Influence of KNO_3, $CaCl_2$ and $MgSO_4$ Concentrations on Growth and Cichoric Acid Accumulation in Hairy Root Culture of Purple Coneflower (*Echinacea purpurea* L.)

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Abstract

Background: Purple coneflower (*Echinacea purpurea* L.) is one of the most important medicinal plants. Hairy root culture of *Echinace,* represent a valuable alternative to field cultivation for the production of bioactive secondary metabolites.

Objective: The present study investigates the effects of KNO_3, $CaCl_2$ and $MgSO_4$ concentrations on growth and the biosynthesis of cichoric acid in *Echinacea purpurea* hairy root culture.

Methods: *Agrobacterium rhizogenes* strain R15834 was used for hairy root induction. The experiment was performed with 0.5 g of fresh hairy roots (about 2 cm long segments) per 100 ml Erlenmeyer flasks containing 30 ml of hormone-free liquid 1/2MS salts with B5 vitamins supplemented with different amounts of KNO_3 (3131, 3939, and 4747 mg/l), $CaCl_2$ (85, 170, and 340 mg/l) and $MgSO_4$ (370 (control), 600, and 1600 mg/l). After 4 weeks, growth parameters and cichoric acid amounts were assessed.

Results: Results indicated that the highest fresh (99.69 g/l) and dry weight (6.91 g/l) of hairy roots were achieved using 3131 mg/l KNO_3. Fresh and dry weights of hairy roots in various $CaCl_2$ and $MgSO_4$ concentrations were not significantly different from those of control. The highest amount of cichoric acid (30.55 mg/g DW) was produced by 1600 mg/l $MgSO_4$ treated hairy roots after 4 weeks.

Conclusion: The present work demonstrated the effectiveness of modified 1/2MS salts supplemented with B5 vitamins medium for improving hairy root growth and *in vitro* production of cichoric acid.

Keywords: Cichoric acid, *Echinacea purpurea* L., Fresh weight, Hairy roots, Macro-elements

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Introduction

Echinacea purpurea L. is a traditional North American perennial medicinal herb that gained international popularity because of its nutraceutical and medicinal properties [1]. The potential active compounds in *Echinacea* are caffeic acid derivatives (CADs), alkamides, polysaccharides and glycoproteins that exhibit various clinical effects such as antioxidative, antibacterial, antifungal properties and are used for treating common cold, respiratory and urinary diseases [2]. Several CADs such as cichoric acid, chlorogenic acid, caftaric acid and caffeic acid have been identified in *Echinacea* species [3]. Cichoric acid (major compound in *Echinacea purpurea*) has shown phagocytic, antihyaluronidase activity [4], inhibited HIV-1 integrase and replication [5] and immunostimulatory properties [6]. Commercial production of *Echinacea* has been limited by a range of issues including contamination of plant materials by microorganisms, pollution from the environment, variability of active components and lack of pure, standardized plant material for biochemical analysis [3, 7]. In recent years, plant organ cultures have become an alternative to whole plants for the production of valuable bioactive compounds [8]. Great efforts have been focused on transformed hairy roots [3, 9, 10, 11, 12]. Hairy roots, the results of genetic transformation by *Agrobacterium rhizogenes* [13], have attractive properties for secondary metabolite production. The fast growth in hormone-free culture medium, genetic as well as biochemical stability, low doubling time, eases of maintenance and ability to synthesize a range of secondary metabolites offers some advantages of hairy root culture [14, 15, 16]. Moreover, hairy roots are an organ culture, but their ability to secondary metabolites production is higher than in intact plant [17], cell suspension and callus cultures [14]. The conditions for culturing *Echinacea purpurea* hairy roots have effects on growth and cichoric acid production [3, 9, 10]. Therefore, to maximize the usefulness of hairy roots at the industrial level, it is essential to optimize different physical and chemical factors during hairy root production. The objective of the present study was to investigate the effects of the various KNO_3, $CaCl_2$ and $MgSO_4$ concentrations on hairy root growth in *Echinacea purpurea* L. In addition, phytochemical responses of hairy roots (cichoric acid accumulation) were determined.

Materials and methods

Plant material and culture conditions

Echinacea purpurea seeds were obtained from Pakanbazr Co. (http://www.pakanbazr.com/). The seeds were washed with tap water and were surface-sterilized in 70% (v/v) ethanol for 1 min. The seeds were washed with sterile distilled water, and were immersed in 5% (v/v) sodium hypochlorite solution containing one drop of Tween 20 (Merck) per 50 ml for 20 min and then washed three times (5 min) with sterile distilled water. The cultivation was done in glass bottles with autoclavable plastic caps (5.5 cm in diameter, 8 cm in height, and 250 ml in volume) containing 50 ml hormone-free Murashige and Skoog (MS) [18] medium supplemented with 2% (w/v) sucrose and 0.6% agar-agar (Merck). The pH of the medium was adjusted to 5.8 by addition of HCL or KOH

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prior to autoclaving. Cultures were kept at 25 ± 2 ºC under a 16-h photoperiod provided by cool white fluorescent with a light intensity of 60 µmol m^{-2} s^{-1}.

Leaves (Lamina plus petiole) of 60-day-old plantlets obtained from *Echinacea purpurea* seeds were used as explants for induction of hairy roots. *Agrobacterium rhizogenes* strain R15834 (a gift from Dr. M. Karimi, Gent University, Belgium) was used for induction of hairy roots in *Echinacea purpurea* explants. *A. rhizogenes* were grown to mid-log phase (A_{600} = 0.6) at 26 ºC on an incubator shaker in liquid Luria–Bertani medium (10 g/l tryptone, 5 g/l yeast extract, and 10 g/l NaCl, pH 7.0). The bacteria cells were centrifuged (2500 rpm, 10 min) and resuspended at a cell density of A_{600} = 1.0 in liquid inoculation medium containing half strength MS salts and B5 vitamins with 30 g/l glucose. The leaf explants were infected by being dipped into an *A. rhizogenes* suspension for 5 min and then were blotted dry on sterile filter paper to remove excess *Agrobacterium*; then incubated in the dark at 25 ºC on agar-agar solidified half-strength MS with B5 vitamins medium. After 3 days of co-cultivation with bacteria, the leaf explants were transferred to a hormone-free medium that contained half strength MS salts and B5 vitamins, 30 g/l sucrose and 300 mg/l cefotaxime. The procedure was repeated three times to ensure that no bacterial cell colony survived. Visible roots in *Echinacea purpurea* were formed after 10–12 days upon infection with *A. rhizogenes*. The hairy roots were formed mainly on the petiole of the infected leaves. After repeated transfers to fresh medium, rapidly growing hairy roots were obtained. For confirmation of the transformed nature of these hairy roots, presence of the Ri plasmid-derived *rol*B gene was verified through PCR amplification (data not shown). The *rol* genes in the Ri-plasmid in *A. rhizogenes* are responsible for the induction of hairy roots in various plant species. The isolated hairy roots were maintained by subculturing at 21-day intervals in 50 ml hormone - free 1/2MS salts with B5 vitamins and 30 g/l sucrose in 100 ml Erlenmeyer flasks on a rotary shaker (115 rpm). The subcultures were carried out at 25 ºC in dark. These roots were maintained for about 2 years in the liquid medium.

Treatment with various MS macro-element concentrations

The hairy roots were transferred into 100 ml Erlenmeyer flasks containing 30 ml of liquid hormone - free MS medium with half strength salts and B5 vitamins supplemented with different concentrations of KNO_3 (3131, 3939, and 4747 mg/l), $CaCl_2$ (85, 170, and 340 mg/l) and $MgSO_4$ (370 (control), 600, and 1600 mg/l). The pH of the medium was adjusted to 5.8 prior to autoclaving. The experiment was performed with 0.5 g of fresh roots (about 2 cm long segments) per flask. The cultures were maintained on a shaker (115 rpm) at 25 ºC in dark for 28 days. Three flasks were used for each treatment. After 4 weeks, all hairy roots were harvested, and growth parameters were assessed.

Determination of growth parameters, pH and content of cichoric acid

The growth of hairy root cultures was measured by determining fresh weight (FW), dry weight (DW), percentage dry weight, and growth ratio. For fresh weight (FW) determination, the hairy root cultures were gently pressed on filter paper to remove excess water and weighed. The samples were then

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dried in darkness at 40 ºC for 24 h and dry weight (DW) was measured. In addition, medium pH value, the percentage of browning (1 = Brown, 2 = Light brown, 3 = Cream, 4 = Cream to white, 5 = White) and the content of cichoric acid were measured. Each treatment was performed in triplicate. The growth ratio was calculated from the dry weight using the following equation: Growth ratio = (Final DW – Initial DW) / Initial DW [19]. The pH was measured in the biomass-free culture medium.

Analytical procedures

Extraction and quantification of CADs from the dried hairy root cultures were carried out. Hairy root samples were ground to a fine powder using a mortar and pestle, and then 20 mg powder samples were extracted for 30 min in 1 ml MeOH and 0.1% phosphoric acid (70:30, v/v) in an ultrasonic water bath (LC 130H, Elma, Germany) at RT. Samples were centrifuged for 5 min at 10000 rpm. The supernatants were filtered and transferred to new vials. Cichoric acid amount was analyzed by high-performance liquid chromatography (HPLC). Equal amounts (50 µl) of each replication manually were injected into the HPLC system (Knauer, pump K-1001) equipped with a C18 column (250×4.6 mm, pore size 5 µm; Teknokroma). The mobile phase was an isocratic 30% (v/v) acetonitrile: 70% water containing 0.1% (v/v) phosphoric acid at rate of 1.75 ml/min for 10 min. The UV detector K-2501 (Knauer) was set at 330 nm. Reference standard of cichoric acid was purchased from Sigma-Aldrich (Germany). The quantification of cichoric acid (tR = 4.03 min) was determined on the basis of peak area with comparison to the above-mentioned standard. Concentration of cichoric acid was measured on a dry weight basis (mg/g).

Statistical analysis

The data was analyzed statistically by the SPSS software ver. 18.0. It was used the fully randomized statistical design, and differences among the means were determined using Duncan's multiple range test at $P<0.05$. Nonparametric test (Kruskal-Wallis ANOVA) was used for statistical analysis of hairy root color. Results are presented as mean ± standard errors of three replications.

Results

There were notable differences among various MS macro-elements concentrations in terms of growth parameters (fresh weight, dry weight, and growth ratio), medium pH value, and the content of cichoric acid. The highest fresh (99.69 g/l) and dry weight (6.91 g/l) of hairy roots were achieved by the use of 3131 mg/l KNO_3; whereas the lowest fresh weight (17.67 g/l) of hairy roots was achieved at very high concentration of KNO_3 (4747 mg/l) in 1/2MS with B5 vitamins medium. As shown in table 1 and Figures 1 and 2 it was found that lower KNO_3 concentration (3131 mg/l) in medium could be optimal concentration for *Echinacea purpurea* hairy root growth compared with other treatments. However, incorporation of very high concentration of KNO_3 resulted in the decline of fresh weight, dry weight, and growth ratio (17.67 g/l, 1.37 g/l and 0.30, respectively). Fresh and dry weights of hairy roots in various $CaCl_2$ concentrations were not significantly different from that of control (P value > 0.05, Table 1). No additional increments were

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Table 1- Effects of KNO_3, $CaCl_2$ and $MgSO_4$ concentrations on growth parameters and medium pH of *Echinacea purpurea* hairy roots after 4 weeks of culture

Treatment (mg/l)		Fresh weight (g/l)	Dry weight (g/l)	Percentage of dry weight	pH
Control		25.23 ± 0.64 c *	1.28 ± 0.07 c	5.06	6.64 ± 0.04
KNO_3	3131	99.69 ± 14.12 a	6.91 ± 0.73 a	6.93	5.77 ± 0.03
KNO_3	3939	48.40 ± 4.17 b	3.83 ± 0.16 b	7.91	6.06 ± 0.04
KNO_3	4747	17.68 ± 0.70 c	1.37 ± 0.05 c	7.74	6.45 ± 0.03
$MgSO_4$	600	27.41 ± 1.38 c	1.31 ± 0.03 c	4.77	6.59 ± 0.02
$MgSO_4$	1600	31.95 ± 5.72 c	1.73 ± 0.39 c	5.41	6.64 ± 0.03
$CaCl_2$	85	23.49 ± 1.44 c	1.16 ± 0.05 c	4.94	6.68 ± 0.04
$CaCl_2$	170	22.99 ± 1.83 c	1.27 ± 0.03 c	5.50	6.73 ± 0.12
$CaCl_2$	340	22.53 ± 1.53 c	1.24 ± 0.07 c	5.49	6.67 ± 0.01

* Mean ± standard error of three replicates.

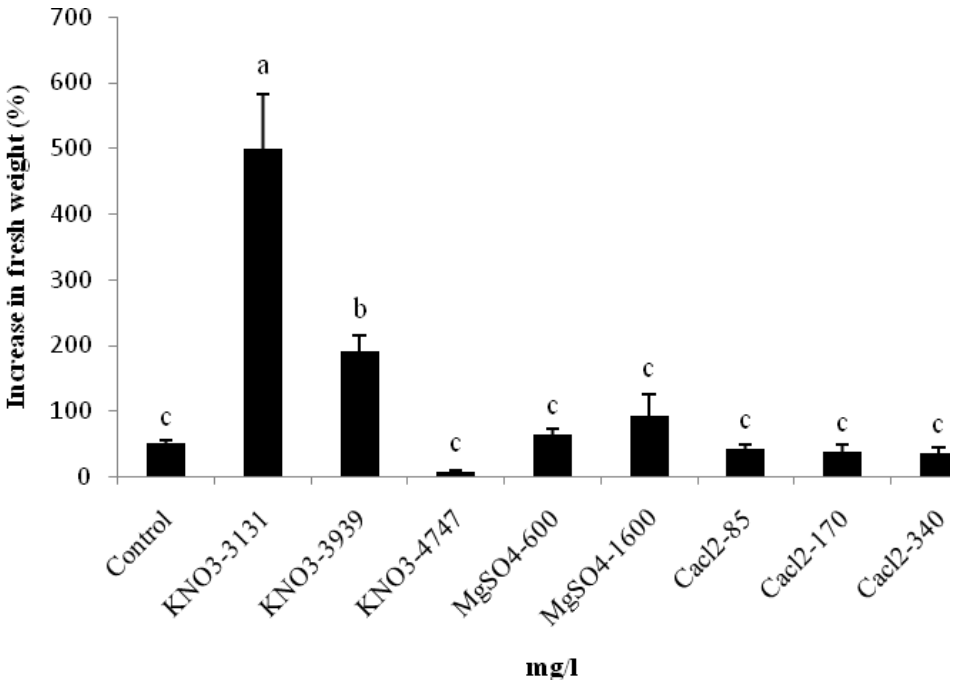

Figure 1- Effects of different MS macro-elements concentrations on increasing in fresh weight of *Echinacea purpurea* hairy roots

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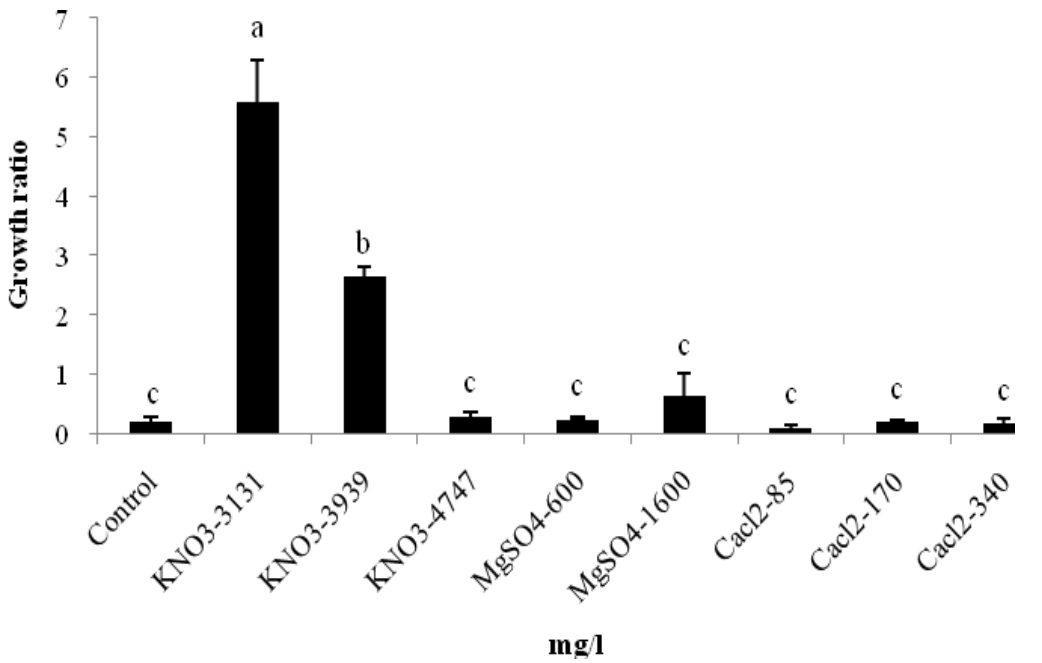

Figure 2- Effects of MS macro-elements concentrations on hairy roots growth in *Echinacea purpurea*

observed in fresh weight, dry weight and growth ratio with $MgSO_4$ beyond 600 and 1600 mg/l. The effects of MS macro-elements concentrations on the accumulation of cichoric acid in the hairy roots of *Echinacea purpurea* were presented in Figure 3. When the $CaCl_2$ concentration was increased from 85 mg/l to 340 mg/l, the content of cichoric acid decreased from 29.83 mg/g DW to 19.74 mg/g DW. Further, increase in the concentration of KNO_3 in contrast with control, reduced the accumulation of cichoric acid in hairy roots. The highest amount of cichoric acid was produced by 1600 mg/l $MgSO_4$ treated hairy roots after 4 weeks, which was 30.55 mg/g DW (Figure 3). Results obtained from statistical analysis using Kruskal-Wallis Test indicated that there were significant differences among various MS macro-elements concentrations in terms of hairy root color (Table 2). Results showed that medium containing 3131 mg/l $KN0_3$ with highest mean rank (26) had the best effect on color of hairy roots (White color) and 4747 mg/l KNO_3 had the lowest mean rank on color of hairy roots (Brown color) (Figure 4). Also, treatment containing 1600 mg/l $MgSO_4$ had lower mean rank on color of hairy roots than control. To better understand the influence of used macro-elements on growth of hairy roots and secondary metabolite, medium pH value was investigated. Table 1 shows the changes of medium pH value in *Echinacea purpurea* hairy root culture after treating by various MS macro-elements. In treatment with 3131 mg/l KNO_3, which produced the highest fresh weight of hairy roots, pH value significantly decreased in comparison with the other treatments.

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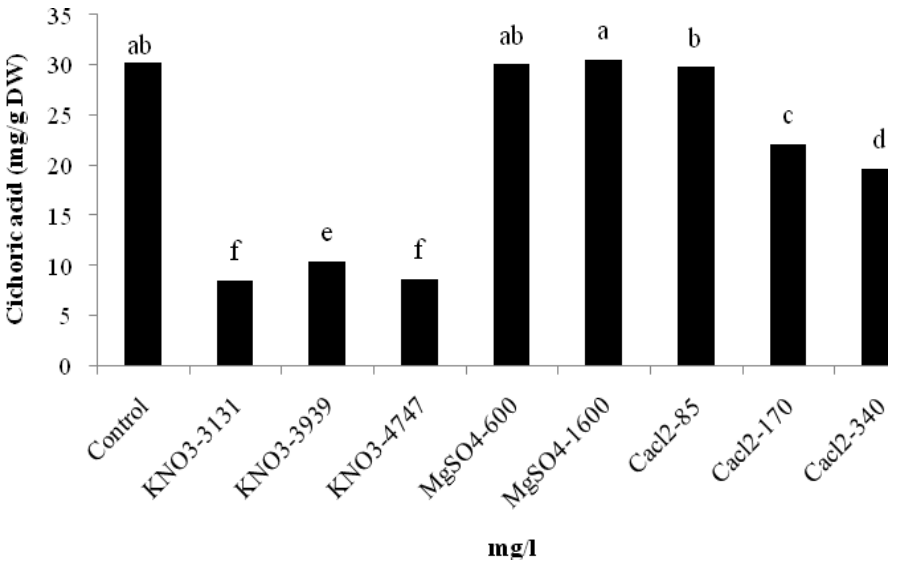

Figure 3- Effect of different MS macro-elements concentrations on cichoric acid content in *Echinacea purpurea* hairy root culture

Table 2- The result of Kruskal-Wallis Test in measured character

	Hairy roots color
Chi-square	23.927
df	8
Asymp. Sig.	0.002

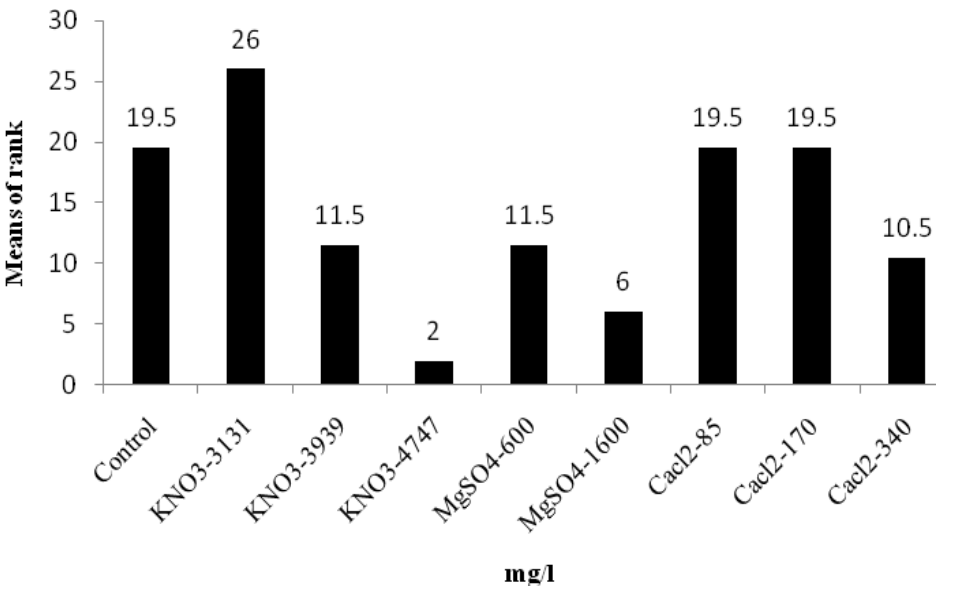

Figure 4- Ranking of different MS macro-elements concentrations on *Echinacea purpurea* hairy roots color

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Discussion

Hairy root culture offers new opportunities and good prospects for *in vitro* production of valuable plant secondary metabolites. The present work demonstrated the effectiveness of some macro-element concentrations (KNO_3, $CaCl_2$ and $MgSO_4$) in hairy root culture of *Echinacea purpurea*. Nitrogen is the essential mineral element required in the greatest amount in plants. Roots can utilize mineral nitrogen (NO_3^- and NH_4^+); however, nitrate (NO_3^-) is the most important source of nitrogen for root development [20]. The interesting finding of this investigation was an increasing 3.95 fold in fresh weight of hairy roots treated with 3131 mg/l (31 mM) KNO_3 over the control and decreasing in cichoric acid production. This decreasing in cichoric acid production was probably due to the increasing total nitrogen content in comparison with control (1/2MS medium). Lower total nitrogen content within limits often benefited secondary metabolites accumulation [19]. The results showed that the hairy roots growth was in inverse proportion to the increase of KNO_3 concentration in medium. Findings of Chashmi et al. (2008) in *Atropa belladonna* hairy roots indicated that 95 mM concentration of KNO_3 in MS medium, decreased root fresh weight; while the highest amount of atropine was produced in 35 mM KNO_3 [21]. Results indicated that two treatments containing 3131 mg/l KNO_3 and 85 mg/l $CaCl_2$ had the highest and lowest growth ratio (5.59 and 0.11, respectively). The growth rate of hairy roots depended on linear root extension, the formation of new growing points, and a secondary increase in root diameter [9]. In the present study, the different concentrations of $CaCl_2$ didn't show significant effects on growth parameter in comparison to the control, indicating that the growth of hairy roots was neither inhibited nor stimulated by $CaCl_2$. The increase of $CaCl_2$ concentration in media decreased significantly cichoric acid content. High level of Ca^{2+} in the medium activates peroxidases, which are involved in the degradation of secondary products [22]. The conditions for culturing *Echinacea purpurea* hairy root cultures were reported to have effects on growth and cichoric acid production [3, 9, 10]. It has been previously reported that nutrient medium treatment could be effective for enhancing growth and secondary metabolite synthesis in hairy root cultures [20].

Conclusion

In conclusion, quantitative and qualitative changes in fresh weight and cichoric acid yields of coneflower hairy roots were achieved by modification of nitrate concentrations in the medium. The optimum KNO_3 concentration for the growth and biomass production of *Echinacea purpurea* hairy roots was 3131 mg/l in 1/2MS medium. The findings suggest some macro-elements, as KNO_3, $CaCl_2$ and $MgSO_4$, can be used as an abiotic elicitor to improve secondary metabolite production in hairy root cultures. Also, the present work demonstrated the effectiveness of hairy root cultures of *Echinacea* for the *in vitro* production of cichoric acid, which is gaining interest due to its antiviral and inhibition activity against the integrase of HIV-1.

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