



Adventitious Root Development and Secondary Metabolites Accumulation by Auxin in *Cichorium intybus* L.

Roghayeh Fathi¹ , Mehdi Mohebodini^{2*} , Esmail Chamani³

- 1- Ph.D. Student Horticultural Sciences, Department of Horticultural Sciences, Faculty of Agriculture and Natural Resources, University of Mohaghegh Ardabili, Ardabil, Iran
- 2- Associate Professor, Department of Horticultural Sciences, Faculty of Agriculture and Natural Resources, University of Mohaghegh Ardabili, Ardabil, Iran
- 3- Professor, Department of Horticultural Sciences, Faculty of Agriculture and Natural Resources, University of Mohaghegh Ardabili, Ardabil, Iran

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Corresponding Author:

Mehdi Mohebodini

Email:

Mohebodini@uma.ac.ir

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ABSTRACT

Introduction: Adventitious root cultures of medicinal plants are a source of secondary metabolites of pharmaceutical importance, and are considered as an alternative method for clonal propagation and germplasm conservation in medicinal plants. Chicory (*Cichorium intybus* L.) is a medicinal plant from *Asteraceae* and is used in traditional medicine to promote appetite and digestion. This plant contains many important metabolites including chicoric acid, inulin, scolimine, coumarin and flavonoids. In the current research, an efficient protocol has been developed for adventitious root culture on MS medium supplemented with different concentrations of Indole-3-acetic acid (IAA) and α -Naphthalene acetic acid (NAA).

Materials and Methods: The seeds were surface-sterilized with 50 ml l⁻¹ sodium hypochlorite for 20 min, subsequently with 700 ml l⁻¹ ethanol for 90 s. The surface-sterilized seeds were inoculated against the MS medium and cultures were incubated at 25 \pm 2 $^{\circ}$ C under fluorescent light for a cycle of 16 h light and 8 h dark per day. The leaves explants of 28-day-old in vitro plantlets were used as explants. For root initiation, IAA (0, 0.2, 0.4 and 0.6 mg l⁻¹) and NAA (0, 0.5, 1 and 1.5 mg l⁻¹) were used. After four weeks, the well-established roots were separated. To determine the best medium of composition for growth of roots, approximately 100 mg fresh weight of adventitious roots were cultured in MS liquid medium with different concentrations of IAA (0, 0.5, 1 and 1.5 mg l⁻¹) and NAA (0, 0.5, 1 and 1.5 mg l⁻¹).

Results and Discussion: According to the results, among the different concentrations of IAA, the highest root induction (72.5 percent), root number (4.75), and root branch (10.08) were exhibited by 1.5 mg/L IAA. Among different NAA levels, the highest root induction (88.88 percent), and root number (8.04) were observed in 1.5 mg/L NAA and was not significantly different from 0.5 and 1.5 mg/L NAA. This hormone at concentration of 1.5 mg/L, induced the highest root branching (18.42 per explant). The highest fresh weight (0.74 g) and dry weight (0.062 g), growth index (6.51), and phenol (4.1 mg/g DW) were obtained in MS liquid medium containing 0.5 mg l⁻¹ NAA in combination with 0.5 mg l⁻¹ IAA, and Flavonoid content in 270, 300 and 330 nm wavelengths was higher (60.26, 85.88 and 98.53 μ g g⁻¹ DW) in the roots obtained from 1 mg l⁻¹ of NAA in combination with 1 mg l⁻¹ of IAA. Increasing NAA concentrations induced callus mediated root formation and produced a lower number of adventitious roots. By using IAA, adventitious roots were initiated, but the frequency and average number of roots initiated were lower when compared with NAA.

Conclusion: Adventitious roots obtained by different concentration of auxins are a suitable tool for the production of plant secondary metabolites due to their genetic stability, and generally, show a fast growth rate. This study describes the protocol for adventitious root induction which could further be useful for the production of secondary metabolites and biomass.



Introduction

Medicinal plants are a major source of life-saving drugs and bioactive compounds. These compounds currently extracted from plants are used as food additives, pigments, dyes, insecticides, cosmetics, perfumes and fine chemicals (Sanchez et al., 2019). Chicory (*Cichorium intybus* L.) is a medicinal plant, known to grow as a weed on roadsides. It is used in traditional medicine to promote appetite and digestion (Vahabinia et al., 2019). Over 100 individual and important compounds have been identified from this medicinal plant, the majority of which are from the roots (Cova et al., 2019). Chicory root extracts have anti-bacterial and hepatoprotective effect (Carazzone et al., 2013). The fresh root is bitter, with a milky juice. To obtain roots of a larger size, the soil must be rich, light and well manured. Under cultivation, the root becomes large and fleshy with a thick rind and is employed extensively when roasted and ground for blending with coffee (Hazra et al., 2002). The root of chicory contains a number of important metabolites such as chicoric acid, inulin, sesquiterpene lactones, coumarins, flavonoids and vitamins. It is used as antihepatotoxic, antiulcerogenic, antiinflammatory, appetizer, digestive, stomachic, depurative, and diuretic (Peng et al., 2019). Inulin is used to replace sugar and reduce the calories of food. Due to its non-digestibility, it is suitable for consumption by diabetics (Carazzone et al., 2013). Pharmacological investigation of the root extract of chicory revealed its immunomodulatory, antitumor and anticancer properties (Hazra et al., 2002). *In vitro* adventitious root culture system is an effective tool for plant metabolites production, and also an experimental system for secondary metabolic pathway elucidation studies (Moradi et al., 2019). Moreover, growth of root culture is highly advantageous, as it is an alternative method for clonal propagation and germplasm conservation in medicinal plants (Srivastava et al., 2019). The *in vitro* production of medicinal compounds can become possible through root culture under precisely controlled physical and chemical conditions (Ali et al., 2018). Root cultures can be used in many ways including studies of carbohydrate metabolism, mineral nutrient

requirements, essentiality of vitamins, and other growth regulators. Root cultures grow rapidly, are relatively easy to prepare and maintain, show a low level of variability, and can be easily cloned to produce a large supply of experimental tissue (Srivastava et al., 2019).

In the field of secondary metabolite production, hairy root cultures, induced by *Agrobacterium rhizogenes* has been also reported as an ideal production system for plant metabolites production (Georgiev et al., 2007). Fathi et al. (2018) reported hairy roots induction in *C. intybus* by different *A. rhizogenes* strains and the maximum hairy root induction and number of roots were observed from 5-day-old cotyledons explants. They suggested 3 days co-culture of explant and *Agrobacterium* was more efficient in inducing hairy root phenotype in transformation (Fathi et al., 2018). However, compared with hairy root induction, adventitious roots culture system is safer and easier for management (Nakayasu et al., 2018).

The quality and quantity of the secondary metabolite, collected from wild and field grown plants, are often fluctuating and heterogeneous, depending upon the environmental conditions. Infestation, diseases and the application of pesticides would additionally decrease the quality of the plant materials (Gerth et al., 2007). These problems can be overcome by using an *in vitro* production technique. Plant cell suspension, hairy and adventitious root cultures are used as natural “bioactive substance factories” to produce plant-derived compounds (Hahn et al., 2003). By these techniques, secondary metabolites are isolated and produced in large quantities, as well as of high qualities, and are stable and produced rapidly in an adapted culture medium. Therefore, production of secondary metabolites under *in vitro* conditions has become an active field of research (Sato et al., 2001). Adventitious roots have been successfully induced in many plant species and cultured for the production of high value secondary metabolites of pharmaceutical, nutraceutical, and industrial importance (Murthy et al., 2008). In the present paper, the effects of the different levels of strengths of auxin on growth and secondary metabolites production

in adventitious root culture of *C. intybus* is described.

Materials and Methods

Plant material

The current research was conducted in 2017 at University of Mohaghegh Ardabili. The seeds were surface-sterilized by rinsing them in water mixed with fungicide benomyl (20 mg l⁻¹) for 30 min and later sterilized with 50 ml l⁻¹ sodium hypochlorite for 20 min subsequently with 700 ml l⁻¹ ethanol for 90 s. The MS basal medium supplemented with 30 g l⁻¹ sucrose and 5.5 g l⁻¹ agar was autoclaved at 1.06 kg cm⁻² pressure and 121 °C for 20 min. The surface-sterilized seeds were inoculated against the medium, and cultures were incubated at 25 ± 2 °C under fluorescent light for a cycle of 16 h light and 8 h dark per day.

Adventitious root induction

The leaf explants of 28-day-old in vitro plantlets were cultured in MS medium containing 0, 0.2, 0.4 and 0.6 mg l⁻¹ of indole-3-acetic acid (IAA), and 0, 0.5, 1, 1.5 mg l⁻¹ *a*-naphthalene acetic acid (NAA) alone. A medium devoid of auxins was used as control. All media were adjusted to pH 5.8 with 0.1 M NaOH before gelling with 5.5 g agar l⁻¹ and autoclaved at 121 °C for 20 min. The cultures were maintained at 25 ± 2 °C under a 16/8 h (day/night) photoperiod provided by cool white fluorescent lamp with a light intensity of 4000 lux. The cultures were refreshed every 2 weeks. The data were collected after 4 weeks for the percent of adventitious roots induction and mean number of roots.

Adventitious Root Biomass Determination

For fresh biomass accumulation, fresh roots (2–2.5 cm in long and 100 mg per flask) were transferred aseptically into 250 ml Erlenmeyer flasks containing 20 ml of MS liquid medium supplemented with combinations of growth regulators, NAA (0, 0.5, 1 and 1.5 mg·l⁻¹) and IAA (0, 0.5, 1.0 and 1.5 mg·L⁻¹). The cultures were incubated on a rotary shaker at 80 rpm at 25 ± 2 °C under dark condition. 35 days later, these roots were carefully washed with sterile distilled water and pressed

gently on filter paper to remove excess water and finally weighted. Similarly, for dry biomass determination, roots were dried in an oven at 30 °C and finally weighted. Fresh and dry biomass of adventitious roots were expressed in gram/liter.

Determination of secondary metabolites content

The content of phenol was determined by the Folin-Ciocalteu method (Soland and Laima, 2001). For this purpose, 1 mL of 95% ethanol was added to 10 mg of dried hairy roots of powdered clones and control; the solution was then subjected to extraction for 48-72 hours. Upon centrifuging at 6000 rpm for 10 min, 0.5 mL of the sample extract solution was mixed with 0.5 mL of 95% ethanol. Then, 250 µl of diluted folin reagent (1:10) and 500 µL of 5% Na₂CO₃ were added to the extract, and the mixture was shaken thoroughly. Subsequently, the solution was diluted to 3 ml with distilled water and mixed well. After incubation at 23 °C in dark for 1 hour, absorbance spectra of the samples were recorded using a spectrophotometer (Jenway 6305, English) operated at 725 nm. Gallic acid standard curve was used to investigate the number of samples.

Flavonoids were measured using the Krizek et al. (1998) method. For this purpose, 2 g of dried root were weighed and mixed with 3 ml of acid ethanol. It was then passed through a filter paper and placed in a warm water bath at 80 °C for 10 minutes. After cooling the samples, the absorbance by spectrophotometer was read at 270, 300 and 330 nm in three wavelengths. The extinction coefficients formula was used to determine the amount of flavonoids.

Statistical analysis

All experiments were performed based on a completely randomized design (CRD). All of the experiments were replicated three times. The data were subjected to analysis of variance test. The means were compared using Duncan's multiple range tests at a 5% level of significance. The results were analyzed statistically using SPSS 16 software.

Results and Discussion

Adventitious root initiation by IAA

The MS supplemented with NAA and IAA

initiated adventitious roots and showed significant results at the 1% probability level (data not shown). The protrusions were formed from cut ends and veins after two weeks of inoculation (Figure 1). The results of the experiments showed that the highest root induction (72.5 percent), root number (4.75), and root branch (10.08) were exhibited by 1.5 mg/L IAA (Figure 2, 3, 4). Among different NAA levels, the highest root induction (88.88 percent) and root number (8.04) were observed in 1.5 mg/L NAA which was not significantly different

from 0.5 and 1.5 mg/L NAA. This hormone at concentration of 1.5 mg/L, induced the highest root branching (18.42 per explant) (Figure 2, 3, 4). Increasing NAA concentrations induced callus mediated root formation and produced a lower number of adventitious roots. By using IAA, adventitious roots were initiated, but the frequency and average number of roots initiated were lower when compared with NAA. The results demonstrated that NAA is a more effective treatment for triggering the induction of adventitious roots from explants of *C.intybus*.

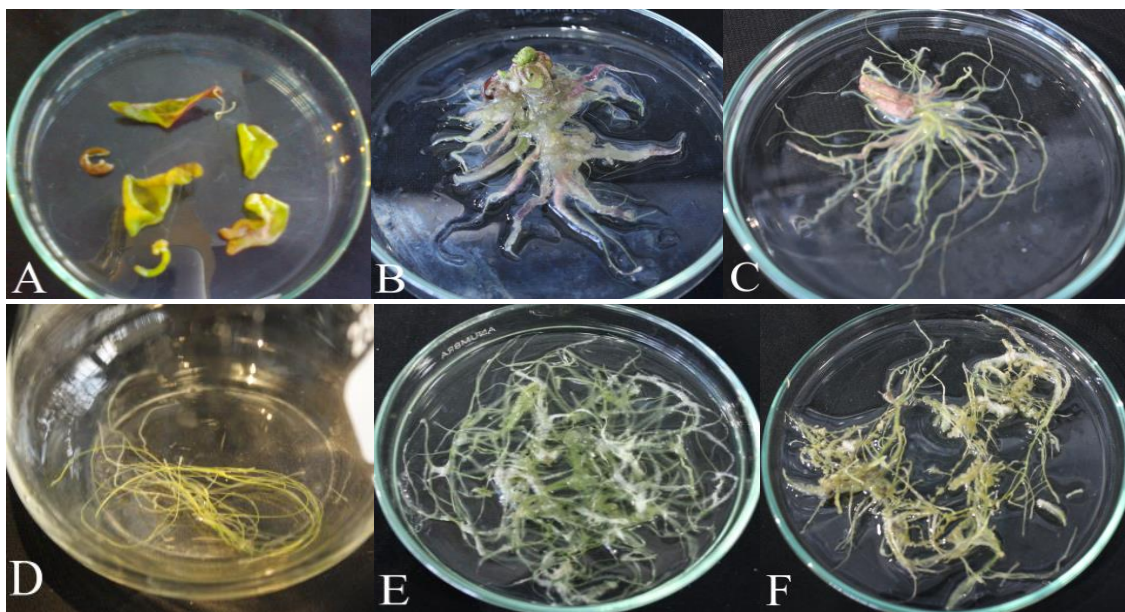


Figure 1. Effects of NAA and IAA on adventitious root induction, A: Without hormone, B: Adventitious root induction by 1.5 mg/L NAA, C: Adventitious root induction by 0.6 mg/L IAA. D: Control root culture, E: Adventitious root culture in NAA, F: Adventitious root culture in IAA

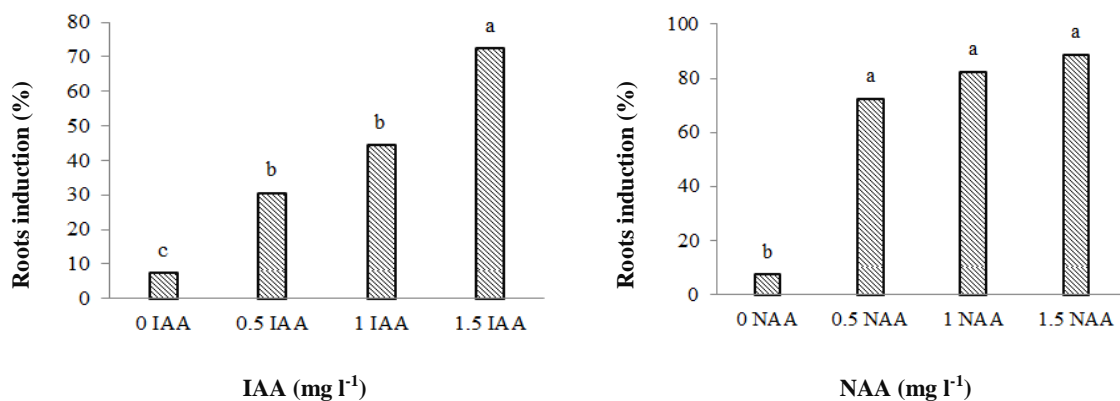


Figure 2. The effects of hormone on roots induction

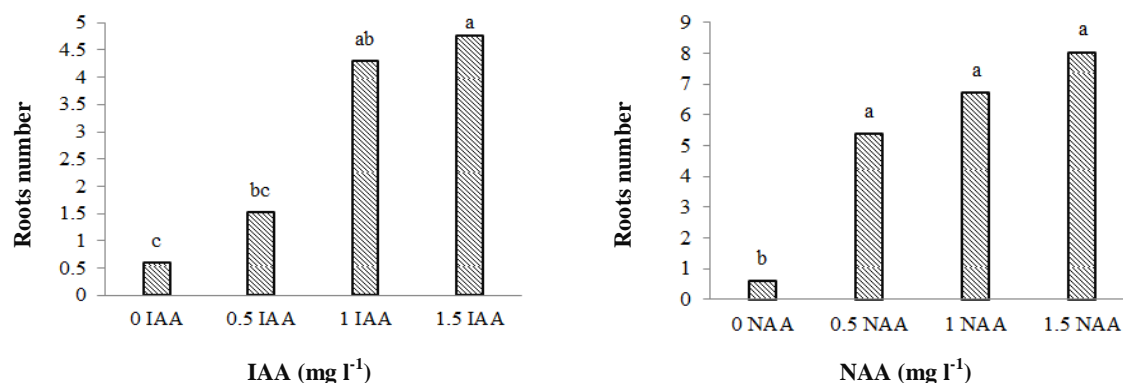


Figure 3. The effects of hormone on roots number

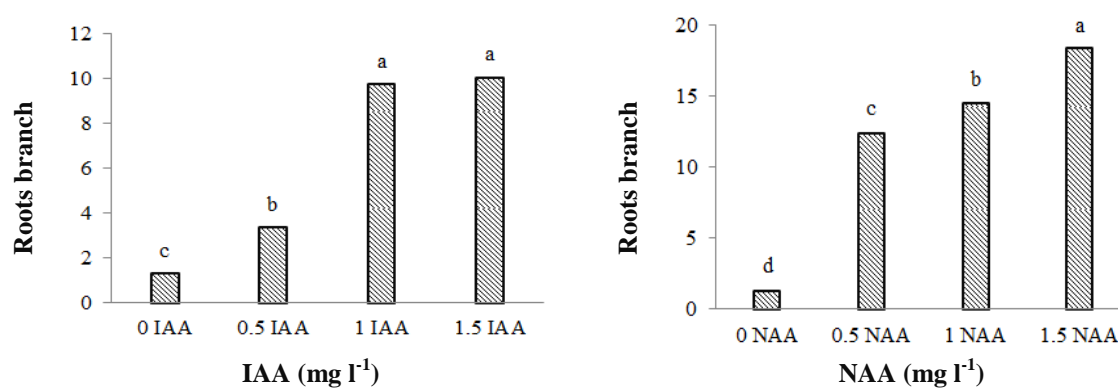


Figure 4. The effects of hormone on roots branch

The adventitious root induction technique has been applied to economically important plants for a secondary metabolite production, like *Panax ginseng* (Le et al., 2018), *Oplopanax elatus* (Han et al., 2019) *Taxus baccata* (Rowinsky et al., 1990), and *Iris germanica* (Akashi et al., 2005). Mainly, auxins are involved in most aspects of root development, therefore, the responses of various concentrations of auxins were tested for the purpose of root initiation and elongation. The vein-associated cells were stimulated and actively divided in response to auxins, which function as pluripotent stem cells, which can be switched into roots (Rose et al., 2006). Higher concentrations of NAA were shown to have the highest rooting efficiency. This could be explained by the fact that auxins at high concentration may possess an elicitor for cell elongation (Hussein et al., 2012). Many studies revealed that the addition of auxins liquid medium stimulates adventitious

root induction in high-valued medicinal plants. Lee et al. (2015) reported that lower concentration of NAA accelerates the adventitious root production in *Aloe vera*. Lee et al. (2011) observed that the addition of NAA and IAA to the medium induced adventitious roots in *Lycopersicon esculentum*. Hussein et al. (2012) found that the application of NAA is the best option to produce adventitious rooting in *Eurycoma longifolia*. Contrary to previous reports, in this investigation, different concentrations of NAA induced optimum adventitious rooting response in *Prunella vulgaris* L. liquid culture. Therefore, it is concluded that the addition of 1.0 or 1.5 mg l⁻¹ NAA ensures the best medium for adventitious root production in *P. vulgaris*

Adventitious Root Biomass Determination

An amount of 0.1 g of roots inoculum was inoculated against liquid medium containing different concentrations of NAA

and IAA (0, 0.5, 1.0 and 1.5 mg l⁻¹). This experiment showed significant results at the 1% probability level (Table 1). Maximum adventitious root biomass accumulation (0.74 g) was observed after 35 days of root culture in liquid medium containing 0.5 mg l⁻¹ of NAA in combination with 0.5 mg l⁻¹ of IAA. The addition of 1 mg l⁻¹ NAA and IAA also induced higher accumulation of biomass (0.65 g), but lower than 0.5 mg l⁻¹ of NAA and IAA. Other treated cultures also produced optimum biomass after 35 days (Figure 5). It shows that the production of adventitious root biomass in liquid culture is PGRs dependent. After 35 days, 0.5 mg l⁻¹ NAA and IAA treated culture showed a fourfold increment in biomass. The growth study of the roots in shake cultures showed that the dried weight of the adventitious roots increased rapidly in 35 days. The 0.062 g/L drymass was produced in MS medium supplemented with 0.5 mg l⁻¹ of NAA in combination with

0.5 mg l⁻¹ of IAA followed by 0.055 g/L on MS medium with 1 mg l⁻¹ NAA and IAA (Figure 5). Maximum growth index (6.51) was observed 0.5 mg l⁻¹ of NAA in combination with 0.5 mg l⁻¹ of IAA (Figure 6). Phenolic contents were estimated in harvested roots from different experiments. The highest average content was 4.1 mg/g DW in 0.5 mg l⁻¹ of NAA and 0.5 mg l⁻¹ of IAA followed by 1 mg l⁻¹ of NAA in combination with 0.5 mg l⁻¹ of IAA (3.98 mg/g DW) and 1 mg l⁻¹ of NAA and 1 mg l⁻¹ of IAA after 35 days of culture (Figure 7). The results of the flavonoid measurement showed significant differences between flavonoid content in the root resulted from the hormonal experiment in comparison with medium control. Flavonoid content in 270, 300 and 330 nm wavelengths was higher (60.26, 85.88 and 98.53 µg g⁻¹ DW) in the hairy roots obtained from 1 mg l⁻¹ of NAA in combination with 1 mg l⁻¹ of IAA (Table 2).

Table 1. Analysis of variance of the effect of NAA and IAA on roots growth and secondary metabolites

Source of variation	df	Means of square						
		Fresh weight	Dry weight	Growth index	Phenolic content	Flavonoid 270 nm	Flavonoid 300 nm	Flavonoid 330 nm
NAA	3	0.36**	0.002**	39.31**	1.97**	290.75**	647.15*	1177.59**
IAA	3	0.035*	0.0002**	4.19**	1.65**	466.32*	757.47*	777.53*
NAA × IAA	9	0.06**	0.0004**	6.97**	0.44**	371.42*	427.12*	790.82**
Error	32	0.009	0.0005	0.89	0.122	61.51	189.94	223.48

*, **: significant at 5% and 1% probability level.

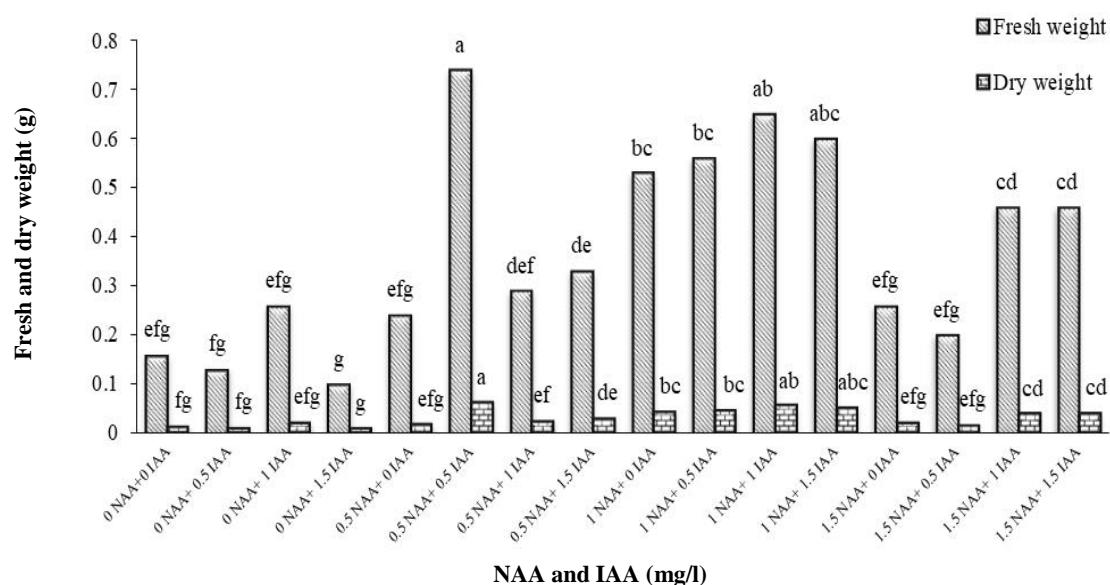


Figure 5. The effects of interaction between NAA and IAA on fresh and dry weight

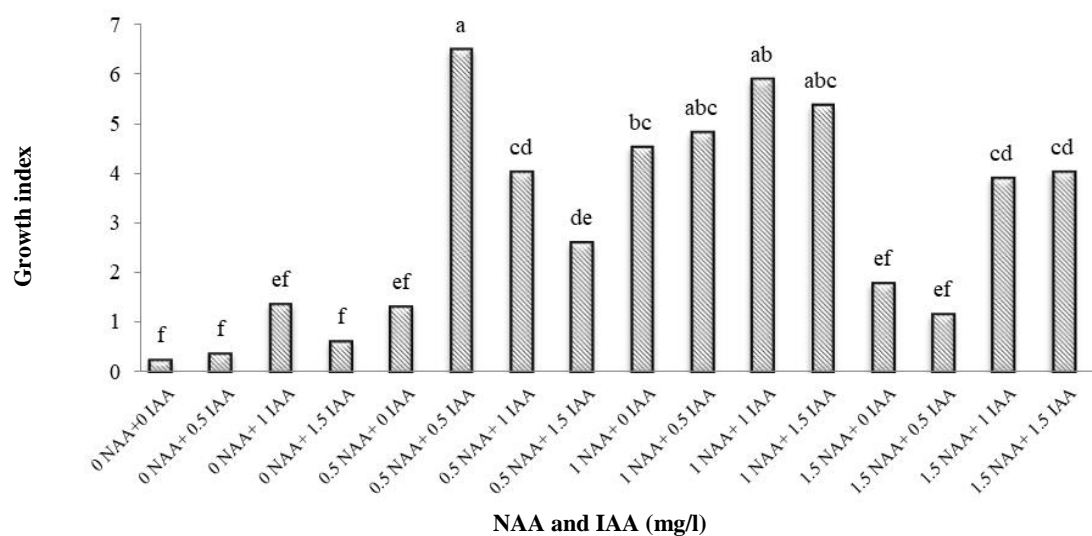


Figure 6. The effects of interaction between NAA and IAA on growth index

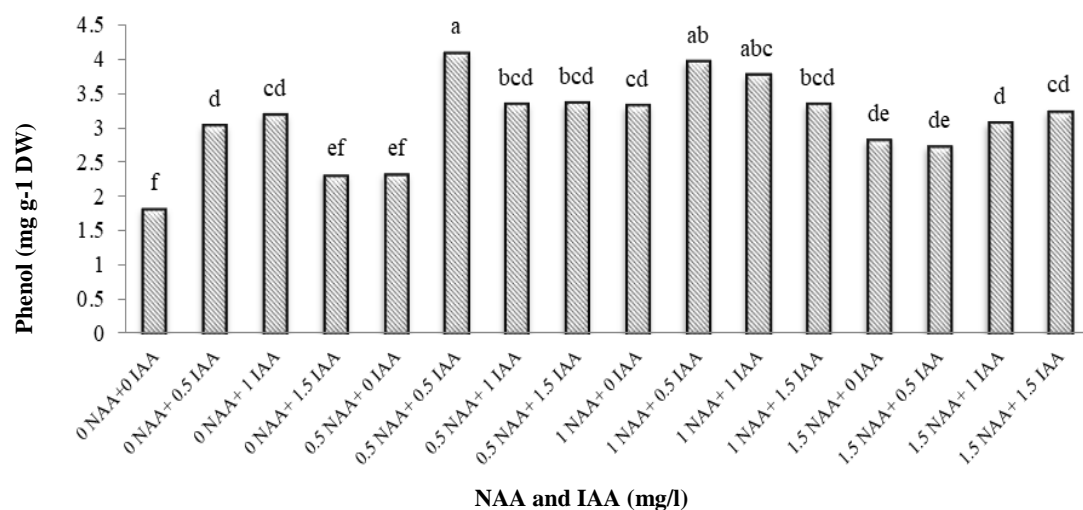


Figure 7. The effects of interaction between NAA and IAA on phenol content

Table 2. Mean comparisons of the effects of NAA and IAA on flavonoid content

NAA (mg l ⁻¹)	IAA (mg l ⁻¹)	Flavonoid 270 nm (µg/g DW)	Flavonoid 300 nm (µg/g DW)	Flavonoid 330 nm (µg/g DW)
0	0	29.9 ^{bcd}	38.12 ^c	44.35 ^{cde}
0	0.5	31.95 ^{bcd}	51.96 ^{bc}	57.06 ^{cde}
0	1	23.56 ^d	36.31 ^c	42.11 ^{de}
0	1.5	25.69 ^{cd}	34.33 ^c	41.09 ^e
0.5	0	24.56 ^d	37.52 ^c	54.66 ^{cde}
0.5	0.5	58.24 ^a	70.79 ^{ab}	92.22 ^{ab}
0.5	1	31.04 ^{bcd}	48.67 ^{bc}	58.54 ^{cde}
0.5	1.5	37.72 ^{bcd}	56.22 ^{bc}	55.62 ^{cde}
1	0	22.51 ^d	39.83 ^c	41.65 ^e
1	0.5	36.47 ^{bcd}	56.86 ^{bc}	60.27 ^{cde}
1	1	60.26 ^a	85.88 ^a	98.53 ^a
1	1.5	30.97 ^{bcd}	48.02 ^{bc}	54.61 ^{e-g}
1.5	0	40.58 ^{bc}	56.39 ^{bc}	72.36 ^{bc}
1.5	0.5	42.83 ^b	50.84 ^b	58.43 ^{cde}
1.5	1	54.14 ^{a-c}	49.38 ^{bc}	71.07 ^{bcd}
1.5	1.5	28.77 ^{bcd}	48.71 ^{bc}	63.67 ^{cde}

Numbers followed by the same letter are not significantly different ($P < 0.05$).

In this study different media were tested in order to optimize adventitious root growth and secondary metabolites accumulation because this either reduced or enhanced rooting behavior among different species (Cui et al., 2010). Medium content has an important regulatory role in repressing the transcription of photosynthetic genes (Sheen et al., 1999), also induced changes in growth, physiology and secondary metabolite content in adventitious roots (Baque et al., 2010). The protrusions were formed from cut ends and veins after two weeks of inoculation (Figure 3) and *In vitro* adventitious root culture showed a high rate of proliferation and active secondary metabolism (Hahn et al., 2003). Therefore, a substantial increase in phenol and flavonoid content was detected in the adventitious roots grown in MS medium supplemented with NAA in combination with IAA. The results are in agreement with earlier studies on *Lobelia inflata* (Yonemitsu et al., 1990), *Fagopyrum esculentum* (Lee et al., 2015), and *Withaniasomnifera* (Murthy et al., 2008). Adventitious-root cultures of *Iris germanica* on liquid medium accumulated 4-5 fold higher contents of isoflavone, aglycones, and glucosides in 3-week-old liquid cultures (Akashi et al., 2005). The results of root induction in *Papaver somniferum* showed that the concentration of 5.0 mg/mL of yeast extract elicitor had the most impact on the growth rate of the roots (Siahmansour et al., 2018). The present study was a description, drawing upon testing different auxins for *in vitro* secondary metabolites production from adventitious root cultures. The produced phenol content was 2.25 folds higher than that of the natural plant, depending on the medium content. The study will be helpful for commercial-scale production of secondary metabolites using bioreactors.

Phenol and Flavonoids are widely distributed in various plant organs, and their positive effects on human health are mostly

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- Ali, H., Khan, M. A., Ullah, N., & Khan, R. S. (2018). attributed to their antioxidant property (Lin and Du, 2018). Numerous studies have revealed that flavonoid and anthraquinone accumulation in root cultures are improved by hormones. For instance, Lee et al. (2015) confirmed that 50 μ M NAA is optimal for the enhancement of flavonoid production in adventitious root cultures of *Eleutherococcuskoreanum*. Hwang et al. (2013) also determined a maximum content of total flavonoids at 20 μ M MeJA and 5 μ M IBA with the adventitious root culture of *Codonopsis lanceolate*. In this regard, Perassolo et al. (2017) indicated that elicitation with 100 μ M NAA resulted in massive anthraquinone accumulation in the hairy root culture of *Rubiatinctorum*. These findings indicate that a suitable amount of NAA should be used in a culture system to maximize metabolite production. Our elicitation strategy involved treatment of *C.intybus* with NAA and IAA which produced the highest amount of total phenol and flavonoids.
- Conclusion**
- Adventitious roots obtained by different concentration of auxins are suitable tools for the production of plant secondary metabolites due to their genetic stability, and generally, they show fast growth rate. The present study described the protocol for adventitious root induction which could further be useful for the production of secondary metabolites and biomass. In this research the highest root induction and root number were observed in 1.5 mg/L NAA and the highest fresh weight and dry weight, and phenol were obtained in MS liquid medium containing 0.5 mg l⁻¹ NAA in combination with 0.5 mg l⁻¹ IAA.
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