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The Effect of Some Plant Growth Regulators on Cell Biomass in the Cell Suspension Culture of *Calendula officinalis* L. and *Calendula arvensis* L. Species

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Abstract: ***The cell suspension culture C. officinalis and C. arvensis plants having medicinal and economical important was carried out in the four different MS medium which supplemented with different concentrations of NAA:BAP (1:1, 0.5:5 mg/l) and IAA:BAP (1:1, 0.5:5 mg/l)under sterile conditions. For this purpose, four months old calli which grown in the callus cultures were passed into the cell suspension culture. In this cell suspension cultures, cell counting and measurement of fresh/dry weight process were realized for two Calendula species during 40 days. This process repeated once in a five days. All of the research results were evaluated with Tukey Multiple Comparison Test.***

Keywords: *Calendula officinalis*, *Calendula arvensis*, cell suspension culture, plant growth regulators

1. Introductıon

Calendula officınalıs (pot marigold) L., known for its ornamental plant characteristics, is a medicinal plant which is belonging to Asteraceae (Compositae) family. The species grows to 20 up to 40 cm height and has 20 varieties. Its flower appears yellow [1]. Its chemical constituents include triterpene glycosides, triterpene alcohols, flavonol glycosides, essential oil, polysaccharides and fatty oil [2]. Many studies have reported that the plant have pharmacological effects such as anti-cancer [3; 4; 5; 6], anti-microbial [7; 8; 9; 10; 11], anti-leishmanial [12; 13], anti-HIV [14], antioxidants [15;16;17], cytotoxic, anti-tumor [18; 3; 19], anti-viral [20], anti-inflamatuar [21; 19], oedema diuretic [22], hypoglycemic [23], uterotonic [24], lymphocyte activator effect [3], in venous ulcer treatment [25] and for biligenic function [26].

It was known that the in vitro cultures has some advantages. Some of this advantages is secondary metabolite production, the cell proliferation, etc. under sterile and controlled conditions during culture period in the laboratory [27].

The callus was defined the accumulation containing undifferentiated/unorganized parenchymatic cells that was occurred at the injured areas of undifferentiated cells and tissues [28]. The callus culture was state that the beginning of cell suspension culture [29]. It was clarified that the auxin and cytokinin amounts was important in the in vitro cultures [30]and the equal ratio of auxin/cytokinin was caused the callus formation [31]. It was explained for the in vitro culture that the different biotic (elicitation, etc.) and abiotic (plant growth regulators, metal ions, etc.) stimulating compounds added in the nutrient medium [32; 33; 34; 35].

The cell suspension culture can be prepared from explant from differentiated tissue or callus with undifferentiated cell mass [36]. Transition from undifferentiated structures to cell suspension cultures requires a shorter time compared to the transition from differentiated plant parts. Therefore, it is indicated that callus is preferred as starting material in cell suspension cultures [37]. This process is achieved by varying concentrations two major plant growth regulators (auxin and cytokinin) in the nutrient medium [38]. Under appropriate conditions, callus cells can continue to grow in the suspension cultures without differentiation [39].

The objective of myresearch was to evaluate the effect of different auxin:cytokinin combinations which added to MS medium to enhance cell biomass induction capability of C. officinalis and C. *arvensis* species in the cell suspension culture. Therefore, the cell suspension culture would be an important step to obtain medically important secondary metabolites containing of C. officinalis and C. *arvensis* species.In this research Ifocused on the establishment of the cell suspension cultures and development of optimal conditions in suspension cultures for cell biomass growth.

2. Experımental Work

2.1. Plant Material

In this research two species of Calendula were used as a plant material. Certificated seeds of C. *officinalis* and C. *arvensis* were bought from Ceylan Agricultural Company in Turkey.

2.2. MS Medium Prepearing

The MS mediums was prepared as ¼ strength and supplied with different combinations and concentration of auxin and cytokinin, pH adjustedto 5.80 and 8 g/l agar, 15g of sucrose were added to the MS medium[40]. The materials used in the laboratory and the MS nutrient mediums were autoclaved for 15 min.

In order to begin the cell suspension cultures, it was prepared the four different MS mediums which supplemented with the

different concentrations of auxin (NAA, IAA) and cytokinin (BAP)[41].

2.3. Calli Growth and Cell Suspension Culture

Calli growth were established as described from [41].After the 4th callus subculture 120 days old calli of C. *officinalis* and C. *arvensis* species were transferred to the cell suspension culture correlated with MS1, MS3, MS4, MS6 nutrient mediumAt the 0. culture initiation of cell suspension, the transfer of calli was performed as 1 g. into the 100 ml of erlenmeyer flask. The cell suspension cultures of two Calendula species were placed in the orbital shaker adjusted to 110 rpm (Table 1).

Table 1: The MS nutrient mediums used the cell suspension cultures

Medium	Auxin (mg/l)	Cytokinin (mg/l)
	NAA (mg/l)	BAP (mg/l)
MS1	1	1
MS3	0,5	5
	IAA (mg/l)	BAP (mg/l)
MS4	1	1
MS6	0,5	5

2.4. The Growth Parameters of Cell Suspension Cultures

It was determined the culture period reached the maximum cell viability and the maximum fresh/dry weight in the 0. culture of cell suspension. For this purpose, the cell counting and the measurement of fresh/dry weight was carried out. So, the cell suspension cultures were taken into the 1th subculture before the cell deaths began.

The cell counting was carried out to determine the cell viability in every 5 days during the 40 days from the 0. day of the cell suspension cultures of C. *officinalis* and C. *arvensis* species. 1 ml sample which taken from the cell suspension culture was colored with 1 ml of methylene blue and so the sample volume was completed to 2 ml. Cell counting were realized with Thoma chamber for the viability test. Thus, the average cell viability (%) was detected.

The measurements of fresh and dry weight was performed to determine the cell growth of the suspension cultures. For this purpose, the vacuum filtration system be composed of filtration pump (Lab 312, DrVAC-300), filtration flask and sieve with a pore size of 100 µm (Sartorius) was utilized.The once in every 5 days from 0. day during 40 days, it was filtered by the vacuum filtration system via milipore filter by taking 5 ml of sample between the 0. culture and 1th culture of cell suspension cultures. With this way, the fresh weight and the liquid medium was separated from each other.The fresh weight (g/l) was determined by weighting the cell weight remaining onto the filter on a precision scale. The fresh weight by isolating from the cell suspension cultures was dried by standing in the room temperature until the stable callus biomass was obtained (2 days). The results of measurements was recorded by weighting the drying cells on a precision scale. The measurement of fresh and dry weight was practiced in three repetition. The results of cell viability, fresh and dry weight measurement was evaluated with Tukey Multiple Comparison Test.

3. Results and Dıscussıon

3.1. Results of The Cell Suspension Culture

In the 0. culture of cell suspension culture, it was observed that the nutrient medium was clear, become blurred, turned to brown blurring at the 0., 15. and 30. day, respectively.Besidesit was observed that 0., 15. and 30. day of the cells was not seperated, seperated partially and seperated from each other almost completelyin the cell suspension 0. culture, respectively. At the end of cell suspension 4. culture (120 days), photography of the cell biomass in the MS1, MS3, MS4, MS6 nutrient mediums was showed at the Figure 1.

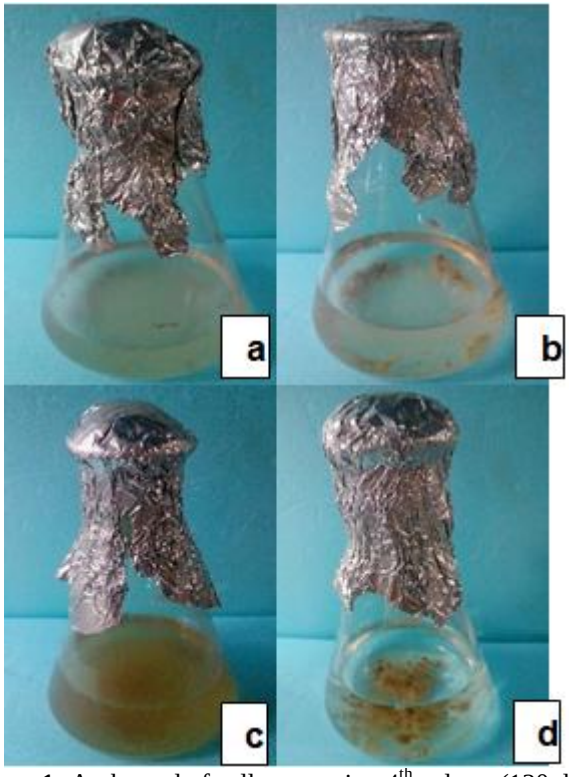

Figure 1: At the end of cell suspension 4th culture (120 days) a) MS1, b)MS3, c) MS4, d) MS6 nutrient mediums

Table 2. Descriptive statistics and Tukey Multiple Comparison Test results according to day, nutrient medium with regard to percent cell viability (%)

Day	Nutrient medium									
	MS6		MS4		MS3		MS1			
	$\bar{X} \pm S_{\bar{X}}$		$\bar{X} \pm S_{\bar{X}}$		$\bar{X} \pm S_{\bar{X}}$		$\bar{X} \pm S_{\bar{X}}$			
0. day	77.170 ± 1.320Cg		81.460 1± 1.340Bf		82.220 ± 0.290Bf		85.250 ± 1.080Af			
5. day	79.770 ± 1.180Cf		83.570 ± 1.190Be		84.730 ± 1.110Be		87.910 ± 1.040Ae			
10. day	82.440 ± 1.030De		84.615 ± 0.000Cde		86.616 ± 0.982Bd		88.709 ± 0.834Ae			
15. day	84.921 ± 0.892Dd		86.453 ± 0.806Cc		89.775 ± 0.758Bc		91.399 ± 0.641Ad			
20. day	89.412 ± 0.636Dbc		91.017 ± 0.543Cb		94.742 ± 0.516Bb		96.906 ± 0.438Ab			
25. day	90.863 ± 0.552Dab		93.843 ± 0.463Ca		96.906 ± 0.438Ba		98.589 ± 0.352Aa			
30. day	91.166 ± 0.534Da		94.095 ± 0.445Ca		97.099 ± 0.411Ba		98.686 ± 0.327Aa			
35. day	88.974 ± 0.662Dc		91.964 ± 0.487Cb		95.579 ± 0.435Bab		97.629 ± 0.336Aab			
40. day	82.090 ± 0.883De		85.960 ± 0.533Ced		89.724 ± 0.449Be		93.428 ± 0.337Ae			

Note 1. Differences between nutrient medium averages shown with different capital letters in the same day is important ($p \leq 0,05$).
Note 2. Differences between day averages shown with different small letters in the same nutrient medium is important ($p \leq 0,05$).

3.2. Results of The Growth Parameters of Cell Suspension Cultures

The cell counting, the measurement of fresh and dry weight was carried out during 40 days in the cell suspension 0. culture (40 days) of C. *officinalis* and C. *arvensis* species and determined the average cell viability (%) and the average fresh and dry weight (g/l) was specified in the cell suspension 0. culture.

Because of the alkaline feature of methylene blue, methylene blue was colored the asidic compartment of cell (the nucleic acid in the cell nucleus and the organelle of cell). According to this method, while the cells colored the light blue compared to the nutrient medium was counted as the alive cell, the cells colored the dark blue was counted as the dead cell.

Day X nutrient medium interaction of average cell viability (%) in the cell suspension culture (MS1, MS3, MS4, MS6 nutrient mediums) was indicated in Table 2.

Day X plant species of average cell viability (%) Tukey Multiple Comparison Test results in the cell suspension culture (MS1, MS3, MS4, MS6 nutrient mediums) was indicated in Table 3.

Table 3: Descriptive statistics and Tukey Multiple Comparison Test results according to day and plant species with regard to percent cell viability

Day	Plant Species	$\overline{X} \pm S_{\overline{X}}$
0. day	C. *officinalis*	82.890 ± 1.230Af
	C. *arvensis*	80.170 ± 1.050Bf
5. day	C. *officinalis*	84.960 ± 1.110Ae
	C. *arvensis*	83.030 ± 1.140Be
10. day	C. *officinalis*	86.352 ± 0.938Ad
	C. *arvensis*	84.837 ± 0.787Bd
15. day	C. *officinalis*	88.954 ± 0.937Ac
	C. *arvensis*	87.320 ± 0.871Bc
20. day	C. *officinalis*	93.571 ± 0.955Ab
	C. *arvensis*	92.468 ± 0.944Bb
25. day	C. *officinalis*	95.513 ± 0.931Aa
	C. *arvensis*	94.588 ± 0.935Ba
30. day	C. *officinalis*	95.701 ± 0.904Aa
	C. *arvensis*	94.822 ± 0.912Ba
35. day	C. *officinalis*	94.030 ± 1.030Ab
	C. *arvensis*	93.040 ± 1.060Bb
40. day	C. *officinalis*	88.380 ± 1.280Ac
	C. *arvensis*	87.220 ± 1.360Bc

Note 1. Differences between plant species averages shown with different capital letters in the same day is important ($p \leq 0,05$).
Note 2. Differences between day averages shown with different small letters in the same day is important ($p \leq 0,05$).

Results of the cell counting made, the average percentage cell viability was increased till the 25th day, reached a constant value between 25th and 30th day and was begun to decrease after 30th day in the cell suspension culture of C. *officinalis* and C. *arvensis*. It was revealed that the maximum of average percentage cell viability was acquired in the 30th day and from the MS1, MS4, MS3, MS6 nutrient medium, respectively. It was found that day X plant species X nutrient medium triple interaction p value is 0.9784 and it is not statistically significant.

The cell viability (%) graphs composed as regards to the cell counting in the cell suspension culture was indicated for C. *officinalis* and C. *arvensis* species (Figure 2-3).

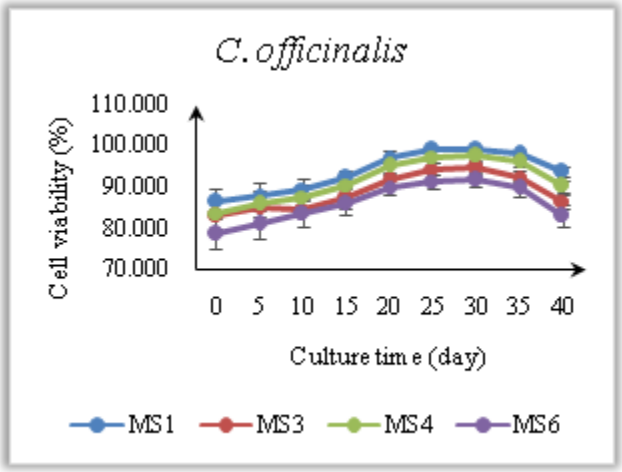

Figure 2: The cell viability (%) in the cell suspension culture of C. *officinalis* species

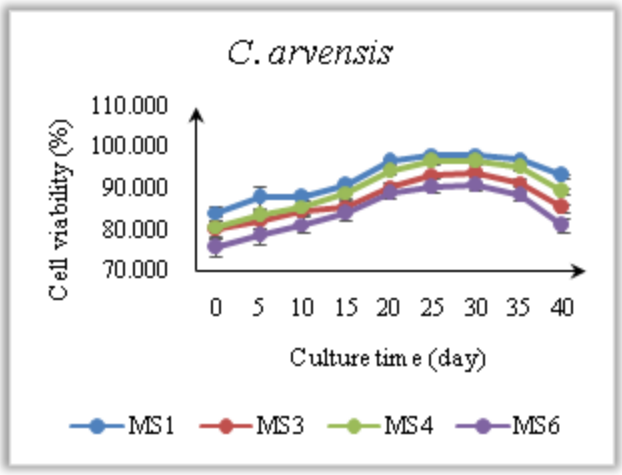

Figure 3: The cell viability (%) in the cell suspension culture of C. *arvensis* species

The fresh and dry weight was increased from the 0. day to the 30. day, reached to the maximum value on the 30. day and decreased till on the 40. day from this point. The maximum fresh and dry weight was gotten from the MS1, MS4, MS3 and MS6 nutrient medium, respectively.

It was decided that the fresh and dry weight of C. *officinalis* species in the MS1 nutrient medium of the cell suspension culture was to be 115.07 g/l (fresh weight) and 56.176 g/l (dry weight) on the 30th day, while decreased to 109.06g/l (fresh weight) and 49.349 g/l (dry weight) on the 35. day. Otherwise, it was confirmed that the fresh and dry weight of C. *arvensis* species in the MS1 nutrient medium of cell suspension culture was to be 112.07 g/l (fresh weight) and 56.286 g/l (dry weight) on the 30. day, while decreased to 107.21 g/l (fresh weight) and 54.244 g/l (dry weight) on the 35. day. It was found that plant species X nutrient medium X day triple interaction of fresh/dry weight (g/l) is significant statistically (Table 4-5). In terms of fresh weight, difference between all day averages in all plant species and all nutrient medium is statistically important ($p \leq 0,05$). That is, all of the average fresh weight in all days is different from each other. This wasn't lettered in the table (Tablo 4). In terms of dry weight, differences between day averages except for shown

romen numeral is statistically significant in the same nutrient medium and same plant species (p≤0,05) (Table 5).

Plant species X nutrient medium X day triple interaction of fresh weight (g/l) during the cell suspension 0. culture of C. *officinalis* and C. *arvensis* species was indicated in Table 4.

Table 4. Descriptive statistics and Tukey Multiple Comparison Test results according to day, plant species, nutrient medium with regard to fresh weight

Plant species	C. *officinalis*						C. *arvensis*					
Nutrient medium	MS6	MS4	MS3	MS1	MS6	MS4	MS3	MS1				
	$\bar{X} \pm S_{\bar{X}}$	$\bar{X} \pm S_{\bar{X}}$	$\bar{X} \pm S_{\bar{X}}$	$\bar{X} \pm S_{\bar{X}}$	$\bar{X} \pm S_{\bar{X}}$	$\bar{X} \pm S_{\bar{X}}$	$\bar{X} \pm S_{\bar{X}}$	$\bar{X} \pm S_{\bar{X}}$				
0. day	50.051 ± 0.001Aa	50.040 ± 0.004Aa	50.055 ± 0.002Aa	50.054 ± 0.001Aa	50.048 ± 0.003Aa	50.052 ± 0.007Aa	50.064 ± 0.010Aa	50.068 ± 0.019Aa				
5. day	53.051 ± 0.001Ca	54.046 ± 0.002Bb	54.058 ± 0.003Ba	55.057 ± 0.003Aa	53.056 ± 0.003Da	54.207 ± 0.161Ba	54.063 ± 0.003Ca	55.102 ± 0.003Aa				
10. day	57.064 ± 0.006Da	61.055 ± 0.006Ca	62.077 ± 0.003Ba	65.055 ± 0.005Ab	57.094 ± 0.007Da	61.058 ± 0.003Ca	62.048 ± 0.002Ba	65.168 ± 0.003Aa				
15. day	65.065 ± 0.006Da	73.055 ± 0.007Ca	74.070 ± 0.003Bb	80.052 ± 0.002Aa	64.058 ± 0.003Da	73.090 ± 0.003Ca	75.167 ± 0.006Ba	79.099 ± 0.004Ab				
20. day	78.050 ± 0.001Db	91.054 ± 0.003Ca	92.069 ± 0.003Ba	100.06 ± 0.001Aa	80.073 ± 0.002Da	90.034 ± 0.002Cb	91.054 ± 0.002Bb	100.06 ± 0.006Aa				
25. day	84.063 ± 0.005Db	98.053 ± 0.006Ca	102.07 ± 0.004Ba	110.06 ± 0.007Ab	88.059 ± 0.003Da	97.024 ± 0.003Cb	101.06 ± 0.002Bb	110.53 ± 0.001Aa				
30. day	86.064 ± 0.004Db	101.05 ± 0.005Ca	106.07 ± 0.004Ba	115.07 ± 0.006Aa	90.034 ± 0.003Da	99.056 ± 0.004Cb	104.04 ± 0.004Bb	112.07 ± 0.003Ab				
35. day	84.044 ± 0.004Db	95.043 ± 0.003Ca	99.085 ± 0.006Ba	109.06 ± 0.005Aa	85.272 ± 0.034Da	94.216 ± 0.001Cb	99.108 ± 0.002Ba	107.21 ± 0.003Ab				
40. day	72.039 ± 0.006Db	80.040 ± 0.004Ca	82.077 ± 0.003Ba	89.070 ± 0.005Aa	72.416 ± 0.002Da	79.206 ± 0.002Cb	82.098 ± 0.003Ba	87.108 ± 0.002Ab				

Note 1. Differences between nutrient medium averages shown with different capital letters in the same species and same day is important (p≤0,05).
Note 2. Differences between plant species averages shown with different small letters in the same day and same nutrient medium is important (p≤0,05).

Table 5. Descriptive statistics and Tukey Multiple Comparison Test results according to day, plant species, nutrient medium with regard to dry weight

Plant species	C. *officinalis*						C. *arvensis*					
Nutrient medium	MS6	MS4	MS3	MS1	MS6	MS4	MS3	MS1				
	$\bar{X} \pm S_{\bar{X}}$	$\bar{X} \pm S_{\bar{X}}$	$\bar{X} \pm S_{\bar{X}}$	$\bar{X} \pm S_{\bar{X}}$	$\bar{X} \pm S_{\bar{X}}$	$\bar{X} \pm S_{\bar{X}}$	$\bar{X} \pm S_{\bar{X}}$	$\bar{X} \pm S_{\bar{X}}$				
0. day	10.009 ± 0.003Aa	10.021 ± 0.004Aa	10.042 ± 0.003Aa	10.052 ± 0.003Aa	10.119 ± 0.003Aa	10.108 ± 0.004Aa	10.108 ± 0.003Aa	10.327 ± 0.003Aa				
5. day	11.079 ± 0.001Ca	12.021 ± 0.003Ba	12.071 ± 0.004Ba	13.040 ± 0.003Aa	11.111 ± 0.005Ca	12.052 ± 0.002Ba	12.175 ± 0.003Ba	13.269 ± 0.002Aa				
10. day	14.010 ± 0.002Ca	15.024 ± 0.003Ba	15.142 ± 0.004Ba	16.189 ± 0.002Aa	14.008 ± 0.002Ca	15.011 ± 0.003Ba	15.057 ± 0.002Ba	16.093 ± 0.003Aa				
15. day	22.220 ± 0.002Da	26.199 ± 0.002Ca	27.092 ± 0.002Bb	30.159 ± 0.001Aa	21.060 ± 0.002Db	26.287 ± 0.003Ca	28.178 ± 0.003Ba	29.840 ± 0.006Aa				
20. day	37.791 ± 0.005Db	45.727 ± 0.003Ca	47.537 ± 0.003Ba	50.193 ± 0.002Ab	39.029 ± 0.003Da	45.033 ± 0.002Cb	45.928 ± 0.004Bb	51.091 ± 0.004Aa				
25. day	42.428 ± 0.004Db	48.007 ± 0.003Cb	50.262 ± 0.002Bb	55.018 ± 0.003Ab	43.400 ± 0.003DaI	49.218 ± 0.002CaI	51.053 ± 0.026Ba	56.208 ± 0.001AaI				
30. day	43.085 ± 0.002Db	49.068 ± 0.002Cb	51.324 ± 0.002Bb	56.176 ± 0.002Aa	43.785 ± 0.083DaI	50.102 ± 0.041Ca	52.101 ± 0.011Ba	56.286 ± 0.045AaI				
35. day	34.182 ± 0.002Db	41.734 ± 0.003Cb	44.269 ± 0.005Bb	49.349 ± 0.004Ab	41.240 ± 1.330Da	49.197 ± 0.018CaI	50.328 ± 0.212Ba	54.244 ± 0.036Aa				
40. day	27.256 ± 0.016Db	33.528 ± 0.004Ca	36.569 ± 0.003Ba	43.210 ± 0.003Aa	27.837 ± 0.004Da	32.968 ± 0.004Cb	33.427 ± 0.004Bb	39.235 ± 0.015Ab				

Note 1. Differences between nutrient medium averages shown with different capital letters in the same species and same day is important ($p \leq 0,05$).
Note 2. Differences between plant species averages shown with different small letters in the same day and same nutrient medium is important ($p \leq 0,05$).

Plant species X nutrient medium X day triple interaction of dry weight (g/l) during the cell suspension culture of C. *officinalis* and C. *arvensis* species was indicated in Table 5.

The changing amount of fresh and dry weight of C. *officinalis* species was indicated in the Figure 4-5.

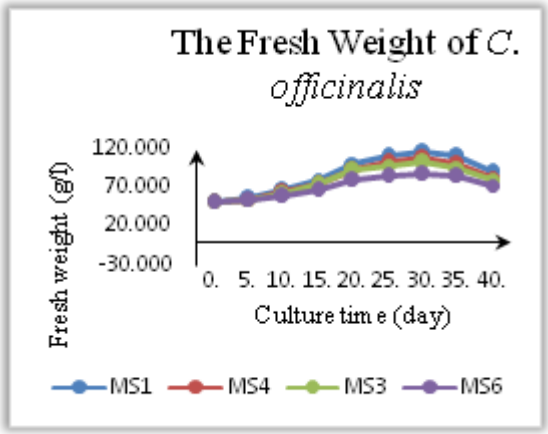

Figure 4: The fresh weight (g/l) in the cell suspension culture of C. *Officinalis*

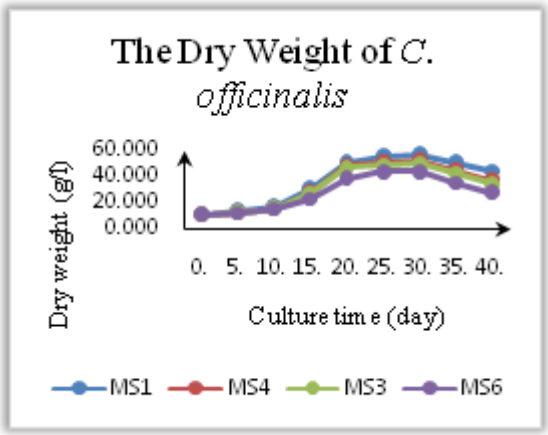

Figure 5: The dry weight (g/l) in the cell suspension culture of C.*officinalis*

The changing amount of fresh and dry weight of C. *arvensis* species was indicated in the Figure 6-7.

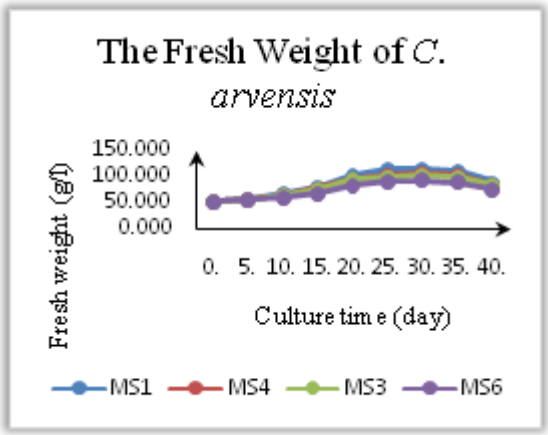

Figure 6: The fresh weight (g/l) in the cell suspension culture of C. *arvensis*

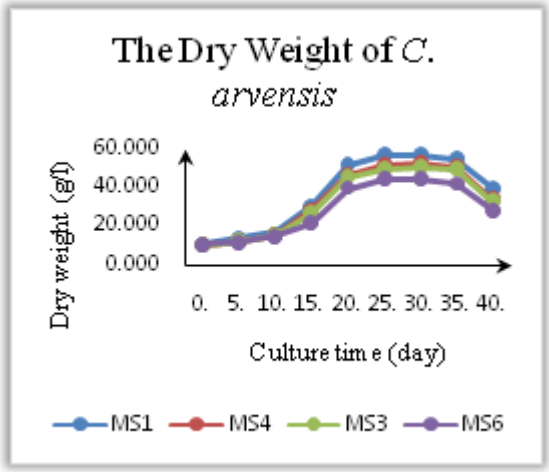

Figure 7: The dry weight (g/l) in the cell suspension culture of C. *Arvensis*

According to the measurement of cell viability (%), fresh and dry weight, it was proven that the cell suspension culture of C. *officinalis* and C. *arvensis* species (MS1, MS3, MS4, MS6 nutrient medium) was to be lag phase between on the 0.-5.day, log phase between on the 5.-25. day and the death phase on the 25.-30. day (the fresh and dry weight increased very little on the 25.-30. day). In reference to cell viability counting and the measurement of fresh/dry weight, it was determined that the cell viability and fresh/dry weight was begun to decrease after the 30th day. As a result of this, the subcultures was carried out by repeating in the every 30 days.

The cells in suspension culture can exhibit greater rates of cell division than cells in callus culture[42; 43; 44; 45; 46].To optimize biomass production of an important medicinal plant, C. *officinalis* and C. *arvensis*, I studied the impact of adding factors such as the plant growth regulators (i.e., NAA, BAP) on the growth of biomass of C. *officinalis* and C. *arvensis* in cell suspension culture at 5-day intervals during 120 days. Nguyen and Paek found that MS media containing 2,4-D is optimal for culturing P. ginseng in cell suspension culture. Thus, the results obtained in the present study are interesting because most cell suspension cultures of ginseng cells previously documented required 2,4-D, which is unsuitable for pharmaceutical and food industrial use due to its potency as an herbicide and a carcinogen. Generally, cytokinins and auxins promote cell division and cell expansionn in plant cell suspension cultures[47; 48; 49; 50].As general information, [51]reported that auxin and cytokinin are key regulators of plant secondary growth. A study by[52]also demonstrated the essential role of cytokinins in the cell cycle and primary metabolite formation in crop plant cell suspension cultures.

In contrast to [53], I established that the fresh and dry weight was increased and decreased with parallel to each other. I identified that the fresh and dry weight wasn't increased during the cell suspension culture (40 days). Otherwise, [53]was indicated that the fresh weigth was increased during the cell suspension culture (45 days). Consistent with my research,[53] was stated that the cells were died in the cell suspension culture after a while. Otherwise, [53] was defined that the fresh and dry weight was increased in the logarithmic phase up to the 20. day in the cell suspension culture done with C. *officinalis* cotyledon and hypocotyl. In contrast

to[53], I specified that the fresh and dry weight wasn't increased until the end of cell suspension culture (40 days).

Myresearch results were parallel with [54]in respect to the color of cell suspension cultures differentiated according to the culture days.Consistent with myresearch results, [55] reported that light conditions increase cell clustering in C. *officinalis* cell suspension cultures. It was determined that the cell amounts dispersed in the nutrient mediums and the size of aggregates increased during three months.

4. Conclusion

In conclusion, cell proliferation has been achieved from callus by administration of NAA, BAP and IAA to the cell suspension cultures.The growth parameters of the cultures were detected by determining the period in which cell suspension cultures of C. *officinalis* and C. *arvensis* species had the highest cell viability and the maximum cell weight.The most appropriate period for the production of excess secondary metabolite, which may be the next stage of my study, could be determined.Plant growth regulators, which may be a stress factor in the nutrient medium, are also associated with the amount of secondary metabolite.

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