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ORIGINAL ARTICLE

Investigation of the Alizarin content in Medicinal-industrial Madder (*Rubia tictorum* L.) Root

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ABSTRACT
Rubia tinctorum L. (madder) as a pharmaceutical plant has proven to have various medicinal usages. Madder root has preventive effect upon urinary and menstrual disorders and its extract has proven effective in treating kidney and bladder stones. Many compounds have been extracted from the roots of madder which most important of them is alizarin. In order to evaluate some growth characteristics and alizarin concentration in this plant, this study was conducted in a research farm in Isfahan, Iran. The experiment was set up in a completely randomized block design with sixteen treatments and three replications for each treatment using nitrogen and phosphorous fertilizers. The growth of madder increased on the certain level of nitrogen and phosphorous application, the content of alizarin elevated about 102% for nitrogen and 30% for phosphorous toward the control ($p < 0.01$). Three important nutrients under the study consist of nitrogen, phosphorus, potassium. Fertilization (high level of nitrogen (180 kg ha^{-1}) and lower level of phosphorous (60 and 120 kg ha^{-1})) could improve N, P and K concentration.
Keywords: Alizarin, Rubia tinctorum, growth parameters.

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INTRODUCTION

Rubia tinctorum L. (madder) is a member of Rubiaceae family. Madder root has been used medicinally to obviate the urinary and menstrual disorders [1, 2]. Furthermore, extracts from *R. tinctorum* are effective for the treatment of kidney and bladder stones [3, 4].

Many compounds have been extracted from the roots of *R. tinctorum* by various analytical methods which the most important among them is HPLC. There are several anthraquinone (a kind of secondary metabolites) in the roots and rhizomes of *R. tinctorum* [5] such as alizarin, purpurin, lucidin, rubiadin and their derivatives [3,2]. It has been reported that these anthraquinones have pharmacological activities such as anticancer, antimicrobial, antifungal and antioxidant activities [6].

Alizarin is a durable red dye that it is commonly used in textile and leather industry therefore production and application of completely natural products instead of chemicals that were used in the past is important [7].

Iran ranks among the main producers of this plant [8] that it has been cultivated in arid and semiarid regions of Iran. These areas are frequently affected by high salinity [9].

Plants yield and their productivity can be developed by several factors such as suitable soil and selection of resistant species to undesirable production conditions [10]. Allowing the salt tolerant plant such as *rubia tinctorum* to grow in less productive lands such as salty soils in competitive of those growing on normal lands, has economic benefits.

N as an essential element playing a key role in plant life cycle and is required for chlorophyll, proteins, nucleic acid and amino acids production [11]. Furthermore nitrogen is a component of many enzymatic proteins regulating some processes related to plant growth [12]. Therefore, nitrogen strongly affects the crop yield, biomass and development [13].

Phosphorous as an essential macronutrient has an important role in the biochemical and physiological processes [14].

Interactions of nitrogen with P enhance root system development, good productivity and other plant functions resulting in regulation in crop yield and its quality [15].

The main goal of this study was to investigate the alizarin content and the traits related to growth of madder under two mineral nitrogen and phosphorous application and if we can improve the yield, growth and root pigments of this industrial and medicinal plant by fertilizer application to gain a high mass of this plant. No such studies have been conducted before and we performed investigations on *rubia tinctorum* for the first time.

MATERIALS AND METHODS

This experiment was conducted during 2013, 2014 in Roodasht research station located at 65 kilometers east of Isfahan, Iran.

The intact seeds of *R. tinctorum* (local cultivar of Esmat) were supplied from Yazd Agricultural Research and Natural Resources Center, Iran. Then sterilized seeds were planted in the rows with 15 cm depth and 40 cm distance. Four levels of nitrogen fertilizer (0, 60, 120 and 180 kg N ha^{-1}) and four levels of phosphorous fertilizer 0, 60, 120 and 180 kg P ha^{-1}) as the source of urea and triple super phosphate respectively, were applied before planting. For investigation of madder growth in salt conditions the field was irrigated by the saline water (Ec=10 ds/m) once per each 10 days. After six months of growth, *R. tinctorum* was harvested and its foliage and roots collected and transported to the laboratory where they were washed with tepid water and after evaporation of water from their surface, fresh weights of leaf and root of madder were recorded. After this process the same leaves and roots dried at $70^{\circ C}$ for 48 h to determine their dry weights. 10 roots of each plant were selected randomly for measuring diameter by digital culis and then mean of them recorded for each replicate. The determination of alizarin was conducted according to the Shin method [16]. First 0.5 g of dried root powder was mixed with 10 mL of methanol then it was leaved in bain-marie (50˚C) for 1 hour. After that the samples were centrifuged in 4000 rpm for 20 min and the topping solution was decreased under pressure. The residual solution was solved in 1mL of methanol and finally the absorption of alizarin was read at 572 nm wavelength. For getting the concentration of this dye (mg/g DW), the standard curve of alizarin was used.

Relative water content (RWC) as an indicative for water status demonstrates the effects of osmotic adjustment (OA) that is a forceful mechanism for protection of cellular hydration [17].

RWC was measured according to the method of Smart and Bingham [18]. First the fresh weight of ten leaf discs from each plant was recorded then these leaves were floated for 4 hours on distilled water to determine turgid weight. At last, the leaves were dried in oven with 85˚C for 24 hours. Finally RWC was calculated according to this formula:

RWC (%) = [(FW-DW)/(TW-DW)]×100

FW: Fresh Wight

DW: Dry Weight

TW: Turgid Weight

The dried root (in 70°C for 48 hours) were grinded and after wet digestion by adding sulfosalicylic acid and hydrogen peroxide, determination of the elements were conducted by Kjeldahl method for Nitrogen, flame photometry for Potassium and colorimetery by ammonium vanadate for phosphorous [19].

This study was conducted based on completely randomized designs with sixteen treatments and three replications. The results were statistically analyzed with SAS 9.1 software and the difference among treatments was analyzed by using Duncan's Multiple Range test at 1% and 5% probability level.

RESULT AND DISCUSSION

Alizarin

The alizarin content was increased significantly by nitrogen and phosphorous application. Results showed that the highest alizarin content among different concentrations of nitrogen application was obtained for 120 kg N ha^{-1} which was 102% more than control effect and the lowest alizarin content was seen in control ($p < 0.01$). Analysis of variance for the effect of phosphorous showed that 60 and 120 kg P ha^{-1} respectively had the highest impact on the alizarin content increasing near the 30%. Phosphorous in the higher rates (180 kg ha^{-1}) had reducing influence on the alizarin concentration in comparison to other rates of nitrogen. The highest alizarin content was gained from the interaction between nitrogen and phosphorous in the rate of 120 kg N ha^{-1}+60 kg P ha^{-1}, which was 277% more than non application of these fertilizers.

RWC

Leaf RWC was significantly affected by nitrogen and phosphorus applications. The highest RWC was obtained in application of 180 kg N ha^{-1} about 40% more than the control. The results showed that phosphorous fertilizer in a certain level (120 kg ha^{-1}) increased RWC and then in higher rates of P it was reduced.

The interaction between nitrogen and phosphorous fertilizers also in the rate of 120 kg N ha^{-1}+120 kg P ha^{-1} on RWC increase had the highest influence and lowest RWC was observed in application of 0 kg N ha^{-1} + 180 kg P ha^{-1}.

Saneoka *et al.* [20] asserted that nitrogen application increases synthesis of proteins that result in increasing cell wall thickness and absorption of extra water via protoplasm becomes more, hence relative water content will ameliorate.

Leaf RWC increased with the increase in nitrogen application [21]. In rice plants with high nitrogen fertilizer, RWC was higher than control [17].

Namvar *et al*., [22] reported that increasing in nitrogen fertilizer caused increasing in RWC but whit highest rate of nitrogen application, RWC diminished significantly. Kaya *et al*. [23] reported that P application resulted in a little increase in both RWC and dry weights.

Table 1. Analysis of variance for nitrogen and phosphorus fertilizer effects.

Source of variation	df	Alizarin	RWC	Leaf dry weight	Leaf fresh wt.	Root dry wt.	Root fresh wt.	Root diameter	Root length	N	P	K
R	2	2044.72ns	320.27ns	0.035ns	0.27*	0.006ns	0.014ns	0.48ns	6.57ns	0.02ns	0.005ns	0.012ns
N	3	38456.17**	806.74*	0.16**	0.26*	0.04ns	0.05**	2.07*	18.56**	0.33**	0.03**	0.1**
P	3	14809.48**	615.02ns	0.04*	0.29**	0.003ns	0.006ns	5.49**	34.72**	0.03**	0.022**	0.03**
N*P	9	14665.07**	845.94**	0.055**	0.12ns	0.02ns	0.04**	0.92ns	1.73ns	0.04**	0.017**	0.04**
Error	30	1597.16	237.00	0.012	0.05	0.015	0.01	0.66	4	0.007	0.003	0.005
CV (%)		19.96	25.92	13.53	14.54	36.75	25.32	11.59	7.43	11.54	18.81	4.37

ns= non significant, ** = $p < 0.01$ and * = $p < 0.05$

Table 2. Comparison means for interaction effect of nitrogen and phosphorous fertilizers on evaluated factors in madder.

K (%)	1.57cde	1.59b-e	1.63bcd	1.19f	1.58cde	1.48e	1.59b-e	1.56cde	1.69abc	1.77a
P (%)	0.26c-f	0.28cd	0.26c-f	0.16f	0.27cde	0.28cd	0.34bc	0.17ef	0.24c-f	0.41ab
N (%)	0.57ef	0.49e	0.58ef	0.9abc	0.52ef	0.5ef	0.66de	0.63def	0.63def	0.82bc
Root length (cm)	23.66d	23.66d	25.66bcd	28abc	26.5a-d	25.66bcd	29.33ab	30a	25.5bcd	25.16cd
Root diameter (mm)	5.7de	6.7b-e	6.93b-e	7.66a-d	6.72b-e	6.56b-e	7b-e	7.79abc	6.41cde	6.16de
Root fresh weight (kg)	0.19e	0.4bcd	0.39bcd	0.42bcd	0.5abc	0.41bcd	0.39bcd	0.48abc	0.64a	0.43bcd
Root dry weight (kg)	0.2b	0.32ab	0.26ab	0.3ab	0.43ab	0.33ab	0.35ab	0.39ab	0.43ab	0.34ab
Leaf fresh weight (gr)	1.43cd	1.45cd	1.97ab	1.37d	1.46cd	1.51bcd	1.89abc	1.77a-d	1.56bcd	1.54bcd
Leaf dry weight (gr)	0.75b-e	0.71cde	0.86bc	0.64de	0.66cde	0.6e	0.85bcd	0.84bcd	0.74b-e	0.72cde
RWC (%)	49b-e	48.33b-e	71.66abc	27.66e	45cde	72abc	54.66a-e	75.33ab	39.66de	39.33de
Alizarin (mg/gDW)	93.61fg	167.43def	166.94def	85.91g	280.53bc	334.64ab	146.69efg	141.81efg	180.74de	353.61a
Nitrogen×P hosphorous (kg ha^{-1})	N0P0	N0P1	N0P2	N0P3	N1P0	N1P1	N1P2	N1P3	N2P0	N2P1

N2P2	283.09bc	80.33a	0.84bcd	1.88abc	0.49a	0.55ab	6.8b-e	27.66abc	0.76cd	0.44a	1.68abc
N2P3	223.61cd	72abc	0.76b-e	1.38d	0.23b	0.28de	7.4bcd	28.66abc	0.85bc	0.34bc	1.67abc
N3P0	193.61de	79a	0.77b-e	1.58bcd	0.21b	0.3cde	8.07ab	26bcd	1.05a	0.18def	1.52de
N3P1	118.3efg	62.66a-d	1.26a	2.11a	0.34ab	0.41bcd	6.38cde	28abc	0.95ab	0.32bc	1.72ab
N3P2	241.3cd	71abc	0.94b	1.81a-d	0.27ab	0.37b-e	6.86b-e	28.5abc	0.95ab	0.29c	1.67abc
N3P3	190.95de	62.66a-d	0.93b	2.05a	0.28ab	0.31cde	9.1a	29abc	0.86bc	0.42bc	1.72ab

Means by the uncommon letter in each column are significantly different.
N0=0 kg ha^{-1}, N1=60 kg ha^{-1}, N2=120 kg ha^{-1}, N3= 180 kg ha^{-1}
P0=0 kg ha^{-1}, P1=60 kg ha^{-1}, P2=120 kg ha^{-1}, P3= 180 kg ha^{-1}

Leaf dry weight
Analysis of variance showed that nitrogen in the 0, 60 and 120 kg ha^{-1} had no significant effect on leaf dry weight but high rate of nitrogen fertilizer (180 kg ha^{-1}) caused increasing in leaf dry weight about 32% more than control. The effect of phosphorous on dry weight of leaf was significant. By increasing phosphorous fertilizer to a certain level (120 kg ha^{-1}) leaf dry weight increased and then in application of 180 kg P ha^{-1} decreased. Interaction of nitrogen and phosphorous in the rate of 180 kg N ha^{-1} +60 kg P ha^{-1} showed highest leaf dry weight and in application of 60 kg N ha^{-1}+60 kg P ha^{-1} was the lowest.

Leaf fresh weight
The highest Leaf fresh weight was obtained by application of nitrogen in the rate of 180 kg ha^{-1} and there was no significant difference between N0, N1 and N2. Among the P rates, the highest leaf fresh weight was obtained from 120 kg P ha^{-1} by about 24% more than control on the other hand P application in the certain level had significant effect on increasing of leaf fresh weight but after that with increasing the level of P fertilizer to 180 kg.ha^{-1}, the leaf fresh weight will be decreased. Interaction of nitrogen with phosphorous fertilizer showed no significant effect on leaf fresh weight.

Root dry weight
The effect of both nitrogen and phosphorous application on root dry weight was insignificant and also interaction between N and P had no significantly effect on root dry weight.

Root fresh weight
Nitrogen fertilizer in the rate of 60 and 120 kg ha^{-1} had similar influence on root fresh weight and increased it 34% compared with control.

Phosphorous fertilizers had insignificant effect on root fresh weight. Results showed that maximum root dry weight was obtained by utilization of 120 kg N ha^{-1}+0 kg P ha^{-1} and the lowest root fresh weight was obtained in no application of both nitrogen and phosphorous fertilizers (N0P0). Our results are in agreement with Moniruzzaman *et al*. [24] that reported the root fresh weight of plants increased with increasing N level up to a certain level and then decreased. The minimum root fresh weight was found in control

Root diameter

Increasing nitrogen rate to 180 kg N ha^{-1} resulted in root diameter increase about 13% compared to control. The highest root diameter was observed in the utilization of 180 kg P ha^{-1} too. Analysis of variance for the interaction between nitrogen and phosphorous fertilizers showed no significant effect on root diameter.

Root length

Statistical analysis for the effect of nitrogen fertilizer on root length showed that the highest root length was obtained from 60 and 180 kg.ha^{-1} N and the lowest was from control. Results show that phosphorous significantly influenced on root length. By increasing P rates to 120 and then 180 kg P ha^{-1}, root length will increased too. Interaction between nitrogen and phosphorous fertilizers had insignificant effect on root length of *Rubia tinctorum*.

These results correspond with Moniruzzaman *et al.* [24] which reported that with an increase in the nitrogen levels, the root length increased too in comparison to the control.

The observed increases in the diameter and length of root can be considered as a mechanism for giving more nutrient solution and water.

Shortage of P caused decreasing in root diameter and increasing in specific root length [25]. Our results were in agreement with Moniruzzaman *et al*., [24] that found root diameter increased with increasing N level up to a certain level and then decreased. The lowest diameter of root was found from N0.

For optimum acquisition of N, plants resort to these compensative strategies: exploration of greater soil volume or soil solution due to increasing root length or surface area by expanding root diameter [26] thus in consequence of increasing root diameter there is greater capacity for water transportation and nutrient absorption, in addition these roots are more resistant [27].

Nitrogen application along with phosphorous increased root diameter and root length. The minimum root diameter and length was observed in control [28].

N content

Comparison means based on Duncan's multiple range tests showed that N concentration increased due to 180 kg N ha^{-1} application near 48% compared with control. Phosphorous fertilizer in the rate of 180 kg P ha^{-1} increased N content 17% rather than P0, P1 and P2 treatments. Highest N content was observed in 180 kg N ha^{-1}+0 kg P ha^{-1} and the lowest N concentration was obtained in the application of 0 kg N ha^{-1}+60 kg P ha^{-1}.

Khogali [29] showed by increasing the levels of nitrogen up to 80 kg ha^{-1}, percentage of plant nitrogen increased significantly. Lopez-Bellido [30] reported that by application of nitrogen fertilizer, the nitrogen uptake increased in sugar beet.

P content

By increasing N fertilization to a certain level (120 kg N ha^{-1}) P concentration in the root of madder increased 50% over the control. However, the application of phosphorous fertilizer in the lower rates (60 and 120 kg P ha^{-1}) caused increasing in P uptake by about 37% compared to control. In the interactive effect of nitrogen and phosphorous fertilizers the highest average of P was obtained for 120 kg N ha^{-1}+120 kg P ha^{-1} and the lowest P content was obtained for 0 kg N ha^{-1}+180 kg P ha^{-1} (P <0.01). Khogali [29] reported nitrogen had an increase in content of phosphorous in beet. Rashid and Iqbal [31] showed that absorption of phosphorous increased in application of phosphorous fertilizer.

Leaf N and P concentration increased with P-fertilization whereas N-fertilization only improved N concentration in leaves [32]. Haileselassie [33] in wheat found that increasing in levels of nitrogen and phosphorous fertilizer agitated nitrogen and phosphorous uptake but a current of decrease in both nitrogen and phosphorous absorption took place in higher application levels of each fertilizers.

However, there is not always a better growth in higher nutrient concentrations. An optimal nutrient balance results in a proper growth [32].

K content

With an increase in nitrogen rate, K content has increased, the minimum value of K content being observed in control. But phosphorous fertilizer in lower levels contain 60 and 120 kg P ha^{-1} showed higher K content compared with control. Effect of interaction between nitrogen and phosphorous fertilizers was significant (table 2), the maximum K content was given from 120 kg N ha^{-1}+60 kg P ha^{-1} and the minimum value was seen in utilization of 0 kg N ha^{-1}+180 kg P ha^{-1} (P <0.01).

Conclution

It can be concluded that, fertilization affected alizarin content, growth traits and mineral element concentrations in the root of *Rubia tinctorum*. This suggests that the increase in alizarin concentration in the use of fertilizers can be applied for production of alizarin at a mass scale to produce the dye for textile and leather coloring, cosmetic materials and food industry. We observed a significant positive correlation between nutrient uptake and growth parameters of *Rubia tinctorum*. In current investigation, the increased uptake of mineral elements may have been due to the improved root system and soil exploration by roots for absorption of water and mineral nutrients [34, 35].

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