent, pure 10 ml of the medium for each of the sterilized culture tubes and then put it in the autoclave device at 121 ºC , under 15 inch Ib / sq for 15 minutes and then get it out from the device and they becomes ready for cultivation.

3) Seed collection and sterilization:

Thevetia neriifolia Juss seeds were brought from the (seeds bank) in AL-Diwaniya city. Initially the thick coat that surrounding the seeds were removed (Fig. 1), then place a suitable quantity of seed in the flask and wash with distilled water three times to get rid of the dust and impurities attached, and transfer to the air laminar flow cabinet and sterilize it with 15% sodium hypochlorite with stirring for 7 minutes, then rinse with distilled water for one minute for three times and then sterilize with 70% ethyl alcohol and shake for 30 seconds and then rinse with distilled water for one minute, one for three times to remove ethyl alcohol, and

then put the seed in a petri dish container on a sterile filter paper in order to remove the water (Awika and Rooney, 2004).

4) Callus Induction

Plant tissue culture technique was used for the induction of callus from Thevetia seeds, were the seeds grown on the nutrient medium by using different combinations of growth regulators (2,4-D) and (BA).

5) Seeds Cultivation

After MS medium preparation and seeds sterilization, one seed was taken and cultured in each tube of container on 10 ml of MS medium (Fig. 2) with different concentrations of plant growth regulators (2,4-D) with concentration of (0.5,1,and 1.5) mg/L and (BA) concentration (0.5 , 1, and 1.5) mg/ L and at a rate of 10 replicate for each combination of 2,4-D and BA for the purpose of induction the callus and then incubated under (1000 Lux) pear 16 hours lighting and 8 hours darkness daily , at a temperature of 25±2 ºC

6) Determinate the Fresh Weight

The callus appeared on the seeds after 28 days of cultivate the seeds on the MS medium, after 45 days the callus was completed. The fresh weight of the callus was calculated for all the concentrations of 2,4-D and BA used in the experiment, the fresh weight was measured by using the sensitive electrical balance after removing the residue of the medium attached to the callus by washing with distilled water.

7) Determination of dry weight:

Place fresh callus in Petri dishes, each dish containing 6 pieces of callus tissue for both combinations of 2,4-D and BA, and the dry weights of the callus were determined after drying in the oven at a temperature of 40 ºC for 24 hours.

8) Statistical analysis:

The results were analyzed according to the complete random design (C.R.D) through a laboratory experiment and the extraction of the values of the least significant difference of the workers and the interaction between them for the weights of dry and dry according to the selection of the least significant difference (L.S.D) at the level of probability 0.05 (AL-Rawi and Khalaf Allah, 2000). The present study was conducted in the plant tissue culture laboratory of the Department of Biotechnology / Faculty of Biotechnology – AL-Qasim Green University, for the period 1/9/2018 - 1/3/2019.

Fig. 1 : Seeds of *Thevetia neriifolia* Juss that surrounding by thick coat .

Fig. 2 : Seeds cultivation on the medium (MS)

Results and Discussion

1) Effect of interference between BA and 2,4-D concentrations in fresh and dry weight to induce callus of *Thevetia* :

The effect in fresh weight

As shown in table 1, and (Fig. 3), the concentrations of BA and 2,4-D have a significant effect on the induction of seeds callus of *Thevetia*, when the interaction between growth regulators with concentrations of 1+1 mg/L, BA and 2,4- D is achieved the highest fresh weight of callus is (2.39 g), followed by the combination 1 BA mg/L and 1.5mg /L 2,4-D, as it was the weight of callus (1.92 g) while the fresh weight of the callus was decreased at (1.5 BA + 0.5 2,4-D) mg/L and reached (0.73 g) significantly different from all 2,4-D and BA treatment.

Table 1 : Effect of interference between BA and 2,4-D concentrations in fresh weight of *Thevetia* callus

Fresh weight (g)	Concentrations mg/L	
	BA	2,4-D
1.26	0.5	0.5
1.76	1	
0.73	1.5	
1.64	0.5	1
2.39	1	
1.07	1.5	
1.47	0.5	1.5
1.92	1	
0.87	1.5	
0.252	L.S.D. (0.05)	

The effect in dry weight

In the same direction, dry weights showed different values in table (2), the highest dry weight of the callus was (0.226 g) at the interaction between BA and 2,4-D at a concentration of (1 + 1) mg / L, which differ significantly from the other treatments, the lowest dry weight of the mixture between the concentrations at 1.5 mg/L BA and 2,4-D 0.5 mg / L was (0.07 g) and differed significantly from all the treatments BA and 2,4-D, except for the interference treatment (1.5 BA + 1.5 2,4-D) mg/L.

Table 2 : Effect of interference between BA and 2,4-D concentrations in dry weight of *Thevetia* callus.

Dry weight (g)	Concentrations mg/L	
	BA	2,4-D
0.119	0.5	0.5
0.172	1	
0.07	1.5	
0.159	0.5	1
0.226	1	
0.09	1.5	
0.138	0.5	1.5
0.185	1	
0.08	1.5	
0.0329	L.S.D. (0.05)	

The ratio between the growth regulators of Auxins–cytokinins that added to the nutrient medium MS has their role in obtaining the best fresh and dry weight of the *Thevetia* callus in the nutrient medium due to the physiological balance between Auxins and cytokinins, on the other hand increase in the concentration of Auxins and cytokinins at the expense of the other, that affects on the induction of the callus and reduces its growth (Mineo, 1990). (Centeno *et al.,* 1996) noted the need for a hormonal balance between Auxins and cytokinins in the induction and growth of the callus, which explains the increase in the fresh and dry weight of the induced calls from *Thevetia* seeds. (Carew and Krueger, 1977) reported that the combinations BA and 2,4-D are necessary for the induction and growth the Callus in the *Catharanthus roseus* plant, the addition of both growth regulators to the nutrient medium is important and necessary to induction the callus, where Cytokinin acts as a key to initiating cellular division. The difference in the response of plant parts grown due to the added ratios of Auxin to cytokinin may be due to the difference in the content of these internal parts of the hormones containing it, this, affects the optimal concentration of the callus induction of Auxin and cytokinin, or both, when added to the nutrient medium (Goodwin, 1985). (Schmulling and Schafer, 1997) reported that gene expression there can be a noticeable change in the response of cytokinins, where the cytokinins genes are often regulated by the addition of hormones such as Auxins, the production of these genes plays an important role in the variety of biologic processes such as Cell division and growth of green plastids. The reason for the increase in fresh weight is due to the presence of BA in the medium of growth culture due to the role of the cytokinins in the division of cells and the produce of the important proteins and increase the absorption of water and the growth of callus (Mohamed, 1990). As for Auxin it has a direct role in cell expansion by increasing certain enzymatic activities that are responsible for cell wall flexibility and increased permeability (Mohamed, 1990). These results are consistent with (Rezaeian, 2011) who reported that the highest growth of the *Thevetia* callus was determined by adding concentration 1mg/L of 2,4-D within 45 days Compared to other treatments. As well as (Jaime, 2014) who reported to the possibility of induction and increasing the callus amount of *Dianthus caryophyllus* L. plant when using combinations (1 mg/L BA + 1mg /L 2,4-D) on the nutrient medium MS.

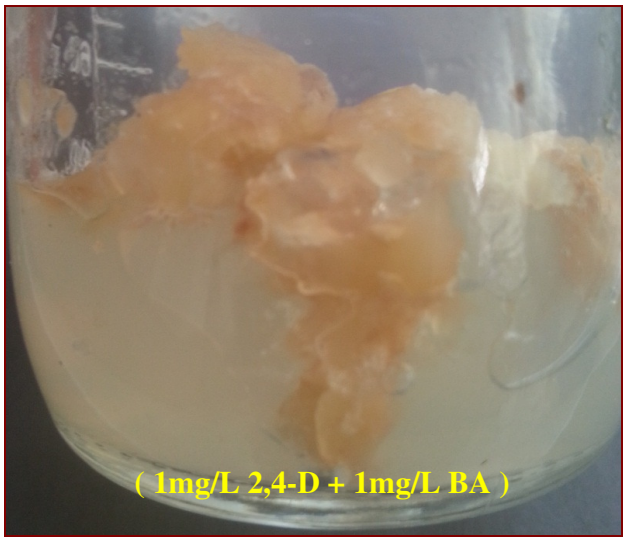

Fig. 3 : Interference between concentration of (2,4-D and BA) for Callus of *Thevetia* plant on the MS medium

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