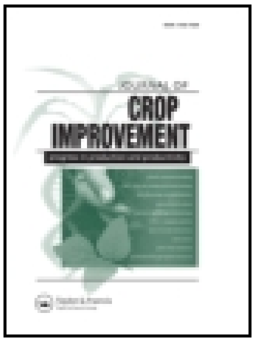

Journal of Crop Improvement

ISSN: 1542-7528 (Print) 1542-7536 (Online) Journal homepage: http://www.tandfonline.com/loi/wcim20

Qualitative response of roselle to planting methods, humic acid application, mycorrhizal inoculation and irrigation management

Hamid-Reza Fallahi, Morteza Ghorbany, Mahsa Aghhavani-Shajari, Alireza Samadzadeh & Amir Hassan Asadian

JOURNAL OF CROP IMPROVEMENT
http://dx.doi.org/10.1080/15427528.2016.1269378

Taylor & Francis
Taylor & Francis Group

Qualitative response of roselle to planting methods, humic acid application, mycorrhizal inoculation and irrigation management

Hamid-Reza Fallahi[a], Morteza Ghorbany[b], Mahsa Aghhavani-Shajari[c], Alireza Samadzadeh[a], and Amir Hassan Asadian[d]

[a]Department of Agronomy and Plant Breeding, Faculty of Agriculture, University of Birjand, Birjand, Iran; [b]Department of Biology, Faculty of Science, University of Birjand, Birjand, Iran; [c]Agronomy and Plant Breeding Department, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran; [d]Agronomy and Plant Breeding Department, Sarayan Faculty of Agriculture, University of Birjand, Birjand, Iran

ABSTRACT

Crop management is an important factor affecting the quality of medicinal plants. Therefore, objectives of our investigation on roselle (*Hibiscus sabdariffa* L.) were: 1) To identify an appropriate planting method in semi-arid regions and 2) to study crop production under water-deficit conditions using eco-friendly techniques. We specifically investigated the effects of planting methods (direct sowing vs. transplanting) (experiment 1, in a randomized complete-block design), as well as effects of water regimes [irrigation after pan evaporation of 100 mm (normal irrigation) and 200 mm (deficit irrigation)], humic acid application (0 and 4 kg ha $^{-1}$) and mycorrhizal inoculation (*Glomus versiforme*, *Glomus intraradices*, and control) (experiment 2, in a split-split-plot layout) on certain qualitative indices of roselle. The amounts of total soluble solids (TSS), anthocyanin content, and maturity index for direct seeding were, respectively, 17%, 15%, and 33% higher than those for transplanting, whereas vitamin C content and total acidity for transplanting treatment were, respectively, 17% and 20% more than those for direct sowing. According to data obtained from the average of the three mycorrhizal treatments, normal irrigation combined with humic acid application increased total acidity (0.88 mg.100 g^{-1}), anthocyanins (67.1 mg.l^{-1}), and vitamin C content (2177 mg.100 g^{-1}) over the control (deficit irrigation and no humic acid application, which had lower acidity (0.53 mg.100 g^{-1}), anthocyanins (38.8 mg.l^{-1}), and vitamin C content (1882 mg.100 g^{-1}). Total phenol and anthocyanins content under mycorrhizal inoculation were relatively higher than under control treatment at both levels of irrigation. G. intraradices produced the largest amount of vitamin C (2353 mg.100 g^{-1}) under deficit irrigation. On average, no-inoculation treatment had higher TSS, pH, and total acidity than any mycorrhizal inoculation treatment. Humic acid application and mycorrhizal inoculation, especially using G. intraradices, showed the highest values of anthocyanins (56.9 mg.l^{-1}) and vitamin C (2309 mg.100 g^{-1}) content. Overall, normal irrigation, combined with humic acid application and mycorrhizal inoculation, partially improved the quality indices of roselle.

ARTICLE HISTORY
Received 29 September 2016
Accepted 4 December 2016

KEYWORDS
Anthocyanin; *Glomus intraradices*; soluble solids; total phenolic compounds; transplanting; Vitamin C

CONTACT Hamid-Reza Fallahi Hamidreza.fallahi@birjand.ac.ir Faculty of Agriculture, Agronomy and Plant Breeding Department, University of Birjand, South Khorasan Province, Birjand.

Introduction

Roselle (*Hibiscus sabdariffa* L.), a member of Malvaceae family, is an annual tropical and sub-tropical herbaceous plant. Calyces (sepals) are the main commercial organ in roselle, which, because of their unique brilliant red color and flavor, are commonly used in food industry for production of beverages, juices, jams, and syrup (Borrás-Linares et al. 2015). However, its young leaves and tender stems are eaten raw in salads or are added to some dishes in some countries. In addition, the seed of roselle has a substantial amount of oil that is used for industrial purposes and biofuel production. Roselle is also used as human food or roasted as a substitute for coffee. Moreover, many medicinal applications have been developed for roselle around the world (Mohd-Esa et al. 2010).

Red calyces of roselle are a source of anthocyanins (about 1.5 g.100 g dry weight^{-1}) and are rich in vitamin C and other antioxidants, such as flavonoids (gossypetine, hibiscetine and sadderetine) (Duangmal, Saicheua, and Sueeprasan 2008; Fasoyiro, Babalola, and Owosibo 2005b). In addition, they are rich in vitamin A, riboflavin, niacin, calcium, iron, minerals, carotene, lycopene, and dietary fiber (Duangmal, Saicheua, and Sueeprasan 2008; Fasoyiro, Babalola, and Owosibo 2005a, 2005b; Wong et al. 2002). Roselle fruit is highly acidic, with low sugar content. Succinic acid and oxalic acid are the two main organic acids in roselle, whereas glucose is the major sugar present in its fruits (Wong et al. 2002).

Roselle fruit quality and its antioxidant activity can be influenced by management practices, such as nutrition and water availability during growth under field conditions. So far, negative effects of severe water stress on growth, chlorophyll content, anthocyanins levels, and calyx production in roselle have been reported, whereas the effects of well-managed deficit irrigation have been positive on these indices (El-Boraie, Gaber, and Abdel-Rahman 2009; Rahbarian, Afsharmanseh, and Modafea Behzadi 2011). Some studies have also revealed that humic acid can improve the growth, yield, nutrient uptake, chlorophyll content, carotenoids, and leaf carbohydrates in roselle under drought-stress conditions (Sanjari-Mijani 2014; Sanjari-Mijani, Sirousmehr, and Fakheri 2015). In addition, a few investigations have suggested that mycorrhizal inoculation had positive effects on roselle growth, yield and quality, and increased resistance to environmental stresses, such as drought (Aulia et al. 2009; Sembok et al. 2015; Sonar, Kamble, and Chavan 2013).

Direct sowing is the normal method of roselle planting in semi-arid regions, which increases water consumption at early growth stages. In addition, direct sowing delays planting because of cool temperatures during early spring, which are detrimental to roselle germination and seedling growth. Transplanting method is thought to be more suiTable than direct-seeding method, for improving water-use efficiency, which can also allow earlier planting. In literature, there is very little information available on the response of roselle to different planting methods. In a study by Obodai (2007), the effects of three planting methods (spot sowing, drilling, and transplanting) on growth and leaf yield of roselle were evaluated. Her results revealed that transplanting method reduced plant height, stem girth, canopy spread, number of branches, and number of leaves, especially compared with spot sowing; whereas there were no clear differences between planting methods relative to nutrient components of roselle. However, the positive impact of transplanting on growth and qualitative yield of some medicinal plants, such as Moldavian balm (*Dracocephalum moldavica* L.) (Aghaee Okhchlar et al. 2012), pumpkin (*Cucurbita pepo*) (Bahlgerdi, Aroiee, and Azizi 2014), and valerian (*Valeriana officinalis* L.) (Wiśniewski et al. 2016), has been reported despite some decline in the value of valerenic acid in roots (0.25% vs 0.16%) and rhizomes (0.17% vs. 0.11%) of common valerian compared with direct seed-sowing method.

Research on medicinal plant roselle is important, since all parts of the plant have many applications in foods, medicine, and industry. In addition, roselle has relatively low water requirements and thus is adapted to some regions of Iran as well as to many other semi-arid areas around the world. However, so far, only few studies have been done on qualitative and quantitative responses of the plant to agronomic management factors. Roselle is considered a minor crop in the production systems and, as a result, it receives inadequate attention (Obodai 2007). Therefore, the aim of this study was to investigate the effects of planting methods, deficit irrigation, humic acid application, and mycorrhizal inoculation on antioxidant activities (phenolic compounds) and fruit quality (TSS, anthocyanin, and vitamin C) of roselle under field conditions. We chose these specific factors mainly because they can increase water-use efficiency and thus represent sustainable strategies for roselle production under water-deficit conditions. Mycorrhizal inoculation and humic acid application can provide some nutrients for the plant in sustainable crop production agroecosystems.

Materials and methods

Field experiment description

For evaluation of the effects of planting methods, humic acid application, mycorrhizal inoculation, and water-deficit treatments on qualitative indices of roselle, two separate experiments were carried out at the research field of University of Birjand at Birjand in Sarayan county (33°N, 58°E, and 1450 masl) in eastern part of Iran. The experimental site is characterized by semi-arid climate, with an average annual precipitation of 150 mm and mean annual temperature of 17°C.

In the first experiment, the effects of planting methods (direct sowing and transplanting) were evaluated in a randomized complete-block design, using three replications. In transplanting treatment, seeds were sown in a greenhouse for 25 d, and then seedlings were transferred to the field on 18 April 2015. In both treatments, irrigation was done after 200 mm pan evaporation. In addition, plant density and fertilizer application were similar to the second experiment, which is described below.

In the second experiment, studied factors, viz., irrigation regimes, humic acid application and mycorrhizal inoculation, were arranged in a split-split-plot layout with three replications. Water regimes [irrigation after 100 mm (normal irrigation) and 200 mm (deficit irrigation) pan evaporation] were the main plots. Humic acid application levels (0 and 4 kg.ha^{-1}) were subplots, and mycorrhizal inoculation treatments (*Glomus versiforme, Glomus intraradices*, and no-inoculation control) were sub-sub-plots.

Seed planting (using "Saravan" cultivar) was carried out on 20 April 2015, with 10 × 50 cm intra- and inter-row distances. In all plots, 30 ton.ha^{-1} cow manure (pre-planting) and a chemical fertilizer containing micronutrients (2 l.ha^{-1}) (foliar application) were used. The main soil, water, and humic acid physico-chemical characteristics were determined before starting the experiment (Tables 1 and 2). Humic acid was applied through irrigation water two times during vegetative growth of roselle (15 and 35 days after emergence). All plots were irrigated similarly two times during the first week after seed sowing, and then irrigation treatments were applied separately in all plots belonging two different irrigation regimes until November 15 when irrigation was stopped. Two weeks after irrigation was stopped, roselle fruits were harvested separately from each plot for chemical analysis.

Table 1. Characteristics of humic acid (% W/W Total; Brand of Humixtract produced in Spain).

Total humic extract	Humic acids	Polycarboxilic acid	Total organic matter	Calcium oxide	Potassium oxide
70%	38%	32%	70%	1%	10%

Table 2. Properties of experimental site with respect to soil and irrigation water.

Soil properties									
EC† (mS.cm^{-1})	pH	O.C. ‡ (%)	N_{total} (%)	P_{ava} (%)	K_{ava} (%)	Sand (%)	Silt (%)	Clay (%)	Soil texture
2.27	8.49	0.13	0.016	0.0002	0.019	48.5	22.5	29	Loam

Water properties							
EC (mS.cm^{-1})	pH	TDS§ (%)	Ca^{2+} (% as $CaCo_3$)	Mg^{2+} (% as $Caco_3$)	Na^{+}(%)	K^{+} (%)	Cl^{-} (%)
1.3	7.81	0.85	0.0048	0.0051	0.0156	0.00004	0.017

†EC = electrical conductivity.
‡O.C = organic carbon.
§TDS = Total dissolved solids.

Chemical analysis

Calyces belonging to each treatment were dried separately at ambient temperature and under shade in a laboratory, which were later used for chemical analyses. Total anthocyanin content was measured using the pH-differential method, as described by Swain (1965). Total polyphenol content was determined by the Folin-Ciocalteu method explained by Chuah et al. (2008). In addition, Horwitz (1980) method was used to determine ascorbic acid (indophenols method) and total acidity based on citric acid. Total soluble solids (◦Brix) and pH of aqueous extract were determined by use of a refractometer and a pH-meter, respectively (Rezvani-Moghaddam et al. 2013). Maturity index (fruit-quality index) was determined as a ratio of Brix to total acidity (Fallahi, Rezvni Moghaddam, and Nasiri Mohallati 2010).

Soil spore population

To investigate the effects of experimental factors on sporulation, vesicular-arbuscular mycorrhizal spores were separated from the soil via wet-sieving method and centrifugation by sucrose, as described by Shirzad and Ghorbany (2015). Then, the number of spores per 10 g of dried soil was recorded.

Data analysis

Data from the two field experiments were statistically analyzed using SAS 9.1 (SAS Institute 2002). Means were separated via Duncan's multiple range test at 5% level of probability.

Results and discussion

Effect of planting methods on fruit quality

Planting method had a significant effect on most of the qualitative indices (TSS, total acidity, maturity index, anthocyanin, vitamin C, and total phenols) in roselle (Table 3). The amounts of TSS, anthocyanin content, and maturity index in the direct-seeding method were higher than in the transplanting method. However, transplanting method increased vitamin C, total acidity, and total phenolic compounds (Table 4). These differences may be related to differences in the plant establishment time in the soil, differential water relations between plant and soil as well as shock to seedlings caused by sudden environmental changes that accompany the transplanting method. In addition, shifting of plant phenological stages by the transplanting method can cause a change in environmental conditions, e.g., day length, relative humidity, and thermal stress during the growing season, which eventually affect quality and quantity of indices (Aghaee Okhchlar et al. 2012). Overall,

Table 3. Mean squares for the effect of planting methods on calyx quality of roselle.

Source of variation	df	pH	TSS† (°Brix)	Total acidity (mg.100 g dry weight[1])	Maturity index	Anthocyanins content ($mg.l^{-1}$)	Vitamin C ($mg.100\ g^{-1}$)	Total phenol (mg.100 g dry weight^{-1})
Replications	2	0.0050	0.005	0.0024*	0.09	1.64*	1574	0.000005
Planting methods	1	0.0010	0.240*	0.0368**	2.25**	66.68**	186846*	0.00350*
Error	2	0.0028	0.005	0.0001	0.015	0.04	5656	0.00008

*, **Significant at 5%, and 1% probability level, respectively.
†TSS = total soluble solids.

Table 4. Means for effects of planting methods on calyx quality of roselle.

Planting methods	pH	TSS† (°Brix)	Total acidity (mg.100 g dry weight[1])	Maturity index	Anthocyanin ($mg.l^{-1}$)	Vitamin C ($mg.100\ g^{-1}$)	Total phenol (mg.100 g dry weight^{-1})
Direct sowing	4.83[a]‡	2.4[a]	0.64[b]	3.72[a]	42.6[a]	1735[b]	7.680[b]
Transplanting	4.86[a]	2.0[b]	0.80[a]	2.49[b]	36.0[b]	2088[a]	7.694[a]

†TSS = total soluble solids.
‡Means with the same letter(s) within a column are not significantly different (P ≤ 0.05) based on Duncan's multiple range test.

because of accepTable qualitative traits of sepals and more sepal yield from the transplanting method [110 $kg.ha^{-1}$ for transplanting method vs. 62 $kg.ha^{-1}$ for the direct seeding method], we recommend the transplanting method for roselle cultivation. In addition, avoidance of possible risks of early season cold, as well as some water saving from the transplanting method, makes it a preferred strategy for roselle production in semi-arid areas. In a similar study on roselle, which was conducted by Obodai (2007), the amounts of some growth criteria and leaf yield in the transplanting method were lower than in the spot sowing and drilling methods; there were no clear differences in the amounts of nutrients, such as iron, calcium, and phosphorus. The effect of planting methods on quantitative and qualitative indices of sepals was not evaluated in that study (Obodai 2007).

Effect of water and nutrients availability on fruit quality

TSS, pH, acidity, and maturity index

Analysis of variance showed that the effects of all experimental factors were significant on total acidity and maturity index, whereas only irrigation × mycorrhizal inoculation interaction significantly affected TSS and pH (Table 5). Total acidity was higher for plants receiving humic acid (23%) and normal irrigation (17%) than that of the control plants, but mycorrhizal colonization reduced the amount of acidity (Table 6). Accordingly, the lowest acidity was recorded for the factorial combination of deficit irrigation (DI) and no-humic acid application (Table 7) and for DI in conjunction with *G. versiforme* (GV) inoculation (Table 8).

Table 5. Mean squares for the effect of irrigation deficit, humic acid application, and mycorrhizal inoculation on calyx quality of roselle.

Source of variation	df	pH	TSS† (ºBrix)	Total acidity (mg.100 g dry weight^{1})	Maturity index	Anthocyanins content (mg.l^{-1})	Vitamin C (mg.100 g^{-1})	Total phenol (mg.100 g dry weight^{-1})	Number of spores per 10 g soil
Replication (R)	2	0.0002	0.005	0.115	0.68	5.05	81016*	0.0005	1.44
Irrigation (I)	1	0.0002	0.040	0.205**	4.64**	349.8**	70081	0.0002	7.11
Error a	2	0.0016	0.180	0.0015	0.51	84.9	1117	0.00025	0.11
Humic acid (H)	1	0.0014	0.040	0.360**	11.81**	894.7**	381477**	0.0105**	2.77
I*H	1	0.00006	0.187	0.187**	8.95**	4396.4**	865	0.0043**	1.00
Error b	4	0.0029	0.424**	0.0045	0.482	19.6	14112	0.0001^{ns}	3.88
Mycorrhiza (M)	2	0.0017	0.066	0.087**	4.61**	423.6**	743722**	0.0183**	5.86
I*M	2	0.0157**	0.332*	0.155**	5.85**	565.0**	200470**	0.0243**	4.52
H*M	2	0.0009	0.0025	0.360**	7.58**	89.6	391226**	0.0145**	0.86
I*H*M	2	0.0008	0.016	0.279**	0.87*	126.8*	778768**	0.0473**	6.08
Error c	16	0.0021	0.0726	0.0034	0.2206	35.7	20963	0.00049	2.58

*, **Significant at 5% and 1% probability level, respectively.
†TSS = total soluble solids.

Table 6. Means for effects of irrigation regimes, mycorrhizal inoculation, and humic acid application on calyx quality of roselle.

Treatments	pH	TSS† (ºBrix)	Total acidity (mg.100g[1])	Maturity index	Anthocyanin ($mg.l^{-1}$)	Vitamin C ($mg.100\ g^{-1}$)	Total phenols (mg.100g[1])	Number of spores per 10 g soil
Irrigation treatment								
100 mm pan evaporation	4.805[a]‡	2.339[a]	0.85[a]	3.01[a]	51.1[a]	2068[a]	7.696[a]	2.66[b]
200 mm pan evaporation	4.810[a]	2.272[a]	0.70[b]	3.72[a]	44.8[a]	1980[b]	7.697[a]	3.55[a]
Humic acid application								
0 $kg.ha^{-1}$	4.801[a]‡	2.337[a]	0.67[b]	3.94[a]	42.9[b]	1921[b]	7.692[b]	3.38[a]
4 $kg.ha^{-1}$	4.814[a]	2.270[a]	0.87[a]	2.79[b]	52.9[a]	2127[a]	7.702[a]	3.33[a]
Mycorrhizal inoculation								
Glomus intraradices	4.813[a]‡	2.266[a]	0.81[a]	2.95[b]	54.7[a]	2249[a]	7.692[b]	2.75[a]
Glomus versiforme	4.794[a]	2.258[a]	0.68[b]	4.08[a]	45.7[b]	1757[c]	7.709[a]	3.91[a]
Control	4.816[a]	2.391[a]	0.84[a]	3.06[b]	43.5[b]	2066[b]	7.688[b]	2.66[a]

†TSS = total soluble solids.
‡Means with the same letter(s) within a column and treatment factor (irrigation or humic acid or mycorrhizal treatment) are not significantly different ($P \leq 0.05$) based on Duncan's multiple range test.

Table 7. Means for interaction effects of deficit irrigation and humic acid application on calyx quality of roselle.

Irrigation treatment (mm pan evaporation)	Humic acid application ($kg.ha^{-1}$)	pH	TSS† (ºBrix)	Total acidity (mg.100g[1])	Maturity index	Anthocyanin ($mg.l^{-1}$)	Vitamin C ($mg.100\ g^{-1}$)	Total phenols ($mg.100\ g^{-1}$)
100	0	4.79[a]‡	2.30[a]	0.82[a]	3.08[b]	35.0[c]	1961[a]	7.694[a]
	4	4.81[a]	2.37[a]	0.88[a]	2.93[b]	67.1[a]	2177[a]	7.698[a]
200	0	4.80[a]	2.37[a]	0.53[b]	4.80[a]	38.8[b]	1882[a]	7.689[a]
	4	4.81[a]	2.16[a]	0.87[a]	2.65[b]	50.9[c]	2078[a]	7.705[a]

†TSS = total soluble solids.
‡Means with the same letter(s) within a column are not significantly different ($P \leq 0.05$) based on Duncan's multiple range test.

The enhancing effect of humic acid on titraTable acidity was in accordance with findings of Aminifard et al. (2012) on hot pepper (*Capsicum annuum* L.). However, our observation was not in accordance with their report about TSS. Qualitative response of roselle to humic acid application has been shown to depend on the time of humic substance consumption during plant growth (Ferrara and Brunetti 2010). The higher acidity in plants treated with humic acid than in non-treated plants could be attributed to utilization of additional C provided by organic fertilizers for the production of organic acids, which are responsible for the fruit acidity (Aminifard et al. 2012).

In the present study, humic acid application and mycorrhizal inoculation had no significant effect on TSS (Table 6). This result was not in agreement with the observations of Bettoni et al. (2014) on onion and of

Table 8. Means for interaction effect of deficit irrigation and mycorrhizal inoculation on calyx quality of roselle.

Irrigation treatment (mm pan evaporation)	Mycorrhizal inoculation	pH	TSS† (ºBrix)	Total acidity (mg.100g^{1})	Maturity index	Anthocyanin (mg.l^{-1})	Vitamin C (mg.100 g^{-1})	Total phenol (mg.100 g^{-1})
100	*Glomus intraradices*	4.78^{ab}	2.38^{a}‡	0.76^{ab}	3.10^{b}	51.8^{ab}	2147^{ab}	7.707^{a}
	Glomus versiforme	4.83^{a}	2.10^{a}	0.85^{a}	2.92^{b}	47.4^{ab}	1897^{bc}	7.703^{ab}
	Control	4.79^{ab}	2.53^{a}	0.94^{a}	3.00^{b}	51.1^{a}	2162^{ab}	7.678^{b}
200	*Glomus intraradices*	4.84^{a}	2.15^{a}	0.86^{a}	2.81^{b}	57.7^{a}	2353^{a}	7.678^{b}
	Glomus versiforme	4.75^{b}	2.41^{a}	0.51^{b}	5.23^{a}	43.9^{ab}	1618^{c}	7.716^{a}
	Control	4.83^{a}	2.25^{a}	0.73^{ab}	3.13^{b}	32.9^{b}	1971^{abc}	7.698^{ab}

†TSS = total soluble solids.
‡Means with the same letter(s) within a column are not significantly different (P ≤ 0.05) based on Duncan's multiple range test.

Baslam, Garmendia, and Goicoechea (2012) on lettuce. It is important to note that our results were obtained without considering the dilution effect attribuTable to the increased size of mycorrhizal plants, where the economical and biological yields of the inoculated plants, on average, increased by 19% and 27%, respectively, with respect to the control (data presented in Fallahi et al. 2017). The ratio of TSS to total acidity (maturity index) is an indicator of both sweetness and fruit acidity, which provides more information than TSS or acidity alone (Suárez et al. 2010). Our results are in accordance with the observations of Rezvani-Moghaddam et al. (2013), who showed a significant correlation between maturity index and TSS (0.45**; ** = significant at the 1% probability level), pH (−0.47**), and titraTable acidity (−0.84**) in seedless barberry. Maturity index (fruit quality ratio) was 20% more in deficit irrigation compared with normal irrigation and 29% higher in no-humic acid application compared with humic acid application (Table 6). The higher amount of maturity index can be considered a positive aspect in the fruit quality, which shows an appropriate equilibrium between the levels of sugars and acidity, which is essential to maintain the juice quality for industry (Aguado et al. 2012). The highest value of this index was obtained in plants that were affected by deficit irrigation, received no-humic, and were inoculated with GV (Table 9). In addition, similar to the findings of Aguado et al. (2012) on citrus, we concluded that in plants under drought stress, the soluble solids and acidity decreased (2.5% and 18%, respectively). However, because of higher reduction in acidity than soluble solids, an increase in maturity index was observed (Table 6).

Table 9. Means for interaction effect of irrigation treatment, humic acid application, and mycorrhizal inoculation on calyx quality of roselle.

Irrigation treatment (mm pan evaporation)	Humic acid application (kg.ha^{-1})	Mycorrhizal inoculation	pH	TSS† (ºBrix)	Total acidity (mg.100g^{1})	Maturity index	Anthocyanin (mg.l^{-1})	Vitamin C (mg.100 g^{-1})	Total phenol (mg.100 g^{-1})
100	0	*Glomus intraradices*	4.77^{ab}‡	2.36^{a}	0.75^{bc}	3.15^{bc}	39.9^{bcd}	1970^{c}	7.713^{b}
		Glomus versiforme	4.81^{ab}	2.06^{a}	0.51^{e}	4.07^{b}	31.0^{de}	1411^{e}	7.693^{d}
		Control	4.80^{ab}	2.46^{a}	1.22^{a}	2.02^{d}	34.2^{cd}	2500^{a}	7.677^{e}
	4	*Glomus intraradices*	4.79^{ab}	2.40^{a}	078^{b}	3.05^{bc}	65.6^{a}	2323^{ab}	7.700^{cd}
		Glomus versiforme	4.85^{a}	2.13^{a}	1.20^{a}	1.77^{d}	63.9^{a}	2382^{ab}	7.714^{b}
		Control	4.79^{ab}	2.60^{a}	0.66^{cd}	3.97^{b}	73.8^{a}	1823^{c}	7.679^{e}
200	0	*Glomus intraradices*	4.82^{ab}	2.20^{a}	0.58^{de}	3.79^{b}	65.1^{a}	2411^{ab}	7.648^{f}
		Glomus versiforme	4.75^{b}	2.53^{a}	036^{f}	6.88^{a}	45.0^{bc}	1500de	7.739^{a}
		Control	4.83^{ab}	2.40^{a}	064^{cd}	3.72^{b}	42.6^{bc}	1735^{cd}	7.680^{e}
	4	*Glomus intraradices*	4.85^{a}	2.10^{a}	1.14^{a}	1.83^{d}	50.3^{b}	2294^{ab}	7.709^{bc}
		Glomus versiforme	4.75^{b}	2.30^{a}	0.65^{cd}	3.59^{b}	42.8^{bc}	1735^{cd}	7.692^{d}
		Control	4.83^{ab}	2.10^{a}	0.83^{b}	2.54^{cd}	23.2^{e}	2205^{b}	7.716^{b}

†TSS = total soluble solids.
‡Means with the same letter(s) within a column are not significantly different ($P \leq 0.05$) based on Duncan's multiple range test.

Anthocyanins content

Anthocyanin content was affected significantly by all experimental factors (Table 5). Amount of these compounds under normal irrigation, humic acid application, and inoculation with GI and GV increased by 12%, 19%, 20%, and 5% relative to the control, respectively (Table 6). Similar to the results of Sanjari-Mijani (2014), application of humic substances had a positive effect on anthocyanin content in roselle under both irrigation regimes (Table 7). In addition, although mycorrhizal symbiosis between roselle root and fungi under normal irrigation had no increasing effect on anthocyanin content of sepals, both species of fungi increased considerably the amounts of these compounds under the water-deficit condition (Table 8). Enhancing effect of mycorrhizal fungi on plant anthocyanins has been previously reported in lettuce by Baslam, Garmendia, and Goicoechea (2012).

Roselle root inoculation with mycorrhiza and humic acid application had a synergetic effect on the anthocyanin content, where the highest value of anthocyanin was obtained with humic consumption and inoculation with GI, which was 33% more than that for the control treatment (Table 10). The improvement of plant qualitative traits, such as anthocyanins, under simultaneous application of mycorrhiza and humic acid, has previously been shown to be related to improved growth of mycorrhizal mycelium under availability of soil organic matter, including humic substance (Gryndler et al. 2005). In addition, humic substances increase the uptake of water and nutrients by the plant, which improves photosynthesis. Eshghi and Garazhian (2015) showed that increased photosynthetic activities resulted in more carbohydrate production, a part of which was used by mycorrhizal fungi.

Generally, normal irrigation, humic acid application, and inoculation with GI was a superior combination relative to anthocyanin content (Table 11).

Table 10. Means for interaction effect of humic acid application and mycorrhizal inoculation on calyx quality of roselle.

Humic acid application ($kg.ha^{-1}$)	Mycorrhizal inoculation	pH	TSS† (°Brix)	Total acidity ($mg.100g^{1}$)	Maturity index	Anthocyanin ($mg.l^{-1}$)	Vitamin C ($mg.100\ g^{-1}$)	Total phenol ($mg.100\ g^{-1}$)
0	*Glomus intraradices*	4.80[a]‡	2.28[a]	0.66[bc]	3.47[b]	52.5[a]	2191[a]	7.680[b]
	Glomus versiforme	4.78[a]	2.30[a]	0.43[c]	5.47[a]	38.0[a]	1456[b]	7.716[a]
	Control	4.82[a]	2.43[a]	0.93[ab]	2.87[b]	38.4[a]	2117[a]	7.679[b]
4	*Glomus intraradices*	4.82[a]	2.25[a]	0.96[a]	2.44[b]	56.9[a]	2309[a]	7.705[ab]
	Glomus versiforme	4.80[a]	2.21[a]	0.92[ab]	2.68[b]	53.4[a]	2059[a]	7.703[ab]
	Control	4.81[a]	2.35[a]	0.74[ab]	3.25[b]	48.5[a]	2015[a]	7.697[ab]

†TSS = total soluble solids.

‡Means with the same letter(s) within a column are not significantly different ($P \leq 0.05$) based on Duncan's multiple range test.

Table 11. Correlation coefficients among qualitative indices of roselle.

	TSS†	pH	Total acidity	Maturity index	Anthocyanin	Total phenol	Vitamin C
TSS†	1						
pH	−0.53**	1					
Total acidity	−0.09	0.33*	1				
Maturity index	0.45**	−0.47**	−0.84**	1			
Anthocyanin	0.15	0.02	0.02	0.02	1		
Total phenol	0.00	−013	0.02	0.18	−0.26	1	
Vitamin C	−0.12	0.34*	0.67**	−0.65	0.26	−0.23	1

*, **Significant at 5% and 1% probability level, respectively.
†TSS = total soluble solids.

Negative impact of water deficit on anthocyanin content of roselle is similar to the findings reported in a previous study on roselle (El-Boraie, Gaber, and Abdel-Rahman 2009). However, results from a similar study on strawberry revealed differential effects of water deficit on anthocyanins of different cultivars (Giné-Bordonaba and Terry 2016). Anthocyanins have an important role in plant stress responses as strong antioxidant molecules, which protect plants from damage from reactive oxygen species (Ende and El-Esaw 2014). However, reduction in these compounds under deficit irrigation in the present study might be because of poor drought-stress tolerance of roselle variety evaluated.

Vitamin C content

Effects of mycorrhizal inoculation and humic acid application were significant for vitamin C content of roselle sepals (Table 5). On average, vitamin C increased by 10% when the plants received humic acid and by 8% when plants were inoculated with GI (Table 6). The positive effect of humic acid on vitamin C is in agreement with the findings of Aminifard et al. (2012) on hot pepper. Normal irrigation, along with humic acid application, produced the highest amount of vitamin C (2177 mg.100 g^{-1}), whereas deficit irrigation, combined with no humic application, had the lowest value for vitamin C (1882 mg.100 g^{-1}) (Table 7). Changes in vitamin C under drought stress have been shown to depend on cultivar, stress severity, and drought duration (Rosales et al. 2012). In a study on common bean (*Phaseolus vulgaris* L.), Rosales et al. (2012) showed that concentrations of vitamin C, phenols, and flavonoids were similar for both susceptible and resistant cultivars under different drought treatments, suggesting a limited contribution of these antioxidant compounds in the selection of drought-resistant common bean cultivars. Therefore, it is possible that vitamin C in roselle under drought stress might be different for other cultivars not included in this study. In addition, the concentration of other antioxidants, such as superoxide dismutase and catalase (not measured in this study), can be increased in Saravan cultivar, which was evaluated in the current study. Interaction effect of irrigation management and mycorrhizal symbiosis revealed that without considering the dilution effect, GI had the most positive effect on vitamin C content of sepals under water-deficit condition (Table 8). Dilution effect is defined as reducing the concentration of a substance in the plant because of increased biomass, despite the increase in final amount of the desired compound in total biomass. Considering sepal yield (data presented in Fallahi et al. 2017) and concentration of vitamin C, the average amounts of this compound would be 33.5, 20, and 20 mg in sepals of each plant for GI, GV, and control treatments, respectively, if the dilution effects were considered.

Both mycorrhiza species showed a positive effect on roselle quality when the inoculation was made in conjunction with the application of humic acid (Table 10). Eshghi and Garazhian (2015) showed that in the presence of humic acid, higher activity and density of beneficial microorganisms and consequent increase in nutrient uptake and qualitative indices could be achieved than when no humic substance was applied. In this regard, Gryndler et al. (2005) in a study on maize (*Zea mays*) observed that application of humic acid improved root colonization by mycorrhizal fungi. Similar results were obtained in our study, where humic acid application enhanced the amount of mycorrhizal frequency by 20% relative to no-humic acid application (data not presented), although it had no significant effect on sporulation of mycorrhiza (Table 5). This observation might be attribuTable to improved growth and activity of mycorrhizal mycelium in the presence of adequate levels of humic acid, as reported by Gryndler et al. (2005).

Total phenolic compounds

Interaction effects of all experimental factors were significant for total phenol content of roselle sepals (Table 5). Phenolic compounds were partially increased by application of humic acid at both water regimes (Table 7). This result is in line with earlier findings on tomato, where organic nutrition management increased phenolic compounds in fruit (Toor, Geoffrey, and Anuschka 2006). Humic substances have been shown to enhance the expression of an enzyme that catalyzes the first main step in the biosynthesis of phenolic compounds (Canellas et al. 2015). Similar results were obtained for mycorrhizal inoculation, especially using GV, where this treatment had the highest amount of phenol content under the water-deficit condition (Table 8). Our results on the enhancing effect of mycorrhizal symbiosis on production of phenolic compounds are similar to those reported previously by Baslam, Garmendia, and Goicoechea (2012) on lettuce. The amount of phenolic compounds in roselle, in line with earlier findings on onion (Bettoni et al. 2014), was increased by mycorrhizal symbiosis at both the humic acid treatments (Table 10). Moreover, water availability status did not have a considerable effect on total phenols in roselle (Table 6). In this regard, results of Rosales et al. (2012) showed that the concentration of total phenols in plants was heavily dependent on cultivar and duration of water restriction, where there was no significant difference between normal and water-deficit treatments 13 d after water stress, whereas the concentration of phenolic compounds increased considerably 22 d after water restriction. Therefore, it seems that measurement of total phenols in roselle under water stress (irrigation intervals of about 20 d) during summer can produce different results than their measurement at final growth stage (as in the current study). This is because increased relative humidity and reduced air temperature at the end of growth cycle can mitigate the effects of drought stress. In total, although phenolic compounds usually increase under environmental stresses, this is not a fixed rule, so that these compounds may sometimes remain unchanged or even be reduced under stress conditions, as reported by Sayyadi et al. (2015).

Conclusion

Results of this study showed that, based only on qualitative parameters, there was no clear-cut recommendation for appropriate roselle planting method; however, considering enhanced calyx yield and water-use efficiency, it seems that transplanting method would be a preferred strategy for roselle production in drought-affected regions. In addition, on average, deficit irrigation decreased the amounts of TSS, acidity, anthocyanin, and vitamin C content. Moreover, humic acid application and plant inoculation with *Glomus intraradices* increased vitamin C, anthocyanin, and phenolic compounds. Overall, the use of transplanting method, application of humic substances, and mycorrhizal inoculation should be appropriate strategies for roselle production, especially under water-deficit conditions. Our data, as one of the first studies on roselle cultivation around the world, were obtained from a one-year study and can be used as a basis for further experiments. In the future researches, two-season or two-environment studies will be more helpful for better understanding of the roselle response to experimental factors.

Funding

We thank Vice President for Research and Technology, University of Birjand, Iran for the financial support of the project (grant number 1394/D/26676).

References

Aghaee Okhchlar, R., R. Amirnia, M. Tajbakhsh, M. Ghiyasi, and M. B. Alizadeh. 2012. The effect of sowing date and sowing method on quantity characteristics and essential oil content on Moldavian balm (*Dracocephalum moldavica* L.). *International Research Journal of Applied and Basic Sciences* 3 (2):353–61.

Aguado, A., J. Frıas, I. Garcıa-Tejero, F. Romero, J. L. Muriel, and N. Capote. 2012. Towards the improvement of fruit-quality parameters in citrus under deficit irrigation strategies. *Agronomy* 12:1–9.

Aminifard, M. H., A. H, A. M, H. Nemati, and H. Z. E. Jaafar. 2012. Effect of humic acid on antioxidant activities and fruit quality of hot pepper (*Capsicum annuum* L.). *Journal of Herbs, Spices & Medicinal Plants* 18:360–69. doi:10.1080/10496475.2012.713905.

Aulia, R. A., O. Mohamad, M. Marlina, H. Rasli, S. N. Fuad, and A. I. Zainal 2009. "Effects of mycorrhiza on productivity of Roselle." Proceedings of the 8th Malaysia Congress on Genetics, Genting Highlands, Kuala Lumpur, Malaysia, August 4–6.

Bahlgerdi, M., H. Aroiee, and M. Azizi. 2014. The study of plant density and planting methods on some growth characteristics, seed and oil yield of medicinal pumpkin (*Cucurbita pepo* var. *styriaca*, cv. '*Kaki*). *American Journal of Life Sciences* 2 (5):319–24. doi:10.11648/j.ajls.20140205.21.

Baslam, M., I. Garmendia, and N. Goicoechea. 2012. Elevated CO_2 may impair the beneficial effect of arbuscular mycorrhizal fungi on the mineral and phytochemical quality of lettuce. *Annals of Applied Biology* 161:180–91. doi:10.1111/aab.2012.161.issue-2.

Bettoni, M. M., A. F. Mogor, V. Pauletti, and N. Goicoeche. 2014. Growth and metabolism of onion seedlings as affected by theapplication of humic substances, mycorrhizal inoculation andelevated CO_2. *Scientia Horticulturae* 180:227–35. doi:10.1016/j.scienta.2014.10.037.

Borrás-Linares, I., S. Fernández-Arroyo, D. Arráez-Roman, P. A. Palmeros-Suárez, R. Del Val-Díazc, I. Andrade-Gonzáles, A. Fernández-Gutiérrez, J. F. Gómez-Leyva, and A. Segura-Carretero. 2015. Characterization of phenolic compounds, anthocyanidin, antioxidantand antimicrobial activity of 25 varieties of Mexican Roselle (*Hibiscus sabdariffa*). *Industrial Crops and Products* 69:385–94. doi:10.1016/j.indcrop.2015.02.053.

Canellas, L. P., F. L. Olivares, N. O. Aguiar, D. L. Jones, A. Nebbioso, P. Mazzei, and A. Piccolo. 2015. Humic and fulvic acids as biostimulants in horticulture. *Scientia Horticulturae* 196:15–27. doi:10.1016/j.scienta.2015.09.013.

Chuah, A. M., Y. Lee, T. Yamaguchi, H. Takamura, L. Yin, and T. Matoba. 2008. Effect of cooking on the antioxidant properties of coloured peppers. *Food Chemistry* 111:20–28. doi:10.1016/j.foodchem.2008.03.022.

Duangmal, K., B. Saicheua, and S. Sueeprasan. 2008. Colour evaluation of freeze-dried roselle extract as a natural food colorant in a model system of a drink. *Food Science and Technology* 41:1437–45.

El-Boraie, F. M., A. M. Gaber, and G. Abdel-Rahman. 2009. Optimizing irrigation schedule to maximize water use efficiency of *Hibiscus sabdariffa* under Shalatien conditions. *World Journal of Agricultural Sciences* 5 (4):504–14.

Ende, W. V. D., and S. K. El-Esaw. 2014. Sucrose signaling pathways leading to fructan and anthocyanin accumulation: A dual function in abiotic and biotic stress responses. *Environmental and Experimental Botany* 108:4–13. doi:10.1016/j.envexpbot.2013.09.017.

Eshghi, S., and M. Garazhian. 2015. Improving growth, yield and fruit quality of strawberry by foliar and soil drench applications of humic acid. *Iran Agricultural Research* 34 (1):14–20.

Fallahi, H. R., M. Ghorbani, M. Aghhavani-Shajari, A. H. Asadian, and A. Samadzadeh. 2017. Influence of mycorrhizal inoculation and humic acid application on growth and yield of Roselle (*Hibiscus sabdariffa* L.) and its mycorrhizal symbiosis index under deficit irrigation. *International Journal of Horticultural Science and Technology* In Press.

Fallahi, H. R., P. Rezvni Moghaddam, and M. Nasiri Mohallati. 2010. The effect of harvesting date on quantitative and qualitative of barberry (*Berberis vulgaris*). *Iranian Field Crops Research* 8:225–34.

Fasoyiro, S. B., S. O. Babalola, and T. Owosibo. 2005a. Chemical composition and sensory quality of fruit-flavoured Roselle (*Hibiscus sabdariffa*) drinks. *World Journal of Agricultural Sciences* 1 (2):161–64.

Fasoyiro, S. B., S. O. Babalola, and T. Owosibo. 2005b. Chemical and storability of fruit-flavoured (*Hibiscus sabdariffa*) drinks. *World Journal of Agricultural Sciences* 1 (2):165–68.

Ferrara, G., and G. Brunetti. 2010. Effects of the times of application of a soil humic acid on berry quality of Table grape (*Vitis vinifera* L.) cv Italia. *Spanish Journal of Agricultural Research* 8 (3):817–22. doi:10.5424/1283.

Giné-Bordonaba, J., and L. A. Terry. 2016. Effect of deficit irrigation and methyl jasmonate application on thecomposition of strawberry (*Fragaria* x *ananassa*) fruit and leaves. *Scientia Horticulturae* 199:63–70. doi:10.1016/j.scienta.2015.12.026.

Gryndler, M., H. Hrselová, R. Sudová, H. Gryndlerová, V. Rezácová, and V. Merhautová. 2005. Hyphal growth and mycorrhiza formation by the arbuscular mycorrhizal fungus *Glomus claroideum* BEG 23 is stimulated by humic substances. *Mycorrhiza* 15 (7):483–88. doi:10.1007/s00572-005-0352-7.

Horwitz, W. 1980. *Officinal methods for analysis*, 13th ed. Washington, USA: Association of Official Analytical Chemists (AOAC).

Mohd-Esa, N., F. S. Hern, A. Ismail, and C. L. Yee. 2010. Antioxidant activity in different parts of roselle (*Hibiscus sabdariffa* L.) extracts and potential exploitation of the seeds. *Food Chemistry* 122:1055–60. doi:10.1016/j.foodchem.2010.03.074.

Obodai, G. A. 2007. A survey and effect of methods of establishment and spacing on the growth and leaf yield of roselle (*Hibiscus sabdariffa*). M. Sc. Thesis, College of Agriculture and Natural Resources, Kwame Nkrumah University of Science and Technology, 148.

Rahbarian, P., G. Afsharmanseh, and N. Modafea Behzadi. 2011. Effect of drought stress as water deficit and planting density on yield of Roselle (*Hibiscus sabdariffa*) in Jiroft region. *New Findings in Agriculture* 5 (3):249–57.

Rezvani-Moghaddam, P., H. R. Fallahi, M. Aghhavani-Shajari, and M. Nassiri-Mahallati. 2013. Effects of harvest date, harvest time, and post-harvest management on quantitative and qualitative traits in seedless barberry (*Berberis vulgaris* L.). *Industrial Crops and Products* 42:30–36. doi:10.1016/j.indcrop.2012.05.007.

Rosales, M. A., E. Ocampo, R. Rodríguez-Valentín, Y. Olvera-Carrillo, J. Acosta-Gallegos, and A. A. Covarrubias. 2012. Physiological analysis of common bean (*Phaseolus vulgaris* L.) cultivars uncovers characteristics related to terminal drought resistance. *Plant Physiology and Biochemistry* 56:24–34. doi:10.1016/j.plaphy.2012.04.007.

Sanjari-Mijani, M. 2014. Effect of humic acid and drought stress on qualitative and quantitative indices of Roselle (*Hibiscus sabdariffa*) medicinal plant. M.Sc Thesis of Faculty of Agriculture, University of Zabol, Sistan & Balouchestan, Iran.

Sanjari-Mijani, M., A. Sirousmehr, and B. A. Fakheri. 2015. The effects of drought stress and humic acid on some physiological characteristics of roselle (*Hibiscus sabdariffa*). *Agricultural Crop Management* 17 (2):403–14.

SAS Institute. 2002. *The SAS System for Windows*. Cary, NC, USA: Release 9.1 SAS Inst.

Sayyadi, A., J. Ahmadi, B. Asgari, and S. M. Hosseini. 2015. Effect of drought and salinity stresses on phenolic compounds of *Thymus vulgaris* L. *Eco-Phytochemical Journal of Medical Plants* 4 (8):50–61.

Sembok, W. W., N. Abu-Kassim, Y. Hamzah, and Z. A. Rahman. 2015. Effect of mycorrhizal inoculation on growth and quality of roselle (*Hibiscus sabdariffa* L.) grown in soiless culture system. *Malaysian Applied Biology* 44 (1):57–62.

Shirzad, H., and M. Ghorbany. 2015. Survey of arbuscular mycorrhizal fungi symbiosis with cotton root in north Khorasan province. *Iranian Journal of Cotton Research* 2 (2):1–12.

Sonar, B. A., V. R. Kamble, and P. D. Chavan. 2013. Native AM fungal colonization in three *Hibiscus* species under NaCl induced salinity. *Journal of Pharmacy and Biological Sciences* 5 (6):7–13. doi:10.9790/3008-0560713.

Suárez, L., P. J. Zarco-Tejada, V. González-Dugo, J. A. J. Berni, R. Sagardoy, F. Morales, and E. Fereres. 2010. Detecting water stress effects on fruit quality in orchards with time-series PRI airborne imagery. *Remote Sensing of Environment* 114:286–98. doi:10.1016/j.rse.2009.09.006.

Swain, T. 1965. Analytical methods for flavonoids. In *Chemistry and biochemistry of plant pigments*, edited by T. W. Goodwin, 543–44. London, UK: Academic Press.

Toor, R. K., P. S. Geoffrey, and H. Anuschka. 2006. Influence of different types of fertilisers on the major antioxidant components of tomatoes. *Journal of Food Composition and Analysis* 19:20–27. doi:10.1016/j.jfca.2005.03.003.

Wiśniewski, J., M. Szczepanik, B. Kołodziej, and B. Król. 2016. Plantation methods effects on common valerian (*Valeriana officinalis*) yield and quality. *The Journal of Animal & Plant Sciences* 26 (1):177–84.

Wong, P., Y. Salmah, H. M. Ghazali, and Y. B. Cheman. 2002. Physico-chemical characteristics of roselle (*Hibiscus sabdariffa* l.). *Nutrition and Food Science* 32 (2):68–73. doi:10.1108/00346650210416994.