2023, 20(3 Suppl.): 919-927
https://dx.doi.org/10.21123/bsj.2023.7399
P-ISSN: 2078-8665 - E-ISSN: 2411-7986
Baghdad Science Journal

Improving propagation of Echinacea purpurea and its content of some active compounds by using Tyrosine and Salicylic acid in vitro

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Received 08/05/2022, Revised 29/10/2022, Accepted 31/10/2022, Published 20/06/2023

Abstract

The seed propagation is the predominant method of Echinacea propagation, which has been criticized for its time-consuming control over the separation factor and the uncertainty of pathogen-free plants produced by this method. The technology of tissue culture has provided multiple opportunities for the production of secondary metabolites continuously without being restricted to a specific season, due to the possibility of controlling the environmental conditions and the components of the nutrient medium needed by the plant. This study was conducted to investigate the effects of salicylic acid as elicitor and tyrosine as precursor on propagation and some secondary compounds production in coneflower in vitro. The result showed the superiority of 2 mg / liter SA over the other treatments, which gave the highest average number of branches formed reaching 33.6 branches / plant part, number of leaves 33.6 leaves/vegetable part, fresh 1.067g and dry 0.058g weights as well as echinacoside concentration of 291.3427 µg / g DW. However, the treatment 100 mg/l tyrosine gave a maximum of average number of branches 11.80 branches / plant part, number of leaves 11.80 leaves/vegetable part, fresh and dry weight 0.152 and 0.023 g respectively. Concerning echinacoside value, its maximum 318.5203 µg / g DW was recorded with 150 mg/l tyrosine. From this study, we concluded that caffeic acid derivatives such as echinacosides are one of the main phytochemical components of Echinacea extracts. The system of cell culture is a tool for the large-scale, year-round culture of plant cells, so it is a continuous source for the production of active compounds.

Keywords: Active compounds, Cone flower, salicylic acid, Tissue culture, tyrosine.

Introduction

Purple coneflower, is known scientifically as *Echinacea purpurea* (L.)Moench, belongs to the group of herbaceous perennials of Asteraceae family. The species belonging to the genus Echinacea has gained great interest due to its increasing medicinal value, so it has become one of the best-selling herbs in North America [1].

The historical origin of the use of the Echinacea plant in the medical field goes back to the Native Americans, who used it to treat a wide range of diseases, insect stings and snake bites. In addition to infections caused by a bacterial cause such as cold infections, eye infections and rheumatic infections, the dried root and shoot are used either in their raw form or in the form of a powder in making a soothing tea for body pain or in the form of extracts packed in capsules to strengthen the immune system in the human body [2, 3]. The medical efficacy lies in the species belonging to the genus Echinacea by possessing groups of active substances. The first group includes lipophilic constituents such as Alkamides and essential oils, while the second group includes hydrophilic constituents such as Caffeic acid derivatives, Glycoproteins and Polysaccharides[4, 5].The plant extracts of *E.purpurea* have properties that stimulate the immune system in the human body because they contain anti-inflammatory substances that affect the respiratory system and caused by infection with bacteria and viruses, which explains why it is used as an antibacterial against these organisms. The same extracts are also used to eliminate abnormal growths in body tissues, such as tumors and abnormal tissue masses, in addition to the entry of these extracts into the manufacture of ointments that protect the skin from sunburn[6].

Echinacea seeds suffered from dormancy that varies according to the species belonging to the genus Echinacea. Also, the efficiency of Echinacea seeds germination and transplants production is rather low and inconsistent, ranging from non-germination to variable frequency depending on the physiology of the seeds and the environment in which they grow as well as the pH and humidity of the soil[7-9]. *E. purpurea* exhibit lower levels of dormancy than *Echinacea pallida* and *Echinacea angustifolia*[7]. Although the propagation of seeds in Echinacea was a widespread method, this method has many disadvantages, including the lack of certainty that the resulting plants are free of pathogens, as they need a long time to grow, as well as the dormancy of Echinacea seeds. The technology of tissue culture has provided multiple opportunities for the production of secondary metabolites continuously and without being restricted to a specific season, due to the possibility of controlling the environmental conditions and the components of the nutrient medium needed by the plant. The percentage of therapeutically active substances is low in most medicinal plants, which encouraged researchers to find ways to increase the active substances and encourage the plant to produce them by using some catalysts that contribute to building the active substances and the formation of primary or intermediate compounds that enter the structural pathways leading to the production of a particular compound[10].

The use of biological and abiotic extracts to stimulate the formation of secondary compounds is an important advancement strategy, as this has been demonstrated by reducing the process time required to achieve high product concentrations while increasing the yield of the raw material in the medicinal plant [11-13]. One study found that the use of extracts (jasmonic acid and yeast extract) and amino acids (phenylalanine and tyrosine), which are precursors of the culture medium, were effective in increasing the production of secondary metabolites in two echinacea cultivars [14]. It was also found that foliar application with salicylic acid and yeast extract gave positive results in increasing the production of caffeic acid derivatives in the aerial parts of *Echinacea purpurea*[15].

Therefore, this study aims to investigate the effects of salicylic acid as an elicitor and tyrosine as a precursor on propagation and the production of some secondary compounds in coneflower plant in vitro.

Materials and Methods

Plant material

The research was carried out in the Central Laboratory for Plant Tissue Culture in the College of Agricultural Engineering Sciences, as the seeds of *Echineace purpurea* were obtained from the Republic of Iran.

Seeds sterilization

The seeds were surface sterilized by soaking with commercial solution Clorox at a concentration of 1% (containing 6% sodium hypochlorite) for 20 min. and rinsed 7 times with sterile distilled water. All sterilization steps were performed under a sterile air laminar flow hood [16]

Seeds germination

The seeds were grown in the growth room under photoperiod of 16 hours of light and 8 hours of darkness, 1000Lux light intensity and a temperature of 23 ± 2 ° C. Medium of germination was MS free.

Branches stock

The resulting branches were cultured on MS medium supplemented with 0.5 mg/L of benzyl adenine (BA), then the resulting branches were subcultured on the same medium for four weeks until a sufficient number of branches was obtained to proceed next experiment.

Experimental design

To stimulate secondary compounds in the branches, the following experiments were performed.

1- The effect of salicylic acid (SA) in increasing the stimulation of secondary compounds:

Branches were planted on MS medium prepared with different concentrations of phytohormone, Salicylic acid (SA) (0, 1, 2, 3) mg/L. The cultures were incubated in the growth room at a temperature of 23 ± 2 ° C, 16 hours of light and 8 hours of darkness, and 1000 Lux light intensity. The experiment was performed with 25 tubes, each tube contained one branch per each tube was considered a replicate.

2- The effect of the precursor Tyrosine (TY) in increasing the stimulation of secondary compounds:

Branches were planted on MS medium supplemented with different concentrations of the precursor tyrosine 0, 50, 100, 150 mg/L. The cultures were incubated under the same conditions mentioned previously. with 25 tubes, each tube contained one branch, and each tube was considered as a replicate.

Growth parameters

Number and length of branches as well as fresh and dry weights were recorded for each experiment.

Extraction and determination of echinacoside

The extraction and determination of echinacoside was carried out using HPLC assay described by Brown et. al[17]. The mobile phase was delivered to the system at a flow rate of 1.5 ml/min and an injection volume of 50 µl. The detection was carried out by PDA detector 330 nm.

Statistical analysis

The experiments were carried out using a Completely Randomized Design (CRD)with twenty-five replications, and the averages were compared according to the LSD test at a probability level of 0.05 [17,18].

Results

Effect of salicylic acid on average number of branches, length of branches and number of leaves of Echinacea plant:

The results of Table 1, indicate that the addition of salicylic acid to the branches multiplication media of Echinacea led to a significant increase in the average number of branches and leaves formed, up to a concentration of 2 mg / liter, which gave the highest average number of branches formed reached 33.6 branches / plant part compared to the control treatment, which gave 2.2 branches. The average number of leaves was 33.6
leaves/vegetable part compared to the control treatment which gave 3.4 leaves/vegetable part. As for the length of the branches, the concentration of 1 mg/liter was significantly superior to the control treatment, as it gave the highest mean of the length of the branches reached 8.1 cm compared to the control treatment 4.2 cm.

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https://dx.doi.org/10.21123/bsj.2023.7399
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Table 1. Effect of salicylic acid on the average number of branches, length of branches and number of leaves of *E. purpurea*.

Salyclic acid mg/L	The average number of branches	The average long of branches	The average number of leaves
0.0	2.2	4.2	**3.4**
1.0	28.2	8.1	**28.2**
2.0	33.6	6.1	**33.6**
3.0	23.8	6.6	**23.8**
LSD 0.05	12.39	1.72	**2.38**

The effect of salicylic acid on the average wet and dry weight of *E . purpurea* :

The results of Table 2 show that the concentration of 2 mg/L of salicylic acid was superior to the rest of the concentrations in increasing the fresh and dry weights of Echinacea plants compared to the control treatment. It was given the highest average dry weight of 0.058 g. and fresh weight 1.067 gm compared with control treatment and 0.145 gm, respectively.

Table 2. Effect of salicylic acid on the average fresh and dry weights of *E . purpurea*.

Salyclic acid mg/L	The average fresh weight	The average dry weight
0.0	0.145	0.013
1.0	1.050	0.052
2.0	1.067	0.058
3.0	1.022	0.047
LSD 0.05	0.108	0.011

Effect of tyrosine acid on average number of branches, length of branches and number of leaves of Echinacea plant:

The results of Table 3 indicate that the addition of tyrosine to the branches multiplication media of Echinacea plant led to an increase in the average number of branches and leaves as well as the length of the branches formed, up to a concentration of 100 mg / liter. At this concentration, the highest average number of branches formed reached 11.8 branches / plant part, compared to the control treatment 2.20 branches / plant part. Also, the treatment 50 and 100 mg/L gave the highest average in length of branches 6.00 cm. The lowest long length of branches 4.20 cm was in case of control treatment. As for the number of leaves, the treatment 100 mg/L from tyrosine gave the highest average number of leaves, which amounted to 11.8 leaves/vegetable part, compared to the control treatment, which gave 3.40 leaves/plant part

Table 3. Effect of tyrosine acid on the average number of branches, length of branches and number of leaves of *E. purpurea*.

Tyrosine acid mg/L	The average number of branches	The average long of branches	The average number of leaves
0.0	2.20	4.20	**3.40**
50	8.60	6.00	**8.60**
100	11.80	6.00	**11.80**
150	8.40	5.00	**8.40**
LSD 0.05	3.547	2.614	**3.522**

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https://dx.doi.org/10.21123/bsj.2023.7399
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The effect of tyrosine acid on the average fresh and dry weight of *E. purpurea*:

The results in Table 4, indicate that the concentration of 100 mg/L of tyrosine was superior to the rest of the concentrations and control in increasing the fresh and dry weights of Echinacea. The lowest treatment in giving average fresh weight was at 150 mg/L, which amounted to 0.093 g. The concentration of 100 mg/L was significantly superior in giving it the highest average dry weight of 0.023 g compared to the 150 mg/L treatment which gave the lowest average dry weight of 0.007 g.

Table 4. Effect of tyrosine acid on the average fresh and dry weights of *E. purpurea*.

Tyrosine acid mg/L	The average fresh weight	The average dry weight
0.0	0.145	0.013
50	0.215	0.011
100	0.512	0.023
150	0.093	0.007
LSD 0.05	0.108	0.008

The effect of salicylic acid on echinacoside E. *purpurea*:

The results of Table 5 show that the addition of the growth regulator salicylic acid to the multiplication media of branches of Echinacea plant led to a significant increase in the concentration of the echinacoside compound. The treatment of 2 mg / liter recorded the highest concentration on the basis of dry weight amounted to 291.3427 µg / g dry weight, while the lowest concentration of echinacoside compound was amounted to 33.5313 µg / g.

Table 5. Effect of salicylic acid on echinacoside in dry **weigt** of ***E. purpurea*(µg / g DW**.)

Salicylice acid mg/L	The average of echinacoside concentration µg / g DW.
0.0	33.5313
1.0	39.3837
2.0	291.3427
157.5833	3.0
LSD 0,05	0.03503

Effect of tyrosine acid on echinacoside in *E. purpurea*:

The results of Table 6, show that the addition of tyrosine acid to the multiplication media of branches of Echinacea plant led to a significant increase in the concentration of the echinacoside compound. The treatment of 150 mg / liter estimated the highest concentration on the basis of dry weight amounted to 318.5203 µg / g in dry weight, while the lowest concentration of echinacoside compound was amounted to 33.5313 µg / g in dry weight.

Table 6. Effect of tyrosine acid on echinacoside in dry weight of *E. purpurea* (µg / g DW)

Tyrosine acid mg/L	The average of echinacoside concentration(µg / g DW)
0.0	33.5313
50	116.4033
100	269.5800
150	318.5203
LSD 0.05	0.03474

Discussion

It is clear from the above that the growth regulator, salicylic acid and the amino acid tyrosine have an effective role in improving the vegetative and physiological growth indicators in the tissue culture of the media equipped with it. In agreement with the results shown in Table 1, Bakry et al [19], and Talaat et al [20], reported an increase in the number of lateral branches, their height and the number of leaves by the presence of tyrosine. The external addition of salicylic acid also had a positive effect on the entry and transfer of ions, as well as on physiological processes [20] in addition to contributing in the increase in carbohydrate synthesis and the increase in cell division in the apical meristem and as a result an accumulation of dry matter occurs during the development of storage organs [21, 22], which in turn led to an increase and a decrease in wet and dry weight. This was similar to the results recorded in Table 2. Data recorded in Tables 3 and 4 may be due to the decrease in growth characteristics Vegetative when high concentrations of the amino acid tyrosine led to tissue tension, which caused cell toxicity and consequently a decrease in the growth characteristics of vegetative parts. Several field studies have shown that salicylic acid has a clear effect on the production of secondary compounds in medicinal plants [23, 24]. According to secondary compound type in medicinal plants, salicylic acid is involved in signal transduction cascades of plant defense response[25, 26].The secondary compounds most responsive to eliciters induction were caffeic acid derivatives, as well as phenols and flavonoids during the up-regulation of phenylalanine ammonia lyase (PAL) and the defensive response[23]. Salicylic acid significantly altered the amount of caffeic acid derivatives such as cichoric acid, chlorogenic acid, caftaric acid, cynarin and echinacoside[15]**.** In the present study, addition of 1.0 to 2.0 mg/l salicylic acid to multiplication cultures resulted in increasing echinacoside content, Table 5. Research and studies concluded that the treatment of medicinal plants with salicylic acid led to an increase in the concentration of this acid within the plant due to the occurrence of a response to cell signals that regulate the expression of defense genes that encode enzymes related to the pathway of phenylpropanoid production[27]. Activity of the main enzymes of the metabolic pathway for phenylpropanoid production was returned to phenylalanine ammonia lyase and chalcone synthase [28, 29]. The Improvement in the accumulation of echinacea production in echinacea may be due to the activation of genes related to the biosynthesis of caffeine derivatives.

The aromatic amino acid, tyrosine, form the basis of C6-C3 phenylpropane units through the shikimate pathway (the pathway provides many aromatic compounds in plants including caffeic acid derivatives such as echinacoside). In the present study, addition of 50 to 150 mg/l tyrosine to multiplication cultures resulted in increasing echinacoside content, Table 6. One study concluded that the use of high levels of tyrosine acid improved the activity of the phenylalanine ammonia-lyase gene it is the enzyme at the entry point of the phenylpropanoid pathway, which yields a variety of phenolic compounds [30, 31]. Also, one study demonstrated that the use of tyrosine levels from 50 to 150 mg/L had a significant effect on the natural products (derivatives of caffeic acid) in *Echinacea purpurea* [12]. This observation agreed with our results in Table 6**.**

Conclusion

Caffeic acid derivatives such as echinacoside are the principal phytochemical constituents of Echinacea extracts. The system of cell culture is a tool for the large-scale culture of plant cells, and is considered as a continuous source for producing active compounds. There are some strategies including precursors feeding and elicitor's treatment in cell cultures in the synthesis of active compounds.

Maximum average number of branches, leaves number, dry and fresh weights have been recorded by adding either 2 mg/L salicylic acid as elicitor or 100 mg/L tyrosine as precursor to multiplication medium. However, the highest echinacoside concentrations are found in branches grown on either 2 mg/L salicylic acid or 150 mg/L tyrosine to the medium. So, this work recommends to supply the MS medium with 0.5 mg/L BA and 2 mg/L salicylic acid to reach high growth parameter and echinacoside concentration.

Author's Declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours. Furthermore, any Figures and images that are not ours have been included with the necessary permission for re-publication, which is attached to the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee in University of Baghdad

Author's Contribution Statement

L. K. J. and Z. M. A. wrote the manuscript. Z. M. A. performed the statistical analysis. H. S. H., L. K. J. and Z. M. A. discussed the results and contributed the final manuscript.

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https://dx.doi.org/10.21123/bsj.2023.7399
P-ISSN: 2078-8665 - E-ISSN: 2411-7986
Baghdad Science Journal

تحسين اكثار نبات الاكينيسيا الوردية ومحتواها من بعض المركبات الفعالة باستخدام حامض التيروسين وحامض السالسيلك خارج الجسم الحي

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[3]قسم العقاقير، كلية بغداد للعلوم الطبية، بغداد، العراق.

الخلاصة

تعد طريقة الاكثاربالبذورهي الطريقة السائدة في اكثار نبات الاكينيسيا الوردية ، يعاب عليها انخفاض نسبة الحصول على نباتات بسبب مايحكمها من عوامل منها عامل الفصل الذي يستغرق وقتًا طويلاً فضلا عن عدم التاكد من خلو النباتات الناتجة من مسببات المرضية. لقد أتاحت تقنية زراعة الأنسجة فرصًا متعددة لإنتاج مركبات الايض الثانوي بشكل مستمر ودون التقيد بموسم معين ، نظرًا لإمكانية التحكم في الظروف البيئية ومكونات الوسط الغذائي الذي يحتاجه النبات. أجريت الدراسة الحالية لمعرفة تأثير حامض الساليسيليك والتايروسين كبادئ ومعرفة مدى تاثيرهما على الاكثار وإنتاج بعض المركبات الثانوية في نبات الاكينيسيا خارج الجسم الحي ، وأظهرت النتائج تفوق المعاملة السالسيلك بتركيز 2 ملغم / لتر ، بتسجيلها اعلى متوسط لعدد الفروع المتكونة 33.6 فرع / جزء نباتي ، وعدد الأوراق 33.6 ورقة / جزء نباتي ، ووزن طازج وجاف 1.067 غم ، 0.058 غم على التوالي ، وتركيز إلايكنوكوسايد 291.3427 ميكروجرام / جرام وزن جاف ، بينما سجلت معاملة 100 ملغم / لتر من التايروسين الحد الأقصى لمتوسط عدد الأفرع 11.80 فرع / جزء نبات ، عدد الأوراق 11.80 ورقة / جزء نباتي ، وزن طازج وجاف (0.152 ؛ 0.023 جم) على التوالي ولكن في تركيز إشنكوسايد كانت معاملة 150 ملغم / لتر من التايروسين هي الافضل اذ سجلت 318.5203 ميكروغرام / جرام وزن جاف. هدفت هذه الدراسة إلى أن مشتقات حمض الكافيك مثل إشنكوسايد هي أحد المكونات الكيميائية النباتية الرئيسية لمستخلصات إلاكينيسيا و ان نظام زراعة الخلايا هو أداة لاستنبات الخلايا النباتية على نطاق واسع وعلى مدار العام، والتي تعد مصدرًا مستمرًا لإنتاج مركبات الايض الثانوية.

الكلمات المفتاحية: مركب الاكينوكوسايد، الوردية، حامض السالسيلك، خارج الجسم الحي، حامض التايروسين.