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Effect of total nitrogen content and $\text{NH}_4^+/\text{NO}_3^-$ ratio on biomass accumulation and secondary metabolite production in cell suspension culture of *Salvia nemorosa*

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Abstract

Woodland sage (*Salvia nemorosa*) is an important native medicinal plant in Iran and is considered as a rich source of phenolic and flavonoids compounds. The present research was conducted with respect to the optimization of medium for biomass accumulation and secondary metabolite production in cell suspension culture of *S. nemorosa*. In this research the effects of total nitrogen content (0, 15, 30, 60, 90, and 120 mM) and $\text{NH}_4^+/\text{NO}_3^-$ ratio (0:60, 10:50, 20:40, 30:30, 40:20, 50:10, and 60:0) were studied in the Murashige and Skoog Medium on biomass growth, total phenolic, flavonoid and rosmarinic acid contents. The maximum accumulation of fresh biomass (294.80 g/l) and total phenolic content (76.61 mg GAE/g DW) was obtained in the medium supplemented with 90 mM nitrogen. The highest rosmarinic acid content (16.41 and 16.16 mg/g DW) was recorded in the medium containing 30 and 60 mM nitrogen. Increasing total nitrogen above 30 mM resulted in a decline in rosmarinic acid production. The ammonium to nitrate ratio also affected the biomass growth and secondary products accumulation. The highest fresh biomass accumulation (296.52 g/l), total phenolic content (87.30 mg GAE/g DW) and Rosmarinic acid content (18.43 mg/DW) were recorded in 10:50 ratio of $\text{NH}_4^+/\text{NO}_3^-$. Increasing the NH_4^+ level or complete elimination of it from culture medium reduced the rosmarinic acid and total phenolic content of *S. nemorosa*.

Our findings revealed that Woodland sage cell suspension needs both nitrogen forms, but ammonium is required at low concentration and nitrate at high levels. The results of the current study are beneficial for medium optimization for the establishment of large scale and bioreactor assisted cell culture of woodland sage for the production of phenolic acids.

Key Words: Ammonium, Nitrate, Rosmarinic acid, Suspension culture, Woodland sage.

INTRODUCTION

Woodland sage (*Salvia nemorosa* L.) is a perennial herbaceous species belonging to the Lamiaceae family and is widely distributed in diverse regions of Iran (Mahdieh *et al.*, 2018). It is an underestimated plant at present, but it may be a promising plant species for the future (Kaprinayak and Fari, 2019). *S. nemorosa* is traditionally employed in folkloric medicine to stop bleeding, treatment of stomach ache, diarrhea, hemorrhages, and furuncles (Božin *et al.*, 2012; Bahadori *et al.*, 2017). Like other *Salvia* species, woodland sage is a rich source of phenolic compounds, such as rosmarinic acid, caffeic acid and ferulic acid (Bahadori *et al.*, 2017; Bayat and Moghadam, 2019).

Secondary metabolites are a group of organic substances produced by plants in response to environmental stress, as a part of their defense mechanism. These compounds show diverse biological activities and are used as pharmaceuticals, agrochemicals, flavors, fragrances, colors, biopesticides,

and food additives in daily human life (Murthy *et al.*, 2014). Secondary metabolites are often produced at low levels (less than 1% dry weight), and their production depends significantly on the physiological and developmental stage of the plant (Oksman-Caldentey and Inze, 2004). The production of secondary metabolites via field cultivation of plants has various disadvantages, such as low yields, fluctuations in concentrations due to environmental and seasonal variations and lack of year-round production (Murthy *et al.*, 2014; Yue *et al.*, 2016). Therefore, plant cell cultures have emerged as attractive alternatives for the production of secondary products. It overcomes most of the barriers in the field production of secondary metabolites and provides a reliable system (Ochoa-Villarreal *et al.*, 2016; Efferth, 2019).

Several factors, such as composition of culture medium, exogenous plant growth regulators, precursor feeding, and elicitation, affect the biomass accumulation and yields of bioactive compounds in plant cell culture (Cui *et al.*, 2010; Praveen *et al.*, 2011). Plant tissue culture medium provides macro and micronutrients, carbon source, amino acids, and essential vitamins. It plays a crucial role in cell growth and the production of secondary metabolites by controlling enzyme activity, gene expression, and the shift between primary and secondary metabolic pathways (Karwasara and Dixit, 2011). Nitrogen is the most important macronutrient in the culture medium, which is supplied in the form of ammonium and nitrate. Total nitrogen concentration and its available forms in the culture media play a decisive role in the propagation and secondary metabolites generation (Ivanova and Staden, 2009; Sivanandhan *et al.*, 2015). Changes in secondary metabolite production rates through modification in total nitrogen content, ammonium to nitrate proportion and type of nitrogen source have been reported in *Orostachys cartilaginea* (Zhang *et al.*, 2017), *Withania somnifera* (Sivanandhan *et al.*, 2015), *Gymnema sylvestris* (Praveen *et al.*, 2011), *Calendula officinalis* L. (Legha *et al.*, 2011), *Pueraria tuberosa* (Karwasara and Dixit, 2011) and *Bacopa monnieri* (Naik *et al.*, 2010).

To our knowledge, despite the high medicinal value of *S. nemorosa*, no studies have been conducted on the production of secondary metabolites in this plant under *in vitro* condition. On the other hand, only a few reports have described cell suspensions from other *Salvia* species as a source of secondary metabolites. It is primarily because of their low metabolite yields, slow growth, and low biomass density (Marchev *et al.*, 2014). Thus, in the present study, we have investigated the effects of total nitrogen content and NH_4/NO_3 ratio on biomass accumulation and secondary metabolite

production in cell suspension culture of *S. nemorosa*.

MATERIALS AND METHODS

Plant material and establishment of suspension culture

The seeds of *S. nemorosa* were collected from Khansar, Isfahan Province (33° 10' 00" N, 50° 23' 00" W) and validated by Herbarium of Research Institute of Forests and Rangelands of Iran. Seeds were surface sterilized and placed on half-strength Murashige and Skoog (MS) basal media (Murashige and Skoog, 1962) without any hormones. Twenty-eight days after seed germination, adult and fully expanded leaves were excised from the plantlets and used as explant source for callus induction. For callus initiation, aseptic leaves were cut into 1×1 cm pieces and incubated on the MS medium supplemented by 16 μM 2, 4-D, and 8 μM BA and solidified by 5.8 g/l plant agar (Plant agar, Duchefa Biochemie, Netherlands). To obtain callus suitable for suspension culture, freshly initiated calli were subcultured three times by two-week intervals on the same medium (Figure 1-A and B). Suspension cultures were initiated from 10-week old friable calli by transferring 4 g to 250 ml Erlenmeyer flasks containing 100 ml of liquid MS medium amended by 16 μM 2, 4-D, and 8 μM BA, 3% sucrose. The pH of the culture medium was adjusted to pH 5.75 by adding 1 M HCl or 1 M KOH before autoclaving. The inoculated flasks were placed on a rotary orbital shaker at 110 rpm and incubated at 25±2 °C in the dark. The results of our earlier experiments (data not shown) indicated that after 20 days, the cultures entered deceleration phase, which is probably due to the reduction of nutrients in the medium. Thus, the cultures were subcultured every 20 days by transferring 20 ml of the culture into 80 ml of fresh medium. Sustainable fine suspensions were obtained after three subculture cycles. The initial inoculum density of each flask was about a 2.9 g/l fresh weight of cells.

Experiment 1

In this experiment effect of total nitrogen content on biomass accumulation and total phenolic, flavonoid and rosmarinic acid contents were evaluated. Cell suspension cultures were established as above and MS basal medium was manipulated with respect to total nitrogen content to 0, 15, 30, 60, 90 and 120 mM. To do this, the concentration of NH_4NO_3 and KNO_3 salts were modified in constant proportions.

Experiment 2

The object of the second experiment was to examine the effects of different ammonium to nitrate ratio on

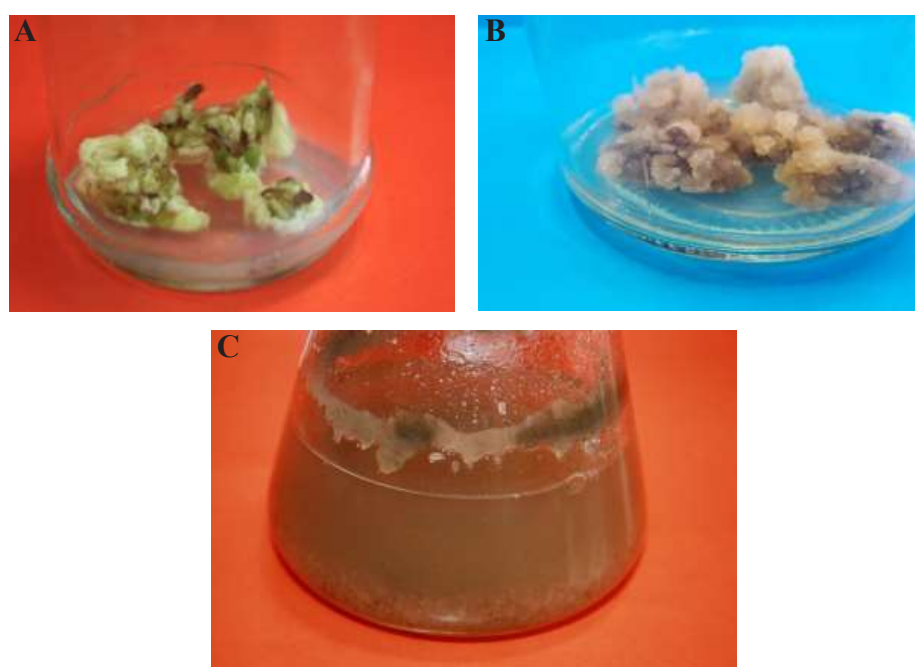


Figure 1. **A:** Callus initiation from leaf explants (14 days after inoculation), **B:** Callus proliferation (14 days old), **C:** Established cell suspension culture in *S. nemorosa* at deceleration phase (24 days).

biomass accumulation and total phenolic, flavonoid, and rosmarinic acid contents in the Woodland sage suspension culture. The ratio of $\text{NH}_4^+/\text{NO}_3^-$ in the MS medium containing 60 mM standard total nitrogen was manipulated to 0:60, 10:50, 20:40, 30:30, 40:20, 50:10, and 60:0 mM. To balance the ratio of ammonium to nitrate in the medium, NH_4NO_3 salt was excluded from the MS basal medium, and NH_4Cl was added as the source of ammonium.

Determination of cell biomass

Cells were separated from the liquid medium by filtration using Whatman No. 1 filter paper. The dry weight of the cells was recorded after drying at 60 °C for 48 h (Deepthi and Satheeshkumar, 2017). The dry biomass weight was used to determine the growth curve by sampling in individual flasks every four days. The specific growth rate was calculated according to Sujanya *et al.* (2008) by Equation 1:

$$(1) \quad \text{SGR} = \frac{(\text{maximum cell DW}) - (\text{Inoculum DW})}{(\text{Culture time in week}) \times (\text{Inoculum DW})}$$

Extraction

The harvested cells were oven-dried at 40 °C for 48 h and ground into a fine powder. About 500 mg of the powder was added to 10 ml methanol and extracted by sonication for 30 min at room temperature. The extracts were then filtered through Whatman no.1 filter papers. The solvent of the extracts was removed

by a rotary evaporator. Then, 1 mg of dried extract was dissolved in 1 mL methanol and kept for further analyses (Attaran Dowom *et al.*, 2017).

Determination of bioactive compounds

The total phenolic content (TPC) of samples was measured spectrophotometrically according to Wojdyło *et al.* (2007) method. Briefly, 100 µl of the above mention prepared extract was thoroughly mixed in a test tube with 2 ml water and 200 µl Folin-Ciocalteu reagent for 3 min, and the mixture was incubated with 1 ml of 20% (w/v) sodium carbonate solution at room temperature for one h. The absorbance of extracts was measured at 765 nm. The concentration of total phenol was expressed as mg of Gallic acid equivalent (GAE) per gram dry weight. Total flavonoid content (TFC) in the harvested cells was determined with the procedure described by Attaran Dowom *et al.* (2017). About 0.5 ml of methanolic extract was mixed with 1.5 ml of methanol, 0.1 mL of 10% (w/v) aluminum chloride, 0.1 ml 1 M potassium acetate and 2.8 ml of distilled water. The reaction mixture was allowed to stand at room temperature for 30 min. The absorbance of the mixture was measured at 415 nm against a blank without the extract. The content of total flavonoids was expressed as mg quercetin equivalent (QUE) per g dry weight. Rosmarinic acid content in samples was determined by a spectrophotometric method described by Öztürk *et al.* (2010). Briefly, the reaction mixture was prepared

by adding 200 μ l extract solution and 200 μ l zirconium (IV) oxide chloride solution to 4.6 ml ethanol. After 5 min, the absorbance was recorded at 362 nm against a blank reagent. Appropriate concentrations (0–18 ppm) of rosmarinic acid standard solution was prepared for the construction of calibration curve. Rosmarinic acid content in the extracts was expressed as mg per g dry weight.

Culture condition and statistical analysis

The initial inoculation density of cultures was adjusted to 3–3.5 g per 100 ml medium for all experiments. All the cultures were incubated at 25 ± 2 °C in the dark and kept under continuous agitation at 110 rpm in a rotary orbital shaker. Our earlier results demonstrated that the suspension cultures of *S. nemorosa* established in the MS medium entered the stationary phase after 28 days of inoculation. Thus, cells were harvested after 28 days, and fresh weight, dry weight, total phenolic, flavonoid and rosmarinic acid contents were measured. All Experiments were conducted in a completely

randomized design (CRD) with four replications. Each flask served as one replication. Statistically significant means were compared using Duncan's multiple range tests at $P \leq 0.05$. Data were analyzed using statistical programs SAS (Version 9.4).

RESULTS AND DISCUSSION

Experiment 1

The results of the analysis of variance showed that overall nitrogen levels significantly affected the fresh and dry biomass accumulation, total phenolic acids, flavonoids, and rosmarinic acid contents (Table 1). The growth curve (Figure 2) and specific growth rate curve (Figure 3) of cultures was drawn based on the biomass dry weight. The biomass growth of cell suspension cultures typically undergoes four stages, namely lag, exponential, deceleration, and stationary phases (Yang *et al.*, 2019). In the present study, cultures in all treatments except zero nitrogen concentration remained in the initial lag phase for 12 days, and then they entered

Table 1. Analysis of variance for effect of total nitrogen on biomass growth and bioactive compounds in cell suspension culture of *S. nemorosa*.

Source of variation	df	Mean of square				
		FW	DW	Total phenolic content	Total flavonoid content	Rosmarinic acid content
Treatment	5	35810.66*	372.49*	1669.87*	9.29*	14.03*
Errors	18	224.44	0.67	1.2064	0.17	10.58
Coefficient of variation (%)	-	8.16	7.56	6.06	9.10	7.10

*: represent significant difference at ($P \leq 0.05$).

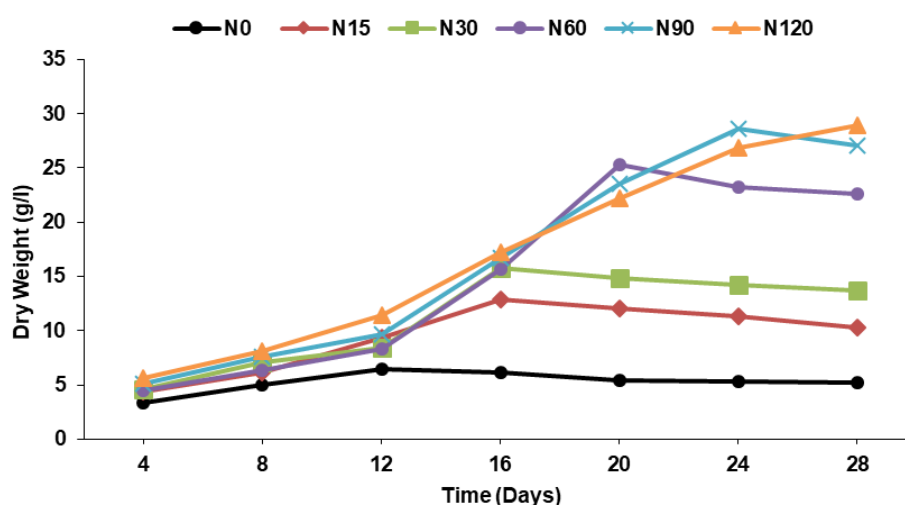


Figure 2. Pattern of Dry biomass accumulation during cell suspension culture of *S. nemorosa* in response to different nitrogen concentration.

the exponential phase. By increasing the total nitrogen concentration in the medium from 15 to 120 mM, the duration of the exponential phase also extended from 4 to 16 days (Figure 1). The maximum increase in dry biomass was reached on day 28, 24, 20, 16, and 16 for nitrogen concentrations at 120, 90, 60, 30 and 15 mM, respectively. Afterwards, the rate of growth was stable, followed by a gradual reduction in cell density and cultures entered the stationary phase except for the 120 mM nitrogen treatment. The exponential phase is a stage of cell suspension culture where the most proliferation and biomass accumulation of cells occur (Murthy *et al.*, 2014). Several factors such as nitrogen, phosphate, carbon source and plant growth regulators levels could influence cell division and proliferation in this phase (Ramachandra Rao and Ravishankar, 2002). Among these factors, nitrogen plays the most crucial role in biomass growth of cell and organ cultures, because it was found to affect the level of proteinaceous or amino acid products in cells that are crucial for cell division and proliferation (Ramage and Williams, 2002). Manipulation of the nitrogen level could be useful in increasing or decreasing the biomass accumulation in

the exponential phase (Murthy *et al.*, 2014; Yue *et al.*, 2016). The results of our study showed that an increase in nitrogen availability in the culture medium led to an increase in biomass accumulation by remaining in the exponential phase. This could be due to the more availability of nitrogen to the cells at this phase. Our findings revealed that total nitrogen content plays as a limiting factor in the biomass growth of woodland sage suspension culture. Increasing the total nitrogen concentration in the medium up to 120 mM resulted in enhancing fresh and dry weight in the cultures. However, the highest fresh weight (319.32 g/l) was recorded at 90 mM treatment, the maximum dry weight (28.91 g/l) was observed in the medium amended by 120 mM nitrogen (Table 2). Here an increase in dry weight was observed with a rise in the total nitrogen content from 90 to 120 mM, while the fresh weight decreased. This difference may be explained by the fact that at concentrations above 90 mM, the rate of cell division decreases, but cells utilize excess nitrogen to synthesize proteins and other macromolecules, thereby their dry matter content increases (Sujanya *et al.*, 2008; Naik *et al.*, 2010). The reason for the decrease

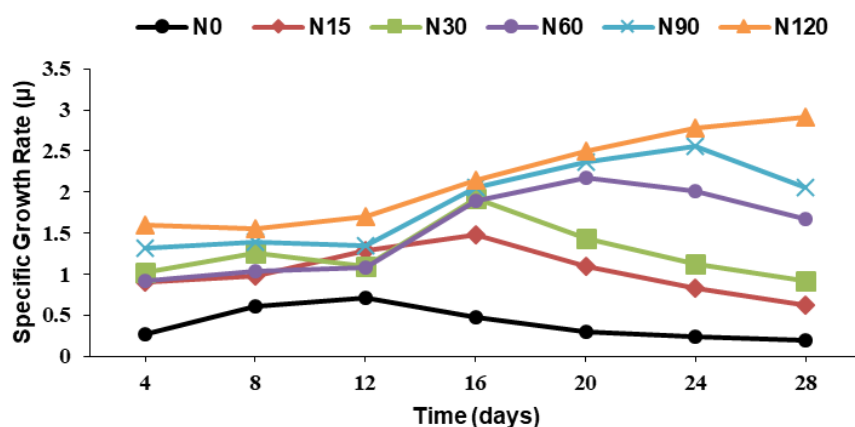


Figure 3. Specific growth rate of cell suspension culture of *S. nemorosa* in response to different nitrogen concentrations.

Table 2. Effect of total nitrogen concentration on biomass growth and bioactive compounds in cell suspension culture of *S. nemorosa* at the end of culture (28 days).

Nitrogen concentration (mM)	FW (g/l)	DW (g/l)	Total phenolic content (mg GAE/g DW)	Total flavonoid content (mg QUE/mg DW)	Rosmarinic acid content (mg/g DW)
0	76.10 ^e	5.22 ^f	25.62 ^f	2.26 ^d	11.36 ^e
15	147.34 ^d	10.26 ^e	33.17 ^e	3.67 ^c	13.75 ^d
30	192.71 ^c	13.72 ^d	51.75 ^d	4.23 ^c	16.16 ^a
60	272.16 ^b	22.76 ^c	62.46 ^c	5.11 ^b	16.41 ^a
90	319.32 ^a	27.02 ^b	76.61 ^a	6.07 ^a	15.36 ^b
120	294.80 ^b	28.91 ^a	70.22 ^b	6.24 ^a	14.80 ^c

Different letters in each column show significant differences according to the Duncan's multiple range test ($P \leq 0.05$).

in cell growth at low concentrations of nitrogen can be attributed to an increase in the production of lethal products and other limiting factors under nutrient deficiency that result in cell death (Praveen *et al.*, 2011; Sahraroo *et al.*, 2016). Also, the maximum specific growth rate of the cells (2.91 g/l per day) was observed in the medium supplemented by 120 mM nitrogen. Overall, the specific growth rate of the cells raised with increasing nitrogen concentration (Figure 2). It means that as the level of total nitrogen increases, more dry matter accumulates in the cells (Sahraroo *et al.*, 2016). Similar to our results, improvement in biomass accumulation in response to elevating nitrogen level have been reported in cell suspension culture of *Azadirachta indica* (Sujanya *et al.*, 2008), *Pueraria tuberosa* (Karwasara and Dixit, 2011), and *Gymnema sylvestre* (Praveen *et al.*, 2011).

An initial increase in total phenolic content (1.3-fold) and total flavonoid content (1.62-fold) was observed when nitrogen was supplied to the culture medium, with respective peak values of 76.61 mg GAE/g DW and 6.41 mg QUE/g DW on 90 mM and 120 mM treatments, respectively. Our results show that there is a direct correlation between biomass accumulation and TPC and TFC production. Raising the total nitrogen content in the culture medium resulted in increase in TPC and TFC through expanding biomass. Cultures grown on the medium containing 15 and 30 mM nitrogen, showed a 2.75 and 4.52-fold increases in rosmarinic acid production, respectively, which was significantly higher than the nitrogen-free medium. Rising nitrogen levels from 30 to 60 mM did not have a notable effect on the RA production, and a further addition in nitrogen concentration reduced the RA quantity in the cultures. Although the effect of source and level of nitrogen on the performance of secondary products accumulation in cell cultures has been reported, its precise role in

this field has not been clearly explained. It should also be noticed that the effect of nitrogen on secondary metabolites production is species-dependent and may exhibit different responses (Ilieva and Pavlov, 1999). Hakkim *et al.* (2011) reported that the content of RA in *Ocimum sanctum* suspension culture increased as cells grew and reached the maximum at the stationary phase. Similar results were reported by Sahraroo *et al.* (2016) in cell suspension of *Satureja khuzistanica*. The stationary phase in the cell culture is the stage when biomass growth ceases, but cells remain metabolically active. In most suspension systems, higher bioactive accumulation occurred in this phase (Krzyzanowska *et al.*, 2011; Jaishankar and Srivastava, 2017). In the present study, the cultures supplemented with 30 and 60 mM nitrogen entered the stationary phase (Figure 1), but cultures amended by 90 and 120 mM nitrogen remained in biomass accumulation state. Accordingly, the decrease in RA production at 90 and 120 mM nitrogen concentrations can be attributed to the growth status of the cultures. In an investigation on *Gymnema sylvestre* suspension culture performed by Praveen *et al.* (2011), a 50 percent reduction in the NH_4NO_3 concentration resulted in the highest (11.35 mg/g DW) gymnemic acid content. Yu *et al.* (2001) reported that lowering the concentration of NH_4PO_3 to half in *Panax ginseng* adventitious root culture led to improving ginsenoside accumulation. This finding is in agreement with our results.

Experiment 2

The results from the analysis of variance indicated that the relative amounts of ammonium and nitrate salts added to the culture medium had a significant influence on fresh and dry biomass accumulation, total phenolic acids, flavonoids, and rosmarinic acid contents in cell culture of woodland sage (Table 3). In the present research, when NH_4^+ concentration was reduced to a

Table 3. Analysis of variance for effect different $\text{NH}_4^+/\text{NO}_3^-$ ratio on biomass growth and bioactive compounds in cell suspension culture of *S. nemorosa* at the end of culture (28 days).

$\text{NO}_3^-/\text{NH}_4^+$ (mM)	FW (g/l)	DW (g/l)	Total phenolic content (mg GAE/g DW)	Total flavonoid content (mg QUE/g DW)	Rosmarinic acid content (mg/g DW)
60:0	267.74 ^b	29.79 ^a	74.20 ^b	6.68 ^a	17.64 ^b
50:10	296.52 ^a	27.46 ^b	87.30 ^a	6.14 ^b	18.04 ^a
40:20	259.11 ^b	21.76 ^c	66.71 ^c	5.37 ^c	16.30 ^c
30:30	232.37 ^c	18.56 ^d	57.62 ^d	44.20 ^d	15.28 ^d
20:40	198.20 ^d	12.89 ^e	47.60 ^e	3.75 ^e	13.17 ^f
10:50	164.94 ^e	10.32 ^f	36.22 ^f	3.34 ^{e†}	12.98 ^e
0:60	156.61 ^e	7.13 ^g	34.77 ^f	3.07 ^f	12.47 ^e

*: represent significant difference at ($P \leq 0.05$).

minimum of 10 mM and NO_3^- level was maximized to 50 mM, the highest accumulation of fresh biomass with 296.52 g/l was achieved. However, increasing nitrate concentration to 60 mM and elimination of ammonium from the culture medium reduced the fresh biomass, but the highest quantity of dry biomass (29.79 g/l) was recorded in this treatment. The specific growth rate in suspension cultures is depicted based on dry biomass accumulated during the time of culture (Sujanya *et al.*, 2008). As shown in Figure 4, the specific growth rate of cultures increased by increasing the nitrate and decreasing the ammonium levels in the medium. It means that *S. nemorosa* cell culture, out of the two available forms of nitrogen in the culture medium, prefers nitrate to produce dry matter. The growth curve of cultures (Figure 5) shows that at ratios 10:50 and 0:60 mM of $\text{NH}_4^+/\text{NO}_3^-$, the duration of

exponential phase in the cultures was extended more than traditional $\text{NH}_4^+/\text{NO}_3^-$ (20:40) balance in the MS medium. Also, in the treatments with 40:20, 50:10, and 60:0 ammonium to nitrate ratios, the duration of the exponential phase declined. These results suggest that nitrate levels in the *S. nemorosa* cell culture can act as a limiting factor for biomass growth. Ammonium and nitrate are the principal forms of inorganic nitrogen sources for both *in vivo* and *in vitro* growing plants. The ratio of the $\text{NH}_4^+/\text{NO}_3^-$ have been shown to markedly affect the biomass growth in the plant cell and organ cultures (Ivanova and Staden, 2009; Poothong and Reed, 2016). Praveen *et al.* (2011) reported that in cell suspension cultures of *Gymnema sylvestre* the highest accumulation of biomass was observed in the MS medium with $2 \times \text{KNO}_3$ which accumulated biomass of 15.30 g/l DW and the minimum amount of

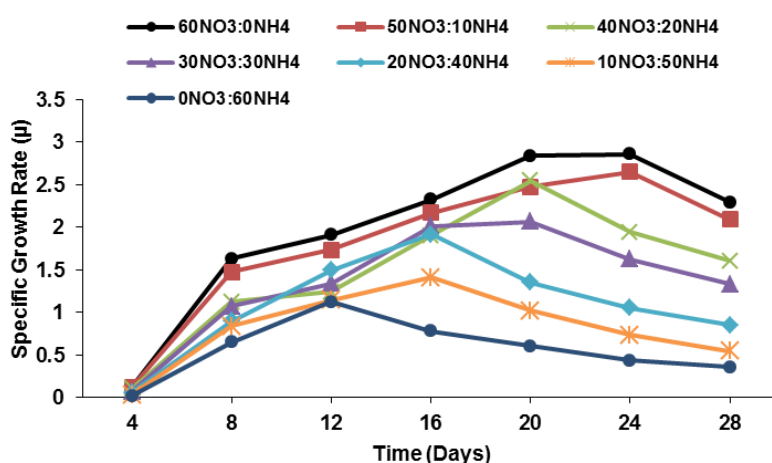


Figure 4. Specific growth rate of cell suspension culture of *S. nemorosa* in response to different ammonium to nitrate ratios.

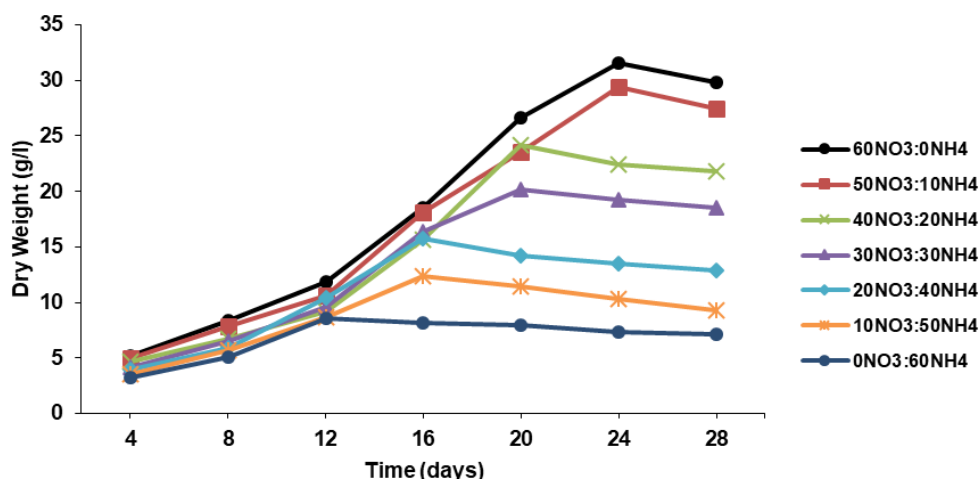


Figure 5. Pattern of Dry biomass accumulation during cell suspension culture of *S. nemorosa* in response to different ammonium to nitrate ratios.

biomass production was also recorded in the medium supplemented with $2 \times \text{NH}_4\text{NO}_3$. In another research on the suspension cultures of *Withania somnifera* conducted by Nagella and Murthy (2010), a two-fold increase in the KNO_3 and NH_4NO_3 concentration in the MS medium resulted in the highest and lowest biomass accumulation, respectively. Similar results were reported by Naik *et al.* (2010) on the adventitious shoot cultures of *Bacopa monnieri*. The reduction of nitrate to 1/2 or 1/4 with respect to the normal level in B_5 medium resulted in decreasing fresh weight in the cell culture of *Satureja khuzistanica* (Sahraroo *et al.*, 2016). Here are also several reports on the positive effect of raised $\text{NH}_4^+/\text{NO}_3^-$ ratio on enhancement of the adventitious shoot multiplication in *Aloe polyphylla* (Ivanova and Staden, 2009), *Malus hupehensis* (Dong *et al.*, 2015), *Rubus germplasm* (Poothong and Reed, 2016), *Withania somnifera* (Sivanandhan *et al.*, 2015). Our results are consistent with the mentioned reports.

The results of our study indicated that the inorganic nitrogen source treatments had significant effects on secondary metabolite production in cell culture of *S. nemorosa*. The maximum total flavonoid content (6.68 mg QUE/g DW) was found with 0:60 mM of $\text{NH}_4^+/\text{NO}_3^-$ ratio treatment, which was 1.2-fold higher than of the traditionally used ammonium to nitrate ratio (20:40 mM) in the MS medium (Table 4). Our findings revealed that when nitrogen was supplied only as a nitrate form to the woodland sage culture medium, the highest TFC was produced; this amount was significantly reduced by increasing the ammonium content in the culture. The highest production of total phenolic compounds and rosmarinic acid content was recorded in the medium supplemented by 10:50 mM $\text{NH}_4^+/\text{NO}_3^-$ with 80.30 mg GAE/g DW and 8.43 mg/ DW, respectively. Our results showed that with lowering ammonium concentration in the medium, the accumulation of TPC and RA improves, but when ammonium was eliminated from the medium, a decreasing trend was observed in

the production of these compounds. These outcomes suggest that the optimal culture medium for the production of secondary compounds with the phenolic structure in woodland sage culture should include high levels of nitrate with negligible levels of ammonium. Alteration of bioactive compounds production through modification in the ratio of ammonium to nitrate ratio in the culture medium is well defined in the literature. In agreement with our results, Sahraroo *et al.* (2016) demonstrated that both forms of mineral nitrogen are crucial for rosmarinic acid production in the flask culture of *Satureja khuzistanica*, but they have not the equal importance. In their study, the RA content declined when nitrate in the culture medium was reduced to 1/2 or 1/4 than the standard level in the B_5 medium. The importance of nitrate for the production of RA is highlighted by Ilieva and Pavlov (1999), who reported a twice enhancement in RA content followed by raising the 1.2-fold concentration of nitrate ions in the suspension culture of *Lavandula vera*. Also, Ilieva and Pavlov (1997) stated that the beginning of intensive biosynthesis of RA by *L. vera* cell culture was correlated with the ammonium ion limitation in the culture. Increasing the ammonium concentration in the tissue culture medium led to the acidification of the medium. The availability of most mineral nutrients to plantlets decreases when medium pH drops below five and therefore, their growth is restricted (Ivanova and Staden, 2009). Ammonium-induced changes in growth and development are related to alterations in hormonal balance. High levels of NH_4^+ can alter the internal content of auxin and cytokinin in plant tissues. Ammonium feeding has been shown to lead to a suppression of auxin content in plant tissues, while the highest levels of cytokinins are observed on $\text{NO}_3^-/\text{NH}_4^+$ mixtures, not on NH_4^+ alone. (Britto and Kronzucker, 2002). The positive effect of increasing the ratio of nitrate to ammonium on the *in vitro* production of secondary metabolites in other species, such as *Gymnema sylvestre* (Praveen *et al.*, 2011), *Calendula*

Table 4. Effect of different $\text{NH}_4^+/\text{NO}_3^-$ ratio on biomass growth and bioactive compounds in cell suspension culture of *S. nemorosa*.

Source of variation	df	Mean of square				
		FW	DW	Total phenolic content	Total flavonoid content	Rosmarinic acid content
Treatment	6	11441.48*	308.29*	1545.82*	11.98*	21.49*
Errors	21	102.45	0.42	0.47	0.17	0.17
Coefficient of variation (%)	-	8.49	9.60	8.18	8.29	7.90

Different letters in each column show significant differences according to the Duncan's multiple range test ($P \leq 0.05$).

officinalis (Legha *et al.*, 2011), *Pueraria tuberosa* (Karwasara and Dixit, 2011), *Withania somnifera* (Nagella and Murthy, 2010; Sivanandhan *et al.*, 2015), *Plantago lanceolata* (Gonda *et al.*, 2014), has also been reported in the support of our results. Contrary to our results, Naik *et al.* (2010) reported that maximum bacoside A content was obtained in 1:2 ammonium to nitrate ratio in cell culture of *Bacopa monnieri*. Rajasekaran *et al.* (1991) reported a twofold increase in Pyrethrin production in cultures of *Chrysanthemum cinerariaefolium* by complete elimination of nitrate in the medium. These reports indicated that the effect of different ratios of $\text{NH}_4^+/\text{NO}_3^-$ on the production of secondary metabolites varies depending on the plant species and the biosynthetic pathway of the metabolite.

In conclusion, our present research on cell suspension cultures of *S. nemorosa* revealed that both the biomass and secondary metabolite accumulation were affected by total nitrogen content and $\text{NH}_4^+/\text{NO}_3^-$ ratio in the culture medium. The maximum accumulation of fresh biomass and total phenolic content was obtained in the medium amended with 90 mM nitrogen. It was also shown that increasing the total nitrogen content above 30 mM had a negative effect on Rosmarinic acid production. Woodland sage cell suspension needs both nitrogen forms, but ammonium is required at low concentrations. The 5 : 1 ratio was obtained as an optimum balance of ammonium to nitrate for biomass accumulation, TFC, TPC and RA production. Increasing in NH_4^+ level or complete elimination of it from culture medium reduced the rosmarinic acid and total phenolic content of *S. nemorosa*. The findings of the current study are beneficial for medium optimization for the establishment of large scale and bioreactor assisted cell culture in woodland sage for the production of rosmarinic acid and other phenols and flavonoid compounds. Also, based on our findings, future research could be conducted for optimization of nitrogen to carbon ratio as well as the concentration of other macro nutrients such as potassium and calcium in order to achieve an economic protocol for the production of phenolic compounds in the *S. nemorosa* suspension culture.

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