Influence of biotic elicitor Aspergillus niger on salicylic acid products in callus cultures of Calendula officinalis L. plant

Muthana Mohammed Ibrahim 1 , Rabab Majead Abed 2 and Farah Qasim Ali 3

1,2,3 University of Diyala - College of education for pure science – Department of Biology
Email : rabab.majead81@gmail.com

Abstract. The study was carried out in the college of education for pure sciences / department of biology / plant tissue and cell culture laboratory to study the effect of biotic elicitors Aspergillus niger in the production of salicylic acid in callus cultures of Calendula officinalis L. plant. The results showed the best induction of the explants, which included the leaves and cotyledon obtained from culturing on the solidified MS medium supplemented with 2.0 mg.L\(^{-1}\) of 2,4-Dichlorophenoxy acetic acid (2,4-D) interacted with 0.5 mg.L\(^{-1}\) Kinetin, which reached the highest fresh weight of callus was 2.2350 and 2.7125 g, respectively, after four weeks culture. The callus induction from the leaves is compact, in contrast to the callus induced by the cotyledon, which is characterized its friable.

1. Introduction

Calendula officinalis L. is one of the plant of Asteraceae family, it is an aromatic plant that is classified in terms of its growth into annual winter ornamental plants [1]. The Mediterranean Sea it is the original habitat for the cultivation of Calendula officinalis L. plant and it also spreads in Eastern and Southern Europe, it is also grown in North America, Eastern Europe, Germany and India [2]. Plant height ranges between 30-60 cm, the leaves are thick and rectangular with reticulate venation, both the top and bottom surfaces of the leaf were puberulent [3]. The plant has extensive uses in the field of herbal medicine flowers are used in the treatment of smallpox, measles, jaundice, constipation and reduced bleeding during menstruation [4].

Tissue culture was used for many medicinal plants to producing secondary metabolism compounds with biological activity production, as this technology provides suitable conditions for the development of tissue culture throughout the year [5], as well as the availability of rapid production of secondary metabolites away from restriction of the agricultural season or the need for large areas of agriculture [6]. The production of some secondary metabolites compounds can be induced by exposing the plant to a biotic or abiotic factors [7]. Fungi were one type of biotic elicitor and Aspergillus niger fungi was one of fungi that used in this field [8]. The Aspergillus niger fungus has an important role in the field of biotechnologies for the production of chemicals, enzymes and medical drugs [9]. This fungus is a widespread fungus and represents a high percentage of natural air microbes, it is characterized by black conidia so it is also called black mold [10].

Salicylic acid is one of the most important secondary metabolites that play an important role in increasing plant resistance to many diseases [11]. Also it has a role in the overall occurrence of
acquired resistance that occurs because of a pathogen in a particular part of the plant and result in the induction of resistance in other parts of the plant [12]. Therefore, the present study aimed to study the effect of different concentrations of Aspergillus niger fungus extract and for different periods on production of salicylic acid in callus cultures of Calendula officinalis L. plant.

2. Materials and methods

- **Calendula officinalis** L. seeds are supplied by the Danish company SAKATA. The seeds were first washed by immersing them in the washing powder solution for 5 min. to remove the suspended material then left under running water for 15 min., the seeds were then sterilized by immersing them in sodium hypochlorite solution at the concentration of 6% 15 min., the seeds were then washed with sterile water 3 times at a rate of 3 min. each time. Sterilized seeds were germinated on the surface of 30 mL from agar-solidified MS(hormone-free) medium [13], and maintained in the dark for three days and when germination was transferred to the growth chamber at 25 ± 2°C and the light at 2000 lux with a daily 16h photoperiod, the seedling is completed 3 weeks old for use in subsequent experiments.

The leaves and cotyledon were excised from 3 weeks old plantlets, and sliced into lengths of 1.0 cm, then transferred to agar-solidified MS medium supplemented with different concentrations of 2,4-D (0.5, 1.0, 1.5, 2.0) mg.L⁻¹ interacted with 0.5 mg.L⁻¹ Kinetin (Kin) to study the effect of these growth regulators and type explant in the induction of callus. The MS medium without plant growth regulators was used as control.

The experiment carried out with Complete Random Design (CRD) with 5 replicates per treatment. Samples were kept in the conditions of the growth chamber referred to previously, and the changes in the plant part were followed until the shape disappeared and the callus formation was established. The fresh weight of callus was determined by calculating the weight difference between the glass bottles and their contents before and after re-culture of the callus at the same development medium and during 30 days of growth period.

- **Aspergillus niger** fungus was isolated from the air depending on the method described in [14]. The diagnosis was based on the morphological and reproductive characteristics of fungi [15]. For preparation the extract of A. niger fungus the extract was prepared according to the method described by [16].

- For study the effect of Aspergillus niger extract in the growth of callus and production salicylic acid Pieces of callus induced from cotyledon leaf with 1 gm. were transferred to glass bottles contains 20 ml of 2.0 mg.L⁻¹ of 2,4-D interacted with 0.5 mg.L⁻¹ of Kin. different concentrations of A. niger extract have been added to it which were (0, 1.0, 1.5, 2.0) mg.L⁻¹. The treatments were kept in the growth chamber for 30 days after which the fresh weights of the callus were measured.

To estimate the content of the callus of salicylic acid, callus were drying by leaving it in the air for 48 h. then grinding it using a ceramic mortar, samples were placed after crushing in plastic bottles until extraction. Salicylic acid extraction was performed according to grand method which described in [17], and the process of separation and diagnosis of salicylic acid was done using high performance liquid chromatography (HPLC) type UFLC SHMADZU Japanese. Using a metal column with specifications which were:

| Stationary phase C18 | Column length 250 mm |
|----------------------|----------------------|
| Inner diameter of column 5.4 mm | Diameter of filling granules 5 μm |

Table 1. shows the conditions for diagnosis of salicylic acid according to report in [18].

Table 1. diagnosis conditions of salicylic acid by HPLC.
Mobile phase 10 water : 90 acetonitrile
Speed of the mobile phase 2 ml / minutes
Size of the injected sample 5
Degree of separation temperature 35 C°
Type of Detector UV light at 320 nm

Callus content of salicylic acid was measured according to the standard curve shown in Figure 1.

Figure 1. Standard curve of salicylic acid.

The amount of salicylic acid was calculated according to the following equation:

\[
\text{Percentage of compound} = \left( \frac{T}{S} \right) \times 100
\]

\( T \) = Curved area of the compound in the tested sample
\( S \) = Curved area of the standard compound

3. Results and discussion

- The results in Table 2. showed highest fresh weight of callus which induced by cotyledon was affected clearly by concentration 2.0 mg.L\(^{-1}\) of 2,4-D interacted with 0.5 mg.L\(^{-1}\) of Kin supplementation which was 2.7125 g with 100% induction contrasted with control treatment which did not show any response. While the fresh weights were in other treatments 0.5, 1.0 and 1.5 mg.L\(^{-1}\) of 2,4-D interacted with 0.5 mg.L\(^{-1}\) of Kin were 0.3460, 1.9920 and 2.1500 g respectively, with 100% induction for each treatment, Also the results showed that the callus induced from cotyledon is characterized by its friable strength and light green color.

Table 2. Effect of different concentration of 2,4-D + 0.5 mg .L\(^{-1}\) Kin on fresh weight and the percentage of induction for callus indicted from cotyledon of Calendula officinalis L. plant.

| Concentration of 2,4-D + 0.5 Kin | Explant Induced | percentage of induction (%) | Fresh weight (g) |
|---------------------------------|-----------------|-----------------------------|-----------------|
| 0.0                             | 0               | -----                       | ----- d         |
| 0.5                             | 5               | 100                         | 0.3460 c        |
| 1.0                             | 5               | 100                         | 1.9920 b        |
| 1.5                             | 5               | 100                         | 2.1500 ab       |
| 2.0                             | 5               | 100                         | 2.7125          |
The fresh weight of callus which induced by leaves was affected clearly by concentration 2.0 mg.L\(^{-1}\) of 2,4-D interacted with 0.5 mg.L\(^{-1}\) of Kin supplementation which recorded highest fresh weight of callus that 2.2350 g with 100% induction contracted with control treatment which did not show any response Table 3. The results in this table also showed no significant differences between treatment 1.0 and 1.5 of 2,4-D in the fresh weight which recorded (1.4828 and 1.6920) g respectively with 100% induction for each one. While a treatment with 0.5 of 2,4-D was recorded lowest value for fresh weight of callus which was 0.7180 g with 100% induction.

**Table 3. Effect of different concentration of 2,4+0.5 mg .L\(^{-1}\) Kin on fresh weight and the percentage of induction for callus indicted from leaves of *Calendula officinalis* L. plant.**

| Concentration of 2,4-D + 0.5 Kin | Explant Induced | percentage of induction (%) | Fresh weight (g) |
|-------------------------------|-----------------|-----------------------------|-----------------|
| 0.0 | 0 | ------ | ------ d |
| 0.5 | 5 | 100 | 0.7180 c |
| 1.0 | 5 | 100 | 1.4280 b |
| 1.5 | 5 | 100 | 1.6920 b |
| 2.0 | 5 | 100 | 2.2350 a |

Auxins and Cytokinins were most used in callus formation and play an important role in induced of secondary metabolites production. And they work together with specific concentrations on emergence of callus, cell division, elongation of tissues and plant organs [19]. 2,4-D was one type of Auxins which play important role on enhanced elongation, division and callus proliferation of cells [20]. In [21] was explained that the success of callus induction from leaves and cotyledon in medium that containing growth regulators attributed to totipotency for cell. Genetic structure of plant cells, level of hormones and vitamins, compatibility of the medium with growth regulators. It is also shown that the best part of the plant to induce callus is root and leaves perhaps because they were the main sites for construction of many essential compounds such as hormones and enzymes, along with secondary metabolic compounds. The study [22] showed there is a positive correlation between the concentration of auxin and fresh weight of callus that inducted from Base and middle parts of young leaves base and middle parts of young leaves of dwarf cardinian plant *Gardenia jasminoides* Elli, fresh weight increased by increasing the growth regulator concentration 2,4-D.

The results in Table 4 showed no significant differences between the fresh weights of callus that treated with different concentrations of *Aspergillus niger* fungus extract (0.0, 1.0, 1.5, 2.0) mg . L\(^{-1}\) after 30 days of planting. The highest rate of fresh weight of callus was 1.5170 g in the treatment of Concentration 1.5 mg . L\(^{-1}\) of *A. niger* extract which did not differ significantly from the control treatment that was 1.5140 g. The results also showed a decrease in callus fresh weight in the treatment of 2.0 mg . L\(^{-1}\) of *A. niger* extract which was 1.4050 g.

**Table 4. Effect of different concentrations of *Aspergillus niger* fungus extract on callus fresh weight of *Calendula officinalis* L. plant.**

| Concentration of fungus extract (mg.L\(^{-1}\)) | Fresh weight (g) |
|--------------------------------------------|-----------------|
| 0.0 | 1.5140 a |
| 1.0 | 1.4740 a |
| 1.5 | 1.5170 a |
| 2.0 | 1.4050 a |
The absence of significant differences between the weights of callus in all treatments after 30 days on agriculture may be attributed to the role of fungus, which may be limited to stimulating the defense processes of the plant and the production of secondary metabolites without the effect of cell weight [32].

- The result in Table 5. Shows the presence of salicylic acid in the callus after 30 day of growth on MS medium that supplement with 2.0 mg L\(^{-1}\) of 2,4-D interacted with 0.5 mg L\(^{-1}\) Kin. The highest content of salicylic acid was in the treatment with 2.0 mgL\(^{-1}\) of A. niger fungus extract which was 1.1470 mg.gm\(^{-1}\) compared with control treatment that 0.4280 mg.gm\(^{-1}\). other concentration of fungus extract had a positive effect in increasing the amount of salicylic acid which were (0.7258, 0.9545) mg.gm\(^{-1}\) for treatment 0.1 and 1.5 2.0 mg.L\(^{-1}\) of A. niger fungus respectively. Curves in the figure 2. show the recorded data from the injection of samples into HPLC in terms of retention time for the standard, which increased by increasing the concentration of fungus extract as shown by curves A,B,C,D in Figure 2.

### Table 5. Effect of different concentrations of Aspergillus niger fungus extract on callus content of salicylic acid for Calendula officinalis L. plant after 30 day from culture.

| Concentration of fungous extract (mg. L\(^{-1}\)) | Ret. Time | No. of dilutions | Area under curved | Amount of salicylic acid (mg.g\(^{-1}\)) |
|-----------------------------------------------|-----------|-----------------|------------------|----------------------------------------|
| 0.0                                           | 3.117     | 7               | 146450           | 0.4280                                 |
| 1.0                                           | 3.133     | 11              | 428352           | 0.7258                                 |
| 1.5                                           | 3.172     | 10              | 326600           | 0.9545                                 |
| 2.0                                           | 3.152     | 12              | 392434           | 1.1470                                 |
| Standard sample                               | 3.131     | 2               | 113084           | 100                                    |

The above results show the addition of different concentrations of A. niger fungus extract, it has an effect on increasing the accumulation of salicylic acid in the callus, which increased by increasing the concentration of fungus extract, and reached the highest content at the concentration 2.0 mg. L\(^{-1}\). In [21] that explained that plant cells give higher productivity to secondary metabolites when they are under stress, and when they agglomerated with each other. The concentration of the biotic factor, the duration of exposure to it, age and condition of the tissue caltuer have an important role in the production of secondary metabolites in callus [25]. This is confirmed by the results of the current study and another study which showed that the addition of Aspergillus niger extract in concentration 2.0 mg. L\(^{-1}\) to callus culture of Marsilea quadrifolia led to increased plant growth and the concentration of carbohydrates and protein.
Figure 2. the curves of salicylic acid by HPLC in cotyledon callus for *Calendula officinalis* L. plant after 30 day from culture which traded with different concentrations of *Aspergillus niger* fungus extract.

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