Alleviatory activities in mycorrhizal tobacco plants subjected to increasing chloride in irrigation water

Ali Reza Safahani Langeroodi,[1] Farshad Ghooshchi,[2] Teena Dadgar[3]

[1]Department of Agronomy, Payame Noor University, Tehran; [2]Department of Agronomy, Varamin-Pishva Branch, Islamic Azad University, Varamin; [3]Department of Biology, Gorgan Branch, Islamic Azad University, Gorgan, Iran

Abstract

The effects of presence and absence of arbuscular mycorrhizal (AM+ and AM-) fungus (AMF) *Glomus intraradices* on agronomic and chemical characteristics of field-grown tobacco (*Nicotiana tabacum* L.) Virginia type (cv. K-326) plants exposed to varying concentrations of chloride 10, 40, 70 and 100 mg Cl L^{-1} (C1-C4) were studied over two growing seasons (2012-2013). Mycorrhizal plants had significantly higher uptake of nutrients in shoots and number of leaves regardless of intensities of chloride stress. The cured leaves yields of AM+ plants under C2-C4 chloride stressed conditions were higher than AM- plants. Leaf chloride content increased in line with the increase of chloride level, while AMF colonised plants maintained low Cl content. AM+ plants produced tobacco leaves that contained significantly higher quantities of nicotine than AM- plants. AM inoculation ameliorated the chloride stress to some extent. Antioxidant enzymes like superoxide dismutase, catalase, ascorbate peroxidase, and glutathione reductase as well as non-enzymatic antioxidants (ascorbic acid and glutathione) also exhibited great variation with chloride treatment. Chloride stress caused great alterations in the endogenous levels of growth hormones with abscisic acid showing increment. AMF inoculated plants maintained higher levels of growth hormones and also allayed the negative impact of chloride. The level of 40 mg L^{-1} in combination with arbuscular mycorrhizal can be considered as the acceptable threshold to avoid adverse effects on Virginia tobacco.

Correspondence: Ali Reza Safahani Langeroodi, Department of Agronomy, Payame Noor University, 19395-3697 Tehran, Iran.
Tel: +98.9113711990.
E-mail: safahani_ali@yahoo.com

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Introduction

Soil salinity problems arose during the last year in coastal plains of Mediterranean areas, caused mainly by the poor quality of the irrigation water and increasing noticeably during the dry season (Selvakumar *et al*., 2014; Sifola and Postiglione, 2002).

Excessive quantities of chloride in the cured leaf reduce the rate of burn and cause certain adverse effects such as increased hygroscopicity, dinginess, uneven colours and undesirable odors in cured tobacco leaves (Karaivazoglou *et al*., 2006). Salt stress causes physiological drought to plants, imbalance in nutrient composition and excessive toxicity due to Na and Cl ions thereby leading to reduction in osmotic potential of plants, disruption of cell organelles and their metabolism. These ultimately affect plant growth and reduce the yield.

Arbuscular mycorrhizal fungi (AMF) are associated with the roots of over 80% terrestrial plant species (Smith and Read, 1997) including halophytes, hydrophytes and xerophytes. AMF have been shown to promote plant growth and salinity tolerance by many researchers; They promote salinity tolerance by employing various mechanisms, such as enhancing nutrient acquisition (Abeer *et al*., 2015), producing plant growth hormones, improving rhizospheric and soil conditions (Selvakumar *et al*., 2014), increased root hydraulic conductivity, enhanced water uptake due to extraradical hyphae, osmotic adjustment that promotes turgor maintenance and accumulation of antioxidant compounds (Colella *et al*., 2014) and altering the physiological and biochemical properties of the host (Gamalero *et al*., 2010). In addition, AMF can improve host physiological processes like water absorption capacity of plants by increasing root hydraulic conductivity and favourably adjusting the osmotic balance and composition of carbohydrates (Kumar *et al*., 2015; Ruiz-Lozano, 2003). This may lead to increased plant growth and subsequent dilution of toxic ion effect (Daei *et al*., 2009). These benefits of AMF have prompted it to be a suitable candidate for bio-amelioration of saline soils.

To date, no information is available about the interaction between of AM fungi and high chloride concentration in irrigation water on the agronomical and physiological responses of tobacco. Therefore, the purpose of this research was to determine the effect of different levels of chloride stress and AM inoculation on various morpho-biochemical parameters of tobacco. This study may be helpful in order to further understand salt tolerance mechanisms in AM plants.

Materials and methods

Field preparation and application of experimental treatments

Field experiments were conducted in the Tirtash Tobacco Research Institute (36 45´N; 53 44´E, altitude: 14 m) in northern Iran over two years (2012-2013). Some physicochemical characteristics of the soil used are, sand 47%, silt 24.6%, clay 28%, lime 2.2%, pH 7.4, N 0.13%, EC 2.5 dS m^{-1}, low organic carbon status (0.40%), total N (0.06%), and available P (6.5 mg kg^{-1}) and available K status (187.5 mg kg^{-1}), field capacity 26.4 % dry weight and wilting point 14.9 % dry weight.

The soil was kept fallow for a year to reduce indigenous mycorrhizal fungi and decompose the root fragments of previous crop to eliminate propagules. Since mycorrhizal spore propagules extracted from the native soil were extremely low (1-2 per kg), no attempt was made to fumigate the soil. Vermiculite-based mycorrhizal inoculum carrying arbuscular mycorrhizal (*Glomus intraradices* Schenck & Smith; in recent years known as *Rhizophagus irregularis*; Schußler and Walker, 2010), which is known to colonize the roots of *N. tabacum* L. (Cosme and Wurst, 2013) fungus was applied in a tobacco nursery at a rate of 100 g m^{-2} just prior to sowing seeds on sowing lines marked at a distance of 5 cm apart. The inoculum was originally cultured in maize roots; heavily colonized roots (100 g) carrying the propagules (spores, infected roots, soil) were diluted in sterile vermiculite (1 kg). The mycorrhizal and non-mycorrhizal tobacco nurseries were maintained separately. The fertility status of the nursery soil was similar to that of the experimental soil. The inoculated and non-inoculated tobacco plants were irrigated once every 2-3 days. Tobacco roots were tested for mycorrhizal colonization at the end of 4 weeks after inoculation (28 days after sowing). After establishment of the symbiosis (approximately 40% root colonization), mycorrhizal and non-mycorrhizal tobacco plants were transplanted in the main field at a spacing of 0.5 m between plants and 1 m between rows, in during early June of two years (2012 and 2013). On an average, 2 plants per square meter were maintained in each plot measuring a dimension of 10 m length × 6 m width (60 m^2). Also, all the experimental plots were surrounded with earth dikes, and a distance of 3 m between plots was left bare in order to prevent the lateral spread of water. The tillage practices, including cultivation and disking, were the common conventional practices in the region.

The plants were drip irrigated with four level of chloride included: 10, 40, 70 and 100 mg Cl L^{-1} (C1-C4). Chloride was added to the water as $CaCl_2$. The fact that the10 mg Cl L^{-1} concentration in water is considered very low and without adverse effects on tobacco (Karaivazoglou *et al*., 2006) led us to the decision to take this chloride concentration as control.

Before transplanting 53 kg ha^{-1} of P and 125 kg ha^{-1} of K were added to the top 0.2 m of soil. N fertilizers of 120 kg ha^{-1} were distributed as follows 50% at transplanting as ammonium sulphate (21% N) and 50% as ammonium nitrate (26% N) at five side dressing. The latter was split into applications, one at seedling establishment and one at the beginning of rapid stem elongation.

During the first half of August, when approximately 50% of plants per plot were flowering, the plants were topped at a height of 24-25 leaves per plant. An average of about 64 plants per treatments was harvested from the central part of each plot (32 m^2) to determine yield.

Rainfall and temperatures during the cultivation period (May-September) in the two experimentation years are shown in Table 1. Rainfall and temperature during two years of experiments were similar and in accordance with the regional average. The same number (10) of irrigations (same amount of chloride) was applied in each year.

A 2×4 factorial randomized block design included two mycorrhizal treatments (with AM, AM+ or without AM, AM-) and four chloride levels in irrigation water (C1-C4) replicated four times on agronomic and chemical properties of Virginia tobacco (cv. K-326; The selected cultivar is the highest quality commercial cultivars in the north of Iran).

Data collection

Photosynthesis measurement

Carbon exchange rate (CER), transpiration rate (E) and stomatal conductance (gs) were measured by an infrared gas analyser (Li-6400; LI-COR, Lincoln, NE, USA) on four replications per treatment from 9:30 to 10:40 am at a sunny day before harvest. Measurements were recorded when the total coefficient of variation was less than 0.5%.

Growth measurements and biochemical analysis

Ten plants were randomly selected from each experimental plot in each replication at flowering stage and the following parameters were recorded: dry shoot mass, dry root mass, number of leaves, percentage mycorrhizal root colonization, pigments and plant growth regulators, antioxidative enzymes and non-enzymatic. Water use efficiency (WUE), was calculated by dividing the total leaves yield (kg ha^{-1}) by the quantity of water consumed inclusive of precipitation (mm) as indicated by Boyer (1995) and mycorrhizal dependency at each chloride level. Mycorrhizal dependency (MD) or response to mycorrhizal colonization was calculated for plants in each chloride treatment by using the following formula (Gerdemann, 1975):

$$MD = \frac{\text{Dry weight of AM + plant at a particular level of chloride}}{\text{Dry weight of AM − plant at the same level of salinity}} \times 100$$

Table 1. Monthly distribution of the number of irrigations, irrigation volumes, rainfall and temperature in the two years of study.

Month	Irrigation (n)		Volume (mm)		Rainfall (mm)		Mean temperature (°C)	
	2012	2013	2012	2013	2012	2013	2012	2013
May	-	-	-	-	70	45	18.1	18.4
June	2	1	63	30	40	36	21.8	23.2
July	5	5	138	170	25	15	25	26.3
August	2	3	78	101	3	0	25.3	25.5
September	-	-	-	-	42	40	20.8	21.5

Irrigation water supplied at transplanting (average of 22 mm) was not included.

The AM fungi spore count in native field soil was minimal (~3 spores 100 g^{-1} air-dried soil). Root colonization by AM was determined by preparing root samples at 1 g in each experimental unit according to the method of Philips and Hayman (1970), and roots were stained using the Gridline- Intersect Method (Giovannetti and Mosse, 1980).

Each plant was extracted from the soil by digging a trench around it 0.3 m^2 by 0.6 m deep and removing it as a block. The roots were washed well to remove all traces of soil and the plants then separated into leaves, stalks and roots. The fresh weight of the sample plant parts was recorded and the samples were then dried to a constant weight in an oven at 70°C whereon dry weight was then recorded.

Leaves from control and Cl-stressed plants were excised at flowering stage to measure relative water content (RWC) and osmotic potential (Ψs) according to Turner (1981) and Martinez-Ballesta *et al*. (2004), respectively.

Extraction and quantification of pigments and plant growth regulators

Indole acetic acid (IAA) and abscisic acid (ABA) were extracted and purified as described by Kusaba *et al*. (1998). The method described by Lee *et al*. (1998) was followed for extraction and estimation of gibberellic acid (GA3) by gas chromatograph-mass spectrometer (GC-MS). Photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoids) in the leaves were determined as the method described by Moran (1982).

Free proline content and Electrolyte leakage (EL) in plant material were determined as the method described by (Bates *et al*., 1973; Dionisio-Sese and Tobita, 1998) respectively.

Extraction and estimation of antioxidative enzymes and non-enzymatic, such as ascorbate peroxidase (APX), superoxide dismutase (SOD), glutathione reductase (GR), catalase (CAT), ascorbic acid (AsA), glutathione (GSH) and oxidized glutathione (GSSG) were measured according to Nakano and Asada (1981), Van Rossum *et al*. (1997), Carlberg and Mannervik (1985), Luck (1974), Law *et al*. (1983) and Anderson (1985), respectively.

Cured leaf yield and chemical composition

Leaf yield was determined from an average of 64 plants per plots harvested from the central part of each plot. All plants were harvested by hand, in 5 primings, by removing 4-5 leaves each time at weekly intervals starting 6 weeks after transplanting. The harvested leaves, cured in typical oven for Virginia tobacco. Yield of cured leaves was determined at standard moisture content of 19% for each of the four stalk positions.

Minerals and chemicals composition of leaf chloride content was analysed using the standard AOAC (1997) method. Total N was analysed employing the Kjeldahl procedure (Bremmer and Mulvaney, 1982). Nicotine and reducing sugars were measured using CORESTA recommended methods No. 35 (CORESTA, 1994a) and No. 38 (CORESTA, 1994b), respectively. In addition, K, P and Mg were determined. K was determined by flame emission spectroscopy, P by the molybdenum blue-ascorbic acid method (Olsen and Sommers, 1982), Ca and Mg by atomic absorption spectroscopy.

Statistical analysis

Yield and other agronomic and chemical traits were subjected to analysis of variance (ANOVA). However, since the same number (10) of irrigations (same amount of chloride) was applied in each year, the response to chloride was relatively similar from year to year, as well as Bartlett's test and the combined analysis of the two growing seasons were applied. Bartlett's X^2 test showed that combining the data from both years was acceptable. In the analysis that follows, all values given are the averages of the data for the 2 years combined. Means were compared using least significant difference test at 5% level.

Table 2. Mean of cured leaf yield, water use efficiency, mineral contents in leaf, number of leaves, shoot dry weight, root dry weight, percentage root colonisation, mycorrhizal dependency, nicotine, reducing sugar and proline of mycorrhizal and non-mycorrhizal tobacco plants exposed to varying concentrations of chloride (average of two growing seasons, 2012-2013).

Treatment	Root colonisation (%)	MD (%)	Leaves per plant	Root dry weight (g plant^{-1})	Shoot dry weight (g plant^{-1})	Yield of cured leaves (kg ha^{-1})	WUE (kg m^{-3})	Mg (%)	P (%)	K (%)	N (%)	Nicotine (%)	Reducing sugar (%)	Proline (mg g dry wt^{-1})
AM+														
C1	67^{a}	120^{a}	24.2^{a}	29.9^{a}	354.5^{a}	2329.00^{a}	1.66^{a}	0.66^{a}	0.18^{a}	2.2^{a}	2.10	1.41^{a}	11.1^{c}	2.14^{c}
C2	59^{b}	119^{a}	23^{ab}	25.8^{b}	327.4^{ab}	2297.66^{a}	1.64^{a}	0.64^{a}	0.17^{ab}	2.1^{ab}	2.09	1.23^{ab}	12.5^{bc}	3.76^{b}
C3	50^{c}	115^{b}	21^{b}	24.1^{bc}	295.1^{bc}	2021.56^{b}	1.57^{ab}	0.60^{b}	0.16^{b}	2.0^{b}	2.05	1.2^{b}	13.3^{b}	4.89^{b}
C4	43.3^{d}	113^{b}	18.5^{c}	23.2^{c}	290.2^{c}	1971.02^{b}	1.40^{b}	0.59^{b}	0.14^{c}	1.8^{c}	2.01	1.15^{b}	15.7^{a}	6.18^{a}
LNSD	6.1	3	2.2	1.8	35.2	66.3	0.19	0.03	0.02	0.2	ns	0.19	1.5	1.16
AM-														
C1	-	-	21^{a}	25.5^{a}	295.4^{a}	1998.21^{a}	1.44^{a}	0.61^{a}	0.12^{a}	1.6^{a}	2.00^{a}	1.09	8.1^{b}	1.73^{b}
C2	-	-	16.2^{b}	21.7^{b}	278.7^{ab}	1899.32^{b}	1.35^{a}	0.54^{b}	0.11^{a}	1.5^{a}	1.90^{ab}	1.03	10.2^{a}	2.80^{ab}
C3	-	-	12^{c}	19.1^{bc}	256.8^{bc}	1785.74^{c}	1.23^{b}	0.51^{bc}	0.09^{b}	1.3^{b}	1.85^{b}	1.08	10.9^{a}	3.23^{a}
C4	-	-	11^{c}	18.2^{c}	244.5^{c}	1734.11^{c}	1.20^{b}	0.49^{c}	0.08^{b}	1.2^{b}	1.80^{b}	1.1	11.3^{a}	3.45^{a}
LSD			3.7	2.8	30.5	80.7	0.11	0.04	0.02	0.2	0.17	ns	1.8	1.27
ANOVA														
C	*	*	*	*	*	*	*	ns	ns	ns	ns	ns	*	*
AM	**	**	**	**	**	**	**	*	*	*	*	*	*	*
C×AM	*	*	*	*	*	*	*	*	*	*	*	*	*	*

MD, mycorrhizal dependency; WUE, water use efficiency; Mg, magnesium; P, phosphorus; K, potassium; N, nitrogen; AM+, mycorrhizal; C1, 10 mg Cl L^{-1}; C2, 40 mg Cl L^{-1}; C3, 70 mg Cl L^{-1}; C4, 100 mg Cl L^{-1}; LSD, least significance difference; AM-, non-mycorrhizal; ANOVA, analysis of variance; C, chloride; AM, arbuscular mycorrhizal. ns, not significant; *P≤0.05; **P≤0.01. $^{a-d}$Means with different letters are significantly different at P≤0.05.

Results and discussion

Mycorrhizal colonisation and mycorrhizal dependency

Twenty eight day old mycorrhiza-inoculated tobacco seedlings had 40% colonisation at the time of transplanting while non-inoculated seedlings registered only 1-2% colonisation. After a month of exposure to varying concentrations of chloride level in the main field, none of the tobacco plants in the non-inoculated treatments were colonised by the AM when examined during the experimentation (Table 2). The highest AM root colonisation was observed in C1 treatment, and it decreased significantly with increasing chloride levels (Table 2). Previous research has shown that salinity, not only affects negatively the host plant but also the AMF. It can hamper colonisation capacity, spore germination, and growth of fungal hyphae and hyphal spreading after initial colonisation (Kumar *et al*. 2010, 2015; Miransari, 2010).

The data presented in Table 2 reveal that the mycorrhizal dependency (MD) of tobacco plants were significantly reduced by increased chloride levels (Table 2). In AM tobacco, the highest MD values belonged to C1in comparison with other chloride levels. A decrease in MD at higher chloride level (C2-C4) could be due to the inhibitory effect of chloride on AM fungal growth and spore density (Kumar *et al*., 2015).

Plant growth parameters

The treatments of increasing chloride level significantly decreased all the growth attributes such as number of leaves, dry shoot mass and dry root mass of both AM- and AM+ tobacco plants (Table 2). However, in growth parameters, AM+ plants were higher than AM- plants regardless of concentrations of chloride level. The highest chloride level, AM+ plants were comparable to C1, AM- plants.

In *Vigna unguiculata* L, Abeer *et al*. (2015) demonstrated that length as well as fresh and dry biomass of shoot and root declined with the increasing salinity. Exposure to stress reduces hydraulic conductivity and disturbs extension of cell wall causing considerable decline in morphological attributes of plants (Selvakumar *et al*., 2014). An increased root length and density or an altered root system morphology, as enhancing soil exploration and water extraction, have been hypothesised as potential mechanisms for the improved stress resistance of mycorrhized plants (Gamalero *et al*., 2010; Candido *et al*., 2013, 2015).

Cured leaf yield and water use efficiency

The cured leaf yield and water use efficiency (WUE) of tobacco decreased significantly under increasing concentrations of chloride. Conversely, mycorrhizal inoculation enhanced the tobacco leaf production and WUE regardless of concentrations of chloride level (Table 2). Mycorrhizal colonisation improved growth, water status, nutrient content, yield and quality of tobacco leaf when exposed to varying concentrations of chloride level. The 2-year field study suggests that AM inoculation improves salinity tolerance of tobacco plants as a secondary consequence of enhanced nutritional status of the host plant, especially N and P.

Tobacco leaf yield decreased with salinity, in agreement with many studies on tobacco (Karaivazoglou *et al*., 2005; Sifola and Postiglione, 2002). Reduced yield under chloride stress treatments could be attributed to $CaCl_2$ increasing the osmotic potential of the solution and the activity of Cl in the root zone (Karaivazoglou *et al*., 2005, 2006). Those changes may have affected plant growth and, consequently, yield through their effects on plant-water relationship and on nutritional imbalances (Abeer *et al*., 2015). Direct effects of salinity include accumulation of salt in old leaves, which may hasten leaf death. This prevents the supply of assimilates or hormones to the growing regions which eventually affects the plant growth. The improved nutritional status and relative water content caused by mycorrhizal colonisation would have alleviated salinity impacts and promoted tobacco leaf production under varying concentrations of chloride. Because mycorrhizal treatments consistently increased leaf yields under varying concentrations of chloride, WUE of AM plants were much higher than control plants.

In this study, mycorrhizal tobacco plants had higher WUE values compared with non-mycorrhizal plants, as indicated by lower water loss and higher RWC, probably because of improvement of water absorption capacity by AM fungus.

Our results also confirmed the water status (WUE and RWC) of tobacco plants positively correlated with photosynthesis activity. Mycorrhizal plants showed higher levels of photosynthesis activity indicating that AM symbiosis had a positive impact on mass flow of water to the leaf surface, and an increased water absorption by extraradical hyphae (Zhu *et al*., 2011). On the other hand, AM inoculated plants had better water status, which allow host plants to sustain higher stomatal conductance and transpiration rate (Table 3), consequently reducing leaf epidermal resistance and improving photosynthetic activity.

Leaf mineral composition

The effect of chloride on the content of K, N, Mg and P was not significant, although a slight decreasing trend was recorded with the increase of chloride in irrigation water (Table 2). Colonisation of AMF caused increase in these mineral ions as compared to control non-AMF plants and also reduced the chloride stress induced impact to marked extent (Table 2). It is well known that salinity causes nutrient imbalance in plants. AMF help plants to uptake more nutrient. Chloride ion has antagonistic relationship with several other ions like K. In present study higher concentration of chloride and lowered concentrations of other ions like K, P, Mg under increasing of concentrations of chloride is in concurrence with the findings of Bilgili *et al*. (2011) for canola and Abeer *et al*. (2015) for cowpea. AMF not only reduced the deleterious effect of excess chloride by reducing its uptake but also caused a significant increase in uptake of other important mineral elements like K, P and Mg. AMF colonisation in wheat significantly increased the shoot concentrations of P, K, and Zn whereas decreased Na and Cl concentrations (Daei *et al*., 2009).

Regression analysis between levels of chloride in irrigation water and chloride concentrations in the leaves showed that chloride concentrations in leaves had a linear response to rates of irrigation water chloride (Figure 1), the rate of linear increase of leaf chloride concentration was higher in AM-plants than AM+ plants. Chloride concentration in leaves can be predicted with the equations are shown in Figure 1, according to chloride level in irrigation water and arbuscular mycorrhizal inoculation. It is considered that an acceptable Virginia tobacco should contain less than 1% of chloride. Leaves with higher chloride concentration are of poor quality with reduced burning rate (Sifola and Postiglione, 2002). Based on the above results it is preferable to use irrigation water with chloride concentration below 25 mg L^{-1} since at this level the chloride concentration in the leaves remained around 1%. On the other hand, the chloride level of 40 mg L^{-1} in irrigation water in combination with AMF can be considered as the threshold upper limit. In such high concentrations the use of AMF are recommended, because keep the leaf chloride concentrations around the acceptable level (Figure 1).

Nicotine and reducing sugar

In the present study, reducing sugar content was significantly enhanced with an increase in chloride level from C1 to C4, regardless of mycorrhizal treatments (Table 2). AM+ plants had considerably higher amount of reducing sugar compared to AM- plants (Table 2). Increasing chloride level enhanced soluble carbohydrate concentration in tobacco regardless of mycorrhizal treatments and is mainly because, carbohydrate plays a crucial role in maintaining osmotic balance in plant exposes to salt stress and hence protects plant from adverse salt effect (Datta and Kulkarin, 2014). Under increasing chloride level, AMF colonisations in tobacco increased reducing sugar accumulation, which is required for better osmoprotection [this kind of data is supported by Datta and Kulkarni (2014)].

The effect of chloride on nicotine concentration of leaves was not significant and showed inconsistent trend (Table 2). The nicotine concentration in the leaves of the tobacco was enhanced by AMF regardless of the chloride levels (Table 2). Similar inconsistent results and a very slight effect of chloride on nicotine content in Virginia, Burley, and Maryland tobacco have been reported by others (Collins and Hawks, 1993).

In our study the direction of microbial effects on nicotine concentrations in the leaves was often associated with the direction of their effects on N concentration, and independent of leaves yield. AMF is known to incorporate inorganic N outside the roots into amino acids, and translocate it from the extraradical to the intraradical mycelium as arginine (Cosme and Wurst, 2013), which is a precursor of nicotine (Fritz *et al*., 2006). This may be the reason why in our set up the positive effects of AMF on leaves N concentration were associated with the greatest increases in nicotine.

Photosynthetic activity, chlorophyll contents and carotenoids

Increasing of concentrations of chloride in irrigation water markedly decreased their carbon exchange rate (CER) and E in the AM- plants (Table 4). Chloride stress decreased their g_s in all plants. Mycorrhizal plants had higher CER, E and gs than the AM- plants under all concentrations of chloride level (Table 4).

Significant reduction in photosynthesis was found in salt-stressed tobacco (Sifola and Postiglione, 2002). In the present study, Increasing of concentrations of chloride also reduced the CER, E and gs in both AM- and AM+ plants. However, mycorrhizal plants had significantly higher CER, E and gs than the non-mycorrhizal plants under varying concentrations of chloride level (Table 4). A number of studies have demonstrated that, during abiotic stress, mycorrhizal plants often maintain higher gas exchange rates than non-mycorrhizal plants (Ruiz-Lozano, 2003). These positive effects may also have accounted for the enhanced plant growth of AM-colonised plants, most probably by enhancing CO_2 fixation under salt stress. The higher values of photosynthetic

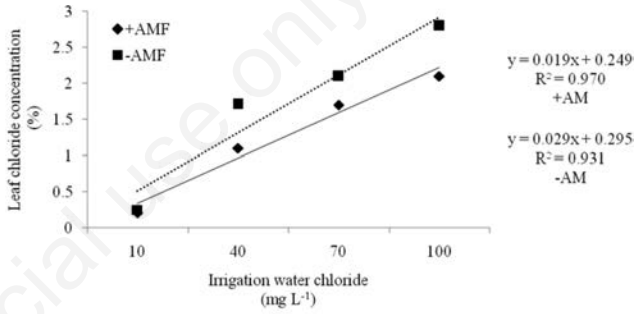

Figure 1. Correlation between levels of irrigation water chloride and chloride concentrations of leaves in presence and absence of arbuscular mycorrhizal fungi (average of two growing seasons, 2012-2013).

Table 3. Mean of catalase, ascorbate peroxidase, superoxide dismutase, ascorbic acid, reduced glutathione, oxidised glutathione, and glutathione reductase in leaf of mycorrhizal and non-mycorrhizal tobacco plants exposed to varying concentrations of chloride (average of two growing seasons, 2012-2013).

Treatment	SOD (EU mg^{-1} protein)	CAT (EU mg^{-1} protein)	APX (EU mg^{-1} protein)	GR EU mg^{-1} (protein)	ASA (n Molg fresh wt^{-1})	GSH (n Mol g fresh wt^{-1})	GSSG (n Mol g fresh wt^{-1})
AM+							
C1	115^{c}	125^{d}	4.5^{c}	8.1^{c}	5.5^{a}	97.2^{c}	25.7^{c}
C2	136^{b}	147^{c}	6.7^{b}	9.1^{c}	4.7^{b}	153.1^{b}	55.2^{b}
C3	140^{ab}	175^{b}	8.1^{a}	13.0^{b}	4.3^{b}	174.5^{ab}	64.2^{b}
C4	149^{a}	195^{a}	8.9^{a}	14.2^{a}	4.1^{b}	201.7^{a}	87.5^{a}
LSD	10	15	1.2	1.1	0.7	37.1	21.2
AM-							
C1	95^{c}	101^{c}	4.1^{c}	7.1^{b}	4.8^{a}	87.7^{c}	28.2^{c}
C2	120^{b}	133^{b}	6.2^{b}	8.5^{b}	3.1^{b}	145.1^{b}	44.8^{bc}
C3	133^{a}	154^{a}	7.1^{ab}	10.3^{a}	2.8^{b}	165.1^{ab}	59.4^{b}
C4	141^{a}	160^{a}	7.7^{a}	11.2^{a}	2.7^{b}	189.9^{a}	78.5^{a}
LSD	11	17	1.4	1.5	0.9	38.3	25.1
ANOVA							
C	*	*	*	*	*	*	*
AM	*	*	*	*	*	*	*
C×AM	**	**	**	**	*	*	*

SOD, superoxide dismutase; EU, endotoxin units; CAT, catalase; APX, ascorbate peroxidase; GR; glutathione reductase; ASA, ascorbic acid; GSH, reduced glutathione; AM+, mycorrhizal; C1, 10 mg Cl L^{-1}; C2, 40 mg Cl L^{-1}; C3, 70 mg Cl L^{-1}; C4, 100 mg Cl L^{-1}; LSD, least significance difference; AM-, non-mycorrhizal; ANOVA, analysis of variance; C, chloride; AM, arbuscular mycorrhizal. *P≤0.05; **P≤0.01. $^{a-d}$Means with different letters are significantly different at P≤0.05.

activity in mycorrhizal plants indicate that the photosynthetic apparatus of these plants is less damaged by the salt stress imposed (Aroca *et al*., 2013).

AM symbiosis enhanced the concentrations of chlorophyll a, chlorophyll b and carotenoid in all concentrations of chloride level (Table 4). The stress of increasing concentrations of chloride significantly decreased the concentrations of chlorophyll a, b and carotenoid (Table 4). Reduction in chlorophyll contents due to increasing of chloride level is in line with the findings of Karaivazoglou *et al*. (2005), Kumar *et al*. (2015), and Abeer *et al*. (2015), who have reported a considerable decline in chlorophyll contents of *Nicotiana tabacum*; *Jatropha curcas* and *Vigna unguiculata* plants exposed to salinity stress. AMF inoculation increases chlorophyll content because of its direct influence on the uptake of Mg, which is an important component of chlorophyll pigment (Abeer *et al*., 2015).

Membrane stability index and proline content

Electrolyte leakage from the cellular membranes of tobacco plants increased considerably under increasing of chloride level (Table 4). However, application of AMF checked the electrolyte leakage significantly in the AM-inoculated plants exposed to chloride stress (Table 4). Proline accumulation increased in AMF inoculated as well as chloride stressed plants as compared to control (Table 2). However increase was more conspicuous under chloride stressed plants. Inoculation of AMF in chloride stressed plants further enhanced the accumulation of proline (Table 2). More decrease of electrolyte leakage in AM-colonised tobacco than in non-AMF ones seem to be related to a high accumulation of proline in shoot of the plants. proline may act as osmolyte and stabilising protein.

Membrane permeability usually appraised as electrolyte leakage is a key indicator of membrane integrity in plants subjected to stress conditions (Datta and Kulkarni, 2014). Mycorrhizal colonisations in plants lowered electrolyte leakage concentration in tobacco plants. Hence it can be suggested that, mycorrhizal associations in tobacco helped to improve membrane structure and its stability under chloride stress condition. Similar type of finding was observed when mycorrhizal *Acacia Arabica* and *Lycopersicon esculentum* plants were allowed to grow under saline condition and had less membrane permeability over non-mycorrhizal plants (Datta and Kulkarni, 2014; He *et al*., 2007).

In many plants, various solutes such as proline have been shown to accumulate during salinity. Their accumulation might be of importance by regulating cytosolic pH and NDA/NDAH rate, stabilising proteins and scavenging hydroxyl radicals protecting cells from the adverse effect of ROS (Abeer *et al*., 2015). Similar results were reported by Kumar *et al*. (2015), who postulated that the proline level increases in the stressed AM- *Jatropha curcas* plants.

Osmotic potential and relative water content

The leaf relative water content (RWC) and osmotic potential of AM+ and AM- tobacco plants altered significantly (Table 4). The RWC decreased progressively with increasing concentrations of chloride level and consequently, osmotic potential also decreased in order to maintain turgor potential values, which were even increased with concentrations of chloride (Table 4). However, chloride stressed AM+ tobacco plants maintained higher RWC regardless of concentrations of chloride in irrigation water and were comparable to C1 AM- plants. Mycorrhizal inoculation increased osmotic potential and consequently decreased turgor potential for AM+ tobacco plants.

Improved water uptake as a result of AMF is possibly due to the direct influence of AMF hyphae on the root morphology and the improved N and P nutritional status (Wu *et al*., 2014). The P content of mycorrhizal tobacco plants was consistently higher than non-mycorrhizal plants regardless of intensities of chloride stress. A close relationship between P content and salt tolerance has been reported earlier (Selvakumar *et al*., 2014).

Table 4. Mean of chlorophyll a, chlorophyll b, carbon exchange rate, stomatal conductance, transpiration rate, relative water content, membrane stability index, osmotic potential, and carotenoids in leaf of mycorrhizal and non-mycorrhizal tobacco plants exposed to varying concentrations of chloride (average of two growing seasons, 2012-2013).

Treatment	CER (µmol m^{-2} s^{-1})	g_s (cm s^{-1})	E (mmol H_20 m^{-2} s^{-1})	Chlorophyll a (mg g fresh wt^{-1})	Chlorophyll b (mg g fresh wt^{-1})	Carotenoids (mg g fresh wt^{-1})	RWC (%)	MSI (%)	Ψs (MPa)
AM+									
C1	8.4^{a}	1.90^{a}	11.5^{a}	1.251^{a}	0.340^{a}	0.188^{a}	96.82^{a}	93.71^{a}	-1.33
C2	7.9^{a}	1.81^{ab}	10.9^{ab}	1.107^{b}	0.324^{ab}	0.164^{b}	89.74^{ab}	88.25^{a}	-1.45
C3	6.4^{b}	1.76^{bc}	10.1^{b}	0.987^{bc}	0.309^{b}	0.140^{c}	83.01^{bc}	73.17^{b}	-1.65
C4	5.9^{b}	1.65^{c}	9.4^{b}	0.850^{c}	0.285^{c}	0.114^{d}	78.85^{c}	71.12^{b}	-1.85
LSD	0.7	0.13	1.1	0.14	0.017	0.021	9.1	8.5	ns
AM-									
C1	6.3^{a}	1.69^{a}	8.7^{a}	0.997^{a}	0.286^{a}	0.138^{a}	90.12^{a}	87.12^{a}	-1.43^{a}
C2	5.0^{b}	1.48^{b}	8.07^{ab}	0.77^{b}	0.271^{a}	0.125^{a}	83.10^{ab}	68.58^{bc}	-2.55^{b}
C3	4.8^{b}	1.37^{b}	7.21^{bc}	0.514^{c}	0.204^{b}	0.098^{b}	74.85^{b}	57.12^{cd}	-2.63^{b}
C4	4.1^{b}	1.2^{c}	6.3^{c}	0.451^{c}	0.174^{c}	0.045^{c}	69.5^{b}	49.32^{d}	-2.9^{b}
LSD	1.1	0.16	1.3	0.22	0.013	0.023	8.3	15	1.1
ANOVA									
C	*	*	*	*	*	*	*	*	*
AM	*	*	*	*	*	*	*	*	*
C×AM	**	*	*	*	*	*	*	*	*

CER, carbon exchange rate; g_s, stomatal conductance; E, transpiration rate; RWC, relative water content; MSI, membrane stability index; Ψs, osmotic potential; AM+, mycorrhizal; C1, 10 mg Cl L^{-1}; C2, 40 mg Cl L^{-1}; C3, 70 mg Cl L^{-1}; C4, 100 mg Cl L^{-1}; LSD, least significance difference; AM-, non-mycorrhizal; ANOVA, analysis of variance; C, chloride; AM, arbuscular mycorrhizal. *P≤0.05; **P≤0.01. $^{a-d}$Means with different letters are significantly different at P≤0.05.

Antioxidant enzymes and non-enzymatic activities

Results regarding activities of antioxidant enzymes are depicted in Table 3. Chloride stress caused a significant increase in activities of antioxidant enzymes studied and increase was consistent with the increase in concentration of chloride (Table 3). AMF alone increased the activities of SOD, CAT, GR and APX (Table 3). In combination with chloride treatment AMF inoculation further enhanced the activities of antioxidant enzymes studied.

Results pertaining to the combined effect of chloride and AMF on ASA, GSSG and GSH are depicted in Table 3. Increasing concentrations of chloride level reduced ASA content while as GSSG and GSH was increased. However inoculation of AMF caused considerable increase in these attributes (Table 3). AMF inoculation in chloride stressed plants further enhanced the contents of GSSG and GSH (Table 3).

Antioxidant enzymes play an important role in scavenging of reactive oxygen species and hence averting the oxidative stress induced damaging effects on several sensitive molecules like proteins nucleic acids and lipids. In our results increase in activities of SOD, CAT, GR and APX due to chloride stress is in concurrence with the findings of Abd_Allh *et al*. (2015) for *Sesbania sesban*. SOD is involved in scavenging of superoxide radicals into water and hydrogen peroxide (Mittler, 2002). H_2O_2 produced is converted into water and oxygen either by CAT and APX (Mittler, 2002). Increased activities of antioxidant enzymes in AMF plants support the findings of Abd_Allh *et al*. (2015) for *Sesbania sesban* and Latef and Chaoxing (2011) for tomato. GR, APX, reduced glutathione (GSH), oxidized glutathione (GSSG) and ascorbic acid (ASA) are the important components of ascorbate-glutathione pathway which is actively involved in scavenging of ROS (Mittler, 2002). Ascorbate-glutathione cycle involves a series of redox reactions where the net electron flow is from NADPH to H_2O_2 resulting in the conversion of H_2O_2 into water. Increased activity of GR helps in enhanced production of reduced glutathione. Reduced glutathione produced from the reduction of oxidised glutathione acts as electro donor during the conversion of dehydroascorbate (DHA) into ASA and ASA acts a electron donor in conversion of H_2O_2 into water and oxygen (Mittler, 2002). Decrease in content of ASA and increase in GSH found in our study is in concurrence with the findings of Abd_Allh *et al*. (2015) for *Sesbania sesban*.

Table 5. Mean of indole acetic acid, gibberellic acid, and abscisic acid in leaf of mycorrhizal and non-mycorrhizal tobacco plants exposed to varying concentrations of chloride (average of two growing seasons, 2012-2013).

Treatment	IAA (n Mg fresh wt^{-1})	GA (n Mg fresh wt^{-1})	ABA (n Mg fresh wt^{-1})
AM+			
C1	474.36^{a}	215.33^{a}	71.33^{c}
C2	350.96^{b}	160.98^{b}	110.45^{bc}
C3	332.39^{b}	120.83^{c}	145.10^{b}
C4	277.96^{c}	63.33^{d}	209.80^{a}
LSD	40	21	29
AM-			
C1	433.26^{a}	131.66^{a}	110.40^{d}
C2	300.12^{b}	98.10^{ab}	175.49^{c}
C3	290.03^{b}	73.96^{b}	250.23^{b}
C4	128.26^{c}	14.13^{c}	376.45^{a}
LSD	55	30	38
ANOVA			
C	*	*	*
AM	*	*	*
C×AM	*	*	*

IAA, indole acetic acid; GA, gibberellic acid; ABA, abscisic acid; AM+, mycorrhizal; C1, 10 mg Cl L^{-1}; C2, 40 mg Cl L^{-1}; C3, 70 mg Cl L^{-1}; C4, 100 mg Cl L^{-1}; LSD, least significance difference; AM-, non-mycorrhizal; ANOVA, analysis of variance; C, chloride; AM, arbuscular mycorrhizal. *P≤0.05. $^{a-d}$Means with different letters are significantly different at P≤0.05.

Endogenous phytohormone

Drastic decline was observed in endogenous levels of IAA and GA3 due to chloride stress (Table 5). Inoculation of AMF not only increased the growth hormone levels but also ameliorated the chloride induced deleterious effects (Table 5). However ABA levels decreased due to AMF inoculation while increased under chloride stress conditions (Table 5).

Chloride stressed tobacco plants showed drastic decline in the endogenous synthesis of IAA, and GA3 while as AMF inoculated plants showed higher contents of these growth regulators. Endophytic fungi cause increase in endogenous levels of IAA and GA (Abd_Allah *et al*., 2015). ABA plays an important role in plant responses to abiotic stresses including salinity. As expected, we found out that ABA levels in non-colonised and AM plants increased as a consequence of salinity. Previous studies showed that AM symbiosis can alter the levels of ABA in the host plant and that, under salinity stress, the levels of ABA are lower in AM-colonised than in non-colonised plants (Aroca *et al*., 2013), as we found here under non-saline conditions. These results, together with those of other physiological parameters, support that AM symbiosis improves plant fitness. Babu *et al*. (2012) demonstrated that salinity stressed tomato plants showed increment in the concentration of ABA and IAA leading to better adaptation of tomato to salt stress. Hamayun *et al*. (2010) observed that soybean cultivars subjected to salt stress exhibited increase in ABA and decrease in GA3 synthesis.

Conclusions

It is shown here that AM symbiosis alleviates the negative effects of salt stress in tobacco plants by altering the hormonal productions and affecting plant physiology in the host plant, allowing plants to grow better under these unfavourable conditions. The results confirm the potential of arbuscular mycorrhizas in protecting host plants against unfavourable environmental conditions and pave the way for applying AM symbiosis in sustainable agriculture in Mediterranean conditions. Due to the variability of plant response to mycorrhizal treatments requires, however, additional multi-year studies on a wider range of tobacco genotypes are needed. A further indication emerging from this study is that, under the climatic conditions of Northern Iran, the optimum chloride level in irrigation water for acceptable Virginia tobacco is below 25 mg L^{-1}, whereas the level up to 40 mg L^{-1} in combination with AMF can be considered as the upper threshold limit.

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