Effect of nitrogen and phosphorous fertilizers on the yield and secondary metabolites of medicinal plant *Rubia tinctorum* L. under salinity conditions

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Abstract

In this study the effect of four levels of nitrogen fertilizer (0, 50, 100, 150) kg/ha and four levels of super phosphate triple (0, 50, 100, 150) kg/ha were investigated on *Rubia Inctorum* L. (madder) under salinity stress. A factorial experiment was carried out in a research farm in Roaddasht, Isfahan based on complete randomized blocks with three replications. The results of the variance analysis showed that increase in flavonoids content (at 330 nm) was statistically meaningful under various levels of phosphorous fertilizer and interaction of phosphorous with nitrogen (P≤0.01). Flavonoid content was also significantly increased under various levels of nitrogen (P≤0.05). Moreover, the amount of proline in the leaves of madder showed significant variation under different levels of phosphorous, nitrogen, and the interaction of nitrogen and phosphorous at P≤0.01. Also the performance of shoots was significantly affected by different levels of phosphorous and its interaction with nitrogen at P≤0.01. Flavonoids contents at 330 nm wavelength varied between 0.78 to 0.411 nanometer and while the maximum of this flavonoid level was observed under the application of 100 kg/ha nitrogen and phosphorous, the minimum was related to 150 kg/ha of phosphate and the control (0 kg/ha nitrogen). This suggests that by increase in phosphorous and decrease in nitrogen fertilizers, phenolic compounds are reduced in the plants. The maximum and minimum proline contents were observed under 150 kg/ha nitrogen and phosphorous treatment and control, respectively. The performance of shoots varied ranging from 1.75 to 0.75 kg/m^2. Maximum performance was recorded under 150 kg/ha nitrogen and phosphorous treatment and the decrease in phosphorous negatively affected plant performance. The results therefore suggest that yield and secondary metabolites increased with an increase in nitrogen and phosphorous treatment in the salt stressed madder.

Keywords: salinity; yield; proline; phenol

Zamani, Z., H. Zeinali, J. Masood Sinaki and **H. Madani.** 2014. 'Effect of nitrogen and phosphorous fertilizers on the yield and secondary metabolites of medicinal plant *Rubia tinctorum* L. under saline conditions'. *Iranian Journal of Plant Physiology* 4 (2), 949-955.

Introduction

Salinity is one of the most important abiotic stresses that affect agricultural plants and decrease their yields. About %33 of the irrigated fields around the world and %50 of the fields in Iran is faced with the salinity problem (Anonymous, 1997). Modification of saline soils is too costly and difficult. However, as the reaction of various cultivars to salinity is not the same,

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Received: May, 2013
Accepted: January, 2014

using resistant cultivars and farming techniques can help making the best use of saline soils (Dadashi et al., 2007). Among different cultivars, resistance to salinity is variable at different levels of growth and due to different physiologic properties and also salinity tolerance mechanism which play potential role in improving yields under salty stress (Haghniya, 1992).

Salinity is a soil condition by high content of soluble salts. The problem of soil salinity which is increasing stresses plants in two ways: high concentrations of salts in the soil make it harder for roots to extract water, and high concentrations of salts within the plant can be toxic (Munns and Tester, 2008).

Rubia Tinctorum L. is highly resistant to salinity. Farming method, planting time, harvest year, and seed density rate are among the measures to improve yields in this plant (Morovvati et al., 2010). The use of chemical fertilizers for crops production in dry and semi-dry areas where the level of fertility of soil is low is unavoidable. Therefore, for optimum production of *Rubia Tinctorum* L. the use of manure and chemical fertilizers is necessary. Although studies on the nutrition requirements of this plant are few and far in between, in recent years some research was carried out on the influence of this plant on the physicochemical properties of the saline soils in Iran. Results of a study in Ardekan showed that growing *Rubia Tinctorum* L. improved the physicochemical properties of the sodium salty soils. In fact, the speed of penetration of water in the soil increased by 20 times in the first year (Karimi, 2010). Manure requirement of madder is between 40 to 50 tons per hectare and the required phosphate is between 300 to 400 kg/ ha (Mirabzadeh ardakani, 2009).

Nitrogen plays various roles in plants and is an important element in plant nutrition. Nitrogen is necessary for the synthesis of amino acids which are the constituent parts of proteins. These acids also take part in protoplasm formation and cell division. If the available nitrogen for the plant is low, the plant is not able to make proteins for metabolic and structural processes and maintaining the desired growth level (Barker and Pilbeam, 2006). In salty soils nitrogen metabolism inside the plant is disturbed and this is mostly due to negative osmosis of soil solution and the reduction in the ability of the plant to use water. Not only the amount but also the time of application and the kind of nitrogen fertilizer influence its efficiency. Application of nitrogen fertilizer in saline soils results in the maximum output comparable with crops from non saline soils.

In saline soil, nitrogen fertilizer is applied after irrigation because salinity increases in the region where the plant root grows. The accumulated minerals reach the maximum level prior to irrigation and this prevents nitrogen mineralization and its accessibility for the plant. Spraying nitrogen solution (%3 urea solution, 20 kg/ha nitrogen) in the saline soils is particularly economical and useful.

This method is very important especially in the area where the quality of the irrigation water is poor and with each irrigation lots of minerals enter the soil. Water is saved at least for one irrigation turn, and this prevents the minerals from entering into the soil with irrigation water. Leaf feeding and surface distribution of nitrogen with %75 and %25 of the total nitrogen, respectively produce the best results. Generally, from among the common nitrogen fertilizers, urea has a better result than ammonium sulfate in low and medium saline soils whereas it is better to use nitrate fertilizers in highly saline soils (Kafi, 2010).

Application of phosphorous in saline soils increases the crops. Leaves in saline soils that suffer from the phosphorous deficiency become necrotic and the lower leaves turn red. Application of phosphorous removes these symptoms. Similar to nitrogen, plant reaction to phosphorous fertilizer is restricted to low and medium levels of salinity (Kafi, 2010). Phosphorous plays an important role in carbohydrate metabolism and the need for sufficient phosphorous in salty environment has to do with the role of this element in the ion accumulation adjustment and the ion coding inside the cells (Gibson, 1988). A number of research studies have been carried out on the changes in accumulation of flavonoid compounds which are one of the most important secondary metabolites in different plants under various stresses (Derksen and Beek, 2002). The main role of the leaf flavonoids is to protect the photosynthesis cells against destructive ultraviolet rays (Liacoura et. al., 2001). Flavonols are the most important kind of flavonoids (Winkel-Shirley, 2002). Plant pigments content were determined in different tolerant and sensitive plant varieties at wide range of salt concentrations (Sarwat and El-Sherif, 2007). Some experiences confirm antioxidant role of flavonoids in leaf tissues. These compounds operate through accumulation in the epidermal cell vacuoles in leaves and in close relationship with vacuole peroxidase enzyme for detoxification of hydrogen peroxide coming from the other cells or cell parts in plants. The most important compatible osmosis protecting solutions are glycerin, betaine, proline, and poly alleles (Rontein et. al., 2002). Proline accumulation has a positive and direct relationship with improving plant resistance to water shortage and salinity (Saneoka et al., 2004). Proline accumulation in cytoplasm acts like an osmoticum in protecting macromolecule structures in the environment where ion balance is disturbed (Nayyar, 2003). The loss of mycosporin-like amino acid (MAA) in favor of flavonol metabolism is a strong evidence that flavonoids did not likely serve a primary UV-B screening function during the evolution of early land plants(Agati And Tattini. 2010).

Phosphorous and nitrogen are the most important nutrient elements for plants playing a significant role in increasing the crop yield. However, for further production and achieving higher income, farmers are using them more and more. On the one hand, overusing these elements in farms in addition to increasing the cost of production causes environmental pollution problems and crops quality reduction. On the other hand, the ever increasing of application of chemical fertilizers has a negative effect on soil resulting in an imbalance in the soil nutrients. This in turn, results in the yield decreases. In most developed countries nitrogen, phosphorous, and potassium ratio is 100, 45, and 35, respectively. This ratio in Iran is approximately 100, 111, 3, respectively and the farmers use more phosphate than other fertilizers (Saleh Rastin, 2004).

The aim of this research was to investigate the effect of different fertilizer treatments on the shoots and roots yield performance and the amount of secondary metabolites in *Rubia Inctorum* L. under salinity stress.

Materials and Method

This research was designed and carried out in Rooddasht Salinity Research Station in Isfahan Agricultural Research Center during 2011 and 2012. Sixteen fertilizer treatments were investigated through a factorial design with 2 factors and 3 replications. The first factor was nitrogen ferl izer wi th 4 levels including 0, 50, 100, and 150 kg/ha urea chemical fertilizer. The second factor on the other hand was phosphorous ferl izer at 4 levels including 0, 50, 100, and 150 kg/ha super phosphate triple chemical fertilizer. All fertilizers were applied before planting. The experimental field was irrigated by the salty water (Ec=10 ds/m) during the growth period .

To measure the shoot performance, the shoots from one square meter of each plot were harvested and weighed with a scale. Measures were recorded based on kg/ha. In order to measure the root performance, 30 cm^2 of the field was tilled with a spade and the roots were taken out, collected and transported to the laboratory where they were washed with lukewarm water and their fresh weight was recorded. The roots were then dried in shadow for 15 days aer which they were weighed using a scale to obtain dry root weight.

Flavonoids were measured using a spectrophotometer based on the method described by Krizek et al. (1998). The absorpon was read at 330 nm. Proline contents were measured using the method of Bates et al. (1973). Proline solution in toluene phase was submitted to spectrophotometry and its content was read at 520 nm. Using the proline standard curve, the amount of this compound was calculated based on µM per grams fresh weight. Data collected for each characteristic were submitted to the analysis of variance using Duncan multi range test (P≤0.05). Statistical analyses were carried out by SAS, MSTATC software.

Results

Analysis of variance (Table 1) showed significant differences in dry root weight and leaf proline content at P≤0.01. Also shoot flavonoid contents showed significant variations under different phosphorous fertilizer levels as well as interaction of effects of nitrogen and phosphorous fertilizers at P≤0.1 and P≤0.05, respectively (Table 1).

Mean comparison of different levels of phosphorous fertilizer application showed that the maximum and minimum proline contents were achieved under 150 kg/ha and 0 kg/ha phosphorous fertilizer, respectively (Table 2). Also, the maximum and minimum dry root weights were recorded under 100 and 150 kg phosphorous fertilizer, respectively. This means that applying excessive fertilizer reduced root performance. Moreover, applying 100 kg phosphorous fertilizer resulted in maximum flavonoid level; however, phosphorous fertilizer application more than 100 kg/ha reduced flavonoid content.

Table (3) compares mean effect of nitrogen fertilizer where maximum dry root weight was achieved at 100 kg/ha fertilizer beyond which no significant effect was recorded on this attribute. Maximum flavonoid and proline were recorded under 150 kg/ha fertilizer treatment. Minimum flavonoid and proline were observed under control and 50 kg/ha nitrogen fertilizer treatment. This suggests that increase in fertilizer up to 150 kg/ha also increased leaf flavonoid.

Mean comparison of interaction between nitrogen and phosphorous showed that the

Table 1
Analysis of variance of characteristics of madder treated with urea and triple super phosphate fertilizer under salinity stress

Source of Variance	df	Flavonoid 330 (μM/g)	Proline	Root Dry Weight (g)	Shoot Yield (kg)
repetition	2	0.0051	0.00000096	27.58	0.117
nitrogen	3	0.0174*	0.00000362**	105.572**	0.0914 ns
phosphorous	3	0.0618**	0.0000337**	569.66**	0.36**
nitrogen* phosphorous	9	0.0245**	0.00003801**	551.829**	0.258**
error	30	0.051	0.00004	16.42	0.046

Ns,*, and ** show not significant, significant at P≤0.05, and significant at P≤0.05, respectively.

Table 2
Mean effect of various levels of phosphorous fertilizer on the characteristics of *Rubia tinctorum* L. under salinity conditions

Phosphate	Proline (μM/g)	Flavonoid 330 (μM/g)	Root Dry Weight (g)	Shoot Yield (kg)
0	0.135c	0.557b	1.44b	1.093b
50	0.136b	0.610ab	1.415b	1.275a
100	0.134c	0.65a	1.414b	0.883c
150	0.138a	0.484c	1.873a	1.21ab

Different letters refer to significant differences at p≤0.05 using Duncan multi range test .

Table 3
Mean effect of various levels of nitrogen fertilizer on the characteristics of *Rubia tinctorum* L. under salinity conditions

Nitrogen	Proline (μM/g)	Flavonoid 330 (μM/g)	Root Dry Weight (g)	Shoot Yield (kg)
0	0.136a	0.521b	33.929b	1.02b
50	0.135b	0.608a	35.58b	1.06ab
100	0.135b	0.578ab	39.97a	1.2a
150	0.136a	0.595a	33/46a	1.17a b

Different letters refer to significant differences at p≤0.05 using Duncan multi range test.

maximum and minimum root performance was recorded for the control and 50 kg/ha nitrogen and phosphorous fertilizer treatment, respectively. Variation in shoot performance ranged between 1.74 and 0.75 kg/ha. Maximum performance was achieved by 150 kg/ha nitrogen and phosphorous treatment and with a cut in phosphorous fertilizer, performance also decreased. Variaon in flavonoid content at 30 nm ranged between 0.78 and 0.411 and the maximum and minimum contents were observed at 100 kg nitrogen and phosphorous fertilizers and 150 kg phosphorous and 0 kg nitrogen fertilizers, respectively. This suggests that with an increase in phosphorous and decrease in nitrogen fertilizers, phenolic compounds in leaves are reduced (Table 4).

Discussion

In order to find adequate amount of nitrogen and phosphorous fertilizers for the crops it is important to study the plant response to various amounts of nitrogen and phosphorous fertilizers under different salinity levels. Application of too much fertilizer can increase salinity of the soil around roots and negatively affect the plant growth and performance. Optimal fertilization is therefore necessary for achieving desirable performance in the crops.

Nitrogen and phosphorous are the most important nutrients for plants performance. However, in an attempt to get better yields and therefore gain more income, farmers are using more and more of these fertilizers which in addition to incurring extra expenses, pollute the environment and reduce the quality of the agricultural products. In fact, increase in the use of chemical fertilizers has negative effects on soil and disturbs its nutritional equilibrium. In most developed countries the allowance of nitrogen, phosphorous, and potassium fertilizers used are 100, 45, and 35 kg/ha. This rao in Iran is much disproporonate on the order of 100, 111, and 3 kg/ha and the farmers use too much phosphate fertilizer (Saleh Rasn, 2004).

Many studies have shown that saline soil management improves quality and quantity of the plant performance (Khan et al., 1995). Improvement in the performance of the plants feeding on the nutrients in saline soils depends on the salinity level and the nutrition content of the soil (Drihem and Pilbeam, 2002). Interaction of effects of salinity and nutrients on plant performance and their absorption of nutrients are studied on several plants (Papadopoulos and Rending, 1983). Application of nitrogen fertilizer is important in all soil types particularly in saline soils (Flores et al., 2001).

Results of the present study showed that

Table 4
Interaction of effects of nitrogen and phosphorus on the characteristics of *Rubia tinctorum* L. under salinity conditions

Nitrogen and Phosphorus	Flavonoid 330 (µM/g)	Proline (µM/g)	Root Dry Weight (g)	Shoot Yield (kg)
0-0	0.544def	0.132f	92.71d	0.81bc
50-0	0.553cde	0.139bc	181a	1.36abc
100-0	0.577cde	0.135de	105.4cd	0.812bc
150-0	0.411f	0.139bc	48e	1.103abc
0-50	0.486ef	0.14b	179.31a	1.373abc
50-50	0.68abc	0.134e	173.16a	1.063bc
100-50	0.719ab	0.131f	136.73b	0.93bc
150-50	0.547def	0.136d	161.1a	0.89bc
0-100	0.564cdf	0.134e	113.3bcd	1.4abc
50-100	0.508cde	0.134e	117.8bc	1.25abc
100-100	0.78a	0.138c	70.1e	1.03bc
150-100	0.46ef	0.135de	104.02cd	1.12abc
0-150	0.63bcd	0.131f	129.4bc	0.78bc
50-150	0.7ab	0.135de	118.5bc	1.42ab
100-150	0.526def	0.134e	50.52e	0.75c
150-150	0.518def	0.142a	124.63bc	1.74a

Different letters refer to significant differences at $p \leq 0.05$ using Duncan multi range test using too much of the phosphorous fertilizer reduced root performance. This is contrary to the results of a study reported by Khalil et al. (1967) who believed that salinity - through limiting root growth - reduces phosphorous absorption by the plant. Therefore, they believed that application of phosphorous fertilizer increases its absorption by the plants and consequently helps improve plant growth. Salt stress can reduce phosphorous intake by plants even though in some studies the absorption was reduced or remained unaffected. In saline soils, availability of phosphorous is not only affected by ionic bounds that reduce the activity of this mineral, but also it is affected by calcium complexes that control phosphorous solubility (Graa n and Gr ieve, 1999). There are also studies reporting that phpsphorus concentration increases in salt sensitive rice cultivar but decreases in salt tolerant cultivars of e.g. rice (Kumar et al. (2008).

Root performance of madder is 15 – 20 tons/ha of which %10 - %12 is the pigment. The yield in 3 and 4-year-old plants is more than in 2-year-old plants. As mean dry root performance of 2- year - old plants is 8 – 10 tons/ha and 3- year - old plants produce 10 -15 tons/ha dry roots, harvesting 2 - year - old plants is not economic (Rezaei et al., 2010). In our study, due to highly saline soil, flavonoid and proline contents were increased. Kovacik et al. (2007) in their study on the effect of nitrogen stress on chamomile showed the aggregation of flavonoids in the stressed plants. Increase in proline level keeps turgor pressure and reduces cell membrane damage; therefore, through osmotic adjustment adaptation to salinity and water deficit is increased (Pandey and Agarwal, 1998). Studies showed that in saline conditions, application of nitrogen improves the plants' salinity tolerance (Ravikovitch and Yoles, 1971). Furthermore, this effect depends on the plant, salinity level, and environmental conditions (Grattan and Grieve, 1999). Researchers maintain that adding nitrogen fertilizer can reduce some salinity related problems while using too much nitrogen can increase the salinity level (Ravikovitch and Porath, 1967). Regarding the water shortage and salinity problems in Isfahan, particularly in the eastern region of the province in Rooddasht, madder is suggested as a salinity tolerant crop which can tolerate saline water up to 13EC. Madder can be used as forage as well as a medicinal and industrial plant. Many applications have been found for this valuable plant in recent decades. These include medicine, biochemical research, food industry (as an anti oxidant, essential oil, antibacterial etc.), and textile industry. As vast areas of the country are suitable for this crop, there is more room for research studies on this plant.

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