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Effect of Nitrogen Form on Trifoliate Orange (*Poncirus trifoliata*) and Sour Orange (*Citrus aurantium*) Plants Grown Under Saline Conditions

Louloudia KOULIMBOUDI*, Anna PAPAFILIPPOU, Maria TZANOUDAKI and Christos CHATZISSAVVIDIS
Laboratory of Pomology, Department of Agricultural Development, Democritus University of Thrace, 68 200 Orestiada, Greece
*Corresponding author: k_loula@yahoo.gr

Abstract

The effect of the form of nitrogen (N) on the vegetative growth and the chemical composition of trifoliate orange plants (*Poncirus trifoliata*) and sour orange plants (*Citrus aurantium*) irrigated with Hoagland nutrient solution with or without NaCl combined with three forms of N (nitrate, ammonium and their combination) was studied. At the end of the experiment, it was found that the trifoliate orange plants were more sensitive to salinity, since the weight of the fresh matter and the concentration of chlorophyll in the leaves were negatively affected. The highest values in the weight of the leaves and roots of the trifoliate orange plants were observed in the treatments with ammonium N, while the highest concentration of chlorophyll was observed in the treatments with a combination of nitrate and ammonium N under normal conditions. Furthermore, the highest values in the FW of the roots and shoots as well as in the chlorophyll units in the basal and top leaves were found in the sour orange plants which received a combination of nitrate and ammonium N under salinity conditions. Generally, N forms had different effects on the two genotypes in many cases. Finally, the inclusion of NaCl in the nutrient solution increased Na concentration in the leaves, the shoots and the roots of the two genotypes, whereas K concentration was reduced.

Key words: nitrogen forms, salinity, sour orange, trifoliate orange, citrus

Introduction

Salinity has existed in nature for centuries before the appearance of humans, constituting a threat to agriculture in certain parts of the planet for over 3,000 years (Flowers, 2006). The term refers to a high concentration of ions (as a rule Na^+ and Cl^-), mainly in the roots.

The origin of salts found in the ground can be attributed to the type of rocks and minerals, salty lakes, irrigation with water of low quality, high underground water levels, bad soil drainage, low precipitation, area topography, the existence of impermeable ground strata, high evapotranspiration and the upward movement of water (Therios, 1996). In general, saline soil problems are more intense in areas with semi-arid and arid climates, where high values of evapotranspiration and low levels of rainfall are found, which are insufficient for washing away salts in the ground. A major problem for agriculture is secondary soil salinisation due to the use of water of low quality for irrigation. The factors mentioned above have led to the fact that 50% of irrigated areas are adversely affected by high salt concentration (Zhu, 2001), often as a result of secondary salinisation. Salinity causes billions of dollars of damage worldwide (Pitman and Lauchli, 2002), while hundreds of thousands of hectares of cultivated land are taken out of production every year (Martinez Beltran and Licona Manzur, 2005).

Salinity constitutes one of the most serious abiotic stresses with harmful consequences on productivity, especially of sensitive species, such as citrus fruits (Storey and Walker, 1999, Ben-Hayyim and Moore, 2007, Navarro et al., 2014), where osmotic, ionic and secondary oxidative stress is observed (Lee et al., 2013). The

accumulation of salts in the root zone reduces the yield of plants through the increase of osmotic pressure and the reduction of water availability (Zhu, 2001, Munns, 2002). These stresses impair basic metabolic functions (Munns, 2002, Loreto et al., 2003), while toxicity may lead to leaf drying and reduce the total photosynthetic surface, which in turn leads to a drop in the production of photosynthetic products and, more generally, a change in the production of organic compounds, necessary for maintaining growth (Munns, 2002). The rate of leaf fall due to salinity is the factor which determines the survival of the plant (Laüchli and Grattan, 2007). Furthermore, in citrus fruits, salinity may likely cause damage to the plants resulting in nutritional imbalances, which are attributed to the limited absorption of some elements, such as K (Romero-Aranda et al., 1998) and N (Chatzissavvidis et al., 2008).

However, there are significant differences in salinity resistance between different rootstocks in citrus fruits (Walker and Douglas, 1982, Behboudian et al., 1986). Thus, for example, the trifoliate orange exhibits resistance to salinity which relates to the reduction of Na^+ ion transfer to the tissues of the stems (Storey and Walker, 1999), while the sour orange is considered to display a relative sensitivity (Ben-Hayyim and Moore, 2007).

The resistance of plants to salinity is due to the exclusion of toxic ions, the accumulation of ions in the vacuoles of the plant cells and osmotic regulation through the production of substances which maintain osmotic pressure (Grieve and Walker, 1983). At the same time, one of the most important characteristics determining salinity resistance in citrus fruits is their ability to limit toxic ion accumulation in the tissues relative to the volume of water absorbed through transpiration and to prevent the movement of ions in the leaves (Balal et al., 2012, Banüls et al., 1997, Moya et al., 2003). Furthermore, salinity resistant citrus species respond immediately to the aforementioned stress by promoting the expression of the transporter genes, while simultaneously reducing the metabolic procedures inside the plant (Brumos et al., 2009). Increasing the resistance of citrus plants to salinity is one of the most significant goals of their cultivation (Navarro et al., 2014). The creation of plant genotypes which will achieve sufficient nutrition in soils poor in available nutrients is an environmentally friendly approach, which limits the degradation of land by reducing the use of agricultural machinery and minimizing the application of chemical substances in crops (Thongbai et al., 1993).

Materials and Methods

One-year-old trifoliate orange (*Poncirus trifoliata* (L.) Raf.) and sour orange (*Citrus aurantium* L.) plants were used. Bare-rooted plants were decapitated at a height of 15cm, all lateral shoots were removed, and they were weighed and planted in 5L plastic bags. A sand-perlite medium was used at a ratio of 1:1 by volume. The plants were grown in the greenhouse of the Department of Agricultural Development of the Democritus University of Thrace (longitude: 26°31, latitude: 41°29), under natural lighting and an average temperature of 24.7°C. During the first 29 days, the plants received 0.5 L of water every 2 days. Then, plants of similar size were selected and divided into six treatments. Afterwards, every two days the plants were irrigated with 0.2 L of a half-strength modified Hoagland nutrient solution (Table 1). More specifically, each of the first three treatments included one of three forms of nitrogen (NO_3^- - N, NH_4^+ -N, and a combination of the two forms at a ratio of 1:1.6) respectively, while the other three treatments included one of the three forms of N each, combined with NaCl. In every treatment, the total N concentration was 2.6 mol/m^3. Also, the plants received distilled water every 15 days in order to wash away salts. The experiment lasted for 57 days, until the first severe symptoms of salinity (marginal necrosis of basal leaf blades) were observed.

At the end of the experiment, measurements were taken of the fresh matter weight (FW) of the leaves, the stems and the roots of the plants, as well as the length of the lateral shoots. Additionally, K and Na concentrations in the leaves, stems and roots were determined with a flame photometer (Jenway), P was determined using the vanado-molybdo-phosphate yellow colour method with a Shimadzu spectrophotometer, while chlorophyll was determined in SPAD units (Opti-Sciences, CCM-200, USA) in mature top and basal leaves. Furthermore, the efficiency of the use of K (KUE) and P (PUE) was calculated as the quantity of biomass produced per element unit. The JMP 8 application was used for the statistical processing of the data.

Table 1. Ingredients used in the Hoagland nutrient solution.

Ingredients (mM)	Treatments*					
	A	B	C	D	E	F
KH_2PO_4	0.40	0.40	0.40	0.40	0.40	0.40
$MgSO_4 * 7H_2O$	0.25	0.25	0.25	0.25	0.25	0.25
$Ca(NO_3)_2$	1.00	-	0.50	1.00	-	0.50
KNO_3	0.60	-	-	0.60	-	-
KCl	-	0.60	0.50	-	0.60	0.50
$CaCl_2$	-	1.00	0.50	-	1.00	0.50
NH_4Cl	-	2.60	1.60	-	2.60	1.60
NaCl	-	-	-	50	50	50

*A: no NaCl + NO_3^--N, B: no NaCl + NH_4^+-N, C: no NaCl + NO_3^-/NH_4^+-N, D: NaCl 50 mM + NO_3^--N, E: NaCl 50 mM + NH_4^+-N, F: NaCl 50 mM + NO_3^-/NH_4^+-N

Results and Discussion

As far as leaf FW in the trifoliate orange is concerned, under no salinity, it exhibited its highest value in the treatment which received the ammonium form of N (NH_4^+-N) (7.64 g), while out of the treatments irrigated with NaCl, the highest value was found in the ones that received NH_4^+-N and the combination of NO_3^-/NH_4^+-N (5.46 and 5.15 g, respectively) (Figure 1A). The decrease in leaf FW in the trifoliate orange, in the presence of NaCl, is in line with the findings of Simpson et al. (2014), who, after a study of two trifoliate orange hybrids found that salinity led to a reduction of the standing leaf area. In the sour orange, the highest values were observed in the treatments with NH_4^+-N and NO_3^-/NH_4^+-N, both under salinity and in the control treatment. It is known that the form of N affects growth rate and nutrient uptake (Edwards and Horton, 1982). With few exceptions, higher growth rates are achieved with a combination of the two forms of N, whose favourable concentrations depend on the total concentrations of the administered elements (Marschner, 1995). On the whole, the leaf and root FW in the sour orange were statistically significantly higher (P<0.001) than the corresponding weights in the trifoliate orange.

Additionally, salinity reduced the root FW in the trifoliate orange, while, under these conditions, better results were observed in the treatment which received NO_3^-/NH_4^+-N (7.07 g FW) (Figure 1B). Under salinity, high concentrations of Na and Cl affected negatively plant growth (Morais et al., 2012). In the absence of NaCl, the application of different N forms did not seem to affect the root FW of one leguminous species. In the case of the sour orange, a significant reduction was noted in the root FW in the treatment that received NH_4^+-N in the presence of NaCl (9.61 g).

However, the addition of NaCl, as well as different N forms did not cause statistically significant differences in the stem FW, or the length of the lateral shoots of the two genotypes (Figure 2). The value of the first parameter ranged from 5.10 to 8.05 g for the trifoliate orange and from 6.05 to 8.69 g for the sour orange. At the same time, the highest value of the lateral shoot length in the trifoliate orange was 18.22 cm and in the sour orange 14.83 cm, without any statistically significant differences between the two rootstocks.

The results from the chlorophyll measurement (in SPAD units) of the basal leaves (Figure 3A) showed that the values for the trifoliate orange ranged from 36.93 in the treatment which received NO_3^--N with NaCl, to 81.80 in the treatment that received NO_3^-/NH_4^+-N without NaCl. On the other hand, salinity and the administered form of N did not contribute to the statistically significant differentiation of the values in the case of the sour orange. Concerning chlorophyll measurements (SPAD units) of top leaves (Figure 3B), values for the trifoliate orange had a wider range, from 42.02 to 59.65, whereas they fluctuated between 23.50 and 33.30 for the sour orange. The use of NaCl had a positive effect on the chlorophyll of the sour orange leaves in the treatments which received NH_4^+-N and a combination of NO_3^-/NH_4^+-N. However, previous studies showed that salinity had contributed to a drop in chlorophyll concentration in the sour orange (Roussos et al., 2013). Additionally, in the

trifoliate orange, the highest value was found in the treatment with the combination NH_4^+/NO_3^--N, with and without salinity. Overall, the SPAD chlorophyll values in the leaves of the trifoliate orange were statistically significantly higher (P<0.001) compared to those of the sour orange.

However, the values of chlorophyll with regard to FW, as well as standing leaf area, were not significantly affected in the various treatments (Figure 4). The largest concentration of chlorophyll in terms of leaf FW of the trifoliate orange was found in the treatment with NO_3^-/NH_4^+-N without NaCl (1.37 mg/g), while, in the sour orange, it was found when providing NH_4^+-N with NaCl (1.24 mg/g). The same treatments also gave the highest concentrations of chlorophyll in terms of standing leaf area in both the trifoliate and sour orange (no data presented).

After the determination of Na concentration in the leaves, it was observed that the plants that grew without NaCl had a Na concentration under 0.53%, while the ones irrigated with NaCl exhibited values over 1.18% (Figure 5). At the same time, the values of this parameter were statistically significantly higher in the trifoliate orange than in the sour orange in all the treatments with NaCl, which is contrary to the conclusion that Simpson et al. (2014) reached when comparing Na ion accumulation in sour orange plants and trifoliate orange hybrids. Generally, the sour orange is considered to sufficiently exclude Na ions compared to other commercial rootstocks (Syvertsen and Garcia-Sanchez, 2014). In the roots, Na concentration under salinity ranged from 1.29 to 1.38% for the sour orange and 1.16 to 1.31% for the trifoliate orange (Figure 6A), exhibiting higher values in both rootstocks compared to the treatments without NaCl. Similar results were reached by Kostopoulou et al. (2014), where Na concentration in sour orange roots increased by 42.3% after the addition of NaCl. The lowest Na concentration among the treatments with NaCl, was observed in the roots of trifoliate orange plants which received NO_3^--N. Also, Na concentrations in the stems of both genotypes with the addition of NaCl ranged between 0.28 and 0.31% (Figure 6B), with the exception of the trifoliate orange treatment irrigated with NO_3^--N, which exhibited the statistically lowest concentration of the element (0.24%). Na concentrations in the leaves, the roots and the stems of the two genotypes were statistically significantly higher (P<0.001) under salinity compared to the control treatment. Kostopoulou et al (2014) arrived at a similar conclusion when studying mandarin plants grafted onto *C. aurantium* and *Swingle citrumelo* rootstocks exposed to salinity.

Potassium concentrations in sour orange leaves ranged from 1.08 to 1.26%. The highest K concentration was found in the control treatment with NO_3^--N. Additionally, a significant reduction in K concentration is observed when applying NO_3^--N, or NH_4^+-N in the sour orange in the presence of NaCl. The existence of Na and Cl ions in the substrate may affect the uptake of nutrients through antagonistic interactions and by affecting membrane selectivity (Ruiz et al., 1997). With regard to trifoliate orange plants, the addition of NaCl in the nutrient solution led to a significantly lower K concentration (1.12 to 1.14%), compared to the treatments without NaCl (1.29 to 1.48%) (Figure 7A). NaCl causes a decline in K concentration in the roots of both genotypes. In the majority of studies referring to the toxic effect of NaCl, a drop in K^+ concentration is observed due to competition with Na^+ in plant roots (Cerda et al., 1995). Additionally, K concentration values in the roots of the trifoliate orange were two to five times higher than the corresponding values in the sour orange (Figure 7B). Inclusion of ammonium N in the nutrient solution led to an increase in K concentration in the trifoliate orange control plants, as also found by Chatzissavvidis et al. (2007) for three Greek olive cultivars. Overall, under salinity, K concentration exhibited a drop in the leaves and the roots of the two genotypes.

Potassium concentration in trifoliate orange stems grown under normal conditions ranged between 0.49% (NO_3^-/NH_4^+-N) and 1.12% (NO_3^- -N) (Figure 8A). Furthermore, the most significant decline in K concentration was found in the treatment that received NO_3^--N with NaCl. Conversely, plants irrigated with NO_3^-/NH_4^+-N and NaCl showed an increase in K concentration compared to the corresponding treatment under normal conditions. In the sour orange, the value of the above parameter exhibited a statistically significant decrease with the addition of NaCl, as mentioned by Kostopoulou et al. (2014). The lowest value of K concentration in the said plant was observed when administering NO_3^--N and NaCl.

The addition of NaCl led to a statistically significant increase in K use efficiency (KUE) in the two genotypes (Figure 8B). Limited K uptake is

potentially counterbalanced in part by its storage for the production of biomass (Hafsi et al., 2007). Moreover, perhaps the availability of the osmotically active Na^+ ions is what causes K^+ ions to be channeled into other metabolic pathways, such as the increase of photosynthesis (Speer & Kaiser, 1991). The statistically significantly lowest KUE was observed when treating the plants with NO_3^--N for the trifoliate orange or NH_4^+-N for the sour orange, under non-saline conditions. At the same time, the statistically significantly highest KUE in both the trifoliate orange and the sour orange was found in the treatment with NO_3^--N and NaCl (152 and 196, respectively). Overall, genotypic differences were observed in terms of this parameter and more specifically, its values were statistically significantly higher in the sour orange than in the trifoliate orange in all the treatments. Saykhul et al. (2013) reached a similar conclusion after studying three olive cultivars in a hydroponic experiment.

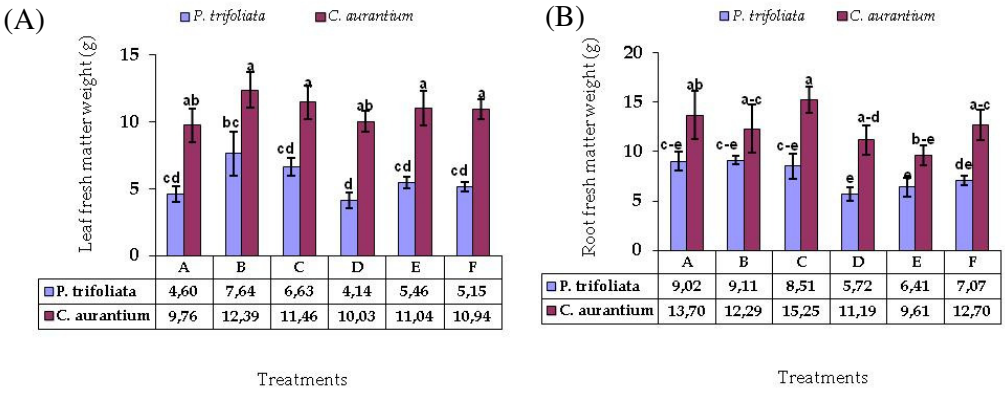
In sour orange leaves there were no statistically significant differences in P concentration between the treatments as it ranged from 0.14 to 0.17% (Figure 9A). P concentrations in the trifoliate orange ranged between 0.17 and 0.22% in the treatments without NaCl, while under salinity, they were found to be between 0.24% and 0.27%. NH_4^+-N fertilisation without NaCl favours P absorption, as it has been observed in a previous study (Torres de Claasen and Wilcox, 1974), while similar results were reached by Chatzissavvidis et al. (2007) for two olive cultivars. In total, it was observed that by using NaCl, P concentration increased significantly in trifoliate orange leaves.

Additionally, in the sour orange, most treatments did not show significant variations in P concentration in the roots (0.13 - 0.16%) (Figure 9B). An exception was the treatment that received NO_3^--N (0.10%) under salinity, possibly due to the competition of P with NO_3^- and Cl^-. Tsampardoukas et al. (2009) reported a similar conclusion while studying the effects of various N forms on ungrafted olive plants. In the trifoliate orange plants, P concentrations were predominantly higher compared to the sour orange. Therefore, P concentration ranged from 0.12 to 0.20% while salinity increased P concentration considerably in plants that received NO_3^--N.

As far as P concentration in sour orange stems is concerned, its highest value was found in the treatment irrigated with NH_4^+-N and no NaCl (0.11%), while the addition of NaCl caused a decrease in P concentration in the stems (Figure 10A). However, in the trifoliate orange, the presence of NaCl in the treatment with NH_4^+-N led to a significant increase in the concentration of the element in the stems. At the same time, the values of this parameter in the treatments irrigated with NO_3^-/NH_4^+-N remained unchanged under normal or saline conditions.

Plants differ genetically in their ability to absorb, transfer and accumulate nutrients during adaptation to environmental stresses (Shahbaz Akhtar et al., 2010). Specifically, with regard to P, genotypic differences have been found in terms of its use efficiency (Castillo et al., 2013, Trehan, 2009). However, in contrast to KUE, this parameter of P did not exhibit variations relating to the presence of NaCl, the application of different forms of N or the plant species. In the trifoliate orange, PUE values were found between 639 and 900, while in the sour orange they ranged between 686 and 990 (Figure 10B).

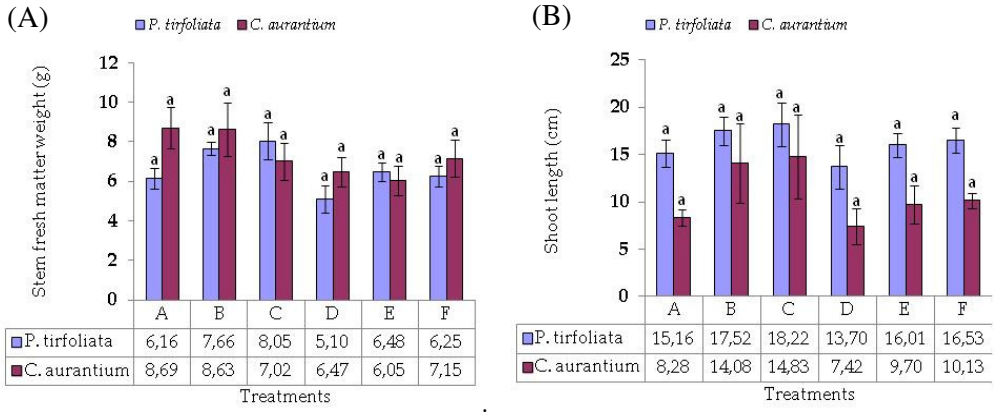
In conclusion, the trifoliate orange showed greater sensitivity to salinity than the sour orange, as leaf FW and chlorophyll concentrations were affected to a greater degree. Also, the inclusion of NaCl in the nutrient solution caused significant variations in the uptake of Na, K and P, while the three N treatments did not affect growth and the chemical composition of the plants in the same way. Due to the great importance of these two genotypes as rootstocks in agriculture, it is deemed necessary to further study their behaviour under salinity and while using various nutritional methods.



	A	B	C	D	E	F
P. trifoliata	4,60	7,64	6,63	4,14	5,46	5,15
C. aurantium	9,76	12,39	11,46	10,03	11,04	10,94

	A	B	C	D	E	F
P. trifoliata	9,02	9,11	8,51	5,72	6,41	7,07
C. aurantium	13,70	12,29	15,25	11,19	9,61	12,70

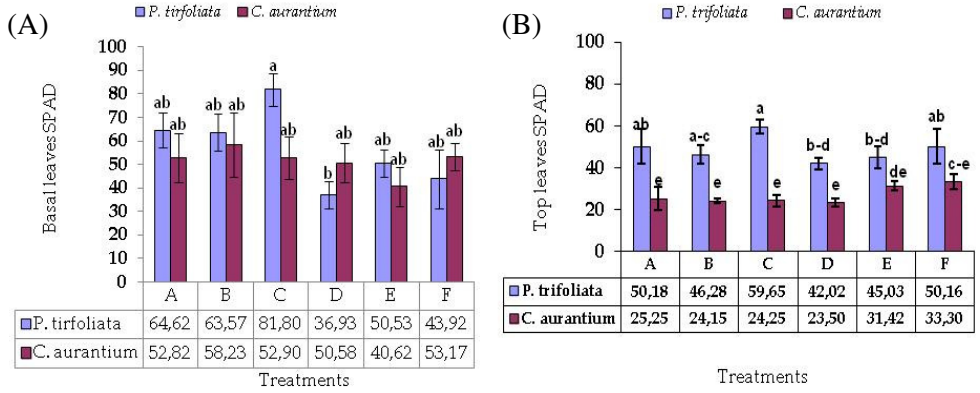
Figure 1. Fresh matter weight of leaves (A) and root (B). All the values are averages of six replicates. The different letters in the same column show statistically significant differences among the six treatments and the two genotypes (P<0.05).



	A	B	C	D	E	F
P. tirfoliata	6,16	7,66	8,05	5,10	6,48	6,25
C. aurantium	8,69	8,63	7,02	6,47	6,05	7,15

	A	B	C	D	E	F
P. tirfoliata	15,16	17,52	18,22	13,70	16,01	16,53
C. aurantium	8,28	14,08	14,83	7,42	9,70	10,13

Figure 2. Fresh matter weight of stems (A) and shoot length (B) of the plants *P. trifoliata* and *C. aurantium*.



	A	B	C	D	E	F
P. tirfoliata	64,62	63,57	81,80	36,93	50,53	43,92
C. aurantium	52,82	58,23	52,90	50,58	40,62	53,17

	A	B	C	D	E	F
P. trifoliata	50,18	46,28	59,65	42,02	45,03	50,16
C. aurantium	25,25	24,15	24,25	23,50	31,42	33,30

Figure 3. SPAD readings in basal leaves (A) and top leaves (B) of the plants *P. trifoliata* and *C. aurantium*.

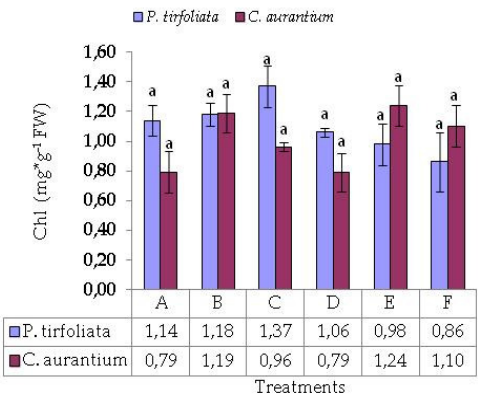


Figure 4. Leaf chlorophyll concentration of the plants *P. trifoliata* and *C. aurantium*.

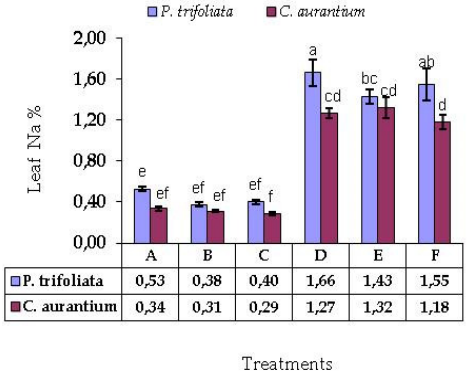


Figure 5. Leaf Na concentration of the plants *P. trifoliata* and *C. aurantium*.

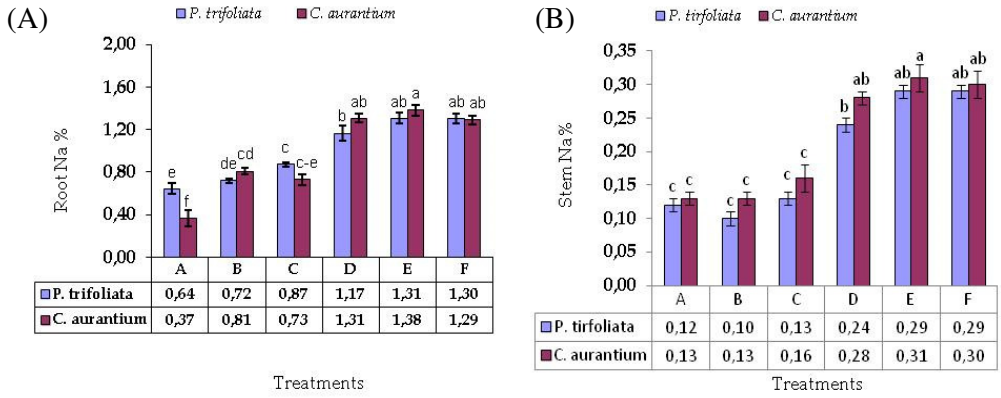


Figure 6. Na concentration in root (A) and stems (B) of the two genotypes.

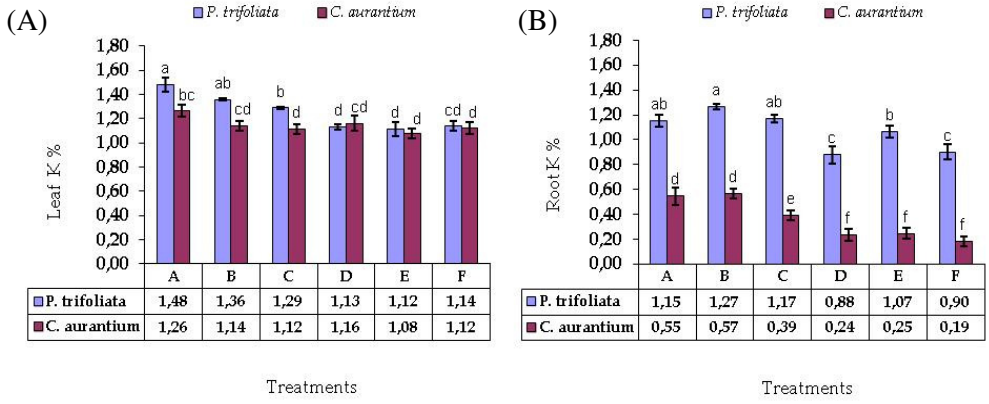


Figure 7. K concentration in leaves (A) and root (B) of the two genotypes.

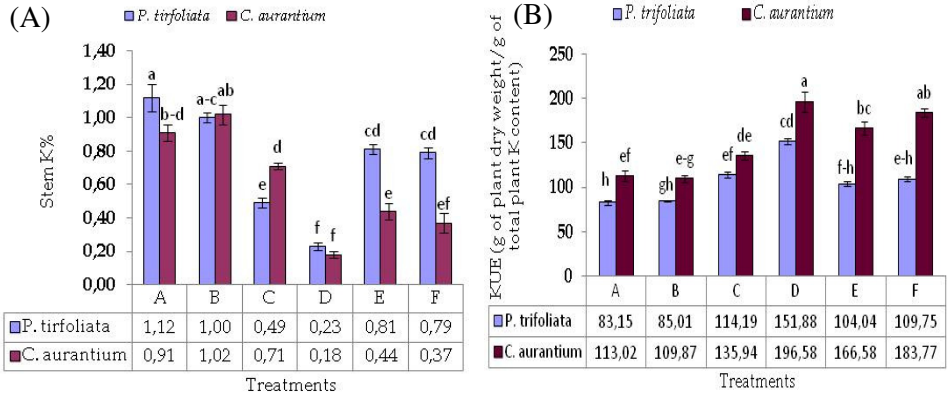


Figure 8. K concentration of stems (A) and KUE (B) of the two genotypes.

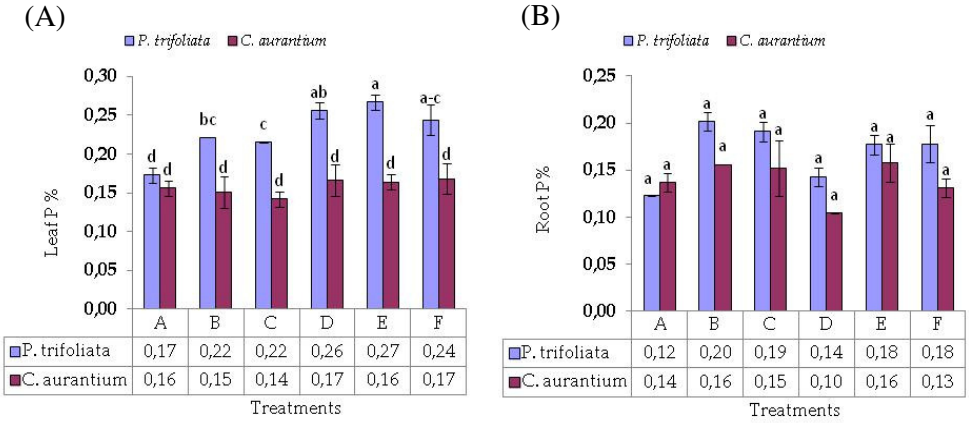


Figure 9. P concentration in leaves (A) and roots (B) of the two genotypes.

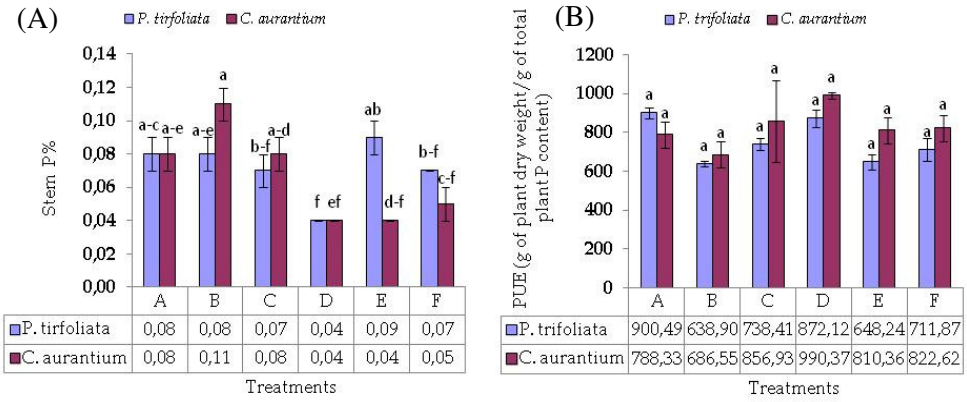


Figure 10. P concentration of stems (A) and PUE (B) of the two genotypes.

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