SAKARYA ÜNİVERSİTESİ FEN BİLİMLERİ ENSTİTÜSÜ DERGİSİ
*SAKARYA UNIVERSITY JOURNAL OF SCIENCE*

**e-ISSN:** 2147-835X
**Dergi sayfası:** http://dergipark.gov.tr/saufenbilder

Geliş/Received
11.08.2016
Kabul/Accepted
21.11.2016

Doi
10.16984/saufenbilder.283920


# Effects of Salt Stress and Inoculation Ratios in Cell Cultures of *Rubia tinctorum* L.

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**ABSTRACT**

In this study, salt stress was implemented to callus and cell suspension cultures of *Rubia tinctorum* L. at 100 mM and 200 mM concentrations and the effects on fresh and dry weights (g), relative dry weight ratios, and alizarin and purpurin contents (mg/g dry weight) of biomass were determined. Due to an increase in salt concentrations, biomass obtained from callus and cell suspension cultures were decreased. In callus cultures, due to the low viability and proliferation rate of the callus cells, the biomass showed no significant increase. Alizarin and purpurin contents decreased as the salt concentrations in callus cultures increased, but amounts of these compounds increased in cell suspension cultures. Inoculation ratios were also found effective on fresh and dry weights, and alizarin and purpurin contents of the samples tested. 1:2 inoculation ratio caused higher alizarin and purpurin contents in cell suspension cultures, but biomass amounts were found higher at 1:4 inoculation ratio. The highest alizarin (3.183 mg/g) and purpurin (1.153 mg/g) contents were detected in the sample that had 1:2 inoculation ratios and was obtained from the medium at 200 mM NaCl concentration.

**Keywords:** alizarin, callus, cell suspension, purpurin, *Rubia tinctorum* L., salt stress.

# *Rubia tinctorum* L. Hücre Kültürlerinde Tuz Stresi ve Inokülasyon Oranlarının Etkileri

**ÖZ**

Bu çalışmada, *Rubia tinctorum* L. kallus ve hücre süspansiyon kültürlerine 100 mM ve 200 mM konsantrasyonlarda tuz stresi uygulanarak elde edilen biyokütlelerdeki yaş ve kuru ağırlıklar (g), bağıl kuru ağırlık oranları (mg/g kuru ağırlık) ve alizarin ve purpurin içerikleri üzerine etkileri belirlenmiştir. Tuz konsantrasyonundaki artışa bağlı olarak, kallus ve hücre süspansiyon kültürlerinden elde edilen biyokütle azalmıştır. Kallus kültürlerindeki hücrelerde düşük canlılık ve çoğalma oranına rağmen, biyokütle belirgin bir düşüş göstermemiştir. Kallus kültürlerinde tuz konsantrasyonu arttıkça alizarin ve purpurin içeriği azalmıştır, ancak bu bileşiklerin miktarı hücre süspansiyon kültürlerinde artmıştır. Yapılan denemelerde inokülasyon oranlarının yaş ve kuru ağırlık miktarı ve alizarin ve purpurin içeriği üzerine etkili olduğu tespit edilmiştir. Hücre süspansiyon kültürlerinde 1:2 inokülasyon oranı daha yüksek alizarin ve purpurin içeriği sağlarken, 1:4 inoküsyon oranında biyokütle miktarı daha fazladır. En yüksek alizarin (3.183 mg/g) ve purpurin (1.153 mg/g) içerikleri 1:2 inokülasyon oranında ve 200 mM NaCl içeren besin ortamından tespit edilmiştir.

**Anahtar Kelimeler**: alizarin, kallus, hücre süspansiyonu, purpurin, Rubia tinctorum L., tuz stresi.

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## 1. INTRODUCTION (GİRİŞ)

*Rubia tinctorum* L., commonly known as wild madder, is a perennial shrub of Rubiaceae family and mostly grows in Europe and Asia [1]. The main components are di- and trihydroxyanthraquinones, alizarin, purpurin and their derivatives, ruberythric acid (alizarin-primeveroside), pseudopurpurin, and lucidin-primeveroside [2]. The rhizomes and roots are used as natural dye sources, and the red color of madder comes from the alizarin component [3]. In Turkey, *R. tinctorum* L. rhizomes and roots are traditionally used as natural dyes in fabrics and handwoven carpets.

Because these components have been reported to exhibit various pharmacological and biological activities, including anticancer, antimalarial, antimicrobial, antifungal, antioxidant, bactericidal and spasmolytic activities, madder is also used in medicine [4]. Furthermore, due to their heat and light resistant characteristics, anthraquinones have potential use in the food processing industries [5]. In Turkey, *R. tinctorum* L. rhizomes and roots are traditionally used as natural dyes in fabrics and handwoven carpets [6]. *R. tinctorum* L. also contains flavonoids, compounds with antioxidative effects. Commercial importance and a need for renewable resources of valuable chemicals have lead to attempts in developing alternative systems for their production [7]. Anthraquinones have been obtained by *in vitro* cultures of Rubiaceae plant species. Different approaches to increase their production have been tested, ranging from media optimization to elicitation [8, 9, 10, 11].

To our knowledge, the effect of salt stress on *R. tinctorum* L. callus and cell culture systems have yet to be studied. The aim of this study was to determine the effects of salt stress on alizarin and purpurin productions in callus and cell cultures of *R. tinctorum* L. The interactions between biomass accumulation and secondary metabolite production were also investigated.

## 2. MATERIALS AND METHODS (MATERYAL VE METOD)

Alizarin (1,2-dihydroxy-9,10-anthraquinone) and purpurin (1,2,4-trihydroxy-9,10-anthraquinone) were obtained from Chromadex (Irvine, CA, USA). Acetonitrile and methanol of HPLC grade were purchased from Merck (Darmstadt, Germany). Pure water used in the analysis was prepared by using in-house nanopure water system (Sartorius Arium 611, Sartorius-Stedim, Goettingen, Germany). Ethyl acetate, sodium carbonate ($Na_2CO_3$), hydrogen chloride (HCl) and trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany).

*In vitro* grown *R. tinctorum* L. plantlets (Fig. 1a) were obtained from the Plant Cell and Tissue Culture Laboratory of Ege University, Faculty of Engineering, Bioengineering Department (Izmir, Turkey). *R. tinctorum* L. plantlets were subcultured in intervals of four weeks in MS [12] medium supplemented with 0,5 mg/l indole-3-butyric acid (IBA), 3% sucrose, and 0,7% agar. The pH of the medium was adjusted to 5,8 with 0,5 M NaOH and 0,5 M HCl. The medium was autoclaved at 121ºC, under a pressure of 1.2 kg/cm$^2$ for fifteen minutes. All cultures were incubated at 24±1ºC in the dark.

In the fourth week of cultivation, stem explants (approximately 0,5 - 1 cm in length), incised from internode parts of the sterile plantlets, were placed onto three semi-solid MS media in order to maintain the callus induction. This experiment was conducted with three repetitions and 20 stem explants were used in each repetition. These three media (Table 1) contained two different concentrations of NaCl (*control medium-M*: 0 mM NaCl; *M1 medium:* 100 mM NaCl; *M2 medium:* 200 mM NaCl) and were supplemented with 0,1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0,5 mg/l BA, 0,5 mg/l kinetin (6-furfurylaminopurine; KIN), 3% sucrose, and 0,7% agar. The pH of the media was adjusted to 5,8 and they were autoclaved and incubated as mentioned above.

Calli, grown on the explants, were placed onto the fresh medium and subcultured in intervals of 7 days to identify increases in fresh weight. Growth curves were generated with regards to these increases.

**Table 1:** Semi-solid media (M, M1, M2) compositions used for callus initiation.

| Semi-Solid Media | Basal Medium | Plant Growth Regulators and Contents | NaCl Content |
|---|---|---|---|
| M (Control) | MS | 0.1 mg/l 2,4-D<br>0.5 mg/l BA<br>0.5 mg/l KIN | 0 mM |
| M1 | MS | 0.1 mg/l 2,4-D<br>0.5 mg/l BA<br>0.5 mg/l KIN | 100 mM |
| M2 | MS | 0.1 mg/l 2,4-D<br>0.5 mg/l BA<br>0.5 mg/l KIN | 200 mM |

In order to identify the biomass production without subculturing, 1 g calli were placed onto M, M1 and M2 media. After four weeks, fresh weights of calli were recorded. To determine dry weights, calli were freeze-dried and their dry weights were also recorded**.** Considering the dry weight/fresh weight ratios (relative dry weight ratios) [13], water contents of the biomass was assessed and the quality of the samples were compared**.**

Two grams of calli, obtained from the semi-solid M medium on the fourth week of the culture, were

transferred to 50 ml of liquid M medium (*ML medium*), which had the same composition as the control medium-M without agar. After four weeks, liquid medium with cell clumps and single cells (Fig. 1d) were filtered through a 30-mesh sieve. Cell clumps, remained on the sieve, were freeze-dried. Filtrate, consisting of suspended cells, was diluted with the ratio of 1:2 and 1:4 using liquid ML, M1L and M2L media (compositions of the media are given in Table 2) to establish suspension cultures. After suspension cultures were established, living cell numbers per ml were counted in an interval of 7 days using Neubauer hemocytometer and Tripan Blue dye. The growth curve of suspension cultures was also identified to determine the growth characteristics of the living cells in liquid medium.

**Table 2:** Contents and inoculation ratio in ML, M1L and M2L liquid media.

| Media (sample codes) | Content of Media | Inoculation Ratio[A] |
|---|---|---|
| ML-A | M (NaCl-free) | 25/50 (1/2) |
| ML-B | M (NaCl-free) | 25/100 (1/4) |
| M1L-A | M1 (M + 100 mM NaCl) | 25/50 (1/2) |
| M1L-B | M1 (M + 100 mM NaCl) | 25/100 (1/4) |
| M2L-A | M2 (M + 200 mM NaCl) | 25/50 (1/2) |
| M2L-B | M2 (M + 200 mM NaCl) | 25/100 (1/4) |

[A]Inoculation Volume (ml) / Total Volume (ml)

Lyophilized (Christ Alpha 1-4 LD, Braun, Melsungen, Germany) and powdered madder samples were extracted four times for 10 min with 10 ml methanol using an ultrasonic device (Ultrasonic LC 30). Each extract was filtered, combined and evaporated to dryness under vacuum at 60°C (SpeedVac Concentrator, Savant SPD 121P, Thermo Scientific). The residues were hydrolyzed by refluxing with 10 ml of 5% HCl for 1 hour. All hydrolyzed samples were neutralized with 1 M $Na_2CO_3$ and extracted four times with 10 ml ethyl acetate. Each extract was combined and evaporated to dryness under vacuum at 60°C.

U-HPLC analysis was based on aglycones formed by hydrolysis of the corresponding glycosides: All of the obtained samples were dissolved with 10 ml methanol and then analyzed. Chromatographic separations were performed on a Thermo Hypersil C-18 100 µm × 2.1 µm × 3 µm column (Thermo Fisher Scientific Inc., Massachusetts, USA) using Accela U-HPLC system equipped with PDA detector, autosampler, quaternary pump and column oven (Thermo Fisher Scientific Inc., Massachusetts, USA). Chromatography was carried out using a mobile phase consisting of 0.01% TFA in water (solvent A) and acetonitrile (solvent B). The U-HPLC method is shown in Table 3. The flow-rate during the analysis was 750 µl/min and peaks were detected at 250 nm. Calibration curves were established by dissolving 10 mg alizarin and purpurin with 100 ml HPLC grade methanol and 6 level calibration curves were prepared by serially diluting this stock solution 2-fold with methanol (100–3.125 mg/ml). The correlation coefficient of the calibration curves, calibration range, limit of detection (LOD), limit of quantification (LOQ), and $R^2$ for alizarin and purpurin are reported in Table 3.

For estimation of fresh and dry weights of biomass, experiments were arranged in a randomized factorial design and each experiment was repeated three times in callus cultures and twice in suspension cultures. Each value is the mean±SE of the replications. Data was analysed with SPSS 17.0 and MSTAT-C using one way ANOVA and the post hoc tests were performed using LSD test at $p<0.05$.

**Table 3:** U-HPLC method used for alizarin and purpurin analyses and range, linear fit equation, LOD, LOQ and $R^2$ for alizarin and purpurin.

| Time (min) | A (%) | B (%) | Flow (µl/min) |
|---|---|---|---|
| 0.00 | 73 | 27 | 750 |
| 2.38 | 73 | 27 | 750 |
| 7.21 | 50 | 50 | 750 |
| 9.67 | 45 | 55 | 750 |
| 9.68 | 5 | 95 | 750 |
| 10.87 | 5 | 95 | 750 |
| 10.88 | 73 | 27 | 750 |
| 12.07 | 73 | 27 | 750 |

| Compounds | Range | Linear Fit Equation* | LOD | LOQ | $R^2$ |
|---|---|---|---|---|---|
| **Alizarin** | 3.125 - 100 | $y^A = 6.20847e-006\ x^B + 0.589354$ | 0.589354 | 1.768062 | 0.998595 |
| **Purpurin** | 3.125 - 100 | $y^A = 8.79204e-006\ x^B + 0.847002$ | 0.847002 | 2.541006 | 0.998995 |

[A] y: concentration (µg/ml), [B] x: area

## 3. RESULTS AND DISCUSSION (SONUÇLAR VE TARTIŞMA)

Callus formation was seen on the edges of stem explants on the fourth day of culture and became clearly visible at the end of the first week (Fig.1a). The color of the callus tissue was initially yellow (Fig. 1b) in the M (control) medium however, it turned to much darker hues of orange with prolonged culture period (Fig. 1c and 1d).

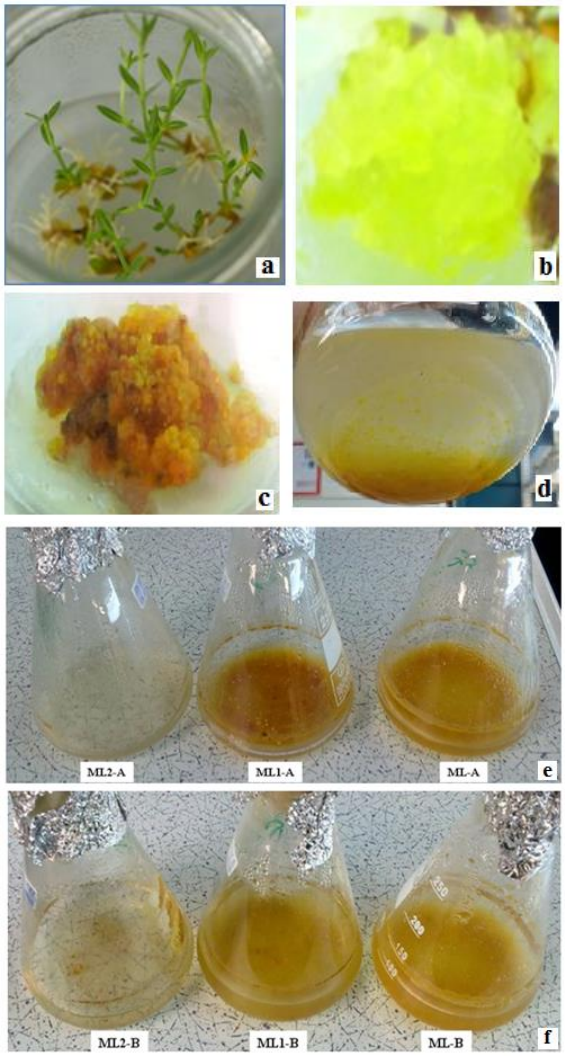


**Figure 1: (a)** *In vitro* grown *R. tinctorum* L. plantlets, **(b)** yellow callus initiated on stem explants, **(c)** orange-colored callus in M media on the fourth week of the culture, **(d)** suspension culture of *R.tinctorum* L. in the M media after four weeks, **(e)** Suspension cultures in ML2-A, ML1-A, ML-A and **(f)** ML2-B, ML1-B and ML-B.

Callus initiation percentages of stem explants were determined in M, M1 and M2 media. In M medium, callus initiation was observed on all stem explants (100 %). In M1 medium, callus initiation ratio was 57,78 % and in M2 medium only 6,67% of stem explants formed callus. In the growth curve of callus cultures (Fig. 2), it was observed that the growth rate was very high in the M medium. Between the 7$^{th}$ and 35$^{th}$ days, callus cultures showed the fastest growth rate. In the M1 medium, the biomass increased between the 7$^{th}$ and 21$^{st}$ days, and then became stable. In the M2 medium, the biomass remained the same for the whole culture period.

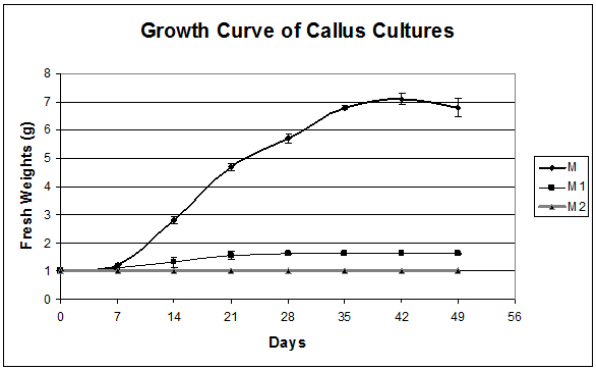


**Figure 2:** Growth curves of callus cultures in intervals of 7 days in semi-solid M, M1 and M2 media.

Due to the increasing salt concentrations, there was a decrease in the biomass obtained from unsubcultured callus cultures (Table 4), and the color of the biomass turned from orange to brown. Fresh weights and dry weights of callus cultures were also in reverse correlation with the increasing NaCl concentrations.

The highest biomass accumulation was observed in the M (control) medium (8,21 g fresh weight; 0,180 g dry weight). In the M1 medium, the biomass accumulation was found to be 1,80 g of fresh weight and 0,125 g of dry weight. In the M2 medium, no significant biomass increase was observed. Salt stress did not significantly affect the relative dry weight ratios in callus cultures, and the data shows that there was no quality difference due to the NaCl concentrations in the callus cultures.

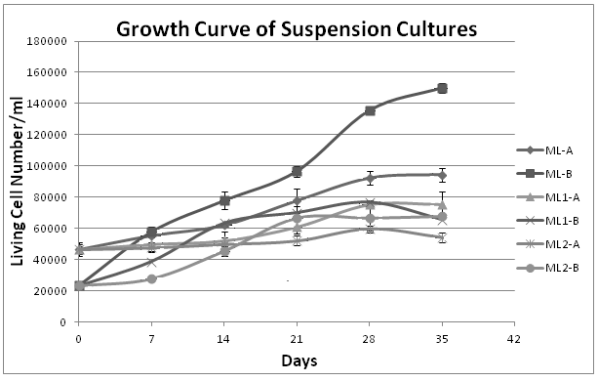


**Figure 3:** Living cell numbers per ml in liquid ML-A, ML-B, M1L-A, M1L-B, M2L-A and M2L-B media.

The growth curve of suspension cultures (Fig. 3) obtained from ML-A, ML-B, M1L-A, M1L-B, M2L-A and M2L-B media showed that the fastest increase in the number of living cells were observed in the ML-B medium (1:4 inoculation ratio). 1:4 diluted samples (ML-B, M1L-B and ML2-B) showed faster increase in

living cell numbers than 1:2 diluted samples (ML-A, M1L-A and ML2-A). Increasing NaCl concentration enhanced the coloration in suspension cultures (Fig. 1e and 1f**)**. The darkest cell biomass was observed in the ML2-A and ML2-B samples.

Increasing salt concentrations caused a decrease in biomass in suspension cultures (Table 5). 1:4 diluted samples, ML-B, M1L-B and M2L-B, had higher fresh and dry weights than ML-A, M1L-A and M2L-B, respectively. The highest fresh weight (96,40 g/L) was observed in the ML-B media, while the highest dry weight (7,430 g/L) was observed in the ML1-B media. Although no significant effect of increasing NaCl concentration on relative dry weight ratios was seen in callus cultures, NaCl concentration and inoculation volume has affected the water contents and quality of suspension culture biomass. ML1-B medium had the highest relative dry weight ratio (0,1626) amongst the obtained samples.

In U-HPLC analyses of callus cultures, NaCl concentrations were found effective on both alizarin and purpurin productions ($p<0.05$). The highest values of alizarin and purpurin were obtained from the M medium (2,87 mg/g and 0,43 mg/g dry weights). Increasing NaCl concentration caused decreases in both purpurin and alizarin amounts. There was also a positive correlation between the biomass decrease and alizarin and purpurin production in callus cultures.

As NaCl concentrations increased, the viability and proliferation ratios of callus cultures decreased (Table 4). In regards to the decrease in cell viability in the callus tissue, biosynthetic ability was also decreased as a result of salt toxicity, and alizarin and purpurin productions were lower in the salt-free medium.

**Table 4:** Fresh and dry weights of calli biomass in the three media, and their alizarin and purpurin contents on the fourth week of culture ($LSD_{FW}$ = 0.1672, $LSD_{DW}$ = 0.02825, $LSD_{RDW}$= 0.03629, $LSD_{Alizarin}$ = 0.02825, $LSD_{Purpurin}$ = 0.006318, $p<0.05$).

| Media | FW[A] (g) | DW[B] (g) | RDW[C] Ratio | Alizarin (mg/g DW) | Purpurin (mg/g DW) |
|---|---|---|---|---|---|
| **M** | 8.21±0.16 a | 0.180±0.02 a | 0.0219±0.002 a | 2.87 a | 0.43 a |
| **M1** | 1.80±0.21 b | 0.125±0.01 b | 0.0694±0.011 a | 1.98 b | 0.20 b |
| **M2** | 1.12±0.09 c | 0.052±0.01 c | 0.0464±0.006 a | 1.32 c | 0.12 c |

Values within the columns followed by small letters are significantly different at the 0.05 level by LSD's test.
Each value is the mean ± S.E. of 3 repetitions.
([A]FW: Fresh Weights; [B]DW: Dry Weights; [C]RDW Ratio: Relative Dry Weight Ratio)

**Table 5:** Fresh and dry weights, and relative dry weight ratios of suspension cultures and the alizarin and purpurin contents of the suspended cells ($LSD_{FW}$ = 0.8115, $LSD_{DW}$ = 0.1548, $LSD_{RDW}$= 0.1094, $LSD_{Alizarin}$ = 0.05626, $LSD_{Purpurin}$ = 0.02813, $p<0.05$).

| Liquid Media (sample codes) | FW[A] (g/L) | DW[B] (g/L) | RDW[C] Ratio | Alizarin (mg/g DW) | Purpurin (mg/g DW) |
|---|---|---|---|---|---|
| ML-A | 41.60±3.00 b | 5.810±0.112 b | 0.1406±0.0129 ab | 0.681 d | 0.102 d |
| ML-B | 96.40±5.42 a | 5.758±0.330 b | 0.0596±0.0001 cd | 0.860 c | 0.113 d |
| ML1-A | 25.60±2.60 c | 3.164±0.014 c | 0.1248±0.0122 abc | 0.864 c | 0.137 c |
| ML1-B | 46.40±4.61 b | 7.430±0.401 a | 0.1626±0.0248 a | 0.844 c | 0.069 e |
| ML2-A | 0.82±0.03 d | 0.028±0.007 e | 0.0339±0.0073 d | 3.183 a | 1.153 a |
| ML2-B | 1.46±0.34 d | 0.118±0.020 d | 0.0888±0.0345 bc | 2.915 b | 0.499 b |

Values within the columns followed by small letters are significantly different at the 0.05 level by LSD's test.
Each value is the mean ± S.E. of 2 repetitions.
([A]FW: Fresh Weights; [B]DW: Dry Weights; [C]RDW Ratio: Relative Dry Weight Ratio)

In U-HPLC analyses of samples obtained from suspension cultures, the interaction between NaCl concentrations and inoculation volumes were found to be effective on alizarin and purpurin productions (Table 5). The alizarin and purpurin contents of cell clumps that remained on the sieve were calculated to be 0,218 mg/g and 0,024 mg/g of dry weight (data not shown). Increasing NaCl concentration enhanced both alizarin and purpurin productions amongst the samples tested. The highest alizarin and purpurin productions were detected in the ML2-A sample (3,183 mg/g and 1,153 mg/g dry weights). In this sample, the effect of both salt stress and inoculation ratio caused 14,6 times higher alizarin and 48,04 times higher purpurin productions than the cell clumps that remained on the sieve. The ML2-B sample also showed high alizarin and purpurin contents (2,915 mg/g and 0,499 mg/g dry weights, respectively). Minimum alizarin and purpurin productions were obtained in the ML-A and ML1-B media, respectively. Although these contents were found to be minimum amongst the samples that were tested, alizarin and purpurin contents were 3,1 and 2,9 times higher than the contents of the cell clumps, respectively.

Salt stress, implemented at 100 mM and 200 mM concentrations, was found to be effective in both the callus and suspension cultures. In callus cultures, fresh and dry weights, and alizarin and purpurin contents were in reverse correlation with the rising NaCl concentration. Although fresh and dry weights decreased as a result of salt stress in suspension cultures, alizarin and purpurin contents increased significantly. Hussein et al. (2011) [14] reported that in callus cultures of fenugreek, growth and secondary metabolite accumulations decreased significantly as a result of high salt concentrations. According to Nazif et al. (2000) [15], salt stress led to reduction in biomass however, anthraquinone contents of cultured cells of *Cassia acutifolia* suspension cultures were enhanced. In *Stevia rebaudiana* callus and cell suspension cultures, growth was degreased as a result of salt stress and browning was also observed. Steviol glycoside concentrations increased in both callus and suspension cultures [16]. 150 mmol/L NaCl in MS medium was found the most suitable for hyperin and isoquercitrin accumulation in callus cultures of *Apocynum venetum* L. [17].

As a result of this study, salt stress implementation method for higher alizarin and purpurin production in *R. tinctorum* L. cell suspension cultures was found to be effective. This secondary metabolite production expanding method may be combined with other methods like elicitation, precursor addition, immobilization, etc. that enhance secondary metabolite accumulations. In this way, biomass with higher secondary metabolite contents will be produced.

## ACKNOWLEDGEMENTS (TEŞEKKÜR)

The authors are thankful to Prof. Dr. Erdal Bedir and İsmail Hakkı Akgün (Ege University, Turkey) for their kind support to this study.

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