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Effects of NaCl applications on root growth and secondary metabolite production in madder (*Rubia tinctorum* L.) root cultures

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Abstract: Madder (*Rubia tinctorum* L.) is a valuable plant rich in anthraquinones having dying properties and biological activities. This study was carried out to determine the effect of sodium chloride (NaCl) applications on the root growth and secondary metabolite accumulation in adventitious roots of madder. For this aim, adventitious roots derived from stem explants in *in vitro* conditions were cultured in MS medium containing different concentrations of NaCl (0, 1, 2, 3 and 4 g/l) for 7 days. Then roots were evaluated in terms of root growth index, total AQ, alizarin, purpurin and total phenolic contents. Based on the results, root growth decreased in line with the elevating level of NaCl while secondary metabolite accumulation significantly increased with NaCl applications compared to the controls. It was determined that NaCl at 3 g/l concentration was the most effective application in terms of total AQ, alizarin, purpurin and phenolic accumulation.

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1. INTRODUCTION

Plants are major sources of valuable secondary metabolites used in pharmaceutical, cosmetic, perfumery and food industries because of both their antioxidant, antimicrobial and biologic activities and their colour, flavor and fragrance properties [1]. In recent years involving commercial importance of secondary metabolites has increased the interest in alternative techniques such as cell and tissue and organ culture in the production of bioactive substances in plants [2]. As an alternative to traditional methods, plant tissue culture techniques play vital roles in the production of desirable compounds from plants [3]. Production of secondary metabolites by cell and tissue cultures has a lot of distinct advantages including independent from seasonal and geographical constraints, reliable, simpler and more predictable production compared to *in vivo* in the whole plant [1]. Moreover, *in vitro* secondary metabolite production also provides an important strategic advantage in order to remove the danger of extinction in the plants collected from nature.

Madder (*Rubia tinctorum* L.) is a perennial plant rich in anthraquinone (AQ) derivatives including alizarin and purpurin in its roots and rhizomes. AQs with biological activities and dye

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properties are very valuable compounds in the textile, food and pharmaceutical industries [4-9]. In madder, in order to obtain the highest amount of AQs, roots and rhizomes of 3 years old plants collected from nature are used. Continuously wild-collecting of plants from nature can cause the extinction of plant in the near future [10]. For this reason, *in vitro* secondary metabolite production ensures significant advantages not only provide short-term and high-yield production of the required AQs but also prevent the risk of plant extinction. Furthermore, it is possible to enhance secondary metabolite accumulation by applying some external applications to plant cells, tissues and organs in *in vitro* conditions. Salt is one of the most important external applications that can be applied to *in vitro* cultures to increase valuable bioactive metabolite accumulation. The previous studies indicated that salinity influenced the growth, secondary metabolite production and their composition in cell, tissue and organ cultures of different plants [11-15].

Phenolic compounds defined as organic metabolites containing benzene ring are also another valuable compounds used in food, cosmetic, perfumery and pharmaceutical industries Because of having antioxidant and biological activities [16].

This study was carried out to determine the effect of different concentrations (0, 1, 2, 3 and 4 g/l) of sodium chloride (NaCl) applications on growth index, total AQs, alizarin, purpurin and total phenolic contents in adventitious roots of madder under *in vitro* conditions.

2. MATERIAL AND METHODS

2.1. Plant Materials and Induction of Adventitious Roots

In this study, internode parts of shoots of three-year-old madder were used as plant materials. After washing tap water, shoots were surface-sterilized in 20% (v/v) commercial sodium hypochlorite solution supplemented with 0.1% Tween 20 for 10 min, then rinsed three times with sterilized water. For obtaining the adventitious roots from internode parts, the method of Kubota et al. [17] was used. Briefly, internode parts of shoots (1 cm long) were planted horizontally in MS medium [18] containing 20 g/l sucrose, 2 g/l gelrite agar, 2.5 mg/l indole acetic acid (IAA) and 0.1 mg/l kinetin and cultured 4 weeks. The induced adventitious roots were cultured in the MS liquid medium of the same composition for 4 weeks, then maintained in the MS liquid medium containing 30 g/l sucrose. Adventitious roots were subcultured two times using same media after every 4 weeks. Cultures were grown at 25±1 ºC in the dark and liquid cultures were agitating on an orbital shaker at 100 rpm.

2.2. NaCl Applications

About 250 mg of adventitious roots were transferred to 30 mL of MS liquid medium, containing 30 g/l sucrose, in 100 mL flasks and maintained at 25°C on a shaker (100 rpm) in a growth chamber under dark conditions. NaCl at 1, 2, 3 and 4 g/l concentrations was added to the root cultures at 7 days after inoculation. Control was supplemented distilled water as in stock solutions of NaCl. After 7 days, adventitious roots were harvested, washed with distilled water, weighed and used in the analyses. Experiments were performed in triplicate and three flasks were used for each replication.

2.3. Growth Index

The harvested roots were washed several times with distilled water, soaked with tissue paper to remove the surface water. The growth index of the roots was calculated using the following equation:

$$\text{Growth index} = \frac{(\text{fresh weight of harvested roots} - \text{fresh weight of inoculated roots})}{\text{fresh weight of inoculated roots}}$$

2.4. Determination of AQs

AQs were extracted from the roots by the method of Schulte et al. [19]. Briefly washed roots were dried at 50 ^{o}C until a constant weight and dried root samples were ground into a fine powder using a mortar and pestle. Samples were extracted twice with boiling 80% ethanol until the tissues were colorless. The ethanol fractions were pooled and filtered by using 0.45 µm Whatman micro filters.

The quantitate analyses of total AQ, alizarin and purpurin in the roots were done by using a PG Instruments spectrophotometer (T70 Plus Dual Beam/ Arlington, USA). The content of total AQ was calculated from the absorbance values of the extract at 434 nm, using a molar extinction coefficient of alizarin (ε434=5.5) [19]. The absorbances of alizarin and purpurin at 572 and 516 nm, respectively were measured and their amounts were calculated by the calibration curves of the standard compounds Alizarin and purpurin contents were performed by the method of Shin [20] and expressed as mg/g DW. Data presented are the average of three measurements.

2.5. Determination of Total Phenolic Content

Total phenolics of the root samples were extracted twice with 70% ethanol containing 0.2% hydrochloride acid in an ultrasonic water bath. Total phenolic contents were determined spectrophotometrically according to the Folin-Ciocalteu colorimetric method [21], calibrating against gallic acid standards and expressing the results as mg gallic acid equivalents (mg GAE/g DW). Data presented are the average of three measurements.

2.6. Statistical Analyses

Data were performed by using analysis of variance (ANOVA) using SPSS 16.0 for Windows Software Package and the means were separated by Duncan's multiple range tests.

3. RESULTS AND DISCUSSION

The results pertaining to the effect of NaCl on growth index of the adventitious roots under salt stress during the culture period of 7 days is as shown in Figure 1. The growth index significantly decreased in parallel with the increase in the salt concentrations (p<0.05). The highest growth index was found in the control roots as 0.36 while the lowest value (0.21) was obtained from the roots treated with 4 g/l of NaCl. Based on the results it can be noticed that NaCl applications had negative effect on the adventitious root growth of madder. Similarly, as reported before by Nartop et al. [15], the stress induced by salt reduced biomass of callus and suspension cultures in madder. Previously reported studies have shown that increased NaCl applications decrease the growth of *in vitro* cultures in several plants, such as Fenugreek calli, Cassia acutifolia and Panax ginseng [11, 22, 23]. The excessive intake of NaCl causes reduction of growth, even cultures can lose their survival. This may be due to the increased osmotic pressure, specific ion toxicity and ionic imbalances as mentioned previously by Munns [24]. Because NaCl stress lead to cellular dehydration, which causes osmotic stress and removal of water from the cytoplasm to vacuoles [25].

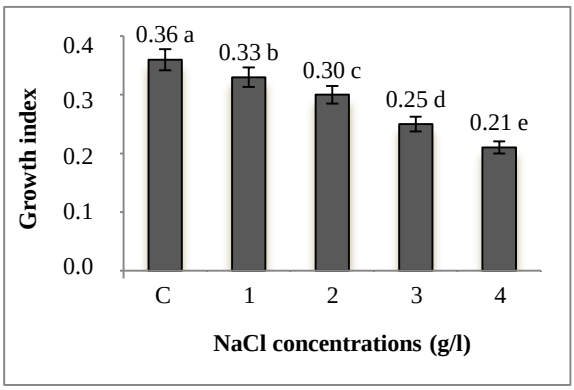

Figure 1. Effect of NaCl applications on the growth index of roots.

Amount of alizarin, an AQ derivative, also changed depending on the NaCl applications. All NaCl applications were significantly increased the alizarin accumulation in adventitious root cultures compared to control roots (p<0.05). As shown Figure 2(b), the highest alizarin content (3.79 mg/g) was found in 3 g/l of NaCl treated roots. The application of NaCl also improved the content of purpurin, another important AQ derivative, significantly (Figure 2(c)) and the highest purpurin content was determined in the roots treated with 3 g/l of NaCl as 0.69 mg/g. When NaCl concentration increased to 4 g/l, purpurin content decreased according to that of 3 g/l of NaCl. Stress applications significantly enhanced the total phenolic contents as compared to controls (Figure 2(d)). Maximum phenolic content (26.82 mg/g) was measured from the roots raised under 3 g/l of NaCl while the lowest level (13.19 mg/g) was obtained from the control roots.

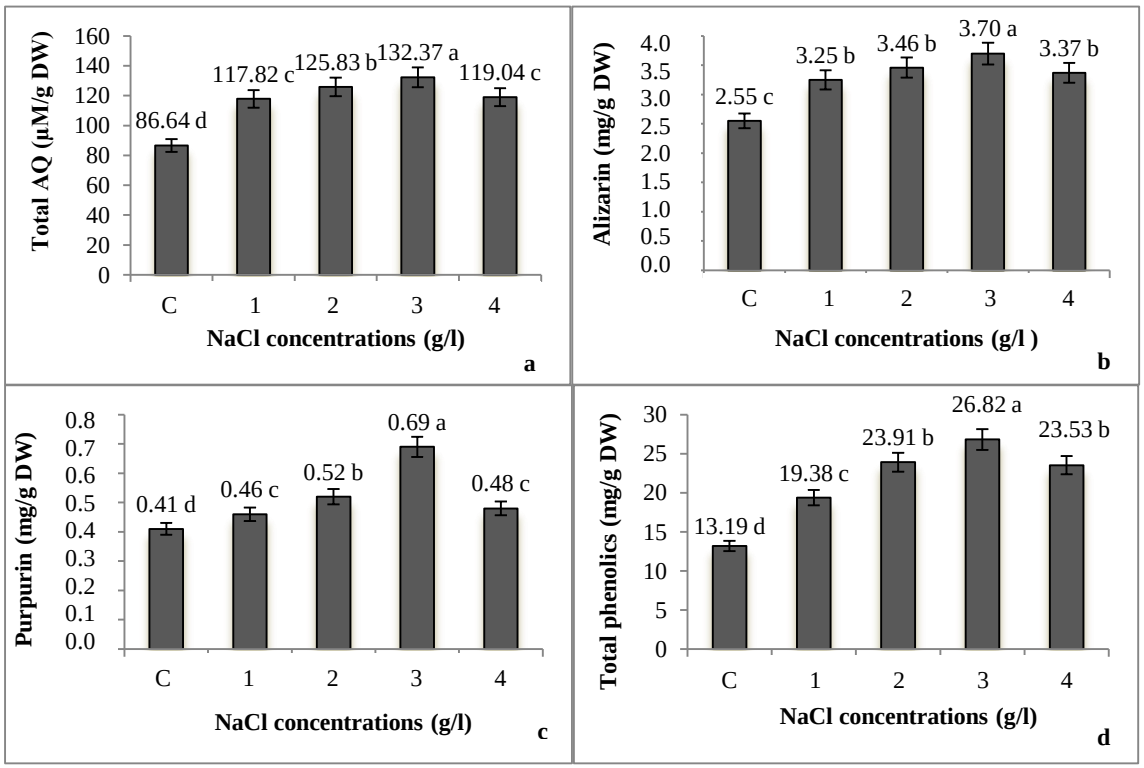

Figure 2. Effect of NaCl applications on secondary metabolites a) total AQ content b) alizarin content c) purpurin content d) total phenolic content.

According to our results, it was determined that all the applications increased the amount of the corresponding secondary metabolites significantly, and the highest values were obtained from the roots treated with 3 g/l of NaCl. Furthermore high concentrations of NaCl decreased

both growth of cultures as well as secondary metabolite biosynthesis. Similarly, Hussein and Aqlan [11] reported significant decline in the growth and secondary metabolite accumulations of fenugreek callus cultures under high concentrations of NaCl. Salt stress reduced growth of adventitious roots, whereas accumulation of AQs and phenolics were enhanced compared to controls. The reverse correlation between growth and biosynthesis of secondary metabolites was also reported by several authors [26-28]. In another study, the effect of NaCl on alizarin and purpurin contents in callus and cell suspension cultures was investigated [15], the highest amounts of alizarin and purpurin in callus cultures were obtained from 100 mM NaCl treatment while the optimal concentration for alizarin and purpurin in cell suspensions was determined as 200 mM NaCl**.** A similar study, Nazif et al. [22] reported that salt stress caused an increase of AQ in *Cassia acutifolia* cell suspension cultures. NaCl leads to cellular dehydration and as a result, the cytosolic and vacuolar volumes decrease. Thus NaCl often causes not only ionic but also osmotic stress and removal of water from the cytoplasm [29]. Plants have developed complex mechanisms such as developing enzymatic and non-enzymatic antioxidant defense mechanisms, for adaptation to the osmotic, ionic and oxidative stresses that are induced by the salt stress [30]. Secondary metabolites as non-enzymatic antioxidants accumulate under different environmental stresses and they acts as membrane stabilizer during abiotic stress [31]. It is well known that exposure to salinity induces the *in vitro* production of secondary metabolites such as phenols, terpenes and alkaloids [13, 14, 23, 32] when applied at the appropriate dose and at the correct stage of cultures.

4. CONCLUSION

As a result of this study, salt stress implementation method for higher toatal AQs, alizarin, purpurin and phenolic production in R. tinctorum L. root cultures was found to be effective. Application of NaCl to adventitious root cultures of madder decreased root growth index but induced AQ and phenolic accumulation when it was applied the appropriate concentration. In this study, the highest production of AQs and phenolic substances in the secondary metabolite production was found in the 3 g/l of NaCl concentration. To conclude, the salt induced the production of valuable phytochemical in plants and thus NaCl may be a promising compound for use in adventitious root cultures because of its positive effects on secondary metabolite production in madder.

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