

Research Article

Effect of abiotic elicitor methyl jasmonate on production of rutin in callus cultures of *Abutilon hirtum* L.

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Article Info

<https://doi.org/10.31018/jans.v14i4.4006>

Received: September 7, 2022

Revised: December 4, 2022

Accepted: December 9, 2022

How to Cite

Nadir, D. S. and Alwan, A. M. (2022). Effect of abiotic elicitor methyl jasmonate on production of rutin in callus cultures of *Abutilon hirtum* L.. *Journal of Applied and Natural Science*, 14(4), 1493 - 1499. <https://doi.org/10.31018/jans.v14i4.4006>

Abstract

A significant therapeutic plant from the Malvaceae family is *Abutilon hirtum* (L.). It is frequently used in traditional medicine to treat a variety of illnesses. Rutin is a flavonoid molecule with commercial value that has anticancer, nutritional, and anti-ageing properties. This research project was carried out to evaluate the effect of abiotic elicitor Methyl Jasmonate (MeJA) at concentrations 0.0, 0.01, 0.02, 0.03 mmol.L⁻¹ on accumulation of rutin by callus cultures of *Abutilon hirtum* L. after 30 days of growth on Murashige and Skoog (MS) medium to which 2000 µg.L⁻¹ 2,4-Dichlorophenoxy acetic acid (2,4-D) + 500 µg.L⁻¹ Kinetin (Kin) were added. The results showed that the best callus stems cultivated on MS growth medium fortified with 2000 µg.L⁻¹ 2,4-D + 500 µg.L⁻¹ Kin, recorded the highest fresh weight of 2.887 g after four weeks. This induced callus was characterized by its friable texture. The data of rutin detection in cultures of callus obtained from stems explant of *A. hirtum* L., using High-Performance Liquid Chromatography (HPLC) indicated the presence of rutin in these cultures by comparison with the rutin standard samples containing a precisely known concentration of a substance for use in quantitative analysis.. Treatment with 0.03 mmol.L⁻¹ of Methyl Jasmonate gave the best rutin amount of 5.606 mg.g⁻¹ was much higher than the control treatment of 1.569 mg.g⁻¹. These data clearly indicated that callus cultures are a potential continuous and constant source of rutin, as a secondary metabolite, and as an alternative to field plants.

Keywords: *Abutilon hirtum* L., Abiotic elicitors, Callus cultures, MeJA, Rutin.

INTRODUCTION

Abutilon hirtum L. is a perennial herb belonging to Malvaceae plant family (Brenzel, 1995). It grows naturally in tropical and subtropical regions of America, Africa and Australia (Nithya *et al.*, 2016). Hamed *et al.* (2017) reported that leaves of *A. hirtum* L. have cytotoxic activity. However, its seeds are used as an expectorant, helpful for piles, and as a laxative in chronic cystitis, gonorrhoea and gleet. Leaves water extract is used to treat wounds, bladder inflammations, diabetes mellitus and ulcers (Gomaa *et al.*, 2018), because water extract contains secondary metabolites that give it pharmaceutical and medical importance. These secondary metabolites include phenols, tannins, flavonoids (Hamed *et al.*, 2015 a), rutin and essential oils (Ali *et al.*, 2009, Kassem, 2007). It also contains sterols that have antibacterial, antioxidant and anticancer properties (Hamed *et al.*, 2015 b, Hassanein *et al.*, 2011).

Plant cell, tissue and organ culture are considered a typical fast source for producing a huge amounts of medical and pharmaceutical compounds *in vitro*, being affected by the environmental constraints and are not interfered by other compounds, as what would occur when they are isolated from the whole plant (Ramawat and Merillon, 2019). Secondary metabolic compounds are recently considered important sources of pharmaceutical, cosmetic and other industrial products (Jan *et al.*, 2021). Biosynthesis and accumulation of secondary metabolites occur in plant cell and tissue cultures when exposed to different types of stresses like elicitors and signal molecules (Nadir *et al.*, 2021 a). However, Murthy *et al.* (2014) showed that the use of MeJA as an abiotic elicitor for *in vitro* cell culture, as a new technique for the production of secondary metabolites through the activation of the enzyme, the induction of the expression of genes that are related to defense and excessive production of secondary metabolites.

Secondary metabolites include a class of organic phenolic compounds called rutin, and can be precisely classified as a flavonol composed of quercetin in addition to a molecule of disaccharides formed from glucose and rhamnose (Meinhart *et al.*, 2020). Caglayan *et al.*, (2019), mentioned that rutin is good food and medicine substituent. Rutin pharmaceutical activities are related to many properties such as anti-inflammatory and antioxidant (Abarikwu, *et al.*, 2017; Gorbani, 2017). Chen *et al.* (2014) demonstrated that rutin is a protection agent against pulmonary injuries and anti-chronic inflammation of colon, besides lowering the oxidative stress levels and brain inflammation. Rutin is also used for the treatment of kidney vascular hypertension (Kaur and Muthuraman, 2016). Chen *et al.* (2021) reported that rutin was used for the treatment of dementia.

The present study aimed to extract, identify and estimate rutin from callus cultures of *Abutilon hirtum* L., and investigate the influence of adding methyl jasmonate MeJA at various amounts to culture media on the quantity of rutin produced by the induced callus cultures of this plant.

MATERIALS AND METHODS

The seeds were collected from Rania village in Sulaymaniyah Governorate, Iraq. The work was done in the Laboratory of Plant cell and Tissue culture, Department of Biology-College of Education for Pure Sciences-University of Diyala, Iraq. Seeds of *A. hirtum* were used to grow the plant in our laboratory. Therefore, no need to mention the source of the plant seeds of *A. hirtum* L. were sterilized by washing under running tap water for 15 minutes in order to eliminate the soil particles, then immersed in sodium hypochlorite solution (% of active chloride as %6) at a ratio of one volume of the sterilizing material: one volume sterilized water for 15 minutes with shaking. The seeds were then washed 3 times with sterilized distilled water, at an average of 5 minutes / each time, to remove the residual of the sterilizing material (Udayakumar *et al.*, 2013). The sterilized seeds were kept on filter papers to dryness. They were then grown on the surface of 20 ml of solid MS medium (Murashige and Skoog, 1962), free of the growth regulators, in glass jars of 250 ml volume with 5 seeds / jars. The samples were kept in darkness for 3 days to mimic the natural growth conditions. Once the growth started, the samples were transferred to a successive light/darkness system of 16 h light / 8 h darkness with a light intensity of 2000 Lux for 3 weeks.

MS medium was prepared according to the method described (Nadir, 2021 b). Its pH was adjusted to pH 5.7-5.8. Stems were taken at a length of 1 cm / piece⁻¹ from sterile seedlings as an explant for callus induction after 3 weeks of growth. They were transferred to glass jars of 250 ml containing 50 ml of MS induction medium

supplemented with 0.0, 500, 1000, 1500, 2000 µg.L⁻¹ 2,4-D + 500 µg.L⁻¹ Kin. Ten (10) explants per treatment were cultured. The samples were left in the development room at the conditions previously described at light intensity of 2000 Lux (Ahmad *et al.*, 2020). The changes in the plant part were monitored until it lost its shape and changed completely to a callus. The percentage of response and callus fresh weight were determined after 30 days by the differences between the gross weight of the glass jars with their contents, and their weight after removal of their contents, depending on the theoretical scale. The degree of callus induction response together with the observations about callus texture and color were monitored.

A stock solution of 10 mM MeJA (Sigma-Aldrich) was prepared in 50% ethyl alcohol (V/V), filtered and sterilized with a millipore of 0.22 µm diameter prior to its addition to callus cultures (Ji *et al.*, 2019). In order to investigate the effect of abiotic elicitor MeJA, on the growth of callus cultures and their contents of rutin, segments from induced callus, each weighing 0.5 g were taken from the callus and transferred to glass jars containing 50 ml Murashige and Skoog medium fortified with 2000 µg.L⁻¹ 2,4-D + 500 µg.L⁻¹ Kin, for which (0.0, 0.01, 0.02 and 0.03) mmol.L⁻¹ MeJA stock solution were added and incubated for 30 days (Govindarjan *et al.*, 2015).

Rutin was extracted from callus cultures according to the method previously described by Boligon *et al.* (2015). 40 µL of the extracted sample was injected into High-Performance Liquid Chromatography HPLC, and the concentration of each component of the crude extract was estimated by comparing the samples area with that of the standard. Isolation and identification of rutin extracted from the callus of *A. hirtum* L. were carried out using HPLC, as described by Boligon *et al.* (2015). Rutin identification was carried out at the Ministry of Science and Technology/ Baghdad laboratories, depending on the standard sample's retention time. The concentration of crude rutin was estimated according to the following equation:

$$\text{Sample concentration} = (\text{Area of the sample} / \text{Area of standard}) \times \text{concentration of the standard} \times \text{number of dilution} \quad \dots \text{Eq.1}$$

Statistical analysis

The statistical analysis was performed by using Duncan test with a probability of 0.05. The results with the same letters do not show significant differences.

RESULTS AND DISCUSSION

In the present study, the efficiency of seed surface sterilization of *A. hirtum* L. by commercial bleach, 6% sodium hypochlorite (NaOCl) appears to be a suitable sterilizing material. The efficiency was 100% in terms of

producing healthy seedlings on the solid MS medium free from the growth regulators. The produced seedlings of 21 days were used as a source of the explants obtained from stems for callus induction.

The results (Table 1 and Fig. 1) demonstrated that stems planted on MS growth medium fortified with suitable growth regulators were much better regarding the suitability on induction of callus than the control. Murashige and Skoog medium supported with 1500 $\mu\text{g.L}^{-1}$ and 2000 $\mu\text{g.L}^{-1}$ 2,4-D, stimulated callus induction and achieved the highest value of callus induction of (100%), compared with the control treatment of (0%) (Fig. 1 A, D, E). The induction percentage declined to 50% at treatment 500, 1000, $\mu\text{g.L}^{-1}$ 2,4-D (Fig. 1 B, C). The results also revealed that the best callus fresh weight data were at the treatments of 1500 $\mu\text{g.L}^{-1}$ and 2000 $\mu\text{g.L}^{-1}$ 2,4-D (2.088, 2.887 g.piece⁻¹ respectively)

exceeding the treatments with of 500, 1000, $\mu\text{g.L}^{-1}$ 2,4-D which were (0.416, 0.481 g.piece⁻¹ respectively). The control treatment recorded the least value of 0.055 g.piece⁻¹. The control's explants were characterised as swollen, whilst explants planted on growth (MS) medium enforced with 2000 $\mu\text{g.L}^{-1}$ 2,4-D lost their shape. However, callus has a friable texture and pale greenish color. There was a complete loss of explant shape, and transformation of explant to a segment of callus at the end of 30 days of incubation, on media containing 500, 1000, 1500 $\mu\text{g.L}^{-1}$ 2,4-D. The callus had a solid texture and pale green colour.

Auxin is very important for callus induction because it increases cell activity through polarisation of the nutritional elements and increasing of their metabolic activities, which lead to an increase callus size and weight, in addition to the role created by the hormonal combi-

Table 1. Percentage of callus induction and the average callus fresh weight derived from seedlings stems of *Abutilon hirtum* L. grown on MS medium enriched with different concentrations of 2,4-D + 500 $\mu\text{g.L}^{-1}$ Kin

Concentrations of growth regulators ($\mu\text{g.L}^{-1}$)		Induction (%)	Average fresh weight (g.piece ⁻¹)
2,4-D	Kin		
0.0		0	0.055 d
500		50	0.416 c
1000	500	50	0.481 c
1500		100	2.085 b
2000		100	2.884 a

10 pieces were used per treatment; Data with the same letters have no significant statistical differences according to Duncans test at the probability level of 0.05.

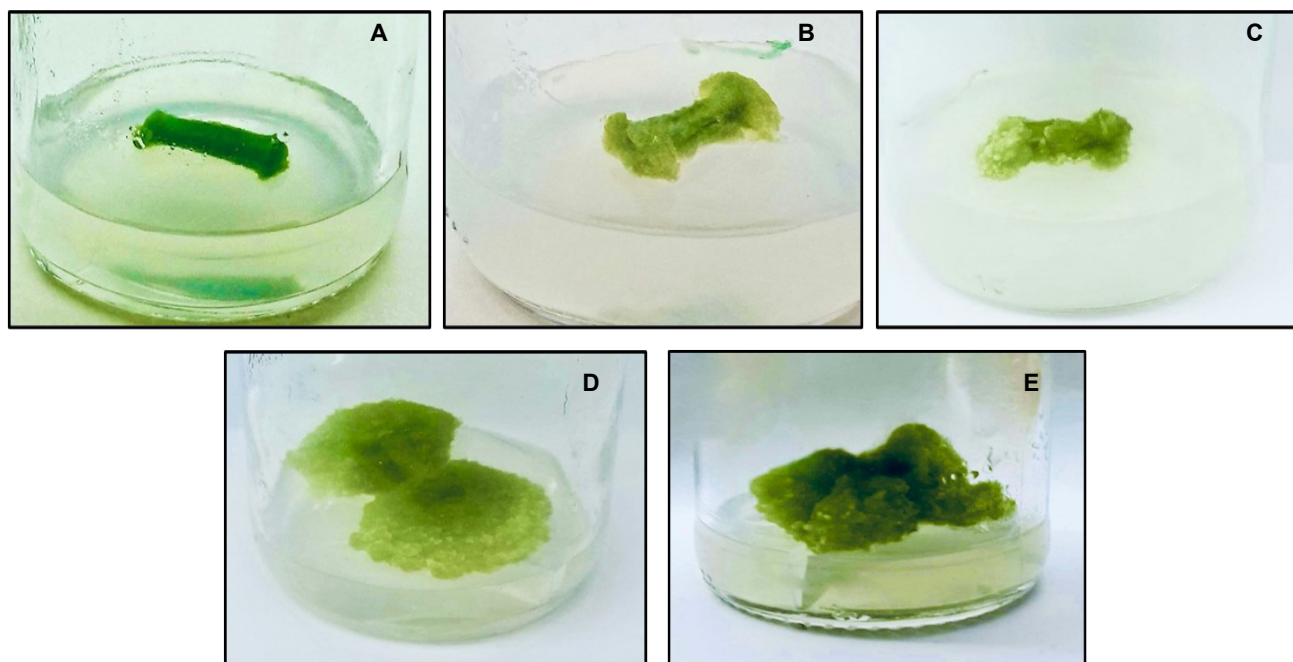


Fig. 1. Effect of different concentrations of 2,4-D + 500 $\mu\text{g.L}^{-1}$ Kin on callus induction from seedlings stems of *Abutilon hirtum* L. (A): Control treatment showing stimulated callus induction of (0%) (B): 500 $\mu\text{g.L}^{-1}$ 2,4-D showing stimulated callus induction of (50%) (C): 1000 $\mu\text{g.L}^{-1}$ 2,4-D showing stimulated callus induction of (50%) (D): 1500 $\mu\text{g.L}^{-1}$ 2,4-D showing stimulated callus induction of (100%) (E): 2000 $\mu\text{g.L}^{-1}$ 2,4-D showing stimulated callus induction of (100%).

Table 2. Influence of various MeJA concentrations on fresh weight (g) of the induced callus from stems of *Abutilon hirtum* L. after 30 days.

MeJA Concentrations (mmol.L ⁻¹)	Callus fresh weight (g)
0.0	4.776 a
0.01	5.050 a
0.02	5.360 a
0.03	3.146 a

Results followed by the same letter do not significantly differ according to Duncan's test.

nations between the percentage of the balanced concentrations of auxin and cytokinins (Issa *et al.*, 2019). Auxins expand cell walls and increase RNA metabolism. RNA has a major role in synthesising the important proteins required for cell division, reproduction, and cell wall expansion (Ljung, 2013). It was reported by Tahir *et al.* (2011) that 2,4-D is a very active auxin, which stimulates a potent response resulting in callus formation that helps cells to divide and elongate.

Both regulations of protein and metabolism of carbohydrates are affected by cytokinins, and therefore, lead to the stimulation of cells division (Liao *et al.*, 2015); that is, on top to their major role in the increment of cells division, due to increasing the ability of the meristemic and parenchymal cells to divide. These cells, which lost their specialization and converted to meristematic cells, led to increase tissue volume of cultured explants grown on sterilized nutritional media (Ruzicka *et al.*, 2009).

Data on the growth of 0.5 g induced callus cultures of stems on solid MS medium enriched with 2000 µg.L⁻¹ 2,4-D + 500 µg.L⁻¹ Kin, to which 0.0, 0.01, 0.02, 0.03 mmol.L⁻¹ MeJA, as elicitor added, are given in Table 2. This shows no significant differences between the callus fresh weight of induced treatments by adding differ-

ent MeJA concentrations, compared with the recorded amount of fresh callus after 30 days of incubation. Highest callus fresh weight was 5.360 g at 0.02 mmol.L⁻¹ MeJA, which decreased 4.776, 5.050, 3.146 g at the addition of 0.0, 0.01, and 0.03 mmol.L⁻¹ MeJA, respectively. The resulting callus had a friable texture and greenish color.

Saeed *et al.* (2017) reported no significant variations among fresh callus weights at the end of 30 days of incubation, despite different MeJA concentrations as abiotic elicitors. This can be attributed to MeJA effect on induction of the biosynthetic processes, as a typical defense response of the plant, such as the production of phytoalexins without affecting cells' weight. See *et al.* (2011) pointed out that adding MeJA was not necessary for the enhancement of cell growth but was needed to stimulate the rapid accumulation of anthocyanin production by *M. malabathricum* in cell cultures.

- Many studies reported that adding different MeJA concentrations to cell cultures media or spraying MeJA on the plants at different growth periods increased fresh or dry weight. This increment was low and insignificant compared with the control treatment (Exposito *et al.*, 2010, Zhang *et al.*, 2015 and Divya *et al.*, 2014).

Data related to the identification of rutin by using HPLC (Table 3) demonstrated the presence of rutin in cultures to which 2000 µg.L⁻¹ 2,4-D + 500 µg.L⁻¹ Kin were added after 30 days of growth regarding the retention time of the standard sample (Fig. 2 B). The addition of MeJA as an abiotic elicitor has a potent effect on increasing rutin concentration in callus within cultures planted on MS medium enforced by the same concentration of the growth regulators to which 0.01, 0.02, 0.03 mmol.L⁻¹ MeJA were added. Rutin concentration increased directly with MeJA concentration, a concentration of 1.918 mg.g⁻¹ shown at 0.01 mmol.L⁻¹ MeJA, which increased to reach a value of 3.586 mg.g⁻¹ at 0.02 mmol.L⁻¹ MeJA. The highest significant rutin value

Table 3. Retention time and concentration of rutin isolated from callus cultures of *A. hirtum* L., grown on MS medium enriched with 2000 µg.L⁻¹ 2,4-D + 500 µg.L⁻¹ Kin, to which 0.01, 0.02, 0.03 mmol.L⁻¹ of MeJA were added

Rutin source	Number of dilutions	Retention time (minute) for rutin	Area under the curve of the rutin (mAU x minutes)	Concentration of rutin (mg.g ⁻¹)
Standard rutin	-	3.15	208371	100
Induced callus from stems after 30 days (control)	80	3.17	114705	1.569 d
Induced callus from stems + 0.01 mmol.L ⁻¹ MeJA.	100	3.19	115166	1.918 c
Induced callus from stems + 0.02 mmol.L ⁻¹ MeJA.	100	3.18	236628	3.586 b
Induced callus from stems + 0.03 mmol.L ⁻¹ MeJA.	100	3.19	38377	5.606 a

VThe values within the same column, followed by the same letter do not differ significantly according to Duncans test.

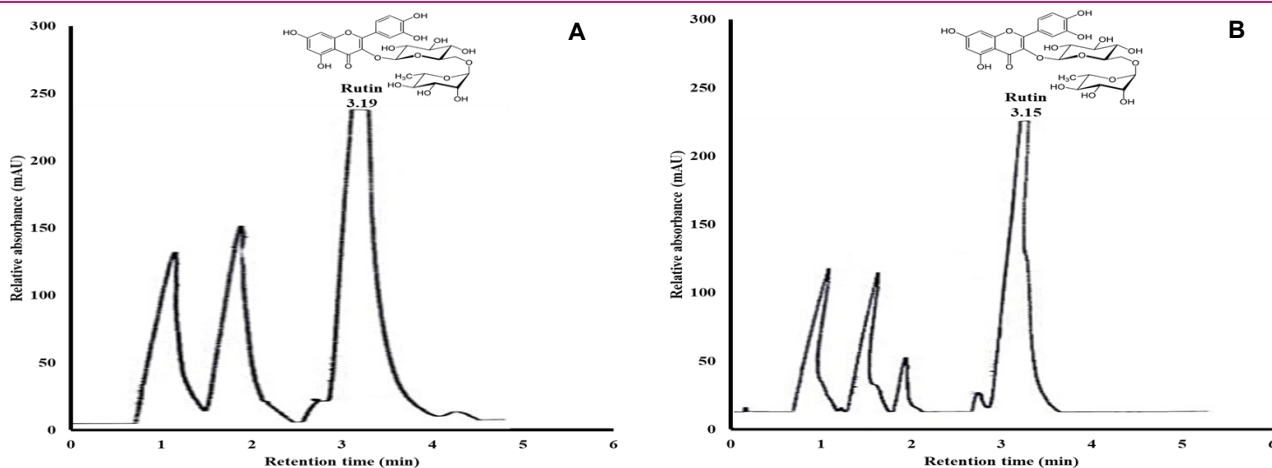


Fig. 2. Curves showing data of rutin : (A). Rutin standard curve, (B). Rutin extracted from callus cultures of *A. hirtum* L., maintained with $2000 \mu\text{g.L}^{-1}$ 2,4-D + $500 \mu\text{g.L}^{-1}$ Kin, to which 0.03 mmol.L^{-1} MeJA concentration added

(5.606 mg.g^{-1}) was recorded at 0.03 mmol.L^{-1} MeJA (Fig. 2 A) compared with control treatment 1.569 mg.g^{-1} . The chromatograms were produced by laboratories of the Iraqi Ministry of Science and technology. The results (Table 3) showed that adding different MeJA concentrations to callus culture as an abiotic elicitor affects the accumulation and increases rutin production in callus cultures. Rutin concentration gradually increased with the increase of MeJA concentration until it recorded the highest rutin concentration at 0.03 mmol.L^{-1} MeJA. This means that the cell gives its highest productivity when it is at a settled state, under stress and condensed with each other. Different parameters can affect the production and accumulation of secondary metabolites, by callus, including the incubation period with the elicitor, its concentration, age, state of the culture and nutrient formation (Ramawat and Merillon, 2019). In addition to the usage of MeJA as an active abiotic elicitor in phenylpropanoid biosynthesis and stimulation of primary Phenylalanine ammonia-lyase (PAL) enzyme activity, which converts amino acid phenylalanine to cinnamic acid 4-hydroxylase and isomerase enzymes. These enzymes have a major role in the production of different coumarins and the expression of related genes in the formation of these compounds (Saini *et al.*, 2014; Ali *et al.*, 2015).

Conclusion

The present study concluded that callus induced from stems explant of *A. hirtum* L. grown on MS medium supplemented with $1500 \mu\text{g.L}^{-1}$ and $2000 \mu\text{g.L}^{-1}$ 2,4-D achieved the highest value of callus induction. Auxin is very important for callus induction. No significant differences were observed between callus fresh weight of induced treatments, to which received variable amount of MeJA concentrations added, compared with that of control. The resulting callus had a friable texture and green color. However, adding MeJA to callus cultures

of *A. hirtum* L. strongly affected rutin production. Rutin concentration gradually increased with the increase of MeJA concentration. The highest rutin concentration was reached when 0.03 mmol.L^{-1} MeJA was added. This is the first time MeJA is used as an abiotic elicitor to stimulate the cells of *A. hirtum* L. by using plant tissue culture technology in callus cultures.

Conflict of interest

The authors declare that they have no conflict of interest.

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